Suppression of Vinculin Expression by Antisense Transfection Confers Changes in Cell Morphology, Motility, and Anchorage-Dependent Growth of 3T3 Cells

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Abstract. The expression of vinculin, a major component of adhesion plaques and cell-cell junctions, is markedly modulated in cells during growth activation, differentiation, motility and cell transformation. The stimulation of quiescent cells by serum factors and the culturing of cells on highly adhesive matrices induce vinculin gene expression, whereas the transformation of fibroblast and epithelial cells often results in decreased vinculin expression (reviewed in Rodríguez Fernández, J. L., B. Geiger, D. Salomon, I. Sabanay, M. Zöller, and A. Ben-Ze'ev. 1992. *J. Cell Biol.* 119:427). To study the effect of reduced vinculin expression on cell behavior, 3T3 cells were transfected with an antisense vinculin cDNA construct, and clones displaying decreased vinculin levels down to 10-30% of control levels were isolated. These cells showed a round phenotype with smaller and fewer vinculin-positive plaques localized mostly at the cell periphery. In addition, they displayed an increased motility compared to controls, manifested by a faster closure of "wounds" introduced into the monolayer, and by the formation of longer phagokinetic tracks. Moreover, the antisense transfectants acquired a higher cloning efficiency and produced larger colonies in soft agar than the parental counterparts. The results demonstrate that the regulation of vinculin expression in cells can affect, in a major way, cell shape and motility, and that decreased vinculin expression can induce cellular changes reminiscent of those found in transformed cells.

MICROFILAMENTS are major cytoskeletal components of eukaryotic cells involved in determining cell shape, dynamics, and adhesion. At cell junctions, either with other cells, or with the extracellular matrix (ECM),[1] microfilaments are linked to transmembrane receptors of the cadherin and integrin families by cytoplasmic adherens junction (AJ) plaque proteins (Burridge et al., 1988; Geiger and Ginsberg, 1991; Tsukita et al., 1992). Vinculin is a major and ubiquitous, AJ plaque component (Geiger, 1979, 1989; Burridge et al., 1988), whose complete sequence (Cotou and Craig, 1988; Price et al., 1989) and the protein domains involved in its interaction with other junctional proteins were determined (Bendori et al., 1989; Jones et al., 1989; Westmeyer et al., 1990).

While vinculin belongs to the group of "household" structural proteins, its expression may be modulated in a variety of cell types in culture and in vivo. For example, cultured fibroblasts regulate vinculin mRNA and protein synthesis in response to changes in cell contacts and shape (Ungar et al., 1986; Bendori et al., 1987). Vinculin organization and expression are also regulated during differentiation of a variety of cell types including granulosa cells (Ben-Ze'ev and Amsterdam, 1987), adipocytes (Rodríguez Fernández and Ben-Ze'ev, 1989), smooth muscle cells in culture (Belkin et al., 1988), and migrating epithelial cells in vivo (Zieske et al., 1989). In addition, vinculin organization (Ridley and Hall, 1992) and vinculin gene expression are rapidly and transiently stimulated in quiescent 3T3 cells after serum-factor stimulation (Ben-Ze'ev et al., 1990; Bellas et al., 1991), and in regenerating rat liver (Glück et al., 1992). Moreover, the level of vinculin is reduced or even undetectable in certain malignant cells of fibroblastic and epithelial origin which also have a diminished microfilament system (Raz et al., 1986; Rodríguez Fernández et al., 1992a). The molecular basis for the regulation of vinculin expression and its relationship to the observed cellular changes are still largely elusive (Ben-Ze'ev, 1991).

The possible physiological significance of the regulation of vinculin levels was recently addressed by the overexpression of vinculin in normal and transformed cells, followed by examination of the consequent effects on cell behavior (Rodríguez Fernández et al., 1992a, b). Increased vinculin expression in 3T3 cells resulted in extensive spreading with abundance of stress fibers and adhesion plaques (Geiger et al., 1992a), accompanied by a dramatic decrease in cell mo-

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1. *Abbreviations used in this paper:* AJ, adherens junction; ECL, enhanced chemiluminescence; ECM, extracellular matrix.

tility (Rodríguez Fernández et al., 1992b). Elevation of vinculin expression in malignant fibroblastic and epithelial cells which express diminished levels of vinculin, resulted in the suppression of the tumorigenic ability and anchorage independence of these cells (Rodríguez Fernández et al., 1992a).

In this study, we examined the effect of a forced reduction in vinculin levels on the structural and kinetic properties of nontransformed cells. The level of vinculin was specifically suppressed in 3T3 cells by antisense vinculin cDNA transfection, and clones stably expressing vinculin at levels between 10–70% of those detected in 3T3 cells were isolated. We show here that cells with low vinculin levels are poorly spread, displaying fewer and smaller adhesion plaques. Furthermore, these cells showed increased motility, and produced large colonies in soft agar. These results suggest that decreased vinculin expression in 3T3 cells can affect cell morphology and growth properties in a mode reminiscent of that found in anchorage independent tumor cells.

Materials and Methods

The Antisense Vinculin Expression Vector NIV-pJ4Ω

A 1.06-kb fragment (Ben-Ze'ev et al., 1990), coding for amino acids 198–549 of mouse vinculin, was inserted in the antisense orientation into the expression cassette of the pJ4Ω expression vector (Shaulsky et al., 1991). In this vector, transcription of the cDNA is controlled by the Moloney murine leukemia virus long terminal repeat promoter-enhancer sequence (Mo-MuLV LTR), whereas the SV40 small t antigen intron, and the SV40 T antigen polyadenylation sequences provide signals for RNA processing. This mouse vinculin antisense expression vector was designated NIV-pJ4Ω (Fig. 1).

Cell Culture and Transfection

Balb/C 3T3 clone A31 cells were grown in DME plus 10% calf serum (Gibco Laboratories, Grand Island, NY). The cells were cotransfected with the NIV-pJ4Ω vector and with a neomycin resistant gene (neo^r) by the calcium phosphate precipitation method and colonies resistant to 400 μg/ml G-418 (Geneticin; Gibco Laboratories) were isolated.

Growth in semisolid medium was determined by seeding 10^4 cells per 35-mm diam dish in 0.85 ml of medium containing 10% serum and 0.3% bacto-agar (Difco, Detroit, MI) on top of a solid layer of 2.5 ml of 0.5% bacto-agar in the same medium. Plates were incubated for 2 wk at 37°C in a 9% CO_2-containing humidified incubator. The number of colonies containing >50 cells was determined microscopically.

