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Misdirected vimentin messenger RNA alters cell morphology and motility

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SUMMARY

Localized messenger RNAs were first observed as embryonic determinants that altered development when mislocalized. In recent years localized mRNAs have been found for several cytoskeletal proteins, including actin, vimentin and several microtubule associated proteins. We sought to determine whether redirecting mRNA for a cytoskeletal protein to an inappropriate address would alter cellular phenotypes. To do so we generated vimentin mRNAs with a myc epitope tag and the β-actin 3′ untranslated region (3′ UTR) as a localization signal. When misdirected vimentin mRNAs are expressed in either fibroblasts or SW13 cells, cells develop numerous, extremely long processes; these cells also move more slowly to enter a wound of the monolayer. In situ hybridization revealed that the misdirected mRNA was often localized in the processes, in contrast to endogenous vimentin mRNA. The processes usually contained actin distal to the transgenic vimentin and microtubules proximal to it. SW13 cells lacking vimentin produced fewer and shorter processes, suggesting a dominant negative effect that involves recruitment of endogenous vimentin. Control experiments that transfected in constructs expressing tagged, correctly localized vimentin, or β-galactosidase that localized through the β-actin 3′ UTR, indicate that neither the shape nor the motility changes are solely due to the level of vimentin expression in the cell. This is direct evidence that the site of expression for at least one cytoskeletal mRNA alters the phenotype of the cell in which it is expressed. Messenger RNA localization is proving to be as essential for the normal maintenance of somatic cell phenotypes as embryonic determinants are for embryogenesis.

Key words: Vimentin, mRNA location, Cell shape, Cell movement

INTRODUCTION

Localized messenger RNAs are fundamental to multicellular life. Localized mRNAs in oocytes (Capco and Jeffrey, 1982; Weeks et al., 1985; Rebagliati et al., 1985) act as embryonic determinants that alter development when mislocalized. The last fifteen years have seen many localized mRNAs identified and their times of action during embryogenesis related to their role in development. Localized messenger RNAs determine anterior-posterior (Gavis and Lehman, 1992, 1994) and dorsal-ventral axes, as well as control many steps of early morphogenesis. This extensive literature has recently been reviewed (Micklem, 1995; Morgan and Mahowald, 1996; Melton, 1987, 1991).

In a surprising parallel to this embryonic localization, several cytoskeletal proteins have localized mRNAs, including actin, vimentin and several microtubule associated proteins (Fulton, 1993; Kislauskis and Singer, 1992; Lawrence and Singer, 1985; Singer, 1992). Cytoskeletal mRNAs are sometimes localized with submicron precision (Cripe et al., 1993); in some cell types as many as seven different addresses exist for cytoskeletal mRNAs (Morris and Fulton, 1994).

Although altered mRNA patterns for messages such as *bicoid* and *nanos* affect embryogenesis, much less is known about the functional consequences of cytoskeletal mRNA patterning. Function of some kind is suggested by the frequent association of mRNA positioning with development: during neuronal development (Hannan et al., 1995; Steward, 1995; Johannessen et al., 1995), myogenesis (Hill and Gunning, 1993; Bassell et al., 1994; Morris and Fulton, 1994; Fulton and Alftine, 1997), and the growth response to serum (Latham et al., 1994). However, the only example to date of a functional consequence of patterned mRNA for cytoskeletal proteins is seen with actin. Delocalization of actin mRNA by antisense oligonucleotides makes cells lose polarity and reduces movement (Kislauskis et al., 1994). We sought to modify the position of the mRNA encoding the intermediate filament protein vimentin to determine whether the extensive and precise patterning that has been seen for that mRNA is functionally relevant for the cell.

MATERIALS AND METHODS

Construction and names of plasmids

To create the misdirected construct MVβ, the plasmid Bluescript with six tandem repeats of the myc epitope (obtained from DiMario; Heine et al., 1993) was digested with *Nco*I and *Bam*HI. Using pMV6.2,

which contains the full-length mouse vimentin gene including the 3′ UTR (provided by Capetanaki, 1990), as template, the vimentin coding sequence was PCR amplified using appropriate forward (5′-CCTTCGAAGCCATGGCTA-3′) and reverse (5′-GTGGGAT CCTTATTCAAG-3′) 5′ and 3′ primers. The resulting product was cut with *Nco*I and *Bam*HI and ligated into the pBluescript/*myc* plasmid. The resulting *myc*-vimentin fragment was excised by *Sal*I and *Bam*HI digestion. The plasmid pRSV620 (provided by Kislaukis et al., 1993) contains the β-galactosidase sequence and the 3′ UTR of β-actin (called here **Z**β (Lac***Z***-*β*-actin 3′ UTR) for convenience); it was cut with *Sal*I and *Bam*HI to remove the β-galactosidase insert. The fragment containing *myc*-vimentin sequence was inserted between the 5′ and 3′ UTRs of Zβ. The final construct consists of the parent pRSV vector, the ***M****yc* and ***V***imentin coding sequences and the 3′ UTR of *β*-actin (**MV**β). MVβ is under the control of the RSV promoter; the myc tag is at the amino-terminal end of the vimentin protein.

To make the construct containing ***M****yc*-tagged ***V***imentin with the ***V***imentin 3′ UTR (**MVV**), pMV6.2 was used as the template to PCR amplify the vimentin 3′UTR using a forward primer (5′-ACGGATCCTAAAAATTGCACACACTTGGTG-3′) to create a 5′ *Bam*HI site and a reverse primer (5′-AGCCGCGGTGAAGCAG-TAACAAGTTGGTCA-3′) to create a 3′ *Sac*II site. The MVβ construct was cut with *Bam*HI and *Sac*II to remove the β-actin 3′ UTR, and the fragment with the vimentin 3′ UTR was then inserted into it to generate MVV.

Cell culture

Embryonic fibroblasts were cultured from 12-day chicken embryos (Isaacs et al., 1989). Bob Evans (University of Colorado, Denver) kindly provided two lines of SW-13 adrenal cells. SW-13/c1.1 cells ($SW\text{-}13^{+}$) contain, while most SW-13/c1.2 cells ($SW\text{-}13^{-}$) lack, a detectable vimentin filament system. These lines are grown in a 1:1 mixture of F12:DMEM with 5% FBS and 10 mg/ml gentamicin.

All cell types were grown in 35 mm culture dishes which contained three or four 12 mm coverslips. Coverslips were washed in 100% methanol for fifteen minutes and autoclaved in 0.5% bovine tendon collagen and dried. Cells were plated at a concentration of 0.2-0.6×10^{6}/dish. All experiments were performed on cells grown on coverslips. Cells were prepared for transfection as described (L'Ecuyer et al., 1995). Transfections were performed using unilamellar liposomes containing Lipofectamine™ (Gibco/BRL) and published protocols (Hawley-Nelson et al., 1993).

