The histone mRNA 3' end is required for localization of histone mRNA to polyribosomes

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ABSTRACT

The final step in mRNA biosynthesis is transport of the mRNA from the nucleus to the cytoplasm. Histone genes from which the 3' stem-loop has been deleted are transcribed to give RNAs with heterogeneous 3' ends. These RNAs are localized in the nucleus and are stable. Addition of the histone 3' processing signal either on short (<250 nts) or long (>1000 nts) transcripts restores 3' processing and transport of the mRNA to the cytoplasm. In addition chimeric histone-U1 snRNA genes which produced RNAs with either histone or U1 3' ends were analyzed. Transcripts which ended with U1 snRNA 3' ends were not efficiently localized to polyribosomes. However, transcripts containing the same sequences including the snRNA 3' end followed by the histone 3' end were present in the cytoplasm on polyribosomes. Taken together these results suggest that the histone 3' end is required for export of histone mRNA to the cytoplasm and association of the mRNA with polyribosomes.

INTRODUCTION

The synthesis of messenger RNA in eucaryotic cells requires a number of modifications to the initial transcript, which occur during and after transcription. These modifications include addition of a 5' cap, formation of the 3' end of the mRNA, and for genes containing introns, assembly into hnRNP particles and removal of the intervening sequences. Only mature mRNAs are then exported to the cytoplasm where they can be translated into protein. Thus there must be a mechanism for selecting mature mRNAs for transport to the cytoplasm or retaining unprocessed RNAs in the nucleus.

Replication-dependent histone mRNAs differ from other mRNAs in that they lack a poly(A) tail at the 3' end. Instead they end in a highly conserved stem-loop sequence. Histone genes also are the only class of genes encoding mRNAs which lack intervening sequences (1). The 3' end of the histone mRNA is

formed by an endonucleolytic cleavage which requires U7 snRNP (2–4). Thus the only processing step required in histone mRNA biosynthesis is 3' end formation.

The functions of the 3' end of mRNAs are not completely understood. The 3' poly(A) tail has been implicated in several functions of mRNA (reviewed in ref. 5 including protecting the mRNA from degradation (6). Changes in the length of the poly(A) tail which occur as part of a developmental program are correlated with the recruitment of mRNAs into polyribosomes (7–9). Recently the poly(A) tail has been directly implicated in translation initiation (10–12).

The 3' stem-loop at the end of histone mRNA is involved in several aspects of regulation of histone mRNA metabolism. The efficiency of 3' processing is regulated during the cell cycle (13–15) and contributes to the level of expression of individual histone mRNAs (16). The stem-loop is also the only sequence required for regulation of histone mRNA degradation (17). Histone mRNA is rapidly degraded at the end of S-phase after cessation of DNA synthesis (15, 18). Here we report that the 3' end of histone mRNA is also required for export of the RNA to the cytoplasm. Removing the stem-loop from the histone gene results in transcripts which are retained in the nucleus. Substituting the last 10 nucleotides of U1 snRNA for the histone 3' end results in defined transcripts with the snRNA 3' end which are not associated efficiently with polyribosomes. These results suggest that another function for the histone 3' end is to direct the mRNA to the polyribosomes.

MATERIALS AND METHODS

Construction of altered histone genes. The genes used for subcloning in these experiments were the mouse histone H3.2–614 (19), H2b–221 (20), H2a–614 (21) genes and the mouse U1b genes (22, 23). The following truncated genes were constructed: the H2b–221 gene was truncated by digestion with *Ava*I at codon 92 and cloned into the *Xcy*I site of the polylinker of pUC18 to give the gene H2b (273). The complete H3.21–221

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Figure 1. Chimeric genes used in these studies. **A.** The histone genes with truncated 3' ends or with the position of the 3' end signal altered are shown. **B.** The chimeric U1-histone genes are shown. These genes have the mouse U1b promoter and first 5 nucleotides of coding sequence, portions of the histone H2a coding region and the U1b 3' end starting at either nucleotide 117 or 156 of the coding region. The histone H2a-614 3' end was placed 3' of the U1 end in the UHUH genes. The restriction enzymes sites used for S1 nuclease mapping are shown: *Ava*I; *Nar*I; *Nco*I; *Sal*I. □ histone coding region; □ histone untranslated regions; ▭ plasmid sequences; □ globin polyadenylation signal; — promoter sequences; ■ U1 sequences.

gene was also present on this clone (24). The H3.2-614 gene was truncated at the *Sal*I site at codon 58 and inserted into pUC18 at the *Sal*I site in the polylinker to give the H3(174) gene. A 65 nt fragment containing the H2a-614 3' end signal (16) was attached to the H3(174) gene to produce the H3(174)SL gene. The H2a-614(560)SL clone had the 3' end signal moved 560 nucleotides 3' of the gene. This gene has been described previously (25). The H3.21polyA gene has the mouse α -globin polyadenylation site in place the histone 3' processing signal and has been described (26). Some of the chimeric U1-histone genes have been previously described (27). All the genes used in this study are diagrammed in Figure 1.

Introduction of genes into cells. The altered genes were introduced into tk⁻ mouse L cells by cotransfection with the herpes virus thymidine kinase gene using the calcium phosphate procedure as previously described (24) or into CHO cells by cotransfection with the pSV2neo gene using the polybrene procedure (28) as previously described (29). Pools of stable transformants were selected and grown as mass cultures. The L cell transfections yielded multi-copy number transformants while the CHO cell transfections resulted in low copy number transformants.

