FIBRONECTIN EXPRESSION IS DETERMINED BY THE

GENOTYPE OF THE TRANSFORMED PARENTAL CELL

IN HETEROKARYONS BETWEEN NORMAL

AND TRANSFORMED FIBROBLASTS

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ABSTRACT

The expression of fibronectin, a cell surface-associated transformation-sensitive glycoprotein, was studied in hetero- and homokaryons of normal and SV40-transformed human fibroblasts. In immunofluorescence, fibroblast homokaryons had an intense surface-associated and intracellular fibronectin fluorescence similar to that of normal fibroblasts. Transformed cells and their homokaryons had a minimal surface-associated and a weak intracellular fibronectin fluorescence. In heterokaryons formed between transformed and normal fibroblasts, the expression of fibronectin fell within 24 h to the level of the transformed cell homokaryons. The change was detectable already at 3 h after fusion and was gene-dose dependent. These results show that the transformed genotype determines fibronectin expression in the heterokaryons.

KEY WORDS   fibronectin · heterokaryon · malignant transformation · phenotypic regulation · fibroblast

Hybrids between normal and transformed cells have been useful in the study of phenotypic expression and interaction of normal and transformed genotypes. Malignancy as studied by inoculation of cells into animals is often suppressed in hybrids (15, 44). On the other hand, hybrid cells in vitro can express either a transformed (3, 4), a normal (41), or an intermediate phenotype (20, 22, 43). Chromosome loss from hybrid cells (23, 42) makes it, however, difficult to determine whether the observed phenotypic changes are because of gene regulation or loss of genetic material.

In heterokaryons, fused cells which contain nuclei of different origin, the genomes of the parental cells remain separate. Before the heterokaryons divide and form mononuclear hybrid cells, no chromosomes are lost. Thus, possible phenotypic changes in heterokaryons more likely result from regulation of gene function (6, 7, 8, 50).

We have analyzed the expression of fibronectin in heterokaryons to investigate the interaction between normal and transformed genotypes in phenotypic expression. Fibronectin is a glycoprotein with a mol wt of 220,000 (11, 35). It occurs in immunologically cross-reactive forms in plasma (29) and in connective tissue (18, 32). In cultured fibroblasts, it is characteristically expressed as a cell surface-associated protein (10, 29, 39, 48), which is greatly reduced after viral transformation (10, 36). The change in fibronectin expression correlates with the tumorigenicity of transformed

cells in vivo (2) and with their decreased adhesion and altered morphology seen in vitro (1, 47, 49). Analogously, surface-associated fibronectin appears on cells when temperature-sensitive Rous sarcoma virus-transformed chick fibroblasts are grown at a temperature nonpermissive for transformation (13, 27). Furthermore, embryonal carcinoma cells of mouse teratocarcinoma begin to express surface-associated fibronectin as they differentiate into nonmalignant endoderm-like cells (38). Based on these findings, fibronectin has been regarded as the most reliable marker distinguishing normal and transformed cells (2, 35).

In the present study, we demonstrate that surface-associated fibronectin is lost from heterokaryons of normal and transformed fibroblasts. This indicates that fibronectin expression is determined by the genotype of the transformed parental cell.

MATERIALS AND METHODS

Cell Cultures

Human embryonic body wall fibroblasts were cultivated in RPMI 1640 medium and used while in their 10th to 20th passage in vitro. VA13, an established line of SV40-transformed Wi38 human fibroblasts (14), was cultivated in Eagle's minimal essential medium. As a marker for the transformed state, the expression of the SV40-induced T antigen was used. The VA13 cells were positive for T antigen as detected by immunofluorescence (17). The media were supplemented with 10% fetal calf serum (Gibco Biocult, Glasgow, Scotland), penicillin (100 IU/ml) and streptomycin (50 μg/ml). The cells were mycoplasma-free as tested by DNA-staining (30).

Labeling of Cells

POLYSTYRENE LABELING OF CELLS: Cells were identified by cytoplasmic labeling with polystyrene particles (37). Fibroblast monolayers were incubated with small polystyrene particles (diameter 0.5 μm, 25 μl of the suspension supplied by the manufacturer per 90-mm diameter Petri dish; Polysciences, Inc., Warrington, Penn.) for 24 h. Nonphagocytized particles were removed by washing the monolayer three times with 10 ml of phosphate-buffered saline (PBS, pH 7.2) and by subsequent centrifugation (twice, 400 g for 5 min) of the cells detached with trypsin. The pelleted cells were plated on glass cover slips (0.2 × 10⁶ cells/50-mm diameter Petri dish) 24 h before fusion.

