

# SURFACE DISTRIBUTION OF LETS PROTEIN IN RELATION TO THE CYTOSKELETON OF NORMAL AND TRANSFORMED CELLS

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## ABSTRACT

The organization of LETS protein on the surface of NIL8 hamster cells has been examined by immunofluorescence staining. The distribution of LETS protein was found to depend on the culture conditions; in subconfluent, low-serum arrested cultures the LETS protein is predominantly located at the cell-substrate interface and also in regions of cell-cell contact, whereas in dense cultures the cells are surrounded by a network of LETS protein fibrils. Transformed derivatives of these cells exhibit only sporadic staining for LETS protein, in the form of short intercellular bridges.

Agents that cause alterations in cell shape and cytoplasmic filaments have been used to explore the relationship of LETS protein to the internal cytoskeletal elements. Reciprocally, perturbations of the cell surface were examined for their effects on internal filaments. The arrangement of microtubules seems to be unrelated to the presence of LETS protein in the cells studied. Actin microfilament bundles and LETS protein respond in a coordinate fashion to some perturbants but independently with respect to others. The patterns of staining for LETS protein are consistent with an involvement in cell-to-cell and cell-to-substrate adhesion.

**KEY WORDS** LETS protein · actin · tubulin · immunofluorescence · adhesion

Transformation of fibroblasts by RNA and DNA tumor viruses and a variety of other agents is associated with changes in cellular properties which may be related to the cell surface, e.g., loss of anchorage and serum dependence for growth, contact inhibition of movement, altered transport of metabolites, increased agglutinability by lectins, and mobility of cell membrane components. Most strikingly, there is a dramatic change in cell shape,

from a fusiform to a more rounded or polygonal outline. Transformed cells fail to flatten and spread out in sparse culture or when serum deprived. This change in cellular morphology is accompanied by alterations in culture morphology. In general, normal cells tend not to pile up or overlap and do not grow detached from the substratum, whereas transformed cells can do so. Many of these features can be considered to be related to the adhesive properties of the cells (1, 26, 46).

Changes in the cell surface on transformation

of fibroblasts can also be detected at the biochemical level. One of the most consistently observed alterations is loss or marked reduction in amount of a large surface glycoprotein, LETS protein (19, 20), also referred to as Z (37), SF antigen (42), and CSP (49). A correlation of tumorigenicity in vivo with the absence of LETS protein has recently been described with a series of transformed cell lines (7); and although a few examples exist where this correlation does not hold (19, 18, 32), these observations may have some bearing on the role of LETS in vivo.

The role of LETS protein in normal cell functions is therefore of some interest. There are indications that it could play a part in cell-substrate adhesion. Addition of LETS protein to transformed cells results in increased attachment of cells to the substratum and resumption of an apparently normal cell and culture morphology (3, 48, 50). The distribution of LETS on such treated cells is very similar to that on normal fibroblasts (3).

Another alteration observed in transformed cells is that they contain a less well-organized cytoskeleton. For example, the arrangement of actin microfilaments seen under the plasma membrane of normal cells by electron microscopy is not seen in transformed cells (15, 27, 28), and the bundles of actin filaments ("cables") detected by immunofluorescent staining in normal well-spread fibroblasts are absent or reduced in their transformed derivatives (33, 45). Similarly, microtubules are reportedly less numerous in transformed cells (5, 10). There is a close correlation between loss of actin-containing structures and of LETS protein from transformed cells. Both are most abundant in growth-arrested normal cells and greatly reduced by transformation or proteolysis (20). Readdition of LETS protein to transformed cells results in reappearance of actin microfilament bundles (3, 47). The possibility arises, therefore, that the changes in these two parameters are related in some way.

In this paper we describe a detailed study of the distribution of LETS protein on normal and transformed hamster cell lines and the effect of various perturbations on the organization of LETS protein in relation to cell shape and adhesion. We also address the questions of the relationship of the surface arrangement of LETS protein to the internal cytoskeletal organization, by examining in parallel the distribution of LETS protein and

of actin and microtubules during modifications of the cell surface or of the cytoskeleton.

## MATERIALS AND METHODS

### *Cells and Culture Conditions*

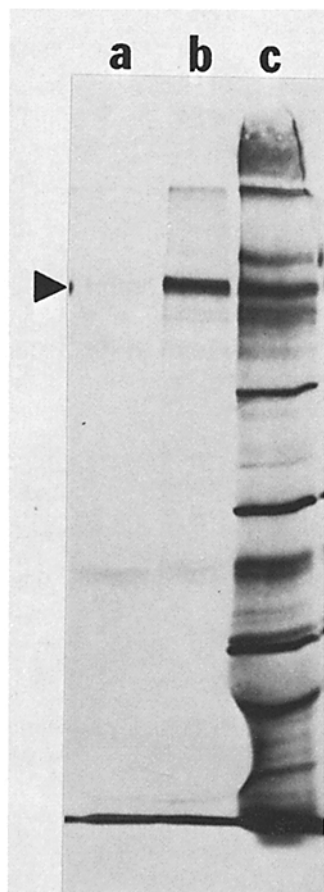
The normal hamster cell line NIL8 and a derivative transformed by hamster sarcoma virus (NIL8-HSV6) were grown in plastic Petri dishes (BioQuest, BBL & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md.) in Dulbecco's modified Eagle's medium (DME) plus 5% fetal calf serum (FCS) (Flow Laboratories, Inc., Rockville, Md.). For immunofluorescence studies, the cells were replated by trypsinization (0.06% at 37°C for 10 min) onto 12 mm<sup>2</sup> glass cover slips (Gold Seal, Scientific Products, Bedford, Mass.) in 35- or 60-mm Petri dishes, at various densities and serum concentrations as indicated in the Results section. After replating onto cover slips, cells were allowed to reattach and, unless otherwise stated explicitly, were not used until the 3rd day after plating.

