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Involvement of the 5′-leader sequence in coupling the stability of a human H3 histone mRNA with DNA replication

(gene expression/cell cycle/mRNA degradation/fusion gene)

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ABSTRACT **Two lines of evidence derived from fusion gene constructs indicate that sequences residing in the 5′-nontranslated region of a cell cycle-dependent human H3 histone mRNA are involved in the selective destabilization that occurs when DNA synthesis is terminated. The experimental approach was to construct chimeric genes in which fragments of the mRNA coding regions of the H3 histone gene were fused with fragments of genes not expressed in a cell cycle-dependent manner. After transfection in HeLa S3 cells with the recombinant plasmids, levels of fusion mRNAs were determined by S1 nuclease analysis prior to and following DNA synthesis inhibition. When the first 20 nucleotides of an H3 histone mRNA leader were replaced with 89 nucleotides of the leader from a *Drosophila* heat-shock (hsp70) mRNA, the fusion transcript remained stable during inhibition of DNA synthesis, in contrast to the rapid destabilization of the endogenous histone mRNA in these cells. In a reciprocal experiment, a histone-globin fusion gene was constructed that produced a transcript with the initial 20 nucleotides of the H3 histone mRNA substituted for the human β-globin mRNA leader. In HeLa cells treated with inhibitors of DNA synthesis and/or protein synthesis, cellular levels of this histone-globin fusion mRNA appeared to be regulated in a manner similar to endogenous histone mRNA levels. These results suggest that the first 20 nucleotides of the leader are sufficient to couple histone mRNA stability with DNA replication.**

The human histone genes are a moderately repeated gene family that encode the major structural proteins of chromatin. It has been well established that histone gene expression and DNA replication are temporally and functionally coupled. The synthesis of most histone proteins (1–6) and the steady-state levels of histone mRNAs (7–12) are closely correlated with DNA synthesis in the S phase of the cell cycle. At the natural end of S phase or following inhibitor-induced termination of DNA synthesis, there is a coordinate and stoichiometric decrease in histone mRNA levels and histone protein synthesis (8–13). The rapid loss of histone mRNA under these conditions is in contrast to minimal changes in nonhistone mRNA levels. The selective destabilization of histone mRNA during DNA synthesis inhibition is post-transcriptionally mediated; destabilization is not dependent on transcription (11) but requires protein synthesis (11–15). The cellular and molecular basis for histone mRNA turnover, however, remains unresolved.

To address molecular mechanisms operative in the selective destabilization of histone mRNAs, we are attempting to identify regions of a cloned, cell cycle-dependent human H3 histone gene (16, 17) that are involved in the destabilization of its transcripts. Our approach is to construct fusion genes, in which fragments of the mRNA coding regions of the cloned human H3 histone gene are fused with fragments of other genes not expressed in a cell cycle-dependent manner. After transfection into HeLa S3 cells, levels of fusion mRNAs are analyzed in the presence or absence of DNA synthesis inhibitors. For our initial studies we used a *Drosophila* heat shock gene, *hsp70*, that is rapidly induced and efficiently transcribed at elevated temperatures in homologous as well as in a number of heterologous cell types (18). Heat shock conditions were employed that induce activation of the *hsp70* promoter and at the same time have minimal effects on cellular processes associated with DNA synthesis (19). We report here the construction of a *Drosophila hsp70*-human H3 histone fusion gene that can be induced by elevated temperatures in HeLa cells. This gene encodes a chimeric mRNA in which the first 20 nucleotides of the H3 histone mRNA leader region are replaced by a portion of the hsp70 mRNA leader. Unlike endogenous human H3 histone mRNAs, this fusion transcript does not appear to be destabilized in cells treated with hydroxyurea to inhibit DNA synthesis.

In a reciprocal experiment, the first 20 nucleotides of the H3 histone leader region were fused with a human β-globin gene segment. Globin mRNA stability in HeLa cells is not sensitive to inhibition of DNA replication. However, transcripts of the recombinant gene containing the histone leader segment are destabilized when DNA synthesis is blocked by hydroxyurea. Thus, the first 20 nucleotides of a human H3 histone mRNA appear to be sufficient to couple mRNA stability to DNA replication.

