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Association of the class V myosin Myo4p with a localised messenger RNA in budding yeast depends on She proteins

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SUMMARY

Asymmetric distribution of messenger RNAs is a widespread mechanism to localize synthesis of specific protein to distinct sites in the cell. Although not proven yet there is considerable evidence that mRNA localisation is an active process that depends on the activity of cytoskeletal motor proteins. To date, the only motor protein with a specific role in mRNA localisation is the budding yeast type V myosin Myo4p. Myo4p is required for the localisation of *ASH1* mRNA, encoding a transcriptional repressor that is essential for differential expression of the *HO* gene and mating type switching in budding yeast. Mutations in Myo4p, in proteins of the actin cytoskeleton, and in four other specific genes, *SHE2-SHE5* disrupt the daughter-specific localisation of *ASH1* mRNA. In order to understand if Myo4p is directly participating in mRNA transport, we used in situ colocalisation and coprecipitation of Myo4p and *ASH1* mRNA to test for their interaction. Our results indicate an association of Myo4p and *ASH1* mRNA that depends on the activity of two other genes involved in *ASH1* mRNA localisation, *SHE2* and *SHE3*. This strongly suggests a direct role of Myo4p myosin as a transporter of localised mRNAs, convincingly supporting the concept of motor-protein based mRNA localisation.

Key words: RNA localization, Myosin V, Intracellular transport

INTRODUCTION

Eukaryotic cells need to localise specific sets of proteins to distinct sites within the cell. Proteins are generally sorted co- or posttranslationally (reviewed by e.g. Görlich, 1997; Schatz and Dobberstein, 1996), but there is an increasing number of proteins that are localised because their corresponding mRNAs are targeted to specific sites within the cytoplasm (Bogucka Glotzer and Ephrussi, 1996; Singer, 1992; St Johnston, 1995).

mRNA localisation occurs in organisms as diverse as amoeba (Han et al., 1997), yeast (Long et al., 1997; Takizawa et al., 1997), algae (Bouget et al., 1995), insects, and vertebrates (Micklem, 1995). In principle, mRNA localisation can be achieved by selective local RNA protection, mRNA anchoring or mRNA transport (St Johnston, 1995). Although not proven yet it has been suggested that many mRNAs are localised by active transport (Ainger et al., 1993; Arn and Macdonald, 1998; Bashirullah et al., 1998) or a combination of transport and anchoring (Sundell and Singer, 1991; Yisraeli et al., 1990).

Anchoring and transport require a functional microfilament or microtubule cytoskeleton (Hesketh, 1996; Singer, 1992). Microtubule-dependent mRNA localisation has been observed in *Drosophila* and *Xenopus* oocytes (Pokrywka, 1995; Yisraeli et al., 1990), neurons (Gao, 1998; Knowles et al., 1996), or oligodendrocytes (Ainger et al., 1993). Actin-dependent localisation has been reported for fibroblasts (Sundell and Singer, 1991), amoeba (Han et al., 1997) or yeast (Long et al., 1997; Takizawa et al., 1997). In a few cases, both filament systems are involved in mRNA localisation, most likely in the two subsequent steps of mRNA transport and mRNA anchoring (Yisraeli et al., 1990).

It is generally assumed that active movement of mRNAs involves motor proteins that move along cytoskeletal filaments. Both actin-dependent myosin and microtubule-dependent kinesin or dynein motors have been implicated in mRNA transport (Nasmyth and Jansen, 1997). Localisation of *Drosophila oskar* RNA, a microtubule-dependent process, is mimicked both temporarily and spatially by the movement of a kinesin-β-galactosidase fusion protein, implicating a so far unknown kinesin as a motor involved in *oskar* transport (Clark et al., 1994). However, recent studies on the in vivo movement of a GFP- (green fluorescent protein-) Exuperantia fusion protein that might be a component of *bicoid* ribonucleoprotein particles in *Drosophila* suggested the action of several different microtubule-dependent motors during cytoplasmic mRNA transport (Theurkauf and Hazelrigg, 1998). Movement of myelin basic protein (MBP) mRNA-containing particles in oligodendrocytes occurs with a speed consistent with that of kinesins (Ainger et al., 1993). Furthermore, depletion of kinesin leads to a block of MBP-RNA transport in such cells (Carson et al., 1997). However, the molecular nature of

microtubule-dependent motor proteins that transport mRNAs remains elusive.

The only actin-dependent motor protein with a specific role in mRNA localisation is the budding yeast Myo4p (Long et al., 1997). Myo4p is a class V unconventional myosin (Titus, 1997). Class V myosins have so far been implicated in vesicle transport in yeast, squid, chicken and mouse (Govindan et al., 1995; Mermall et al., 1998; Prekeris and Terrian, 1997; Titus, 1997). However, there is no indication for a role of Myo4p in vesicle transport (Haarer et al., 1994). In contrast, deletion of *MYO4* results in a severe defect in localisation of *ASH1* mRNA (Long et al., 1997).