### *Antisera*

LETS protein was prepared as follows. NIL8 cells were grown to confluence in roller bottles. They were then rinsed and changed to fresh medium containing FCS or FCS which had been depleted of high molecular weight proteins by treatment with 30% ammonium sulfate. The cells were cultured in the fresh medium for 2-4 days. During this time, they released large amounts of LETS protein into the medium. This was purified by precipitation with 30% ammonium sulfate and G-200 Sephadex chromatography (3). To free the LETS protein of residual contaminants it was run on preparative SDS-polyacrylamide gels; the LETS protein band was detected by Coomassie blue staining, this was cut out, and the protein was eluted electrophoretically and concentrated by ethanol precipitation. The preparative electrophoresis was repeated if necessary to obtain a single band. The electrophoretically purified LETS protein was emulsified with complete Freund's adjuvant and injected into rabbits at several subcutaneous dorsal sites. 2-3 wk later the injection was repeated with incomplete adjuvant. Blood was tested, and further booster injections were given if necessary. Rabbits were bled at 5-10 day intervals. Generally, 80-500  $\mu$ g protein were used per injection. Active antisera have been obtained with as little as two injections of 80  $\mu$ g each.

The antisera raised against SDS-gel purified LETS protein did not react with fetal calf or calf serum by double immunodiffusion but gave a single line of identity when tested against purified LETS protein, NIL8 whole cell lysates, or NIL8 culture medium. If the cells were iodinated before preparation of lysate or conditioned medium, the precipitation line was labeled. Since Ouchterlony double-immunodiffusion was not a sensitive test

for minor contaminating activities in the serum, the antisera were tested by radioimmunoprecipitation analysis (Fig. 1). Metabolically labeled NIL8 cells, or conditioned media, were precipitated by indirect precipitation and analyzed on SDS-polyacrylamide gels. A single major band, comigrating with iodinated LETS protein was precipitated from conditioned medium by immune sera. Only traces were precipitated by preimmune sera. Small amounts of radioactivity in bands migrating just ahead of LETS protein were sometimes but not always



**FIGURE 1** Immunoprecipitation with antiserum to LETS protein. NIL8 cells were cultured in [<sup>35</sup>S]methionine for 24 h and the conditioned medium harvested and centrifuged. Aliquots were incubated with preimmune and immune rabbit antisera followed by goat anti-rabbit IgG. The washed precipitates and a starting sample were analyzed on SDS-polyacrylamide slab gels by radioautography. (a) Preimmune precipitate; (b) immune precipitate; and (c) equivalent amount of labeled medium. Note the complete precipitation of LETS protein (arrowhead) and only trace precipitation of one or two other labeled proteins.

seen. These are probably degradation fragments of LETS protein (our unpublished data). Similarly, precipitation from cell extracts yielded only material comigrating with iodinated surface LETS protein. These data establish that the antisera do not react with components of serum in the cell culture medium but do react with a single protein having properties identical with those of surface LETS protein.

Rabbit antibody to calf thymus actin was the generous gift of Drs. E. Lazarides and K. Burridge (Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.). The antigen had been extracted from SDS-polyacrylamide gels as described by Lazarides and Weber (25), and the antiserum obtained had been purified by two cycles of ammonium sulfate precipitation. This partially purified IgG preparation was used at a dilution of 1 in 120 in phosphate-buffered saline (PBS).

Rabbit antibody to tubulin was a generous gift of Drs. G. Fuller and B. R. Brinkley (University of Texas, Houston, Texas) and was prepared against glutaraldehyde cross-linked bovine brain tubulin (14). Use of this antibody has been reported (5). In the experiments reported here, the anti-tubulin antibody was used at a dilution of 1:20 in PBS.

#### *Immunofluorescence Staining*

The methods used were adapted from Lazarides (24). For surface staining, cells grown on cover slips were used live or after formaldehyde fixation. For fixation, cells were rinsed by dipping into PBS at room temperature, drained but not dried, and immersed in 3.5% formaldehyde (a 1 in 10 dilution in PBS of the AR grade, Mallinckrodt Inc., St. Louis, Mo.) for 30–60 min at room temperature. The cells were rinsed again and could then be stored at 4°C in PBS. Fixed cells were usually stained within 24 h but could be used after periods up to 7 days without undergoing significant deterioration, except that after storage they had a greater tendency to be permeable to antibodies whereas freshly fixed cells excluded the antisera.

Cells were treated with acetone after formaldehyde fixation to make them permeable to internal staining; the cover slips were placed first for 2 min in a 1:1 mixture of acetone and water at -20°C, transferred to cold acetone for 5 min, returned to 1:1 acetone/water for 2 min, and then rinsed in PBS at room temperature. Acetone extraction was carried out just before staining; extracted cells were not stored. Unless otherwise stated, cells were routinely stained for LETS protein after formaldehyde fixation only, and for actin and tubulin after formaldehyde fixation and acetone treatment.

**ANTIBODY STAINING:** After rinsing in PBS, the first antiserum was added (20  $\mu$ l per cover slip), and the cells were incubated for 30 min in a moist chamber at 37°C. The cells were then rinsed by repeated vigorous dipping into PBS and incubated for a further 30 min with 20  $\mu$ l of fluorescein-conjugated goat antiserum to

rabbit IgG (Miles-Yeda, Rehovot, Israel). After further thorough rinsing in phosphate-buffered saline (PBS), the cells were washed gently and briefly in water, to remove as much salt as possible, and were mounted in Gelvatol 20/30 (a gift of Monsanto Co., St. Louis, Mo.) prepared according to Rodriguez and Deinhardt (38).

Diluted sera were spun at 2,000 rpm for 10 min in a bench centrifuge immediately before use, and commercial conjugated serum was passed through a 0.22- $\mu$ m Millipore filter (Millipore Corp., Bedford, Mass.) before use in order to remove aggregates and nonspecifically staining background material.

Routine controls included using preimmune sera and normal fluorescent goat serum, and omission of the first antibody, as noted in the text. Cover slips were examined by phase-contrast and epifluorescent illumination in a Zeiss Universal microscope. Photographs were taken on Kodak Plus X film. The bar on the micrographs denotes 50  $\mu$ m throughout, unless explicitly stated otherwise.

### *Pretreatment of Cells*

As indicated in the Results section, cells were pretreated in some experiments with a variety of reagents before fixation and staining.

NP-40 (Nonidet P-40, Shell Chemical Co., New York) was used at concentrations of 0.01, 0.10, and 1.00% vol/vol in PBS with or without the addition of 2 mM EDTA and 2 mM PMSF. Cells were incubated for 15 min at room temperature and then stained without fixation, or fixed in formaldehyde and then stained. After NP40 extraction, it was not necessary to use acetone to make the cells permeable. Trypsin (10,000 U/mg; Sigma Chemical Co., St. Louis, Mo.) was used at concentrations of 1–10  $\mu$ g/ml in PBS. The cells were incubated at room temperature for times as indicated in the text. Trypsinization was stopped by transferring the cover slips to a solution of soybean trypsin inhibitor (20  $\mu$ g/ml) for 5 min, rinsing in PBS, and then fixing in formaldehyde.