MATERIALS AND METHODS

Cloning and Plasmid Purification. DNA fragments (histone or globin), isolated either by electroelution or from low melting temperature agarose gels, were ligated into appropriate vectors using T4 DNA ligase essentially as described by supplier (New England Biolabs), and the ligation products were used to transfect *Escherichia coli* HB101. Plasmid DNA was isolated by an alkaline lysis procedure (20, 21) and purified by CsCl/ethidium bromide equilibrium density gradient centrifugation (22).

Transfection and Short-Term Transient Expression in HeLa S3. Transfection was performed according to Gorman *et al.* (23). Calcium phosphate/DNA complex was prepared as described by Graham and van der Eb (24) by using 10 μg of plasmid DNA and 10 μg of salmon sperm DNA as carrier. For cells transfected with the H3 histone-β-globin fusion gene, pSVβ$_2$ΔHEN, incubation was continued in a moist atmosphere of 5% CO_2/95% air at 37°C for 40–48 hr before harvest. Cells transfected with the heat shock human H3

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Abbreviation: bp, base pair(s).

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histone fusion construct pSP1519H3 were incubated at 37°C for 18–20 hr after glycerol shock. The 37°C medium (Eagle's minimal essential medium plus 10% fetal calf serum) was then replaced with the same medium at 42°C, and the cells were placed in a 42°C, moist, 5% CO_2/95% air atmosphere incubator for 2–3 hr before harvest.

Formation of Cell Lines Containing Stably Integrated Copies of pSP1519H3. HeLa monolayers were transfected as above but with equal molar quantities of pSP1519H3 and pSV2NEO (25) (10 μg of each) without using carrier DNA. Selection of cells expressing the gene was carried out essentially as described by Southern and Berg (25) using G418 (GIBCO).

To test for heat inducible expression of the heat shock-histone fusion gene, the medium of cells growing on plates at 37°C was changed to prewarmed 42°C medium, and then cells were incubated at 42°C for varying lengths of time before harvest.

RNA Isolation and S1 Nuclease Analysis. RNA was isolated as described (26, 27). S1 nuclease assays were carried out by a modification of the method of Berk and Sharp (28, 29).

RESULTS

Construction and Expression of a Heat Shock-Histone Fusion Gene. A recombinant gene was constructed by fusing the 5′-regulatory elements and the initial 89 nucleotides of the mRNA leader from a *Drosophila hsp70* gene with a human H3 histone gene from which the 5′-flanking sequences and the first 20 nucleotides of the leader had been deleted. The mRNA transcribed from this chimeric gene should differ from the normal human H3 histone message only in a portion of the 5′-untranslated leader region. This difference, the substitution of 89 nucleotides of the *Drosophila hsp70* leader for the first 20 nucleotides of the histone mRNA, will distinguish the fusion transcript from endogenous histone mRNA.

The *hsp70* control elements were obtained by inserting a 2-kilobase *Pst* I fragment from pG3 (30) into pUC 8. The resulting plasmid (SP1), was then cleaved with *Hin*dIII and ligated with a 780-base-pair (bp) *Hin*dIII fragment from pST519 (17). This fragment contains a complete human H3 histone structural gene except for the 5′ control elements and the initial 20 nucleotides of the nontranslated mRNA leader. The plasmid containing the histone gene in the appropriate orientation (pSP1519H3) is compared with the pST519 human histone gene in Fig. 1.

To determine whether the *Drosophila hsp70*-human H3 histone fusion gene is heat inducible, we transfected HeLa cells with pSP1519H3 and subjected the cells to heat shock at 42°C for various lengths of time. The relative levels of *Drosophila hsp70*:human H3 histone fusion mRNA present in the transfected cells after each time period were determined by S1 analysis (Fig. 2). The probe used was a 5′-end-labeled *Sma* I fragment of pSP1519H3 that extends 240 nucleotides into the transcribed region of the fusion gene (Fig. 1). This