ASH1 codes for a transcriptional repressor required to repress the *HO* gene in *Saccharomyces cerevisiae*, thereby regulating mating-type switching (Bobola et al., 1996; Sil and Herskowitz, 1996). Ash1 protein is primarily accumulating in the nuclei of daughter cells that have been generated by budding process from the mother cell. Since mother cells are depleted of Ash1p, *HO* is expressed and these cells will undergo mating type switching. The asymmetric distribution of Ash1 protein stems from the daughter-specific localisation of *ASH1* mRNA (Long et al., 1997; Takizawa et al., 1997). Targeting of *ASH1* mRNA to the distal tip of the daughter cell occurs at the end of anaphase and involves sequences in the 3′ untranslated region (3′UTR) of *ASH1* mRNA. Localisation depends on an intact actin cytoskeleton and five *SHE* genes, one of which, *SHE1* is identical to *MYO4* (Jansen et al., 1996). Deletion of *MYO4* or *SHE2-4* result in a failure to localise *ASH1* mRNA to the daughter cell tip whereas deletion of another *SHE* gene, *SHE5/BNI1* leads to mislocalisation of *ASH1* mRNA to the yeast bud neck (Long et al., 1997).

So far there is no evidence for an association of Myo4p and *ASH1* mRNA during localisation at anaphase. We wanted to investigate if Myo4p is actually associated with *ASH1* mRNA and could serve as a motor protein in mRNA transport. We have used a combination of in situ hybridisation and indirect immunfluorescence to detect Myo4p and *ASH1* mRNA in the same cell. In addition, we have applied co-immunprecipitation to look for an association of Myo4p and *ASH1* mRNA under various conditions. We present data showing that Myo4p and *ASH1* mRNA colocalise at the time of *ASH1* mRNA transport and that they can be found associated in vivo. Both colocalisation and association depend on the proper function of two other She proteins, She2p and She3p.

MATERIALS AND METHODS

Yeast strains and methods

All yeast strains used in this study (Table 1) were derived from W303 (Rothstein, 1983). Cells were grown in standard yeast media. Yeast transformation was carried out according to the method of Gietz and Schiestl (1995).

The strain carrying a myc9-epitope tag prior to the *MYO4* stop codon was created as follows: A cassette containing 9 myc epitopes and the *TRP1* marker from *Kluveromyces lactis* (Shirayama et al., 1998) was amplified using the primer pair *MYO4*-1stepF (5′-TTAGCTACTGTCAGTAAAATTATAAAATTAGACAGAAAATCC-GGTTCTGCTGCTAG-3′) and *MYO4*-1stepR (5′- ATACATATATAC-ATATATGGGCGTATATTTACTTTGTTCTTACCTCGAGGCCAGA-AGACT-3′). The amplification product was targeted by homologous recombination into the 3′ region of the *MYO4* locus of strain K4451 to create an in-frame fusion of the nine myc epitopes and *MYO4*. This generated strain RJY349. The *PEP4* gene of RJY349 was disrupted with a *pep4::URA3* construct (Zachariae and Nasmyth, 1996), resulting in strain RJY376. Other strains carrying multiple myc-epitopes at their carboxytermini have been described elsewhere (Jansen et al., 1996; Zachariae et al., 1996). All myc-epitope strains were transformed with a 2μ-plasmid carrying the *ASH1* gene (Long et al., 1997).

Table 1. Yeast strains

Name	Relevant genotype	Source
K4451	*Mat**a**, HO-ADE2, HO-CAN1*	Jansen et al. (1996)
K5380	*Matα, HO-ADE2, HO-CAN1, SHE3-myc6*	Jansen et al. (1996)
K5705	*MAT**a**, HO-ADE2, she2::URA3, MYO4-myc6*	Jansen et al. (1996)
K5706	*MAT**a**, HO-ADE2, she3::URA3, MYO4-myc6*	Jansen et al. (1996)
K5707	*MAT**a**, HO-ADE2, bni1::URA3, MYO4-myc6*	Jansen et al. (1996)
K6502	*MAT**a***, YEplac181-*ASH1*	Long et al. (1997)
K6915	*Mat**a**, pep4::URA3, bar1::hisG, CSE1-myc9::HIS3*	Zachariae et al. (1996)
RJY336	*Matα, HO-ADE2, HO-CAN1, SHE3-myc6*, YEplac181-*ASH1*	K5380 transformed with C3319 (Long et al., 1996)
RJY349	*Mat**a**, HO-ADE2, HO-CAN1, MYO4-myc9::TRP1(K.lactis)*	K4451 transformed with myc9-tagged MYO4 3′ region (see Materials and Methods)
RJY375	*Mat**a**, bar1::hisG, CSE1-myc9::HIS3, pep4::URA3* YEplac 181-*ASH1*	K6915 transformed with C3319 (Long et al., 1996)
RJY376	*Mat**a**, HO-ADE2, HO-CAN1, MYO4-myc9::TRP1(K.lactis), pep4::URA3* YEplac 181-*ASH1*	RJY375 transformed with pTS15 and C3319 (Long et al., 1996)
RJY411	*MAT**a**, HO-ADE2, she2::URA3, MYO4-myc6* YEplac181-*ASH1*	K5705 transformed with C3319 (Long et al., 1996)
RJY412	*MAT**a**, HO-ADE2, she3::URA3, MYO4-myc6* YEplac181-*ASH1*	K5706 transformed with C3319 (Long et al., 1996)
RJY413	*MAT**a**, HO-ADE2, bni1::URA3, MYO4-myc6* YEplac181-*ASH1*	K5707 transformed with C3319 (Long et al., 1996)

All strains were isogenic derivatives of W303 (also called K699) whose full genotype is *MAT**a**, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+*.

