EXPRESSION OF MICROTUBULE NETWORKS IN NORMAL CELLS, TRANSFORMED CELLS, AND THEIR HYBRIDS

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ABSTRACT

Microtubules play an important role in several cellular functions including cellular architecture and chromosome movement in cell division. Tubulin which polymerizes to form mictotubules can be purified to homogeneity and used to raise antisera. Antisera prepared against porcine or chicken tubulin reacts well with mammalian tubulin. We have examined normal and transformed cells of mouse and human origin for microtubules by indirect immunofluorescence methods. Extensive networks of microtubules (MN) are easily detectable in normal and some transformed cells. The fixation procedure employed and the morphology and the cellular attachment properties seem to determine the ease of detection of MN in these cells. Cells derived from tumors and exhibiting several transformed phenotypes contained MN comparable to those of normal cells. Hybrids between transformed mouse cells and normal human cells were examined. They showed a variability in morphology, but all contained MN. These hybrids exhibited several transformed phenotypes. We conclude that in the cell lines we have examined there is no correlation between the transformed phenotypes and the organization of tubulin.

KEY WORDS microtubules · immunofluorescence · transformed phenotypes · somatic cell hybrids

Of the fibrous structures present in mammalian cells, the most prominent are 6-nm microfilaments, 10-nm filaments and 25-nm filaments. The largest of these structures are microtubules and could constitute 5–15% of the soluble protein in cultured cells (10, 14). Microtubules are involved in such essential functions as cellular movement, chromosome movements in mitosis and meiosis, and in intracellular movements of organelles (for reviews see references 13 and 20). The basic subunit of the microtubule is tubulin, which has a mol wt of 110,000 daltons. The native form of tubulin found in cytoplasm is a heterodimer of two polypeptide chains, α and β (11). Because of the relative abundance of this protein in cells, it is possible to obtain large amounts of purified tubulin. Antibodies against purified tubulins have been obtained and were shown to react with tubulin from evolutionarily distant species; for example, antisera raised against tubulin from sea urchin sperm flagellar outer doublets react with human microtubules (23).

Mammalian cells in culture have been stained with antibody against tubulin, and extensive arrays of microtubules can be seen to radiate from the nucleus to the cell boundary (1, 23). After the discovery that transformed cells lack or have diminished microfilament bundles or actin cables (16), several investigators examined normal and transformed cells by electron microscopy and in-

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direct immunofluorescence microscopy for alterations in the cytoplasmic microtubule complex or microtubule networks (MN). Brinkley et al. (1) and Miller et al. (12) have tested transformed cells, normal cells, and their somatic cell hybrids for MN after fixation with formaldehyde. They concluded that transformed cells have diminished MN and that the hybrids can be classified into four groups according to the extent of MN they possess. They have correlated the different degrees of MN in these hybrids with two different parameters of transformation. Similar observations with transformed cells have been made by other investigators (see reference 5 for example). Other investigators (6, 15, 19) claim that there are no differences in MN between normal and transformed cells. To resolve these differences, we have tested several cell lines for MN after different methods of fixation and present the results here. Our results indicate that transformed cells, normal cells, and their cell hybrids all contain MN and that the differences that some previous investigators have observed might be due to the fixation procedure and cellular morphology. We failed to find any correlation with transformed phenotypes and extent of MN in these cells.

MATERIALS AND METHODS

Cells

PG19 is an hypoxanthine transferase (HPRT)-deficient mouse cell line derived from a mouse melanoma (8, 9) and was kindly provided by Dr. R. Kennett (University of Pennsylvania). GM1429 is a human fibroblast line with the chromosomal composition 46, X, rcp(X; 9)(q13; q32) and was purchased from the Institute for Medical Research (Camden, N. J.). HT1080 cells are derived from a human sarcoma (18) and were obtained from the American Type Culture Collection. All these cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO H-21; Grand Island Biological Co., Grand Island, N. Y.) supplemented with penicillin and streptomycin, 10% fetal bovine serum (Flow Laboratories, Rockville, Md.) and glutamine.