Immunofluorescence

Cells were stained for myc and LacZ proteins by immunofluorescence following a published procedure (Cripe et al., 1993). *Myc* was detected with monoclonal antibody 9E10 at 1:100 dilution. β-galactosidase was detected by monoclonal antibody 40-1a (Developmental Studies Hybridoma Bank) at a 1:1500 dilution. Secondary antibody for both myc and lacZ, an antibody tagged with fluorescein and directed against mouse IgG (Cappel), was used at 1:2000 dilution.

To stain for microtubules (tubulin), cells were rinsed with MT buffer, pH 6.9 (0.1 M pipes, 1 mm EGTA, 29% glycerol), and extracted for 1 minute in MT buffer and 0.5% Triton X-100 at 24°C. Cells were washed in MT buffer and then fixed at –20°C in methanol for 5 minutes, rinsed three times, rehydrated and primed with PBS and 0.1% BSA. Priming the cells reduces non-specific background staining and aids in the preservation of structural proteins. Cells were incubated in a 1:100 dilution of the monoclonal anti-tubulin antibody E7 (Developmental Studies Hybridoma Bank) in PBS and 0.1% BSA for 30 minutes at 37°C. After rinsing and repriming, cells were incubated with a FITC tagged secondary antibody for 30 minutes at 37°C. Cells were rinsed, reprimed and labeled for 5 minutes at 24°C with antibody 9e10, rinsed, primed and then incubated for 5 minutes at 24°C in a 1:2000 dilution of secondary antibody IgG-RITC.

To stain for actin and vimentin, cells were washed with PBS, pH 7.4, fixed in 3.7% formaldehyde in PBS for 10 minutes at 24°C and then rinsed with PBS. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes at 24°C, rinsed three times, then primed in PBS and 1% BSA for 5 minutes at 24°C. Actin was labeled with Oregon Green 488 Phalloidin (Molecular Probes) diluted 1:50 in PBS and 1% BSA for 20 minutes at 24°C. Cells were rinsed, reprimed and incubated with the primary antibody (9e10 or 40-1a) for 30 minutes at 24°C. After rinsing and repriming, cells were incubated in RITC labeled IgG secondary antibody for 30 minutes at 24°C followed by three PBS rinses.

Cells were viewed under epifluorescent optics using a Bio-Rad 1024 laser scanning confocal microscope. PPD mounting medium was used to reduce sample fading. Image layout was done on a Silicon Graphics Indy workstation and printed on a Tektronix Phaser II SDX dye sublimation printer.

Process assays

Stained cells were examined by epifluorescent microscopy and each cell was scored for positive staining of transfected gene product, either myc tagged vimentin or β-galactosidase, and for the number of cellular processes. Only the processes that were longer than the average cell body diameter were counted. Approximately 450 cells per sample per experiment were counted. Cell counts were used to calculate the percentage of cells with a given number of processes. The graphs represent four experiments.

Wound assays

For wound healing experiments, cells were plated onto coverslips at 0.20 or 0.15×10^{6} cells per dish, transfected, and the cell monolayer on each coverslip was wounded twice; once, at either 8 or 20 hours before fixation; and again, 0 hours (immediately) before fixation. Wounds were made by lightly scraping a sterile razor blade back and forth across the coverslip until a clean wound, about 0.3 mm wide, was formed. The wounded monolayers were fixed at 48 or 72 hours after transfection and stained using immunofluorescent techniques described earlier. After staining, cells were examined in a Zeiss Axioskop. The average number of positively transfected cells per field of view near the wound edge was determined for both wounds on each coverslip by aligning a 63× field of view tangent to the wound edge at random sites along its length. All of the positively staining cells with nuclei in these fields were counted, and this number was divided by the number of fields examined along that wound edge. The wound edge was defined as a line extended along the nuclei of the cells which had moved farthest into the wound, disregarding the occasional single cells in the middle of the wound that had been missed during the wounding procedure.

For each coverslip, the average number of positively staining cells near the wound edge at 8 or 20 hours after wounding was divided by the number of cells there at 0 hours to normalize data for transfection efficiency. This was calculated for 13-17 different coverslips, and the data were averaged to produce the graph in Fig. 4. Sample sizes are shown in Table 1. Levels of significance shown in Table 2 were determined using the Mann-Whitney U-test.

In situ hybridization

For in situ hybridizations, a 420 base pair probe to the six tandem repeats of c-myc was generated by polymerase chain reaction using pMyc-xlc23 (Heine et al., 1993) as the template. We used a 5′ 20 nucleotide primer (5′AATTAACCCTCACTAAAGGG-3′) and a 3′ 20 nucleotide primer (5′TAATACGACTCACTATAGGG-3′). The PCR reaction was performed for 34 cycles with the extension cycle at 72°C for 3 minutes, the melting cycle at 94°C for 30 seconds, and the annealing cycle for 30 seconds at 55°C. The reaction contained 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dCTP, 0.16 mM dTTP, and 0.04 mM digoxigenin-11-dUTP (Boehringer Mannheim Biochemicals, Indianapolis). Incorporation of digoxigenin was confirmed by dot blot hybridization of the probes and detection with anti-digoxigenin-

alkaline phosphatase antibodies (Boehringer Mannheim Biochemicals). On agarose gel electrophoresis, the labeled probe migrated more slowly than the control fragment lacking digoxigenin, consistent with its higher molecular mass. Because the probe hybridized to the myc tag, it uniquely reveals mRNA from the transfected plasmid and not endogenous vimentin mRNA.

To stain for mRNA and protein by in situ hybridization and immunofluorescence, cells are rinsed two times in 24°C PBS^{++} (PBS, 1 mM $MgCl_2$, 2 mm EGTA) then once in 4°C PBS^{++} before fixation in 3.9% formaldehyde in methanol for 30 minutes at –20°C. Cells are then quenched in 0.1 M glycine, 0.2 M Tris-HCl, pH 7.9, for 10 minutes at 24°C followed by rehydration in PBS and 5 mm $MgCl_2$ for 10 minutes at 24°C. After equilibration in 50% formamide/2× SSC for 10 minutes at 24°C, cells are hybridized with 200 ng probe in 50% formamide, 2× SSC, 1% BSA, 0.1% SDS and 10 mM ribonucleoside-vanadyl complex (NEB) for 1 hour at 37°C. Samples are then rinsed twice with 50% formamide/2× SSC for 10 minutes at 37°C and three times with 1× SSC for 10 minutes at 24°C. Cells are then primed in buffer A^{++} (150 mM NaCl, 100 mM Tris-HCl, pH 7.5, plus 0.3% Triton X-100, 1% BSA) for 5 minutes at 24°C. Samples are then incubated with the primary antibody against myc in buffer A^{++} for 30 minutes at 24°C. Cells are then rinsed three times in buffer A (150 mM NaCl, 100 mM Tris-HCL, pH 7.5) and reprimed in buffer A^{++}. Cells are incubated with 1:100 dilution of anti-digoxigenin-RITC (a sheep Fab from Boehringer Mannheim Biochemicals) and a 1:250 dilution of anti-IgG-FITC (Cappel) for 30 minutes at 24°C, and rinsed three times in buffer A^{++} for 5 minutes at 24°C.

