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Targeting of a chimeric human histone fusion mRNA to membrane-bound polysomes in HeLa cells

(signal peptide/subcellular location/posttranscriptional regulation)

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ABSTRACT **The subcellular location of histone mRNA-containing polysomes may play a key role in the posttranscriptional events that mediate histone mRNA turnover following inhibition of DNA synthesis. Previously, it has been shown that histone mRNA is found primarily on free polysomes that are associated with the cytoskeleton. We report here the construction of an *Escherichia coli* pBR322 β-lactamase signal peptide–human H3 histone fusion gene. The fusion transcript is targeted to membrane-bound polysomes and remains stable following interruption of DNA replication. Relocating mRNA within the cell may provide a procedure for studying the posttranscriptional regulation of gene expression.**

Secreted and cell-surface proteins generally contain an NH_2-terminal extension referred to as a signal peptide. Signal peptides have several general features, such as a charged amino acid within the first 5 amino acids at the NH_2 terminus, a stretch of 10–20 hydrophobic amino acid residues in the middle, a proline within 4–8 amino acids from the signal sequence cleavage site, and an alanine at the cleavage site (for review, see ref. 1). Transport of a secreted protein begins with the translation of the signal peptide. As the signal peptide emerges from the ribosome a signal recognition particle (SRP), composed of six polypeptides and a 7S RNA molecule, binds to the ribosome and arrests translation (2–4). The SRP-ribosome/mRNA complex attaches to the membrane of the endoplasmic reticulum by associating with the SRP receptor (docking protein) (5). When membrane bound, the SRP dissociates from the ribosome, translation resumes, and the protein is cotranslationally transferred into the lumen of the endoplasmic reticulum. The translocation of mRNA coding for proteins destined for export to the membrane of the endoplasmic reticulum prevents the synthesis of secreted proteins in the cytoplasm and in effect partitions the cell into two major subcellular compartments: free polysomes and membrane-bound polysomes.

The biosynthesis of histones, the major structural proteins of chromatin, is temporally as well as functionally coupled to DNA replication (6–10). At the natural termination of S phase as well as following interruption of DNA synthesis, there is a rapid and selective reduction in histone protein synthesis with a concomitant and stoichiometric decrease in steady-state levels of histone mRNA. Selective destabilization of histone mRNA in the absence of DNA replication appears to be mediated at the posttranscriptional level (11–17). The kinetics and extent of histone mRNA degradation following inhibition of DNA synthesis are not affected by treatment of cells with RNA synthesis inhibitors. Histone gene transcription is not reduced following inhibition of DNA replication (18), and modifications in the chromatin structure of a cell cycle-dependent human H4 histone gene that are associated with S phase (DNase I hypersensitivity, S1 nuclease sensitivity, and nucleosome spacing) occur in control and in hydroxyurea-treated cells (19, 20). However, to date, mechanisms involved in histone mRNA turnover are largely unknown.

A viable mechanism to account for histone mRNA turnover must incorporate the ability of nucleases to preferentially utilize histone mRNAs as substrates. This mechanism may involve the recognition of histone mRNAs by a histone-specific nuclease, the activity or availability of which is modified as a function of DNA replication. Alternatively, the effectiveness of histone mRNAs as substrates for nucleases with broad specificity may be related to recognition of sequences or structural elements of histone mRNAs that become accessible when DNA replication is interrupted.

Regardless of the specific mechanism that is operative, the questions arise as to whether the subcellular location of histone mRNA-containing polysomes is functionally related to the coupling of histone mRNA stability with DNA replication and whether subcellular localization can provide for the sequestering and/or concentration of macromolecules involved in histone mRNA turnover. Consistent with such reasoning, results from *in vitro* translation and nucleic acid hybridization analyses indicate that, although histone mRNAs are found predominantly on nonmembrane bound polysomes (21, 22), these histone mRNA-containing polysomes are associated with the cytoskeleton (23). This association with the cytoskeleton might provide a structural basis for the localization of histone mRNAs in specific regions of the cytoplasm. Signal peptides may serve as useful tools to target histone mRNA to membrane-bound polysomes, a subcellular location not normally occupied by histone mRNA. We report here the construction of a β-lactamase (EC 3.5.2.6) signal peptide–human H3 fusion gene that codes for a histone mRNA that associates with membrane-bound polysomes when introduced into HeLa S3 cells. The histone fusion mRNA remains stable during DNA synthesis inhibition. Relocating mRNA within a cell may provide an approach to study the mechanisms involved in posttranscriptional gene regulation.