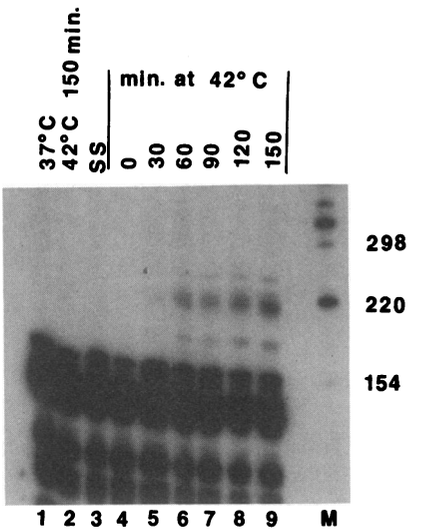

FIG. 2. S1 nuclease analysis of heat-shock-induced *Drosophila* hsp70-human H3 histone fusion mRNAs and endogenous human H3 histone mRNAs. Total cellular RNA (100 μg) was hybridized with a 5′-^{32}P-end-labeled DNA probe derived from a *Sma* I fragment of pSP1519H3 (Fig. 1). After a 3-hr incubation at 53°C, the hybridization reaction mixture was subjected to S1 nuclease digestion, and the ^{32}P-labeled, protected fragments were analyzed by electrophoresis through a 6% polyacrylamide/8 M urea gel. Lanes: 1 and 2, untransfected HeLa cell controls; 3, salmon sperm DNA-transfected cells incubated at 37°C; 4–9, pSP1519H3-transfected cells heat shocked for the times indicated (in min) at 42°C; M, ^{32}P-labeled *Hin*fI digest of pBR322. Sizes of fragments are given in base pairs.

fragment can be distinguished from the 151-nucleotide fragment protected by endogenous transcripts from the H3 histone gene represented in pST519. Both the unmodified endogenous H3 histone mRNAs and the fusion gene transcripts can thus be measured simultaneously in the same RNA preparation. S1 nuclease protected fragments of 185, 210, and 240 bp are detectable after 30 min of heat shock and continue to increase during incubation at 42°C (Fig. 2). Transcripts able to protect these probe fragments are absent in untransformed HeLa cells at either 37°C or 42°C, in cells transformed with salmon sperm DNA, or in nonheat-shocked, transformed cells. The largest fragment protected by heat-induced RNA (240 bp) corresponds to a transcript correctly initiated at the cap site of the *Drosophila* gene. The two additional fragments are derived from internal cleavage by S1 nuclease within the very AT-rich, heat-shock leader sequence (30) rather than from transcripts initiated at different sites.

A family of fragments decreasing in size from the 151 nucleotides expected for the endogenous H3 histone mRNA is influenced neither by heat shock nor by the transfection procedure. These fragments represent endogenous cell cycle-

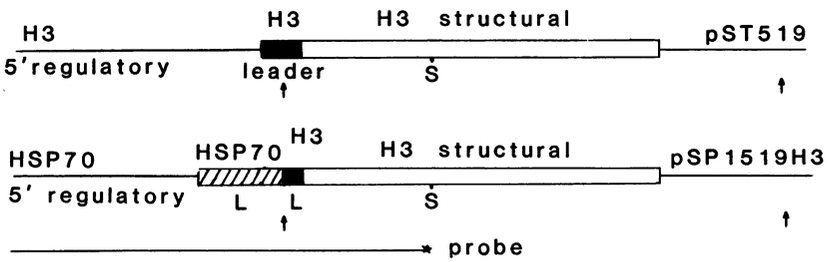

FIG. 1. Comparison of fused gene sequences in pSP1519H3 with cloned human H3 histone sequences in pST519. S, *Sma* I restriction site; ↑, *Hin*dIII restriction site; darkened boxes, H3 histone leader sequences; hatched boxes, hsp70 leader sequences; open boxes, H3 histone translated sequences. Also shown is the 5′-^{32}P-end-labeled probe used in S1 assays.