Combined in situ hybridisation and immunfluorescence

Yeast cells that had been grown overnight in SDC-leucine medium were diluted into fresh YEPD and grown for 5 hours at 30°C. Logarithmically growing cells were fixed with formaldehyde and spheroplasted as described by Long et al. (1997). Spheroplasts were transferred onto poly-L-lysine coated coverslips and dehydrated with ice-cold ethanol. After rehydration in 5× SSC, prehybridisation and hybridisation with a digoxigenin- (DIG-) labelled *ASH1* antisense probe were performed according to the method of Takizawa et al. (1997). Following hybridisation, coverslips were washed twice in 2× SSC/40% formamide and blocked twice in blocking buffer (1× PBS/10% fetal calf serum/0.1% Triton X-100). Spheroplasts were incubated for two hours with sheep anti-DIG antibody (Boehringer Mannheim, Germany), followed by a one hour incubation with rabbit anti-sheep-CY3 (Dianova, Hamburg, Germany) and mouse monoclonal anti-myc antibodies (9E10; Evan et al., 1985). The final step involved a one-hour incubation with donkey anti-rabbit-CY3 (Dianova, Hamburg, Germany) and goat anti-mouse-Alexa488 (Molecular Probes, Oregon, USA) antibodies. Spheroplasts were mounted in 90% glycerol and observed under an Olympus BX60 fluorescence microscope. Pictures were taken with a Hamamatsu 4742-95 camera plus ‘Openlab’ software package

(Improvision, Heidelberg, Germany) and resulting images were mounted using Adobe Photoshop software.

Immunprecipitation and mRNA detection

3×10^8 logarithmically growing cells were disrupted with glass beads in 200 µl breakage buffer (BB: 50 mM Hepes-KOH pH 7.3, 50 mM potassium acetate, 2 mM magnesium acetate, 0.1% Triton X-100, 5% glycerol) containing a protease inhibitor cocktail (Boehringer Mannheim, Germany) and 0.5% BSA. Extracts were cleared by centrifugation (10 minutes at 5000 ***g***). Monoclonal anti-myc antibody 9E11 (Evan et al., 1985) was added to the cleared extracts and incubated for 1 hour on ice following incubation with anti-mouse IgG_2a magnetic beads (Dynal, Hamburg, Germany) at 4°C. Beads were washed three times with BB lacking BSA. Pellets were extracted with phenol-chloroform, ethanol precipitated, resuspended in RQ1 DNase buffer, and treated with RQ1 DNase (Promega, Madison, USA). The remaining RNA was extracted, precipitated, and resuspended in H_2O. RT-PCR was performed with 1 µl RNA as template using the 'Access'-RT-PCR kit (Promega) and the conditions suggested by the manufacturer. The number of amplification cycles was adjusted to avoid reaching a plateau phase during PCR. For amplification of *SIC1* and *ASH1* RNAs, we used 25 cycles whereas 22 cycles were used for amplification of *ADH1* RNA. The following primers were chosen for amplification: *ASH1*-2a: 5′-TACATGGATAACTGAATCTC-3′, *ASH1*-2b: 5′-CAGGATGACCAATCTATTGC-3′ (amplifying a 247 bp product from +1 to +248 of the *ASH1* 3′UTR), *SIC1*+: 5′-TCTC-CGAAAAATGACGCCA-3′, *SIC1*-: 5′-TCGTTCCAGAAACTTTT-TT-3′ (amplifying a 316 bp product from –285 to +31 in respect of the *SIC1* stop codon), *ADH1*-3: 5′-CGGTGGTGAAGTCTTCATTG-3′, *ADH1*-4: 5′-GTGTCAACAACGTATCTACCA-3′ (amplifying a 360 bp product of *ADH1* ORF just prior to the stop codon).

RESULTS

ASH1 mRNA colocalises with Myo4p and She3p at the end of anaphase

Although suggested by it's myosin nature, it has not yet been proven that Myo4p is directly involved in *ASH1* mRNA transport. Myo4p and the She3 protein accumulate in the daughter cell throughout the cell cycle from late G_1, when the daughter is generated as bud until late mitosis (Jansen et al., 1996). In contrast, *ASH1* mRNA is transported to the daughter cell tip only at the end of anaphase (Long et al., 1997). Since Myo4p can be detected in the growing daughter cell much earlier than *ASH1* mRNA is expressed, an alternative function that has been discussed is the transport of a so far unknown mRNA anchor to the daughter cell tip that later sequesters *ASH1* mRNA (Long et al., 1997).

We therefore wanted to localise Myo4p and She3p together with *ASH1* mRNA. To do so, we set up a combination of in situ hybridisation and indirect immunfluoresence. Since it has previously been shown that the myc epitope recognized by the 9E10 monoclonal antibody is compatible with the harsh treatment of yeast cells during in situ hybridisation (Long et al., 1997), we used myc-epitope tagged versions of Myo4p (Myo4p-myc) and She3p (She3p-myc) to detect the proteins. For this purpose, proteins containing nine or six myc epitopes at the carboxy terminus were expressed from the endogenous locus. Both epitope-tagged alleles replaced the corresponding wild-type alleles and were fully functional (see Materials and Methods and data not shown).