RESULTS

Altered morphology in embryonic fibroblasts with misdirected vimentin

We sought to determine whether redirecting mRNAs to an inappropriate address would alter cellular phenotypes. To answer this question, we generated vimentin mRNAs with an epitope tag from the *myc* gene and the β-actin 3′ untranslated region (3′ UTR) as a localization signal. In chick fibroblasts, this 3′ UTR sends heterologous messages to the cell's leading edge (Kislauskis et al., 1993). To misdirect vimentin mRNA within the cell, we expressed this *myc* tagged vimentin construct, MVβ, in chick fibroblasts. After expressing MVβ for 24 hours, some cells appeared largely normal, with somewhat more vimentin at the periphery than in normal cells. However, many cells develop several long processes (Fig. 1a,d). This is consistent with synthesis and subsequent assembly of vimentin at the cell periphery into highly aberrant structures.

By 48 hours many of these cells had developed aberrant bundles of vimentin fibers, winglike processes and extremely long extensions of the cytoplasm. An example of such a process is visible in Fig. 1b,e. Cells expressing misdirected vimentin mRNA for 72 hours often had multiple, extremely long cellular processes, many cell diameters in length. These processes were sometimes extensively branched.

For controls, we determined whether expression of extra, correctly localized vimentin mRNA was sufficient to increase processes, by expressing myc tagged vimentin mRNA directed through the normal vimentin 3′ UTR (MVV). Cells expressing this construct were largely normal in morphology and had fewer and shorter processes than those expressing misdirected vimentin mRNA (Fig. 2a). To show that expression from this promoter had not caused the morphology seen with MVβ, we also expressed β-galactosidase without the β-actin 3′ UTR in these cells. These control cells extended few processes (Fig. 2b). None of the processes were of the extreme length seen at 72 hours in cells with misdirected mRNA. To eliminate the possibility that mRNAs competing for binding at the β-actin sites had caused this morphology, we also expressed β-galactosidase targeted through β-actin 3′ UTR in these cells (Zβ). These resembled the pRSVZ control cells (no image shown).

To quantitate the effect of misdirected mRNA, the number

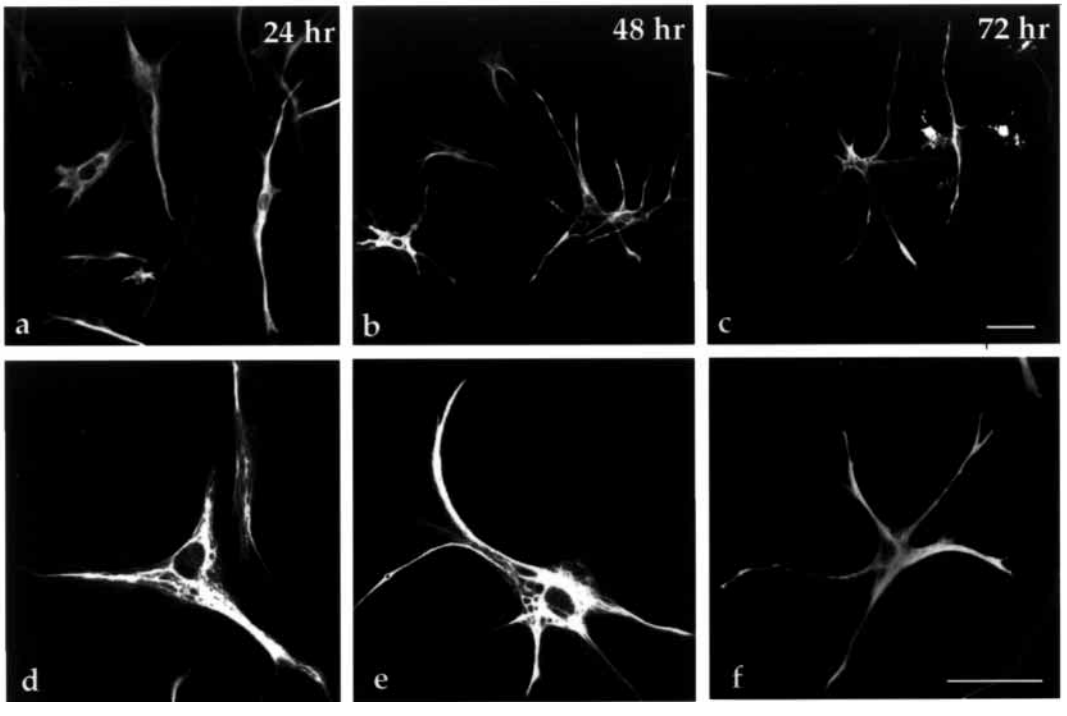

Fig. 1. Cell shapes at different times after transfection with MVβ. Fibroblasts expressing misdirected vimentin for 24 (a,d), 48 (b,e) or 72 (c,f) hours and stained for the myc tag on vimentin, show multiple long processes which are often branched. Bar, 50 μm.

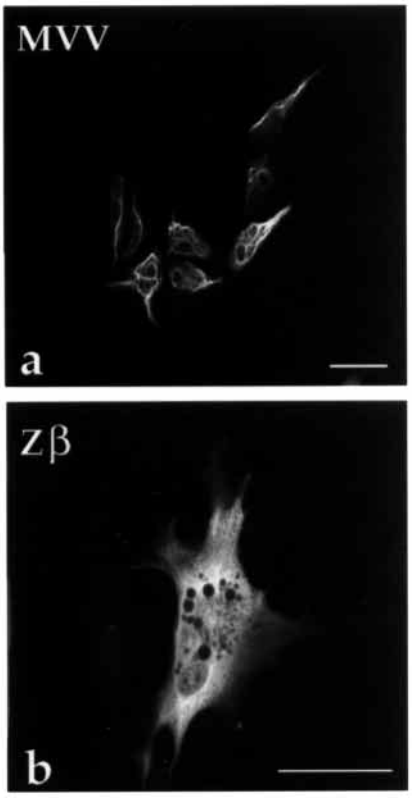

Fig. 2. Controls for transfection with MVβ. (a) Fibroblasts expressing correctly directed vimentin (MVV) for 48 hours and stained for the myc tag on vimentin, show few, short processes that are never branched. (b) Fibroblasts expressing Zβ (mislocalized LacZ, which competes at actin mRNA binding sites) have normal cell shapes. Bar, 50 μm.