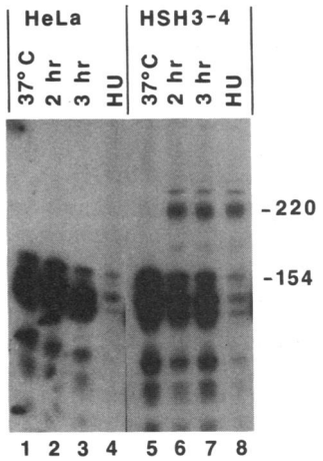

FIG. 3. S1 nuclease analysis of *Drosophila* hsp70-human H3 histone fusion mRNA levels in a cell line containing stably integrated pSP1519H3 DNA. Total cellular RNA (25 μg) was analyzed using the *Sma* I probe shown in Fig. 1. Protected fragments were resolved by electrophoresis through a 6% polyacrylamide/8.3 M urea gel. Lanes 1–4, HeLa cell RNA; lanes 5–8, HSH3-4 cell RNA. Lanes: 1 and 5, cells maintained at 37°C; 2 and 6, heat-shocked cells at 42°C for 2 hr; 3 and 7, heat-shocked cells at 42°C for 3 hr; 4 and 8, heat-shocked cells at 42°C for 3 hr, with hydroxyurea (75 μg/ml) added at the end of the 2nd hr of heat shock.

dependent H3 gene transcripts, because they are diminished by more than 90% following inhibition of DNA synthesis (see Fig. 3).

The results shown in Fig. 2 show that the human H3 histone gene, which is normally cell cycle-dependent, has been placed under control of a temperature regulated promoter. The mRNA encoded by this heat shock-histone gene contains a modified 5′-untranslated leader sequence. The involvement of 5′-leader sequences in H3 histone mRNA stability can, therefore, be directly addressed.

To investigate the stability characteristics of *Drosophila* hsp70-human H3 histone fusion mRNA, we constructed cell lines containing stably integrated pSP1519H3 DNA. Monolayer cultures of HeLa cells were cotransfected with pSP1519H3 and pSV2NEO. Of 45 colonies selected for resistance to the antibiotic G418, six exhibited detectable levels of the hsp70-H3 histone fusion mRNA after heat shock. One of these expressing lines, HSH3-4, was utilized for further studies.

Steady-state levels of histone mRNAs are reduced by approximately 90% when DNA synthesis is inhibited by various drugs (1–14, 31). This phenomenon is primarily the result of rapid and specific destabilization of histone mRNA (8, 11, 13, 15). The effect of the inhibition of DNA synthesis on steady-state levels of hsp70-H3 histone fusion mRNA in HSH3-4 cells was investigated following heat shock. Levels of fusion mRNA in cells incubated in the presence or absence of hydroxyurea were measured by S1 analysis (Fig. 3). Consistent with the results obtained from the short-term transient expression experiment, the heat shock-histone fusion RNA accumulated over a period of 3 hr following the temperature shift. Addition of hydroxyurea at 2 hr had no effect on the level of the fusion mRNA. However, the endogenous H3 histone mRNA decreased as anticipated to less than 10% of control.

These results show that substitution of 89 nucleotides of *Drosophila* hsp70 mRNA leader for the first 20 nucleotides of the histone leader yields an mRNA in which stability is no longer coupled with DNA replication. This suggests that the determinant of DNA replication-dependent mRNA stability may reside in the first 20 nucleotides of the H3 mRNA leader. Alternatively, the heat-shock mRNA leader segment might disrupt the structure of an element elsewhere in the histone mRNA that is important for control of stability.

Construction and Expression of a Human H3 Histone-Human β-Globin Fusion Gene. To address further whether histone mRNA stability is functionally related to the first 20 nucleotides of the H3 histone mRNA, a histone-globin fusion gene pSVβ$_2$ΔHEN was constructed (ref. 17 and Fig. 4). In this recombinant gene the initial 20 nucleotides of the H3 histone leader were substituted for the globin leader. The globin gene was chosen for these constructs because (*i*) it is a small, well-characterized gene, (*ii*) globin is normally expressed in nonproliferating cells, (*iii*) expression is not inhibited by interference with DNA replication (32), and (*iv*) the globin gene can be transcribed under the influence of histone promoter elements (17). The histone-globin fusion gene, pSVβ$_2$ΔHEN, is described (17).

