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# Multiple Types of mRNA-Cytoskeleton Interactions

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Nearly all actively translated mRNAs are associated with the cytoskeleton in HeLa cells and the nature of this association is poorly understood. To gain insight into this association, we have examined and compared the cytoskeleton-mRNA interactions of a signal peptide-histone fusion mRNA (membrane-bound polysomal mRNA) to those of endogenous histone mRNA (nonmembrane-bound polysomal mRNA). We report here the detection of a cytoskeleton attachment site within the signal peptide-histone fusion mRNP/mRNA nucleotide sequence that is not present in wild-type histone mRNA or in HLA-B7 and chorionic gonadotropin-α membrane-bound polysomal mRNAs. These results support the possibility that there are multiple mechanisms for the attachment of specific classes of mRNAs to the cytoskeleton.

The cytoskeleton is a complex network composed of many protein structures including microtubules, microfilaments, and intermediate filaments [for review see 1]. The cytoskeletal scaffold is located throughout the cytoplasm of eukaryotic cells and participates in cell motility, cell shape, and the intracellular transport of macromolecules and organelles. In addition, several lines of evidence suggest that the cytoskeleton plays a role in the translation process. First, nearly all actively translated mRNAs are associated with the cytoskeleton in HeLa cells [2–4]. Second, in poliovirus- and adenovirus-infected cells the transition from host protein synthesis to viral protein synthesis coincides with the exchange of host mRNA with viral mRNA on the cytoskeleton [5,6]. Third, maternally inherited mRNAs become translated at the time they attach to the cytoskeletal structure in sea urchin oocytes [7]. Lastly, the dose-dependent release of mRNA from the cytoskeleton into the soluble phase by cytochalasin D (CD) in HeLa cells parallels the extent of inhibition of protein synthesis [4].

The nature of the attachment of polysomal mRNA to the cytoskeleton is not known. It appears to be independent of the ribosomes since fluoride, high salt, or heat shock treatment results in the partitioning of the ribosomal subunits into the soluble

phase while the mRNA is retained on the cytoskeleton [2,3,8]. The localization of a 5′ cap binding protein with the cytoskeleton suggests that the association of mRNA with the cytoskeleton can occur through the 5′ cap structure of the mRNA [9]. In an earlier study evidence was presented that suggested mRNAs are associated with the cytoskeleton through the 3′ poly-A tail [10]. The findings that poliovirus uncapped mRNAs and poly-A minus histone and reovirus mRNAs are associated with the cytoskeleton suggest that the association may also occur through an internal region of the mRNA or that it varies with respect to each individual mRNA species; however, no direct evidence for this mechanism has been previously described [11–13].

Penman and coworkers reported that the disruption of microfilaments with cytochalasin D releases poly-A+ RNA from the cytoskeleton into the soluble phase of the cytoplasm in a dose-dependent manner [4]. Subsequent studies on specific mRNAs demonstrated a heterogeneous pattern for the attachment of mRNAs to the cytoskeleton [14]. Nonmembrane-bound polysomal mRNAs, classically defined as "free" polysomal mRNAs and generally involved in the synthesis of intracellular proteins [for review see 15], are efficiently released from the cytoskeleton with cytochalasin D [14]. In contrast, membrane-bound polysomal mRNAs, the class of polysomes generally involved in the synthesis of exported proteins destined for the cell surface or secretion [for review see 15], remain attached to the cytoskeleton after cytochalasin D treatment [14]. Membrane-bound polysomes appear to be attached to the cytoskeleton through at least two distinct sites: a cytochalasin D–sensitive site (site I) and a puromycin-sensitive site (site II) [14]. Presumably the CD-sensitive site in membrane-bound polysomal mRNAs is similar or identical to the CD-sensitive site associated with nonmembrane-bound polysomal mRNAs. The puromycin-sensitive site appears to involve the association of the nascent polypeptide and/or ribosome with the remnant protein structure of the endoplasmic reticulum that survives the detergent extraction step during the isolation of the cytoskeleton [3]. To release membrane-bound polysomal mRNAs from the cytoskeleton, both attachment sites must be disrupted as seen during CD and puromycin co-treatment; either drug treatment alone fails to release membrane-bound polysomal mRNA [14].

