Intracellular distribution of histone mRNAs in human fibroblasts studied by *in situ* hybridization

(cell cycle/nuclear proteins/S phase/WI-38 cells/actin mRNA)

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ABSTRACT   We have used *in situ* hybridization to study the intracellular distribution of mRNAs for cell cycle-dependent core and H1 histone proteins in human WI-38 fibroblasts. Because histones are abundant nuclear proteins and histone mRNA expression is tightly coupled to DNA synthesis, it was of interest to determine whether histone mRNAs are localized near the nucleus. Cells were hybridized with tritiated DNA probes specific for either histone H1, histone H4, actin, or poly(A)$^+$ mRNA and were processed for autoradiography. In exponentially growing cultures, the fraction of histone mRNA-positive cells correlated well with the fraction of cells in S phase and was eliminated by hydroxyurea inhibition of DNA synthesis. Within individual cells the label for histone mRNA was widely distributed throughout the cytoplasm and did not appear to be more heavily concentrated near the nucleus. However, histone mRNA appeared to exhibit patchy, nonhomogeneous localization, and a quantitative evaluation confirmed that grain distributions were not as uniform as they were after hybridizations to poly(A)$^+$ mRNA. Actin mRNA in WI-38 cells was also widely distributed throughout the cytoplasm but differed from histone mRNA in that label for actin mRNA was frequently most dense at the outermost region of narrow cell extensions. The localization of actin mRNA was less pronounced but qualitatively very similar to that previously described for chicken embryonic myoblasts and fibroblasts. We conclude that localization of histones in WI-38 cells is not facilitated by localization of histone protein synthesis near the nucleus and that there are subtle but discrete and potentially functional differences in the distributions of histone, actin, and poly(A)$^+$ mRNAs.

Histones are a class of highly abundant nuclear proteins that play a fundamental role in the packaging and expression of the eukaryotic genome (reviewed in refs. 1 and 2). For most histones, the coupling of protein synthesis with DNA synthesis during the S phase of the cell cycle is well established (3–6). This cell cycle-dependent synthesis of core and H1 histones is controlled primarily by the level of available histone mRNA, since stable histone mRNAs accumulate beginning in early S phase and are rapidly and selectively degraded when DNA synthesis is completed or inhibited (7–12). Histone mRNA transcripts undergo little nuclear processing and appear on cytoplasmic polysomes within minutes after they are transcribed (13). In turn, histone proteins are transported to their site of function in the nucleus within several minutes of being synthesized in the cytoplasm (3, 4).

A study from the Steins' laboratory (14) has shown that histone mRNAs are physically associated with the 1% Triton-resistant cytoskeletal framework of the cell, as has been shown for several non-membrane-bound mRNAs (15–17). Hence, non-membrane-bound polysomes are not necessarily "free" to diffuse throughout the cytoplasm. Moreover, recent work by Lawrence and Singer (18) using *in situ* hybridization to cytoskeletal mRNAs has shown that specific non-membrane-bound mRNAs can exhibit distinct and nonrandom patterns of intracellular localization and that the distribution of a specific mRNA may be related to the distribution of the corresponding protein. For example, in motile chicken myoblasts and fibroblasts actin mRNA is heavily concentrated in the lamellipodia, structures in which actin protein undergoes rapid polymerization during cell locomotion (19). In the case of the histones, the potential functional significance of intracellular mRNA distribution is further suggested by the observation that mRNA stability properties change markedly when chimeric histone mRNAs are targeted to the membrane-bound polysomes (20).

The tight coupling of histone mRNA stability to DNA synthesis, the rapid transport of newly synthesized histones into the nucleus, and the recent demonstrations of specific mRNA localization near the site of protein polymerization collectively raise the question of whether histone mRNA might be concentrated around the nucleus. In the work presented here we used *in situ* hybridization to analyze histone mRNA expression at the single-cell level to determine the general intracellular distribution of mRNAs for cell cycle-dependent human histone H1 and H4 mRNAs in S-phase cells. The *in situ* hybridization technique employed was previously optimized for preservation of cellular RNA and morphology (21), and the cells chosen for study were human diploid WI-38 fibroblasts, whose flattened morphology is particularly amenable to analysis and whose histone mRNA expression has been investigated by conventional filter and solution hybridization techniques (22, 23). Finally, the distribution of actin mRNA in WI-38 cells was also evaluated, both to compare it to histone mRNA and to extend previous observations concerning the distribution of this mRNA to another cell type.