We initially established that transcription of the histone-globin fusion gene in HeLa S3 cells is initiated at the *in vivo* mRNA start site of the H3 histone gene. This was determined by S1 nuclease analysis using a 5′-end-labeled *Acc* I fragment that spans the initiation region and extends 3000 nucleotides upstream. Subsequently, we used the 5′-end-labeled *Hin*dIII fragment shown in Fig. 5*A* that spans the entire globin region of the chimeric gene. This probe allows detection of spliced and unspliced forms of the fusion gene transcript. Both forms of the fusion RNA are observed 47 hr after transfection of actively proliferating cultures (Fig. 5*B*). Hydroxyurea inhibition of DNA synthesis in transfected cells causes a rapid decrease in levels of the fusion transcripts (80% decrease of unspliced and 65% decrease of spliced). The presence of the first 20 nucleotides of the H3 histone mRNA leader in the fusion transcript, therefore, appears to render the mRNA sensitive to inhibition of DNA synthesis. This interpretation is further supported by the fact that transcripts of a human β-globin gene with an intact 5′ leader are insensitive to hydroxyurea treatment when transfected into HeLa S3 cells (Fig. 5*C*).

The initial 20 nucleotides of the H3 histone mRNA leader also confer other biochemical properties of cell cycle-dependent histone mRNA stability on the histone-globin fusion transcript. It has been well documented that hydroxyurea-mediated histone mRNA destabilization is prevented by inhibition of protein synthesis (11–15). S1 nuclease analysis using the globin mRNA probe (Fig. 5*D*) shows that addition of cycloheximide prior to inhibition of DNA synthesis by hydroxyurea prevents the loss of the spliced form of the histone-globin fusion mRNA. As an internal control (Fig. 5*E*), the same RNA preparations were assayed for the levels of a cell cycle-dependent H4 histone mRNA by using a 5′-end-labeled probe derived from λHHG41 (11). The results clearly indicate that the histone-globin gene transcript and

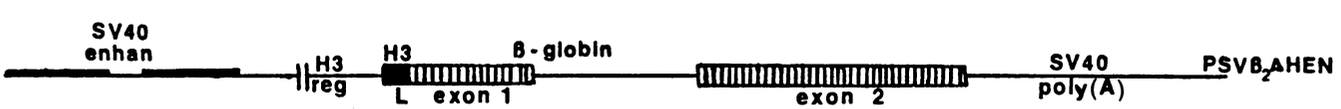

FIG. 4. Structure of pSVβ$_2$ΔHEN consists of the 5′-flanking region, the first 20 nucleotides of the leader sequence from the human H3 histone gene (from pST519) (9, 16, 17), the first and second exons, the first intron of the human β-globin gene, and the simian virus 40 early polyadenylylation sequence. To increase the levels of expression, a simian virus 40 enhancer was inserted upstream from the histone 5′-regulatory sequences.