Immunofluorescence

Cells were grown on glass coverslips, fixed with 3.7% paraformaldehyde in PBS and permeabilized with 0.5% Triton X-100. Vinculin was detected with a broad-range monoclonal antivinculin antibody (antihuman vinculin, Sigma Immunochemicals, St. Louis, MO) as described previously (Bendori et al., 1989). The second antibody was rhodamine-labeled goat antimouse IgG. Actin filaments were stained with FITC-phalloidin (Sigma Chemical Co., St. Louis, MO). The cells were examined with a Zeiss Axiophot microscope.

Cell Motility and Phagokinetic Tracks

Cells were grown to confluence in 24-well dishes. A wound was introduced in the monolayer by the 1 mm-thick tip of a plastic scriber. The medium was changed, and at various times after addition of fresh medium, cells were fixed and stained with Giemsa. The number of migrating cells into a 1-mm² area of the wound was microscopically counted in 5–13 randomly picked areas along the wound for each cell type, as described (Rodríguez Fernández et al., 1992b).

To measure the phagokinetic tracks, coverslips treated with a 1% solution of BSA were coated with colloidal gold (Sigma Immunochemicals) according to Albrecht-Buehler (1977). One thousand cells were seeded on each 20 × 20 mm coverslip and after 24 h of incubation the cells were fixed with

paraformaldehyde (3.7%). The tracks produced by cells were viewed by darkfield microscopy with a 5× objective. The lengths of tracks were determined by projecting individual tracks on a screen, and measuring about 30 randomly selected tracks for each cell type. Differences between means were tested for significance using the Dunnet test (Dunnet, 1955).

PAGE and Immunoblotting

Cells plated on 35-mm diam culture dishes were processed for immunoblotting as described (Harlow and Lane, 1988). Equal amounts of protein were separated by SDS-PAGE under reducing conditions (Laemmli, 1970). Proteins were transferred to nitrocellulose by electroblotting, and vinculin was detected on the blots with a monoclonal antivinculin antibody, followed by anti mouse IgG coupled to an enhanced chemiluminescent probe (ECL, Amersham Corp., Arlington, Heights, IL). Several exposures of the blots to x-ray films were quantitated by a laser densitometer as described (Rodríguez Fernández et al., 1992a).

Two dimensional (2-D) gel electrophoresis of [^{35}S]methionine-labeled cell lysates, using equal amounts of total radioactive proteins in each sample, was performed as previously described (Ben-Ze'ev, 1990).

Southern and Northern Blot Hybridization, and RNAse Protection

The isolation of DNA, restriction endonuclease digestion with EcoRI, 1% agarose gel electrophoresis and Southern blotting onto nitrocellulose membranes (Schleicher and Schuell, Inc., Keene, NH) were performed as described (Sambrook et al., 1989). The ^{32}P-labeling of the probe and hybridization and washing conditions were as described (Rodríguez Fernández et al., 1992a).

Total RNA was extracted by the guanidinium thiocyanate method (Chirgwin et al., 1979). 20 μg of total RNA per lane were separated by electrophoresis on 1.2% agarose gels and 2.2 M formaldehyde gels, transferred to nitrocellulose membranes, and hybridized to ^{32}P-dCTP labeled mouse vinculin and GPDH cDNAs.

RNAse protection was carried out as described by Gilman (1987), with slight modifications. The 1 kb EcoRI mouse vinculin cDNA fragment (Ben-Ze'ev et al., 1990) was inserted into the Bluescript KS11 plasmid (New England Biolabs, Beverly, MA). The orientation of the insert was determined by restriction analysis. The plasmid was linearized with NaeI, and used as template to synthesize the vinculin RNA probe by T7 RNA polymerase. DNase I was used to digest the remaining DNA template. Detection of vinculin antisense RNA transcripts was obtained from total RNA samples hybridized to 5 × 10^5 cpm of labeled probe as follows: the RNA was dissolved in hybridization buffer (80% formamide, 40 mM Pipes pH 6.4, 400 mM NaCl and 1 mM EDTA), denatured at 85°C for 10 min, and hybridized at 42°C overnight. RNAase digestion buffer containing RNAase A (40 μg/ml) and RNAase T1 (2 μg/ml) were added to degrade the unhybridized probe at 37°C for 30 min. RNAases were inactivated by incubating at 37°C with SDS and proteinase K. The RNA was extracted with phenol/chloroform and was separated by electrophoresis on 4% acrylamide gels containing 8 M urea.

Results

Effects of Transfection with Antisense Vinculin cDNA Construct on Vinculin Levels in 3T3 Cells

Balb/C 3T3 cells were transfected with expression vectors designed to produce either the sense or the antisense RNA from a partial mouse vinculin cDNA (Fig. 1), and with the neomycin acetyl transferase gene (neo^r). A total of 50 clones resistant to G418 were isolated after transfection with the antisense vinculin construct, and 10 clones with the sense vinculin construct. The level of vinculin in the various clones was determined by Western blotting with a monoclonal antivinculin antibody of gels loaded with equal amounts of total cell protein. In ∼20% of the clones obtained by antisense vinculin transfection, the level of vinculin was reduced to 10–30% (Fig. 2, lanes 1, 2, 12–14, and Table I), of normal 3T3 levels (Fig. 2, lanes 7 and 8). Clones obtained
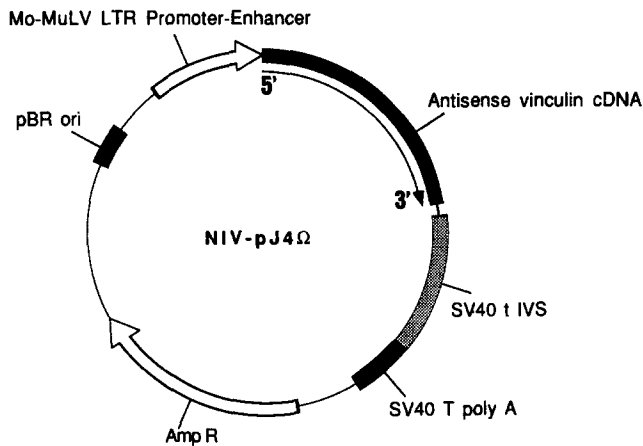
Figure 1. Structure of the mouse vinculin antisense expression vector, NIV-pJ4Ω. The expression vector contains a 1 kb Eco RI fragment of the mouse vinculin cDNA (Ben-Ze'ev et al., 1990) which was inserted in an antisense orientation into the mammalian expression vector pJ4Ω. Transcription of this insert is driven by the Mo-MuLV LTR promoter-enhancer. The SV40 small t antigen intron (*IVS*) and SV40 large T polyadenylation (*poly A*) signal provide RNA processing signals.