The transformed cell monolayers were incubated similarly with large polystyrene particles (diameter, 1 μm). After washing, trypsinization and centrifugation, the cells were used for fusion.

RADIOLABELING: In some experiments the transformed cells were simultaneously labeled with large

polystyrene particles and [³H]thymidine (0.05 μCi/ml for 48 h, sp. act. 6.7 Ci/mmol). These cells were fused with fibroblasts labeled with small polystyrene particles. 24 h later, the cells were fixed and subjected to autoradiography as previously described (31). 99% of the transformed cells had incorporated [³H]thymidine in the nuclei as detected by autoradiography.

Cell Fusion

FUSION WITH SENDAI VIRUS: Fibroblasts cultivated on cover slips in a 50-mm diameter Petri dish were washed once with PBS, and then cooled in 3 ml of Hanks' balanced salt solution at +4°C for 10 min. Virus adsorption was done at +4°C by adding 3,000 haemagglutinating units of Sendai virus (beta propiolactone-inactivated according to Neff and Enders, reference 21, courtesy of Dr. K. Cantell, Central Public Health Laboratory, Helsinki, Finland). After 15 min, the virus-Hanks' suspension was drawn off, and 0.5 × 10⁶ transformed cells in 3 ml of Hanks' solution were added. The transformed cells were allowed to adhere to the virus-treated fibroblast monolayer at +4°C for 15 min. Cell fusion was thereafter induced by incubation at +37°C for 30 min. Finally, the cover slips were carefully washed three times in Hanks' solution and cultivated in medium until fixation.

FUSION WITH POLYETHYLENE GLYCOL: Fibroblasts and transformed cells were prelabeled with polystyrene particles and thereafter plated together on cover slips and cultivated for 24 h before fusion (0.2 × 10⁶ cells of each cell line per 50-mm dish). The cover slip cultures were treated with 50% (wt/vol) polyethylene glycol (PEG, mol wt 1,500; Carl Roth, Karlsruhe, Germany) in PBS for 1 min (24). After PEG treatment, the cover slips were washed three times with PBS and incubated in medium at +37°C until harvest.

Immunofluorescence Staining and Microscopy

FIXATION: For staining of surface-associated fibronectin, cells on cover slips were washed once with PBS and fixed with 3.5% paraformaldehyde (wt/vol, in 0.1 M phosphate buffer, pH 7.2), at room temperature for 30 min. After fixation, the cells were washed twice with PBS. To visualize also intracellular fibronectin, the cell membrane was made permeable to antibodies (17) by treating the paraformaldehyde-fixed cells with Nonidet P40 (BDH Chemicals Ltd., Poole, England; 0.05% vol/vol in PBS, 30 min). After the detergent treatment, the cells were washed five times with PBS.

IMMUNOFLUORESCENCE STAINING OF FIBRONECTIN: Indirect immunofluorescence staining was done with rabbit antiserum against human plasma fibronectin and fluorescein isothiocyanate-conjugated sheep anti-rabbit IgG (Wellcome, Beckenham, England). The purity of the antigen used for immunization, the specificity of the antifibronectin serum, and the

staining techniques have been documented previously (33).

MICROSCOPY: A Zeiss Universal microscope equipped with phase contrast and differential interference contrast (Nomarski) optics and epi-illuminator III RS giving blue excitation light (HBO 200 W lamp, excitation filter BP 455-490, dichroic mirror FT 510, and emission filter LP 520) was used for phase contrast and immunofluorescence microscopy.

Evaluation of Fluorescence

Cells were first identified in phase contrast optics according to the number of nuclei and cytoplasmic labeling with polystyrene particles. The phenotype of the identified cell was then determined from the distribution and intensity of the fibronectin fluorescence. The fluorescence of surface-associated and intracellular fibronectin was evaluated in separate experiments.

As the observer knew whether he evaluated the fluorescence of a heterokaryon or a homokaryon, the possible bias as a result of this was estimated by having one observer first identify a multinucleated cell and another observer independently evaluate the fluorescence of the same cell. The results from an analysis performed by two observers of 100 cells were closely similar ($\pm 3\%$) to those obtained by only one observer. This indicated that it was possible to perform an unbiased evaluation.