Hybrid Cell Lines

The PEP series were obtained by fusion of PG19 cells with BP, a normal diploid fibroblast cell line supplied by Dr. F. Gilbert (University of Pennsylvania). MGM hybrids are derived from fusion of PG19 and GM1429. Cells were fused in monolayer according to the procedure described by Davidson et al. (4). Briefly, $\sim 10^6$ cells each of the two types were plated in a T-25 flask and 24 h later the medium was removed and 3 ml of 50% wt/vol polyethylene glycol (PEG) 1000 (J. T. Baker Chemical Co., Phillipsburg, N. J.) was added. After 1 min the PEG was removed, the cell sheet was washed 3× with DMEM, and fresh medium was added. 24 h later, the cells were trypsinized and plated in a number of T-25 flasks containing DMEM containing 13.6 μg/ml hypoxanthine, 0.19 μg/ml aminopterin, 3.8 μg/ml thymidine (HAT), and 10^{-5} M ouabain. Colonies were isolated by using stainless steel cylinders, and hybrids were maintained in DMEM-HAT.

Chromosome Analysis

The chromosomal composition of the cell hybrids was determined by the following method: Air-dried chromosome preparations were made by conventional methods and stained with Atebrin by the method described by Uchida and Lin (21) and photographed on H & W film with a Zeiss axiomat microscope equipped with epifluorescence optics.

Visualization of Microtubule Networks

Cells were plated on 22 × 22 mm glass cover slips. 24–48 h later, the cover slips were removed and fixed according to one of the three following procedures:

(a) Cover slips containing cells were rinsed in phosphate-buffered saline (PBS, pH 7.2) at room temperature, and treated with 3.7% formaldehyde in PBS for 8–10 min. They were then washed in PBS and immersed in 50% acetone in PBS at 4°C for 3 min. The cover slips were then passed through 100% acetone at −10°C for 5 min, 50% acetone for 3 min, and PBS for 1 min. They were immediately stained for immunofluorescence.

(b) The second method of fixation was that of Osborn and Weber (15). The cover slips containing cells were immersed into cold methanol at −20°C for 5–6 min. They were then rinsed in PBS and stained.

(c) The third method was also described by Osborn and Weber (15). Cells growing on cover slips were washed in microtubule stabilization buffer (MSB; 0.1 M piperazine-*N*, *N*′-bis[2-ethane sulfonic acid] sodium salts adjusted to pH 6.9 with KOH, 1 mM EGTA, 0.1 mM GTP, and 4% polyethylene glycol 6,000) and then incubated for 3–12 min in the same buffer containing 0.5 Triton X-100. The cells were then rinsed in two changes of MSB at 37°C. After this treatment the cover slips were immersed in methanol at −20°C for 5–6 min, rinsed in MSB, and stained immediately. The detergent-resistant cellular structure is referred to as the cytoskeleton.

ANTIBODY STAINING: Antisera were prepared by Dr. V. Kalnins and J. Connolly (University of Toronto) and provided by Dr. M. Kirschner (Princeton University). Porcine or chicken tubulin was purified and antiserum was raised in rabbits (2, 3). The antibody was shown to be specific to tubulin.

Cover slips containing cells fixed by one of the above techniques were placed in a petri dish, and 75 μl of antiserum diluted 1:30 in PBS was added to each and incubated at 37°C for 45–60 min in a humidified atmos-

phere. The cover slips were then washed in PBS, and 75 μl of fluorescein-conjugated goat anti-rabbit IgG (Hyland Diagnostics Div., Travenol Laboratories, Inc.) diluted 1:2 in PBS was added. After incubation for 45–60 min at 37°C, the cover slips were washed and mounted in a solution of 50% glycerol in PBS, pH 7.8. The cells were observed with a Zeiss axiomat microscope equipped with epifluorescence optics and photographed on Kodak Tri-X film. The film was developed with Diafine (Acufine).

RESULTS

Immunofluorescence of Normal Cells

Normal diploid human fibroblasts at early and late passages were tested for MN with antitubulin antibody after fixation. The fibroblasts attach firmly to the substrate and appear very flat (Fig. 1 *A*). Extensive networks of fibers can be seen in these cells. Pretreatment of these cells with colchicine or cold temperature eliminated all such networks. Microtubules are sensitive to colchicine and low temperatures, while other fibrous structures are insensitive (25). Some cells exhibited intense nonspecific nuclear fluorescence (Fig. 1 *B*). This aspect could not be correlated with the particular fixation procedure employed. Brinkley and coworkers (1) have also observed such nuclear fluorescence in formaldehyde-fixed preparations. Fibroblasts treated by any one of the three procedures exhibited extensive MN (Fig. 1 *B–F*). The age of the cell lines or the density of the cell culture had no effect on the display of MN. Mouse 3T3 cells also exhibit extensive MN (results not shown).