Fig. 1 demonstrates that in an anaphase cell, both Myo4 protein and *ASH1* mRNA as well as She3 protein and *ASH1* mRNA are primarily located at the daughter cell tip. Protein staining (shown in green) as well as mRNA staining (shown in red) is detected as particle- or granule-like structures. Similar staining has been reported previously for *ASH1* mRNA (Takizawa et al., 1997). Although there is no exact colocalisation, the extensive overlap of RNA and protein staining suggests that a substantial proportion of Myo4p/She3p and *ASH1* mRNA are spatially associated. In order to assure that colocalisation was not due to antibody crossreactivity, we performed in situ hybridisation/immunfluorescence on cells that do not express a myc-tagged protein but *ASH1* mRNA (Fig. 1I-M). We could not detect any significant immunfluorescence signal in such cells, giving us confidence that the immunfluorescence signal in Fig. 1 originated from the myc-tagged proteins.

We detected colocalisation of *ASH1* mRNA and Myo4p/She3p at the daughter cell tip in about 80% of all cases where an *ASH1* mRNA signal was detectable (23/30 cells for Myo4-myc and 36/46 cells for She3-myc). More importantly, in 10-20% of the observed cells (3/30 cells for Myo4-myc and 8/46 cells for She3-myc) *ASH1* mRNA and the two proteins were detected both at the daughter cell tip and on filamentous structures apparently pointing towards the daughter cell tip (Fig. 1, arrowheads). In a previous work, granular structures containing *ASH1* mRNA have been observed on similar filaments, suggestive of mRNA being transported along actin cables from the mother to the daughter cell (Long et al., 1997).

Myo4p and She3p are associated with *ASH1* mRNA

Colocalisation of *ASH1* mRNA and the two proteins could indicate a physical association of Myo4p/She3p and *ASH1* mRNA, however, it could also be accidental. To verify that colocalisation reflects an association of Myo4p or She3p with *ASH1* mRNA, we applied a combination of immunprecipitation and reverse transcription/polymerase chain reaction (RT-PCR) to analyse if *ASH1* mRNA coprecipitates with the two proteins. Again, we used epitope-tagged versions of Myo4p and She3p. A myc9-tagged version of *CSE1* (Cse1p-myc; Zachariae et al., 1996) encoding a protein involved in nuclear export (Solsbacher et al., 1998) was used as a control. For detection of *ASH1* mRNA by RT-PCR we chose to amplify the 3′ region of *ASH1* mRNA covering the first 247 nucleotides of its 3′UTR since this part of the mRNA has previously been shown to target a *lacZ*/*ASH1*-3′UTR RNA hybrid molecule to the daughter cell (Long et al., 1997). We reasoned that detection of *ASH1* 3′UTR would reflect the presence of the full length *ASH1* mRNA.

The 9E11 antibody equally efficiently precipitates all myc-tagged proteins (Fig. 2A). However, as detected by RT-PCR, the 3′ UTR of *ASH1* is enriched in the immunpellet from a *MYO4*-myc cell extract but not in that from a *CSE1*-myc extract (Fig. 2B, compare lanes 4 and 6). The absence of the corresponding PCR product in a reaction lacking reverse transcriptase indicates that amplification was RNA dependent (Fig. 2B, lanes 7-9). *ASH1* mRNA is also present in an immunpellet from a *SHE3*-myc extract (Fig. 2B, lane 5). In contrast, the *SIC1* mRNA (encoding an inhibitor of B-type cyclin kinases; Schwob et al., 1994) which is also expressed at anaphase, is not enriched in any of the three immunpellets (lanes 4-6). A highly abundant mRNA encoding the alcohol dehydrogenase 1 (*ADH1*) is detected in all pellets but not