Histone mRNAs are naturally localized on nonmembrane-bound polysomes that are associated with the cytoskeleton [13]. Previously we have described the construction of a signal peptide-histone fusion gene that functions in vivo and encodes histone mRNAs that are targeted to membrane-bound polysomes [16]. To investigate further the mechanism of mRNA attachment to the cytoskeleton, we have studied and compared the cytoskeletal association of endogenous histone mRNA (nonmembrane-bound polysomal RNA) with that of signal peptide-histone chimeric mRNA (membrane-bound polysomal RNA). We report here the detection of a cytochalasin D– and puromycin-insensitive cytoskeletal attachment site which is present in signal peptide-histone fusion mRNA but not in endogenous histone mRNA or HLA-B7 and chorionic gonadotropin-α membrane-bound polysomal mRNAs. These results suggest that cytoskeleton attachment sites can be present in an internal region of the mRNA/mRNP nucleotide sequence. In addition, these results support the possibility that there are multiple mechanisms for the attachment of specific classes of mRNAs with the cytoskeleton.

## MATERIALS AND METHODS

### Materials

[$\alpha$-$^{32}$P]dCTP (3,000 Ci/mmol) and [$\gamma$-$^{32}$P] ATP (3,000 Ci/mmol) were purchased from Amersham; ultrapure electrophoresis-grade agarose and Zeta-probe nylon membranes were from Bio Rad; formamide was from BRL; dimethyl sulfoxide and formaldehyde solution (37% w/w) were from Fisher; cytochalasin D (CD), puromycin (Puro), polyoxyethylene sorbitan monopalmitate (Tween 40), sodium deoxycholate (NaDOC), sodium dodecyl sulfate (SDS), Triton X-100, and DNase I were purchased from Sigma; Geneticin (G-418 sulfate), calf serum, horse serum, Eagle's-Modified Minimum Essential Medium (EMEM), and Joklik-modified Minimum Essential Medium (SMEM) were obtained from Gibco.

### Plasmid DNA

The isolation of the cell-cycle-dependent human H3 histone genomic clone ST519 was previously reported [17]. The cDNA clone for the class I histocompatibility antigen HLA-B7 (pDP001) was a kind gift from Dr. Sherman Weissman (Yale University, New Haven, CT) [18]. The cDNA clone for human chorionic gonadotropin-$\alpha$ (phCG$\alpha$) was generously supplied by Dr. John Nilson (Case Western Reserve University, Cleveland, OH) and Dr. John Fiddes (Cal. Bio. Inc., Mountain View, CA) [19]. The signal peptide-histone chimeric gene (SPH3E1) was constructed by fusing the nucleotide sequences coding for the $\beta$-lactamase signal peptide from the *E. coli* plasmid pBR322 into ST519 as previously described [16]. The mutated signal peptide-histone fusion gene (SPH3ATG$^-$) was constructed by changing the ATG translation start codon of SPH3 to TTG according to the site-directed mutagenesis procedure of Zoller and Smith [20].

### Isolation of Cell Lines Expressing SPH3 and SPH3ATG$^-$

HeLa cells grown in suspension culture were seeded at $3 \times 10^6$ cells per 10 cm culture dish in completed EMEM (EMEM supplemented with 5% fetal calf serum, 5% horse serum, 1 mM glutamine, 100 $\mu$g/ml streptomycin, and 100 U/ml penicillin) and incubated overnight at 37°C under 5% $CO_2$. The monolayer cultures were then co-transfected with pSV2neo DNA, which confers resistance to the antibiotic G-418 sulfate, and with SPH3 DNA or SPH3ATG$^-$ DNA (20:1 molar ratio of fusion DNA to pSV2neo DNA) in a calcium phosphate/DNA complex prepared as described by Graham and van der Eb [21]. The cells were glycerol shocked, and 48 h later were seeded into completed EMEM containing 500 $\mu$g/ml active G418 sulfate. The cells were cultured under these conditions for 2–3 weeks, at which time individual colonies that were resistant to G418 sulfate were detected. The resistant colonies were either collected together as a heterogeneous population, referred to as a polyclonal cell culture, or isolated individually as monoclonal cell lines. The cell cultures were screened for expression of the signal peptide-histone fusion genes by S1 nuclease protection analysis (see below). Cell cultures positive for expression of the signal peptide-histone fusion genes were subsequently propagated as suspension cell cultures in completed SMEM (SMEM containing 5% fetal calf serum, 5% horse serum, 100 $\mu$g/ml streptomycin, 100 U/ml penicillin, and 1 mM glutamine) at 3–6 $\times$ $10^5$ cells/ml.

