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mRNA Association With the Cytoskeletal Framework Likely Represents a Physiological Binding Event

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Abstract A multitude of studies has indicated that the vast majority of mRNA and polyribosomes is associated with the detergent-resistant cytoskeletal framework (CSK). However, the nature and purpose of this association remain unclear. To begin unraveling the factors which may mediate this phenomenon, we examined the extent of association of four mRNAs (tubulin, vimentin, actin, and histone mRNA) with the CSKs of NIH 3T3 cells over a wide range of salt concentrations. Results indicate that the vast majority (> 90%) of each of these mRNAs remains associated with the CSK after detergent extraction of cells in low ionic strength buffer (25 mM NaCl). This association is manifest under conditions that cause the complete depolymerization of microtubules but that leave microfilaments and intermediate filaments intact. Even after extensive washing in buffer of approximately physiological ionic strength (150 mM NaCl), 75–85% of these mRNAs still remain associated with the CSK. However, at least 50% of each of these mRNAs can be eluted from the CSK by washing with buffer containing 250 mM NaCl. Not all the mRNAs, though, display the same elution profile. This suggests that different binding sites and/or different binding affinities may exist for different mRNAs. Surprisingly, close to 50% of the polyribosome population remains bound to the CSK despite washing in as much as 1.0 M NaCl. These adherent polyribosomes appear to be of the same size as those that are eluted, allaying the possibility that they are retained by the CSK simply due to size exclusion. Collectively, these data strongly imply that mRNAs are neither weakly adsorbed to the CSK nor physically trapped within the meshwork of cytoskeletal filaments. Instead, mRNA appears to be bound to the CSK via interactions that are, at least partially, ionic in nature and strong enough to exist intracellularly. Moreover, the binding sites for these mRNAs on the CSK are not associated with microtubules, but may be associated with either the microfilament or intermediate filament network.

Key words: polyribosomes, microfilaments, intermediate filaments, mRNA, cytoskeletal framework

Results from cell fractionation studies indicate that the majority of cellular mRNA and polyribosomes is not solubilized by mild extraction with non-ionic detergent, but remains attached to the detergent-resistant meshwork of cytoskeletal filaments and crosslinking proteins termed the cytoskeletal framework (CSK) [1–9]. This has led to the view that mRNA and polyribosomes may be functionally associated with cytoskeletal elements in vivo [11,12]. Specifically, it has been proposed that association of mRNA with the CSK is required for translation to occur [2]. As argued by Fey et al. [13], this may reflect the need to place mRNAs in close proximity to translational cofactors that are also associated with the CSK [7,14–17]. Association with the CSK may also serve to retain mRNAs in specific cytoplasmic locations [18], where their protein products can be effectively incorporated into region-specific organelles or other macromolecular structures [19–22].

However, there is continued speculation that retainment of mRNAs and ribosomes by the detergent-resistant CSK may not reflect a physiological binding event, but merely an artifact due either to weak electrostatic interaction or simple entrapment within the cytoskeletal web. Fueling this speculation is the fact that many of the extraction protocols performed have employed buffers of less than physiological ionic strength (e.g., 100 mM salt or less) in order to avoid salt-induced disassembly of the CSK [1,23]. Therefore, we have developed a procedure for "stabilizing" the CSK and have employed it to assess the extent of mRNA and ribosome association with the CSK over a wide range of salt concentrations. Specifically, we have chosen to analyze select mRNAs that have previously been observed to occupy distinct cytoplasmic locales

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[18,24,25], reasoning that these mRNAs are likely to be attached to structural elements in vivo. The questions we have sought to answer are the following: Do mRNAs and ribosomes remain associated with the CSK at near physiological ionic strength, or greater, thus ruling out the possibility of mere adventitious binding? Can mRNAs and ribosomes be effectively eluted from the CSK, thus demonstrating that trapping, alone, does not produce the observed polyribosome/CSK association? Which elements of the CSK are potential candidates for providing the binding sites for mRNAs and ribosomes?

Results indicate that association of mRNA with the CSK does not result solely from an electrostatic interaction that is too weak to function intracellularly, nor from physical entrapment within the cage of cytoplasmic filaments. Rather, this association appears to be mediated by mRNA/cytoskeletal interactions that are strong enough to operate in the intracellular environment, yet are differentially sensitive to fluctuations in the ionic milieu. In addition, mRNA is retained by the CSK under conditions that promote the disassembly of microtubules, but that leave microfilaments and intermediate filaments intact. Collectively, these findings suggest that mRNA is likely to be associated with the CSK in vivo, and that microfilaments or intermediate filaments may supply the binding sites for mRNA attachment.