by transfection with the vector containing the sense vinculin construct and clones transfected with the neo^r gene had the same level of vinculin as control 3T3 cells. The level of vinculin synthesis by clones obtained after antisense transfection was determined by two-dimensional gel electrophoresis of equal amounts of [^{35}S]methionine pulse labeled proteins (Fig. 3). This analysis suggested that the decrease in vinculin content in the clones shown in Fig. 2 was attributable to a decrease in the synthesis of vinculin (Fig. 3, *B–E*, compare to 3, *A–F*). In addition, Northern blot hybridization with vinculin cDNA showed a comparable decrease in the ~7 kb vinculin mRNA in antisense transfectants (Fig. 6 *I*, compare lanes *4* and *5* with lane *1*).

Transcription of the Antisense Vinculin cDNA

Southern blot hybridization of EcoRI-digested genomic DNA from the antisense transfected clones, using the 1-kb vinculin cDNA probe demonstrated the presence of the transfected vinculin cDNA fragment in the genome of these clones in which vinculin expression was reduced. In control neo^r clones, this vinculin DNA fragment was either absent, or reduced in size (results not shown).

Expression of the mouse vinculin antisense RNA was determined by an RNAse protection assay which generated, after hybridization, a 1 kb protected fragment (Fig. 4 *A*) when hybridized to a 1-kb sense RNA probe synthesized in vitro by T7 polymerase. This 1 kb RNAse protected fragment was present in several individual clones which were shown to have low levels of vinculin (Fig. 4 *A*, lanes *1–3*), but not in a neo^r control clone (Fig. 4 *A*, lane *4*), or when hybridized to tRNA (Fig. 4 *A*, lane *5*). This shows that the antisense cDNA was transcribed into a stable antisense RNA. The presence of antisense RNA in clones with diminished vinculin levels was further suggested by Northern blot analysis showing a very abundant ~2.1 kb vinculin RNA in these clones (Fig. 4 *B*, lanes *2*, *3*, *5*, and *6*), but not in neo^r controls (Fig. 4 *B*, lanes *4* and *7*), or in 3T3 (Fig 4 *B*, lane *1*).
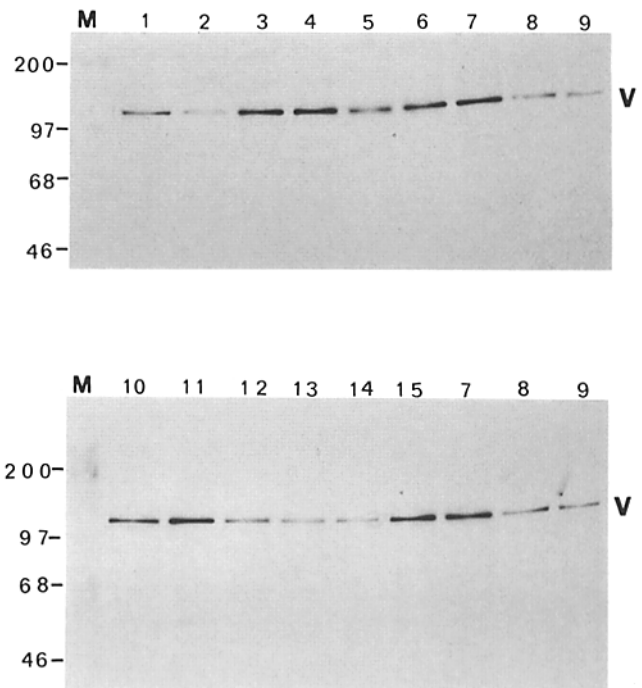

Figure 2. Western blot analysis of cell extracts of Balb/C 3T3 clones transfected with NIV-pJ4Ω. Equal amounts of total cell protein lysates were separated by electrophoresis and blotted onto nitrocellulose. The level of vinculin was determined by quantitative immunoblotting with a broad range antivinculin antibody followed by enhanced chemiluminescence detection with the anti mouse IgG. The following clones were analyzed: (Lane *1*) C51; (lane *2*) C61; (lane *3*) C11; (lane *4*) D31; (lane *5*) D41; (lane *6*) D51; (lane *7*) 3T3 cells; (lane *8*) 3T3 cells 1/4 the amount of protein; (lane *9*) SV40-transformed 3T3 (SVT2) cells; (lane *10*) A11; (lane *11*) A41; (lane *12*) A51; (lane *13*) B51; (lane *14*) B61; (lane *15*) C41. *v*, vinculin; *M*, molecular weight markers.

Table I. Morphology, Motility, and Growth Properties of Antisense-Vinculin Transfected Clones

Cell line	Vinculin levels % of 3T3*	Morphology on plastic	Colonies in agar/10⁴ cells‡	Migration cells/mm²§
3T3	100	flat	30	180 ± 20
D31	108.3	flat	ND	ND
A41	106.7	flat	4	210 ± 60
C11	102.7	flat	ND	150 ± 60
C41	98.8	flat	20	ND
D51	78.9	flat	ND	ND
A11	69.6	flat	ND	ND
D41	58.4	flat	6	ND
C51	43.4	flat	ND	ND
A51	31.6	round	188	400 ± 80
B51	16.6	round	575	460 ± 100
B61	11.0	round	143	360 ± 50
C61	10.3	round	113	ND

* The levels of vinculin were determined by quantitative computerized scanning of the immunoblots shown in Fig. 2. The level of vinculin in 3T3 cells was taken as 100%.

‡ The number of colonies containing >50 cells was determined 2 wk after seeding 10⁴ cells (in duplicates) in agarose-containing medium.