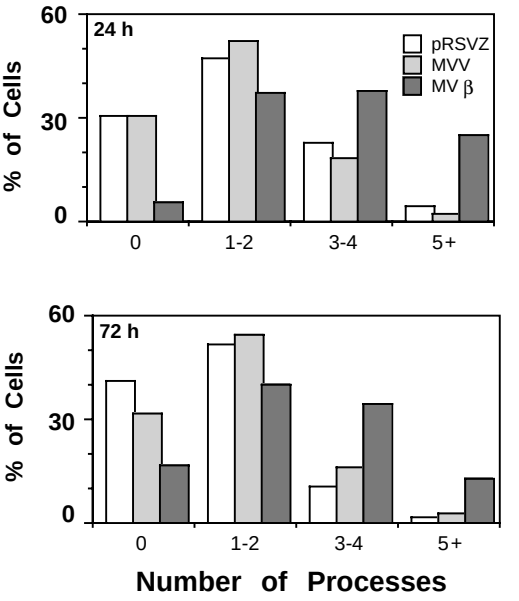

Fig. 3. Number of cell processes as a function of plasmid transfected. Graphs show percentages of cells with a given number of cell processes at given times after transfection. Control, pRSVZ; normally localized vimentin, MVV; misdirected vimentin, MVβ. Cells were scored for positive staining of transfected gene product, either c-myc or β-galactosidase, and for the number of cellular processes. Only processes longer than the average cell body diameter were counted. In each of four experiments, approx. 450 cells per sample were counted. Data reported are the percentages of cells with a given number of processes.

of processes on each cell was counted (Fig. 3); fibroblasts that expressed misdirected mRNA rarely lacked processes, and often had five or more processes. For comparison, a third of the cells expressing either normally localized vimentin mRNA or LacZ localized by the actin 3′ UTR lacked processes, and the remainder typically had one to two processes.

Levels of expression appeared similar for the two constructs. Even brightly staining MVV cells did not acquire the extremely long processes seen with MVβ, while some less brightly staining MVβ treated cells did have them. Both constructs use the same promoter; the mRNAs differ *only* in their 3′ UTRs. Actin and vimentin are both abundant, long lived proteins, so their mRNAs would also be expected to be relatively stable. Actin mRNA typically has a half-life of approx. 5.5 hours (Villarete and Remick, 1996), vimentin mRNA, a half-life of >6 hours. (Lilienbaum et al., 1986). Thus, the quantitative and qualitative differences in fibroblast cell processes do not appear to be due to differences in levels of protein accumulation.

It is important to note that in addition to these quantitative differences in the number and length of processes, there were also qualitative differences. In fibroblasts transfected with MVβ, the processes that were induced were not only extremely long, but they were also often branched, sometimes multiply. The branches often stemmed from a nodule of protein or mRNA. In others, these processes were extended into a flattened veil or cell fragment. These two cell shapes were never observed in cells transfected with either of the control plasmids, MVV or Zβ.

Altered motility in embryonic fibroblasts with misdirected vimentin

Such severe changes in morphology might be expected to alter cell movement. This possibility was tested by transfecting fibroblasts with the three plasmids, allowing cells to express protein and form a confluent monolayer, and then using a scalpel to ‘wound’ the monolayer. Fibroblasts were allowed to move into the open space for 8 or 20 hours. At 48 or 72 hours after transfection, the samples were fixed and stained to indicate whether they had expressed protein from the transfected plasmid. Movement of cells in 20 hours is expressed in Fig. 4, relative to the abundance of transfected cells at the edge immediately after a wound was made. Fig. 4 shows that cells expressing mislocalized vimentin mRNA (MVβ) move significantly more slowly than either the Zβ control or than cells expressing myc tagged vimentin synthesized from correctly localized mRNA. Similar results were seen 8 hours after a wound was made (data not shown).

Cells exhibited considerable variability in their motility, which is more easily seen in scatterplots of the original data (Fig. 5). This variability is inherent to the cells, and not a consequence of expressing extra vimentin, for it is also seen in cells expressing Zβ. The mean values for motility and their standard errors are shown in Table 1. Statistical analysis of the differences in motility between the cells treated with MVβ and

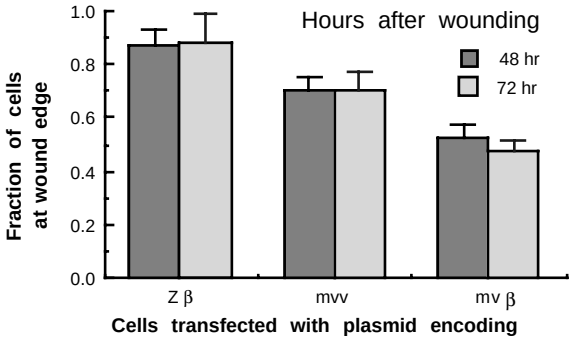

Fig. 4. Cell motility is altered after transfection with MVβ. Cells expressed tagged vimentin for either 48 or 72 hours. Graph shows the proportion of fibroblasts at the wound edge 20 hours after wounding. Cells were transfected with plasmids encoding either β galactosidase localized by actin 3′ UTR (Zβ); normally localized vimentin mRNA (MVV); or misdirected vimentin mRNA, (MVβ). Cells per field at the wound edge were scored for positive staining of transfected gene product, either myc tagged vimentin or β-galactosidase. Staining followed the procedure in Fig. 3.

MVV shows that the differences between MVβ and MVV, or between MVβ and Zβ, are highly significant (Table 2), both at 48 and 72 hours of expressing the transfected proteins. The smaller differences in mobility between MVV and Zβ, the two controls, are significant at a low level at 48 hours, but no longer significant at 72 hours.

Spatial relationship between mislocalized vimentin RNA and vimentin protein

Do these altered patterns of cell shape and motility reflect changes in mRNA position? When cells transfected with MVβ are examined 24 hours after transfection, the most common pattern observed is one in which there is a somewhat peripheral and co-localized distribution of the mislocalized mRNA and transgenic vimentin. In one instance, a deposit of misdirected mRNA was observed at the leading edge with no protein present. A strong association between message and protein at the cell periphery suggests that translation begins very soon after mRNA localization.

Fig. 6 shows the protein and mRNA patterns in cells that have expressed either MVβ, MVV, or Zβ for 48 hours. The panels marked (a), red, show in situ hybridization for mRNA; panels marked (b), green, show the staining for protein. The

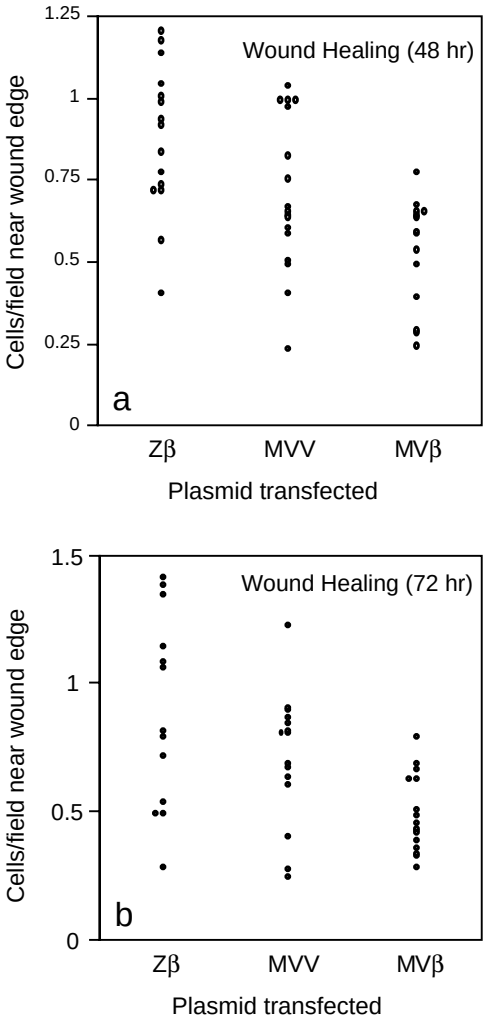

Fig. 5. Cell motility after transfection with MVβ. (a,b) Scatterplots of the data shown in Fig. 4 show the variability of cell motility, which is comparable for the cells transfected with either MVβ or Zβ.