EXPERIMENTAL PROCEDURES

Cell Culture

NIH 3T3 cells were obtained from the American Type Culture Collection (ATCC, Bethesda, MD) and were used throughout the course of the experiments described. The cells were grown in Dulbecco's Modified Eagles Medium (Gibco/BRL, Gaithersberg, MD), supplimented with 10% calf serum (Colorado Serum Co., Denver, CO), sodium pyruvate (0.11 mg/ml), and penecillin/streptomycin, at an atmosphere of 37°C and 5% CO_2, as described by the ATCC. For RNA isolation and Western blot analysis, 1×10^5 cells were plated into 60 mm tissue culture dishes and allowed to grow overnight. For immunofluorescence, 1×10^3 cells were plated onto acid-washed, autoclaved glass coverslips (22 mm^2) and used the next day.

Cellular Fractionation

Cells were separated into detergent-soluble and detergent-insoluble (CSK) fractions as previously described in detail [10]. Briefly, parallel dishes of cells were extracted for 90 seconds in 1.8 ml CSK buffer (10 mM piperizine-*N,N'*-bis(2-ethanesulfonic acid [PIPES]), pH 6.8; 300 mM sucrose; 25 mM NaCl; 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid [EGTA]; 5 mM $MgCl_2$) containing Triton X-100 (0.2% [w/v]), and RNAsin (50 U/ml) to inhibit RNase activity. Where indicated in the text, phalloidin (Sigma Chemical Co., St. Louis, MO) was also included in both the extraction and subsequent wash steps at a concentration of 10 μg/ml. Extraction was performed at 4°C on a rocking platform to ensure complete extraction of each monolayer. After extraction, the solubilized material was removed from each dish of cells, transferred to sterile 15 ml siliconized corex tubes, and placed on ice. The adherent monolayer of detergent-insoluble CSKs was then washed with 1.8 ml of CSK buffer containing phalloidin and NaCl at a concentration of 150 mM, 250 mM, 500 mM, 750 mM, or 1.0 M, for 5 minutes at 4°C with continuous rocking. For immunofluorescence and Western blot analysis, the salt-eluted material was removed and discarded, and the remaining CSK fraction was processed as described below. For RNA analysis, the salt-eluted material was removed from each dish, transferred to siliconized corex tubes, and placed on ice. The salt-washed CSKs were scraped into suspension with 1.8 ml of ice-cold RSB buffer (10 mM Tris-HCl, pH 7.4; 10 mM NaCl; 3 mM $MgCl_2$; 1% Tween 40; 0.5% deoxycholate). Extraction in this double-detergent buffer effectively solubilizes all RNA attached to the CSK [26]. Each CSK suspension was then withdrawn from its respective dish into a 3.0 ml syringe and passed through a 21 gauge needle 3–4 times to generate a homogeneous extract. CSK extracts were then transferred to sterile 2.0 ml siliconized Eppendorf tubes and spun for 1 minute at 15,000*g* (4°C) to pellet the insoluble nuclear matrices. The resulting supernatants were then transferred to sterile 15 ml autoclaved corex tubes and, along with the initial Triton X-100 extracts and salt elutions, were processed for total RNA content.

RNA Isolation

All extracts were diluted with an equal volume of 2X proteinase K buffer (0.2 M Tris-HCl, pH 7.5; 25 mM EDTA; 0.3 M NaCl; 2% SDS; 400 υg/ml proteinase K), adjusted to a common final concentration of 0.65 M NaCl, and placed at

37°C for 30 minutes. NaCl was used here instead of KCl [10] in order to avoid precipitation of the SDS during the proteinase K digestion. The use of one salt over the other did not affect retention of mRNA by the cytoskeletal framework (data not shown). After proteinase K digestion, all samples were phenol/chloroform extracted and then precipated with 2.5 volumes of ethanol at −20°C. Precipitated RNA was washed with 70% ethanol and then dessicated in a Speed Vac (Savant Instruments, Farmingdale, NY). The RNA was then resuspended in 80 υl of sterile water, dispensed into 8 × 10 υl aliquots, and stored at −80°C until used.