Several drugs thought to be active on cytoplasmic filamentous structures were used. These were added directly to the culture medium and incubation was at 37°C. Colchicine (Fisher Scientific Co., Medford, Mass.) and procaine (Sigma) were prepared as concentrated stocks in PBS. Colchicine was used with minimal exposure to light. Cytochalasin B was prepared as a 1-mg/ml stock in ethanol. Addition of ethanol alone had no effect.

The chelating agents, EDTA and ethyleneglycol-bis-( $\beta$ -aminoethyl ether) *N,N'*-tetraacetic acid (EGTA), were used to detach cells from the cover slips by incubation at 37°C. EDTA was used at a concentration of 0.02% in PBS and EGTA at a concentration of 2 mM in PBS. The sulfhydryl reagent, dithiothreitol (DTT, Sigma), was used at concentrations of 1 and 10 mM in PBS or in complete culture medium, as indicated in the Results section.

## RESULTS

### *Distribution of LETS Protein, Actin, and Tubulin in Normal Cells*

The distributions of actin, tubulin, and LETS protein were examined by immunofluorescent staining of cultures of normal NIL8 cells under several different growth conditions.

### *Dense, Arrested Cultures*

Fig. 2 shows staining of dense cultures of NIL8 cells which had been allowed to grow to confluence in 5% FCS without medium change for 3–4 days. After staining of nonpermeabilized cells with antiserum to LETS protein, the most obvious feature was the dense and tangled web of LETS protein surrounding the cells (Fig. 2*a*). It was evident that the network was not confined to individual cell surfaces but extended from cell to cell to form a continuous matrix. In contrast, when cells were stained with preimmune serum, very little staining was seen (Fig. 2*c*) as was also true if fluorescent normal goat serum was used in the second step.

Acetone-treated cells were permeable and when stained with antibodies to actin or tubulin, filamentous arrays were observed in both cases. Antibody to actin stained long, straight fibers running across the cells (Fig. 2*b*), the "stress fibers" or "cables" reported by others (24, 25, 33, 34, 45). Antibody to tubulin stained wavy fibers (Fig. 2*d*), as reported previously for other normal cells (5, 10, 13). Neither the actin nor the tubulin staining was as clearly defined in dense cultures as in well-spread cells (see below). In addition to the fibrous staining, the actin antibody clearly stained the cell boundaries (Fig. 2*c*) and the tubulin antibody stained brightly a short hook-shaped structure at or near the top of the cells (Fig. 2*d*) as reported for other cells (13, 44).

### *Sparse, Arrested Cultures*

Since it was difficult to examine the distributions of LETS protein and actin and tubulin in individual cells in dense cultures, we turned to cells arrested in low serum. These cells are blocked in G<sub>1</sub> of the cell cycle as are those in density-arrested cultures (8), and cultures can be arrested in this way at any cell density. Such cells also have high levels of surface LETS protein (21).

Cells were plated in 0.3% FCS at  $3 \times 10^5$  per 60-mm dish or  $10^5$ /35-mm dish and cultured with-

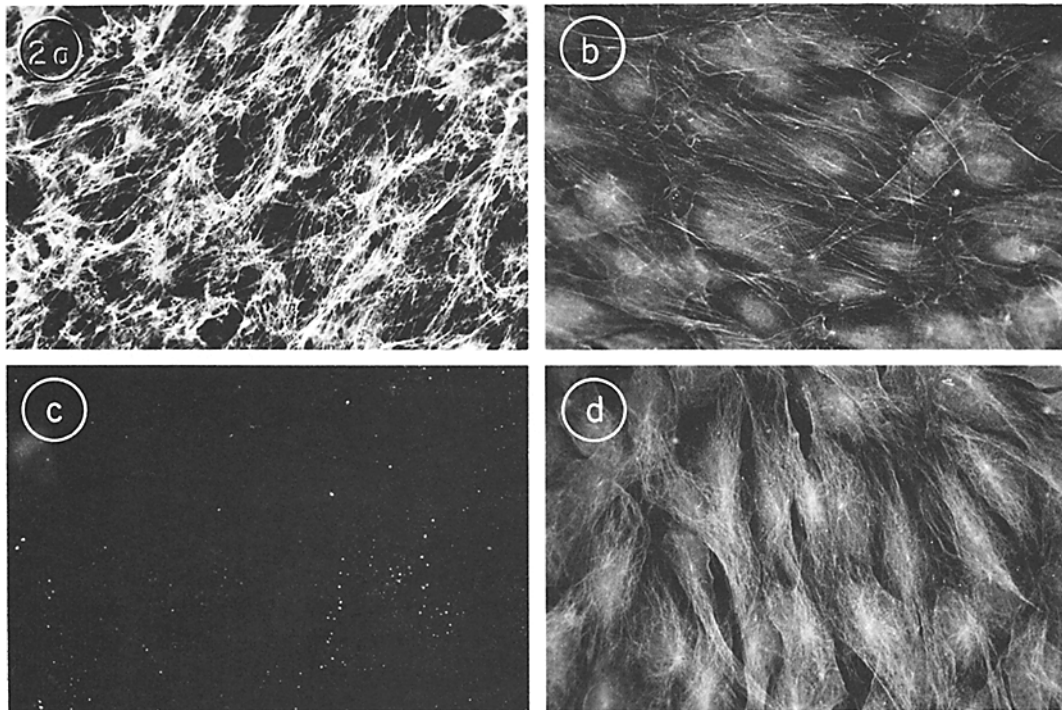


FIGURE 2 Distribution of LETS protein, actin, and tubulin in confluent NIL8 cultures. Cells were seeded in 5% FCS and allowed to grow to confluence before staining at 72 h. (a) Antiserum to LETS protein, no acetone treatment. Note extensive fibrillar network. (b) Anti-actin, acetone-treated. Note poorly visible actin microfilament bundles and marked staining of cell boundaries. (c) Preimmune serum (anti-LETS rabbit), no acetone. (d) Anti-tubulin, acetone-treated. Note wavy fibers. The hook-shaped body can be seen on a few cells but in most cases is above the plane of focus. Magnification same as Fig. 3.