METHODS

Cell Culture. WI-38 human fibroblasts (passage 19) were grown on gelatin-coated glass coverslips in Eagle's minimal essential medium supplemented with 10% fetal bovine serum. Before fixation, cultures of exponentially growing cells were rinsed twice in Hanks' balanced salts solution and then fixed in 4% paraformaldehyde in phosphate-buffered saline with 5 mM MgCl$_2$ for 15 min at room temperature. Where indicated, cells were fixed in 4% glutaraldehyde instead of paraformaldehyde. Coverslips containing cells were then stored in 70% ethanol at 4°C until hybridization.

Abbreviation: LI, localization index.

In some experiments, cells in S phase were labeled in parallel cultures by incubation with [*methyl*-^{3}H]thymidine (0.5 μCi/ml of medium; 1 μCi = 37 kBq) for 15 min just before fixation. In samples in which DNA synthesis was inhibited, cultures were incubated with 10 mM hydroxyurea for 1 hr. To check for retention of total RNA during cell fixation, parallel cultures were incubated with [^{3}H]uridine (10 μCi/ml) for 3 hr before fixation, and then samples were quantitated by scintillation counting.

Probes. The isolation and characterization of λ Charon 4A recombinant phages containing human histone genes have been reported (24). Genomic restriction fragments were subcloned in plasmid pBR322 (9, 25) and contained the following human histone genes: H1 (pFnC16A), H3 (pST519D), and H4 (pF0108A). The actin probe used was a full-length chicken cDNA β-actin clone inserted into pBR322 (26). Plasmid DNAs were nick-translated by standard procedures, using three ^{3}H-labeled nucleoside triphosphates (New England Nuclear, 54–100 Ci/mmol), to a specific activity of 1–3 × 10^7 cpm/μg. Probes nick-translated with [α-[^{35}S]thio]dCTP had specific activities of 1–2 × 10^8 cpm/μg.

Hybridization and Detection. The details and derivation of the hybridization protocol have been published (21, 27). The salient features of this method are that cell treatments that remove cellular constituents, such as proteinase, acid, or acetic anhydride, have been omitted and that incubation in the hybridization solution is short (3 hr) so that any possible diffusion of mRNA is minimized. Cells fixed in paraformaldehyde and stored in 70% ethanol, as indicated above, were rehydrated in phosphate-buffered saline plus 5 mM MgCl$_2$ for 10 min, followed by 0.1 M glycine/0.2 M Tris·HCl, pH 7.4, for 10 min. Cells were then placed in 50% (vol/vol) formamide (Fluka)/2× SSC (0.3 M sodium chloride in 0.03 M sodium citrate buffer, pH 7.0) for 10 min at 60°C prior to hybridization. For each sample, 20 ng of probe DNA, 20 μg of tRNA, and 20 μg of nonspecific competitor DNA (from salmon sperm or *Escherichia coli*) were lyophilized, resuspended in formamide, and melted at 90°C for 10 min. Just before they were placed on the cells, the probe DNA, tRNA, and nonspecific DNA were combined with the hybridization mixture so that the final probe concentration was 1 μg/ml and the final hybridization solution consisted of 50% formamide, 2× SSC, 0.2% bovine serum albumin, 10 mM vanadyl sulfate ribonucleoside complex (28), and 10% dextran sulfate (Sigma). For hybridizations with ^{35}S-labeled probes, 300 mM dithiothreitol was added to the hybridization solution. Cells on coverslips were incubated in 20 μl of hybridization solution for 3 hr at 37°C by putting coverslips cell-side-down on Parafilm. After hybridization, coverslips were placed in 10-ml Coplin jars (VWR Scientific) and rinsed three times with shaking for 30 min each in 2× SSC/50% formamide at 37°C, 1× SSC/formamide at 37°C, and 1× SSC at room temperature. Control samples were incubated with RNase A (100 μg/ml) in 2× SSC for 1 hr at 37°C prior to hybridization. Previous work (18, 21) and other unpublished results from our laboratory have shown that these conditions are sufficiently stringent to provide specific hybridization.

After hybridization and rinsing, samples were dried through a graded series of ethanol solutions, dipped in Kodak NTB-2 emulsion, and stored at 4°C in the dark for 2–3 months for ^{3}H-labeled probes or 1–3 weeks for ^{35}S-labeled probes. At appropriate intervals, slides were developed in D-19 developer and Kodak Fixer and then were Giemsa-stained for viewing at ×1000 magnification with the microscope.