SURFACE-ASSOCIATED FIBRONECTIN: The fibronectin fluorescence of fused cells was compared to that of unfused cells on the same cover slip. The classification of fibronectin expression was based on the difference between the fluorescence intensity of normal and transformed cells. In the transformed, VA13, cells the surface-associated fluorescence was typically restricted to small strands at the edges of the cells or it was completely absent. This pattern was classified as "negative or weak" fibronectin expression. The fluorescence was classified as "moderate" if fibronectin was seen also on the cell body in addition to the fluorescence of peripheral cytoplasmic parts. The expression was classified as "strong" if the fibronectin network expressed on the cell body was as extensive as that of the confluent fibroblasts on the same cover slip. The greatest amount of surface-associated fibronectin is namely expressed by confluent fibroblasts (12). Based on these criteria, the difference between the normal and transformed cell populations was evident (compare unfused fibroblasts with unfused transformed cells in Fig. 9), although the phenotype of individual cells varied.

INTRACELLULAR FIBRONECTIN: Classification of intracellular fibronectin fluorescence was analogously based on a comparison between normal and transformed cells. Most transformed cells had a thin zone of perinuclear, faint fluorescence, which was classified as "weak." In fibroblasts, the intracellular fibronectin often formed large patchy regions of high fluorescence intensity, the area of which equaled two to three times that of the nucleus. This pattern corresponded to "strong" intracellular fluorescence. Fluorescence intermediate between these two patterns was classified as "moderate."

Reaching an unbiased evaluation was facilitated by the fact that the difference between the two main phenotypic classes ("negative or weak," "moderate or strong") which roughly corresponded to the transformed and normal phenotypes was rather obvious.

RESULTS

Identification of Cells

Cells were identified in the phase contrast microscope according to labeling with cytoplasmic polystyrene particles. In visual inspection, the various polystyrene particles could easily be distinguished by focusing to different levels. In photomicrography, the visualization was more difficult because the particles were often located in different planes. The efficiency of the labeling method was evaluated by studying 200 single cells of both cell lines labeled and cultivated separately. Fibroblasts contained ~10-50 small particles per cell. Only 2% of the cells were unlabeled. The transformed cells had ~10-30 large particles per cell. Only 1% of the cells lacked particles. The method could therefore be used for identification of fibroblasts and transformed cells also in cocultivation. After cell fusion, multinucleated cells were identified as homokaryons (particles of one size, Figs. 1 and 2) or heterokaryons (particles of two sizes, Fig. 3).

The reliability of identification based on polystyrene labeling was controlled by [³H]thymidine labeling of the transformed cells before fusion. Autoradiographs were made 24 h after fusion, and the distribution of the [³H]thymidine label was compared to the distribution of polystyrene particles. After fusion with polyethylene glycol, at least 95% of the multinucleated cells could be reliably identified on the basis of polystyrene labeling alone (Figs. 1-3, Table I). Fusion with Sendai virus resulted in even better identification of the multinucleated cells.