Analysis of subcellular localization of mRNAs. Total cell RNA was prepared as previously described (30). Typically 10⁷ to 10⁸ exponentially growing Chinese Hamster Ovary or mouse L cells were used. Cells were fractionated into nuclear, polysomal and cytosol fractions. The cells were trypsinized, and incubated on ice for 10 minutes in swelling buffer (10 mM K(OAc), 1.5 mM Mg(OAc)₂, 10 mM Tris-HCl pH 7.4) to swell the cells. Triton X-100 (final concentration 0.1%) and DTT (final concentration 1 mM) were added and the cells were broken by homogenization using 20 strokes with the tight pestle of a Dounce homogenizer.

Nuclei were collected by centrifugation at 1000×g for 10 min. The nuclei were washed with 1 ml swelling buffer and the supernatants combined to give the cytoplasmic fraction. The cytoplasm was layered on 1.5 ml of a 30% (w/v) sucrose cushion containing swelling buffer and centrifuged for 3 hrs at 36K rpm in a T865.1 fixed angle rotor. RNA was prepared from the pelleted polyribosomes and from the supernatant (cytosol) as previously described (30). In the experiment shown in Figure 2, mouse L cells were homogenized without detergent, resulting in nuclei which were contaminated with cytoplasm.

The transcripts from the different genes were detected and quantified using S1 nuclease mapping as previously described (30). The S1-resistant hybrids were analyzed by gel electrophoresis on 7 M urea-6% polyacrylamide gels and quantified by densitometry. Equal proportions (cell equivalents) from each fraction were analyzed. In some experiments the amount of U1 snRNA in each fraction was measured using the riboprobe protection assay as described previously (31).

RESULTS

Following transcription, the nascent pre-mRNA transcript undergoes a number of processing reactions before it is ultimately transported to the cytoplasm where it is translated. The cell must identify mature mRNAs in the nucleus for export to the cytoplasm. Histone mRNAs provide a simple model system to study this process since they undergo only minimal processing prior to transport to the cytoplasm. Replication-dependent histone mRNAs are short transcripts with a 5' untranslated region of 20-70 nucleotides and a 3' untranslated region of 50-75 nucleotides. To investigate the sequence requirements for localization of a histone mRNA in the cytoplasm we constructed the genes shown in Figure 1.

Figure 2. Subcellular localization of transcripts from the H2b(273) gene. **A.** Mouse L cells transfected with the H2b(273) and H3.21–221 genes in high copy number were fractionated into nuclear and cytoplasmic fractions and RNA prepared. The RNA was analyzed using an S1 nuclease assay using the H2b(273) gene labeled at the 3' (lanes 2–4) or 5' (lanes 5–7) of the *Ava*I site as a probe. The specific activity of the 3' labeled probe was higher than that of the 5' labeled probe. The protected fragments were analyzed by gel electrophoresis and visualized by autoradiography. Lane 1 is pUC18 digested with *Hpa*II. Lanes 2 and 5 show analysis of 10 gm of yeast tRNA. Lanes 3 and 7 are analysis of nuclear RNA. Lanes 4 and 6 show analysis of cytoplasmic RNA. RNA from equal numbers of cells was analyzed in each lane. The multiple protected fragments in lane 3 are due to multiple transcripts with heterogeneous ends. The protected fragments are H2b — protection to the ATG codon by the other endogenous H2b mRNAs; H2b. 1 — protection to the start site of the H2b-221 gene largely due to transcripts from the H2b(273) gene. **B.** The same RNA samples in A were analyzed using the H3.21 gene labeled at the 3' end of the *Sall* site at codon 58. Lane N is nuclear RNA and lane C is the cytoplasmic RNA. RNA from equal numbers of cells was analyzed in each lane. The protected fragments are: H3 — protection of the other endogenous H3 mRNAs to the TAA codon; H3.1 — protection to the 3' end of the H3.21 mRNA largely from the transfected gene; X — protection to the cryptic poly(A) site 3' of the H3.1 gene (34). **C.** Mouse L cells transfected with the H2b(273) gene in low copy number were fractionated into nuclear (N) and cytoplasmic (C) fractions and RNA prepared. The RNA was analyzed with an S1 nuclease assay using the H2b-221 gene labeled at the *Nco*I site at codon 58 as a probe. RNA from equal numbers of cells was analyzed in each lane. The protected fragments are: H2b(273) — protection of the transcripts from the H2b(273) gene to the point of divergence from the probe; H2b — protection of the transcripts from the other endogenous H2b mRNAs to the TAA codon; H2b. 1 — protection to the 3' end of the endogenous H2b-221 mRNA. **D.** Parallel cultures exponentially growing mouse L cells transfected with the H2b(273) gene (lanes 2 and 4) or cells treated with 5 μ g/ml of aphidicolin to block DNA replication (lanes 3 and 5) were treated with aphidicolin for 1 hr and total cell RNA prepared. 10 μ gm of RNA was analyzed at each time using the H2b gene labeled at the 5' end of the *Ava*I site as a probe. 1 μ gm of total cell RNA from untransfected cells was analyzed in lane 1. The protected fragments are: H2b-1 (transcripts from the endogenous H2b-221 gene and the H2b(273) gene (over 90% of these transcripts are from the H2b(273) gene); H2b — transcripts from other mouse H2b genes; H2b(var.) — transcripts from mouse H2b genes which differ from the H2b-221 gene at amino acid 17 (20). The position of the size markers is indicated.

Figure 1A shows the altered histone genes used in this study. The H2b(273) gene and the H3(174) gene were constructed by truncating the complete gene in the coding region. The histone H2a-614 3' end signal was added to the H3(174) gene to give the H3(174)SL gene. The H2a-614(560)SL gene has the 3' end signal placed 560 nucleotides 3' to the normal location, resulting in a 1.1 kb histone mRNA, much longer than normal histone mRNAs. The H3.21polyA gene, which has the globin polyadenylation site attached in place of the histone 3' end signal, has been described previously (26).

