Coupling of replication type histone mRNA levels to DNA synthesis requires the stem–loop sequence at the 3′ end of the mRNA

(transfection/H3/hydroxyurea/serum starvation)

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ABSTRACT The role of the 3′ end of mRNA in coupling between the level of histone mRNAs and DNA synthesis was examined. We introduced modified mouse histone H3 genes into fibroblasts and studied the regulation of several different H3 mRNAs that are not terminated with a normal histone 3′ end. In two cases, the stem–loop sequences were deleted from the mRNAs and replaced either by 3′ sequences flanking the H3 gene or by globin 3′ untranslated region sequences including the polyadenylation signal. In the former case, approximately 50% of the modified mRNA was polyadenylated, whereas in the latter case all of the mRNA had a polyadenylated terminus. In contrast to the normal histone mRNAs, these mRNAs, including the modified polyadenylated form, were stable when DNA synthesis was inhibited with several drugs. The levels of two other histone mRNAs, each containing the stem–loop sequences as an internal part of the mRNA, also were stable when DNA synthesis was inhibited. These results indicate that the posttranscriptional coupling of histone mRNA levels to DNA synthesis requires the presence of the stem–loop sequences at the 3′ end of the mRNA.

The core histone proteins are encoded by a multigene family. In higher eukaryotes there are a number of nonallelic variants of each histone protein. The replication variants are most abundant in rapidly growing cells and (1) that the synthesis of these proteins is coupled to DNA replication (1, 2). Their genes, which are repeated 10–20 times in the mouse genome (3, 4), are clustered in random order. These genes have common structural features including the absence of intervening sequences. They encode mRNAs 400–600 nucleotides long that contain a 3′-terminal stem–loop structure and are lacking a 3′ polyadenylated terminus. The expression of these genes during the cell cycle is controlled at both transcriptional and posttranscriptional levels (5–9).

On the other hand, the replacement variant proteins are synthesized throughout the cell cycle in small amounts (10). Several genes that specify these replacement variant proteins have been isolated and a few features of their sequences are strikingly different from those of the replication-dependent genes. Both the chicken and human replacement H3.3 genes contain intervening sequences and encode polyadenylated mRNAs (11, 12). A chicken H5 gene also encodes a polyadenylated transcript (13). These genes encode mRNAs that lack the 3′-terminal hairpin loop structure. These common features may be characteristic of all replacement variants and may be responsible for their continued expression throughout the cell cycle. Specifically, in contrast to the mRNAs for replication variants, the mRNAs for the replacement variants lacking the 3′-terminal stem–loop but containing poly(A) are stable when DNA synthesis is inhibited with several drugs (8).

We have been interested in determining whether the different 3′ ends—namely, 3′ stem–loop and a poly(A) tail—that distinguish the two types of mRNAs could be responsible for the difference in their regulation during inhibition of DNA replication. In this report, we examine the coupling between DNA synthesis and the expression of several histone mRNAs that have either a deletion of the stem–loop sequences and/or an addition of a polyadenylated terminus.

MATERIALS AND METHODS

Plasmids. The plasmid pH3.21 has been described (14). It contains a chimeric mouse histone H3 gene created by fusing the 5′ end of the H3.2-221 gene and the 3′ end of the H3.1-221 gene. A mutant with the stem–loop sequences deleted was constructed by digesting with Dde I the 770-base-pair (bp) Sal I/HindIII fragment of H3.1-221, which extends from codon 58 to 550 nucleotides 3′ of the gene. Dde I cuts in the middle of the stem–loop sequences (15). The Sal I/Dde I fragment was attached to the 3′ end of the Ava I/Sal I fragment of the H3.2-221 gene to give pHM3.21D. The H3.21 gene was cut with Sst II (codon 132) and then treated with BAL-31 (slow form) to remove 60–90 bases. The DNA was cut with HindIII to excise the 3′ flanking region, which was attached to pHM3.21D. The resulting clone pHM3.21A has a deletion extending 61 bp from the Dde I site in the 3′ direction. An Alu I fragment from the 3′ end of the mouse α2-globin gene was attached to the H3.21D plasmid using HindIII linkers to produce a hybrid H3.21-globin gene. These constructs are shown in Fig. 1.