### Isolation of Cytoskeleton and Soluble-Phase RNA

Exponentially growing HeLa suspension cell cultures were collected by centrifugation and resuspended at $1 \times 10^6$ cells/ml in completed SMEM. Cells were then incubated for 20 min at 37°C with 10 μg/ml CD in DMSO, 0.4 mM Puro in DMSO, 10 μg/ml CD and 0.4 mM Puro, or DMSO alone. The cells were then collected by centrifugation, washed in cold phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM sodium phosphate, pH 6.8), and resuspended in extraction buffer (10 mM Pipes pH 6.8, 100 mM KCl, 2.5 mM $MgCl_2$, 0.3 M sucrose). Triton X-100 was added to a final concentration of 0.5% and the mixture was incubated on ice for 3 min. The extract was centrifuged at 1,500 rpm in an IEC centrifuge at 4°C for 3 min. The supernatant (SOL) was removed and stored at 0°C. The pellet was resuspended in RSB buffer (10 mM Tris–HCl pH 7.4, 10 mM NaCl, 2.5 mM $MgCl_2$) and adjusted to 1% Tween 40 and 0.5% NaDOC. The cytoskeleton was stripped from the nuclei with 15 strokes in a stainless-steel homogenizer with a clearance of 0.002 inch. The nuclei were pelleted by centrifugation at 2,000 rpm for 3 min at 4°C. The supernatant (CSK) was removed and saved for subsequent RNA isolation. Both the CSK and SOL fractions were extracted with phenol/chloroform in the presence of 1% SDS and 0.3 M NaCl. Nucleic acids were precipitated from the aqueous phase with 2.5 volumes of ethanol and 53 mM potassium acetate at −20°C.

Nucleic acids were recovered from the CSK and SOL fractions by centrifugation and resuspended in TCM buffer (10 mM Tris–HCl pH 7.4, 2 mM $CaCl_2$, 10 mM $MgCl_2$). The samples were then digested for 20 min at 37°C with 0.1 mg/ml DNase I that had been previously treated with proteinase K to remove RNase activity [22]. The digested samples were extracted with phenol/chloroform as described above and precipitated in 2.5 volumes of 95% ethanol and 0.25 M sodium acetate at −20°C. The RNA was pelleted by centrifugation, resuspended in double-distilled water, and then quantitated by absorbance at $\lambda_{260/280}$. The quantitation and intactness of the RNA were examined by agarose-formaldehyde gel electrophoresis.

### S1 Nuclease Protection Analysis

Cytoskeleton and soluble fractions were subjected to S1 nuclease protection analysis as described by Berk and Sharp [23]. The S1 probes for analysis of RNAs from SPH3E1- and SPH3ATG-expressing cell lines were the 5′ radiolabeled Sma I fragments from the respective plasmid DNAs. These probes simultaneously detect endogenous H3 histone mRNA (150 nucleotides) and their respective signal peptide-histone fusion mRNAs (280 nucleotides). The RNA (25 μg) was hybridized with the radiolabeled probe in 1× hybridization buffer (0.04 M Pipes pH 6.4, 0.4 M NaCl, 1 mM EDTA, 80% formamide) at 56°C for 3 h. The samples were adjusted to 0.03 M sodium acetate pH 4.6, 0.25 M NaCl, and 1 mM $ZnSO_4$ and digested with 900 units of S1 nuclease at 37°C for 30 min. The S1-digested samples were extracted with phenol/chloroform and precipitated with 2.5 volumes of 95% ethanol at −20°C overnight.

The S1-digested samples were electrophoresed through 6% (w/v) acrylamide–8.3 M urea gels, which were then dried and exposed to pre-flashed XAR5 Kodak film. The distribution of endogenous histone and fusion mRNAs within each fraction was determined by scanning laser densitometric analysis of multiple exposures of autoradiographs in the linear range of the film.