Figure 1B shows the histone-U1 snRNA chimeric genes. The U series of snRNAs (except for U6) are the other class of genes transcribed by RNA polymerase II which are not polyadenylated and lack intervening sequences. To create transcripts which had defined 3' ends which were neither the histone 3' end nor poly(A) sequences, we constructed genes with the U1 promoter and 5 nucleotides of U1 sequence, followed by the histone H2a-614 coding region sequence and then the U1 3' end signal (27). The histone 3' end signal was placed 3' of the U1 3' signal. The use

of the U1 promoter was necessary since U1 3' ends are formed only from transcripts which initiate at the U1 promoter (32, 33). The distal histone 3' end signal resulted in any transcripts which extended past the U1 end forming stable transcripts which ended at the histone 3' end. The UH₅₈₀UH₁₁₇ gene contains the complete histone coding region. The UH₁₄₆UH₁₅₆ gene has only the first 60 nts of the H2a-614 coding region followed by the last 10 nts of the U1 coding sequence and U1 3' end signal followed by the histone H2a-614 3' processing signal. The UH₂₈₁UH₁₁₇ gene lacks the 5' end of the histone coding region, containing only those sequences after codon 93, resulting in a 281 nt transcript which ends at the U1 3' end and a 430 nt transcript ending at the histone 3' end, but does contain an ATG codon in the first 40 nts of the transcript.

Removal of the histone 3' end results in heterogeneous transcripts which are retained in the nucleus. The genes shown in Figure 1 were introduced into mouse L cells or CHO cells and stable transformants were selected. The cells were fractionated into

nuclear and cytoplasmic fractions and RNA prepared from each fraction. In most experiments the cytoplasmic fraction was further fractionated into polyribosomes and cytosol. We assume throughout this paper that the endogenous histone mRNAs are localized predominantly in the polyribosomes. Thus the endogenous histone mRNAs, which are measured simultaneously with the mRNAs from the transfected genes, serve as an internal control for contamination of subcellular fractions. In each fractionation experiment, we analyzed RNAs from equal numbers of cells in each of the subcellular fractions. We also measured the distribution of U1 snRNA in many experiments as a control for leakage from the nucleus. We observed varying degrees of contamination of the nuclear fraction with polysomal material. Under the conditions used for cell fractionation we observed minimal leakage of U1 snRNA from the nucleus (see Figures 7 and 8). If the relative proportions of the mRNAs from the transfected genes and the histone genes are the same in each fraction, then we conclude that the mRNAs from the transfected genes are localized in a similar manner to the endogenous histone mRNAs. If the distribution of the RNAs from the transfected genes differs from the endogenous histone mRNAs, then we conclude that they are localized differently from the endogenous histone mRNAs.

Figure 2 shows the results of transfecting the H2b-221(273) and the H3.21 genes simultaneously into mouse L cells in high-copy number (Figure 2A, B), or in low copy number (Figure 2C). The high copy transfection resulted in very high expression of the genes (29). The transcripts from the H3.21 gene have been described previously (24). The cells were fractionated into nuclear and cytoplasmic fractions using a gentle homogenization technique which left the nuclei contaminated with cytoplasm as indicated by the presence of endogenous mature histone mRNAs in the nuclear fraction.

Using a probe which mapped the 3' end of the transcripts, a large number of transcripts with different 3' ends were observed. These were located almost exclusively in the nuclear fraction (Figure 2A, lanes 3 and 4). The same RNA samples were then mapped with a probe labeled at the 5' end of the Aval site to map the 5' ends of the transcripts. This probe maps transcripts which initiated from the endogenous H2b-221 gene (labeled H2b.1 in Figure 1) and the transfected gene as a 314 nucleotide transcript, and the transcripts from the other endogenous H2b genes as a 273 nt transcript (20). In the high-copy number transfectants 90% of the transcripts with the H2b-221 5' end come from the transfected genes, since the endogenous H2b-221 and H3.1-221 genes are expressed at a very low level, less than 5% of the total H2b and H3 transcripts (20, 24). The majority of the H2b mRNAs from the endogenous genes were found in the cytoplasm, while most of the transcripts which initiated at the 5' end of the transfected H2b(273) gene were present in the nucleus (Figure 2A, lanes 6 and 7).

Figure 2B shows the distribution of the transcripts in the same experiment from the histone H3.21-221 gene which was introduced into the cells together with the H2b(273) gene. Two types of transcripts were produced from this gene, one ending at the histone 3' end and a longer transcript which was polyadenylated at a cryptic polyadenylation site (24, 34). Both of these transcripts cofractionated in parallel with the endogenous histone H3 mRNAs and hence were present in the cytoplasm.

A similar result is shown in Figure 2C using a second cell line which does not contain as many copies of the transfected gene as in Figure 2A. In this experiment we used a probe labeled at

the 3' end of the NcoI site at codon 58 of the intact H2b.1 gene. This probe maps all transcripts from the H2b(273) gene as a 99 nucleotide fragment and the transcripts from the H2b genes as 204 and 249 nt fragments respectively. The cells were fractionated into nuclear and cytoplasmic fractions by a similar procedure, which left the nuclear fraction contaminated with cytoplasm. While about 70% of the endogenous H2b mRNA was present in the cytoplasm, the transcripts from the H2b(273) gene were present exclusively in the nuclear fraction (Figure 2C, lane 3). In the high copy number transfections (Figure 2A) about 40% of the total H2b transcripts were from the transfected genes, while in the low copy number transfection in Figure 2C, less than 5% of the H2b transcripts were from the transfected gene.