Northern Blot Analysis of RNA

A 10 υl aliquot of RNA from all three fractions (representing RNA solubilized by the initial Triton X-100 extraction, RNA displaced from the cytoskeletal framework by salt elution, and RNA that remains associated with the cytoskeletal framework) was used for Northern blotting. As total RNA from all three fractions was resuspended in the same volume (80 υl), this 10 υl aliquot consituted a cellular equivalent representing approximately 1/8 of the total RNA present in the respective fractions. The RNA was separated on 0.8% agarose gels containing 2.2 M formaldehyde, then transferred to nitrocellulose (Bio-Rad Laboratories, Rockville Centre, NY) according to Thomas [27]. Hybridization and washing conditions were as previously described [28]. ^{32}P-labelled probe was prepared by the random priming method of Shank et al. [29], with high specific activity [α-^{32}P]dCTP. The following mRNAs were detected by their respective probes: β-tubulin mRNA (probe constructed from the 1630 base pair Eco RI fragment of the mouse β-tubulin pmβ5 plasmid [30]); histone mRNA (probe derived from the 3.7 Kb Eco RI fragment of the plasmid pAAD.7, which contains the hamster H3.2 gene [31]); actin mRNA (probe constructed from 1.2 Kb Pst I fragment of plasmid pACT-1 [32] and which recognizes both β and γ-actin mRNAs); vimentin mRNA (probe constructed from the 1.2 Kb Eco RI fragment of the rat pVim plasmid [33]). For qualitative determinations of the relative distributions of these mRNAs in the different subcellular fractions, the nitrocellulose blots were exposed to Kodak XAR-5 X-ray film. For quantitative analysis of these distributions, nitrocellulose blots were directly scanned with a Betascope Model 603 Blot Analyzer (Betagen Corp., Waltham, MA). All signals from these blots were in the linear range of detection.

Immunofluorescence

For the analysis of cytoskeletal patterns in unextracted cells, cells were fixed in 4% paraformaldehyde in phosphate buffered saline (pH 7.4) and permeabilized by exposure to acetone for 15 minutes at 4°C. For the analysis of cytoskeletal networks in CSKs, extractions and salt washes were performed essentially as described above and the CSKs fixed in the same manner as unextracted cells. To observe the pattern of actin-containing microfilaments in both unextracted cells and CSKs, the cells (CSKs) were stained directly with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR) according to the protocol supplied by the manufacturer. To observe microfilaments in salt-washed CSKs, rhodamine-conjugated phalloidin was included in the extraction buffer (5 units/ml) in place of nonconjugated phalloidin. This served the dual purpose of both stabilizing the microfilaments against high salt-induced disassembly and allowing them to be visualized by fluorescence microscopy. During the salt washes, however, both rhodamine-conjugated phalloidin (5 units/ml) and nonconjugated phalloidin (1 μg/ml) were used. To visualize microtubules and vimentin-containing intermediate filaments, parallel coverslips of unextracted cells and CSKs (both nonsalt-washed and salt-washed) were first stained with either a monoclonal antibody to α-tubulin (Amersham, Arlington Heights, IL) or a monoclonal antibody to vimentin (Sigma), respectively. Next, cells (and CSKs) were incubated with fluorescein-conjugated goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA). Antibody incubation and washing conditions were performed as described previously [28]. Coverslips were mounted in Mowiol and viewed with a Nikon Optiphot microscope equipped with a 100 W mercury light source for epifluorescence illumination, Zeiss 63X 1.25 numerical aperture oil immersion objective, and Nikon B2 cube. Photomicrographs were recorded with a Nikon UFX-II camera on Kodak TMax film and developed at 3200 ASA.

Western Blot Analysis

Parallel dishes of cells were extracted in the presence of phalloidin (10 υg/ml), as described

above. The soluble material was removed and discarded, and the adherent CSKs were washed with CSK buffer containing phalloidin and 25 mM, 150 mM, 250 mM, 500 mM, 750 mM, or 1.0 M NaCl. The salt-eluted material was also removed and discarded, and the remaining CSKs were then solubilized in 200 μl of 8 M urea and stored at −80°C until used. Cellular equivalents (15 μl) of the solubilized CSKs were separated by SDS-PAGE and transferred to nitrocellulose by electroblotting as previously detailed [34]. Actin and vimentin protein were detected on parallel blots by incubation with monoclonal anti-actin antibody (Amersham) or monoclonal anti-vimentin antibody (Sigma), respectively. The blots were subsequently incubated with polyclonal goat anti-mouse IgG antibody conjugated to alkaline phosphatase (Promega, Madison, WI) and developed with BCIP/NBT following the protocol provided by Promega. Western blots were quantitated using a Visage Model 110 Image Analyzer (Bio Image, Ann Arbor, MI) and corresponding Whole-Band Analysis software in the reflectance mode. All values were in the linear range of standards.

Polyribosome Profile Analysis

Profiles were generated from salt-eluted polyribosomes and CSK-bound polyribosomes using the protocol previously described [28]. Prior to fractionation, cells were treated with 5 μg/ml cycloheximide to stabilize polyribosomes against high salt-induced disassembly. Cellular equivalents from each fraction were resolved on 15–40% sucrose gradients and analyzed with an ISCO Model 185 density gradient fractionator (ISCO, Inc., Lincoln, NE) connected to a Type 6 optical unit and UA5 absorbance monitor.