panels marked (c) show the merged image from the first two images. Panels are shown at slightly different magnifications; scale bars in the figure legends indicate relative size. On the left, a cell transfected with MVβ has developed long processes (only one of which is shown at nearly full length, and another

Table 1. Relative mobility into a wound by cells expressing different plasmids

Time after transfection (hours)	Plasmid DNA	Fraction of cells positive at wound edge* ± s.e.m.	Mean # of positive cells counted/slip	Mean # of fields counted/slip	Number of coverslips counted
48	Zβ	0.87±0.07	97	44	15
48	MVV	0.70±0.06	174	30	17
48	MVβ	0.53±0.04	146	26	14
72	Zβ	0.89±0.10	78	31	13
72	MVV	0.71±0.07	200	23	15
72	MVβ	0.48±0.04	172	21	16

Table shows for each plasmid (controls Zβ and MVV, and the misdirected MVβ) the fraction of the cells expressing the plasmid at the wound edge, relative to the fraction that was there when the wound was made.

*Mean, relative to the number of cells per field at the wound edge, 0 minutes after wounding.

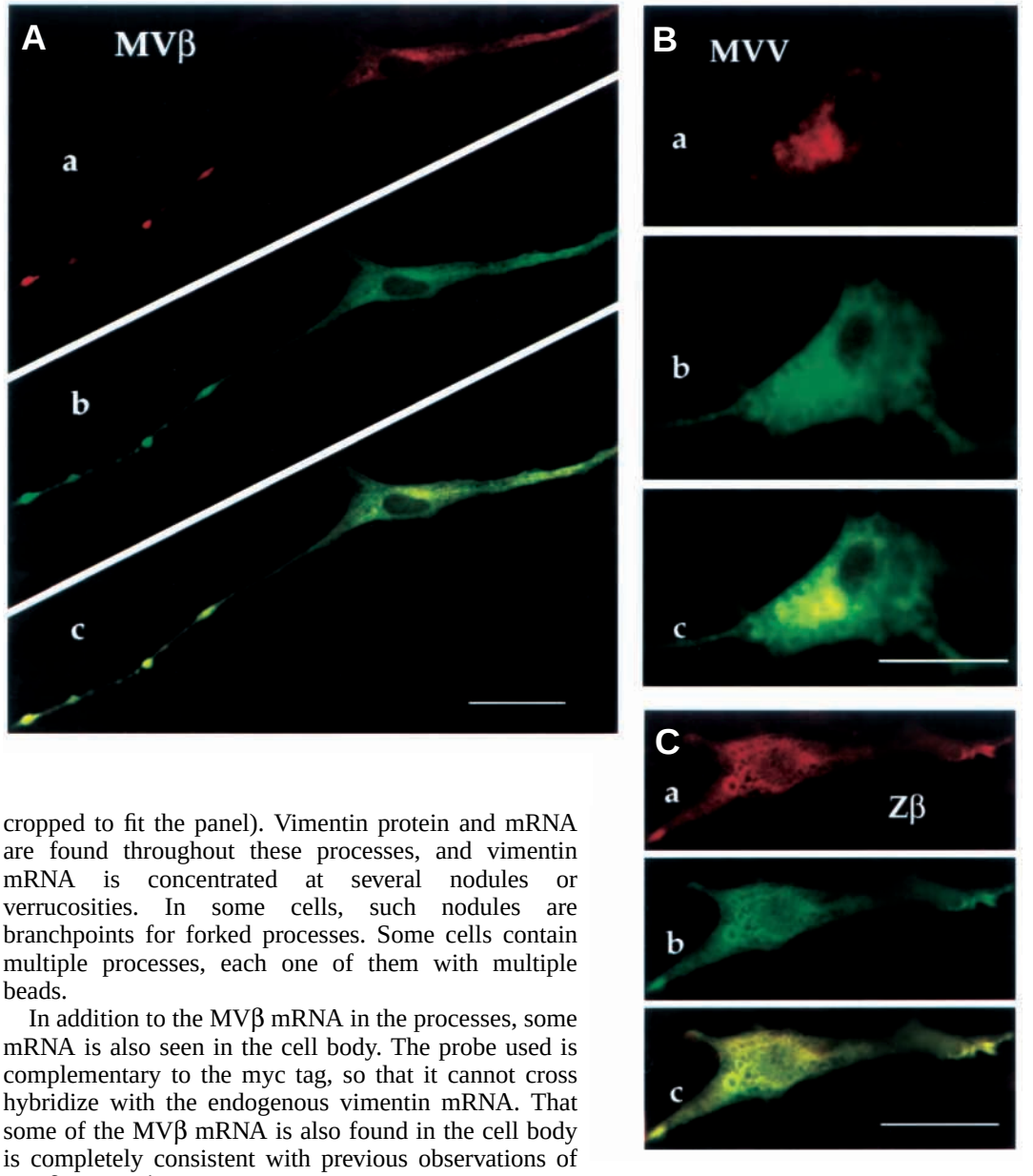

Fig. 6. Location of mRNA and vimentin protein. Each panel is labeled with the plasmid being expressed. Red images show in situ hybridization to display mRNA; green images show protein staining by immunofluorescence. Fibroblasts expressing misdirected vimentin for 48 hours (A,a,b,c), stained green with antibodies to the myc tag on vimentin protein, show multiple long processes. The mRNA (a, red) is shown by in situ hybridization to the myc tag; only the plasmid derived mRNA is labeled. Expression of MVβ leads to long cell processes, beaded with verrucosities that contain both protein and mRNA. In control cells expressing MVV (B,a,b,c), mRNA is largely confined to the pericentrosomal region; cells have few, short processes. In control cells expressing Zβ (C,a,b,c), protein and mRNA are largely colocalized by 48 hours, with some in cell extremities; cell processes are rarely longer than a cell diameter. Bar, 50 μm.

cropped to fit the panel). Vimentin protein and mRNA are found throughout these processes, and vimentin mRNA is concentrated at several nodules or verrucosities. In some cells, such nodules are branchpoints for forked processes. Some cells contain multiple processes, each one of them with multiple beads.

In addition to the MVβ mRNA in the processes, some mRNA is also seen in the cell body. The probe used is complementary to the myc tag, so that it cannot cross hybridize with the endogenous vimentin mRNA. That some of the MVβ mRNA is also found in the cell body is completely consistent with previous observations of the β-actin 3′ UTR. In other studies, it peripherally localizes the mRNA in one to two thirds of the cells at 18 hours after transfection, and even in those cells some of the actin mRNA remains in the cell body (Kislauskis et al., 1993). Therefore, its ability to localize mRNA to the periphery is not absolute.