One explanation of the failure to detect mRNA from the transfected genes in the cytoplasm is that the transcripts which lack defined 3' ends are unstable and are rapidly degraded either in the nucleus or the cytoplasm. To test this possibility we treated cells transfected with the H2b(273) gene with aphidicolin, which blocks DNA replication and also reduces the half-life of polysomal histone mRNA and the rate of histone gene transcription (35, 36). In this experiment we measured the 5' ends of the transcripts from the same cell line analyzed in Figure 2A. In the cells treated with aphidicolin there was a rapid reduction in the endogenous histone H2b mRNA, due both to a decreased transcription rate and a shortened half-life of the histone mRNA (Figure 2D, lanes 3 and 5). The amounts of the transcript from the H2b(273) gene were not significantly decreased (band H2b-1, Figure 2D, cf. lanes 2 and 4 with lanes 3 and 5). The great majority of the transcripts starting at the H2b-1 site are derived from the transfected gene. The transcripts from the transfected gene are not rapidly degraded as a result of inhibition of DNA synthesis since they lack the histone 3' end and are located in the nucleus (37). However, since treatment with aphidicolin reduces the rate of histone gene transcription, if these transcripts were unstable in the nucleus (or transported to the cytoplasm and then rapidly degraded) then the amount of the H2b-1 transcripts would have been greatly reduced after treatment with aphidicolin. Thus the nuclear transcripts from the H2b(273) gene are relatively stable and remain in the nucleus.

Similar results were obtained using a truncated histone H3 gene. The H3(174) gene, which contains the first 220 nucleotides of the H3-614 transcript, was also introduced into mouse L cells. The cells were fractionated into nuclear (N), cytosol (S) and polysomal (P) fractions and RNA from equal numbers of cells from each fraction was analyzed by S1 nuclease mapping. The transcripts from the endogenous and transfected genes were mapped using the intact H3-614 gene labeled at the NcoI site at the ATG codon as a probe. This probe maps the H3(174) transcripts as a 174 nt fragment, transcripts from the endogenous H3 genes as a 408 nt fragment and transcripts from the endogenous H3-614 gene as a 458 nt fragment. As shown in Figure 3A, the transcripts from the H3(174) gene were restricted to the nucleus, while the endogenous H3 transcripts were predominantly found in the polysomal fraction.

To demonstrate that the nuclear transcripts from the H3(174) gene were not simply very unstable, either in the cytoplasm or the nucleus, we treated cells with actinomycin D for 60 min to inhibit RNA synthesis and then examined the amount and localization of the transcripts from the H3(174) gene. Equal amounts of RNA from treated and untreated cells were analyzed in parallel assays (Figure 3B, lanes 1-6) and 3.5 times as much RNA from the actinomycin D treated cells as from the control

Figure 3. Localization of transcripts from the truncated histone H3 genes. **A.** Mouse L cells transfected with the H3(174) gene were fractionated into nuclear (N, lane 1), polysomal (P, lane 2) and cytosolic fractions (S, lane 3) and the RNAs analyzed by a S1 nuclease assay using the H3-614 gene labeled at the NcoI site at the ATG codon as a probe. RNA from equal numbers of cells was analyzed in each lane. The protected fragments are: H3(174) — protection to the point of divergence of the H3(174) gene from the H3-614 gene; H3 — protection of other endogenous H3 mRNAs to the TAA codon; H3-614 — protection of the endogenous H3-614 mRNA. Lane M is pUC18 digested with HpaII. Band P is the undigested probe. **B.** Parallel cultures of mouse L cells transfected with the H3(174) gene either untreated (lanes 1–3) or treated with actinomycin D (lanes 4–6) were fractionated into nuclear (lanes 1,4), polysomal (lanes 2, 5) and cytosolic fractions (lanes 3, 6) and RNA analyzed by S1 nuclease mapping as described in panel A. RNA from equal amounts of cells was analyzed in each lane. Lane M is marker pUC18 digested with HpaII. The protected fragments are labeled as in panel A. **C.** The same RNA samples analyzed in panel B were analyzed again except that RNA from 3.5 times as many cells treated with actinomycin D compared with the control cells was used. **D.** Mouse L cells transfected with the H3(174)SL gene were fractionated into nuclear (N, lane 2), polysomal (P, lane 3) and cytosolic fractions (S, lane 4) and the RNAs analyzed by a S1 nuclease assay using the H3-614 gene labeled at the NcoI site at the ATG codon as a probe. RNA from equal numbers of cells was analyzed in each lane. The protected fragments are: H3(174)SL — protection to the point of divergence of the H3(174)SL gene from the H3-614 gene; H3 — protection of other endogenous H3 mRNAs to the TAA codon; H3-614 — protection of the endogenous H3-614 mRNA. Lane 1 (M) is pUC18 digested with HpaII.

cells was also analyzed (Figure 3C, lanes 1–6). Treatment of cells with actinomycin D reduced the amount of histone mRNA 2- to 3-fold as expected since the half-life of histone mRNA is about 45 min in S-phase cells. The amount of the H3(174) transcript was reduced a similar amount as the endogenous histone transcripts (Figure 3B, lanes 1 and 4). This can be more clearly seen when 3.5 times as much RNA was analyzed from the cells treated with actinomycin D (Figure 3C, lanes 2 and 5). Clearly the H3(174) transcripts were not very unstable or there would not have been detectable H3(174) transcripts in the samples from the actinomycin D treated cells. Thus, as with the truncated H2b transcripts, the H3(174) transcripts were restricted to the nucleus and not simply rapidly degraded after export to the cytoplasm.