Cell Culture and Transfection. Mouse Ltk− cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum as described (14). Cells were transfected either by the standard calcium phosphate transfer protocol (16) or by electroporation (17). Ltk− cells were cotransfected by calcium phosphate with 100 ng of pHSV106 DNA containing the Herpes simplex virus thymidine kinase (tk) gene (18), 10 μg of H3.21(221) DNA, or 10 μg of H3.21A DNA and 10 μg of carrier salmon sperm DNA. Ltk− cells were cotransfected by electroporation with 100 ng of pHSV106 DNA and 10 μg of H3.21-globin DNA. No carrier DNA was used with the electroporation protocol. tk− colonies were selected in medium containing hypoxanthine/aminopterin/thymidine (19). In some experiments, 30 individual colonies were pooled and grown in mass culture. Transfected Ltk− cells were serum starved by plating a logarithmically growing culture at 2×104 cells per 150-mm plate in DMEM containing 10% fetal bovine serum. After 3–4 days, when the cells had grown to 30% of final saturation density, the medium was changed to DMEM with 0.5% fetal bovine serum, and the cells were incubated for 10 days. The cells were stimulated to reenter the cell cycle by exchanging the medium again with DMEM containing 10% fetal bovine serum. DNA synthesis was inhibited in cell cultures that were

Abbreviation: nt, nucleotide(s).
Fig. 1. Structure of H3 histone genes with modified 3' end sequences. Construction of the modified H3 genes is described in Materials and Methods and the text. Stippled boxes denote histone mRNA sequences and lines denote histone flanking sequences. Plasmid sequences are denoted by broken lines. The direction of transcription of the H3 genes is from left to right. (A) Structure of the H3.21(221) chimeric gene was described (14). This figure shows the potential stem-loop structure lying 31 nt past the termination codon (TAA). The Dde I site within the stem–loop sequence was used for construction of the modified 3' ends shown in B and C. (B) Structure of the H3.21A gene containing a deletion beginning at the Dde I site and extending 61 bp in the 3' direction. The open box denotes a 47-bp fragment of pUC9 DNA from the polylinker region. (C) Structure of the H3.21-mouse a-globin gene. Solid box denotes the Alu I fragment from the 3' region of the mouse a-globin gene that includes the polyadenylation signal and cleavage site.

Grown to ~50% of final saturation density by treatment with aphidicolin (Natural Products Division, National Cancer Institute, Bethesda, MD) or with hydroxyurea (Sigma).

RNA Isolation and Analysis. Total cellular RNA was isolated by hot phenol extraction at 6°C as described (20). Polyadenylated [poly(A)] and nonpolyadenylated [poly(A)] RNA fractions were prepared by chromatography on oligo(dT)-cellulose (Collaborative Research, Waltham, MA).

S1 nuclelease analysis with end-labeled probes was performed as described (14). For analysis of the H3.21(221) gene, the H3.21-globin gene, or the H3.21A deletion gene, the respective gene was labeled either at the 3' end of the Sal I site for both the H3.21(221) gene and the H3.21-globin gene, or at the Hpa II site for the H3.21A gene with [a-32P]dCTP using avian myeloblastosis virus reverse transcriptase. Cellular RNA was incubated with the probe at 58°C and the S1 nuclease-resistant DNA fragments were analyzed on 6% polyacrylamide gels in 8 M urea. The dried gels were then autoradiographed. The relative amount of each DNA fragment was determined by scanning the autoradiographs with a Quantimet analyzer.

