Suppression of Tumorigenicity in Transformed Cells after Transfection with Vinculin cDNA

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Abstract. Transfection of chicken vinculin cDNA into two tumor cell lines expressing diminished levels of the endogenous protein, brought about a drastic suppression of their tumorigenic ability. The SV-40-transformed Balb/c 3T3 line (SVT2) contains four times less vinculin than the parental 3T3 cells, and the rat adenocarcinoma BSp73ASML has no detectable vinculin. Restoration of vinculin in these cells, up to the levels found in 3T3 cells, resulted in an apparent increase in substrate adhesiveness, a decrease in the ability to grow in soft agar, and suppression of their capacity to develop tumors after injection into syngeneic hosts or nude mice. These results suggest that vinculin, a cytoplasmic component of cell–matrix and cell–cell adhesions, may have a major suppressive effect on the transformed phenotype.

MALIGNANT transformation is accompanied by diverse cellular manifestations including alterations in cell growth rate, and major structural changes affecting cell adhesion and shape, locomotory activity, and cytoskeletal organization. Morphological changes are perhaps among the most conspicuous features of the transformed phenotype in culture, characterized by rounded cell shape with poorly organized microfilament bundles (Weber et al., 1974; Pollack et al., 1975) and aberrant adhesions (Ben-Ze'ev, 1985; Raz and Ben-Ze'ev, 1987). These changes are often correlated with a reduction in anchorage dependence and the ability of the cells to grow in semi-solid medium such as soft agar or methyl cellulose (Shin et al., 1975). Furthermore, it has been shown that the deterioration or the microfilament system in various malignant cells is accompanied by altered expression of microfilament-associated proteins such as tropomyosin (Matsumura and Yamashiro-Matsumura, 1986), gelsolin (Vandekerkhove et al., 1989), and vinculin (Raz and Geiger, 1982; Raz et al., 1986).

While the alterations in both the growth rate and cell structure are inherent features of the transformed phenotype, the causal relationships between the two are unknown. Are primary changes in the adhesive properties of cells responsible for the loss of adhesion-dependent growth control, or does the altered regulation of proliferation lead to the acquisition of the transformed morphology? In the present study we have directly approached this issue by selectively modulating the levels of vinculin expression in tumor cells, and determined the effect of these changes on the transformed phenotype.

Vinculin is a ubiquitous 117,000-D protein which is associated with adhesion plaques and cell–cell contacts, at the termini of microfilament bundles (Geiger, 1979; Burridge et al., 1988; Geiger and Ginsberg, 1991). Previous studies provided much information on the molecular properties of vinculin, including its complete sequence (Cotou and Craig, 1988; Price et al., 1989), interactions with other junctional proteins (Geiger and Ginsberg, 1991), overall molecular shape (Molony and Burridge, 1985), and microheterogeneity (Geiger, 1982). In addition, it was recently shown that the expression of vinculin may be modulated in cells by exposing them to different environmental stimuli. For example, fibroblasts regulate the expression of vinculin RNA and protein in response to changes in cell density and substrate adhesiveness (Ungar et al., 1986; Bendori et al., 1987); the synthesis and organization of vinculin in smooth muscle cells are both altered after transfer to tissue culture (Belkin et al., 1988); vinculin expression is affected in the course of adipogenesis (Rodríguez Fernández and Ben-Ze'ev, 1989) and steroidogenesis (Ben-Ze'ev and Amsterdam, 1987), as well as during cell migration in vivo (Zieske et al., 1989). Moreover, vinculin gene expression is rapidly, yet transiently, stimulated in quiescent 3T3 cells upon growth activation by serum factors (Ben-Ze'ev et al., 1990; Bellas et al., 1991) and in regenerating rat liver (Glück et al., 1992). Taken together, these results suggested that the regulation of vinculin expression may be involved in a wide variety of cellular processes, yet the cause-and-effect relationships between vinculin regulation and these processes are still unclear (Ben-Ze'ev, 1991).

To determine the effect of changes in vinculin expression on tumorigenicity, we transfected chicken vinculin cDNA into two tumorigenic cell lines which either express vinculin at a reduced level, or no detectable vinculin. These include an SV-40-transformed Balb/c 3T3 cell line, SVT2 (Aaronson and Todaro, 1968), and a malignant metastatic pancreatic adenocarcinoma cell line, Bsp73ASML (ASML), which was derived from a spontaneous rat tumor (Matzku et al., 1983).

We isolated clones of the transfected cell lines expressing different levels of the exogenous chicken vinculin cDNA and studied both their structure and behavior. We show here that the chicken vinculin becomes incorporated into cell-substrate adhesion sites in the transfected cells. Furthermore, in both cell lines, expression of the foreign vinculin at high levels affected cell shape and dramatically suppressed the ability of the cells to form tumors and metastases when injected into experimental animals. This study provides direct evidence that modulation of vinculin levels in malignant cells may radically alter the transformed phenotype.

Materials and Methods

Cell Culture and Transfection

Balb/c 3T3 clone A31 cells and SVT2 cells (Aaronson and Todaro, 1968) which are Balb/c 3T3 cells transformed by SV-40, were grown in DME medium plus 10% calf serum (Gibco Laboratories, Grand Island, NY). The BSp73ASML clone 14 (ASML) (Matzku et al., 1983), which is a highly metastatic rat pancreatic adenocarcinoma cell line, was grown in RPMI medium plus 10% FCS (Biolab, Jerusalem, Israel).

The ability of the tumor cells to grow in soft agar was determined by seeding duplicates of 250, 10^3, and 10^4 cells per 35-mm diameter dish in 0.85 ml of serum-containing medium 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. The number of colonies containing >50 cells was determined microscopically after 5–7 d.

The cells were transfected with a full-length chicken cDNA coding for vinculin. The construct used for transfection was obtained by ligating the EcoRI–BamHI fragment of the cVinl clone (Bendori et al., 1989) containing the 5'-1440 nucleotides, with the EcoRI–BamHI of cVin5 containing the 3'-sequences, including part of the noncoding 3'-end. Previous studies confirmed that the product obtained after expression in eukaryotic cells was indistinguishable from chicken fibroblast vinculin (Bendori et al., 1989). This construct was cloned into the EcoRI site of the polylinker of the pJ4Ω expression vector provided by Dr. V. Rotter of our Institute (Shaulski et al., 1991). The pJ4Ω vector consists of the Mo-MuLV LTR promoter-enhancer sequence, the SV-40 small t-antigen intron, and the SV-40 large T polyadenylation signal in the pBR322 plasmid. The neomycin resistance (neo^r) gene, which was cotransfected with the pJ4Ω construct containing the full-length chicken vinculin, was subcloned into the pSVL expression vector. Cells were transfected by the calcium phosphate precipitation method and colonies resistant to G418, 800 μg/ml (Geneticin; Gibco Laboratories) for SVT2 cells, and 250 μg/ml for ASML cells were isolated.

Immunofluorescence and Electron Microscopy

For immunofluorescence microscopy, cells were cultured on glass coverslips, fixed with 3% paraformaldehyde in PBS, and permeabilized with 0.5% Triton X-100. The paraformaldehyde-fixed ASML cells were sheared off the substrate with a jet of PBS, to expose the ventral cell membrane for better visualizing vinculin organization (Avnur et al., 1983). The anti-chicken vinculin mAb and the broad-range monoclonal antivinculin antibody were those used in a previous study (Bendori et al., 1989; now available from Sigma Chemical Co., St. Louis, MO). The second antibody was rhodamine-labeled goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA). Actin filaments were stained with FITC-phalloidin (Sigma Chemical Co.). The cells were examined by epifluorescence with a Zeiss Axiophot microscope.

For EM, cells were cultured on 35-mm tissue culture dishes. The cells were fixed in situ with 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, postfixed in 0.1% OsO_4, flat embedded in Epon, and sectioned perpendicularly to the plane of the dish. Thin sections were stained with uranyl acetate and examined in a Philips transmission electron microscope, CM 12, at 100 KV.