Among the multinucleated cells, 40-60% were binucleated, 15-35% trinucleated, 5-10% had four nuclei, and 5-15% had five or more nuclei. Neither cell line was preferentially included in the heterokaryons as analyzed in autoradiography (Table II).
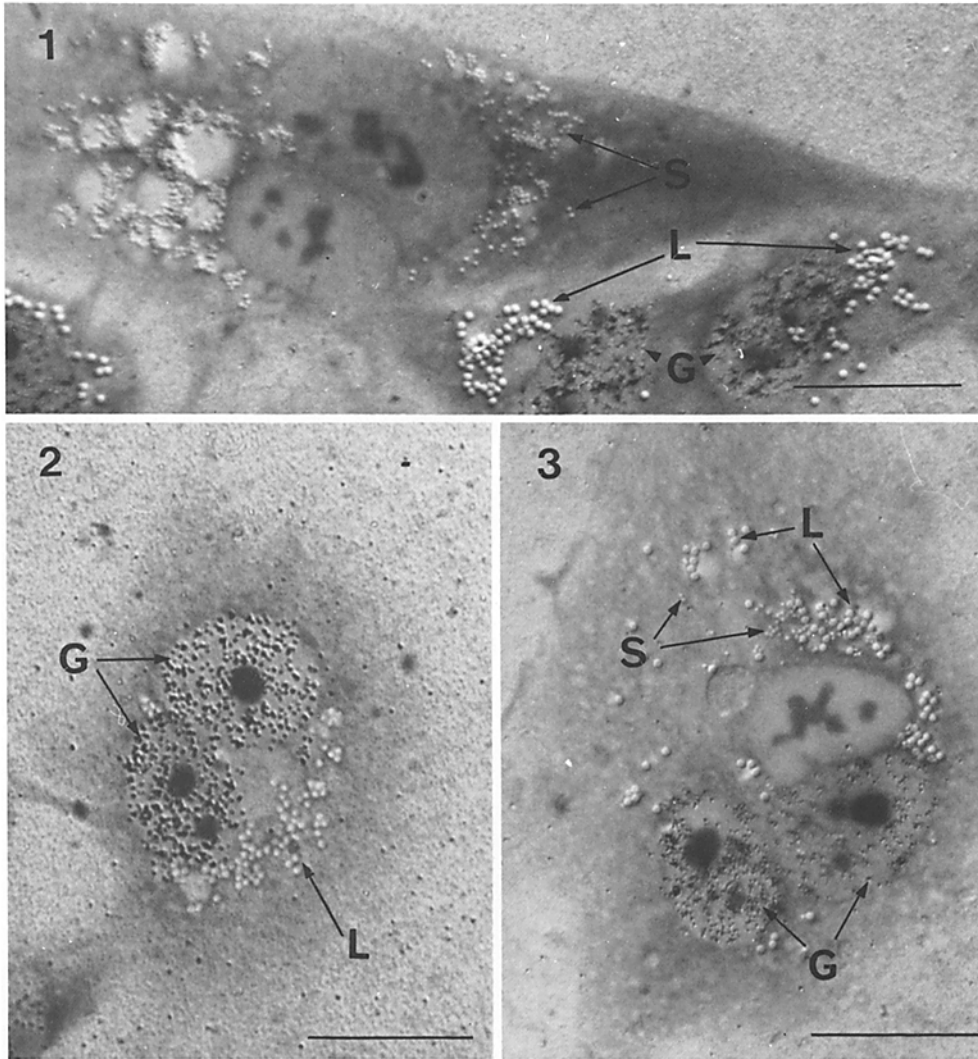
FIGURES 1-3 Autoradiography of fused cultures of fibroblasts and SV40-transformed cells. The fibroblasts were labeled with small polystyrene particles (diameter, 0.5 μm). The transformed cells were labeled with large particles (diameter, 1.0 μm) and with [^{3}H]thymidine. Giemsa stain. Differential interference contrast (Nomarski). × 1,100. Bar, 20 μm.

FIGURE 1 A binucleated fibroblast homokaryon and two unfused transformed cells (lower right) 1 day after fusion. The cytoplasm of the fibroblast homokaryon contains only small (S) particles, and the cytoplasm of the transformed cells only large (L) particles. Silver grains (G) are seen only on the nuclei of the transformed cells.

FIGURE 2 A binucleated homokaryon of transformed cells 1 day after fusion. The cytoplasm contains only large (L) particles, and silver grains (G) are seen on the nuclei.

FIGURE 3 A heterokaryon containing two transformed nuclei and one fibroblast nucleus 1 day after fusion. Both small (S) and large (L) particles are seen in the cytoplasm. Silver grains (G) are seen only on the two nuclei from the transformed cells. The nucleoli of the fibroblast nuclei are irregular in shape. The nucleoli of the transformed nuclei are more regular and round.

TABLE I

Identification of Fused Cells as Heterokaryons or Homokaryons by Simultaneous [³H]Thymidine and Polystyrene Particle Labeling

	Cell type identified with [³H]thymidine labeling		
Cell type identified with polystyrene labeling	VA13 homokaryon	Fibroblast homokaryon	Heterokaryon
VA13 homokaryon	83*	0	0
Fibroblast homokaryon	0	75	4
Heterokaryon	2	5	136
Cell without polystyrene particles	3	0	0

The cells were fixed 24 h after fusion with polyethylene glycol. The nuclear labeling with [³H]thymidine of a multinucleated cell was compared to the cytoplasmic labeling with polystyrene particles of the same cell.

* No. of cells

Fibronectin in Fibroblasts and their Homokaryons

To study surface-associated fibronectin, para-formaldehyde-fixed fibroblasts and their homo-karyons were evaluated. A characteristic abundant, strandlike, surface-associated fluorescence pattern was seen (Fig. 4).

The expression of intracellular fibronectin was analyzed in paraformaldehyde-fixed and NP40-treated cells. In addition to the strandlike, surface-associated fluorescence, the fibroblasts and their homokaryons had also an intense patchy perinuclear fluorescence (Fig. 5).

The distribution of the fibronectin fluorescence detected in fibroblast homokaryons throughout the experiment was similar to that in the unfused fibroblasts. No difference between the particle-labeled and the unlabeled cells could be detected.