RESULTS

Phalloidin Stabilizes the CSK Against Salt-Induced Disassembly

In order to study the effect of different salt concentrations on mRNA binding to the CSK, a protocol first had to be developed to stabilize the microfilaments, which normally disassemble at around 100 mM NaCl [1,23]. This was accomplished by initially extracting the cells in a low ionic strength buffer containing phalloidin, which specifically binds to actin-containing microfilaments and prevents their disassembly [35]. To assess the effectiveness of this protocol, the integrity of the three major filament systems that comprise the CSK (i.e., microfilaments, intermediate filaments, and microtubules) was analyzed by immunofluorescence microscopy after low salt and high washes. Results are shown in Figure 1. It can be seen that both the actin-containing microfilament network and the vimentin-containing intermediate filament network remain intact after extraction/washing in buffer containing 25 mM NaCl, and mirror their respective distributions in unextracted cells. However, the microtubule network is nearly completely disassembled by this extraction protocol. This is most likely due to cold-induced microtubule depolymerization, as extractions performed at 37°C yield microtubule networks that are indistinguishable from those present in unextracted cells (data not shown). As the majority of mRNA remains associated with the cytoskeletal framework when extractions are performed at 4°C [10, and see below], these results indicate that microtubules are most likely not involved in the association of mRNA with the cytoskeletal framework.

Both the microfilament network and the vimentin-containing intermediate filament network remain intact even when the phalloidin-stabilized CSKs are washed with buffer containing 1.0 M NaCl (Fig. 1). Failure to include phalloidin in the extraction buffer, however, results in an almost complete loss of microfilaments when CSKs are washed with 1.0 M NaCl (data not shown). The vimentin-containing intermediate filaments, which normally remain assembled at high salt contrations [36,37], remain intact at 1.0 M NaCl whether or not phalloidin is included.

The relative amount of actin and vimentin protein that remains associated with the CSK after different salt washes was also assessed by immunoblotting. Figure 2 shows that when 3T3 cells are extracted in the presence of phalloidin, subsequent washing of the CSKs with buffer containing as high as 1.0 M NaCl does not cause a notable loss of either actin or vimentin protein. Collectively, these data demonstrate that both the microfilament and intermediate filament networks of phalloidin-stabilized CSKs do not suffer any qualitative or quantitative alterations as a result of exposure to high concentrations of salt. Hence, phalloidin-stabilized CSKs can be used as effective matrices from which to salt-elute mRNAs.