RESULTS

Construction of Histone Genes with Altered 3' End Sequences. The studies described here have been performed by introducing modified mouse histone genes into mouse Ltk- fibroblasts. We used a chimeric mouse histone gene H3.21(221) formed by fusing the 5' end of H3.2-221 and the 3' end of H3.1-221 at the shared Sal I site at codon 58. The construction of this gene has been described (14); it contains a normal stem-loop structure at the 3' end of the gene and ~350 nucleotides of 3' flanking sequence (Fig. 1). Most transcripts produced from this gene are correctly processed to yield an RNA with a normal histone 3' end (14). To investigate the role of the stem–loop sequences in histone RNA regulation, we deleted 61 bp of DNA extending from the Dde I site in the stem–loop sequence into the 3' end to produce the H3.21A gene (Fig. 1) (15). The deletion begins 10 nucleotides (nt) from the normal 3' end of the H3 mRNA. To investigate the consequences of polyadenylation of histone mRNAs, we added a portion of the 3' untranslated region of a mouse a-globin gene, including the polyadenylation signal, to the H3.21(221) gene in place of the stem-loop.

Expression of a Hybrid—H3.21-Globin Gene. To determine whether a polyadenylation signal from another gene could uncouple histone gene expression from DNA synthesis, the hybrid histone–globin gene was introduced into mouse Ltk- cells by electroporation. Southern blot analysis demonstrated that the transfectants contained between one and three copies of the hybrid gene (data not shown).

The expression of the hybrid histone–globin gene was analyzed by S1 nuclease mapping. Total cellular RNA from a cell line transfected with the hybrid histone–globin gene was hybridized to the gene 3'–labeled at the Sal I site at codon 58 (Fig. 2). There were several protected DNA fragments: a 240-nt fragment corresponding to the H3 coding region of the endogenous H3 mRNAs other than H3.1(221); a 117-nt fragment corresponding to H3.2 variant mRNAs which are cleaved by S1 nuclease at codon 96 due to sufficient sequence difference between H3.1 and H3.2 mRNAs; and a 280-nt fragment corresponding to the H3.1(221) mRNA coding region and its 3' untranslated region (4). All of these fragments are derived from endogenous H3 mRNAs present in...
the Ltk– untransfected cell line (Fig. 2D, lanes 3 and 4). In each of the transfected cell lines, an additional 380-nt-protected fragment was observed. This corresponds to a hybrid histone–globin mRNA terminating at the expected globin polyadenylation site. The gene was expressed at high levels, in some cases equivalent to the amount of total H3 mRNA in the cell. The histone mRNA produced was polyadenylated as judged by its binding quantitatively to oligo(dT)-cellulose (Fig. 2A) and it was found to accumulate in the cytoplasm. The expression of the histone–globin gene was investigated under conditions in which the level of endogenous histone mRNAs are known to change. Transfectants were treated with the DNA synthesis inhibitors hydroxyurea and aphidicolin. Fig. 2B shows that the level of the hybrid histone–globin mRNA actually increased after 60 min of drug treatment, whereas the endogenous H3.1(221) mRNA represented by the 280-nt fragment and the other endogenous H3 mRNAs represented by the 240-nt fragment declined. Fig. 2C shows the results of treating the transfected cells with aphidicolin for various lengths of time. The endogenous H3 mRNAs declined within 10 min in the presence of aphidicolin, while the histone–globin mRNA increased after 20 min in the presence of the drug. We do not understand the apparent increase of the histone–globin mRNA when the cells are treated with various drugs.

The relative content of the hybrid mRNA was also examined in resting cells prepared by serum deprivation for 10 days and in cells that were stimulated to reenter the cell cycle by refeeding with serum. The level of the histone–globin hybrid RNA was unaffected by either treatment, whereas exogenous H3 mRNAs declined to low levels during serum starvation and increased after refeeding with serum (Fig. 2D).