§ Cell motility was determined by counting the number of cells which migrated into an area of 1 mm² 16 h after introducing a "wound" into a confluent monolayer.
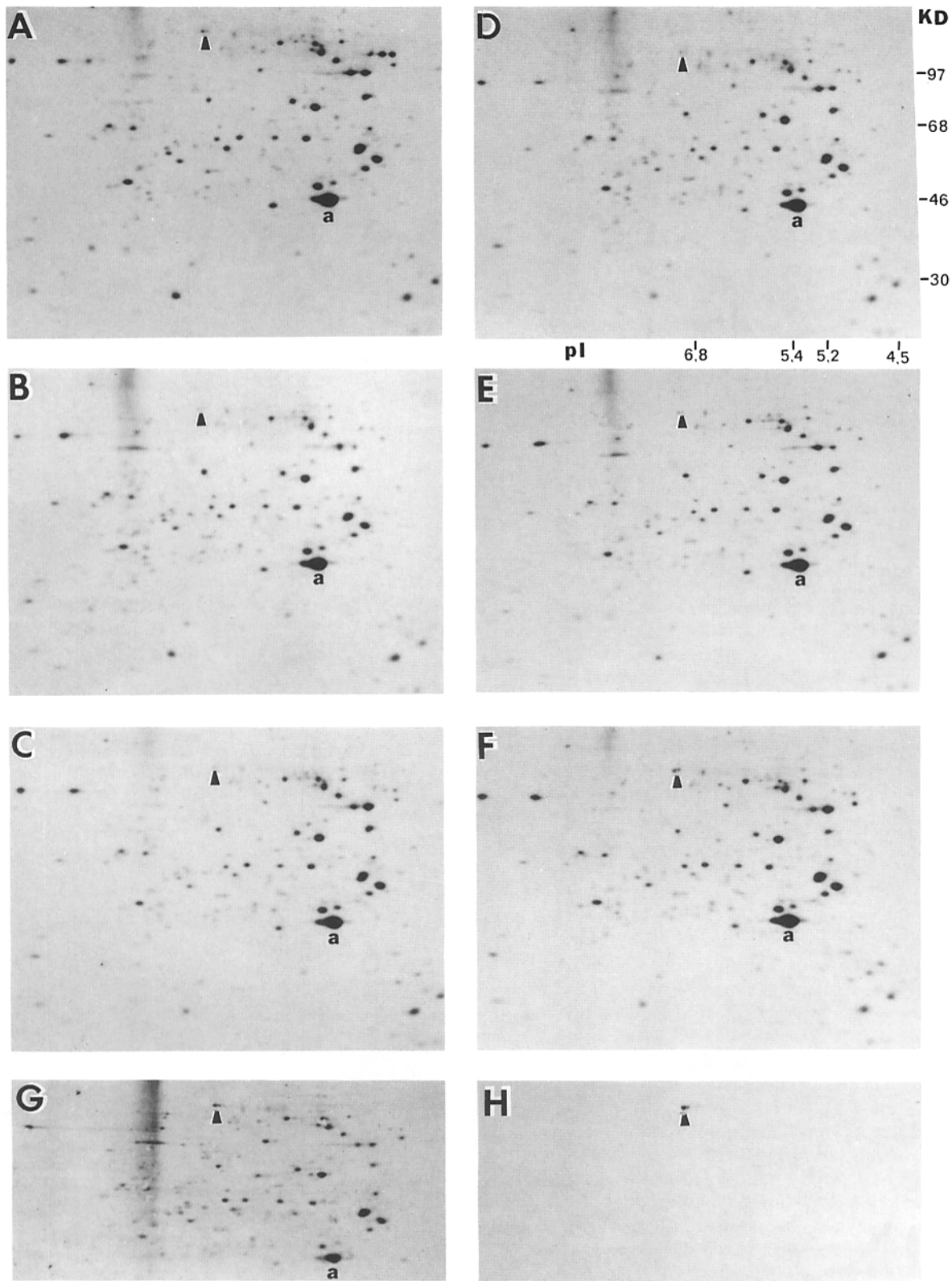
Figure 3. Diminished synthesis of vinculin in antisense vinculin transfected cells. Cells were labeled for 3 h with 100 μCi/ml of [³⁵S]me-thionine and equal amounts of total radioactive cell protein were analyzed by 2-D gel electrophoresis. The clones analyzed were: (*A*) 3T3 cells; (*B*) B51; (*C*) C61; (*D*) B61; (*E*) A51; (*F*) C11 (neoʳ control). (*G* and *H*) Immunoblot detection of vinculin on 2-D gels; (*G*), autora-diogram of a 2-D blot from [³⁵S]methionine labeled cells of clone C11; (*H*) immunoblot with anti vinculin antibody of the blot shown in *G.* The arrowhead points to vinculin; *a*, actin.

A 1 2 3 4 5
- 1.4
- 1.3
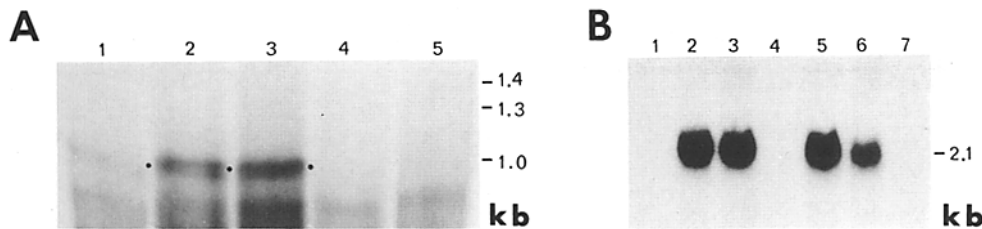- 1.0
k b

B 1 2 3 4 5 6 7
- 2.1
k b

Figure 4. Expression of the antisense vinculin construct. (*A*) A 1 kb EcoRI vinculin cDNA was transcribed and labeled with ^{32}P in vitro using T7 RNA polymerase. 5 × 10^5 cpm of this probe were hybridized in solution with 20 µg of total RNA from the different clones. Unhybridized RNA was digested with ribonucleases A and T1, and the protected RNA was melted and separated by electrophoresis through a denaturing acrylamide-urea gel. The source of RNA was: (Lane *1*) clone B51; (lane *2*) clone B61; (lane *3*) clone C61; (lane *4*) clone D11; (lane *5*) yeast tRNA. (*B*) Total RNA blots containing equal amounts of RNA from 3T3 (lane *1*); and from clones A51 (lane *2*); B51 (lane *3*): C11 (lane *4*); B61 (lane *5*); C61 (lane *6*); C41 (lane *7*) were hybridized with the 1-kb mouse vinculin cDNA probe. In *A*, RNA from antisense transfected clones, showing decreased vinculin, protected an expected 1 kb RNA. In (*B*), a very abundant ∿2.1 kb RNA resulting from transcription of the antisense vinculin RNA and 3′ end SV40 sequences was evident in clones showing decreased vinculin expression.