RESULTS

Exponentially growing human WI-38 fibroblasts were fixed and processed for *in situ* hybridization and autoradiography as described in *Methods*. In initial experiments, samples were hybridized with a mixture of probes for cell cycle-dependent H1, H3, and H4 mRNAs. Hybridization with either ^{35}S- or ^{3}H-labeled probes followed by autoradiography produced a bimodal population of cells in which $\approx$43% of cells exhibited grain densities significantly increased relative to background. Consistent with this result, the fraction of cells in S phase was found to be 44%, as determined by autoradiography of parallel cultures metabolically labeled with [^{3}H]thymidine for 15 min prior to fixation. Both the incorporation of [^{3}H]thymidine into nuclei and the labeling of cells after hybridization with histone probes were eliminated by hydroxyurea inhibition of DNA synthesis prior to fixation, as expected from previous studies using filter and solution hybridization (10, 12). Further confirmation that label over cells hybridized with histone probes represented bona fide detection of histone mRNAs was provided by the lack of label in samples treated with RNase A before hybridization.

The distribution of grains produced by hybridization with a mixture of ^{3}H-labeled probes for the three histone mRNAs was largely cytoplasmic with comparatively little label over the nucleus of the cell. To determine whether individual histone mRNAs exhibited distinct patterns of distribution, cultures were hybridized with probes for H1 and H4 mRNAs separately. Examples of results are presented in Figs. 1–4. With the probe for H1 histone mRNA, $\approx$44% of cells exhibited significant label, with positive cells having 4- to 15-fold more grains per cell than the rest of the population. Hybridization with the H1 probe resulted in label distributed over much of the cytoplasm (Figs. 1 and 2). Generally the area over the nucleus and the outermost region of long cell processes (lamellipodia and filopodia) showed little label (see arrow, Fig. 2). Although most of the cytoplasm was labeled, the distribution of label was not entirely homogeneous, in that some regions of cytoplasm were more densely labeled than others, frequently resulting in a patchy appearance to the overall grain distribution. Control samples treated with RNase before hybridization exhibited little label (Fig. 3). The labeling pattern observed after hybridizations with probes for H4 histone mRNA (Fig. 4) was similar to that for H1 mRNA, although less hybridization was generally obtained with this smaller probe.