### Northern Blot Analysis

Cytoskeleton and soluble RNAs (10 μg/sample) were resolved electrophoretically through 1.5% (w/v) agarose–6% (w/v) formaldehyde gels prepared in MOPS buffer (20 mM MOPS (3-[N-morpholino]propanesulfonic acid) pH 7.0, 5 mM sodium acetate, 1 mM EDTA, 3.7% (w/v) formaldehyde). The RNAs were transferred to Zeta-probe nylon membranes in 20× SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0), and the filters were baked in vacuo at 80°C for 1 h. The filters were prehybridized for 3 h at 43°C in hybridization buffer (50% (w/v) formamide, 5× SSC, 5× Denhardt's (100× Denhardt's is 2% (w/v) polyvinylpyrrolidone, 2% (w/v) Ficoll), 20 μg/ml bovine serum albumin, 50 mM sodium phosphate, pH 7.0, 1% sodium dodecyl sulfate, and 200 μg/ml *E. coli* DNA). Hybridization was for 36–48 h at 43°C with $1 \times 10^6$ cpm/ml of thermally denatured radiolabeled cDNA inserts coding for human chorionic gonadotropin-α and HLA-B7 class I histocompatibility antigen. The filters were washed at 60°C with the following solutions: 1) 5× SSC, 1× Denhardt's; 2) 2× SSC, 0.1% (w/v) SDS; 3) 1× SSC, 0.1% SDS; 4) 0.1× SSC, 0.1% SDS. The filters were dried briefly and exposed to pre-flashed Kodak XAR5 x-ray film at −70°C for varying lengths of time; hybridization was quantitated by scanning laser densitometric analysis of autoradiograms in the linear range of the film.

### Analysis of the Densitometric Results

The distribution of mRNA species between the cytoskeleton and soluble fractions in Figures 2 and 3 was examined by using equal quantities of RNA in each sample. This method of analysis was chosen, rather than using equal volumes of RNA, to demonstrate that the differential attachment of specific mRNAs to the cytoskeleton was a result of the unequal distribution of the mRNA within these fractions and not a consequence of inefficient RNA recovery or loading of RNA onto the S1 or Northern gels. This approach, however, does not directly take into account the unequal distribution of RNA within the control samples or the changes in RNA distribution that occur during CD and/or puromycin treatment. Therefore, the densitometric units need to be adjusted for the yield of RNA that is contained within each of the subcellular fractions. The method for adjusting the densitometric results to reflect the unequal distribution of RNA in the subcellular fractions was as follows:

*Representation of mRNA in Cytoskeleton Fraction*

(X Densitometric Units of Specific mRNA/μg RNA Csk Sample Analyzed in S1 or Northern Gel) (Total μg RNA Recovered in CSK Fraction) = A.

*Representation of mRNA in Soluble Fraction*

(Y Densitometric Units of Specific mRNA/μg RNA SOL Sample Analyzed in S1 or Northern Gel) (Total μg RNA Recovered in SOL Fraction) = B.

% Distribution of Specific mRNA in Cytoskeleton Fraction = (A/(A + B))(100).

## RESULTS AND DISCUSSION

We have recently observed a differential association of nonmembrane-bound and membrane-bound polysomal mRNAs with the cytoskeleton [14]. Nonmembrane-bound polysomes are released from the cytoskeleton into the soluble phase during cytochalasin D treatment, whereas membrane-bound polysomes remain associated with the cytoskeleton. To understand further the differential association of these classes of polysomes with the cytoskeleton, we have examined the cytoskeletal association of a genetically engineered signal peptide-histone fusion mRNA (SPH3E1). The signal peptide-histone fusion mRNA is targeted from nonmembrane-bound polysomes, the natural site of histone protein synthesis, to membrane-bound polysomes [16]. Localization of signal peptide histone fusion mRNA on membrane-bound polysomes and wild-type histone mRNA on nonmembrane-bound polysomes presents the opportunity to study the differential association of these classes of polysomes with the cytoskeleton while examining a specific mRNA species. Figure 1 schematically diagrams the primary structure of endogenous H3 histone mRNA and signal peptide-histone fusion mRNA.