This RNA apparently resulted from the transcription of the antisense cDNA which terminated at the SV40 T polyadenylation signal (Fig. 1).

Phenotype of the Antisense Vinculin Transfected Clones

Phase contrast microscopic analysis showed that the cells of antisense transfected clones displaying diminished vinculin levels were mostly round and poorly spread on the substrate (Fig. 5, *C*, *E*, and *F*). In contrast, the G418-resistant control clones (Fig. 5 *D*) expressing normal vinculin levels as well as cells transfected with the sense vinculin vector, were well spread and had a characteristic fibroblastic morphology, indistinguishable from that of the parental cells. This rounded phenotype was apparent in clones expressing vinculin at a level ∿30% or lower than that of control 3T3 cells (Table I). Clones expressing vinculin at 45%, and more, of the level in 3T3 cells exhibited an apparently normal morphology and vinculin organization. Immunofluorescence analysis of the clones with low vinculin levels revealed a much reduced number and smaller, vinculin-containing adhesion plaques (Fig. 5 *B*), as compared to neo^r control cells which display a developed adhesion plaque system (Fig. 5 *A*).

It was noticed that after several weeks of passage in culture, the affected clones regained a flat morphology (Fig. 6 *B*) and their microfilament and adhesion plaque system apparently recovered (Fig. 6, *C* and *D*). This reversion was observed with essentially all the clones isolated from two independent transfections. Two dimensional gel electrophoresis of [^{35}S]methionine-labeled cell lysates of these revertants showed that the total levels of vinculin in several such clones was similar to that of 3T3 cells (Fig. 6, *F* and *H*, compare with *E* and *G*), and that vinculin RNA levels reverted from the low level found in the affected clones (Fig. 6 *I* lanes *4* and *5*) to the level found in 3T3 cells (Fig. 6 *I*, lanes *1-3*). The results thus reconfirm the close correlation between the level of vinculin and the effect on the morphology of 3T3 cells.

Motility and Growth Properties of Antisense Vinculin-transfected Cells

Previous studies have shown that an increase in the level of vinculin in 3T3 cells results in reduced motility (Rodríguez Fernández et al., 1992*b*). In tumor cells, vinculin overexpression led to a reduced ability of the cells to form colonies in agar, and suppression in their tumorigenic capability in experimental animals (Rodríguez Fernández et al., 1992*a*).

To examine the effects of decreased vinculin levels in 3T3 cells on cell motility we studied the length of phagokinetic tracks produced by individual cells plated on colloidal gold-coated coverslips, and the rate at which the cells migrated and closed a "wound" introduced in a confluent monolayer. The results, summarized in Fig. 7, show that cells which express low levels of vinculin produce, on the average, ∿ twofold longer phagokinetic tracks compared to those of 3T3 cells, or to control neo^r clones. Moreover, the ability of such cells to migrate into an artificial wound in a monolayer was significantly higher than that of control clones (Table I). Thus, decreased vinculin levels confer increased motility on individual cells, as well as on cells moving out into a wound from a monolayer.

Modulation of vinculin levels in cells also had a marked effect on the anchorage dependence of 3T3 cells. Thus, clones expressing low vinculin levels, showed an increased ability to grow in suspension culture (with unusually high cloning efficiency, see Table I), and produced larger colonies in agar (Fig. 8, compare *C* and *D* with *A* and *B*). These properties are similar to those characteristic of transformed 3T3 cells with diminished vinculin levels (Rodríguez Fernández et al., 1992*a*).

Discussion

The expression of antisense RNA in target cells (Izant and Weintraub, 1984; Kim and Wold, 1985; Izant, 1989), is widely used for reducing the expression of a large variety of genes including those encoding cytoskeletal proteins such as nonmuscle actin (Izant and Weintraub, 1985), α-actinin (Schulze et al., 1989), myosin heavy chain (Knecht and Loomis, 1987), myosin light chain (Pollenz et al., 1992), endo B cytokeratin (Trevor et al., 1987), and glial fibrillary acidic protein (Weinstein et al., 1991). This method is particularly advantageous for the study of vital genes whose elimination by targeted homologous recombination could be lethal.