We compared the distribution of histone mRNAs with the distribution of total RNA, poly(A)$^+$ mRNA, and actin mRNA in WI-38 fibroblasts. Acridine orange staining of RNA showed that total RNA, comprising primarily rRNA in ribosomes, was widely distributed throughout the cell (Fig. 8), unlike some cell types in which total RNA and polysomes are often restricted to central regions of the cell (29). Although staining for total RNA was evident throughout WI-38 cells (Fig. 8), the periphery of broad, flat lamellipodia stained less intensely due to the thinness of the cytoplasm in this region. To determine the distribution of total poly(A)$^+$ mRNA, cultures were hybridized with ^{3}H-labeled poly(U). Hybridization to poly(A)$^+$ mRNA produced a uniform distribution of grains over the entire cell (Fig. 7). To provide a quantitative comparison of the extent of localization observed for histone mRNAs and poly(A)$^+$ mRNA, a "localization index" (LI) was determined for samples hybridized with different probes, using the approach described previously (18). This involved determining the ratio of grain densities in the most heavily labeled region and the least heavily labeled region of randomly selected single cells (Table 1). The average LI for poly(A)$^+$ mRNA was 1.5, slightly higher than the theoretical value of 1.0 (which would indicate completely uniform grain distribution) but close to results previously obtained for poly(U) hybridization to chicken myoblasts and fibroblasts (18). In contrast, the LI for cells expressing H1 histone mRNA was 3.6, indicating that this mRNA exhibits a significant degree of nonrandom localization. The LI for the H4 histone mRNA was 3.1, which
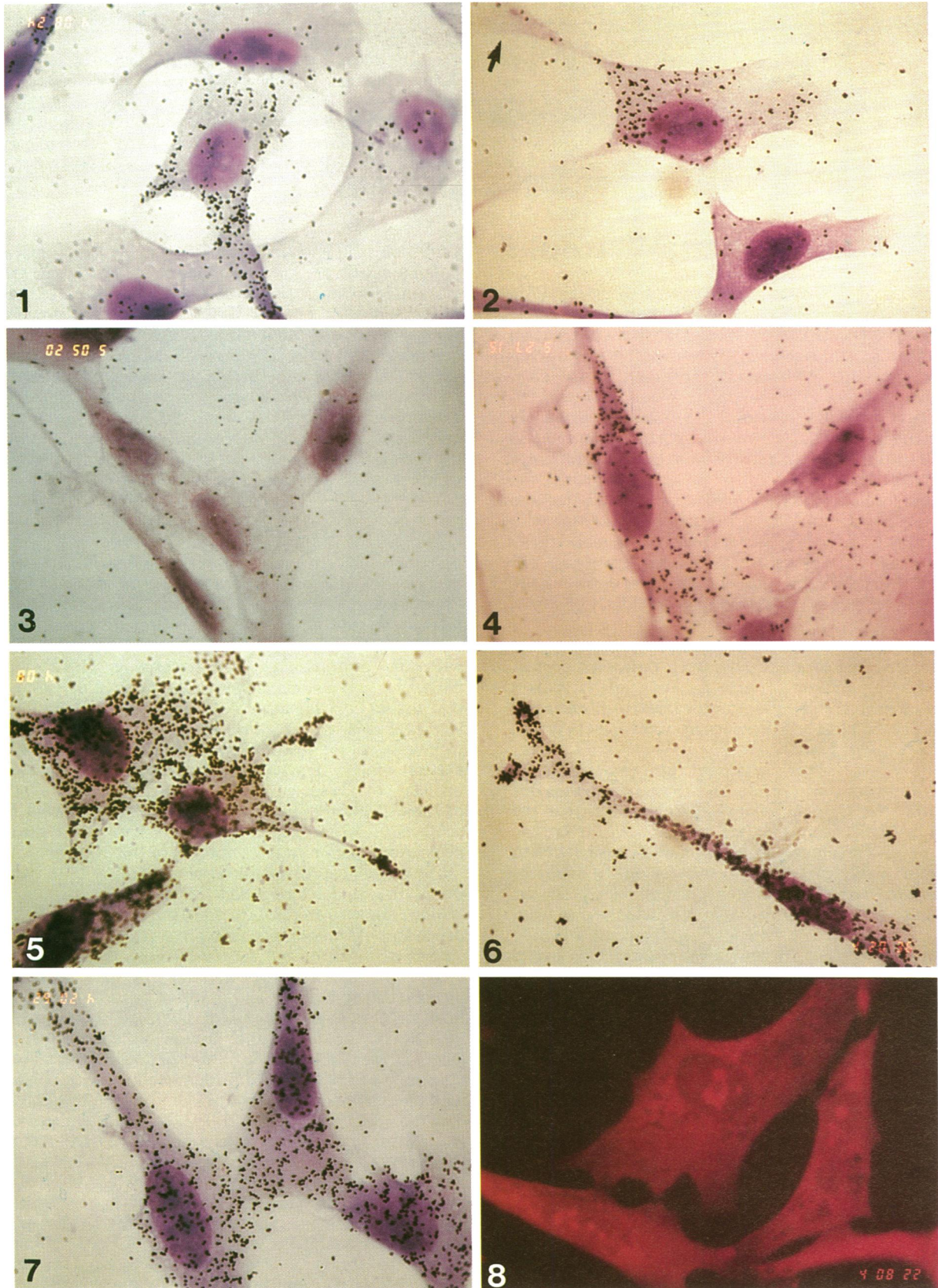
FIGS. 1–8. Autoradiographs of Giemsa-stained WI-38 cells hybridized with tritiated probes and exposed for 3–12 weeks are shown in Figs. 1–7. Figs. 1 and 2: hybridization with H1 probe, showing labeled and unlabeled cells in the same field. Fig. 3: H1 probe with RNase-treated cells, indicating the low level of nonspecific labeling. Fig. 4: hybridization with H4 probe, showing labeled and unlabeled cells. Figs. 5 and 6: hybridization with actin probe. Fig. 7: hybridization with poly(U) probe. Fig. 8: acridine orange staining for total RNA.