Table 2. Statistical analysis of alterations in mobility after transfection

Time after transfection (hours)	Plasmid 1	Plasmid 2	U (2 slower than 1)	p (2 slower than 1)
48	MVV	MVβ	166 (n=14,17)	0.025<P<0.05
48	Zβ	MVβ	188 (n=14,15)	P<0.001
48	Zβ	MVV	179.5 (n=17,15)	0.025<P<0.05
72	MVV	MVβ	185.5 (n=16,15)	0.001<P<0.005
72	Zβ	MVβ	170 (n=16,13)	0.001<P<0.005
72	Zβ	MVV	121.5 (n=15,13)	Not significant

Table shows for each plasmid (controls Zβ and MVV, and the misdirected MVβ) the statistical analysis of the differences observed. The differences between MVβ and the other plasmids are highly significant at all times.

By contrast, the cell shapes for both the MVV and Zβ transfected cells are largely unaffected. Cells transfected with the MVV construct develop short and relatively few processes, similar to those displayed by cells transfected with Zβ. In cells transfected with MVV, the transgenic MVV mRNA is found in two patterns. In roughly a third, the MVV mRNA is distributed more or less evenly throughout the cytoplasm, while the relatively short cell extensions remain nearly devoid of MVV mRNA. In the other two-thirds of the cells, a concentration of MVV mRNA around the cell center toward one side of the nucleus is seen, similar to that previously described for endogenous vimentin mRNA in chicken fibroblasts (Fulton and Alftine, 1995).

The example shown in Fig. 6A was chosen because it has several more processes than a typical cell transfected with

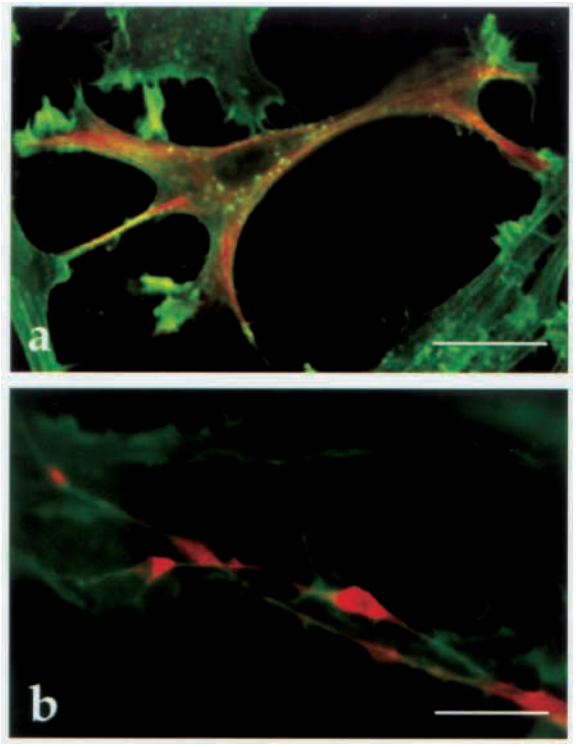

Fig. 7. Distribution of microfilaments after transfection with MVβ. Green shows phalloidin staining for actin; red shows transfected protein staining by immunofluorescence. (a) Actin protein is especially prominent at the cell periphery; myc tagged vimentin expressed from MVβ extends out most of the way into the cell processes, but is not as distal as the actin. Bar, 50 μm. (b) A higher magnification image show that actin protein is present in the cell processes, but less abundant at the nodules or verrucosities; myc tagged vimentin (red) expressed from MVβ is especially abundant at the verrucosities that are rich in MVβ mRNA (seen in Fig. 6A). Bar, 10 μm.

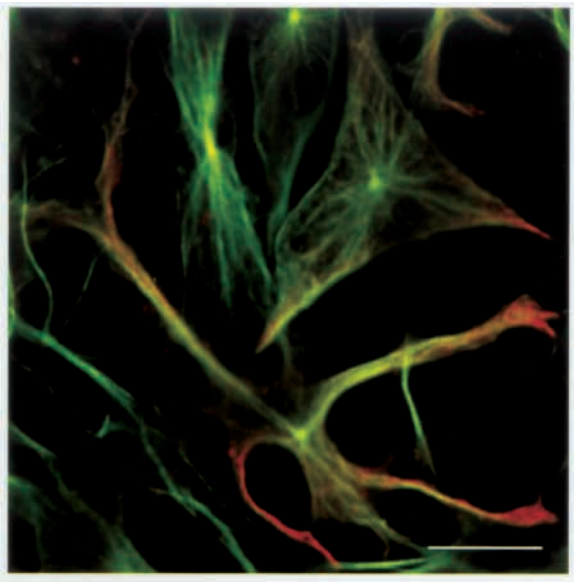

Fig. 8. Distribution of microtubules after transfection with MVβ. Green shows antibody staining for tubulin; red shows transfected protein staining by immunofluorescence. Tubulin protein is especially prominent at the cell center, with a classical radial organization; myc tagged vimentin expressed from MVβ extends most of the way into the cell processes, past the microtubules. Cell at lower left shows extensive processes containing abundant myc tagged vimentin and little tubulin; cell at upper right has a more normal morphology but still shows vimentin that is more peripheral than the microtubules. Several untransfected cells in the field stain only green. Bar, 50 μm.

MVV; yet it still shows the more common mRNA pattern in which vimentin messenger RNA is concentrated near the cell center. This indicates that it is not the numbers of processes that leads to redistribution of vimentin message, but rather the 3′ UTR on the messenger RNA.

In additional control experiments, the Zβ mRNA targeted by the β-actin 3′ UTR is found both out at the periphery and in the cell interior (Fig. 6), but the cell shape remains normal. Thus, simply concentrating extra mRNA at the cell periphery is not sufficient to alter cell shape or motility. In addition, neither does occupying some of the actin mRNA binding sites alter cell shape.

Reorganization of cytoskeletal proteins in embryonic fibroblasts

The long processes that develop in cells expressing the MVβ construct clearly contain the transgenic vimentin protein. They also contain endogenous vimentin protein (not shown). It was of interest to us to determine whether other cytoskeletal elements are found in the cell processes. When cells expressing MVβ are stained to reveal both actin protein and the transgenic vimentin, actin is always found more distally than is the vimentin (Fig. 7). Actin is especially abundant in the small ruffles at the cell edge, where vimentin is absent (Fig. 7a). Actin is also found in the processes between the verrucosities, with relatively little at the verrucosity itself (Fig. 7b). In cells that do not have long cell processes, actin is found in ruffles at the cell periphery, with vimentin protein lying just behind it. Overall, actin organization is similar in cells expressing either MVβ or MVV (not shown), with abundant actin at ruffles and a variable number of stress fibers.