MATERIALS AND METHODS

Construction of the Signal Peptide–Histone Fusion Gene. The construction of the β-lactamase signal peptide–histone fusion gene is outlined in Fig. 1. The first intermediate in the construction of pSPH3E, PSPpSTΔHH/E, involved the placement of the *Escherichia coli* pBR322 β-lactamase signal peptide–globin fusion gene from pSP125e (24) under the transcriptional control of the cell cycle-dependent H3 histone regulatory region from pST519ΔH (25). The 840-base-pair (bp) *Eco*RI/*Hin*dIII fragment from pST519ΔH was isolated electrophoretically and blunt-ended using the Klenow frag-

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Abbreviation: SV40, simian virus 40.

ment of DNA polymerase I. The signal peptide–globin fusion construct was digested with *Bgl* II restriction endonuclease, which cuts once in the untranslated leader region of the fusion gene, blunt-ended with Klenow fragment, and dephosphorylated using calf intestinal phosphatase. The 840-bp blunt-ended fragment from pST519ΔH was ligated into the *Bgl* II blunt-ended site of pSP125e using T4 DNA ligase. The construction of the second intermediate, pSPpSTΔHH/EpST, involved substitution of the chimpanzee globin coding sequences in pSPpSTΔHH/E with the H3 histone coding sequences from pST519 (26). pSPpSTΔHH/E DNA was digested with *Nco* I, which cuts at the signal peptide–globin junction, and treated with calf intestinal phosphatase. pST519 DNA was digested with *Nco* I, which cuts at the ATG translation start codon, and ligated to *Nco* I-cut/dephosphorylated pSPpSTΔHH/E DNA using T4 DNA ligase. The signal peptide–histone fusion gene was subcloned into pUC8 by ligating the 1.1-kilobase-pair *Hpa* I fragment from pSPpSTΔHH/EpST into the *Hinc*II site of pUC8 to form pSPH3. The signal peptide–histone fusion gene in pSPH3 was transcriptionally enhanced by placing the simian virus 40 (SV40) viral enhancer from pSVE108A (27) into the *Eco*RI site in the polylinker of pSPH3 to form pSPH3E1 (containing one enhancer element) and pSPH3E2 (containing two or three enhancer elements).

Transfection and Short-Term Transient Expression in HeLa S3 Cells. HeLa cell monolayers were transfected with 20 μg of DNA, according to Gorman *et al.* (28), in a calcium phosphate/DNA complex prepared as described by Graham and van der Eb (29). The transfected cells were incubated at 37°C, 5% CO_2 in air, for 46 hr following transfection. The cells were harvested and total cellular RNA was isolated as described by Plumb *et al.* (16).