Table 1.   Quantitative summary of histone and actin mRNA distribution in WI-38 cells

mRNA	LI* (range)	% cells with LI > 2.4	Description
Poly(A)$^+$	1.5 (1.0–2.4)	0	Relatively uniform distribution throughout cell.
H1	3.6 (1.4–7.4)	82	Region over nucleus exhibited decreased, rather than increased, label. Label was widely distributed in cytoplasm but frequently patchy. No increase over cell extensions.
H4	3.1 (1.1–5.3)	53	Similar to H1 but patchy appearance was less pronounced.
Actin	2.7 (1.0–5.0)	52	Widely distributed in cytoplasm, but with markedly heavy concentrations in extended lamellipodia and in cell processes contacting other cells.

*LI = (grain density in most heavily labeled region) ÷ (grain density in least heavily labeled region). Numbers represent average for 35–50 randomly selected cells.

also indicates a less homogeneous distribution than observed for poly(A)$^+$ mRNA (Table 1).

The distribution of actin mRNA was also evaluated in WI-38 cells and compared to the distribution of histone mRNA and poly(A)$^+$ mRNA. Representative cells are shown in Figs. 5 and 6. Although actin mRNA was broadly distributed throughout the cytoplasm of most cells, it was commonly observed that in cells extending long narrow lamellipodia, the densest label was over the extreme periphery of the lamellipodia. The nuclear region frequently showed less label, although not in all cells. The average LI for actin mRNA in WI-38 cells was found to be 2.7, reflecting the nonuniform localization observed in approximately half the cells in the population. These results are consistent with previous results indicating that actin mRNA is more highly localized in motile myogenic cells than in less motile cell types (18). Quantitatively, the localization of actin mRNA was less pronounced in WI-38 cells than previously observed for chicken myoblasts and fibroblasts, but the pattern of localization was qualitatively very similar: densest label over cell extensions and in areas where cell processes contact other cells. This pattern of label for actin mRNA differs qualitatively from the pattern of histone mRNA distribution in which the ends of cell extensions were generally not labeled (see arrow, Fig. 2).

While there was slightly less label over nuclei with the poly(U) probe, this was much less pronounced than for results with the histone probes. This indicates that the decrease in label observed in the nuclear area for histone mRNA and, to a lesser extent, for actin mRNA is not due to the thinness of the cytoplasm in this region. In addition, hybridizations with ^{35}S-labeled histone probes produced results similar to those obtained with ^{3}H-labeled probes, with label throughout cytoplasmic regions and comparatively little label over the nucleus. The low level of label over nuclei hybridized with the histone-specific probes is consistent with the rapid processing of histone gene transcripts and the corresponding low level of nuclear histone mRNAs detected by gel blot or solution hybridization analysis (30, 31). Collectively these results suggest that histone mRNA is not as evenly distributed throughout the cell as is total RNA or poly(A)$^+$ RNA and that it exhibits a pattern of distribution distinct from that of actin mRNA. Most important, these results indicate that histone mRNA is not preferentially localized around the nucleus of the cell, wherein the histone proteins reside.

In studying the native configuration of mRNA, it is important to maximize the preservation of cellular RNA in its appropriate morphological context. Because we have found that the quality of fixation in different cell preparations can vary, we took additional steps throughout the course of this work to assure that the morphology and RNA in the cells studied were well preserved. In the experiments reported, two separate preparations of cells fixed in paraformaldehyde were used and, for one of these, the retention of [^{3}H]uridine-labeled total RNA was monitored after hybridization, as previously described (21). In addition, in one experiment

some cell samples were fixed in glutaraldehyde, instead of paraformaldehyde, and the distribution of H1 histone mRNA was found to be similar to that observed for paraformaldehyde-fixed cells. Finally, samples of each cell preparation were stained with antibodies to cytoskeletal elements to demonstrate that the fixation protocol had preserved cytoskeletal filaments and such labile cellular components as microtubules. These analyses indicated that cellular RNA and morphology were well preserved, further supporting the conclusion that histone mRNA, in its native configuration, is distributed throughout regions of the cytoplasm and does not exhibit marked perinuclear localization.

DISCUSSION

The main objective of the work presented here was to evaluate the intracellular distribution of histone mRNAs. Previous work from one of our laboratories (18) has indicated that, in the case of the filamentous proteins comprising the cytoskeleton, their cognate mRNAs are localized in regions where the proteins may be polymerizing. This raises the possibility that nonhomogeneous distribution may be a general property of cellular mRNAs. Since the mRNAs we have studied thus far are for filamentous proteins, it was of interest to investigate the distribution of a message for a noncytoskeletal protein targeted to a specific cellular region. The histones provide such a model. Moreover, since the synthesis of these nuclear proteins is so tightly coupled with DNA synthesis, it was logical to postulate that there may be some nuclear orientation to the synthesis of the proteins.