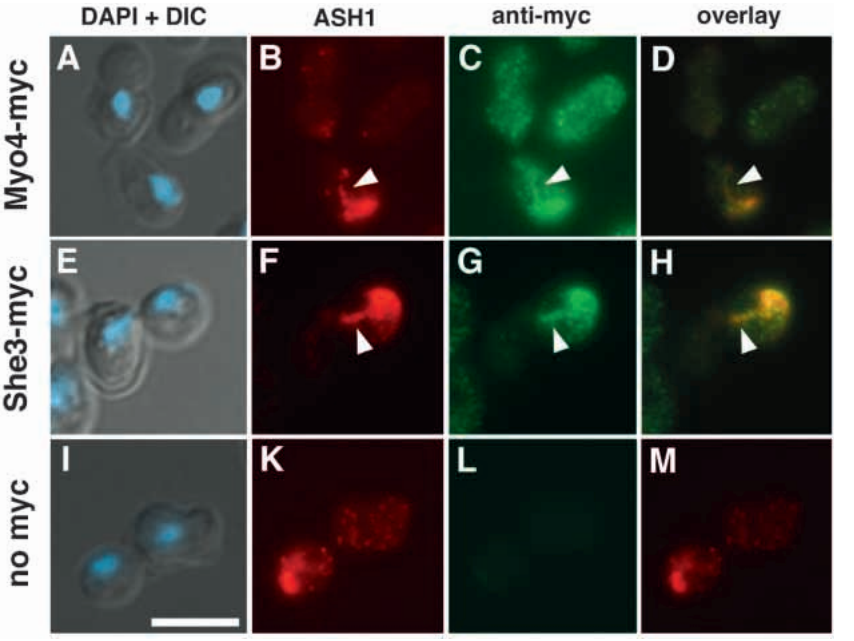

Fig. 1. Colocalisation of *ASH1* mRNA with Myo4p and She3p in anaphase cells. Logarithmically growing cells of strains RJY375 (*MYO4*-myc9, YEplac181-*ASH1*), RJY336 (*SHE3*-myc6, YEplac181-*ASH1*), and K6502 (YEplac181-*ASH1*) were prepared for in situ hybridisation/immunfluorescence as described in Materials and Methods. Typical protein and RNA staining is shown for late anaphase cells with separated nuclei (DAPI staining and differential interference contrast DIC; A,E,I). Both Myo4p-myc (green, C) and She3p-myc (G) localise to a cortical region beneath daughter cell tip and filamentous structures pointing towards the tip (arrowheads). *ASH1* mRNA (red, B and F) localises to identical filaments (D and H) and to the cell tip but staining at the tip is restricted to a narrower region. Bar, 5 μm.

specifically enriched in immunpellets from *MYO4*-myc or *SHE3*-myc extracts as compared to *CSE1*-myc extracts (lanes 4-6). *ADH1* mRNA can be detected in all immunpellets possibly due to unspecific binding of the highly abundant RNA to the affinity matrix used for the immunprecipitation. Similar unspecific binding has been observed for other abundant RNAs that have been tested (data not shown).

Taken together, both in situ detection and coimmunprecipitation data suggest an association of *ASH1* mRNA with She3p and Myo4p.

Association of Myo4p with *ASH1* mRNA is lost in *she2* and *she3* mutants

Mutations in the *SHE1-SHE5* genes result in *ASH1* mRNA mislocalisation and subsequent Ash1 protein synthesis in mother and daughter cell (Bobola et al., 1996; Jansen et al., 1996). In addition, *she3-she5/bni1* mutants but not *she2* mutants disrupt Myo4p localisation to the bud early in the cell cycle (Jansen et al., 1996). Therefore, *she* mutants could in principle fail to localise *ASH1* mRNA by three alternative ways. They could disrupt the association of Myo4p with *ASH1* mRNA, block the localisation of a Myo4p/*ASH1* mRNA complex or affect both processes simultaneously.

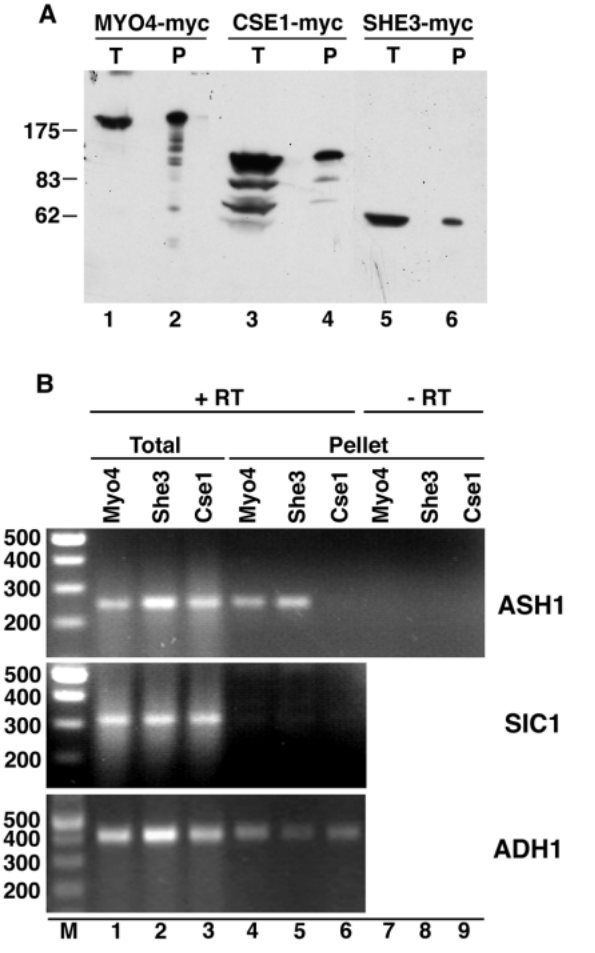

Fig. 2. Coimmunprecipitation of Myo4p, She3p and *ASH1* mRNA. (A) Myc-epitope tagged Myo4p, She3p, or Cse1p were immunprecipiated using monoclonal 9E11 antibody as described in Materials and Methods. 1/20 of total cell extracts (T, lanes 1, 3, and 5) and each immunpellet (P, lanes 2, 4, and 6) were separated on an 8% SDS-PAGE gel, blotted and probed with anti-myc antibody for presence of epitope-tagged proteins. Numbers on the left corresponds to relative molecular mass $\times 10^3$. (B) RNA was extracted from cell extracts (total) and immunpellets (pellet) and used as template for RT-PCR. 247 bp, 316 bp or 360 bp products were amplified using *ASH1*-, *SIC1*-, or *ADH1*-specific primers, respectively. 1/15 of each reaction was separated on a 2% agarose gel and stained with ethidiumbromide. Lanes 1-3: amplification of *ASH1*-3′UTR, *SIC1*, and *ADH1* RNA from total cell extracts. Lanes 4-6: amplification of *ASH1*-3′UTR, *SIC1*, and *ADH1* RNA from *MYO4*-myc, *SHE3*-myc, and *CSE1*-myc immunpellets. Lanes 7-9: no amplification of *ASH1*-3′UTR is seen without reverse transcriptase (RT). Numbers on the left correspond to base pairs.