To examine the association of SPH3E1 signal peptide-histone fusion mRNA with the cytoskeleton, we first prepared HeLa cell lines that express the histone fusion gene, as described in Materials and Methods. Cloned HeLa cell lines were treated with cytochalasin D (10 μg/ml), puromycin (0.4 mM), CD and puromycin, or DMSO alone and then fractionated into cytoskeleton and soluble phases as described by Penman and coworkers [2,3]. Subsequently, RNA from each fraction was isolated and

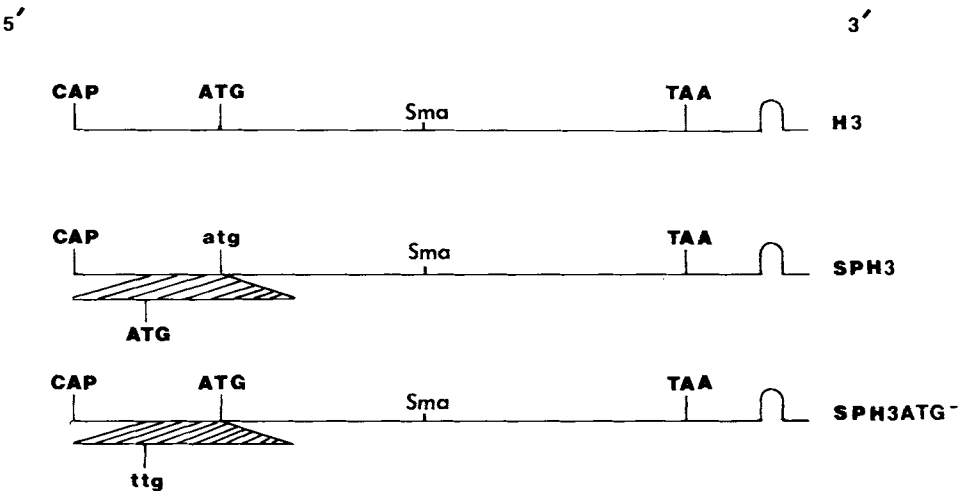


Fig. 1. Schematic diagram of endogenous H3 histone mRNA (H3), wild-type signal peptide-H3 histone fusion mRNA (SPH3E1), and mutated signal peptide-H3 histone mRNA (SPH3ATG$^-$). The endogenous histone mRNA represents transcripts from the cell-cycle-dependent human histone gene ST519 [17]. The construction of the signal peptide-histone fusion genes was previously described [16]. Orientation of mRNA is 5′ to 3′ from left to right. Abbreviations: CAP designates the transcription initiation site; hatched area refers to the nucleotide sequences encoding the signal peptide; ATG is translation start codon; atg depicted in SPH3E1 mRNA is translation start codon for histone coding region; ttg is mutated translation start codon of the signal peptide; Sma is Sma I restriction endonuclease site which is 150 nucleotides 3′ to histone ATG translation start codon; TAA is translation stop codon.