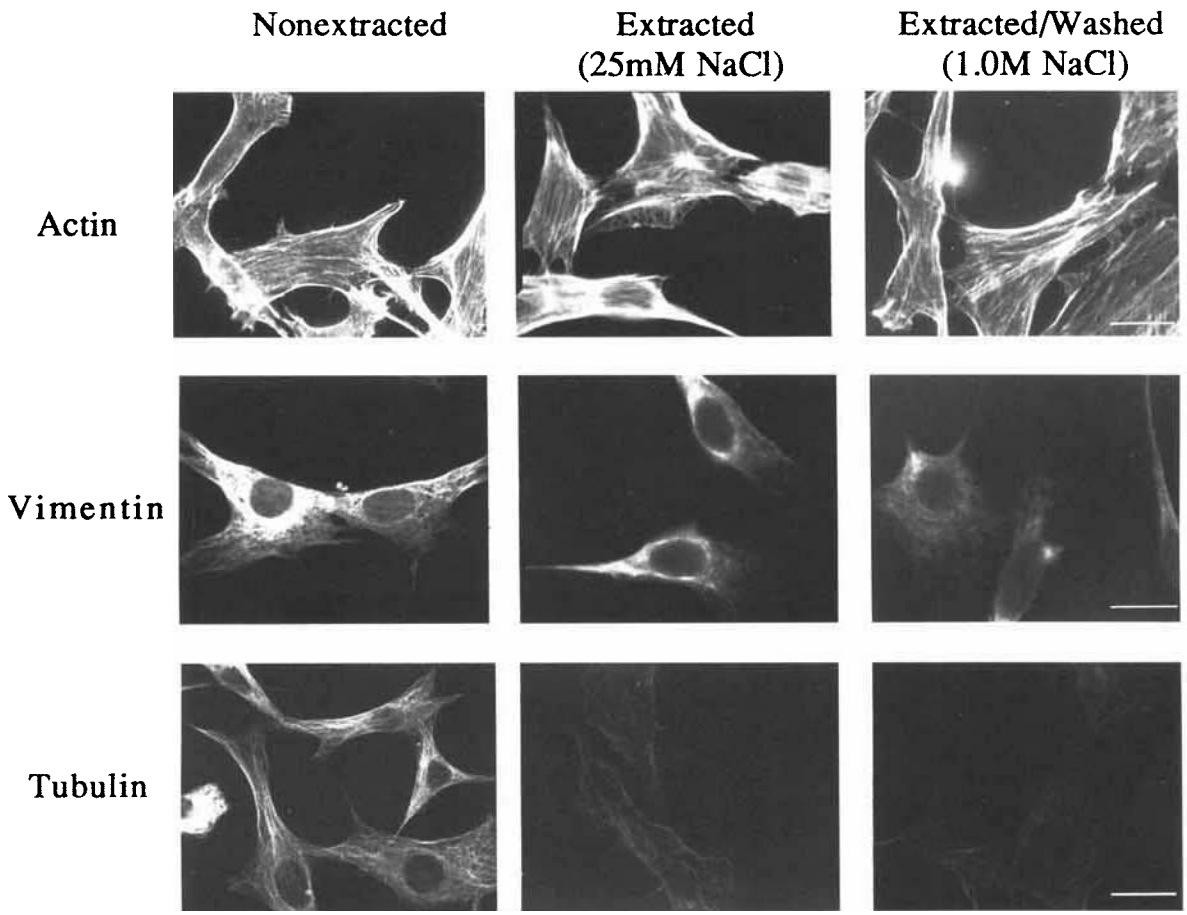

Fig. 1. Stabilization of the CSK against salt-induced disassembly. NIH 3T3 cells were either fixed directly without extraction, or extracted in the presence of phalloidin (10 ug/ml) and then washed with either 25 mM NaCl or 1.0 M NaCl and then fixed. Cells were stained for the presence of microfilaments vimentin-containing intermediate filaments or microtubules using antibodies to actin, vimentin, and tubulin, respectively. The staining protocols are described in Experimental Procedures. Bar = 20 um.

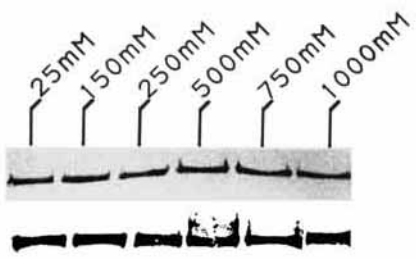

Fig. 2. Western blots of actin and vimentin in salt-washed, phalloidin-stabilized CSKs. Parallel dishes of cells were first extracted in the presence of phalloidin, and then each was washed with a buffer containing one of the indicated NaCl concentrations. Each dish of salt-washed CSKs was then solubilized in 8 M urea and cellular equivalents separated by SDS-PAGE and immunoblotted as described in Experimental Procedures.

Elution of mRNAs From Phalloidin-Stabilized CSKs

To assess the relative affinities of different mRNAs for the CSK, 3T3 cells were first detergent-extracted at low ionic strength in the presence of phalloidin. The resulting CSKs were then differentially eluted with buffers containing increasing concentrations of NaCl and the distributions of four different mRNAs were analyzed in the salt eluates and the corresponding CSK fractions. The mRNAs that were analyzed included tubulin mRNA, actin mRNA, vimentin mRNA, and histone mRNA. These particular mRNAs were chosen because all of them have been shown to display nonhomogeneous cytoplasmic distributions and, therefore, were considered likely to be associated with structural elements in vivo [18,24]. Northern blots of the distributions of these mRNAs are shown in Fig-