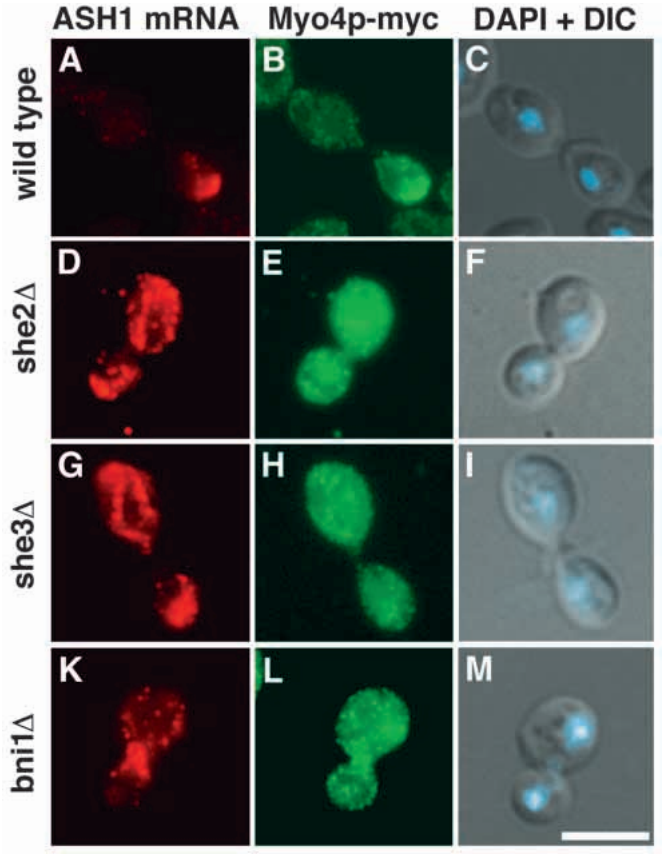

Fig. 3. Colocalisation of Myo4p with *ASH1* mRNA is disrupted by *she2* and *she3* mutants. Exponentially growing cells from RJY375 (wild type), RJY411 (*she2Δ*), RJY412 (*she3Δ*), and RJY413 (*she5/bni1Δ*) were prepared for in situ hybridisation against *ASH1* and Myo4p-myc detection as described in Materials and Methods. (A,D,G,K) *ASH1* mRNA localisation. (B,E,H,L) Myo4p-myc localisation. (C,F,I,M) DAPI staining and DIC. Neither *ASH1* mRNA nor Myo4p-myc specifically localises to the daughter cell tip in anaphase *she* mutant cells. Whereas in a *she5/bni1Δ* mutant partial colocalisation of *ASH1* mRNA and Myo4p-myc is seen at the bud neck (K and L), *ASH1* mRNA particles and Myo4p-myc do not colocalise in *she2Δ* or *she3Δ* mutants (D,E and G,H). Bar, 5 μm.

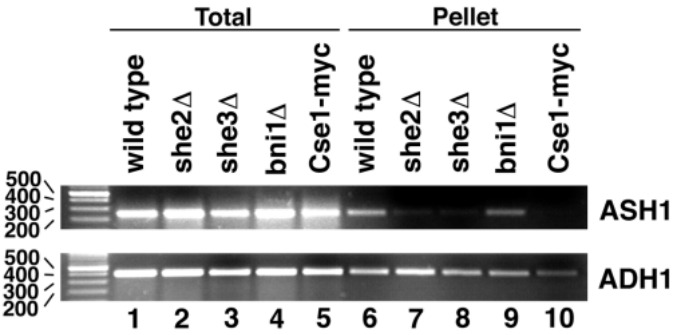

Fig. 4. Association of Myo4p with *ASH1* mRNA depends on *SHE2* and *SHE3*. After immunprecipitation of Myo4p-myc from a wild-type and different *she* mutant strains, RNA was extracted from the Myo4p-myc immunpellet. *ASH1*- or *ADH1*-specific sequences were amplified by RT-PCR from total RNA and RNA in the immunpellet. Lanes 1-5: amplification from total RNA of a 247 bp fragment of *ASH1* 3′UTR or a 360 bp fragment of *ADH1*. Lanes 6-10: Amplification of a 247 bp fragment of *ASH1* 3′UTR or a 360 bp fragment of *ADH1* from RNA co-immunprecipitated with Myo4p-myc. Note that an *ASH1*-specific product is accumulating in the immunpellets of only wild-type or *bni1Δ* mutant strains but not in those of *she2Δ* or *she3Δ* mutants. Numbers on the left correspond to base pairs.