The signal peptide–histone fusion mRNA was detected and

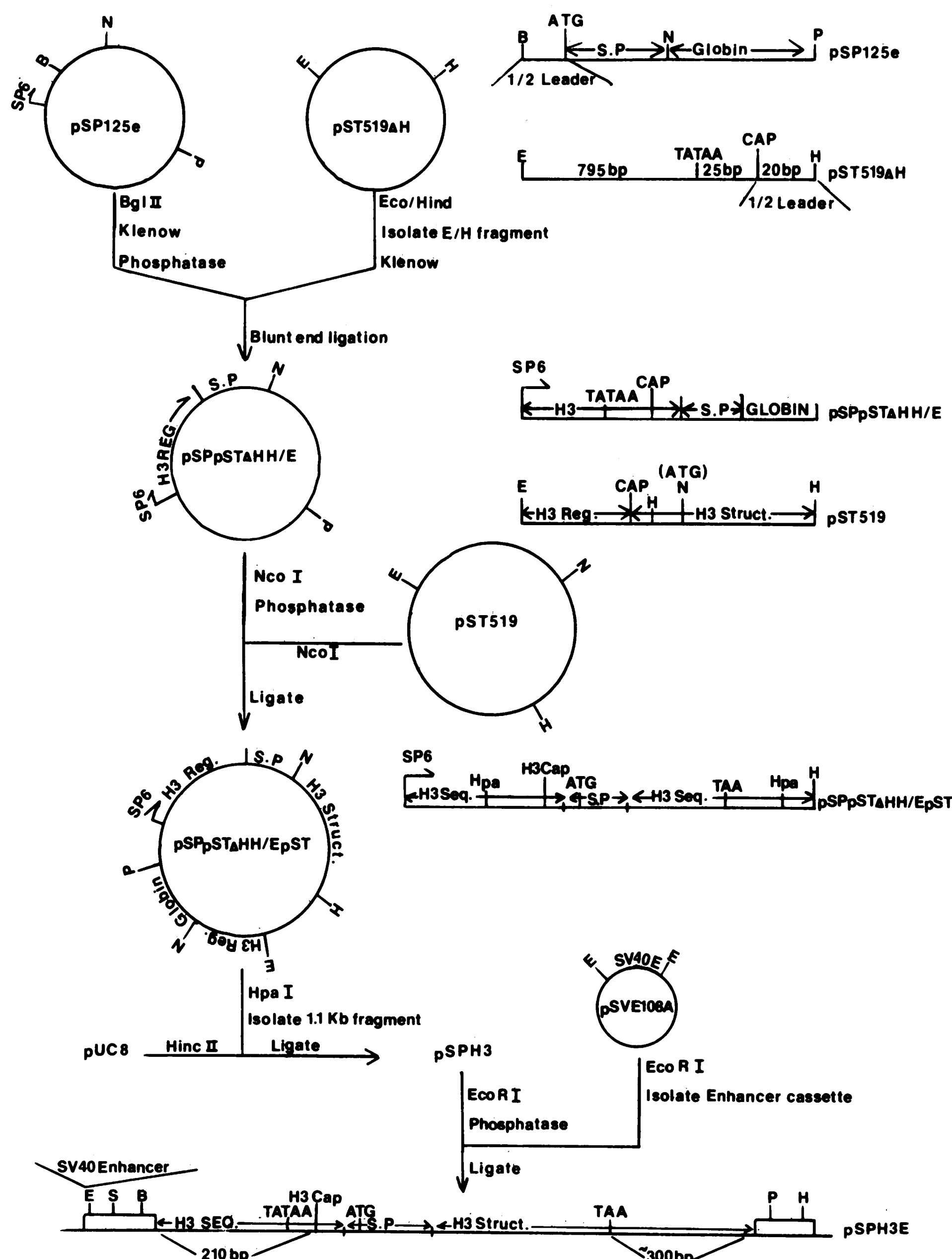

FIG. 1. Outline of the cloning scheme for the construction of the signal peptide–histone fusion gene. Restriction endonuclease sites are as follows: B, *Bgl* II; E, *Eco*RI; H, *Hind*III; N, *Nco* I; P, *Pst* I; S, *Sma* I. S.P, *E. coli* pBR322 β-lactamase signal peptide coding sequences; H3REG, transcriptional H3 histone regulatory region including CCAAT and TATAA consensus sequences; CAP, histone H3 mRNA transcription start site; H3 struct., sequences coding for H3 histone protein; ATG, translation start codon; TAA, translation stop codon; SP6, bacteriophage SP6 promoter; Kb, kilobases.

quantitated by an S1 nuclease protection assay. The 5′ radiolabeled 450-bp *Sma* I fragment from pSPH3E1 was hybridized at 55°C in 80% formamide/400 mM NaCl/40 mM Pipes, pH 6.4/1 mM EDTA to total cellular RNA isolated from the transfected HeLa cells. The hybridized nucleic acids were then treated with 2 units of S1 nuclease per μl and electrophoresed through 6% acrylamide/8.3 M urea. The denaturing gel was dried at 80°C under vacuum and exposed to preflashed XAR-5 Kodak film.