Cell Fractionation, Immunoprecipitation, and PAGE

For immunoprecipitation, cells were labeled for 4 h with 200 μC/ml of [^{35}S] methionine and lysed in RIPA buffer (1% NP-40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 25 mM Tris, pH 8.0). The lysates were clarified by centrifugation at 15,000 *g*, and equal amounts of radioactive proteins (5×10^6 cpm) were incubated with the chicken vinculin-specific antibody. The immune complexes were precipitated with *Staphylococcus aureus* (The Enzyme Center, Boston, MA), and the precipitated proteins were separated by electrophoresis on 8% polyacrylamide gels containing SDS.

Two dimensional (2-D) gel electrophoresis was performed as previously described (Ben-Ze'ev, 1990). 5×10^7 cpm of [^{35}S]methionine-labeled proteins were separated by 2-D gel electrophoresis and transferred to nitrocellulose by electroblotting. The radioactive proteins were visualized by autoradiography of the blot. The positions of the transfected and the endogenous vinculin on the 2-D gels were identified by immunoblotting with the broad-range monoclonal antivinculin antibody, followed by alkaline phosphatase–linked anti-mouse IgG (Promega-Biotec, Madison, WI). The levels of mouse and chicken vinculin, which were separated on the 2-D gels, were determined from the autoradiograms of fluorographed 2-D gels using a laser densitometer and computerized analysis of the images with the QUEST software system as described (Garrels, 1989). About 50–100 random protein spots of the 2-D autoradiograms of each gel were matched by the computer and used for normalizing the different gels. The quantitative determination of the level of vinculin was performed in such normalized gels. Quantitative immunoblotting was carried out using ^{125}I-goat anti-mouse IgG as second antibody.

For cell fractionation, cells grown in 35-mm diameter dishes were labeled overnight with [^{35}S]methionine and extracted with a Triton X-100–containing buffer: 1 ml of buffer containing 50 mM of MES (*N*-Morpholinoethane sulfonic acid) pH 6.1, 2.5 mM EGTA, 5 mM $MgCl_2$, and 0.5% Triton X-100 was added to the culture dishes at room temperature for 2 min. The Triton X-100–soluble fraction was removed and the insoluble fraction, enriched in membrane–cytoskeletal complexes (Ben-Ze'ev et al., 1979) and adhesion plaques (Ball et al., 1986), was scraped into 1 ml of the same buffer. Equal volumes of the two fractions were analyzed by PAGE, followed by immunoblotting with the broad-range antivinculin antibody. The blots were exposed to X-ray film to detect the total protein pattern. Cells were also labeled with [^{35}S]methionine for 3 h and equal volumes of the Triton-soluble and -insoluble fractions were immunoprecipitated with antichicken vinculin antibody and the immunoprecipitates analyzed by polyacrylamide gel electrophoresis.

Northern Blot Analysis

Total RNA was extracted by the guanidinium thiocyanate method (Chirgwin et al., 1979). Northern blots containing 20 μg per lane of total RNA were hybridized (sequentially) with different cDNA probes, which were labeled with ^{32}P-dCTP by the random priming technique (Feinberg and Vogelstein, 1983), as described (Ben-Ze'ev et al., 1990). The following cDNAs were used: a mouse vinculin cDNA (Ben-Ze'ev et al., 1990), a human full-length α-actinin cDNA obtained from D. Kwiatkowski (Harvard Medical School, Boston, MA) (Youssoufian et al., 1990), and a 1-kb mouse talin cDNA isolated in our lab by screening a cDNA library from 3T3-442A preadipocytes in λgt10 (obtained from B. Spiegelman, Harvard Medical School), with a partial chicken talin cDNA obtained from M. Beckerle (University of Utah, Salt Lake City, UT). The 1-kb mouse talin cDNA used corresponds to the COOH-terminal 332 amino acids of mouse talin.

Tumorigenicity and Metastatic Ability Assays

SVT2 cells were injected subcutaneously into syngeneic Balb/c mice (10^6 or 5×10^6 cells per animal). The appearance and size of tumors and survival time were examined daily. The ASML cells were injected into the footpad of syngeneic BDX rats (10^5 and 5×10^5 cells per animal), as described (Matzku et al., 1983), and their survival was monitored. Rats were killed between 40 and 60 d after injection and the lymph nodes (inguinal and paraaortic), as well as the lung tissue, were examined. ASML cells were also injected into nude mice, with 10^5 and 10^6 cells per animal.

Results

Decreased Expression of Vinculin in SV-40–transformed 3T3 and in the Spontaneous Pancreatic ASML Adenocarcinoma

The levels of vinculin expression were determined in two tumor cell lines; an SV-40–transformed Balb/c 3T3 line and in the ASML adenocarcinoma (Fig. 1). In the SVT2 cell line