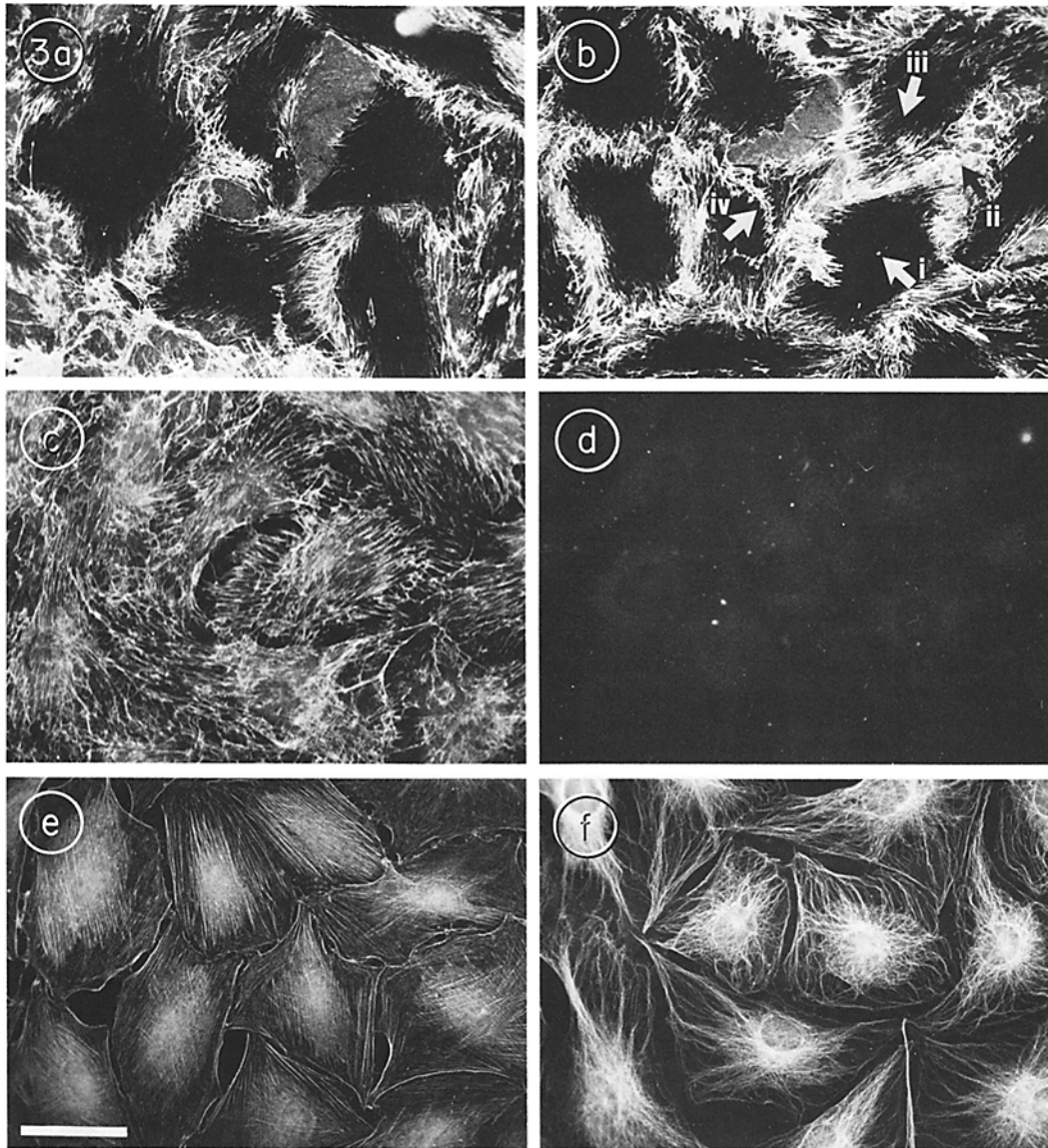
out medium change at 37°C for 3 days. Fig. 3 shows a field of these cells viewed by fluorescence optics (Fig. 3a) after formaldehyde fixation and surface staining for LETS. The most striking feature was that most cells appeared as black shadows surrounded by a fibrous network of LETS protein staining in regions of cell-cell contact. That intact cells occupied these black areas was verified by examining the phase-contrast micrograph of the same field. Staining of a similar field with preimmune serum or replacement of f1-goat anti-rabbit serum with f1-normal goat serum (Fig. 3d) revealed only occasional specks of nonspecific stain.

At high magnification it was evident that LETS protein was present in more than one plane. For example, in Fig. 4, the first level of focus (Fig. 4b) was at the level of the substrate beneath the cell, while the second was above the cell (Fig. 4c).

Fig. 4 also demonstrates that staining of live cells with antiserum to LETS protein gives results