Fibronectin in Transformed Cells and their Homokaryons

In transformed cells and their homokaryons, surface-associated fibronectin was seen only as short fluorescing strands in the cell periphery (Fig. 6), and especially in contact areas of adjacent cells. Only a faint diffuse intracellular fluorescence was detected in transformed cells and their homokaryons (Fig. 7). No difference could be detected between the homokaryons and single cells. Polystyrene labeling did not have an effect on the fibronectin fluorescence.

Fibronectin in Heterokaryons between Normal and Transformed Cells

In heterokaryons the surface-associated fibronectin fluorescence was weak one day after fusion. It was generally similar to the fluorescence of transformed cell homokaryons. Only few fluorescing short strands were seen at the outer edges of the cells (Fig. 4). The intracellular fluorescence was weak in heterokaryons (Fig. 8) and more diffuse than in homokaryons of normal cells (Fig. 5) but somewhat stronger than in transformed homokaryons (Fig. 7).

Kinetics of Fibronectin Expression

HOMOKARYONS OF FIBROBLASTS: 3 h after fusion, the expression of surface-associated fibronectin was reduced in fibroblasts and their homokaryons as compared to unfused cells from cultures not treated with virus or polyethylene glycol. 24 h after fusion, the frequency of positive cells had returned to the normal level of unfused cells and remained stable (Fig. 9). Intracellular fibronectin was not affected by fusion (Fig. 9).

HOMOKARYONS OF TRANSFORMED CELLS: Transformed, VA13 cells and their homokaryons expressed typically low amounts of surface-associated (Fig. 6) and intracellular (Fig. 7) fibronectin. The fibronectin expression remained weak within 3 days after fusion (Fig. 9).

HETEROKARYONS: Changes in fibronecting

TABLE II

Distribution of Nuclei from Transformed and Normal Fibroblasts in Heterokaryons

No. of fibroblast nuclei in individual heterokaryons	No. of VA13 nuclei in individual heterokaryons					Total cell no.
	1	2	3	4	5	
1	25*	10	11	1	0	47
2	10	8	4	1	0	23
3	6	4	4	0	1	15
4	3	1	1	3	0	8
5	0	1	2	0	0	3
Total cell no.	44	24	22	5	1	96

The cells were fixed 24 h after fusion with Sendai virus, and the distribution of [³H]thymidine labeled nuclei from transformed cells was studied by autoradiography.
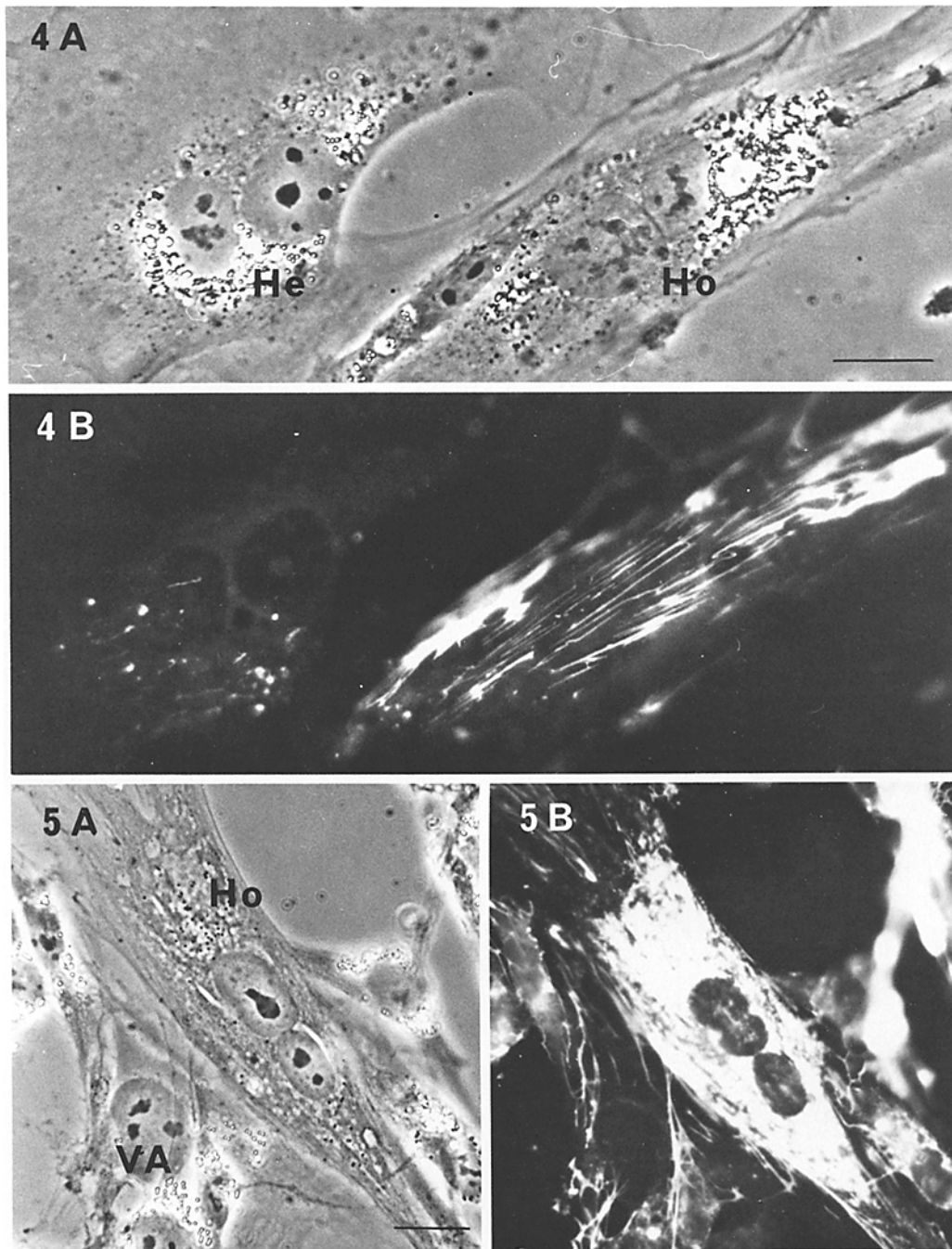
* Number of cells