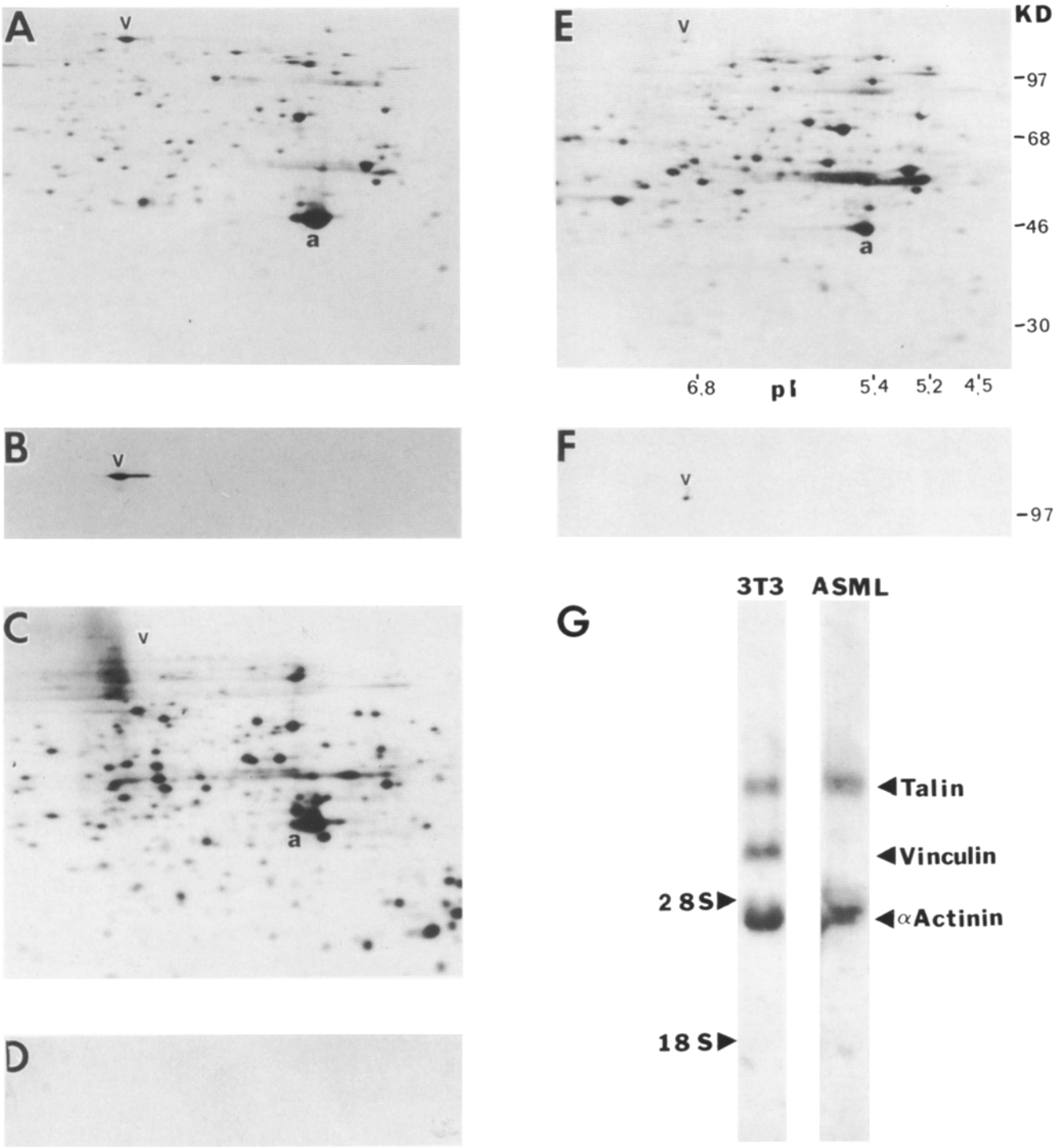

Figure 1. Levels of vinculin expression in 3T3, SVT2, and ASML cells. Cell cultures were labeled for 20 h with 100 μC/ml of [^{35}S]methionine and equal amounts of radioactive total cell protein were separated by 2-D gel electrophoresis and transferred to nitrocellulose. The pattern of total cell proteins was visualized by exposing the blots to X-ray films (*A*, *C*, *E*). The blots were incubated with a broad-range antivinculin antibody followed by alkaline phosphatase–bound anti–mouse IgG (*B*, *D*, *F*). (*G*) Total RNA was extracted from 3T3 and ASML cells and equal amounts of RNA were separated on agarose gel, blotted onto nitrocellulose, and hybridized sequentially with mouse vinculin cDNA, human α-actinin cDNA, and mouse talin cDNA. (*A* and *B*) 3T3; (*C* and *D*) ASML; (*E* and *F*) SVT2. *a*, actin; *v*, vinculin.

(Fig. 1, *E* and *F*), vinculin content was reduced over fourfold when compared to control Balb/c 3T3 cells (Fig. 1, *A* and *B*), as determined by computerized densitometry of the 2-D gels (Fig. 1, *A* and *E*). In the spontaneous rat pancreatic adenocarcinoma ASML cell line, the synthesis of vinculin was undetectable (Fig. 1 *C*), in agreement with a previous report (Raz et al., 1986). The immunoblot assay, using a broad-range antivinculin antibody, was negative in these cells (Fig. 1 *D*), and Northern blot analysis confirmed that while the levels of two adhesion plaque components, talin and α-actinin, were comparable to those in Balb/c 3T3, no detectable vinculin RNA could be demonstrated in the ASML cell line

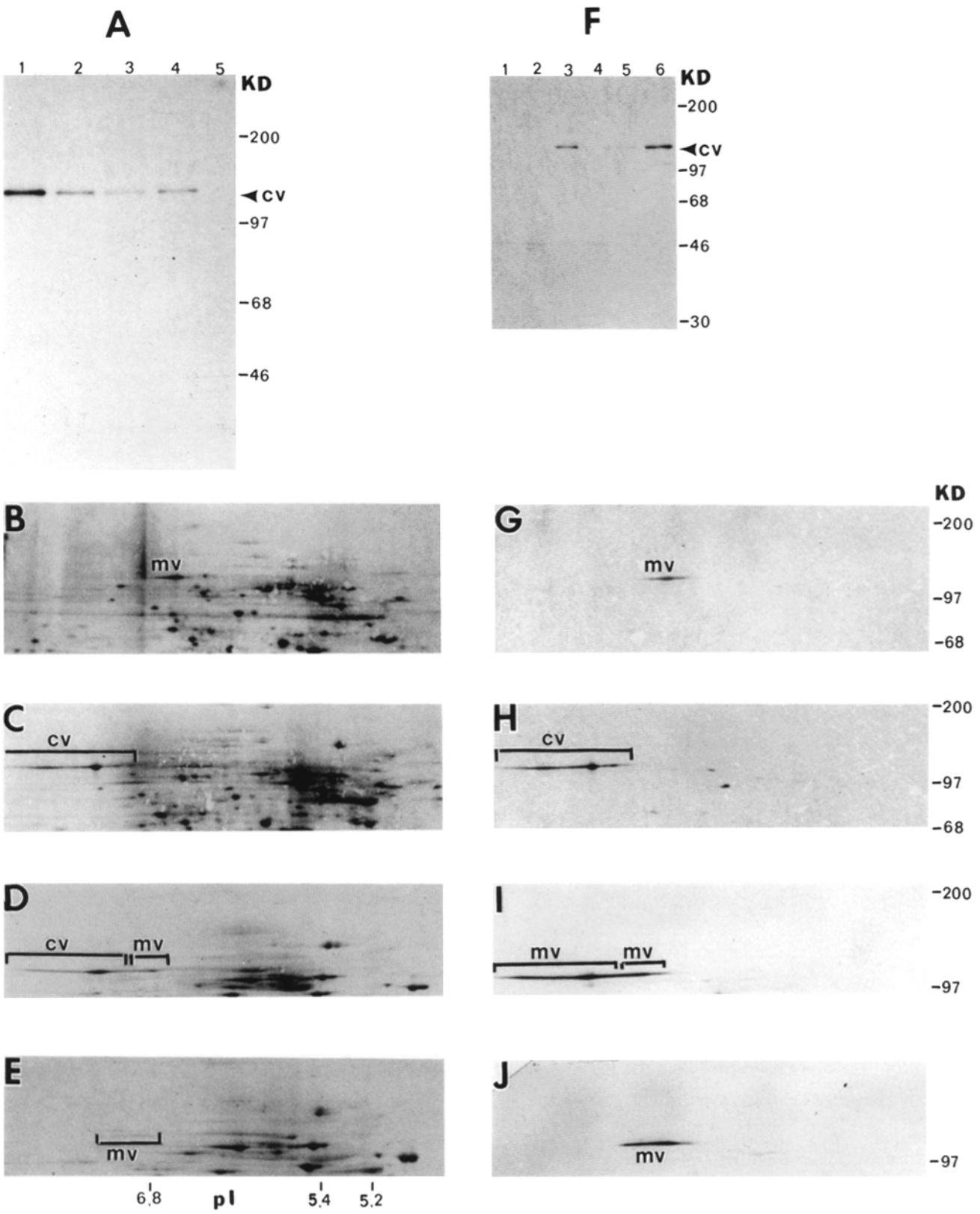

Figure 2. Levels of vinculin expressed in transfected SVT2 and ASML cells. Nontransfected 3T3 cells (*B* and *G*), chicken vinculin–transfected ASML cells (*C* and *H*), chicken vinculin–transfected SVT2 cells (*D* and *I*), and neo^r control SVT2 cells (*E* and *J*) were labeled for 20 h with [^{35}S]methionine. Total cell protein lysates were separated by 2-D gel electrophoresis and blotted onto nitrocellulose. (*B–E*) Autoradiography of the blots. (*G–J*) Immunodetection of vinculin on the same blots with a broad range antivinculin antibody. Individual chicken vinculin–transfected clones of ASML (*A*, lanes *1–5*) and SVT2 (*F*, lanes *1–6*) were labeled for 3 h with 200 μC/ml of [^{35}S]methionine, and 5 × 10^6 cpm of total cell protein were used for immunoprecipitation with an antibody specific for chicken vinculin from each clone. (*A*, lane *1*, *C* and *H*) clone B 12; (*A*, lane *2*) clone B 42; (*A*, lane *3*) clone C 62; (*A*, lane *4*) clone B 31; (*A*, lane *5*) control, untransfected ASML; (*F*, lane *1*) control, untransfected SVT2; (*F*, lane *2*, *E*, and *J*) neo^r SVT2 control; (*F*, lane *3*) clone D 34; (*F*, lane *4*) clone D 41; (*F*, lane *5*) clone D 43; (*F*, lane *6*, *D* and *I*) clone D 44. *cv*, chicken vinculin; *mv*, mouse vinculin.