**Histone Transcripts Containing the Stem–Loop Structure and a Polyadenylated Terminator Are Stable During Inhibition of DNA Synthesis.** Our previous studies of transfected cell lines containing the H3.21 chimere and gene showed that, in addition to a mRNA terminating at the correct site, the transfected species produced a 3' extended transcript, called X, which is 250 nt longer than the normal mRNA. This transcript was polyadenylated and accumulated in the cytoplasm (14, 15). The production of transcript X from the transfected H3.21 gene provided the opportunity to determine whether a histone transcript that contains a normal stem–loop sequence but also contains additional 3' sequences and a polyadenylated terminator is regulated in parallel with normal histone RNA.

Total cellular RNA from a cell line transfected with the H3.21 gene was hybridized to the H3.21 gene labeled at the Sal site at codon 58 (Fig. 3). The mRNA derived from the transfected H3.21 gene has the same 3' end as the endogenous H3.1(221) gene, which is expressed in L cells (Fig. 2D, lane 4) but at low levels (15).

We showed previously that the H3.21 transcript ending at the normal stem–loop structure is regulated coordinately with endogenous histone genes during inhibition of DNA synthesis. To determine whether transcript X was regulated in the same fashion as the normally terminated H3 mRNA, cells were treated with 5 mM hydroxyurea for periods from 15 to 60 min. Total RNA was isolated at each time and analyzed by hybridization to H3.21(221) DNA labeled at the 3' end of the Sal I site (Fig. 3). The 290-nt fragment declined after 15 min and continued to decline by a factor of 5 in the presence of hydroxyurea, in parallel with the endogenous H3 mRNAs represented by the 240-nt fragment. In contrast, the level of the extended transcript X, detected by the 550-nt protected fragment, was unaffected by treatment with hydroxyurea.

**Histone Transcripts with the Stem–Loop Sequences Deleted Are Stable During Inhibition of DNA Synthesis.** The H3.21A stem–loop deletion was constructed by disrupting the expression of a histone mRNA that does not end in the normal 3' hairpin loop. The existence of an extended mRNA, transcript X, produced from the H3.21(221) chimeric gene suggested that transcription proceeds at least 250 nt past the mature histone mRNA 3' end. Results described elsewhere prove that this is the case (15). The normal 3' end must be formed by an endonucleolytic cleavage near the stem–loop structure as has been shown for histone RNAs in other species (21, 22). We expected that the H3.21A gene with the stem–loop sequences deleted would produce large quantities of transcript X.

Total cellular RNA from cell lines transfected with the H3.21A gene was hybridized to the H3.21A gene labeled at the Hpa II site at codon 52. Unexpectedly, the major mRNA species produced from H3.21A was a different extended transcript, called Y, with a 3' end that maps 100 nucleotides past the normal 3' end (408-nt protected fragment; Fig. 4A) (15). The RNA X also was formed in about the same quantity as in the H3.21(221) transfectants. Only 50% of the RNA Y transcripts bound to the oligo(dT)-cellulose, indicating that only a portion of these RNA molecules are polyadenylated (Fig. 4B). The detailed analysis of transcription into the 3' region flanking the H3.1(221) gene and the cryptic processing and polyadenylation sites found there that lead to the production of transcripts X and Y is described in a previous publication (15).

The stability of transcript Y was examined during inhibition of DNA synthesis with hydroxyurea (Fig. 4B). Total RNA was isolated from cells transfected with the H3.21A deletion gene after incubation with 5 mM hydroxyurea for various lengths of time. Both RNA X and RNA Y were stable when DNA synthesis was inhibited. In contrast, the level of the endogenous H3 mRNAs, which are represented by the 240-nt and 280-nt fragments, were reduced to low levels after 30 min of incubation with hydroxyurea.