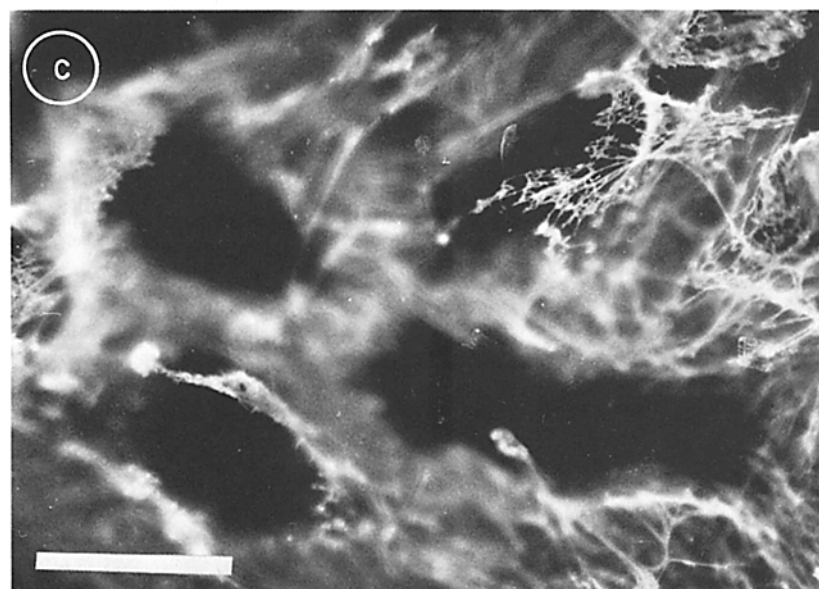
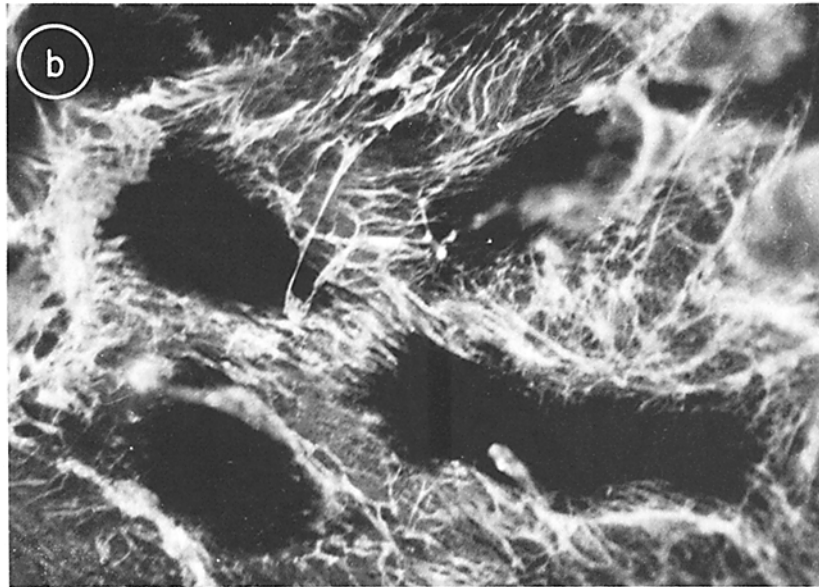
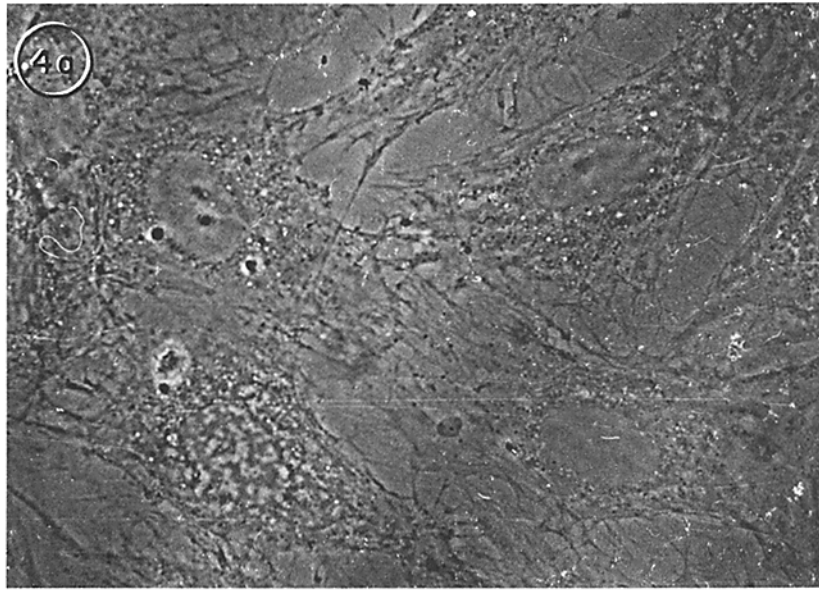
exactly similar to those observed with formaldehyde-fixed cells showing a fibrous network. There was no indication of any patching or capping such as has been observed for certain other surface antigens (e.g., references 11 and 37).

In both fixed and live cells, fibers stained with antiserum to LETS protein extended beyond the cell boundaries (Figs. 3b and 4) and bridged between adjacent cells (Figs. 3b and 4). LETS fibers also extended onto the upper surfaces of individual cells (Figs. 3b and 4). In addition, there were fibers beneath the cells which were not readily stained. They could sometimes be faintly stained within the area covered by the cell (black shadow, Fig. 3a and b, arrows) suggesting that failure to observe staining fibers is attributable to failure of the antisera to penetrate appreciably under the cells. In cells plated at low densities in low serum such that cell-cell contacts were rare, all the cells showed the "black shadow" appearance.



**FIGURE 3** Distribution of LETS protein, actin, and tubulin in NIL8 cultures arrested in low serum. Cells were plated at  $1 \times 10^5/35$  mm plate in DME plus 0.3% FCS, fixed at 72 h and stained for: (a) LETS protein. (b) LETS protein: (i) black shadow area beneath cell; (ii) LETS protein fibrils spanning between cells; (iii) LETS protein fibrils faintly stained underneath cell; and (iv) LETS protein fibrils over cell. (c) LETS protein after fixation and acetone permeabilization. Note absence of black shadows and appearance of LETS protein fibrils distributed throughout the field, i.e., under cells. (d) Stained with antiserum to LETS protein followed by fluorescent normal goat serum. Note absence of fluorescence. (e) Actin: cables and cell periphery stain intensely. Some fluorescence around nucleus. (f) Tubulin: filaments extending to cell boundary and abundant around the nucleus. Bar, 50  $\mu$ m.

**FIGURE 4** Distribution of LETS protein on live NIL8 cells. Cells were plated at  $3 \times 10^5/60$  mm plate in DME plus 0.3% FCS and stained without fixation at 72 h. Photographed with  $63 \times$  oil immersion objective. Three views of the same field: (a) phase contrast (focused on cell edges), (b) bottom of cell (focused on cell edges and substrate), and (c) top of cell (focused on LETS staining fibrils over cell). Bar, 50  $\mu$ m.



Treatment of cells with acetone resulted in abolition of all "black shadows," as shown in Fig. 3c. It is apparent that the network of fibers at the level of the substratum could now be stained, under all the cells. In addition to this network there was also some general fluorescent staining of the cells, and the nuclei could just be seen. These latter two features were also found on staining with preimmune serum after acetone extraction (not shown).

When sparse, arrested cultures, which consist of well-spread cells, were stained with anti-actin antibody, after acetone treatment, strongly stained actin microfilament bundles, similar to those reported by others, were observed (Fig. 3e). The actin bundles were thick and straight, often extending right across the cells, and were predominantly near the base of the cells. Actin staining was also seen around the perimeter of the cells. There was no actin staining if live cells were stained.

Well-spread cells stained with antibody to tubulin showed strongly stained fibers running throughout the cytoplasm, predominantly in a radial pattern (Fig. 3f). The "hook" shaped body at the top of the cells was also observed but is not seen in Fig. 3f owing to the plane of focus.