RESULTS AND DISCUSSION

To begin addressing experimentally the contribution of subcellular location to histone mRNA stability, we have constructed a chimeric gene encoding a cell cycle-dependent human H3 histone protein, which additionally contains sequences coding for the *E. coli* pBR322 plasmid β-lactamase signal peptide. The rationale for this approach was to introduce into HeLa cells a gene encoding an H3 histone mRNA that can associate with membrane-bound polysomes rather than free polysomes and thereby to determine whether the presence of histone mRNA on nonmembrane bound polysomes is requisite for the coupling of histone mRNA stability with ongoing DNA synthesis. Construction of the fusion gene is schematically outlined in Fig. 1. The upstream flanking region of the chimeric gene contains 210 bp of the 5′ regulatory sequences of the cell cycle-dependent human H3 histone gene pST519 including the TATAA and CCAAT consensus sequences. This region is followed by the H3 mRNA cap site and sequences encoding the initial 20 nucleotides of the nontranslated H3 histone mRNA leader. The fusion gene is, therefore, under transcriptional control of the H3 histone gene promoter, and a segment of the H3 leader that has been implicated in the coupling of histone mRNA levels to DNA synthesis is present (25). The H3 histone leader segment is fused to the untranslated leader sequences of the β-lactamase gene. The sequences coding for the entire β-lactamase signal peptide including the ATG translation start codon are present. The H3 histone structural gene is fused in frame to the signal peptide coding sequences and extends ≈300 bp beyond the TAA translation stop codon, including a region that has also been implicated in histone mRNA destabilization when DNA synthesis is inhibited (30). A SV40 enhancer element was incorporated into the upstream *Eco*RI site in the recombinant plasmid to increase cellular levels of the fusion transcript. The mRNA encoded by the fusion gene consists of the first 20 nucleotides of the H3 histone 5′ leader followed by the untranslated leader of the β-lactamase signal peptide, the entire β-lactamase signal peptide coding sequences (including the translation start codon), the entire H3 histone coding region fused in frame to the signal peptide sequences, and the H3 histone 3′ untranslated region (Fig. 2). The junction between the signal peptide coding region and the H3 histone coding region has been sequenced by Sanger's dideoxy method and the reading frame has been conserved (unpublished results). All sequences required for synthesis and processing of the chimeric RNA, as well as for translation of the fusion protein, are present. The signal peptide–H3 histone fusion gene with a single SV40 enhancer element is designated pSPH3E1 and an identical fusion construct containing multiple SV40 enhancer elements in the *Eco*RI site is designated pSPH3E2.

To test for expression of the signal peptide–histone fusion gene in human cells, pSPH3E1 and pSPH3E2 DNA were transfected into HeLa cell monolayers (28, 29). Forty-six hours after transfection, cells were harvested and total cellular RNA was isolated (16). The RNA was subjected to S1 nuclease analysis by a modification of the method of Berk and Sharp (31, 32). The probe used in the S1 nuclease assays was the *Sma* I fragment from pSPH3E1, which was radiolabeled at its 5′ termini (with [^{32}P]ATP by polynucleotide kinase). The probe is complementary to endogenous HeLa histone H3 mRNA from the 5′ end-labeled *Sma* I site within the protein coding region to the signal peptide–histone fusion junction and therefore protects a 130-nucleotide region of the H3 mRNA from S1 nuclease digestion. As seen in Fig. 3, when total cellular RNA from two independent transfections of HeLa cells with pSPH3E1 or pSPH3E2 was analyzed by S1 nuclease digestion, a 130-nucleotide fragment corresponding to endogenous H3 histone mRNA was protected as well as an ≈280-nucleotide fragment corresponding to the signal peptide–histone fusion mRNA species (lanes 3–6). In HeLa cells transfected with salmon sperm DNA, only the 130-nucleotide fragment was detected (lanes 1 and 2). These data demonstrate that the signal peptide–histone fusion gene is capable of expression in HeLa cells and that the mRNA transcribed from this gene is sufficiently stable to be detected in a short-term transient transfection assay.

To determine the class of polysomes with which the signal peptide–histone fusion mRNAs are associated, HeLa cells were transfected with pSPH3E1 and 46 hr later free and membrane-bound polysomes were isolated as described (23). The RNAs were isolated from both classes of polysomes and analyzed by the S1 nuclease protection assay. The *Sma* I fragment of the signal peptide–histone fusion gene (^{32}P, 5′ labeled) was used as the probe. As seen in Fig. 4, ≈30% of the signal peptide–histone fusion mRNA was found in the free polysome fraction and ≈70% was associated with the membrane-bound polysomes (lanes 1 and 2, respectively). This is in contrast to endogenous H3 histone mRNA, which is represented by >90% in the free polysome fraction and <10% in the membrane-bound polysome fraction. The percentage distribution of the histone chimeric mRNA within the polysomal RNA fractions was determined by microdensi-