Immunofluorescence of Transformed Cells

Two different transformed cell lines, one of human and another of mouse origin, were tested for MN. The morphologies of the two cell types are different. Human HT1080 cells are flat and attach firmly to the substratum and contain a well-defined nucleus with cytoplasmic areas clearly distinguishable (Fig. 2 *A*). Mouse PG19 cells (Fig. 2 *D*) are spindle shaped, usually rounded, and are not firmly attached to the substratum. In the PG19 cells, the nucleus occupies a large portion of the cell volume; the cytoplasm is barely discernible. These two cell types were stained for MN after formaldehyde fixation (Fig. 2 *B* and *E*). HT1080 cells display an abundant array of MN, whereas the PG19 cells show a diffuse perinuclear and cytoplasmic fluorescence. To determine whether these differences reflect variations in fixation procedure, we examined cells grown in identical conditions and having identical morphologies but fixed in methanol with or without prior conversion to cytoskeletal preparations. PG19 cells fixed in methanol do not show abundant MN and, in many cases, are indistinguishable from formaldehyde-fixed preparations, while the cytoskeletal preparations show such networks (Fig. 2 *F* and *G*). HT1080 cells display MN under all conditions (Fig. 2 *B* and *C*). Though no quantitation was attempted, there do not seem to be any differences in the density of MN per unit area of cytoplasm in these two cell lines.

The two cell types were tested for transformed phenotypes. HT1080 was derived from a human sarcoma (18) and PG19 was derived from a mouse melanoma (9). Both cell types grow in media containing 2% serum, do not exhibit density-dependent regulation of growth, form colonies in 1.2% methylcellulose, and are capable of forming tumors in immunodeficient nude mice (8; Kucherlapati and Shin, 1979, *Cell* **16**:639–648; and our unpublished results). Thus, the two cell types exhibit identical tumor-related or transformed phenotypes but differ in cellular morphology, nuclear/cytoplasmic ratio, and properties of attachment to plastic or glass surfaces. The fact that MN can be detected in PG19 cells after conversion to cytoskeletons followed by methanol fixation indicates that they do contain cytoplasmic microtubules. The inability to detect the networks after formaldehyde fixation might reflect differences in cell shape, morphology, and attachment properties rather than an intrinsic difference in the organization of microtubules in these cells. It is also possible that formaldehyde differentially affects the MN in different cells.

To determine whether treatment with the microtubule stabilization buffer results in formation of MN, we conducted the following experiment: Human diploid fibroblast cells were kept at 4°C for 1 h to depolymerize the microtubules. The cells were then fixed in methanol or treated with MSB for 1, 3, or 5 min and examined for MN. Cells maintained at 37°C showed abundant MN, while cold-treated or cold-plus-MSB-treated cells did not show any MN. Reincubation at 37°C for 30 min or more restored the MN. Thus, under the conditions we employed, treatment with MSB did not lead to formation of MN.