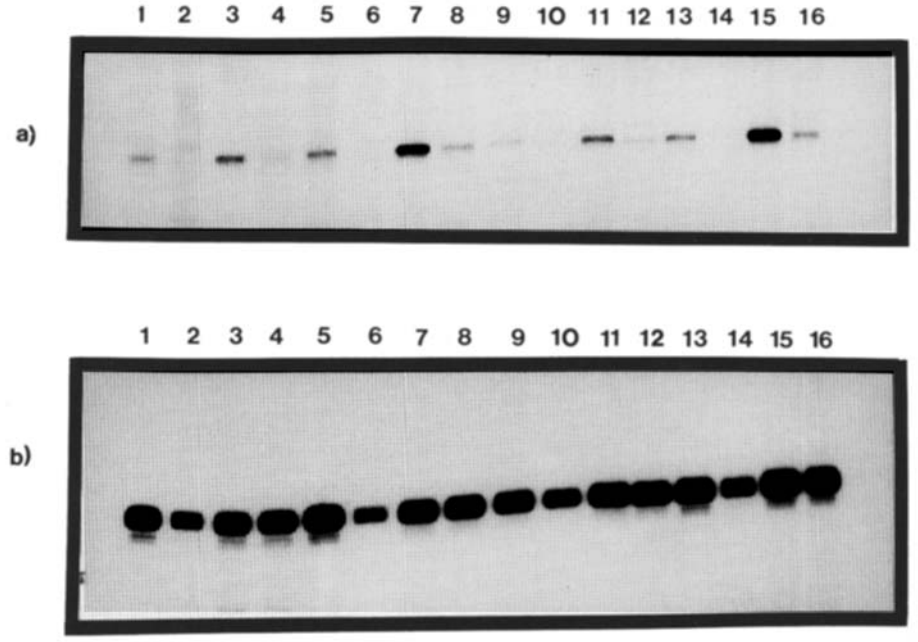


Fig. 2. Cytochalasin D and puromycin co-treatment does not release SPH3E1 or SPH3ATG$^-$ mRNA from the cytoskeleton. Monoclonal cell lines expressing either SPH3E1 or SPH3E1ATG$^-$ fusion genes were treated with cytochalasin D [10 μg/ml], puromycin [0.4 mM], or CD and puromycin for 20 min. Cytoskeleton and soluble-phase RNAs were isolated and subjected to S1 nuclease protection analysis as described in Materials and Methods. **a:** Represents the relative levels of signal peptide-histone fusion mRNAs. **b:** Represents the relative levels of endogenous H3 histone mRNA. **Lanes 1–8:** Represent RNA samples isolated from SPH3E1 expressing cells. **Lanes 9–16:** Represent RNA samples isolated from SPH3ATG$^-$ cell cultures. **Lanes 1, 9:** Control-CSK RNA. **Lanes 2, 10:** Control-SOL RNA. **Lanes 3, 11:** CD-CSK RNA. **Lanes 4, 12:** CD-SOL RNA. **Lanes 5, 13:** Puro-CSK RNA. **Lanes 6, 14:** Puro-SOL RNA. **Lanes 7, 15:** CD/puro-CSK RNA. **Lanes 8, 16:** CD/puro-SOL RNA.

SPH3E1 chimeric mRNA and endogenous H3 histone mRNA levels were determined by S1 nuclease protection analysis. As seen in Figure 2 and Table I, part B, both endogenous histone mRNA and SPH3E1 mRNA are predominantly associated with the cytoskeleton in control cells (92% and >90%, respectively). Cytochalasin D treatment (10 μg/ml) brought about only a limited release of SPH3E1 mRNA from the cytoskeleton, as expected for a membrane-bound polysomal mRNA. Only ~20% of SPH3E1 mRNA compared to ~55% of endogenous histone mRNA was dissociated from the cytoskeleton in CD-treated cells. As described above [14], dissociation of the ribosomes by puromycin during cytochalasin D treatment is necessary for the efficient release of membrane-bound polysomal mRNAs from the cytoskeleton. Surprisingly, CD and puromycin co-treatment failed to release the SPH3E1 mRNA from the cytoskeleton. Greater than 70% of the signal peptide-histone chimeric mRNA and less than 26% of endogenous H3 histone mRNA remained associated with the cytoskeleton after CD and puromycin co-treatment. It is important to note that elevated SPH3E1 mRNA levels were observed in the cytochalasin D–, puromycin-, and the CD-plus-puromycin-treated cells; these increased mRNA levels appear to result primarily from the stabilization of the signal peptide-histone fusion mRNA

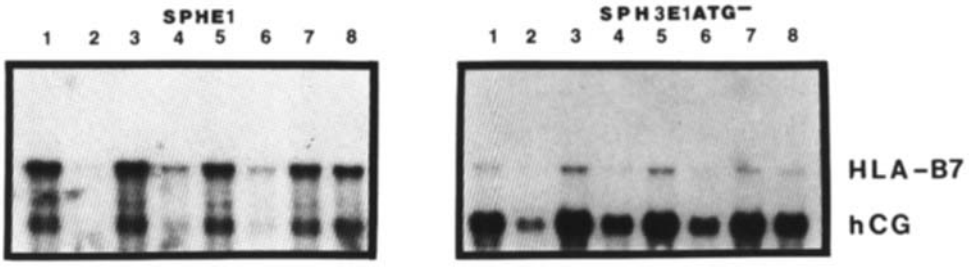


Fig. 3. Endogenous membrane-bound polysomal mRNAs are released from the cytoskeleton during co-treatment with CD and puromycin in SPH3E1 and SPH3ATG⁻ expressing monoclonal cell cultures. Cytoskeleton and soluble phase RNAs (10 μg/sample) were analyzed by Northern blot analysis as described in Materials and Methods. The Northern filters were hybridized with radiolabeled HLA-B7 and chorionic gonadotropin-α (hCGα) cDNAs. **Lane 1:** Control-CSK. **Lane 2:** Control-SOL. **Lane 3:** CD-CSK. **Lane 4:** CD-SOL. **Lane 5:** Puro-CSK. **Lane 6:** Puro-SOL. **Lane 7:** CD/puro-CSK. **Lane 8:** CD/puro-SOL.

during the inhibition of protein synthesis. Histone mRNA steady-state levels increase dramatically during inhibition of protein synthesis [for review see 24]. Furthermore, the increase in signal peptide-histone fusion mRNA levels in the cytoskeleton sample from cytochalasin D–and-puromycin-co-treated cells may also reflect the selective enrichment of the message in this subcellular fraction (Fig. 2).