The relative stability of RNAs X and Y compared with normal H3 mRNA might be due in part to the fact that these transcripts are polyadenylated. However, only 50% of RNA X bound to the oligo(dT)-cellulose, indicating that a substantial fraction of this transcript contained either no
poly(A) or an insufficient length of poly(A) to bind to oligo(dT)-cellulose. To determine the relative stability of nonadenylated RNA Y during hydroxyurea treatment, total RNA was isolated from the H3.21A transfectant before and after 60 min of treatment with hydroxyurea. The RNAs were run on oligo(dT)-cellulose and the amounts of the various transcripts were determined by S1 nuclease protection assays. The results shown in Fig. 4B indicate that the level of nonadenylated RNA Y was unaffected by hydroxyurea treatment. Thus transcripts lacking the stem–loop structure, but containing the rest of the histone mRNA sequences, even when not polyadenylated, are not coordinately regulated during inhibition of DNA synthesis.

Endogenous Histone Transcripts Containing the Stem–Loop and a Polyadenylated Terminator Are Stable During Inhibition of DNA Synthesis. The extended histone transcripts described above were derived from transfected genes. Other studies in our laboratories directed at detecting extended transcripts from endogenous histone genes revealed the existence of an extended polyadenylated H2a.2 mRNA (Fig. 5). Total RNA from mouse L cells was hybridized to the H2a.2(614) gene labeled at the Avai I site at codon 20. There were several protected DNA fragments: 330-nt fragment corresponding to the H2a coding region of H2a mRNAs other than H2a.2(614); and a 400-nt fragment corresponding to the H2a.2(614) mRNA coding region and its 3' untranslated region. In addition, there was a 440-nt fragment corresponding to an extended transcript, H2a-Y, which is only 40 nt longer than the normal 3' end of the H2a.2(614) mRNA. This transcript was polyadenylated as judged by its binding to oligo(dT)-cellulose (lane 3). The H2a-Y transcript is definitely produced from the H2a.2 gene since the amount of the H2a-Y fragment increases when the H2a.2 gene is transfected into cells (unpublished results).

To determine the relative stability of the polyadenylated H2a transcript that contained the stem–loop sequences as an internal part of the mRNA, total RNA was isolated from the L cells before and after 60 min of treatment with hydroxyurea. Part of the RNA was fractionated on oligo(dT)-cellulose and the amounts of the various transcripts were determined by S1 nuclease mapping. The level of the polyadenylated H2a-Y was unaffected by hydroxyurea treatment, while all the endogenous H2a transcripts ending at the normal stem–loop structure declined in the presence of the drug (Fig. 5).

DISCUSSION

The replication-type histone mRNAs are unique among mRNAs in mammalian cells in that, as far as is known, they are the only mRNAs that lack a 3' polyadenylated terminus. Instead they contain a stem–loop structure near the 3' end that is highly conserved in sequence among eukaryotes. These mRNAs are rapidly degraded when DNA synthesis is inhibited with various drugs (23). In contrast, the replacement-type histone mRNAs are stable when DNA synthesis is inhibited, and these mRNAs lack the stem–loop structure at the 3' end and they are polyadenylated (8). These differences between the two types of histone mRNAs suggest that the coupling of replication-type histone mRNA levels to DNA synthesis might be directed by a sequence or structure near the 3' end. Alternatively, the coupling might simply be due to a lack of poly(A). The results presented here strongly support the former hypothesis.

By introducing modified mouse H3 genes into cells we have been able to study the regulation of several different H3 mRNAs that are not terminated with a normal histone RNA 3' end. In each case, the modification results in a mRNA that is not rapidly degraded when DNA synthesis is inhibited with several drugs that reduce the levels of normal histone mRNAs. It should be noted that in each case the modified gene is not altered in the sequences lying 5' of the stem–loop structure and consequently the resulting mRNAs are normal from the 5' end to within 10 nt from the normal end of the mRNA. In two instances, the stem–loop sequences were deleted from the mRNA and replaced either by 3' sequences flanking the H3 gene or by globin 3' untranslated region sequences, including the polyadenylation signal. In the former case, ~50% of the modified mRNA was polyadenylated, whereas in the latter case all of the mRNA had a poly(A) tail. The relative stability of these mRNAs
when DNA synthesis was inhibited could be due to the deletion of the stem–loop sequences, to lengthening of the 3’ untranslated region, or to the addition of poly(A). We believe that most likely the stem–loop sequences are important because in the former case the level of the poly(A)− transcript Y was also unaffected by inhibition of DNA synthesis. It is not likely that the 3’ stability of these mRNAs could be due simply to lengthening of the untranslated region, as another construct we studied with a stem–loop sequence inserted a few nucleotides downstream of site Y produced a transcript that ends at this new stem–loop sequence (24). The level of this extended mRNA declined when RNA synthesis was inhibited.