When transfected cells are double-stained for microtubules and for the transgenic protein, it is clear that the vimentin protein extends past the microtubules into the cell processes, sometimes for considerable distances past the end of the microtubules. In the example shown in Fig. 8, the cells were transfected 24 hours earlier with MVβ. The cell at the upper right has not at this time developed long processes; yet vimentin protein extends past the ends of the microtubules. In the cell at lower left, several long processes have already formed. The vimentin is more distal in these processes, while the microtubules are found principally in the proximal halves.

Altered morphology in SW13 cells with misdirected vimentin

Alterations of cell morphology were not limited to chick fibroblasts. The misdirected vimentin construct was also expressed in SW13 human adrenocarcinoma cells. In the strain of SW13 cells that also expresses an endogenous vimentin gene, MVβ induced many long processes, similar to those that developed in fibroblasts. However, in SW13 cells that did not express endogenous vimentin, MVβ induced processes, but

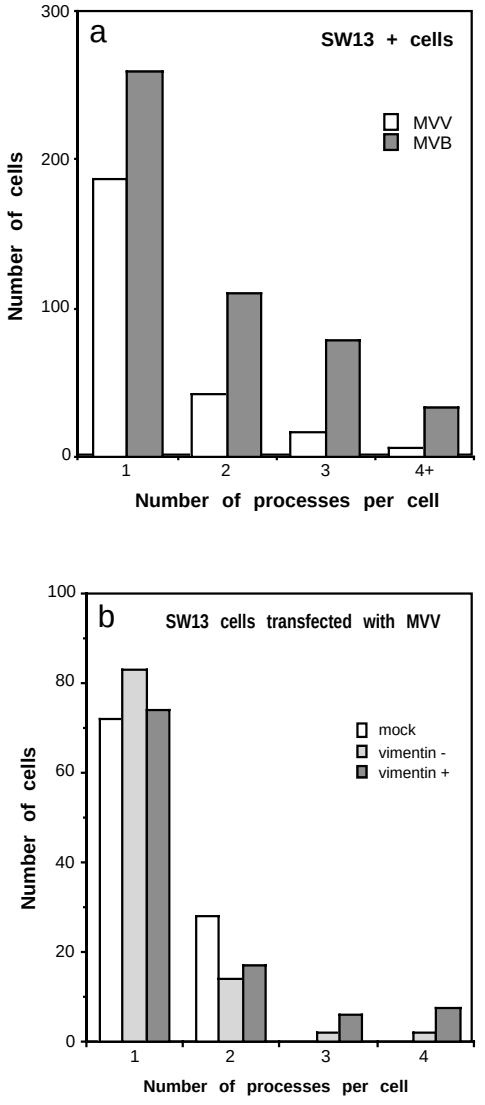

Fig. 9. SW13 cell shape after transfection with MVβ. (a) Number of cell processes in SW13 vimentin$^+$ cells after transfection with either the misdirected MVβ or correctly localized MVV; cells expressing MVβ have more processes than do cells expressing MVV. (b) Number of processes on SW13 cells having or lacking endogenous vimentin after the cells express the appropriately localized MVV; none show excess processes compared to the mock transfected control.

these processes rarely exceeded one or two cell diameters in length and were less frequent (Fig. 9, top graph).

In contrast, vimentin expressed from a correctly located mRNA (MVV) did not induce extra processes. This was the case both in cells that contain some endogenous vimentin protein (vimentin + in the graph) and in the cells lacking vimentin (vimentin – in the graph).

The altered morphology of the SW13 cells expressing MVβ suggested that mobility might also be affected. However, SW13 cells move more slowly than do the embryonic fibroblasts used above, and it proved technically impossible to quantitate their movement. Qualitatively, the cells expressing misdirected vimentin appeared to move more slowly than those expressing MVV or Zβ.

DISCUSSION

What properties of vimentin might make the site of its assembly so important? Vimentin filaments are elastic and highly conserved components of vertebrate cells (Goldman et al., 1996). Vimentin filaments confer unique mechanical properties in vitro to mixtures that include microfilaments and microtubules. Vimentin null mice have defects in the assembly of glial fibrillary acidic protein (Galou et al., 1996), among other deficits, and subtle defects in wound healing (Eckes et al, 1998). Vimentin protein assembles readily into filaments in vitro (Herrmann et al., 1996; Traub et al., 1992; Steinert et al., 1993); however, such filaments are somewhat variable in diameter compared to naturally formed filaments and form so readily that the intermediate steps of assembly are difficult to observe.

Several laboratories have studied vimentin assembly in vivo by microinjecting labeled vimentin protein into a variety of cell types. Such microinjected proteins assemble into filaments, often after passing through structural intermediates such as aggregates in the more distal cytoplasm. However, microinjection studies involve a transient phase with an elevated free monomer concentration. It is difficult to assess how this transient difference in monomer concentration may affect vimentin assembly or dynamics.

Others have studied vimentin assembly in vivo either by expressing transfected vimentin constructs or by fusing cells expressing different vimentin proteins. When vimentin is expressed in cells already containing endogenous vimentin, the newly synthesized vimentin tends to assemble in conjunction with pre-existing vimentin (Ngai et al., 1990). By contrast, if vimentin is expressed in cells devoid of other vimentin protein, the earliest expression is in the distal cytoplasm as isolated rosettes of filaments (Holwell et al., 1997). These come over time to elongate and consolidate in the region of the centriole until a normal vimentin network appears to have assembled.

A different approach fused cells expressing either mouse or human vimentin. In these heterokaryons mouse and human filaments remained distinct for many hours and had largely separate locations at 5 hours. Even at 15 hours individual filaments remained largely as homopolymers when viewed by electron immunochemistry (Avsiuk et al., 1997). This implies that endogenous vimentin, when assembled by the cell in the absence of an additional soluble pool, has relatively slow dynamics. Vimentin's principal mode of redistribution appears to be translocation of intact filaments rather than disassembly and reassembly.

New insights into vimentin filament dynamics in real time came from labeling the amino terminus of vimentin with green fluorescent protein (GFP). In some cell types, GFP-vimentin, expressed stably, cannot assemble into filaments by itself but is capable of coassembly into filaments in the presence of normal vimentin (Ho et al., 1998). In other cell types, during transient transfections, high levels of GFP-vimentin do assemble into filamentous structures without normal vimentin present; these filaments over time acquire largely normal appearing patterns of organization (Prahlad et al., 1998; Yoon et al., 1998). In both cases, video microscopy of the GFP labeled vimentin shows an extensive network in which the majority of components rearrange by translocation (Ho et al., 1998), although a minor fraction appear or vanish within 5 to 10 minutes (Prahlad et al., 1998).

Other requirements for vimentin assembly are clarified by in vitro studies of vimentin from which the carboxy terminus has been deleted (Eckelt et al., 1992; Herrmann et al., 1996). These show that the amino-terminal domain with most of the rod domain can form extended filamentous structures that resemble normal vimentin filaments except for a slight increase in diameter. These in vitro studies confirm that the assembly capability of vimentin is largely contained in the amino-terminal and rod domains, and that residues in the carboxy terminus may regulate diameter and lateral interactions. These in vitro studies did not examine the assembly of copolymers of the carboxy-terminal deletions with full-length vimentin protein. Such coassembly would be highly relevant to the mechanisms of cotranslational assembly.