The overall expression of H1 and H4 mRNAs throughout the culture, as detected in individual cells by *in situ* hybridization, followed the pattern consistent with the cell cycle-dependent expression of these genes described in previous studies of synchronized cell populations using solution or filter hybridization (9–11, 22, 31, 32). The presence of unambiguously positive and negative cells within the same field, and the elimination of all positive cells by pretreatment with RNase, confirmed that autoradiographic detection of label represented bona fide hybridization. Furthermore, the frequency of cells positive for histone messages correlated with the fraction of cells in S phase as judged by [^{3}H]thymidine incorporation into nuclei. In addition, treatment of the cells with hydroxyurea, which inhibits DNA synthesis, eliminated all positive cells.

The grain distribution over single cells was evaluated to determine the intracellular location of histone mRNAs. These results demonstrated that histone mRNAs are present throughout most of the cytoplasm of the cell. Using this approach we found no evidence in WI-38 cells for a heavier concentration of histone mRNA near the nucleus and, in fact, observed less label over the nuclear region. While our results indicate that histone mRNA is widely distributed throughout the cell, it is not as uniformly distributed as poly(A)$^+$ RNA, as indicated by the higher LI for histone mRNA. In most cells hybridized with histone probes, both the area over the nucleus and discrete regions over the cytoplasm showed few

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grains relative to more heavily labeled regions of the cytoplasm. Although there was slightly less label over the nucleus and the periphery of flat lamellipodia with the poly(U) probe, regions with markedly less label were infrequent. Histone mRNA distribution was also qualitatively different from that of actin mRNA, which was widely distributed throughout the cell but with a propensity for heavy concentrations in the "foot" at the extremity of a cell process. The distribution of histone message was unusual compared to our previous reports of localization of mRNAs for filamentous proteins, in that no specific pattern to the placement of labeled and unlabeled cytoplasmic regions could be identified. With the methods applied, we could discern no correlation of grain distributions with any particular morphological structure easily identifiable by phase-contrast microscopy. A possible limitation of tritium autoradiography is that molecules more than 2 μm from the cell surface may not be detected; however, hybridization with ^{35}S-labeled probes also failed to detect high concentrations of mRNA near the nucleus. The specific grain distributions observed suggested clustering of messages within the regions of more densely labeled cytoplasm, but higher resolution, nonisotopic *in situ* hybridizations would be required to confirm this.

The vast majority of histone mRNA present in an S-phase cell is polysomal and undergoing translation (13). Therefore, it does not appear that the rapid synthesis and transport of histones to the nucleus are facilitated by localization of protein synthesis near the nuclear periphery. Recent investigations on the transport of histone fusion proteins into yeast nuclei (37) have shown that a 5 amino acid sequence at the amino terminus of histone H2B is necessary for transport of the protein into the nucleus. Therefore, the amino acid sequence that targets histone proteins to the nucleus appears to be sufficient for their localization. Since it has further been shown that certain histone proteins are not only coregulated in their synthesis but are cotransported as well (37), it may be worthwhile to speculate on the significance of a possible clustering of messages that might facilitate the formation of a histone–histone assembly complex. Investigation of histone message distribution at the electron microscopic level using nonisotopic detection would resolve whether mRNAs for different histones may be colocalized.

The intracellular localization of histone mRNAs has potential significance for understanding not only the coassembly and transport of histones to the nucleus but also the regulation of histone mRNA stability. The control of histone mRNA half-life plays an important role in the tight coupling of histone protein synthesis with DNA synthesis. Upon completion or inhibition of DNA synthesis, histone mRNAs are selectively and rapidly degraded with a half-life of 10 min (11, 12, 36). While it has been proposed that histone mRNA stability is autogeneously controlled by histone protein levels (12, 33–35), the actual mechanism whereby such a rapid and specific mRNA degradation is achieved is still unknown. It has been shown, however, that if the histone mRNA is switched from the cytoskeleton-bound to the membrane-bound compartment, it is no longer cell cycle-regulated (20). This evidence supports the hypothesis that the intracellular distribution of histone mRNA and/or its association with the cytoskeleton is an important component in regulating histone gene expression.

Note Added in Proof. We have recently observed that histone H3 mRNA also exhibits a non-nuclear, non-homogeneous distribution throughout the cytoplasm of S-phase WI-38 cells.

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