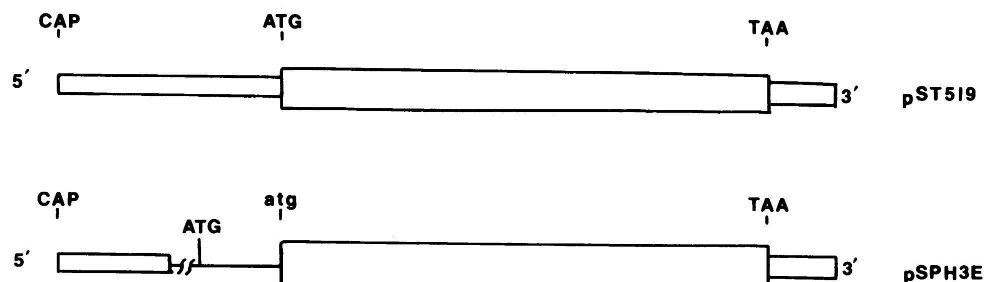

FIG. 2. Diagram of wild-type H3 histone mRNA (pST519) and the β-lactamase signal peptide–H3 histone fusion mRNA. The signal peptide–histone fusion mRNA is essentially identical to the wild-type histone mRNA with the exceptions of the deletion of nucleotides 20–40 of the histone leader and the signal peptide-encoded sequences that have been inserted in frame into the histone gene. The 5′ mRNA sequences (25) and the 3′ mRNA sequences (30) that have been implicated in coupling histone mRNA stability to DNA synthesis are retained in place in the signal peptide–histone fusion mRNA. Boxed areas represent histone H3 mRNA sequences; single-lined areas represent β-lactamase signal peptide-derived sequences.