The inability of CD and puromycin to release SPH3E1 chimeric mRNA efficiently from the cytoskeleton has also been observed in a polyclonal HeLa cell line (Table I, part A). In this case, less than 10% of the SPH3E1 mRNA and ~70% of the endogenous H3 histone mRNA is released from the cytoskeleton during cytochalasin D treatment. Furthermore, cytochalasin D and puromycin co-treatment of polyclonal cell cultures resulted in the release of ~38% of the SPH3E1 mRNA and more than 70% of the endogenous histone mRNA from the cytoskeleton.

To investigate whether the failure to release the signal peptide-histone mRNA from the cytoskeleton with cytochalasin D and puromycin was related to the efficiency of the drug treatments, we studied the association of other, endogenous membrane-bound polysomal mRNAs with the cytoskeleton. Class I histocompatibility antigen HLA-B7 mRNA and chorionic gonadotropin-α mRNA are translated on membrane-bound polysomes and are well represented in HeLa cells [13,14,25]. The distribution of HLA-B7 and chorionic gonadotropin-α mRNAs in the cytoskeleton and soluble RNA samples used in Figure 2 was determined by Northern blot analysis (Fig. 3; Table I, part B). As expected for membrane-bound polysomal mRNAs, HLA-B7 and chorionic gonadotropin-α mRNAs were not released by cytochalasin D treatment alone; over 80% of these mRNAs remained with the cytoskeleton after CD treatment. In contrast, less than 28% of the chorionic gonadotropin-α and HLA-B7 mRNAs remained associated with the cytoskeleton after CD-plus-puromycin treatment which indicates that the drug treatments were effective.

The association of the signal peptide-histone mRNA with the cytoskeleton in CD-and-puromycin-co-treated cells suggests the existence of a cytoskeleton attachment site that is distinct from the site(s) present in endogenous histone mRNA. These results indicate that the site is independent of the ribosomes and nascent polypeptide and is a property of the nucleotide sequence of the message and/or the proteins that interact with the mRNA. Alternatively, the SPH3E1 chimeric mRNA may be effi-

**TABLE I. The Percent of Cytoskeleton-Associated mRNAs Isolated From Cytochalasin D–Puromycin-, and CD/Puromycin-Treated Cells***

| RNA | Control, % | CD, % | Puro, % | CD/puro, % |
|---|---|---|---|---|
| A) SPH3E1 | >95 | 93 | — | 62 |
| Endogenous H3 | 81 | 31 | — | 27 |
| B) SPH3E1 | >90 | 79 | 94 | 72 |
| hCGα | 98 | 81 | 95 | 18 |
| HLA-B7 | 99 | 84 | 93 | 28 |
| Endogenous H3 | 92 | 46 | 90 | 26 |
| C) SPH3ATG− | >90 | 87 | >89 | 77 |
| hCGα | 98 | 81 | 89 | 29 |
| HLA-B7 | 97 | 89 | 88 | 35 |
| Endogenous H3 | 78 | 56 | 79 | 24 |

*The densitometric results from autoradiographs represented in Figures 2 and 3 were normalized for the total yield of RNA from each fraction as described in Materials and Methods. (Note: equal quantities of cytoskeleton and soluble RNAs were analyzed in S1 and Northern assays, which does not take into consideration the unequal distribution of RNA within these fractions or the changes that occur during CD and/or puromycin treatment.) A) Summary of results from polyclonal HeLa cell culture expressing the SPH3E1 gene (autoradiograph not shown). B) Summary of results from monoclonal HeLa cell line expressing the SPH3E1 gene (Figs. 2, 3). C) Summary of results from monoclonal HeLa cell line expressing the SPH3ATG$^-$ gene (Figs. 2, 3).

ciently translated and therefore support re-initiation of translation even in the presence of cytochalasin D and puromycin [note: endogenous histone mRNAs are efficiently translated, 26]. This would result in the synthesis of a portion of the signal peptide which could then serve as an anchor to the cytoskeleton as if the cells were treated with cytochalasin D alone.