Table I. Tumorigenicity of SVT2 Clones Expressing Different Vinculin Levels

Clone	Vinculin level*		Tumor incidence	Growth in agar
	Chicken	Mouse		
			%	%
3T3	0	750	0/6 (0)	<0.01
neo 1	0	ND	6/6 (100)	11.0 ± 0.8
neo 3	0	140	6/6 (100)	13.3 ± 1.0
SVT2	0	135	6/6 (100)	12.3 ± 0.5
D34	344	170	1/6 (16)	5.7 ± 1.0
D43	105	ND	5/6 (83)	ND
D41	80	ND	5/6 (83)	ND
D44	670	160	0/6 (0)	3.6 ± 1.5

* The levels of chicken and mouse vinculin were determined by quantitative computerized analysis of 2-D gels from [^{35}S]methionine-labeled total cell lysates. The numbers represent arbitrary O.D. units.
ND, not done.

(Fig. 1 *G*). Southern blot analysis of genomic DNA from ASML cells using a mouse vinculin cDNA probe, did not reveal an apparent rearrangement of the vinculin gene in these cells (results not shown).

Transfection of SVT2 and ASML Lines with Chicken Vinculin

To study the effect of vinculin on the behavior of SVT2 and ASML cells, the two cell lines were cotransfected with a full-length chicken vinculin cDNA (Bendori et al., 1989) and with the neomycin acetyl transferase gene (neo^r) conferring resistance to the aminoglycoside G418. Resistant colonies were selected and screened by quantitative immunoprecipitation with an antibody that selectively recognizes chicken but not rodent vinculin (Fig. 2 *F*, compare lanes *1* and *2* to lanes *3* and *6*). The levels of chicken vinculin expressed in different transfected clones of either ASML (Fig. 2 *A*) or SVT2 (Fig. 2 *F*) were highly variable. To estimate the content of the exogenous vinculin, relative to the endogenous pool in SVT2 cells, total [^{35}S]methionine-labeled cell lysates of different transfected lines were examined by 2-D gel electrophoresis. The gels were electroblotted onto nitrocellulose paper and the total cellular protein pattern visualized by autoradiography (Fig. 2, *B–E*). The identity of the vinculin spots was confirmed by immunoblotting using a broad-range vinculin antibody (Fig. 2, *G–J*). The levels of chicken and mouse vinculin were determined by quantitative, computerized densitometry of the autoradiograms (Fig. 2, *B–E*),

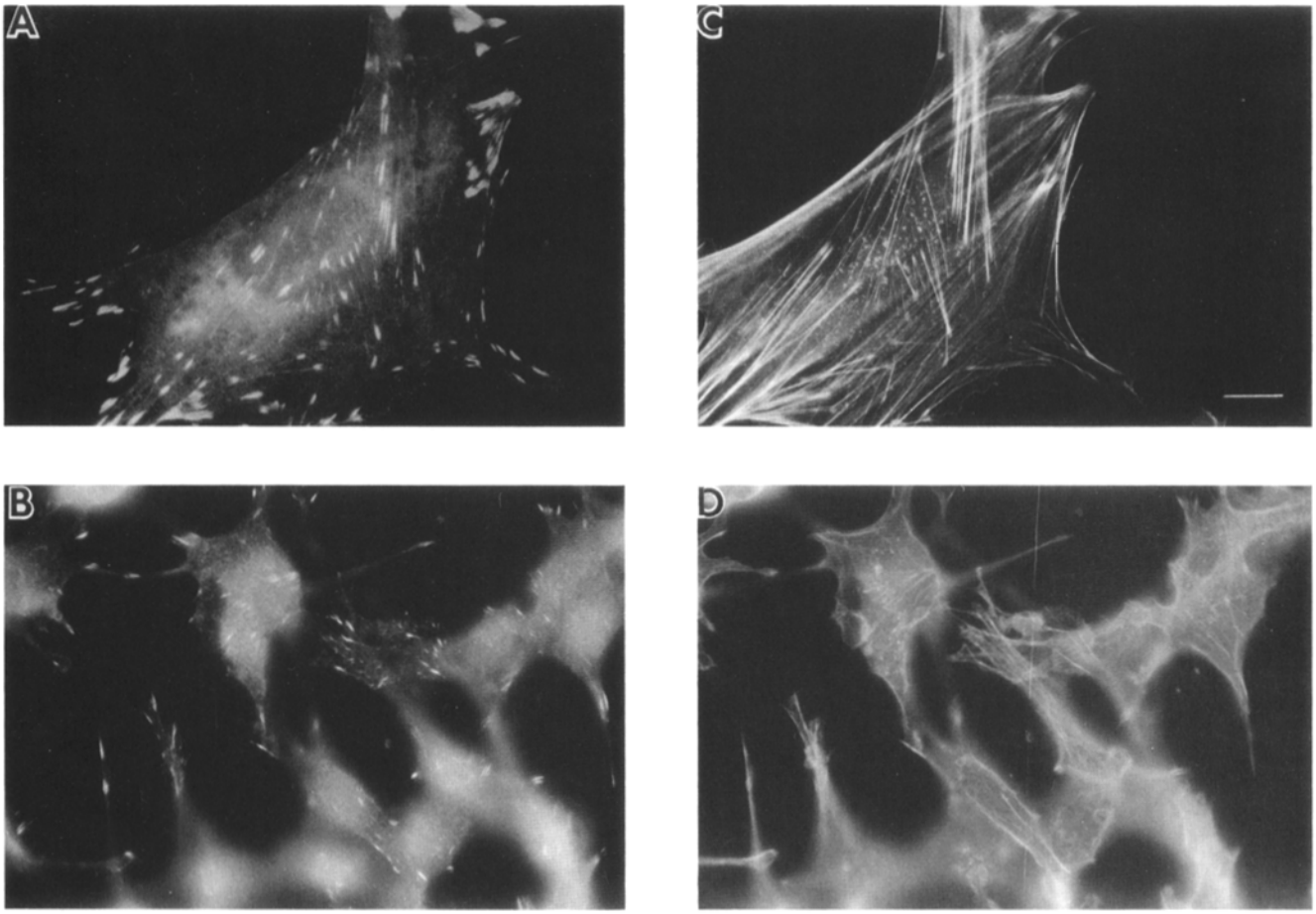

Figure 3. Organization of vinculin and actin in SVT2 and 3T3 cells. (*A* and *B*) Cells grown on coverslips were fixed with paraformaldehyde, permeabilized with Triton X-100, and immunostained with a broad range antivinculin antibody followed by rhodamine-labeled anti-mouse IgG. (*C* and *D*) Actin filaments were visualized with FITC-labeled phalloidin. (*A* and *C*) 3T3 cells; (*B*, *D*) SVT2 cells. Bar, 10 μm.