In this study we have used a 1-kb antisense vinculin cDNA-containing expression vector to transfect 3T3 cells, and demonstrated that the level of vinculin can be reduced down
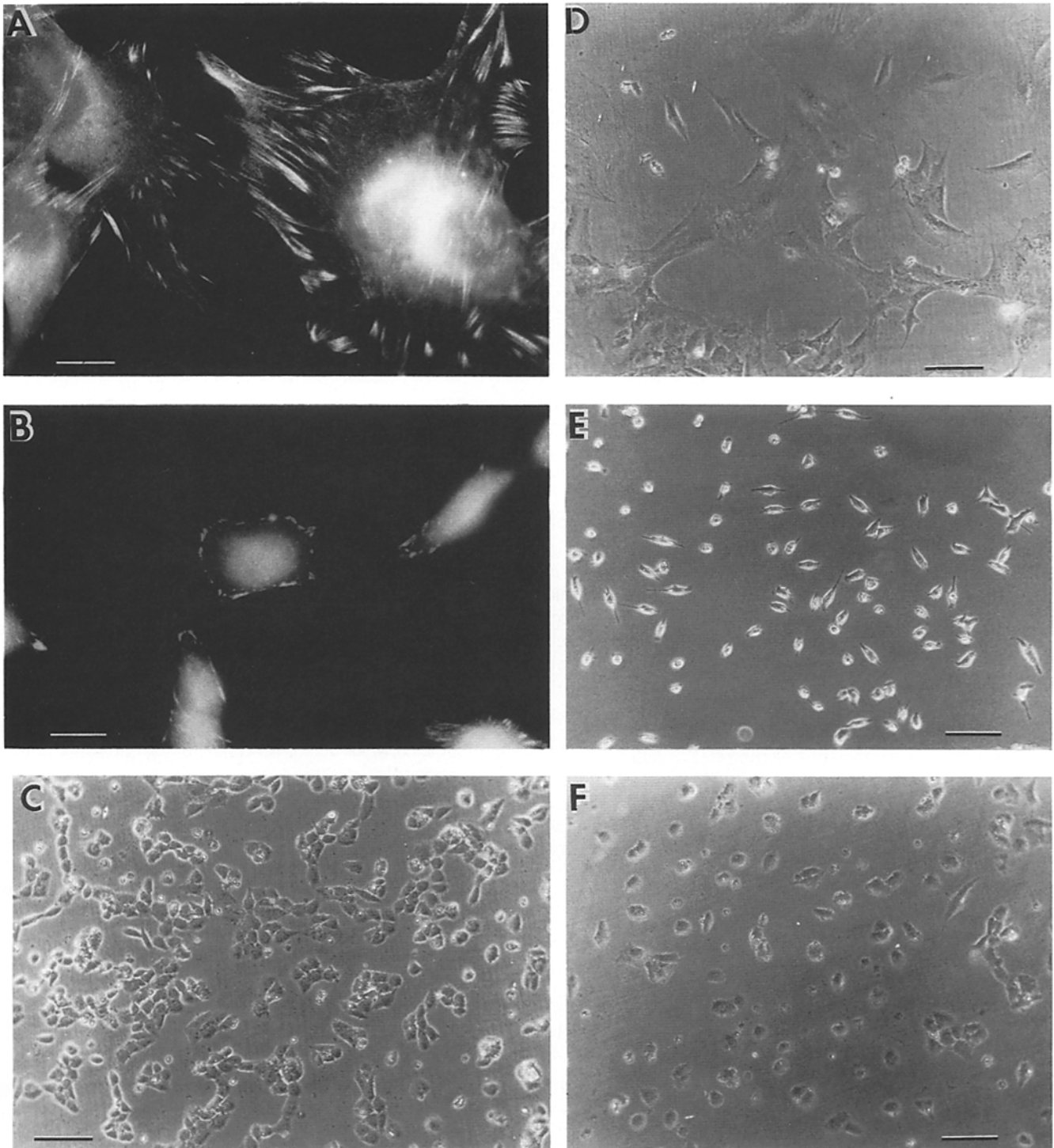
Figure 5. Morphology and vinculin organization in cells expressing antisense vinculin. (*A* and *B*) Cells grown on coverslips were fixed, permeabilized, and immunostained with a broad-range anti vinculin antibody followed by rhodamine-labeled anti mouse IgG. (*A*) clone C11 (neo^r control); (*B*) clone B51. (*C–F*) Cells photographed under a phase microscope. (*C*) Clone B61; (*D*) clone C11; (*E*) clone B51; (*F*) clone A51. The bars in *A* and *B* indicate 10 μm. Bar in *C–F*, 50 μm.

to 10% of the levels normally expressed in these cells. Affected clones were obtained at a rate of ~20% of the number of neo^r colonies, and displayed dramatic changes in cell structure and behavior. They were poorly spread on the substrate, had smaller and fewer vinculin positive plaques, and showed an increased motility and ability to form colonies in soft agar. The analysis of over 120 individual neo^r control colonies did not reveal even one colony with such dramatic phenotypic changes. Computerized analysis of 2-D gels from the affected clones showed no comparable changes in
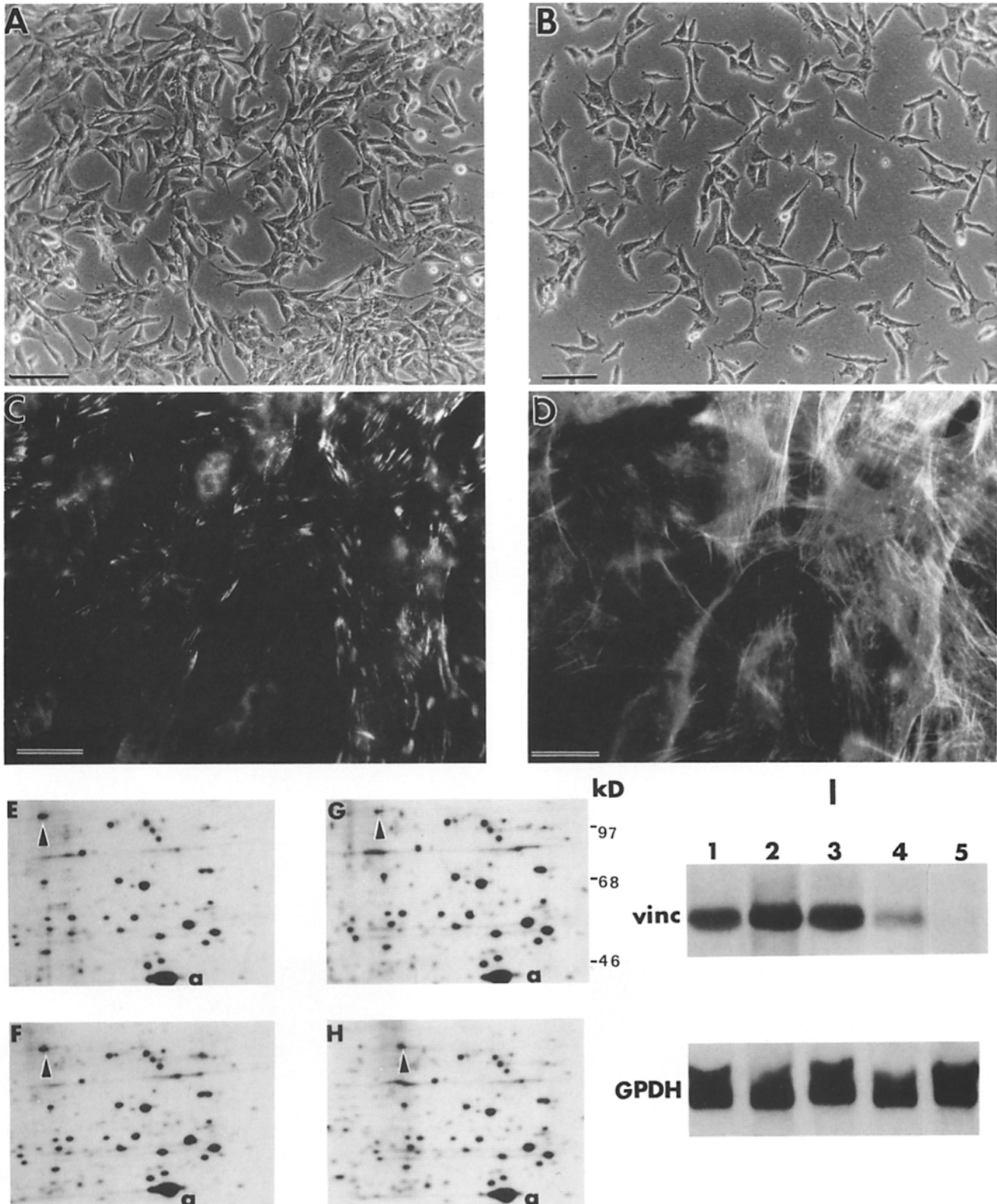
Figure 6. Reversion in phenotype and vinculin expression in 3T3 clones transfected with antisense vinculin. Cells were seeded and examined under a phase microscope (*A, B*), or were fixed and examined by double immunofluorescence for vinculin and actin organization using anti vinculin antibody and rhodamine labeled goat anti mouse IgG (*C*), and FITC-phalloidin for actin (*D*). (*A*) 3T3 cells; (*B*) revertant clone obtained from clone B51. (*E–H*) 2-D gel electrophoresis of equal amounts of [³⁵S]-methionine-labeled total cell protein from: (*E*) 3T3 cells; (*F*) revertant of clone B51; (*G*) neoʳ C11 control clone; (*H*) revertant of clone A51. (*I*) Northern blot hybridization of equal amounts of total RNA with ³²P-vinculin cDNA (vinc) and glyceraldehyde phosphate dehydrogenase cDNA (GPDH) on the same RNA blot. RNA was extracted from: (lane *1*) 3T3 control; (lane *2*) revertant of clone A51; (lane *3*) revertant of clone B51; (lane *4*) clone A51; (lane *5*) clone B51. a, actin; the arrowhead points to the position of vinculin. Bars, (*A* and *B*) 50 μm; (*C* and *D*) 10 μm.
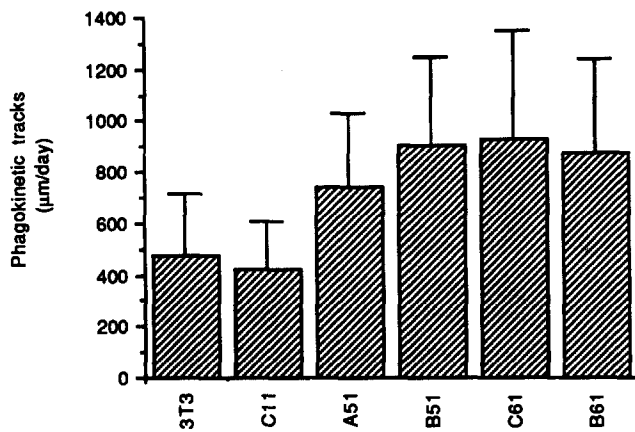
Figure 7. Increased motility of antisense vinculin transfected 3T3 cells. 10^3 cells were seeded on each 20×20 mm coverslip coated with colloidal gold. The phagokinetic tracks produced by individual cells were visualized 24 h after cell seeding by darkfield microscopy. The length of the tracks were determined by projecting $\sim$30 random tracks for each cell line on a screen and by measuring their length. The following cell lines were studied: 3T3; C11, a neo[r] control; A51, B51, C61 and B61, are clones with reduced levels of vinculin (see Table I). Values differ significantly at $p < 0.01$ from the C11 control clone , and at $p < 0.025$ from 3T3 according to Dunnett's test (Dunnett, 1955).