Restoration of the histone 3' restores cytoplasmic localization. The above results suggested that simply removing the 3' end of the histone mRNA resulted in the transcripts being restricted to the nucleus. To demonstrate a positive role for the histone 3' end, we attached a 65 nucleotide fragment from the H2a-614 gene, which includes the sequences required for 3' end formation, to the H3(174) gene to produce the H3(174)SL gene. This gene was introduced into mouse L cells and the cells fractionated into nuclear, cytoplasmic and polysomal fractions (Figure 3D). The

RNAs produced from the H3(174)SL gene cofractionated with the mRNAs from the H3-614 gene, indicating that these transcripts were transported to the cytoplasm and localized on polyribosomes as efficiently as the endogenous histone mRNAs. Thus adding the 30 terminal nucleotides to the H3(174) gene changed the subcellular localization of the transcripts and resulted in most of the transcripts being present on polysomes in the cytoplasm.

Since histone mRNAs are always short (< 700 nt) transcripts, we also moved the 3' end further 3' of the histone gene, creating a large histone transcript with a normal histone 3' end. The H2a-614(560)SL gene has the stem-loop sequence at the 3' end of the intact H2a-614 gene removed and then reattached 560 nts 3' to the gene. This gene is actively expressed and the 1.1 kb transcripts end at the histone 3' end. The RNA from the H2a-614(560)SL gene was primarily found in the cytoplasm on polyribosomes (Figure 4). However, a higher proportion of the H2a-614(560)SL transcripts in steady-state RNA were present in the nuclear fraction compared with the endogenous histone mRNA (Figure 4, lanes N and P). Thus it is likely that the longer transcripts from the H2a-614(560)SL gene were transported to the cytoplasm more slowly than normal histone mRNA, resulting in a higher proportion of the longer transcripts in steady-state

Figure 4. Localization of transcripts from the H2a-614(650)SL gene. Mouse L cells transfected with the H2a-614(650)SL gene were fractionated into nuclear (N), polysomal (P) and cytosolic fractions (S) and the RNAs analyzed by a S1 nuclease assay using the H3-614(650)SL gene labeled at the 3' end of the Aval site at codon 20 as a probe. RNA from equal numbers of cells was analyzed in each lane. Lane T is analysis of total cell RNA from a separate culture transfected with the same gene. The protected fragments are: H2a — protection by the heterologous endogenous H2a mRNAs to the TAA codon; H2a-614 — protection by the endogenous H2a-614 mRNA to the point of divergence from the H2a-614(650)SL gene; H2a(650)SL — protection by the RNA from the H2a-614(650)SL gene.

RNA in the nuclear fraction. These results clearly show, however, that transcripts with an intact histone 3' end are transported to the cytoplasm and localized to polyribosomes, even when they are much shorter (Figure 3B) or longer (Figure 4) than normal histone mRNAs.

Replacing the histone 3' end with a polyadenylation site does not interfere with cytoplasmic localization. The great majority of mRNAs end in a poly(A) sequence rather than the stem-loop structure which is at the 3' end of histone mRNAs. To see whether a poly(A) tail at the 3' end would also allow cytoplasmic localization of histone mRNA, the histone 3' end was removed and replaced with a globin polyadenylation signal to give the H3.21polyA gene. The transcripts were mapped using an S1 nuclease assay with a probe from the H3.21polyA gene labeled at the SalI site at codon 58. This probe maps the endogenous histone H3 mRNAs as a 240 nt fragment and the endogenous RNA from the H3.1 gene as a 280 nt fragment which extends up to the point where the stem-loop was deleted. The transcripts from the H3.21polyA gene protected a 380 nt fragment extending to the polyadenylation site. The distribution of the polyadenylated transcripts paralleled the distribution of the endogenous histone mRNAs, and they were predominantly present in the cytoplasm on polyribosomes, indicating that the poly(A) tail at the 3' end also functions to promote cytoplasmic localization of transcripts from the H3.21 gene (Figure 5). There are other examples of

Figure 5. Localization of transcripts from the H3.21polyA gene. Mouse L cells transfected with the H3.21polyA gene were fractionated into nuclear (N, lane 1), polysomal (P, lane 2) and cytosolic fractions (S, lane 3) and the RNAs analyzed by a S1 nuclease assay using the H3.21polyA gene labeled at the SalI site at codon 58 as a probe. RNA from equal numbers of cells was analyzed in each lane. Total cell RNA was analyzed in lane 4. The protected fragments are: H3 — protection by heterologous endogenous H3 mRNAs to the TAA codon; H3.1 — protection by the endogenous H3.1 mRNA to the point of divergence from the H3.21polyA gene; G(A⁺) — protection by the transcripts from the H3polyA gene.

histone genes encoding polyadenylated mRNAs which use cryptic polyadenylation sites located 3' of the normal histone 3' end (Figure 2) and these transcripts also produce functional histone mRNAs (38, 39).

Transport to the cytoplasm is independent of protein synthesis. Since all the histone mRNAs which are transported to the cytoplasm are efficiently localized on polyribosomes, it is possible that nuclear export and polysomal localization are coupled. Previously we have shown (40) that when mouse myeloma cells are treated with fluorodeoxyuridine to inhibit DNA replication, there is a rapid reduction in histone mRNA levels reaching 5–10% of the control levels by 30 min. If cells are subsequently treated with inhibitors of protein synthesis, which stabilize histone mRNA, then histone mRNA accumulates rapidly approaching control levels within 45 minutes of treatment with cycloheximide (40). Histone mRNA synthesized in the presence of cycloheximide cannot be translated because protein synthesis has been inhibited. In cells treated with cycloheximide there was a significant portion (about 30%) of the histone mRNAs found in the cytosolic fraction. The cytosolic histone mRNA presumably corresponds to the histone mRNA which is synthesized after addition of cycloheximide (Figure 6, lanes 1–3). In cells treated with FdU there is very little histone mRNA, but the residual histone mRNA is preferentially localized on the polyribosomes (Figure 6, lanes 4–6), consistent with the degradation of the mRNA occurring while it is associated with the polyribosomes