Immunofluorescence of Hybrid Cells

Somatic cell hybrids derived from fusion of

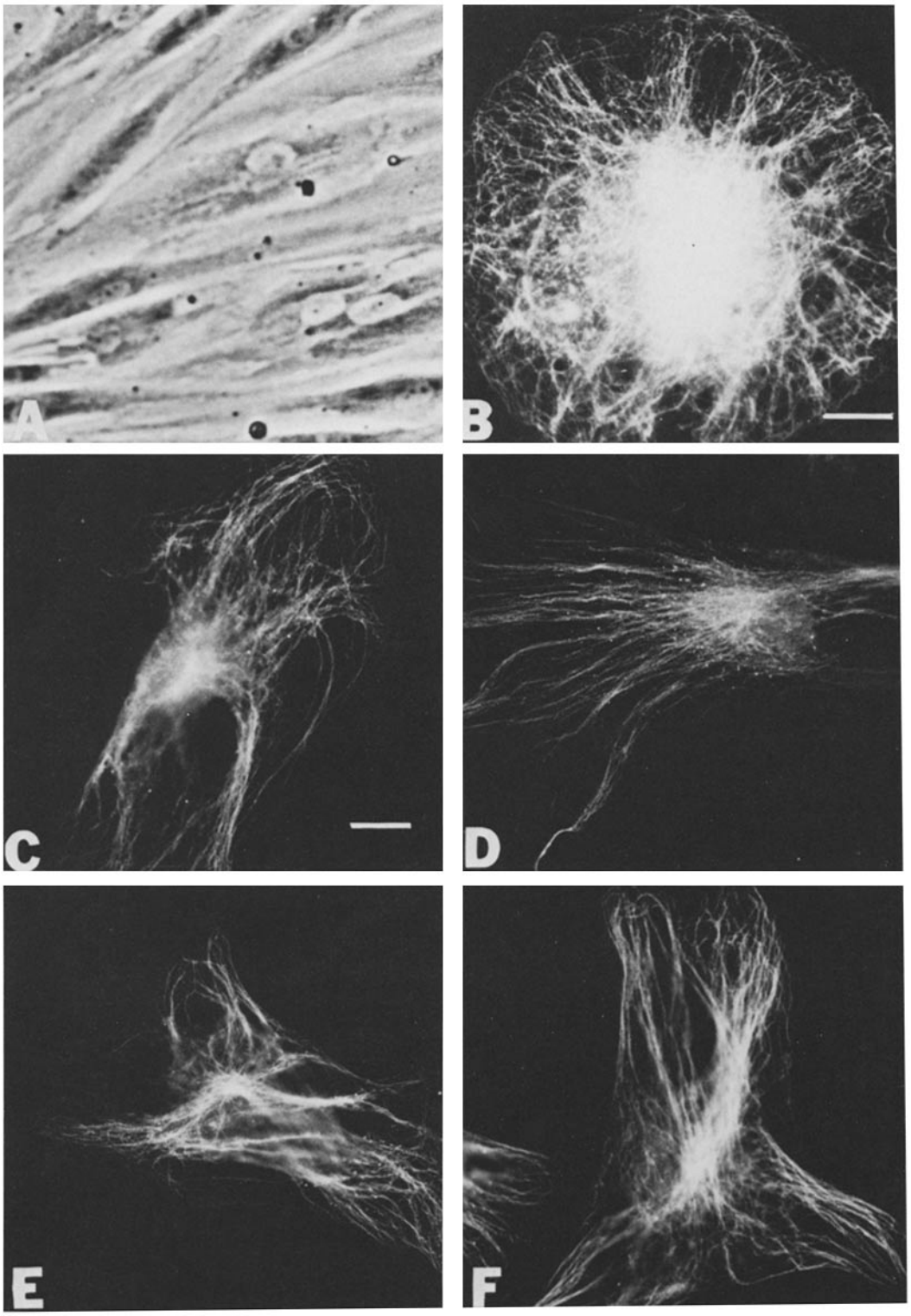

FIGURE 1 Normal diploid human fibroblasts stained with antitubulin antibody by indirect immunofluorescence. (*A*) Phase contrast. (*B*) Cells treated with nonionic detergent to generate cytoskeletons. (*C* and *D*) Cells fixed in formaldehyde. (*E* and *F*) Cells fixed in methanol. Bars, 10 μm.