To address this possibility, we have studied the cytoskeletal association of a mutated signal peptide-histone chimeric mRNA (SPH3ATG$^-$) that is essentially unchanged except that it does not encode a signal peptide. By using site-directed mutagenesis [20], the ATG translation start codon for the signal peptide of SPH3E1 was changed to TTG (Fig. 1). Translation should therefore bypass this altered start codon and initiate at the ATG codon in the histone coding region, resulting in the synthesis of a normal histone protein that does not contain a signal peptide. We have previously verified the mutation by Sanger's dideoxynucleotide sequencing protocol and demonstrated that SPH3E1ATG$^-$ mRNA is associated with nonmembrane-bound polysomes in HeLa cells to the same extent as endogenous H3 histone mRNA, which indicates that the signal peptide was not synthesized in vivo [27]. As calculated from the primary data presented in Figure 2, greater than 90% of SPH3ATG$^-$ mRNA is associated with the cytoskeleton in the control cell culture (Table I, part C). Cytochalasin D and puromycin co-treatment efficiently released chorionic gonadotropin-α and HLA-B7 mRNA from the cytoskeleton but had little or no effect on the association of the SPH3ATG$^-$ mRNA with the cytoskeleton (Figs. 2, 3; Table I). Approximately 77% of SPH3ATG$^-$ mRNA and less than 35% of HLA and hCGα mRNA remained attached to the cytoskeleton under these conditions. Regardless of whether SPH3ATG$^-$ mRNA could carry out limited protein synthesis in the presence of cytochalasin D and puromycin, no signal peptide would be synthesized to anchor the message to the cytoskeleton. This result further supports the proposal that the

nucleotide sequences coding for the signal peptide confer a cytoskeleton attachment site to the histone fusion mRNA which is distinct from the cytoskeleton attachment site(s) of endogenous histone mRNA.

It is not known whether the cytochalasin D– and puromycin-insensitive cytoskeleton attachment site present in SPH3E1 and SPH3ATG$^-$ mRNAs is due directly to the primary nucleotide sequences coding for the signal peptide. The additional site could be due to a change in the overall three-dimensional conformation of the histone fusion mRNA due to the nucleotide sequences coding for the signal peptide. Similarly, the nucleotide sequences encoding the signal peptide could alter the species of proteins associated with the histone fusion mRNP, which could in turn create the cytochalasin D– and puromycin-insensitive attachment site. It should be noted that this artificial fusion mRNA may recognize sites on the cytoskeleton that native mRNAs do not. Nevertheless, this result would still suggest a high degree of specificity in mRNA-cytoskeleton interactions.

The persistence of the association of the fusion message with the cytoskeleton during cytochalasin D treatment and during CD and puromycin co-treatment suggests that this site is independent of intact actin-containing microfilaments. However, we cannot rule out the possibility that a subset of actin filaments exists in a stabilized form during CD treatment and participates in mRNA binding. The signal peptide-histone chimeric mRNA attachment site (cytochalasin D and puromycin insensitive) does not appear to be associated with the microtubules since this cytoskeletal component is removed during the fractionation procedure [3]. The CD- and puromycin-insensitive site expressed by the signal peptide-histone fusion mRNA may be mediated by the intermediate filaments. Previous studies on other cell types have proposed that the association of mRNPs with the cytoskeleton is independent of the microfilaments and may involve the intermediate filaments [12,28]. Furthermore, Scherrer and co-workers have demonstrated by immunofluorescence studies that prosomes, a class of RNP complexes that are associated with translationally repressed mRNAs, are localized on the cytoskeleton in regions that are superimposable with cytokeratin-type intermediate filaments in HeLa cells [29]. It is possible that the nature of the interaction of prosomes with an intermediate filament network may be similar to the interaction of the signal peptide-histone fusion mRNA with the cytoskeleton. The role of the intermediate filaments in the attachment of the chimeric mRNAs to the cytoskeleton remains to be determined.

In summary, a cytoskeletal attachment site has been detected which is present in the signal peptide-histone chimeric mRNAs. This site appears to be independent of polysome structure and is either a direct or indirect result of the nucleotide sequences coding for the signal peptide. There are no known cytoskeleton attachment sites identified to date and the signal peptide-histone chimeric mRNAs, as well as other model fusion mRNAs, should prove useful in providing insight into the elements involved in the attachment of mRNAs to the cytoskeleton. The results presented here are consistent with the possibility that mRNAs are associated with the cytoskeleton in a heterogeneous manner.

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