FIGURE 4 (A) Phase contrast micrograph of a binucleated fibroblast homokaryon (Ho) and a binucleated heterokaryon (He). The cells have been fixed with paraformaldehyde 1 day after fusion. Identification of the cells was based on labeling with cytoplasmic polystyrene particles of two different sizes. At this magnification the particles appear partly aggregated and some are out of focus. (B) An immunofluorescence micrograph of the same field. The fibroblast homokaryon displays the typical strandlike, surface-associated fibronectin fluorescence, whereas the heterokaryon expresses only small fluorescing patches of fibronectin. × 700. Bar, 20 μm.

FIGURE 5 (A) Phase contrast micrograph of a binucleated fibroblast homokaryon (Ho) and several transformed VA13 cells (VA). The cells have been fixed with paraformaldehyde and subsequently treated with NP40. (B) In immunofluorescence, both surface-associated and intracellular fibronectin fluorescence is visualized. In the fibroblast homokaryon, a typical strong perinuclear fluorescence is seen. The transformed VA13 cells have, in addition to few extracellular strands, either a faint or no intracellular fluorescence. × 550. Bar, 20 μm.
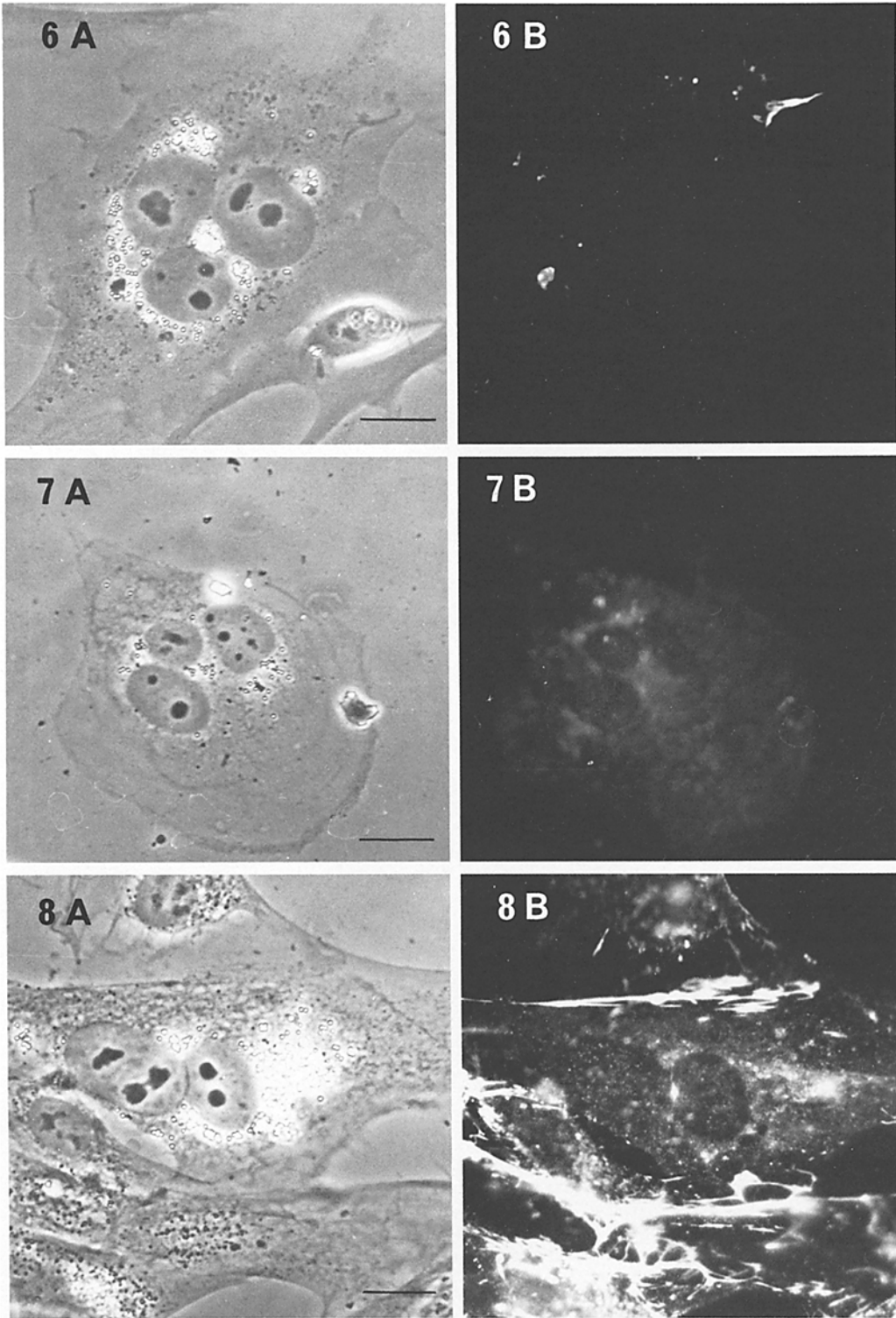
expression were studied by comparing the hetero-karyons to the homokaryons of normal and transformed human fibroblasts. As compared to the fibroblast homokaryons, the frequency of hetero-karyons expressing surface-associated fibronectin was reduced already at 3 h after fusion (Fig. 9). Within 24 h, the frequency of positive heterokar-