Figure 6. Localization of histone mRNAs in cells treated with cycloheximide. Mouse myeloma cells were treated with cycloheximide for 45 min (lanes 1–3), fluorodeoxyuridine for 30 min (lanes 4–6), or fluorodeoxyuridine for 30 min (lanes 4–6), followed by cycloheximide for an additional 45 min (lanes 7–9). The cells were fractionated into nuclear (N), polyribosomal (P) and cytosolic (S) fractions and the amount of histone mRNA analyzed using the H2a-614 gene labeled at the 3' end of the *Ava*I site at codon 20 as a probe. RNA from equal numbers of cells was analyzed in each lane. The protected fragments are: H2a-614 (405 nts) protection by the H2a-614 mRNA; H2a (330 nts) — protection by other H2a mRNAs.

(37). When cycloheximide is added to cells for 45 min starting 30 min after treatment with FdU, the great majority of histone mRNA present in the cell was synthesized after the addition of cycloheximide. This histone mRNA is predominantly found in the cytosolic fraction (Figure 6, lanes 7–9). These results demonstrate that continued protein synthesis is not necessary for transport of the histone mRNA to the cytoplasm. Equally importantly the results demonstrate that during the fractionation procedure, cytosolic mRNAs do not become adventitiously associated with polyribosomes.

Localization of transcripts from chimeric U1-histone genes. The third defined class of 3' ends found on transcripts synthesized by RNA polymerase II are the 3' ends of the snRNA genes. Previously we reported that chimeric U1-histone genes which had the 5' region of the U1 gene followed by the coding and the 3' end of the histone gene yielded transcripts which formed histone 3' ends efficiently and were localized on polyribosomes (27). Here we examine the localization of transcripts from the chimeric U1-histone genes with U1 3' ends. These genes were constructed so that we could examine the fate of RNA polymerase II transcripts which had defined ends, but which did not end in

either a polyA tail or a histone stem-loop at the 3' end. The snRNAs are normally transported to the cytoplasm after transcription where they are assembled into snRNPs and then reimported into the nucleus (41). The chimeric genes we constructed had two 3' end signals, a U1 snRNA 3' end signal followed by a histone 3' end signal and they all produced two types of transcripts, one which ended at the snRNA 3' end and longer transcripts which ended at the histone 3' end.

The $UH_{580}UH_{117}$ gene containing a U1 promoter, the complete histone coding sequences and a U1 3' end followed by a histone 3' end was introduced into CHO cells (Figure 1B). Two types of transcripts, a 580 nt transcript ending at U1 3' end and a 727 nt transcript ending at the histone 3' end, were formed from the $UH_{580}UH_{117}$ gene. Because of the length of the transcript, formation of the U1 3' end is inefficient (D.R.P. and W.F.M., unpublished results). Analysis of transcripts from three independent pools of cells transfected with the $UH_{580}UH_{117}$ gene is shown in Figure 7A. The transcripts with histone 3' ends are found predominantly in polysomes cofractionating with the endogenous histone mRNAs. The transcripts with U1 3' ends were distributed in all three fractions, and less than 30% of the transcripts were found in the polysomal fraction. Analysis of the distribution of the endogenous U1 RNAs using a mouse U1b riboprobe (31) in the same fractionated samples (Figure 7B) showed that the U1 RNAs were localized predominantly in the nuclear fraction. Thus the variable distribution of the transcripts with the U1 3' ends is not a result of general leakage of nuclear components during fractionation. It is clear however that transcripts ending with U1 3' ends are predominantly non-polysomal, whereas transcripts which end at the histone 3' end, are polysomal even though they contain all the same sequences.

We also examined the subcellular localization of transcripts from two other genes which produce RNAs with both U1 and histone 3' ends. The $UH_{146}UH_{156}$ and the $UH_{281}UH_{117}$ genes each produce more transcripts ending at the U1 3' end than at the histone 3' end. The $UH_{281}UH_{117}$ gene, like the $UH_{580}UH_{117}$ gene contains the last 50 nucleotides of U1 RNA, including the Sm binding site, while the $UH_{146}UH_{156}$ gene contains only the last 10 nts of U1 snRNA and none of the secondary structure elements which might allow the cell to 'identify' it as an snRNA. Translation from any of these transcripts could initiate at an ATG codon located within the first 70 nts of the transcript and in the proper context for translation. Figure 8 shows the subcellular distribution of the transcripts from these genes as well as the distribution of the endogenous histone and U1 transcripts for two separate pools of cells. Figure 8A shows that the transcripts from the $UH_{146}UH_{156}$ gene with the U1 3' end were present primarily in the cytosol fraction (Figure 8A, lanes 3 and 6), although there were small amounts of transcripts with U1 ends in the nuclear and polysomal fractions. Fewer than 25% of the transcripts were present in the polysomal fraction. In contrast over 90% of the transcripts ending at the histone 3' end were present in polyribosomes (Figure 8A, lanes 2 and 5) and these were distributed similarly to the endogenous histone mRNAs, which were predominantly localized on polyribosomes (Figure 8B, lanes 3 and 6). The endogenous U1 snRNAs were predominantly found in the nuclear fraction (Figure 8C, lanes 3 and 6) in the same subcellular fractionation.