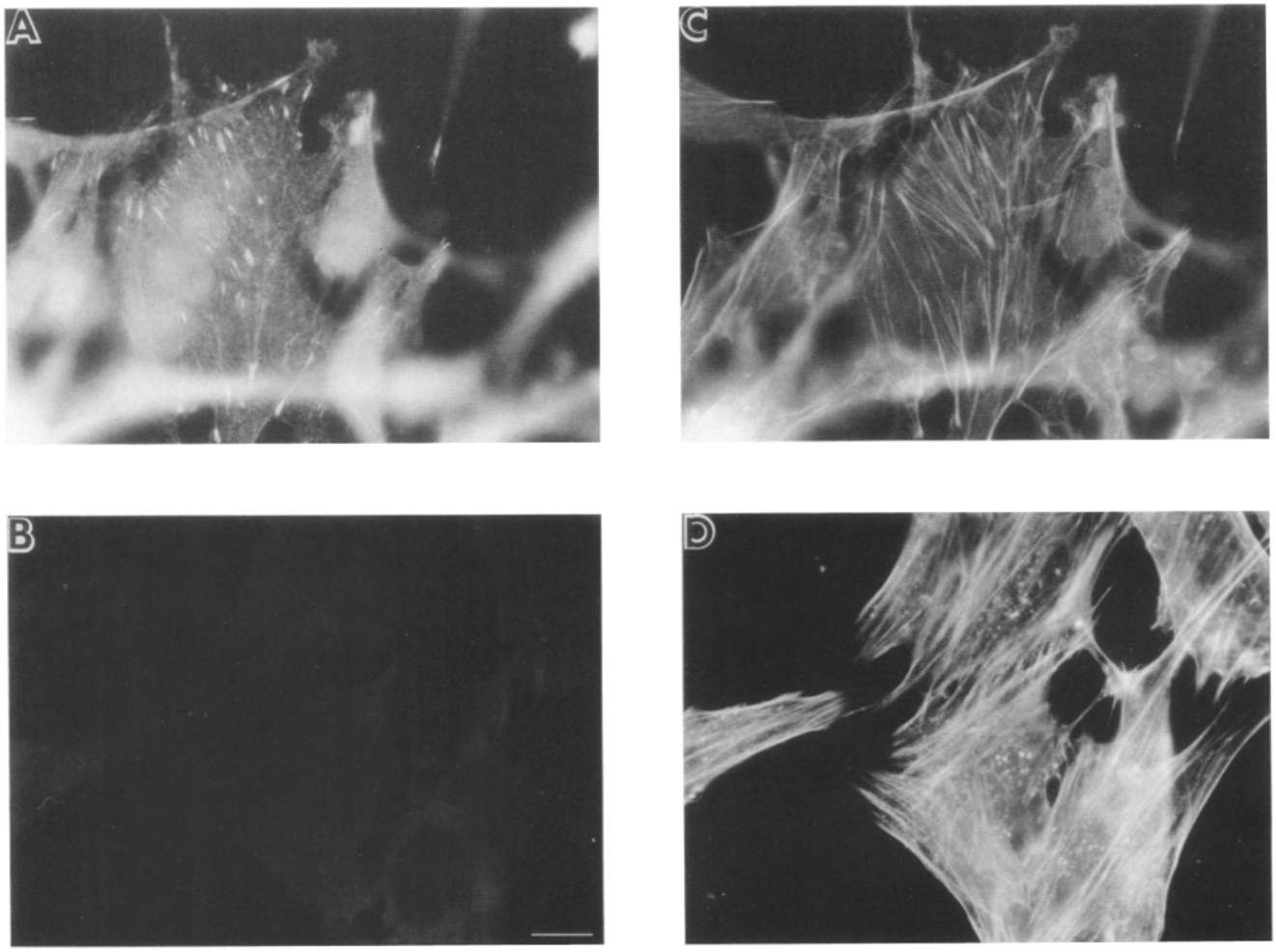

Figure 4. Organization of vinculin and actin in vinculin-transfected SVT2 cells. (*A* and *B*) Cells grown on coverslips were immunostained with an antibody specific for chicken vinculin, followed by rhodamine-anti-mouse IgG. (*C* and *D*) Actin filaments were stained with FITC-phalloidin. (*A* and *C*) Chicken vinculin-transfected SVT2 cells, clone D44; (*B* and *D*) 3T3 cells. Bar, 10 μm.

aided by the fact that chicken vinculin has the same M_r as the mouse protein, but a more basic pI (Fig. 2, *H* and *I*, compare to *G* and *H*). The intensity of the vinculin spots was determined after the density of ~100 random spots was normalized on the different 2-D gels by a computerized program.

In ~70 independent transfected SVT2 clones the levels of chicken vinculin varied between 10% to over 400% of the endogenous vinculin pool. For example, in the transfected clone D 44 (Fig. 2, *D* and *I*), the synthesis of the exogenous vinculin was fourfold higher than that of the endogenous vinculin (Fig. 2, *E* and *J*), and thus comparable to that present in nontransformed Balb/c 3T3 cells (Table I). Similarly, in over 100 different transfected ASML clones, from two different transfections, the levels of the exogenous vinculin varied from 5 to 250% (Fig. 2, *C* and *H*) compared to the vinculin levels in Balb/C 3T3 cells (Fig. 2, *B* and *G*). Analysis of several autoradiograms of SVT2 clones expressing different levels of the transfected chicken vinculin, showed no significant changes in the level of the endogenous mouse vinculin. There were small variations in the growth rate among different clones, but these were not related to the amount of chicken vinculin expressed in the respective cells.

Immunofluorescent Localization of the Transfected Vinculin in SVT2 and ASML Cells

SVT2 cells have a very poorly organized actin filament network (Fig. 3 *D*) with small vinculin-containing adhesion plaques (Fig. 3 *B*), compared to control Balb/c 3T3 cells (Fig. 3, *C* and *A*, respectively). In the transfected SVT2 cells, the exogenous chicken vinculin was localized with a chicken vinculin-specific antibody not recognizing the endogenous mouse vinculin (Fig. 4 *B*). In SVT2 cells, it was found that regardless of its level of expression, the transfected chicken vinculin was associated with adhesion plaques (Fig. 4 *A*) at the termini of actin filaments (Fig. 4 *C*), essentially indistinguishable from the endogenous mouse vinculin (Fig. 3 *B*). Moreover, cells expressing high levels of exogenous vinculin displayed a more flat morphology with focal contacts apparently larger than those of SVT2 cells. In ASML cells which have a rounded shape and do not express detectable levels of vinculin, only a faint background staining was obtained with the broad-range antivinculin antibody (Fig. 5 *B*). In vinculin-transfected ASML cells the foreign vinculin was organized in small patches located along the ventral cell surface (Fig. 5 *A*). Electron microscopic examination of cell