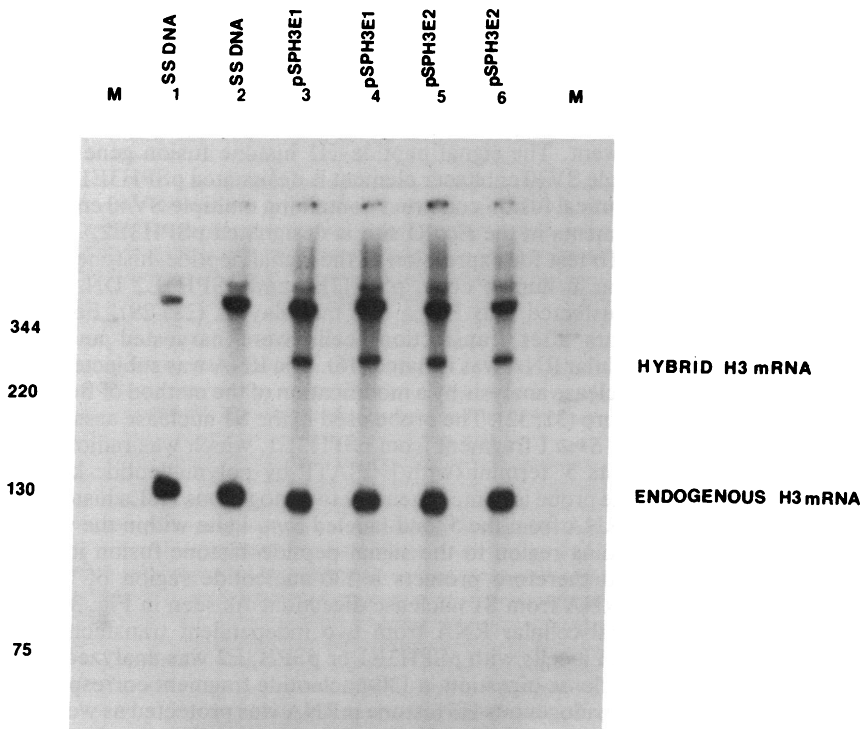

FIG. 3. Expression of the *E. coli* pBR322 β-lactamase signal peptide–H3 histone fusion gene in HeLa cells. The expression of the signal peptide–histone fusion gene was tested in a short-term transient assay and analyzed by an S1 nuclease protection assay. HeLa cell monolayers were transfected with 20 μg of DNA, according to Gorman *et al.* (28), in a calcium phosphate/DNA complex prepared as described by Graham and van der Eb (29). The transfected cells were incubated at 37°C, 5% CO_2 in air, for 46 hr following transfection. The cells were then harvested and total cellular RNA was isolated as described by Plumb *et al.* (16). The 5′ radiolabeled 450-bp *Sma* I fragment from pSPH3E1 was hybridized at 55°C in 80% formamide/400 mM NaCl/40 mM Pipes, pH 6.4/1 mM EDTA to 200 μg of total cellular RNA isolated from the transfected cells. The hybridized nucleic acids were then treated with 2 units of S1 nuclease per μl and electrophoresed through 6% acrylamide/8.3 M urea. The denaturing gel was dried at 80°C under vacuum and exposed to preflashed Kodak XAR-5 film. Lanes 1 and 2, HeLa cells transfected with salmon sperm DNA; lanes 3 and 4, HeLa cells transfected with pSPH3E1; and lanes 5 and 6, HeLa cells transfected with pSPH3E2. Marker lanes (M) are radiolabeled *Hin*fI digests of pBR322 DNA. Each lane represents RNA isolated from an independently transfected cell culture. Values on the left are given in nucleotides.

tometric analysis of the autoradiograms and the values were corrected for total yield of RNA in each subcellular fraction. Though the extent to which the signal peptide–histone fusion mRNA is represented on the membrane-bound polysomes varied from experiment to experiment, we always observed 60–90% of the fusion mRNA in the membrane-bound polysome fraction (n = 6).

We have demonstrated here that addition of a signal peptide coding sequence to an mRNA that is normally translated on free polysomes results in the targeting of the chimeric message to membrane-bound polysomes in intact mammalian cells. These results suggest that the prokaryotic β-lactamase signal peptide is recognized in a eukaryotic cell and that it is sufficient to target histone mRNA to membrane-bound polysomes.