FIGURE 9 Expression of surface-associated (top) and intracellular (bottom) fibronectin in homo- and hetero-karyons of normal and transformed fibroblasts. The height of the bars indicates the percentage of the cells expressing fibronectin at various time points (3 h to 3 days) after fusion. The intensity of the fluorescence is classified as strong (dotted part of the bars) or moderate (open part of the bars). The remaining population expressed weak or negative fibronectin fluorescence (see text). Bars marked Co (control) show the fluorescence intensity of unfused cells 24 h after fusion. The 95% confidence limits (binomial distribution, moderate or strong vs. weak or negative) are indicated. The small numeral below each bar indicates the number of cells studied. 10% of all the cells studied were independently evaluated by two observers.

yons diminished further and almost reached the level of the VA13 homokaryons (Fig. 9). Intracellular fibronectin expression in heterokaryons was little affected in comparison to the fibroblast homokaryons (Fig. 9).

Heterokaryons with Unequal Numbers of Normal and Transformed Nuclei

In homokaryons formed by fibroblasts or transformed cells the number of nuclei had no detectable effect on the expression of surface-associated or intracellular fibronectin as compared to unfused cells.

80 heterokaryons were analyzed to examine a possible gene-dose effect on fibronectin expression in heterokaryons. The nuclei were identified on the basis of nucleolar morphology. The fibroblasts characteristically had irregular nucleoli (Figs. 1 and 3) and the transformed, VA13 cells had condensed and round nucleoli (Figs. 2 and 3).

Heterokaryons with more VA13 than fibroblast nuclei either completely lacked or had a weak surface-associated fluorescence (Table III). On the other hand, in cells with an excess of fibroblast nuclei the fibronectin expression was moderate. In these cells the fibronectin fluorescence occurred especially at the edges of the cells, but it was still clearly less intense than in fibroblast homokaryons (Table III).