These results indicate that the stem–loop sequences are involved in the coupling of histone mRNA levels to DNA synthesis. Perhaps these sequences form a recognition site near the 3’ end of the mRNA, which interacts with factors determining histone mRNA stability. Two further observations are important regarding the mechanism. Two of the mRNAs studied here contain the normal stem–loop sequence as an internal part of the mRNA. Transcript X, produced from the H3.21(221) chimeric gene, has a 3’ end that lies at a cryptic polyadenylation site 250 nt downstream of the stem–loop, and transcript H2a-Y produced from the endogenous H2a.2 gene has a 3’ end that lies at a cryptic processing site only 40 bases downstream of the stem–loop. The fact that the levels of both X and H2a-Y are not coupled to DNA synthesis suggests that the proposed mechanism requires that the stem–loop sequences reside at the 3’ end. Results to be reported elsewhere suggest that the 3’ end sequences must interact in some way with the protein synthesis machinery.

Graves et al. (24) have found that the level of histone mRNAs with normal 3’ ends are not coupled to DNA synthesis if protein synthesis on the mRNA is prematurely terminated more than 300 bases from the 3’ end. All of these results point to the importance of the sequences close to the 3’ end of histone mRNA as having a role in its stability. Results reported recently by others suggest a similar conclusion for certain polyadenylated mRNAs as well (25).

Recent studies indicate that the cell-cycle regulation of histone gene expression involves both transcriptional and posttranscriptional processes (5–9). Hanly et al. (26) have demonstrated that 5’ flanking sequences are required for the transcriptional regulation of a human histone H4 gene. Furthermore, Capasso and Heintz (27) have shown that transcriptional regulation of the H4 gene requires a H4-specific trans-acting factor that interacts with these 5’ distal sequences. Likewise, Artishevsky et al. (28) have demonstrated that 5’ sequences from a hamster H3 gene can confer cell-cycle regulation on a bacterial neomycin-resistance gene when the hybrid gene is introduced into hamster cells. However, the expression of the hybrid gene was not regulated as stringently as that of the endogenous histone genes, suggesting that the 5’ sequences make only a partial contribution, probably transcriptional, to the regulation of histone genes.

Using a similar approach, Morris et al. (29) have reported that the first 20 nt of a human H3 gene fused to a β-globin gene results in coupling of the mRNA stability to DNA replication. This result is contrary to our findings that indicate that a normal 3’ end is required for coupling of histone mRNA stability to DNA synthesis. On the other hand, our results are consistent with experiments by Lüscher et al. (30), who reported that mRNA produced by fusing the simian virus 40 early promoter to a mouse H4 gene is subject to normal posttranscriptional control, presumably due to the control at the level of mRNA degradation. They also observed a read-through transcript from this construct, which extended to a downstream polyadenylation site in the vector. This transcript was not subject to posttranscriptional regulation. More recently, they have localized the relevant control sequences to a small region including the stem–loop sequences (31). These studies suggest that both 5’ and 3’ flanking sequences are involved in determining the cell-cycle regulation of histone genes; 5’ sequences appear to be required for transcriptional regulation, while the 3’ end of the mRNA appears to be important for posttranscriptional regulation.

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