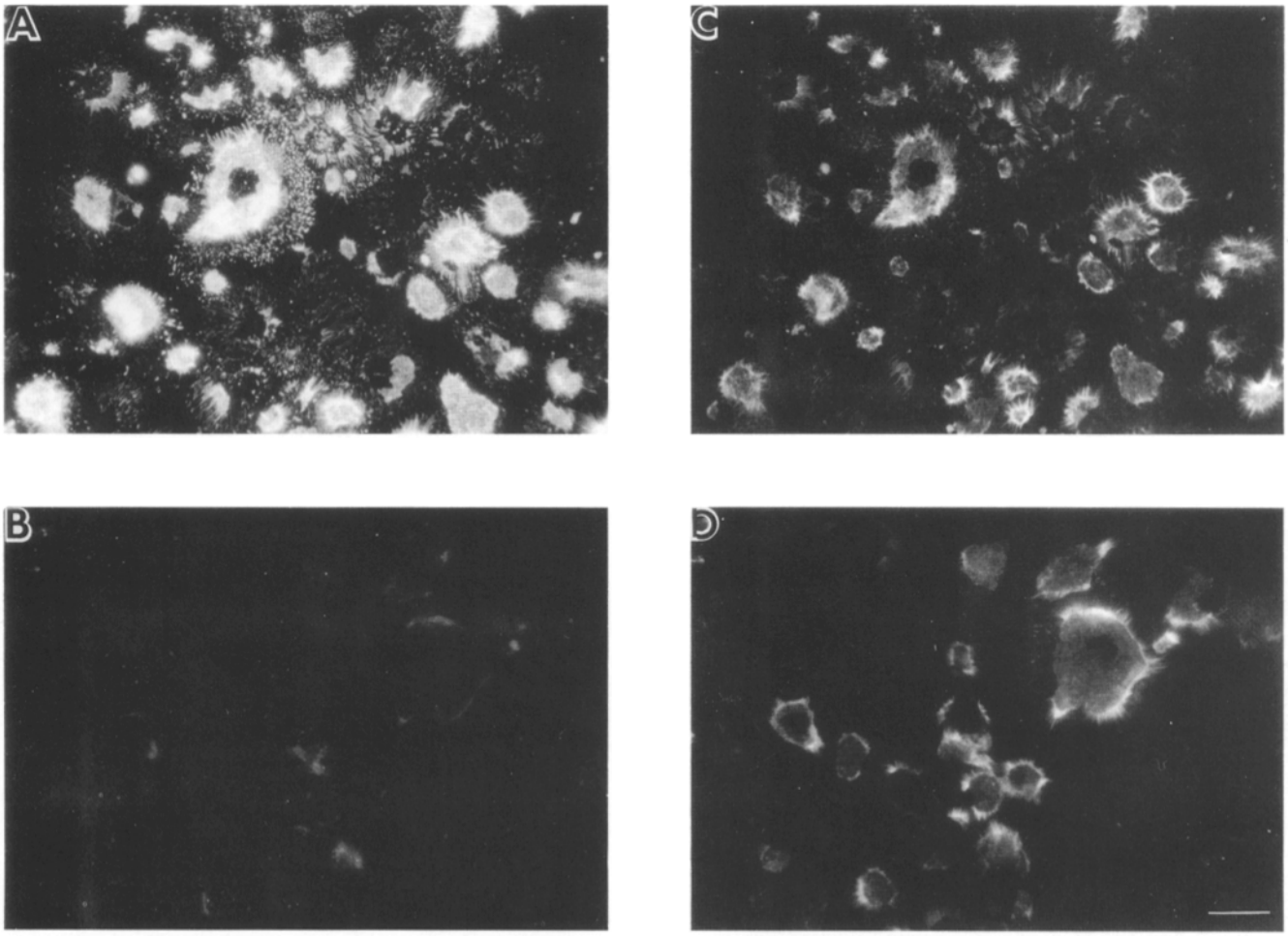

Figure 5. Organization of vinculin and actin in vinculin-transfected ASML cells. (*A* and *B*) Cells grown on coverslips were fixed and sheared off the substrate by a stream of PBS, before further permeabilization with Triton X-100. The cells were immunostained with a broad-range antivinculin antibody, followed by rhodamine-anti-mouse IgG. (*C* and *D*) Actin was visualized with FITC-phalloidin. (*A* and *C*) Chicken vinculin-transfected ASML cells, clone B12; (*B* and *D*) Untransfected ASML cells. Bar, 10 μm.

substrate contacts in control and vinculin-transfected ASML cells, revealed that while the gross morphology did not radically change, vinculin expression resulted in a twofold increase in the average cross-section length of these contact sites (data not shown).

To further determine the relative levels of assembled (Triton X-100 insoluble) versus unassembled (Triton X-100-soluble) vinculin, the content of the protein in the two fractions was determined (Fig. 6). Quantitative immunoblot and immunoprecipitation analyses with equal volumes of these fractions were performed with the various cells, either using the broad-range antivinculin antibody (Fig. 6 *A*), or the chicken vinculin-specific antibody (Fig. 6 *C*). The segregation of mouse vinculin between a Triton-soluble and -insoluble fraction in 3T3 cells (Fig. 6 *A*, lanes *1* and *2*), and of the transfected chicken vinculin in SVT2 cells (Fig. 6 *C*, lanes *1* and *2*) were very similar (70% Triton-soluble, 30% Triton-insoluble). A higher proportion of the transfected chicken vinculin was found in the Triton X-100–insoluble fraction of the ASML transfectants (45%) (Fig. 6 *A*, lanes *6* and *8*, and Fig. 6 *C*, lane *4*) as compared to SVT2 cells (Fig. 6 *C*, lane *2*). The reason for this variation could result from differences in extractability of proteins in the different cell types.

Tumorigenic Properties of Vinculin-transfected SVT2 and ASML Cells

Motivated by the suppressed expression of vinculin in SVT2 and ASML cells, we examined the effect of forced expression of vinculin on the transformed phenotype. SVT2 cells, neo^r clones, as well as several transfected clones expressing different levels of chicken vinculin (Fig. 2 *F*), were injected subcutaneously into syngeneic Balb/c mice, and development of tumors was examined (Table I). Nontransfected SVT2 cells and the neo^r control clones produced palpable tumors within 5–7 d after injection. These tumors reached, in 2–3 wk, a size of 4–6 cm in diameter. In contrast, clones expressing high levels of vinculin (such as clone D 44; Table I) did not produce palpable tumors even 2 mo after injection, at which time the SVT2 and neo^r injected animals had all developed tumors and died. The suppressed ability to form tumors in syngeneic animals by the vinculin overexpressing cells was also reproduced in nude mice injected with the various clones. As can be seen in Table II, the expression of exogenous vinculin did not lead to complete suppression of tumorigenicity, but both the rate of tumor development and the size of the tumors were significantly reduced. Since the shape of the tumors was rounded, the de-

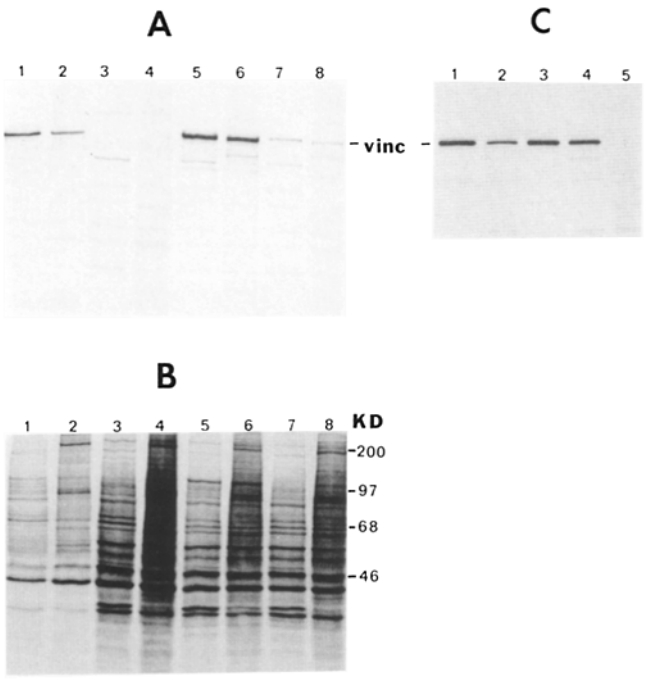

Figure 6. Partitioning of vinculin in the transfected cells between detergent-soluble and -insoluble fractions. 3T3 cells (*A*, lanes *1* and *2*), ASML cells (*A*, lanes *3* and *4*), vinculin-transfected ASML clone B 12 (*A*, lanes *5* and *6*), and clone B 31 (*A*, lanes *7* and *8*) were labeled for 20 h with [^{35}S]methionine. The cells were extracted with Triton X-100 and the level of vinculin was determined in the Triton X-100 soluble- (*A*, lanes *1*, *3*, *5*, *7*) and insoluble-fractions (*A*, lanes *2*, *4*, *6*, *8*) by immunoblotting with a broad-range antivinculin antibody. (*B*) The total protein pattern of the blot shown in *A* was visualized by autoradiography. (*C*) SVT2 cells transfected with vinculin (clone D 34) (lanes *1* and *2*) and ASML transfected with vinculin (clone B 12) (lanes *3* and *4*) were labeled for 3 h with [^{35}S]methionine and the levels of the transfected vinculin in the Triton X-100 soluble (lanes *1* and *3*) and insoluble fractions (lanes *3* and *4*) were determined by immunoprecipitation with a chicken vinculin-specific antibody. (*C*, lane *5*) Precipitation with nonimmune mouse serum of total radioactive ASML clone B 12 cell extracts. *vinc*, vinculin.

crease in tumor mass formed in nude mice injected with clones expressing high levels of the transgene (D 34 and D 44) was six- to eightfold, compared to untransfected SVT2 cells. It is noteworthy that transfected clones expressing the foreign vinculin at levels below those of the residual endogenous protein, were essentially indistinguishable in their tumorigenic ability from the control SVT2 cells in both syngeneic and nude mice. To further characterize the cellular changes induced by the vinculin transfection, we determined the ability of the cells to grow in semi-solid medium. As previously shown (Shin et al., 1975), 3T3 cells cannot grow in agar gels, while SV-40–transformed 3T3 cells readily form colonies in agar. The results shown in Table I indicate that SVT2 clones expressing the highest levels of vinculin had a significantly reduced ability to form colonies in soft agar.