The transcripts ending at the U1 snRNA end from the $UH_{146}UH_{156}$ genes are very short and might only accommodate one ribosome at a time if they were translated and hence might not be present in the polysomal fraction. Arguing against this

Figure 7. Localization of transcripts from the $UH_{580}UH_{117}$ gene. **A.** Three independent pools of CHO cells transfected with the $UH_{580}UH_{117}$ gene were fractionated into nuclear (lanes 1, 4, 7; N), polysomal (lanes 2, 5, 8; P) and cytosolic (lanes 3, 6, 9; S) fractions and the RNAs analyzed using the $UH_{580}UH_{117}$ gene labeled at the 3' end of the *NarI* site as a probe. RNA from equal numbers of cells was analyzed in each lane. The protected fragments are: H2aH — protection by the endogenous hamster H2a mRNAs; U1 — protection by RNAs ending at the U1 3' end; H — protection by transcripts ending at the histone 3' end. Band P is the undigested probe. Lane 10 is pUC18 digested with *HpaII*. **B.** RNA from pools 1 and 2 in panel A was analyzed using a mouse U1b riboprobe (31). This probe detects the CHO U1a RNA as a 80 nt fragment and the minor CHO U1b RNA as a 130 nt fragment. RNA from equal numbers of cells was analyzed in each lane, except for lane 4. Lanes 1–3 are pool 1 and lanes 4–7 are pool 2. Lanes 1 and 5 are nuclear fractions (N), lanes 2 and 6 are polysomal (P) fractions, and lanes 3 and 7 are cytosolic fractions (S). Lane 4 is 100 ngms of total cell RNA.

possibility is the fact that the 210 nt transcript from the H3(174SL) gene is localized efficiently on polyribosomes (Figure 3D). We examined the localization of a third transcript with a U1 snRNA end, the 281 nt transcript derived from the $UH_{281}UH_{117}$ gene. The transcripts from the $UH_{281}UH_{117}$ H gene ending at the histone 3' end were primarily present in the polysomal fraction, while 70% of the transcripts ending at the U1 3' end were not polysomal (data not shown).

Taken together all of these results strongly suggest that the histone 3' end is a dominant signal which aids in transport of RNA to the cytoplasm and participates in the localization of the histone RNA on polyribosomes.

DISCUSSION

The final step in the biosynthesis of functional mRNA is the export of the RNA from the nucleus to the cytoplasm and the association with polyribosomes. Little is known about the mechanism by which the recognition of a mature mRNA and targeting to the cytoplasm is accomplished in the nucleus. Our studies clearly show that for histone mRNA, an essential part of the recognition signal is the stem-loop at the 3' end of the mRNA. This sequence can be replaced by a poly(A) tail but not by an snRNA 3' end, even when the transcript contains the secondary structure normally present at the 3' end of U1 snRNA.

The poly(A) tail at the 3' end of messenger RNAs has a number of functions (5). It is likely that the stem-loop structure at the 3' end of histone mRNA functions similarly in the metabolism of histone mRNA as the poly(A) tail in the metabolism of other mRNAs. The poly(A) tail is a major determinant of mRNA stability, serving to protect the mRNA from degradation (42, 43).

This function of the poly(A) tail requires the poly(A) binding protein (6) which is tightly associated with cytoplasmic poly(A) mRNAs, but apparently not with nuclear poly(A) RNAs (44). Removal of the poly(A) binding protein accelerates degradation *in vitro* (6, 42) and removal of the poly(A) tail may well be a prerequisite for degradation of the mRNA *in vivo* (6). The sequences which control mRNA stability are generally located in the 3' untranslated region of the mRNA (45–47) and hence the poly(A) tail has a non-specific effect on mRNA stability.

A second role of poly(A) has recently been recognized. There is increasing evidence that poly(A) is directly involved in translation of mRNA, probably at the step on translation initiation (12). During *Xenopus* oocyte maturation, there is specific polyadenylation and deadenylation of maternal mRNAs. Most maternal mRNAs are deadenylated and removed from polyribosomes, while the subset of mRNAs which have their poly(A) tails lengthened are preferentially recruited into polyribosomes (7–9). These data are consistent with a role for the poly(A) tail in recruitment of mRNA into polyribosomes.

Experiments with yeast also support a role for poly(A) and the poly(A) binding protein in RNA transport (48) and in translation (10). Munroe and Jacobsen (11) presented biochemical evidence supporting a role for poly(A) in initiation of protein synthesis. Genetic experiments on the role of poly(A) binding protein demonstrated that the loss of poly(A) binding protein had direct effects on protein synthesis, consistent with a role of the 3' end of mRNA in translation initiation (10).

It is probable that the 3' end of histone mRNA plays a similar role in the metabolism of histone mRNA. We have previously shown that the signal regulating histone mRNA stability is the 3' end of the mRNA (17, 37), and Ross and coworkers have shown

Figure 8. Localization of transcripts from the $UH_{146}UH_{156}$ and $UH_{281}UH_{117}$ genes. **A.** Two independent pools of CHO cells (lanes 1–3 and 4–6) transfected with the $UH_{146}UH_{156}$ gene were fractionated into nuclear (lanes 1 and 4, N), polysomal (lanes 2 and 5, P) and cytosolic (lanes 3 and 6, S) fractions. RNA from equal numbers of cells was analyzed by S1 nuclease mapping using the $UH_{146}UH_{156}$ gene labeled at the XbaI site in the 5' untranslated region as a probe. The protected fragments are: U1 — protection by transcripts ending at the U1 3' end; H — protection by transcripts ending at the histone 3' end. Lane 7 (M) is pUC18 digested with HpaII. **B.** The same RNA samples analyzed in panel A were analyzed by S1 nuclease mapping using the H2a-614 gene labeled at the NarI site as a probe. This probe detects the endogenous hamster H2a mRNAs ($H2a_H$). Lanes 2 and 5 are nuclear RNAs (N), lanes 3 and 6 are polysomal RNAs (P) and lanes 4 and 7 are cytosolic RNAs (S). Lane 1 is pUC18 digested with HpaII. **C.** The same RNA samples were analyzed with the mouse U1B riboprobe as in Figure 7C. Lanes 1 and 4 are the cytosolic fractions (S), lanes 2 and 5 are the polysomal fractions (P) and lanes 3 and 6 are the nuclear fractions (N). Only the protected fragment from the hamster U1b RNA is shown.

that histone mRNA is degraded 3' to 5' by a nuclease which is associated with polyribosomes (49, 50). Thus the 3' end of histone mRNA, like the 3' end of other mRNAs, is involved in determining and regulating the half-life of histone mRNA.