DISCUSSION

The major finding of the present study was that the expression of fibronectin, a cell surface-associated, transformation-sensitive protein, was suppressed in heterokaryons between normal and SV40-transformed human fibroblasts. One day after fusion, the expression of surface-associated fibronectin had diminished to the level of the
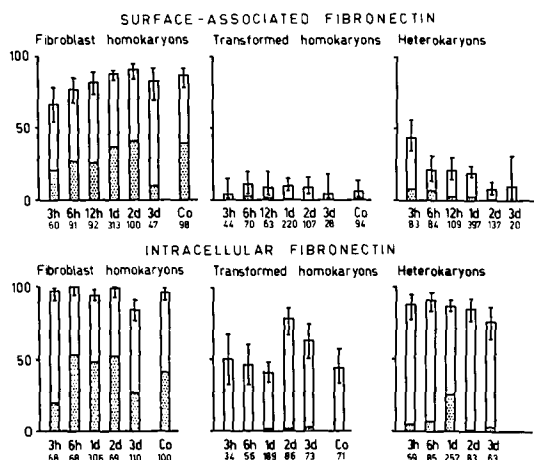
FIGURE 6 (A) Phase contrast micrograph of a trinucleated homokaryon or transformed cells, 1 day after fusion, fixed with paraformaldehyde. (B) In immunofluorescence, surface-associated fibronectin is seen as weakly fluorescing patches. × 550. Bar, 20 μm.

FIGURE 7 (A) Phase contrast micrograph of a trinucleated VA13 homokaryon, 1 day after fusion, fixed with paraformaldehyde and treated with NP40. (B) The intracellular fluorescence is faint. × 550. Bar, 20 μm.

FIGURE 8 (A) Phase contrast micrograph of a binucleated fibroblast-VA13 heterokaryon, 1 day after fusion, fixed with paraformaldehyde and subsequently treated with NP40 for intracellular staining of fibronectin. (B) In immunofluorescence, a few peripheral, extracellular, fluorescing strands can be seen in addition to the faint intracellular fluorescence. × 550. Bar, 20 μm.

Fibronectin expression	Excess of fibroblast nuclei[*]	Excess of VA13 nuclei[‡]
Negative or weakly positive	23[§]	30
Moderately or strongly positive	26	1

30% of the cells were independently evaluated by two observers.

[*] Heterokaryons with one VA13 nucleus and 2 or 3 fibroblast nuclei

[‡] Heterokaryons with one fibroblast nucleus and 2 or 3 VA13 nuclei

[§] No. of cells

transformed cell homokaryons. On the other hand, in fibroblast homokaryons fibronectin expression increased with time. This is in accordance with the continuous accumulation of fibronectin into an extracellular matrix during cultivation of fibroblasts in vitro (2, 5, 9, 19). The results indicate that the transformed genome determines the expression of fibronectin in heterokaryons. This is further supported by our observation that the effect was gene dose dependent.

Most previous studies on the interaction between normal and transformed genomes have been performed with continuously growing hybrid cell lines (see review by Ringertz and Savage, reference 26). In hybrids of normal and SV40-transformed cells, SV40 T antigen expression and tumorigenicity dominated (3, 4, 16). On the other hand, tumors were not induced by mouse cell hybrids which contained various mouse tumor cell genomes (15, 44, 45, 46), indicating that malignancy had been suppressed. After prolonged cultivation of these hybrid cells in vitro, however, chromosomes were lost and malignancy in vivo was re-expressed (15). Furthermore, hybrid cells with an intermediate phenotype have been described (20, 22, 43).

The discrepancies in these results can be a result of the loss of different chromosomes from the hybrid cell lines (42). Also, cell cultivation and conditions applied in tests for tumorigenicity and other transformed characteristics exert a selective pressure on the cell population. Phenotypic changes in continuously growing hybrid cell lines

can thus reflect adaptation or selection rather than genetic regulation.

In heterokaryons, on the other hand, the interacting genomes are intact. Phenotypic changes are therefore more likely a result of regulatory events than gene loss or selection. It is conceivable that such regulation takes place through nucleo-cytoplasmic interaction (6, 7, 8, 25). Genome interactions mediated through a common cytoplasm probably took place also in our experiments, in which phenotypic changes were observed within 3-24 h after fusion. Analogously, SV40 T antigen was transmitted within 1 day to the normal nuclei of heterokaryons between normal and transformed cells (34). In heterokaryons containing chick erythrocyte nuclei, the SV40 T antigen migrated into the activated erythrocyte nuclei during days 2-4 (28). Similarly, fibronectin was lost 3-5 days after fusion from the surface of heterokaryons between chick erythrocytes and human fibroblasts (40).

In conclusion, the present results show that in heterokaryons between normal and transformed human fibroblasts the expression of the phenotype is controlled by the transformed parental cell genome. Whether this is exclusively a result of the dominance of the transformed state or is affected by the state of differentiation of the parental cells should be further studied in heterokaryons formed between fibroblasts and nontransformed cells which do not produce fibronectin.

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