Similar effects induced by vinculin transfection were also noted in the malignant metastatic ASML line. The various transfectants and the parental line were injected into syngeneic BDX rats. Cells injected into the footpad, readily spread through the lymphatic system and formed multiple lung metastases which killed the animals within 35–55 d (Fig. 7). ASML cells expressing the highest levels of chicken vinculin (clones B 12 and A 25), had a dramatically suppressed ability to form metastatic tumors (Table III), and consequently animal survival after injection with these cells was significantly prolonged (Fig. 7). Cells expressing low or moderate levels of the transgene (Fig. 8) formed tumors in the lymph nodes in proximity to the site of injection, but had a significantly reduced capacity to form lung metastases (Table III). Interestingly, we noticed that upon prolonged culture in vitro, revertants can be obtained from the transfected ASML clones which originally expressed high levels of vinculin (five such clones were obtained). Examination of the tumorigenic properties of revertants showed that in addition to the loss of vinculin expression, the cells reverted to the highly malignant phenotype (Fig. 7 and Table III; clone B 12 r). In vinculin-transfected ASML clones isolated from two independent transfections, the tumorigenic ability in nude mice and the ability to grow in soft agar were both di-

Table II. Latency and Growth of Tumors in Nude Mice

Cell line	Tumor diameter after injection day (in cm)*			
	7 d	14 d	21 d	28 d
3T3	0	0	0	0
	(0, 0, 0, 0)	(0, 0, 0, 0)	(0, 0, 0, 0)	(0, 0, 0, 0)
SVT2	0.5	2.1	6.0	
	(0.4, 0.6, 0.5)	(2.0, 2.2, 2.1)	(6.0, 6.5, 5.4)	
D 34	0.2	1.2	3.8	
	(0, 0.3, 0.4)	(1.0, 1.2, 1.3)	(3.6, 4.0, 3.9)	
D 44	0.1	0.9	3.2	
	(0, 0, 0.3)	(1.0, 1.0, 0.7)	(3.2, 3.0, 3.5)	
ASML	0.7	0.9	1.0	1.1
	(0.6, 0.7, 0.7)	(0.8, 0.9, 0.9)	(0.8, 1.1, 0.9)	(1.2, 1.1, 1.0)
neo 4	0.7	0.9	1.0	1.1
	(0.8, 0.6, 0.7)	(0.9, 0.9, 0.8)	(0.9, 1.1, 0.9)	(1.1, 1.3, 1.0)
A 25	0	0	0.3	0.8
	(0, 0, 0, 0)	(0, 0, 0, 0)	(0, 0, 0.4, 0.6)	(0.6, 1.0, 0.8, 0.7)
B 12	0	0	0	0.3
	(0, 0, 0, 0)	(0, 0, 0, 0)	(0, 0, 0, 0)	(0, 0, 0.3, 0.6)

* The size of tumors is expressed as the mean higher outer diameter. As the tumors were spherical in shape, the differences in tumor mass formed in animals by vinculin-transfected cells and controls were much larger (for example, the tumor mass formed by injecting D 44 was about sevenfold smaller at 21 d than that formed by injecting SVT2 cells). Animals injected with D 34, D 44, and SVT2 cells were killed 21 d after injection.

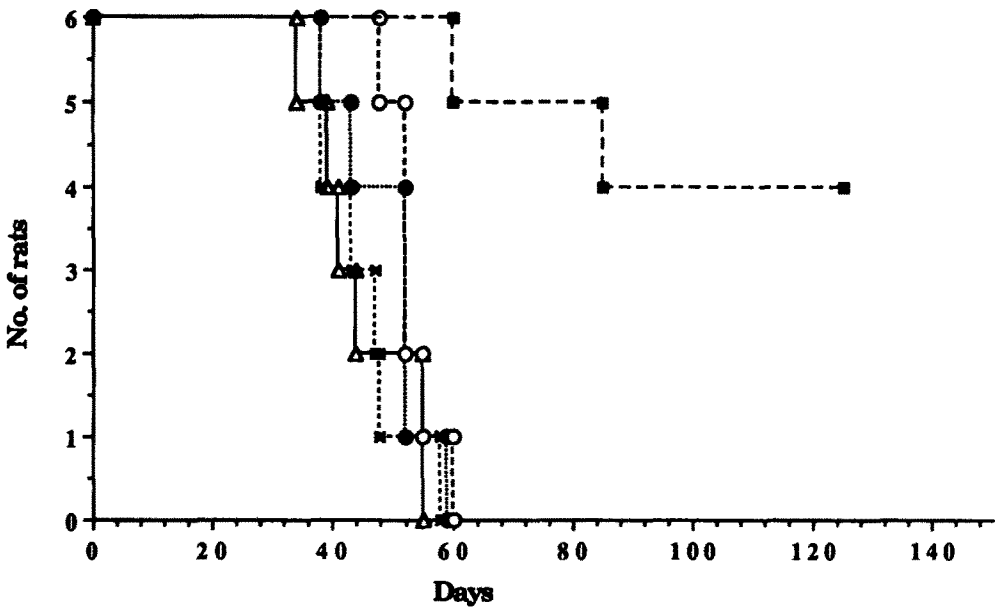

Figure 7. Rat survival after injection with ASML cells expressing different chicken vinculin levels. Groups of six rats were injected in the footpad with 5×10^5 cells of ASML clones expressing different levels of chicken vinculin. The survival time of individual rats, in days, was determined. (Δ----Δ) Untransfected ASML cells; (X----X) clone B 12 r; (■----■) clone B 12; (○----○) clone B 31; (●----●) clone C 62. For levels of vinculin and corresponding tumorigenicity of these clones see Fig. 2 *A* and Table III.

minished in clones expressing the highest levels of vinculin (clones A 25 and B 12; Tables II and III). As the tumors were spherical, the decrease in tumor mass formed in nude mice injected with these cells was more extensive (six- to eightfold) than the two- to threefold decrease in tumor diameter shown in Table II.

Discussion

The primary objective of this study was to assess the specific effect of vinculin on the tumorigenic phenotype. We chose to focus on vinculin owing to several indirect observations including: (*a*) the reduction in vinculin levels observed in several spontaneous and virally-induced tumors (Raz et al., 1986; this study); (*b*) the known effects of malignant transformation on cell adhesiveness in general, and in particular on cell–matrix and cell–cell adherens junctions (Ben-Ze'ev, 1985; Raz and Ben-Ze'ev, 1987); and (*c*) the role of vinculin in the assembly and stabilization of adherens junctions and the associated cytoskeleton (Bendori et al., 1989; Geiger

Table III. Metastatic Spread of BSp73ASML Expressing Different Vinculin Levels

BSp73 ASML clone	Growth in agar	Vinculin level*	Tumor take	Metastatic load at autopsy	
				Lymph nodes	Lung
	%	%			
14	17.8 ± 1.9	0	5/5	Ø 2.5–5.0‡	5/5 Miliary§
neo 4	12.4 ± 0.5	0	5/5	Ø 2.5–5.0	5/5 Miliary
neo 5	ND	0	5/5	Ø 2.5–5.0	5/5 Miliary
A 25	1.4 ± 0.5	100	3/5	Ø 0.5–1.0	1/5 4 nodules 4/5 no metastases
B 12	2.6 ± 0.7	118	2/5	Ø 1.0–2.0	2/5 5–20 nodules 3/5 no metastases
B 12 r	ND	0	5/5	Ø 2.5–5.0	5/5 Miliary
C 13	ND	31	5/5	Ø 1.0–2.0	3/5 Miliary 2/5 no metastases
C 34	ND	30	5/5	Ø 0.5–1.0	2/5 Miliary 3/5 4 nodules
D 26	5.1 ± 1.9	14	5/5	Ø 2.5–5.0	2/5 Miliary 3/5 2–25 nodules
D 37	ND	23	5/5	Ø 2.5–5.0	3/5 Miliary 2/5 3 nodules
A 34	ND	26	5/5	Ø 2.0–5.0	3/5 Miliary 2/5 10–32 nodules
B 51	ND	10	5/5	Ø 2.5–5.0	3/5 Miliary 2/5 9–25 nodules

The table reflects the state at 50 d after injection.
ND, not done.
* The levels of vinculin were obtained by quantitative computerized scanning of the immunoblots shown in Fig. 8. The level of vinculin in the B 12 clone was determined by comparing immunoprecipitations of clone A 25 and B 12 in an independent experiment.
‡ Mean tumor diameter in centimeters.
§ Miliary is >1,000 nodules.