In the case of staining for both actin and tubulin, the fibrous patterns were much more clearly seen in the serum-arrested cells than in the dense, arrested cultures (cf. Figs. 2b and d with 3e and f), although in both cases the cells are arrested in the same phase of the growth cycle. The pattern of staining for LETS protein also differed between serum- and density-arrested cells; the latter showed a network surrounding the cells, whereas the former showed fibers predominantly between neighboring cells and between cells and substratum. Thus, the distributions of all three fibrous structures examined differ depending on the growth conditions. Under neither culture regime could an obvious relationship be discerned between the arrangement of LETS protein at the surface and the actin or tubulin fibers inside. However, such relationships are not ruled out and will be examined further below.

### *Growing Cultures*

Cells were plated in 5% FCS and kept without medium change for 3 days. During this period, cover slips were removed at intervals and stained for LETS protein and actin.

At the earliest times examined (3–4 h), most cells were attached but were round and not markedly spread (Fig. 5a). Attached cells stained with antiserum to LETS protein displayed a ring of bright fluorescence close to the edge of the cell. This could often be resolved into short radial fibrils of LETS protein. The actin was arranged in concentric rings, particularly at the periphery of the cell (not shown).

With increasing time, cells became more flattened and LETS protein staining could readily be seen on the lower surface as small fibrils (Fig. 5b), which were predominantly arranged radially. These cells displayed geometric arrays of actin staining similar to those described by Lazarides (24), and the cell edge also stained for actin.

As the cells were cultured for longer periods, the pattern of LETS fibrils underneath the cells became increasingly complex with fibrils running in various directions under the cells. In cells seeded at low densities ( $3.10^4$  per 60 mm plate) at which cells made only few intercellular contacts, this remained the predominant pattern for 24 h. Virtually all staining was in the region of cell-substrate contact. When cells were seeded at higher density, as in Fig. 5, they began to make contact after about 8 h and LETS protein staining then also appeared in the regions of cell-cell contact (Fig. 5c). Actin filament bundles were readily visible in the cells at early times (Fig. 5d). With increasing time, both LETS fibrils and actin staining became stronger and better defined (Fig. 5e and f). The patterns remained essentially unchanged for the next 12 h.

At 30 h, the cells were mostly in contact with others although subconfluent. The actin bundles were strongly stained, and the cell edges were also well defined (Fig. 5h). The LETS staining was brighter, and the filaments appeared thicker, but there was little change in overall distribution (Fig. 5g). This appearance persisted until about 48 h, at which time the cells were approaching confluence (Fig. 5i and j). Staining of actin was decreased in intensity, and the bundles appeared finer, while staining of the LETS protein network increased in intensity. At 75 h the monolayer was confluent with cells overlapping each other throughout the culture. From the distribution of LETS protein it was impossible to discern cell borders, and there was an apparently continuous network of LETS protein fibrils around the cells. The actin bundles were extremely fine, but the



cell edges were relatively well defined by actin staining (Fig. 5*k* and *l*). Thus, in agreement with earlier biochemical data, the amount of LETS protein increased in confluent cultures. In contrast, actin bundles were most evident in cells at earlier times and were less obvious in confluent cultures.

It appears that LETS protein is most prevalent in arrested cultures, whether sparse or dense, whereas actin microfilament bundles are best observed in well-spread cells, growing or not. In well-spread cells much of the LETS protein is found beneath the cells. When cells are in contact, LETS protein is also found in the contact regions.

#### *Distribution of LETS Protein, Actin, and Tubulin in Transformed Cells*

HSV-transformed NIL cells are somewhat smaller and much less well spread than their normal counterparts. They grow as small colonies with little migratory tendency; and when the culture becomes confluent, the cells also grow in suspension above a monolayer, rather than piling up. These properties could all be related to reduced adhesion.

There was very little anti-LETS staining of NIL-HSV cells grown to confluence in 5% FCS, and no black shadows could be discerned. Occasional areas of the culture stained more than others, and this staining consisted predominantly of small stitches of LETS protein between cells (see Fig. 6*b*). These stitches were shorter than those located at similar sites in NIL cells. Almost no fibers were found over the surface of or beneath cells. A few cells of more fibroblastic shape were occasionally found, and these tended to stain somewhat more for LETS protein around their edges. In sparse cultures (Fig. 6), each individual colony had a uniform appearance; most were completely devoid of LETS fluorescence while stitches of LETS were present on a few, suggesting that individual clones arise with more or less LETS on their surface (cf. Fig. 6*a* and *b*). Both sparse and dense cultures in 5% FCS displayed some nonspecific staining, seen with both immune and preimmune sera (Fig. 6*c*). Staining of NIL-HSV cells after acetone extraction increased the general fluorescence, but this was also nonspecific. No fibers were stained under the cells, either with or without acetone treatment.

The pattern of staining for actin after acetone was quite different from that seen in NIL cells

and in good agreement with previous reports on the lack of visible actin cables in tumor virus-transformed cells. There was profuse internal fluorescence, both apparently unstructured and in discrete speckles. In addition, the cell border was strongly stained, especially over membrane ruffles (Fig. 6*d* and *f*). Some short thin fibrils were seen, and longer filaments were found in the occasional more elongated, atypical cells. Dense cultures stained for actin were not very different from sparse ones. Thus, both the external network of LETS protein and the actin microfilament bundles appear markedly reduced in the transformed cells. Transformed cells stained for tubulin revealed a fine fibrous array (Fig. 6*e* and *g*), that looks quite similar to the pattern seen in dense normal fibroblasts (Fig. 2*d*) although not as striking as that of well spread normal cells. Thus, it appears that in NIL-HSV cells the microtubular organization is not completely disrupted.