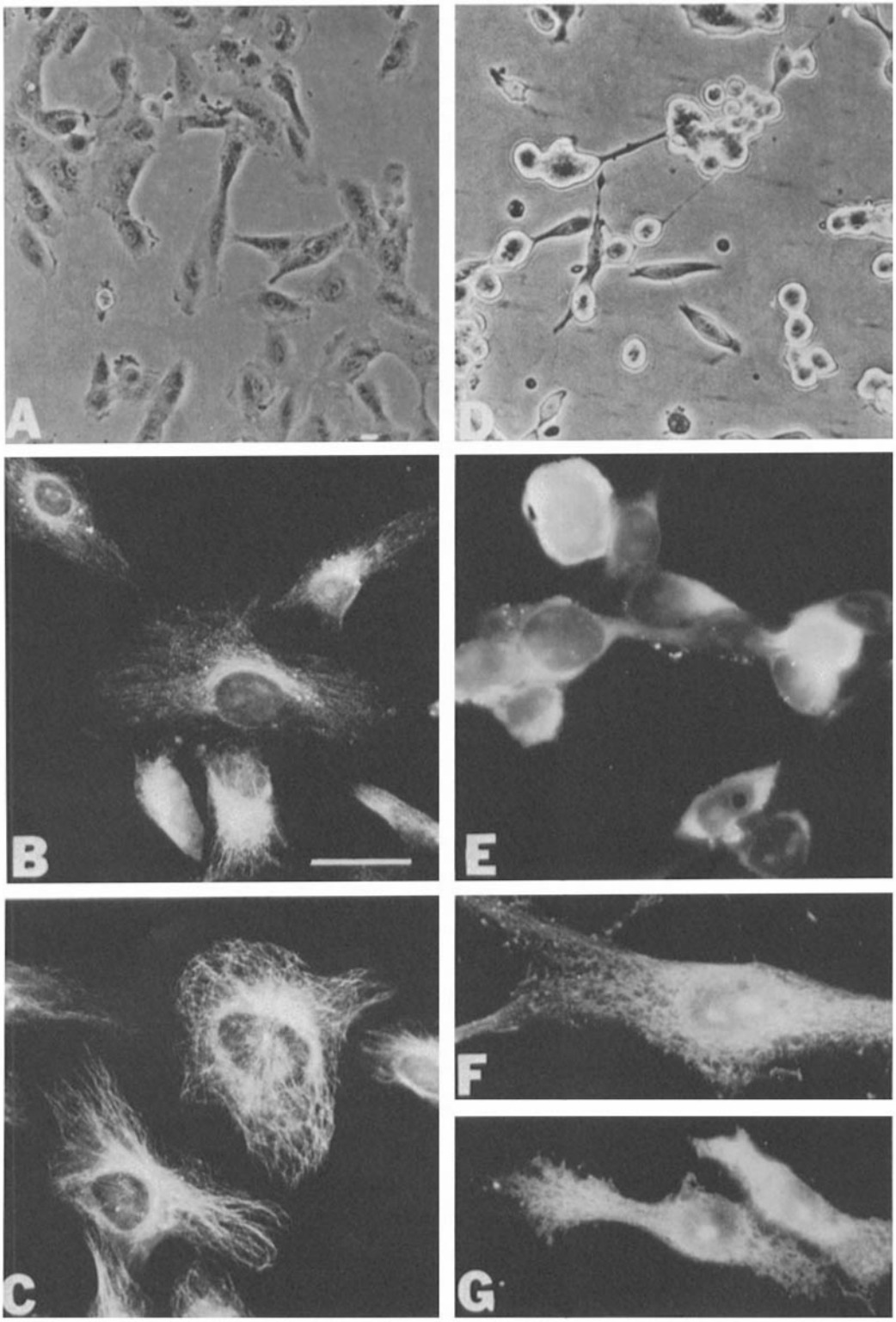

FIGURE 2 Transformed human (HT1080) cells and mouse (PG19) cells stained for microtubule networks. (*A*) Phase-contrast photograph of HT1080 cells. (*B*) HT1080 cells fixed in formaldehyde. (*C*) HT1080 cells fixed in methanol. (*D*) Phase-contrast photograph of PG19. (*E*) PG19 fixed in formaldehyde. (*F* and *G*) PG19 cytoskeletons. Bars, 10 μm.

PG19 and one of two different human diploid fibroblasts were examined for microtubule arrays after fixation with formaldehyde. The stained slides were independently examined by two individuals. The results are presented in Table I. There is a great degree of heterogeneity among the hybrids. Some hybrids were very similar to the PG19 parent, displaying diffuse cytoplasmic fluorescence, while others had well-defined MN similar to those observed in the human diploid fibroblasts; still others can be considered intermediate in the extent of MN. All of the hybrids retained the full mouse genome and partial complements of the human genome. The different patterns of fluorescence did not correlate with the presence or absence of any specific human chromosome (Table I). To determine whether the observed differences after formaldehyde fixation are a function of the fixation procedure, we tested several of the hybrid cell lines for microtubule arrays after preparing cytoskeletons. The results are presented in Figs. 3 and 4. Though there are slight differences in the fluorescence patterns after various fixation procedures, it is clear that all these hybrids contain abundant networks of microtubules. As in the parental cells, the differences in fluorescence after formaldehyde fixations seem to be attributable to the variation in cellular morphology rather than the presence or absence of microtubule arrays.

We have determined that the variability of fluorescence after formaldehyde fixation cannot be correlated with transformed phenotypes. Several of the hybrids were tested for tumorigenicity in nude mice and other aspects of transformation such as serum dependence and ability to form colonies in 1.2% methylcellulose. All hybrids tested exhibited all these tumor-associated phenotypes (Table I and Kucherlapati and Shin, 1979, *Cell* **16**:639–648). These results indicate that there is no relationship between these phenotypes and the variation in immunofluorescence after formaldehyde fixation.