the levels of the microfilament proteins actin, the nonmuscle tropomyosin isoforms and α-actinin, nor in that of the cytoskeletal proteins α and β tubulin, or vimentin.

The phenotypic changes obtained in antisense vinculin transfected clones were apparently unstable, and after five to seven passages in culture the cells re-expressed vinculin RNA and protein at levels comparable to those of control 3T3, and regained the flat morphology. Moreover, cells showing only a moderate decrease in vinculin expression (<50% reduction) displayed a flat phenotype and their motile properties were unaltered. Taken together, the results show that the effects observed are attributable to the decrease in vinculin expression, and further demonstrate the tight relationship between the expression of vinculin and the shape of the cells and their adhesion plaques. Interestingly, Northern blot analysis indicated that the revertants still produced significant levels of RNA corresponding to the antisense cDNA used for transfection. This phenomenon has been reported also in other systems, including studies with cytoskeletal protein genes (Liu et al., 1992; Knecht, 1989; Knecht and Loomis, 1987; Schulze et al., 1989). The mechanisms responsible for reversion of antisense suppression in those systems is still enigmatic. It was suggested that it may involve the induction of the antagonistic effects of the unwindase, or a change in the compartmentalization of the target
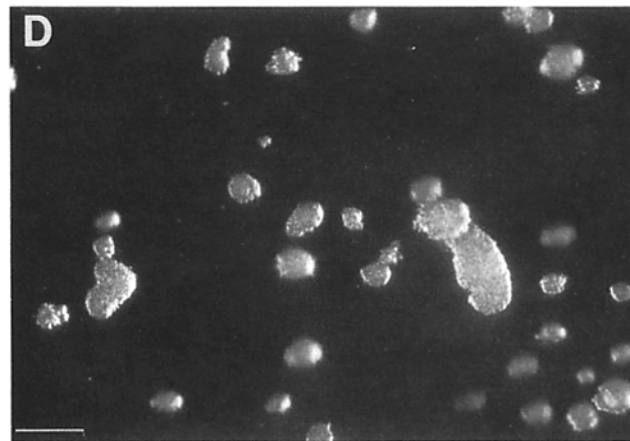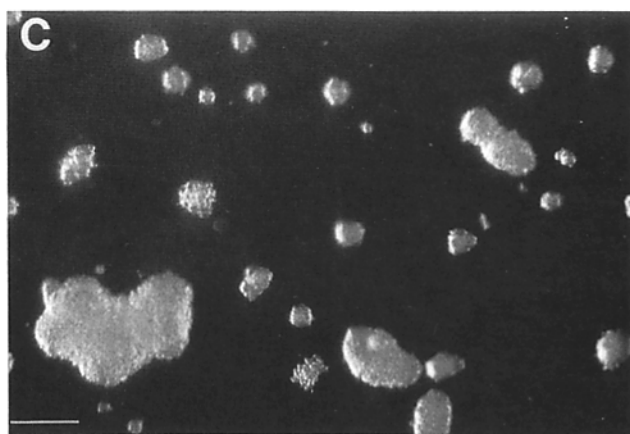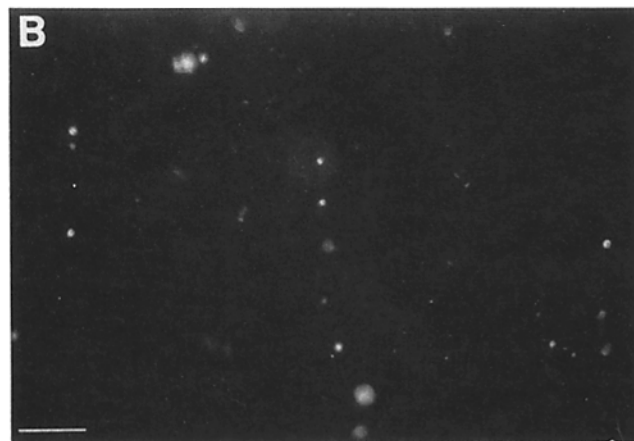