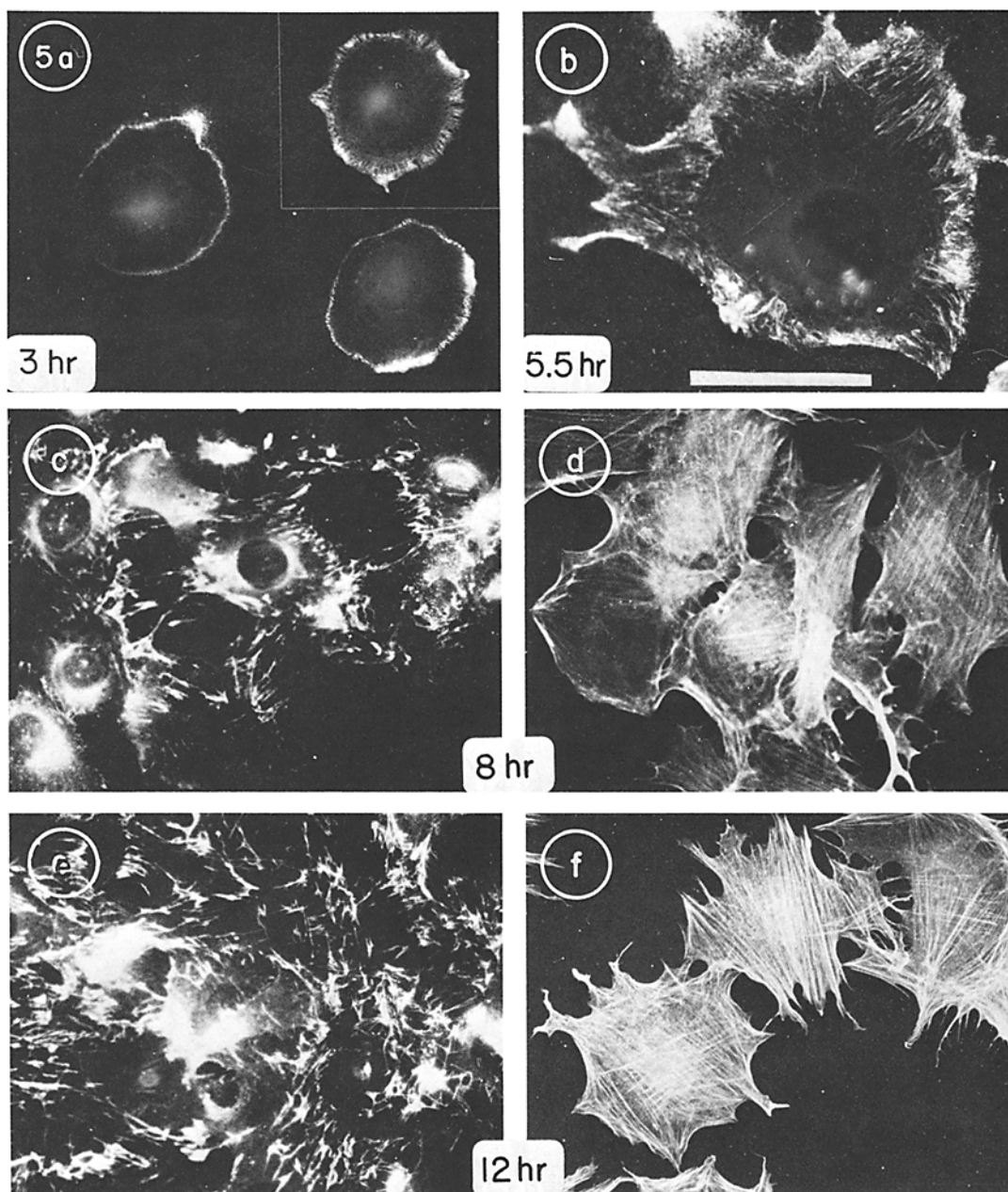
#### *Patterns of Staining in Treated Cells*

The relationship of the external LETS network to the plasma membrane and underlying cytoskeleton has been approached by using selective removal of surface membrane proteins, and subsequent examination of the remaining structure. This has permitted us to ask whether the integrity of the LETS protein network is dependent to any extent upon an intact membrane, and whether perturbations of the surface affect the cytoskeleton. We also examined the effects of drugs known to act on the cytoskeleton for their effects on the distribution of LETS protein.