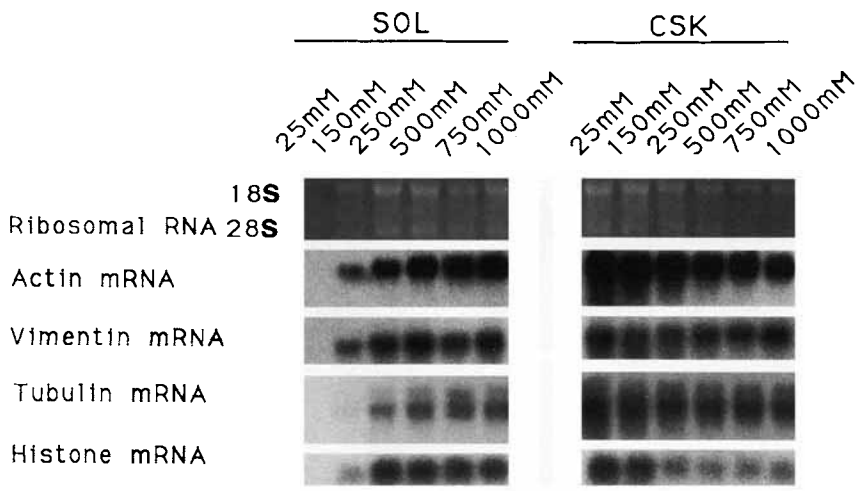

Fig. 3. Northern blot profiles of mRNAs eluted from phalloidin-stabilized CSKs. Parallel dishes of cells were first detergent-extracted in the presence of phalloidin and then washed with CSK buffer containing phalloidin plus one of the indicated concentrations of NaCl. Total RNA was isolated from detergent-soluble and salt-wash fractions, as well as from the corresponding CSKs. Cellular equivalents of RNA were separated by formaldehyde/agarose electrophoresis, blotted, and probed for the indicated mRNAs as detailed in Experimental Procedures.

ure 3, and the percent of each mRNA present in the salt eluates and corresponding CSK fractions is displayed graphically in Figure 4. It can be seen that the vast majority (> 90%) of each of these four mRNAs remains associated with the CSK fraction as a result of the initial detergent extraction (25 mM NaCl). Subsequent elution of the CSKs with 150 mM NaCl, which approximates the salt concentration found intracellularly, causes the release of an additional 15–20% of the total cellular content of each of these mRNAs, leaving approximately 75% still associated with the cytoskeletal framework. A more stringent elution of the CSKs with 250 mM NaCl, though, displaces about ⅓ of total tubulin, actin, and vimentin mRNA and approximately ⅔ of total histone mRNA. Further increases in the NaCl concentration of the elution buffer up to 1.0 M promotes only slightly greater removal of tubulin, actin, and vimentin mRNA, though it results in nearly 80% displacement of histone mRNA. Though only one set of data is shown here, three different analyses of these profiles (performed on replica plates) did not significantly deviate from each other.

The displacement of these mRNAs occurs in the absence of any obvious morphological alteration and without concomitant disassembly of either microfilaments or intermediate filaments (Figs. 1 and 2 show that, under the conditions employed, these filaments are stable in the presence of up to 1.0 M NaCl). That a large fraction of these mRNAs can be displaced from the CSK in a nondestructive manner strongly argues against their being simply physically trapped within the meshwork of cytoskeletal filaments. Furthermore, as the majority of each mRNA is displaced only at salt concentrations greater than that which normally exists intracellularly, this additionally suggests that the association of mRNA with the CSK is strong enough to operate in vivo and is likely to reflect a physiological binding event.

Polyribosome Profiles of Salt-Washed CSKs

Finally, polyribosome profile analysis was performed to determine if mRNAs retained by the CSK are of larger size than those displaced by salt elution. Results are presented in Figure 5. It can be seen that the profile of polyribosomes that remain associated with the CSK in the presence of 1.0 M salt is similar to that of the polyribosomes that are eluted. Since polyribosomes of different size show nearly the same efficiency of elution, this indicates that polyribosome size, alone, does not determine whether mRNAs remain associated with the CSK. It is unlikely, therefore, that mRNAs are retained by the CSK simply due to a sieving effect. Clearly, other factors must be operative in binding the mRNAs to the CSK.

DISCUSSION

We have presented three lines of evidence strongly suggesting that mRNA associated with