In order to understand the role of the *SHE* genes, we wanted to localise *ASH1* mRNA and Myo4p-myc in the same *she* mutant cell. As shown previously, *she2* and *she3* mutants fail to localise *ASH1* mRNA, which results in distribution of *ASH1* mRNA in form of particles or filaments in the cytoplasm (Fig. 3; Long et al., 1997). In contrast, *she5/bni1* mutant cells mistarget *ASH1* mRNA to the bud neck region (Fig. 3K). Myo4p-myc is no longer detected at the daughter cell tip in *she2* and *she3* mutant cells (E and H) whereas in a *she5/bni1* mutant considerable amounts of Myo4p-myc are seen at the bud neck together with *ASH1* mRNA (K and L). Interestingly, a previous report showed that Myo4p is localised at the daughter tip in *she2* mutants (Jansen et al., 1996). A closer inspection of our Myo4-myc immunfluorescence results showed that proper localisation of Myo4-myc to the bud tip can only be seen in cells with small buds. Large budded cells and especially late anaphase cells that express *ASH1* mRNA do not show any Myo4p-myc localisation (data not shown).

Cytoplasmic Myo4p-myc in *she2* and *she3* mutants does not colocalise with *ASH1* mRNA particles suggesting that Myo4p is no longer associated with *ASH1* mRNA. To test this, immunprecipitations of Myo4p-myc were performed from cell extracts of strains mutant for *she2*, *she3*, or *she5/bni1*. *ASH1* mRNA efficiently coprecipitated with Myo4p-myc from wild-type and *bni1Δ* cell extracts suggesting that *ASH1* mRNA is still associated with Myo4p in *bni1Δ* mutants (Fig 4, lane 9). In contrast, in cells lacking She2p or She3p, only little or no *ASH1* mRNA could be detected in the Myo4p-myc immunpellet (lanes 7 and 8) although Myo4p-myc protein levels were similar in immunpellets from wild-type and *she* mutant cells (data not shown). No such differences could be observed in the unspecific precipitation of *ADH1* mRNA (ADH1, lanes 6-10). The result suggests that both She2p and She3p but not She5/Bni1p are required for association of Myo4p with *ASH1* mRNA.

DISCUSSION

Localisation of specific messenger RNAs to distinct sites within the cytoplasm is an important tool in the cell's repertoire of protein sorting. It is generally accepted that the majority of localised mRNAs is actively transported to their destination sites and that transport depends on a proper cytoskeleton and cytoskeleton-associated motor proteins. However, so far only very few candidates for motors involved in mRNA transport have been identified (Arn and Macdonald, 1998).

The yeast unconventional myosin Myo4p is one of these candidates. Myo4p is one of the two class V myosins in yeast (Titus, 1997). The class V subgroup of unconventional actin-dependent motors is believed to be involved in intracellular vesicle transport (Mermall et al., 1998). Such a role is very likely for Myo2p, the second class V myosin in yeast

(Govindan et al., 1995; Johnston et al., 1991). However, there is no evidence for Myo4p being involved in vesicular transport processes. Instead, *myo4* mutants fail to localise a specific mRNA, *ASH1* mRNA to the daughter cell tip during yeast anaphase (Long et al., 1997).

Although proposed (Amon, 1998), it has not been proven that Myo4p is actually transporting *ASH1* mRNA during mitosis. Myo4p as well as She3p, another protein with an essential role in *ASH1* mRNA localisation can be detected at the distal cortex of the daughter cell much earlier in the cell cycle than *ASH1* expression occurs (Jansen et al., 1996). Since Myo4p accumulates at the growing daughter cell's (the bud's) tip from late G_1 to the end of mitosis, an alternative function of Myo4p for RNA localisation has been suggested (Amon, 1998; Long et al., 1997). The myosin could deliver a so far unknown mRNA anchor to the daughter cell's tip early in the cell cycle that later sequesters *ASH1* mRNA to this site. In order to support the idea of Myo4p being actively engaged in *ASH1* mRNA transport, we used two complementary approaches to show an association of the myosin with the mRNA.

Myo4p can be detected to colocalise with *ASH1* mRNA in cells that are just undergoing anaphase. It is important to note that colocalisation of *ASH1* mRNA and Myo4p is not perfect but the two signals are widely overlapping. Such an overlapping localisation has also been detected in a recent approach that used a hybrid mRNA between *ASH1*-3′UTR and lacZ instead authentic *ASH1* mRNA (Bertrand et al., 1998). Overlapping staining could reflect two different states of Myo4p, a mRNA-associated state where mRNA and protein staining overlap and a cargo-free state where Myo4p is detectable without associated mRNA. With our biochemical approach, using coprecipitation of Myo4p and *ASH1* mRNA we are not able to distinguish between such putative two states of Myo4p, mainly for two reasons. Firstly, RNA detection after co-immunprecipitation by RT-PCR is not purely quantitative. However, we had to rely on RT-PCR for detection limit reasons. Secondly, for the immunprecipitation we used a cycling yeast culture containing cells from all stages of the cell cycle. In such a culture, all cells contain Myo4p but only a small proportion of cells express *ASH1* mRNA, enabling us to experimentally access a Myo4p-*ASH1* association only qualitatively. A further analysis of the quantitative nature of Myo4-*ASH1* mRNA association will be a necessary future goal. However, it is evident from our co-immunprecipitation results that Myo4p and She3p are associated with *ASH1* mRNA. It has also been demonstrated that this association is rather specific since another RNA that is expressed during yeast anaphase, *SIC1* is not found to coprecipitate with Myo4p or She3p.