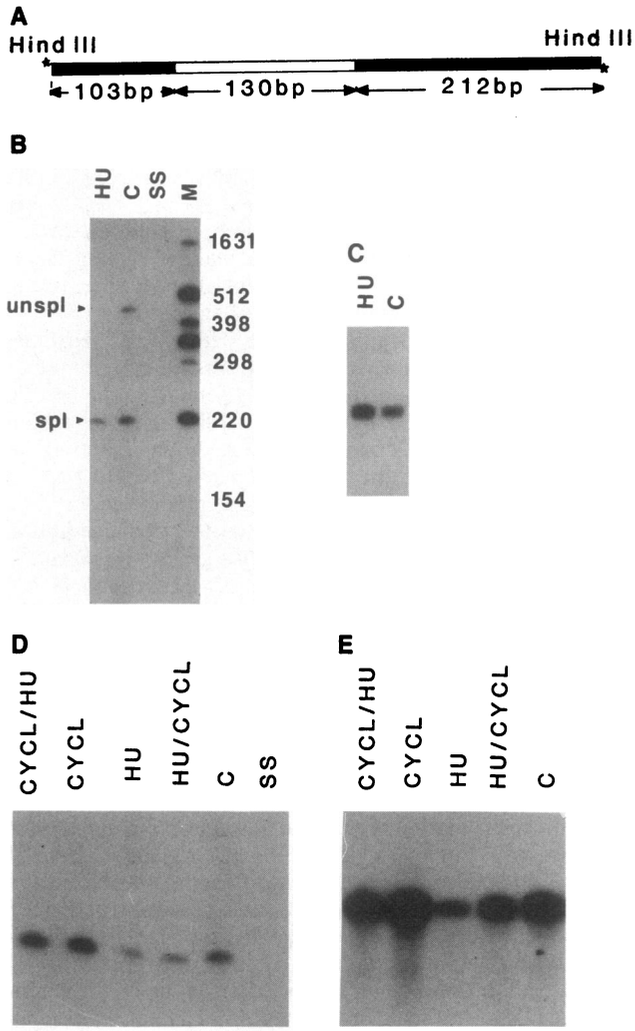

FIG. 5. S1 nuclease assays of cellular levels of a human H3 histone-human β-globin fusion transcript in HeLa cells following inhibition of DNA replication. (*A*) β-globin probe used in S1 assays shown in Fig. 5 *B–D*. The probe is fully protected (445 bp) by unspliced β-globin transcripts, and the spliced β-globin mRNA protects a 212-bp fragment. (*B*) S1 analysis of human H3 histone-human β-globin spliced and unspliced fusion mRNA, from short-term transient expression of pSVβ$_2$ΔHEN transfected into HeLa cells. Total cellular RNA (25 μg) was used for each S1 analysis. Lanes: HU, hydroxyurea was added to the medium (75 μg/ml) at 45 hr after transfection, and the cells were harvested 2 hr later. C, cells were harvested at 47 hr after transfection. SS, cells were transfected with salmon sperm DNA alone and harvested at 47 hr after transfection. The size markers, ^{32}P-labeled *Hin*fI digest of pBR322, are in bp. (*C*) S1 analysis of human β-globin spliced mRNA from short-term transient expression of a complete human β-globin gene transfected into HeLa cells. Total cellular RNA (25 μg) was used in each S1 analysis. Lanes HU and C are in Fig. 5B. (*D*) S1 analysis of human H3 histone-human β-globin spliced fusion mRNA from short-term transient expression of pSVβ$_2$ΔHEN transfected into HeLa cells. Total cellular RNA (25 μg) was used in each S1 analysis. Lanes: CYCL/HU, cycloheximide (10 μg/ml) was added to the medium a 44.5 hr after transfection. Hydroxyurea (75 μg/ml) was added 30 min later, and the cells were harvested at 47 hr after transfection. CYCL, cycloheximide (10 μg/ml) was added to the medium at 44.5 hr after transfection. The cells were harvested at 47 hr after transfection. HU/CYCL, hydroxyurea (75 μg/ml) was added to the medium at 44.5 hr after transfection. Cycloheximide (10 μg/ml) was added to the medium 30 min later, and the cells were harvested at 47 hr after transfection. HU, C, and SS are as in Fig. 5*B*. (*E*) S1 analysis of endogenous H4 histone mRNA in total cellular RNA (25 μg) from the same pSVβ$_2$ΔHEN transfected cells analyzed in *D* above. Lanes are labeled as in *D*. Probe was derived from a cloned human H4 gene from λHHG41 (11).

endogenous histone mRNAs respond to inhibitors of DNA replication and protein synthesis in a parallel manner. The first 20 nucleotides of the H3 histone mRNA leader sequence are, therefore, sufficient to couple mRNA stability with DNA replication.

DISCUSSION

The goal of these studies was to identify specific sequences in histone mRNA responsible for coupling mRNA stability with DNA replication. Two lines of evidence derived from fusion gene constructs indicate that sequences in the 5′-untranslated region of a human H3 cell cycle-dependent histone mRNA are involved in the selective destabilization that occurs when DNA synthesis is terminated. First, a fusion mRNA in which the first 20 nucleotides of the human H3 histone mRNA leader have been replaced with 89 nucleotides of the leader from a *Drosophila* hsp70 mRNA remains stable following inhibition of DNA synthesis. It, therefore, appears that either this fusion mRNA lacks "targeting" sequences for histone mRNA destabilization or that characteristics of the fusion mRNA mask the targeting signal. A second fusion mRNA that contains the first 20 nucleotides of the human H3 histone mRNA leader attached to human β-globin coding sequences appears to be regulated in a manner similar to endogenous human histone mRNA. The level of mRNA from a complete human β-globin gene introduced into HeLa cells does not decline when DNA synthesis is blocked.