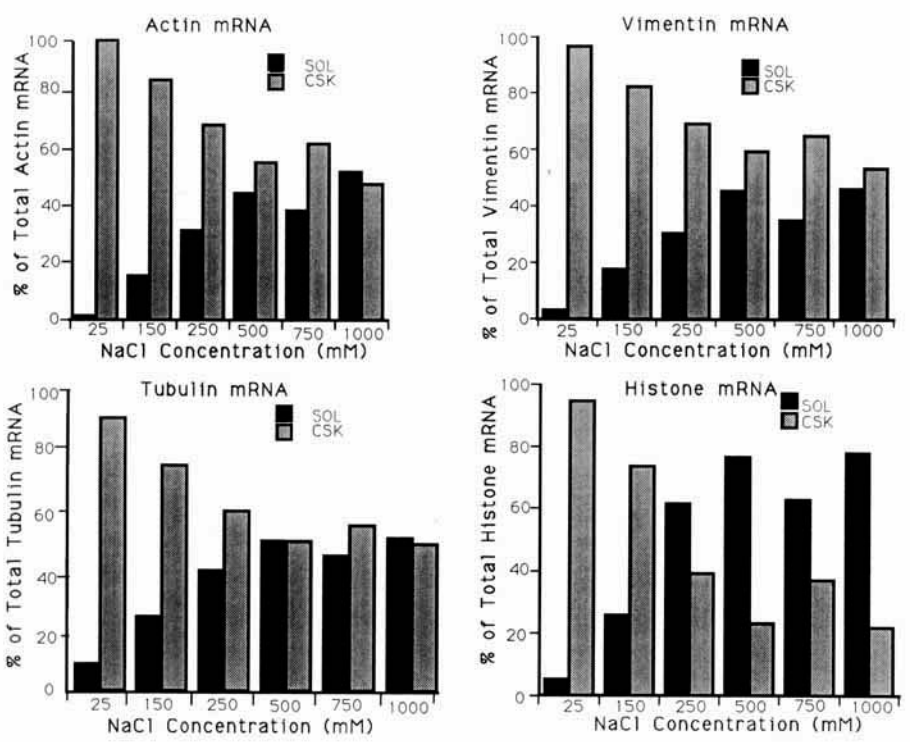

Fig. 4. Quantitative analysis of mRNAs eluted from phalloidin-stabilized CSKs. The absolute amount of radioactivity present in each lane of the Northern blots in Fig. 3 was directly assayed (in cpms) with a Betascope Blot Analyzer. The percentage of each $mRNA_{(actin,\ tubulin,\ vimentin\ or\ histone)}$ that either is displaced (SOL mRNA) or remains CSK-bound after each extraction/salt-wash combination is plotted versus the concentration of NaCl used in the wash, and was calculated in the following manner: First, the fraction of each mRNA that remains CSK-bound after the initial detergent extraction (25 mM NaCl) was quantitated according to the formula CSK-bound cpms/(SOL cpms + CSK-bound cpms) and termed $mRNA_{frac}$. Next, the radioactivity values from the SOL and CSK fractions for each extraction/salt-wash combination were summed for each mRNA (e.g., actin mRNA SOL $cpms_{(150\ mM\ NaCl)}$ + actin mRNA CSK $cpms_{(150\ mM\ NaCl)}$). These sums were then multiplied by $(1/mRNA_{frac})$ to generate the total cpms for each mRNA prior to extraction. The percentage of CSK-bound $mRNA_{(actin,\ tubulin,\ vimentin\ or\ histone)}$ remaining after each extraction/salt-wash combination was quantitated according to the formula CSK-bound cpms/total cpms. The percentage of SOL $mRNA_{(actin,\ tubulin,\ vimentin\ or\ histone)}$ that is eluted after each extraction/salt-wash combination was quantitated as 100% − (% CSK-bound mRNA).

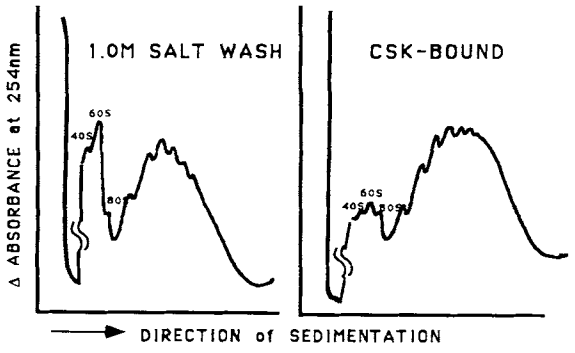

Fig. 5. Polyribosome profiles of salt-eluted and CSK-bound polyribosomes. Cellular equivalents of both SOL and CSK fractions of 1.0 M NaCl-washed CSKs were resolved on 15–40% sucrose gradients as described in Experimental Procedures.

the CSK is neither the result of a weak electrostatic interaction nor a simple trapping by cytoskeletal filaments. First, 75–85% of actin, tubulin, vimentin, and histone mRNAs was found to remain associated with the CSK despite extensive washing in buffer containing a physiological concentration of salt (150 mM NaCl). Second, washing of the CSK with up to 1.0 M NaCl removes about 50–60% of the tubulin, actin, and vimentin mRNAs and approximately 80% of the histone mRNA. Third, mRNA elution is accomplished without concomitant destruction of the filament systems that comprise the detergent-resistant CSK (i.e., microfilaments and intermediate filaments).