TABLE I

Properties of Normal Cells, Transformed Cells, and their Cell Hybrids

Cell line	Formaldehyde Fixation*		Cytoskeletons	Tumors in nude mice	Human chromosomes‡
	OBS 1	OBS 2			
GM1429	+	+	+	−	All
PG19	−	−	+	+	None
PEP6a	+	+	+	+	1, 2, 4, 6, 7, 10, 11, 13, 16, 17, 18, 19, X
PEP7a	+	+	+	+	4, 5, 6, 10, 11, 12, 16, 17, 18, 19, 20
PEP7d3B	−	−	+	NT	7, 13, 14, X
PEP8a	+	+	+	+	1, 2, 3, 4, 5, 6, 7, 8, 10, 11, 12, 15, 16, 17, 20, 21, X
PEP9c	+	+	+	+	6, 7, 18, X
PEP12c	±	±	+	+	2, 3, 4, 6, 7, 8, 9, 10, 11, 13, 14, 15, 16, 17, 20, 21, 22, X
PEP12e	+	+	+	+	2, 6, 7, 8, 9, 11, 17, X
MGM19a	±	−	+	+	1, 2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 16, 17, 18, 20, 22, X
MGM21c	+	+	+	+	1–12, 15–18, 20, 21, X
MGM23c	+	+	+	+	1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 16, 17, 18, 22
MGM39a	−	−	+	+	5, 6, 7, 8, 9, 10, 11, 12, 13, 16, 17, 18, 20

Only chromosomes that were present in >10% of the cells were included. 15–40 cells were examined for each cell line.

NT, Not tested.

* The slides were examined independently by two observers.

‡ Data from Kucherlapati and Shin (1979, *Cell* **16**:639–648).