The specific molecular mechanism by which the leader region of histone mRNA might participate in regulation of stability remains to be resolved. A regulatory factor, activated upon cessation of DNA synthesis, might recognize a specific nucleotide sequence in the leader. The failure of histone mRNA to be destabilized in the presence of protein synthesis inhibitors argues that the factor is a protein that is rapidly turned over or sequestered. The factor might be a nuclease or could direct binding of a nuclease to messages containing the target sequence. Another possible explanation is autogenous control, where the feedback of histone protein itself may serve to recognize histone mRNA-containing polysomes and selectively destabilize histone mRNA. However, inspection of the 5′-leader regions of sequenced histone genes fails to reveal a consensus sequence, despite the fact that stability of all of the mRNAs encoding cell cycle-dependent histones appears to be coordinately regulated (11). Alternatively, the factor might recognize a specific three-dimensional structure common to all cell cycle-regulated histone mRNAs. Putative stem and loop structures can be deduced from the leader sequences of many histone mRNAs. A computer analysis of histone mRNAs sequences and predicted secondary structures might clarify this issue.

A comparison of cell cycle-regulated and constitutively expressed histone mRNA structure would be of particular interest. Bird *et al.* (26) have observed poly(A)$^+$ H4 histone mRNA in both proliferating and nonproliferating rat myoblasts. Wells and Kedes (33) report a similar structure for a human H3 histone mRNA that is present at a basal level throughout the cell cycle. Unlike cell cycle-dependent histone mRNAs, this RNA is polyadenylylated and is encoded by a gene containing at least one intervening sequence. It remains to be determined whether this class of histone mRNA escapes the mechanism responsible for degradation of cell cycle-dependent histone mRNAs because of the absence of a target sequence or structure.

Although we find that endogenous histone mRNA and the introduced histone-globin gene transcript are both sensitive to inhibition of DNA synthesis, the level of the chimeric transcript is affected to a lesser extent (Fig. 5). Since transcription of the histone-globin fusion gene is under the influence of a simian virus 40 enhancer element, accumulation of this mRNA may proceed throughout the cell cycle.

The mechanism responsible for selective destabilization of histone mRNA might only operate during part of the cycle. In exponentially growing cultures, nearly all of the endogenous histone mRNA is in S phase cells, where stability is clearly dependent on continued DNA replication. The residual chimeric mRNA seen during hydroxyurea treatment may represent transcripts present in non-S phase cells. Alternatively, there may be a contribution of sequences outside of the first 20 nucleotides of the 5'-leader sequence to DNA-replication-dependent mRNA stability. Luscher *et al.* (34) have reported that sequences in the 3'-terminal part of a mouse H4 histone gene confer DNA-replication-dependent stability on a chimeric transcript containing 5' sequences derived from the simian virus 40 early gene. The possibility exists that sequences in both the 5' and 3' regions of a histone mRNA influence mRNA stability during the cell cycle or that the determinants of mRNA stability reside at different positions in various members of the histone gene family. A systematic analysis of chimeric gene constructs containing various regions of the different classes of histone genes will be necessary to resolve this question.

Cell cycle-dependent histone genes share a number of properties with the *myc* oncogene (35–37) and the adenovirus encoded *Ela* gene (38–40). All three classes of genes are expressed in a growth-dependent manner; all encode mRNAs with short half-lives; and there is evidence that gene expression is regulated at least in part at the level of mRNA stability. Moreover, turnover of these mRNAs requires ongoing protein synthesis, a property shared with inducible mRNAs encoding interferon (41), interleukin-2 (42), and heat-shock proteins (43, 44). In the case of the myc transcript, which is initiated from 2 promoters, Dani *et al.* (37) observed that cycloheximide had a differential stabilizing effect on the 2.2-kb but not the 2.4-kb myc mRNA species in HL60 cells. Thus, they suggest that the 5'-noncoding region of the myc mRNA might contain an important regulatory target for degradation. It is logical that any gene that is expressed transiently either during a specific portion of the cell cycle or in response to a metabolic signal must produce a transcript that either has an intrinsically short half-life or is subject to posttranscriptional regulation. Further investigations will reveal whether the structural determinants of metabolic stability are localized within the 5'-leader region of most mRNAs or if this property is characteristic of only certain messages.

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