An important finding of our in situ hybridization/immunfluorescence results is the observation of colocalisation of Myo4p/She3p with *ASH1* mRNA not only at the daughter cell tip but also on filamentous structures pointing towards the tip. Similar combinations of daughter tip and filamentous staining have already been observed for *ASH1* mRNA alone (Long et al., 1997). Such staining might reflect mRNA that has been partially transported with the filamentous staining reflecting mRNA transport intermediates. If so, Myo4p and She3p are colocalising with such transport intermediates.

But why can we detect only so few cells (between 10% and 20% of all cells with *ASH1* mRNA signal) with filamentous costaining of *ASH1* mRNA and Myo4p/She3p? There might be three reasons for this. Firstly, only few cells show just filamentous *ASH1* mRNA staining without an already localised signal. Even in cases where such a signal is detectable it is not clear if this reflects mRNA on the move or mislocalised mRNA since in a given wild-type cell population a small number of cells mislocalise *ASH1* mRNA, resulting in improper Ash1 protein sorting (Long et al., 1997). Therefore, such cells were exempted from this analysis. Secondly, we have more often observed Myo4p-myc staining as short filamentous structures in daughter cells under conditions where no in situ hybridisation had been performed prior to Myo4p-myc detection (data not shown). Possibly, a major proportion of filamentous Myo4p-myc staining is lost upon the harsh conditions of in situ hybridisation. Thirdly, transport from the mother to the daughter cell could be a fast process. Bertrand et al. (1998) followed the movement of particles containing a *lacZ*/*ASH1*-3′UTR hybrid RNA in vivo. A single particle moved from the mother cell to the daughter cell tip in about two minutes suggesting that transport intermediates might be very short-lived. Since our in situ approach detects mRNA and proteins only in a steady state situation we might preferentially detect already localised mRNA and protein.

Besides Myo4p, a set of four She proteins is required for proper localisation of *ASH1* mRNA (Long et al., 1997). We have tested the effect of independently depleting three of them on Myo4p-*ASH1* mRNA association. Both colocalisation and coprecipitation approaches give consistent results. An association of the myosin and the RNA is lost in cells lacking She2p or She3p but not in cells lacking She5/Bni1p. These results suggest that She2p and She3p are required for interaction of Myo4p with *ASH1* mRNA. Since She3p is also both colocalising and biochemically associated with *ASH1* mRNA, it could be involved in connecting Myo4 and *ASH1* mRNA, as suggested by Bertrand et al. (1998). Since She3p and Myo4p are colocalising throughout the cell cycle (Jansen et al., 1996) and have been found to interact in a two-hybrid interaction assay (A. Frank and R.-P. Jansen, data not shown), such an interpretation is feasible. However, it is important to note that She3p is also essential for targeting of Myo4p to the daughter cell independently of *ASH1* mRNA's presence (Jansen et al., 1996). This result implicates that She3p could have a dual function in Myo4p localisation and association of Myo4p with *ASH1* mRNA.

In contrast to She2p and She3p, Bni1p presumably functions not in Myo4p-*ASH1* mRNA association but rather in correct targeting of Myo4p and *ASH1* mRNA to the bud tip. Bni1p is involved in several aspects of the actin cytoskeleton's function, including cytokinesis and formation of mating type projections (Evangelista et al., 1997; Kohno et al., 1996). The Bni1 protein interacts with several other proteins that fulfil different actin-associated functions like profilin or Rho proteins. It has been speculated that Bni1 is involved in generation or maintenance of actin cytoskeleton polarity (Tanaka and Takai, 1998). Interestingly, a recent finding that Bni1p can also interact with the translation elongation factor EF1alpha suggests a more direct role of this protein in mRNA metabolism (Umikawa et al., 1998).

Taken together, our data indicate that *ASH1* mRNA is associated with She3p and the type V myosin Myo4p, an association that depends on two other genes involved in *ASH1*

mRNA localisation. If Myo4p directly or indirectly binds to *ASH1* mRNA is not yet clear. However, we assume that direct binding is unlikely. In the case of motor-protein based vesicle transport, a number of proteins is required to connect the motor protein and the vesicle to be transported (Hirokawa, 1998). Similarly, proteins associated with Myo4p might connect RNA and motor protein. A possible candidate for such a protein is She3p since it also associates and colocalises with *ASH1* mRNA.

Our data are consistent with a recent report that analysed the transport of an *ASH1*/*lacZ* reporter mRNA in vivo (Bertrand et al., 1998). Here, a reporter hybrid mRNA was shown to move in form of a single large mRNP particle to the daughter cell. Movement depended on Myo4p and occured with a speed consistent with that of type V myosin (Mooseker, 1995). The large mRNP particle dispersed in *she2Δ* and *she3Δ* mutants, suggesting a role of She2 and She3 proteins in particle formation.

In summary, biochemical and in situ approaches demonstrate Myo4p to be the first motor protein that is directly involved in the transport of a localised mRNA. Analysing the specific role of the She proteins in *ASH1* mRNA-Myo4p association in budding yeast will be of substantial importance for our understanding of how mRNA particles are assembled and moved in other cell types as well (Ainger et al., 1993; Ferrandon et al., 1994).

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