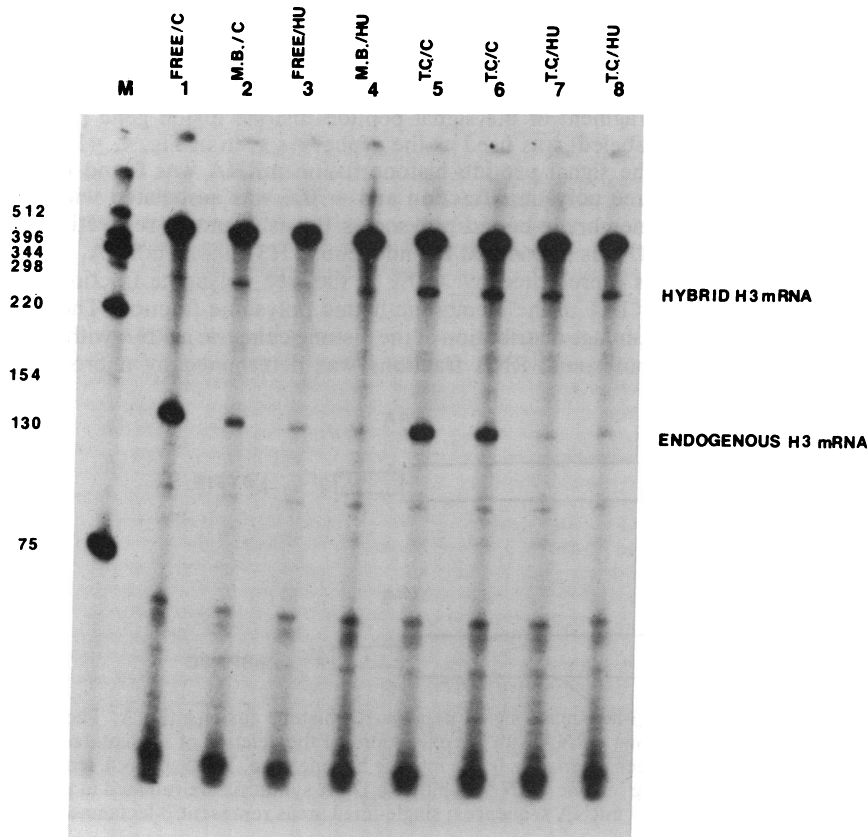

FIG. 4. Stability of the signal peptide–histone fusion mRNA following inhibition of DNA synthesis. HeLa cell monolayers were transfected with pSPH3E1 and cultured as described in the legend to Fig. 2. Forty-six hours after transfection, half of the transfected HeLa cell cultures were treated with 1 mM hydroxyurea in Eagle's minimal essential medium containing 10% fetal calf serum for 1 hr at 37°C, 5% CO_2 in air. Cell cultures were harvested, and free and membrane-bound polysomes were isolated (23). Total cellular RNA from control and hydroxyurea-treated samples was also isolated. The RNA was analyzed by the S1 nuclease protection assay as described in the legend to Fig. 3 using 5 μg of RNA per sample. The distribution of mRNA species within the subcellular fractions was quantitated by densitometric analysis of the autoradiograms. Lane 1, free polysomes from untreated cells; lane 2, membrane-bound polysomes from untreated cells; lane 3, free polysomes from hydroxyurea-treated cells; lane 4, membrane-bound polysomes from hydroxyurea-treated cells; lanes 5 and 6, total cellular RNA from untreated cells; and lanes 7 and 8, total cellular RNA from hydroxyurea-treated cells. Lanes 5–8 represent RNA isolated independently from transfected cell cultures. Marker lane (M) as in Fig. 3. Values on the left are given in nucleotides.

The ability to direct a cell cycle-dependent histone mRNA to membrane-bound polysomes provided the possibility to address the involvement of subcellular location in the coupling of histone mRNA stability with DNA replication. As seen in Fig. 4 (lanes 1–4), endogenous histone mRNA levels were reduced by >90%, as determined by densitometric analysis of the autoradiogram, in HeLa cells following inhibition of DNA replication by treatment with 1 mM hydroxyurea for 60 min. In contrast, inhibition of DNA replication by hydroxyurea treatment does not result in a reduction of fusion message levels on membrane-bound polysomes (lane 4). The finding that the signal peptide–histone fusion mRNA is stable following DNA synthesis inhibition is further supported by the S1 nuclease analysis of total cellular RNA (Fig. 4). The endogenous histone mRNA levels in hydroxyurea-treated HeLa cells were reduced by ≈95% compared with those in untreated HeLa cells (lanes 5–8) (with each lane representing an independent transfected cell culture). In contrast, only a 4% reduction in fusion mRNA levels was measured (lanes 5–8). These results demonstrate that the incorporation of the *E. coli* β-lactamase signal peptide into a human H3 histone gene is sufficient to target the encoded fusion mRNA to the membrane-bound polysomes and to confer stability to the fusion message when DNA synthesis is inhibited.

The recognition of a prokaryotic signal peptide in a eukaryotic cell is supported by *in vitro* and *in vivo* studies (24, 33–35). *Saccharomyces cerevisiae* has been shown to express the *E. coli* pBR325 β-lactamase gene *in vivo* and to process the precursor protein to the enzymatically active, mature protein. The processing of the bacterial preprotein into the mature species has also been demonstrated *in vitro* using a crude yeast extract (36). In addition, it has been reported by Wiedmann *et al.* (37) that *E. coli* plasmid pBR322 β-lactamase mRNA that is synthesized and capped *in vitro*, when microinjected into *Xenopus* oocytes, can be translated into protein that is ultimately secreted from the cell. These results suggest a common mechanism of signal peptide recognition among prokaryotic and eukaryotic organisms.

Based on previous results, inclusion of the histone 5′ leader sequences (25) and the 3′ untranslated sequences (30) should permit the destabilization of the fusion mRNA when DNA synthesis is inhibited. Hence, the finding that the fusion mRNA is stable when DNA replication is inhibited may be functionally related to the change in the subcellular location of the mRNA. The presence of the fusion message on membrane-bound polysomes rather than on free polysomes, where histone mRNA normally resides, may separate the message from the factor(s) that is involved in the selective destabilization of histone mRNA that occurs when DNA synthesis is inhibited. However, we cannot eliminate the possibility that the presence of sequences coding for the β-lactamase signal peptide may contribute to stability properties of the fusion message. The ability to target an mRNA to an otherwise foreign region within the cell could offer the possibility to address the question of what role the subcellular location plays in the posttranscriptional regulation of gene expression. Studies are necessary to define further the role of the subcellular location of histone mRNA, as well as of other factors, in the posttranscriptional regulation of histone mRNA turnover.

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