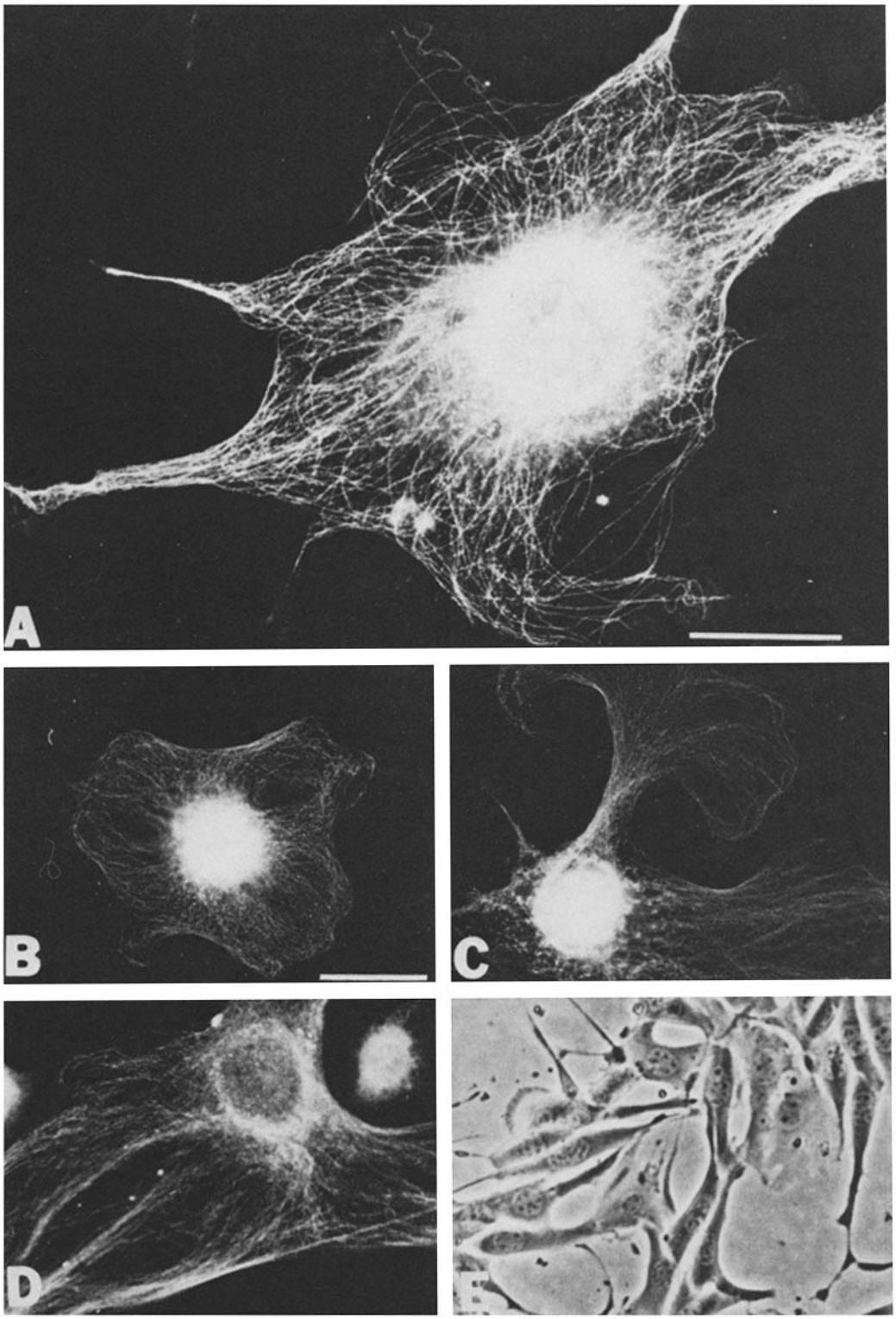

FIGURE 3 Somatic cell hybrid lines PEP9c and PEP12c stained for microtubule networks. (*A*) PEP9c cytoskeleton. (*B* and *C*) PEP9c fixed in formaldehyde. (*D*) PEP12c cytoskeleton. (*E*) PEP12c phase contrast. Bars, 10 μm.