Figure 8. Anchorage independent growth of clones expressing antisense vinculin. Cells were seeded in 0.3% agarose containing medium (10^4 cells per 35 mm diameter dish). Pictures were taken under a phase contrast microscope after 2 wk of incubation at 37°C. (*A*) Clone C11, neo[r] control; (*B*), clone C41, neo[r] clone; (*C*) clone B51; (*D*) clone C61. See Table I for levels of vinculin in these clones. Bar, (*A–D*) 100 μm.

RNA thus making it inaccessible to the antisense RNA (for recent reviews see Inoye, 1988; Erickson and Izant, 1992).

The changes in cell phenotype and growth properties in agar of cells expressing low vinculin levels were not sufficiently stable to allow a long term study of possible effects on the tumorigenic ability of the cells in animals. Nevertheless, the poorly spread phenotype of these clones, and their increased motility and ability to form colonies in agar, are known characteristics of transformed fibroblasts (Stoker et al., 1968; Shin et al., 1975). In agreement with these findings, a dramatic decrease in vinculin expression was detected in SV40-transformed 3T3 cells, and in a spontaneous adenocarcinoma, and the restoration of vinculin to near normal levels in these transformed cells effectively suppressed their tumorigenic ability in animals and decreased their anchorage independence (Rodríguez Fernández et al., 1992a). The present study extends these observations and demonstrates that in normal cells a targeted reduction in vinculin expression was sufficient for conferring changes in cell shape, motility, and in the adhesion-dependent growth ability of the cells. The modulation of vinculin expression may therefore constitute an effective control mechanism for suppressing or allowing cell transformation.

As vinculin is a major component of adhesion plaques and cell junctions, and is known to associate with several other proteins which form and regulate cell adhesion, it is conceivable that changes in vinculin levels exert their effect by affecting signals related to cell adhesion. Studies with truncated adhesion receptors of either the integrin or cadherin type, in which the extracellular domains of the receptors were deleted or modified, showed that these receptors are still recruited to cell–cell and cell–ECM adhesions, provided their cytoplasmic domain is preserved (LaFlamme et al., 1992; Geiger et al., 1992b; Fujimori and Takeichi, 1993). These observations suggest a role for cytoskeletal plaque proteins in an "inside out" regulation of adhesion by controlling the recruitment and assembly of such receptors via their cytoplasmic domains and their binding to microfilaments.

The mechanisms of this regulation are suggested to involve posttranslational modifications, including tyrosine phosphorylation (Volberg et al., 1992; Burridge et al., 1992; Juliano and Haskill, 1993; Guan and Shalloway, 1992). This suggestion is based on the fact that vinculin and other AJ components are substrates for tyrosine kinases which are localized at cell junctions and adhesion plaques (Sefton et al., 1981; Rohrschneider, 1980), and on observations indicating that the activity of such AJ-associated kinases is elevated in transformed cells (Guan and Shalloway, 1992). Moreover, profound morphological and motile changes are induced by inhibiting tyrosine phosphatases in normal cells, and the conditional morphological changes induced in ts mutants for transformation by RSV are reversed by inhibiting tyrosine kinases (Volberg et al., 1991, 1992).

The present study demonstrates that in addition to such posttranslational modifications, changes in the expression of plaque components may provide an alternative mechanism for controlling adhesion-related cell functions. The cooperative model for the assembly of the junctional plaque from soluble cytoplasmic components (Kreis et al., 1984; Geiger et al., 1990, 1992a) would predict that a decrease in vinculin expression may result in an inefficient assembly of adhesion plaque molecules, leading to decreased adhesion, and this in

turn will result in increased motility. In contrast, overexpression of vinculin may facilitate a cooperative and more efficient recruitment and assembly of AJs, and will lead to increased adhesion and decreased motility. The experimental data of this study and of several other studies, in which either vinculin (Rodríguez Fernández et al., 1992b), or other microfilament proteins such as gelsolin (Cunningham et al., 1991) and α-actinin (Glück et al., 1993) were overexpressed, are consistent with these views. For example, the increase in vinculin expression by 20% in 3T3 cells was sufficient for dramatically reducing cell motility (Rodríguez Fernández et al., 1992b), while similar overexpression of the actin severing protein gelsolin, resulted in a significant increase in the chemotactic migration of 3T3 cells (Cunningham et al., 1991).

The molecular mechanisms which link the changes induced in vinculin level with altered cell shape and motility, and the associated alterations in anchorage dependence are not defined yet. A positive correlation between cell locomotion and the stimulation of cell proliferation by growth factors was suggested in both fibroblasts (Heckman et al., 1993) and epithelial cells (Barrandon and Green, 1987). Anchorage dependence is a characteristic property of normal cells, while anchorage independent growth, namely the loss of requirement for cell-ECM adhesion for growth, is considered one of the hallmarks of cell transformation (Stoker et al., 1968; Shin et al., 1975; Ben-Ze'ev et al., 1980; Wittelsberger et al., 1981). Several recent reports have suggested that signals for normal cell growth are transmitted from the ECM through integrins (reviewed by Schwartz, 1992; Juliano and Haskill, 1993), and in transformed cells such signaling may be constitutively activated, as manifested by their anchorage independent growth (Guan and Shalloway, 1992; Juliano and Haskill, 1993; Ben-Ze'ev, 1992). It is possible that changes in the assembly state of the adhesion complex between integrins and the microfilaments, induced by modulating vinculin expression, may confer alterations in the adhesion-related signal transduction, and thereby lead to the observed anchorage independent growth of antisense-transfected cells. Further studies, including the generation of stable clones with vinculin null mutations are needed for elucidating the molecular details of this link between the regulation of AJ protein levels and the control of cell morphology, motility and growth.

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