All of these studies of assembly, both in vitro and in vivo, provide a new and rich context in which to interpret earlier studies of vimentin assembly in vivo (Isaacs et al., 1989). These studies indicated that a fraction of vimentin polypeptides can assemble into filaments during translation. The fraction of peptides that assembled during translation varied from about 10% in red blood cells to about 50% in fibroblasts and muscle cells. This assembly by nascent peptides was called 'cotranslational assembly' and the nascent peptides assembling in this way displayed the same biochemical characteristics of assembly (high salt resistance, sensitivity to arginine) as full length vimentin (Isaacs et al., 1989). Several cytoskeletal proteins appear to assemble this way, but to varying extents (Fulton and L'Ecuyer, 1993). Recently, structural predictions about the protein properties needed to permit such assembly were tested. Elongated proteins or proteins associating longitudinally on elongated structures were identified as potential candidates; tropomyosin, an elongated protein binding to the elongated thin filament proved to assemble during translation to a significant extent (L'Ecuyer et al., 1998).

Necessarily, for polypeptides that undergo their first assembly event as nascent peptides, the site of messenger RNA will be an important aspect of their assembly. For that reason, vimentin messenger RNA location was studied. Vimentin message is localized near the centriole in fibroblasts and reorganizes during cell orientation to remain with the centriole during orientation (Fulton and Alftine, 1995). Vimentin message becomes highly localized in developing muscle cells (Cripe et al., 1993). Its most highly ordered pattern consists of stripes of less than one-half micron in width at the spacing of the sarcomere; this implies a high degree of precision, specificity, and developmental control in message positioning (Morris and Fulton, 1994). A region in the vimentin 3′ UTR (a common site for localization signals) has a highly conserved protein binding domain (Zehner et al., 1997) but its function is not known.

These observations of message position and the previous biochemical observations of cotranslational assembly led to questions about the functional significance of message position. This was addressed directly by generating vimentin messenger RNAs that were mislocalized through the β-actin 3′-UTR. This 3′-UTR directs heterologous messages towards the anterior end of the cell, away from the normal position of vimentin messenger RNA in fibroblasts (Kislauskis et al., 1994).

A likely explanation for the shape changes in cells expressing MVβ is that, after the misdirected vimentin mRNA is transcribed, it is localized to the normal position of actin mRNA, and vimentin is synthesized at the cell periphery. The normal site for vimentin synthesis in chick fibroblasts is near the cell center, around the centrosome (Fulton, 1993). However, in transfected cells expressing misdirected vimentin mRNA a new population of vimentin protein is generated at the cell periphery. This novel depot of vimentin protein may provide a binding site for endogenous vimentin mRNAs, which subsequently become localized increasingly at the cell periphery, leading to yet more vimentin synthesis at the periphery. This would provide a self-amplifying process of misorganization leading to the time dependent phenotype observed.

However, it proved impossible to test this model directly. We attempted to use in situ hybridizations to detect the endogenous vimentin messenger RNA in transfected cells; however, the transgenic vimentin gene, which encodes mouse vimentin, is sufficiently similar to the endogenous chicken gene that, under all conditions that we tested, the transgenic vimentin mRNA also hybridized to our probes. Therefore it was impossible to test this autocatalytic model explicitly.

Is it possible that the changes in cell shape and mobility are due to different levels of vimentin protein in cells expressing MVβ and MVV? This possibility can be rejected for several reasons. First, the two mRNAs differ only in the 3′ UTR, so the stability of the two messages should resemble the sources of the 3′ UTR; these two mRNAs differ insignificantly in half-life, with probably the longer half-life for vimentin mRNA. Second, the presence of long processes did not correlate directly to intensity of staining; some brightly staining MVV cells lacked long processes, while some MVβ cells had processes even though less intensely stained. Third, there were qualitative differences between the two plasmids. Expression of MVV never lead to the highly branched or winglike processes seen sometimes with mislocalized vimentin mRNA, nor did cells with MVV have the nodules of protein seen with MVβ. Fourth, in a different cell type, SW13, MVV did not produce any excess of processes; MVβ only caused processes in cells that also contained endogenous vimentin. Finally, numerous investigators have now expressed additional vimentin in a variety of cell types (Ho et al., 1998; Yoon et al., 1998; Prahlad et al., 1998) and did not observe the processes seen with MVβ.

Does the cotranslational assembly of vimentin greatly limit the significance of these findings? We do not believe so for three reasons. First, only about fifty percent of vimentin peptides assemble during translation; the others assemble afterwards. Second, several cytoskeletal proteins are known to undergo cotranslational assembly (including titin, myosin and tropomyosin), and others are likely to do so. Third, localized messages may be functionally significant in other ways for assembly. For instance, it is the formation of actin nuclei that is the limiting step for polymerization; perhaps localization of actin message increases the local concentration of trimeric nuclei. It may be possible to force vimentin to assemble after translation, by removing a hairpin in the coding region that causes pausing. If so, it will be possible to test explicitly whether message position for vimentin is significant only for cotranslationally assembled protein.

We present here the first case in which an altered phenotype is observed in cells after misdirection of mRNA for a

cytoskeletal protein. The phenotype observed is dominant negative, as it is observed even in cells containing normal mRNAs. The observations on SW13 cells with and without vimentin suggest that the phenotype is amplified by the presence of normal mRNAs. This strong phenotype may directly reflect vimentin's role in motility in this cell type. Because there are no specific drugs active against vimentin, the mechanistic contribution of vimentin in motility is not understood, and may vary depending upon cell type. Several investigators did not detect gross qualitative differences in motility in cells where vimentin was aggregated with antibodies (Klymkowsky, 1981; Gawlitta et al., 1981); no quantitative differences in motility were seen in vimentin null cells expressing vimentin under tetracycline control (Holwell et al., 1997).

However, other investigators have seen vimentin contribute to motility in normal cells (Eckes et al., 1998; Azzarone and Macieira-Coelho, 1984), the enhanced motility of transformed cells (Chu et al., 1996), and the reduced motility of aging cells (Wang, 1985). Since the mechanism of vimentin's contribution is not known, we cannot explicitly connect the observed phenotype to the mechanism. Perhaps the changes in motility seen here are another example of a dominant negative phenotype being more pronounced than a null phenotype.

Several other cytoskeletal mRNAs are highly localized. For one of them, β-actin, delocalization of mRNA leads to loss of cell polarity (Kislauskis et al., 1994). Taken with these results, this strongly suggests that for all cytoskeletal mRNAs that are highly localized, mislocalization would result in altered cellular phenotypes. This suggests that mRNA localization is as essential for the normal maintenance of somatic cell phenotypes as mRNA localization is already known to be in embryogenesis.

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