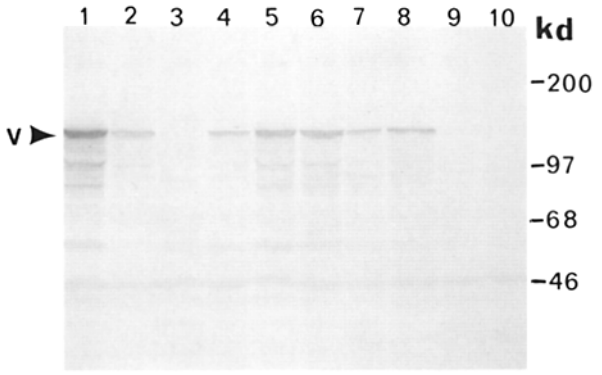

Figure 8. Levels of vinculin in ASML clones used for tumorigenesis and metastasis assays. Equal amounts of protein of total cells lysates were separated by gel electrophoresis and the level of vinculin was determined by quantitative immunoblotting with a broad range antivinculin antibody followed by ^{125}I-anti-mouse IgG. (Lane *1*) Clone A 25; (lane *2*) clone A 34; (lane *3*) clone B 12 r; (lane *4*) clone B 51; (lane *5*) clone C 13; (lane *6*) clone C 34; (lane *7*) clone D 26; (lane *8*) clone D 37; (lane *9*) clone neo 4; (lane *10*) clone neo 5. *v*, vinculin.

and Ginsberg, 1991). The difficulty, however, in the unequivocal interpretation of these earlier observations, was that the causal relationships between vinculin expression, or its modulation, and the acquisition of the transformed phenotype, has not been established. To directly address this issue we selected two tumor lines in which vinculin expression was low or abolished, and determined the effect of expression of this single protein on tumorigenicity in vivo and on the transformed phenotype in culture.

The results indicated that restoration of vinculin in these tumor cells to the normal, or near normal levels, led to the drastic suppression of the tumorigenic ability of the cells. In both cell types this effect correlated directly with the level of expression of the foreign vinculin and with the extent of cell adhesion in culture. It thus appears that vinculin can act as an effective tumor suppressor in these malignant cell lines. To assess the significance of these findings it is essential to address two major questions: (*a*) Is the observed effect strictly unique for vinculin? (*b*) What might be the molecular and cellular mechanism responsible for tumor suppression by vinculin?

As far as the specificity of the effect is concerned, we found that control neo^r clones, as well as clones expressing low levels of the transfected vinculin were essentially as tumorigenic as the parental cell line. In addition, revertants that lost the expression of the exogenous vinculin, regained the tumorigenic properties. Furthermore, when attempting to determine whether in vivo there was a selection for decreased expression of vinculin from the injected cells, we found that cell lines established from the metastatic nodules of animals injected with vinculin-transfected adenocarcinoma cells showed, in several such lines, a decreased level of vinculin (data not shown). To exclude the possibility that high levels of the vector itself affect nonspecifically cell behavior, we have carried out Southern blot analyses. The results indicated that a large copy number of the integrated transfected cDNA did not always correlate with high levels of expression of the transfected vinculin, and only the latter corresponded to the suppression of tumorigenic activity (data not shown). The suppression of tumorigenicity was observed with the vinculin-transfected cells in both syngeneic and nude mice which have a compromised immune system, suggesting that immunological reactions are apparently not the cause for the effect observed. This conclusion is also supported by the fact that vinculin is a highly conserved protein (Price et al., 1989; Weller et al., 1990; Ben-Ze'ev et al., 1990), and thus a poor immunogen, and being an intracellular protein, it is not exposed to the immune system. Moreover, overexpression of another cellular protein, such as a mutant murine p53 cDNA which lacks the nuclear localization signals (Shaulsky et al., 1991), had no effect on the tumorigenicity of SVT2 cells (data not shown). It appears therefore that the observations reported in this study are indeed attributable to the specific increase in the expression of the transfected vinculin.

Another concern is whether the phenotypic effect observed in the vinculin transfectants is due only to an increase in vinculin levels, or to the fact that the transfected protein is derived from a different species. The choice of chicken vinculin for transfection was based on both the availability of the respective cDNA, and on the need for vinculin that may be distinguishable immunochemically from the endogenous rodent protein. While we cannot exclude qualitative differences between the chicken and rodent proteins, all the data obtained suggest that the two are functionally indistinguishable. The chicken and mammalian vinculins both human (Price et al., 1989; Weller et al., 1990) and partial sequences of mouse (Ben-Ze'ev et al., 1990) are highly homologous (>95%). Immunofluorescence studies have shown that the transfected vinculin is associated with cell–matrix adhesions in both cell types and the partitioning of the exogenous vinculin between Triton X-100 soluble and insoluble fractions was identical to that of the endogenous protein.

The mechanism whereby a specific increase in vinculin level suppresses tumorigenicity is not clear. Since vinculin is considered a structural component of cell–cell and cell–extracellular matrix contacts, it is conceivable that it exerts its effect on tumorigenicity by affecting cell adhesion and/or motility. The immunofluorescence and electron microscopic results, showing that the area of cell substrate contacts increase in SVT2 and ASML cells expressing high levels of the transfected vinculin, support this notion. The effect of vinculin on cell motility was also recently demonstrated in Balb/c 3T3 cells, expressing chicken vinculin. It was shown that its transfection into 3T3 cells results in a significant reduction in the locomotory ability of the cells (Rodríguez Fernández et al., 1992). In view of the dynamic nature of focal contacts and cell–cell adhesion assembly (Kreis et al., 1984), an increase in vinculin levels can lead, by promoting a cooperative assembly of focal contact proteins, to a shift in the equilibrium between the soluble and membrane-bound pools of these components, to form more and more stable focal contacts (Geiger et al., 1990). This will result in an increased adhesion and reduced locomotion (Rodríguez Fernández et al., 1992), and thus may bring about a suppressive effect on the tumorigenic properties of the transformed cells.

This notion is in line with recent studies which demonstrated that transfecting into mammalian cells, genes coding for individual microfilament-associated proteins may alter microfilament organization and cell movement. For example, transient transfection, or microinjection, of villin into

mesenchymal cells caused the disruption of stress fibers and the formation of microvilli (Friedrich et al., 1989; Franck et al., 1990). Furthermore, the chemotactic migration of 3T3 cells was significantly increased in cells overexpressing gelsolin by 25% after transfection with the respective cDNA (Cunningham et al., 1991). It was suggested that overexpression of gelsolin may impair the assembly of microfilaments and thus enhance cell migration.

It is noteworthy, that transfections into transformed cells of contact-receptors, which are known to associate with the microfilament system through a vinculin-rich plaque, brought about similar effects on the tumorigenic phenotype of cells: Overexpression of either the fibronectin receptor (Giancotti and Ruoslahti, 1990), or E-cadherin (Vleminckx et al., 1991; Frixen et al., 1991; Novarro et al., 1991), resulted in the suppression of the malignant phenotype. Moreover, in the highly metastatic ASML cells studied here, another adhesion molecule, the CD44 hyaluronate receptor, shows a different splicing pattern compared to the nonmetastatic counterpart clone, Bsp73AS. Transfection of the ASML form of CD44 into the latter cell line was sufficient to confer a highly metastatic phenotype on them (Günthert et al., 1991). The present study demonstrated that, in addition to contact receptors, modulations in the expression of a cytoplasmic component of adherens junctions are capable of effectively suppressing the tumorigenic behavior of cells.

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