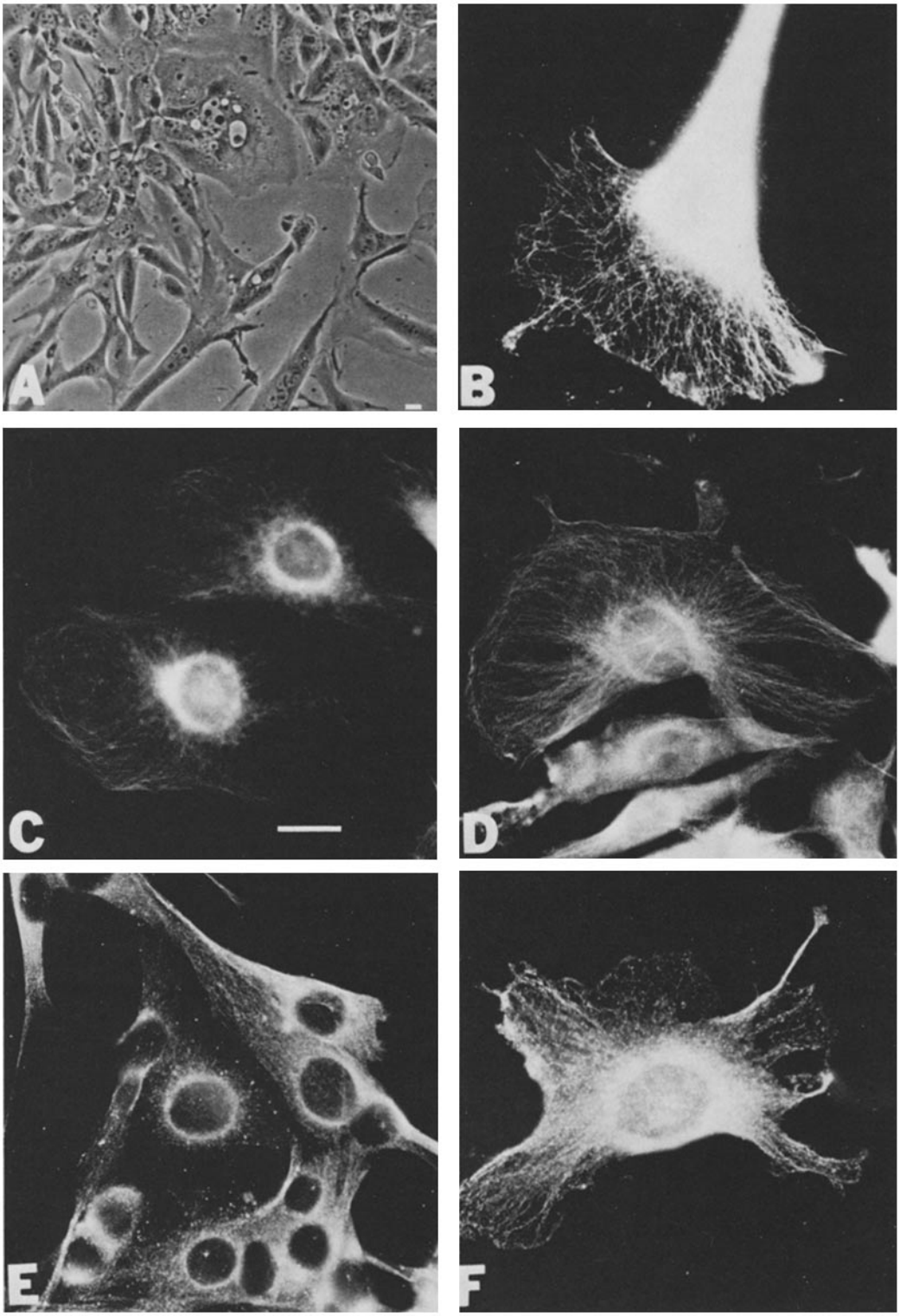

FIGURE 4 Somatic cell hybrid lines. PEP7d3B and MGM21c stained for microtubule networks. (*A*) Phase-contrast PEP7d3B. (*B*) Cytoskeleton of MGM21c. (*C*) Methanol-fixed PEP7d3B. (*D*) Formaldehyde-fixed PEP7d3B. (*E*) Cytoskeleton of PEP7d3B. (*F*) Formaldehyde-fixed PEP7d3B. Bar, 10 μm.

DISCUSSION

We have tested cultured and transformed mouse cells, human cells, and their hybrids for the presence of networks of tubulin by indirect immunofluorescence techniques. Before incubation with the specific antibody, the cells were treated with formaldehyde or methanol. In some instances, the cells were treated to generate cytoskeletons before methanol fixation. Cells fixed in formaldehyde, in general, showed a much less-defined array of networks in all cells, whereas the cytoskeletal preparations exhibited extensive networks of microtubules. The cells that we tested differed in their morphology and their properties of adhesion to the substrate. Human fibroblasts, HT1080 cells, and some hybrids were flat and attached firmly to glass, while PG19 and other hybrids were small, rounded, and attached loosely to the glass. MN are easily detectable in the first group of cells. Though microtubule networks are visible in the second group of cells, photography was difficult because, in any specific focal plane, only a small portion of the microtubules were in focus against a background of diffuse unfocused networks. This property, in addition to the relatively small amount of cytoplasm, seems to be responsible for the difficulty in detecting MN in these cells. It has been argued that formaldehyde destroys microtubule networks (6, 18). The fact that MN can be detected in a number of cell types fixed in formaldehyde makes this unlikely. It is possible that pretreatment with detergent or methanol fixation might render the microtubules more accessible to the antibody or reduce the nonspecific background, permitting easier visualization of the microtubule networks (15).

PG19 and HT1080 cells have been tested by us and other investigators for properties associated with transformed cells. Both of these cell types exhibit many of these properties: they are derived from spontaneous tumors, can grow well in low serum, form colonies in semisolid medium, and grow as tumors in immunodeficient nude mice. We have shown that both these cell types contain extensive networks of microtubules comparable to those in normal diploid fibroblasts which exhibit none of the above-mentioned transformed phenotypes. Thus, we could not find any correlation between the transformed phenotypes and the presence of MN in these cells.

Cell hybrids between transformed and nontransformed cells have been examined for MN after formaldehyde fixation (12). Miller and colleagues classified their hybrids into four categories, depending on the extent of detectable MN in them. Though we have obtained similar results from our hybrids (Table I), these differences disappear if cytoskeletal preparations are examined. Thus, the differences observed in formaldehyde-fixed preparations seem to reflect variations in cellular morphology rather than an intrisic alteration in microtubule networks.

We have demonstrated that cells exhibiting transformed phenotypes contain, at least in many instances, as extensive MN as do normal diploid fibroblasts (Figs. 1–4). These results indicate that transformed phenotypes are not associated with any qualitative differences in tubulin networks. Possible quantitative differences of MN in normal and transformed cells are probably due to factors involved in altering cellular morphology and the ratio of nuclear to cytoplasmic areas. The nature of these factors and their mode of action is not known.

The presence of actin cables has been implicated in maintaining cellular architecture in normal cells. Pollack and colleagues (16) have shown that transformed cells have no or reduced actin cable structure. Despite a report to the contrary (7), there is correlation between the absence of actin cables and several parameters associated with transformation (17). The results presented here and those of Osborn and Weber (15) and Weber et al. (24) indicate that, despite possible functional relationships between actin and tubulin, these substances are probably regulated independently in transformed cells.

Since the completion of this work, a similar report has been published (22).

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