If mRNA association was simply due to adventitious binding, then it should not have survived the extensive salt washings that were performed

(e.g., nearly 50% of actin, tubulin, and vimentin mRNAs remain CSK-associated despite washing in 1.0 M NaCl). Indeed, such avid binding is characteristic of the association of mRNP proteins with mRNA [38]. On the other hand, if mRNA association with the CSK was due solely to trapping within the network of stabilized microfilaments and intermediate filaments, then mRNA should not have been released from the CSK without a concomitant disruption of these filament systems. This is particularly true for tubulin mRNA and histone mRNA, both of which are widely distributed throughout the cytoplasm of fibroblasts [18,24]. If these mRNAs were physically held in place by a web of crisscrossing filaments, it would have necessitated major cytoskeletal restructuring in order to dislodge them. As our results show that little qualitative or quantitative disruption of microfilaments and intermediate filaments occurs during mRNA elution, it is doubtful that these filaments physically restrain any of the mRNAs examined. Instead, it appears as if mRNAs are non-covalently attached to the CSK, either directly or indirectly, through interactions that are strong enough to exist at the average intracellular ionic strength, yet are sensitive to changes in the ionic environment. While these results do not prove that mRNA actually binds to the CSK in vivo, they do infer that it is a likely event. Furthermore, the observation that the unique topological distributions of mRNAs are not disturbed when cells are extracted with non-ionic detergent [25,39,40] is consistent with the concept of mRNA binding to the CSK in vivo.

Particularly intriguing is the finding that only about 50% of each of the cytoskeletal mRNAs is able to be eluted from the CSK under conditions that promote nearly 80% elution of histone mRNA. This differential sensitivity is most probably not due to cytoskeletal mRNAs existing in larger polyribosomes, as polyribosome profile analysis showed that size is not a factor in the association of mRNA with the CSK. Moreover, as all four of the mRNAs examined have been shown to occupy vastly distinct cytoplasmic domains [18,24], it is doubtful that the greater retention of the three cytoskeletal mRNAs is due to their existing in cytoplasmic regions that are uniquely resistant to detergent extraction. It is also unlikely that this heightened association of cytoskeletal mRNAs results from co-translational assembly of nascent cytoskeletal polypeptides with the CSK [19], as polyribosome disassembly does not affect either the extent of mRNA/CSK association [1] or proper mRNA localization [41].

The enhanced CSK retention of the three cytoskeletal mRNAs versus histone mRNA does, however, suggest several other possibilities: cytoskeletal mRNAs may possess higher affinities for common mRNA binding sites on the CSK; cytoskeletal mRNAs may be associated with specific mRNA binding proteins that are more avidly associated with the CSK than are the proteins which bind histone mRNA; cytoskeletal mRNAs may contain specialized sequences which bolster their attachment to the CSK. With regard to this last possibility, it may be that the association of a single mRNA molecule with the CSK is mediated by more than one binding event [42,43]. If this is so, then the absence of a poly(A) tail from the histone mRNA may significantly influence its sensitivity to salt elution. Indirect support for an influential role of the poly(A) tail in attaching mRNA to the CSK is provided by two other, independent, observations: poly(A) sequences are quantitatively retained by the CSK of *Chaetopterus* eggs after exhaustive treatment with RNase A [44], and nonadenylated mRNAs move more rapidly through the cytoplasm of *Xenopus* oocytes than mRNAs with a poly(A) tail, implying that the presence of a poly(A) tail allows for cytoplasmic interactions [45–47].

While it now appears likely that mRNA association with the CSK relects an in vivo binding event, the molecular factors involved in this binding remain to be elucidated. Findings from different laboratories suggest that either actin filaments [6,23,25,26] or vimentin filaments [40,44,48] may provide the binding sites for mRNA attachment. Other studies suggest that another component of the CSK, possibly the microtrabecular lattice, is involved [49–51]. It is also unresolved whether mRNAs are attached directly to the CSK, or indirectly through proteins of messenger ribonucleoprotein particles (mRNPs) [38]. Related to this matter, it is presently not known how the elution of mRNAs is achieved. It could be that high salt causes the direct displacement of mRNAs from their binding sites on the CSK. Alternatively, if mRNAs are attached to the CSK via mRNP proteins, it may be the mRNP protein–mRNA interaction that is disturbed by high salt.

An intriguing hypothesis is that attachment of mRNA to the CSK is mediated by specific

proteins which possess at least two binding sites. One site allows for recognition of specific sequences within mRNAs, and the other promotes interaction with topologically arranged cytoskeletal elements. This could, theoretically, allow for both the generalized binding of all mRNAs to the CSK as well as for unique mRNA distributions. In support of this hypothesis are the recent findings that specific nucleotide sequences present within the 3′ untranslated regions of both *Veg 1* mRNA [52] and *bicoid* mRNA [53] are required for their correct cytoplasmic localization (and presumably for their attachment to structural elements as well). Given that our results indicate that mRNA association with the CSK most likely represents a physiological binding event, we are now in the position to begin identifying nucleotide sequences and isolating the protein factors which promote this interaction.

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