The fact that transcripts with heterogeneous 3' ends are retained in the nucleus could be explained in several ways. It is likely that these transcripts don't terminate normally since we have shown that in the absence of a histone processing signal transcription continues well beyond the end of the histone gene (25). The failure of the transcripts to appear in the cytoplasm is not a result of their being released from the nucleus and then rapidly degraded in the cytoplasm. When RNA synthesis is inhibited by actinomycin D (Figure 3B, C) or when histone RNA transcription is selectively inhibited with an inhibitor of DNA replication (Figure 2D), there was not a rapid loss of the transcripts from the nucleus. This result indicates that the transcripts which accumulate from the truncated genes are stable in the nucleus for a reasonable period of time. When these transcripts are released from the chromatin they may be in a compartment which is not accessible for export to the cytoplasm. Eckner and Birstiel have recently reported that the formation of the histone 3' end by the normal RNA processing mechanism, as opposed to formation by a ribozyme catalyzed reaction, promotes the cytoplasmic localization of histone mRNA (51). Our results with the transcripts from the truncated genes are entirely consistent with their results.

Either a polyadenylation signal or a histone 3' end, even when placed several hundred nucleotides 3' of the normal histone 3' end, restored transport of the transcript to the cytoplasm. A second feature of mRNAs, the 5' cap structure, has been postulated to

serve as a transport signal for RNA polymerase II transcripts (52). Since the transcripts which initiate from the histone promoter presumably have 5' 7-CH₃ guanosine cap structures it is likely that the presence of the 5' cap is not sufficient for transport to the cytoplasm, although it may well be necessary.

Histone 3' ends efficiently localize mRNAs to polyribosomes

The results with the truncated genes suggest a role for the histone 3' end in transport of the RNA to the cytoplasm. The results with the U1-histone chimeric genes strongly support a role for the histone 3' end in efficient association of the mRNA with the polyribosomes. The U1 snRNA genes are transcribed by RNA polymerase II. After transcription the transcripts are normally released to the cytoplasm, where they undergo further processing (53, 54). The 3' end of the RNA is trimmed by removing 2–10 nucleotides, depending on the snRNA (41, 55). The snRNA assemblies with Sm proteins and is methylated to a 2,2,7 trimethylguanosine cap (56). The snRNP is then transported back into the nucleus. Both the binding of proteins to the Sm binding site and the trimethylation of the cap structure are necessary for nuclear localization of the snRNPs (57, 58). Normally snRNA precursors containing a 5' cap structure pass through the cytoplasm without becoming associated with polyribosomes, suggesting that additional features other than the cap structure may be essential for translation.

The chimeric histone-U1 genes utilized in this study each formed two types of transcripts, one ending at the U1 3' end and one, which contains exactly the same sequence plus about 100 nucleotides, which ends at the histone 3' end. Each of the

transcripts from the UH₅₈₀UH₁₁₇ gene could code for a normal histone protein and indeed histone transcripts which are initiated from a U1 promoter give rise to functional histone mRNAs (27). The transcripts from all the U1-histone chimeric genes contain initiation codons in the appropriate context within 70 nts of the 5' end of the transcript. After transcription of the chimeric U1-histone genes, it is likely that the RNAs ending at the U1 3' end are transported to the cytoplasm, since this is the normal fate of snRNA transcripts and the normal 3' sequences are present. The UH₅₈₀UH₁₁₇ and UH₂₈₁UH₁₁₇ genes each contain the Sm binding site, so it is possible that these RNAs might then be transported back into the nucleus. However, the UH₁₄₆UH₁₅₆ gene lacks the Sm binding site and would be expected to remain in the cytoplasm, which is what we observed (Figure 8).

The transcripts from these three genes which contain histone 3' ends were distributed in parallel with the endogenous histone mRNA and were primarily present on polyribosomes. In contrast the transcripts containing U1 3' ends are not efficiently localized to polyribosomes, with less than 30% of the transcripts being found in the polysomal fraction. A significant fraction, >40%, of the transcripts containing the Sm binding site are present in the nucleus, suggesting that they have been imported back into the nucleus.

These results suggest a role for the histone 3' end in export of mRNA from the nucleus and in association of the mRNA with polyribosomes. How the histone 3' end promotes incorporation of a mRNA into polyribosomes is not clear, but the most straightforward way would be to promote translation initiation, as has been suggested for the poly(A) tail (10–12). The role of the histone 3' end in transport to the cytoplasm and localization to polyribosomes, like the function of the poly(A) sequence, is probably mediated by proteins which interact specifically with the histone 3' end. We have recently described a protein which binds the 3' end of histone mRNA (59). This protein is present in both the nucleus and polyribosomes and may be a component of the histone mRNP. This protein is a likely candidate for mediating the transport of the histone mRNA to the cytoplasm as well the localization of the histone mRNA to polyribosomes. It is likely that the histone 3' end plays a parallel role to the poly(A) tail which is clearly important in all aspects of metabolism of the majority of mRNAs.

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