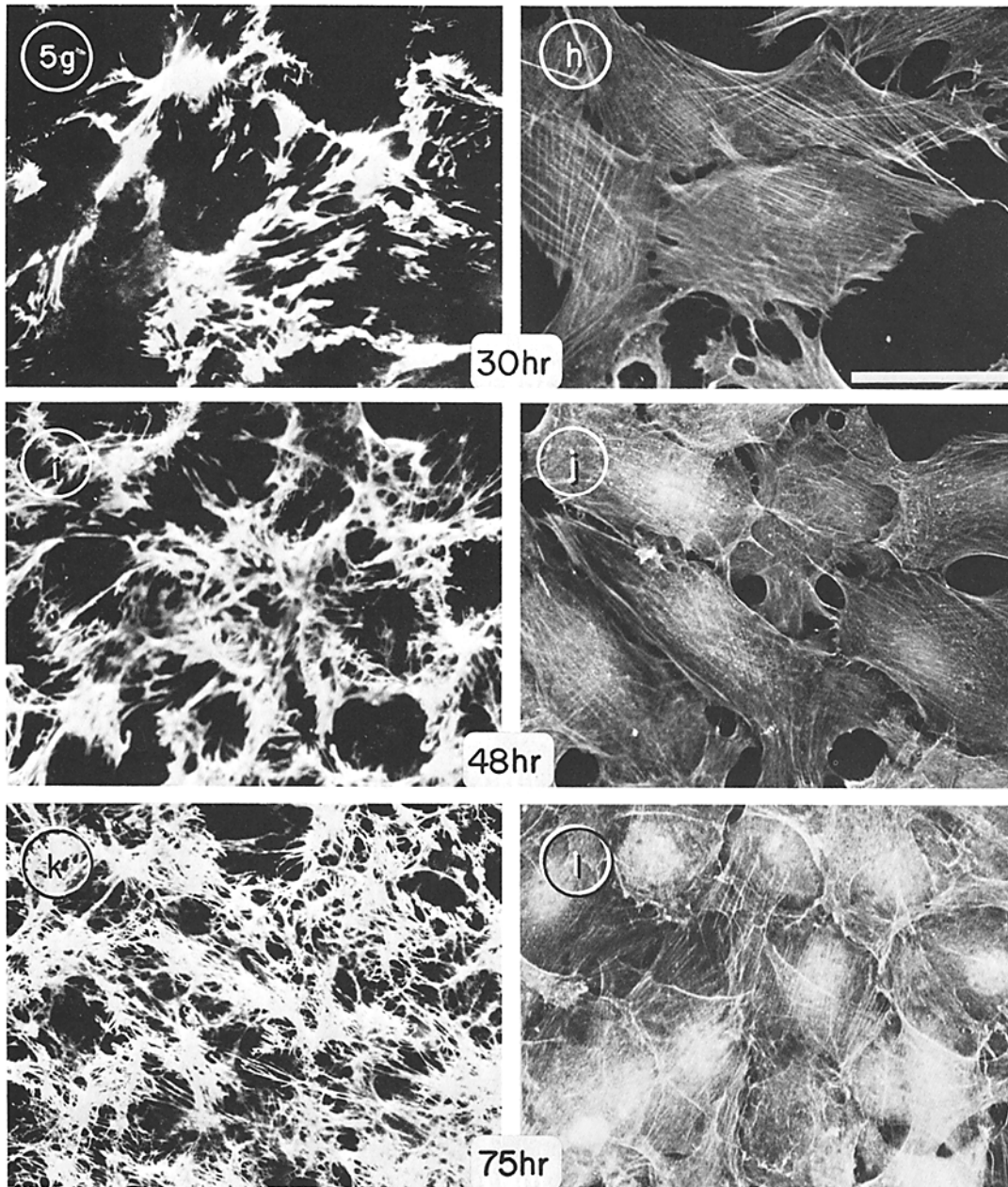
#### *NP-40 Extraction*

We have previously demonstrated that treatment of NIL cells with 0.5–1% NP-40 results in loss of 60–70% of the total cell protein and of most surface proteins detectable by lactoperoxidase iodination, but complete retention, in an insoluble form, of the external LETS protein. In addition, less than 50% of the actin is extracted and some myosin and all of a protein of 58,000 daltons also remain (23).

Cells cultured in low serum were extracted with 0.01 and 0.1% NP-40 and then stained for LETS, actin or tubulin with or without prior formaldehyde fixation. Even at 0.01% NP-40, which extracts less than 2% of the cells' protein, cells were sufficiently permeable to stain internally for actin without acetone treatment. At this low de-

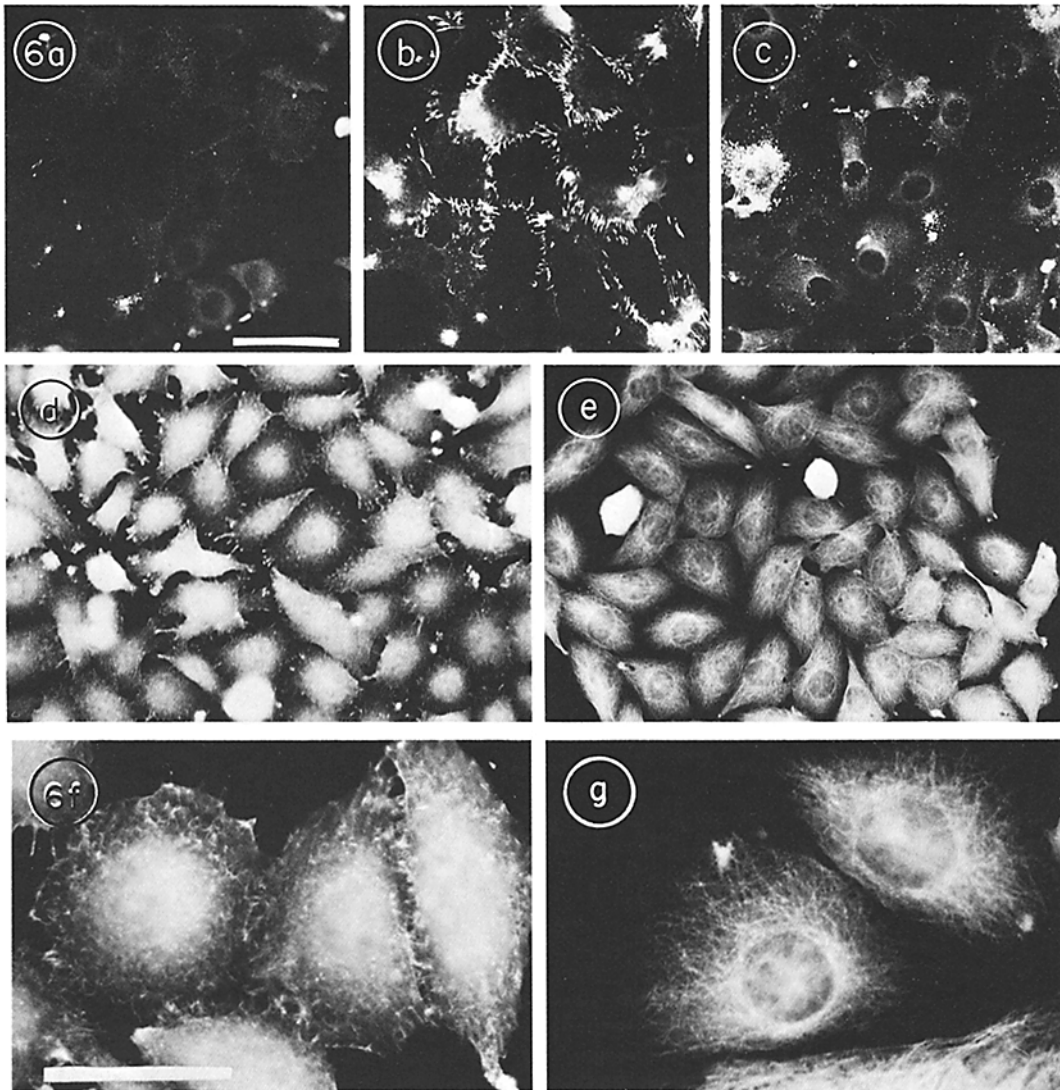


**FIGURE 5** LETS protein and actin organization in growing NIL8 cultures. Cells were plated at  $1.5 \times 10^6/60$  mm dish in DME plus 5% FCS and fixed at intervals as indicated. (*a* and *b*) Stained for LETS protein. Panel *b* is at  $1.58 \times$  higher magnification than other panels. Note short radial fibrils of LETS protein in the early spreading cells. (*c*, *e*, *g*, *i*, and *k*) Stained for LETS protein. (*d*, *f*, *h*, *j*, and *l*) Stained for actin. Note that as cells begin to make contact, LETS protein fibrils appear between cells as well as beneath them. Position of cells in panel *c* can be recognized by the dark circles corresponding with the nuclei. As cultures become denser, LETS fibrillar network becomes denser, and actin staining becomes less striking. Bar,  $50 \mu\text{m}$ .



tergent concentration the LETS staining of the cells was essentially the same as on untreated cells, except that no black shadows were observed, and most cells gave the appearance of having an intact upper and lower layer of LETS (Fig. 7). Likewise, the actin staining was not very different from acetone-treated cells. Occasionally, the ex-

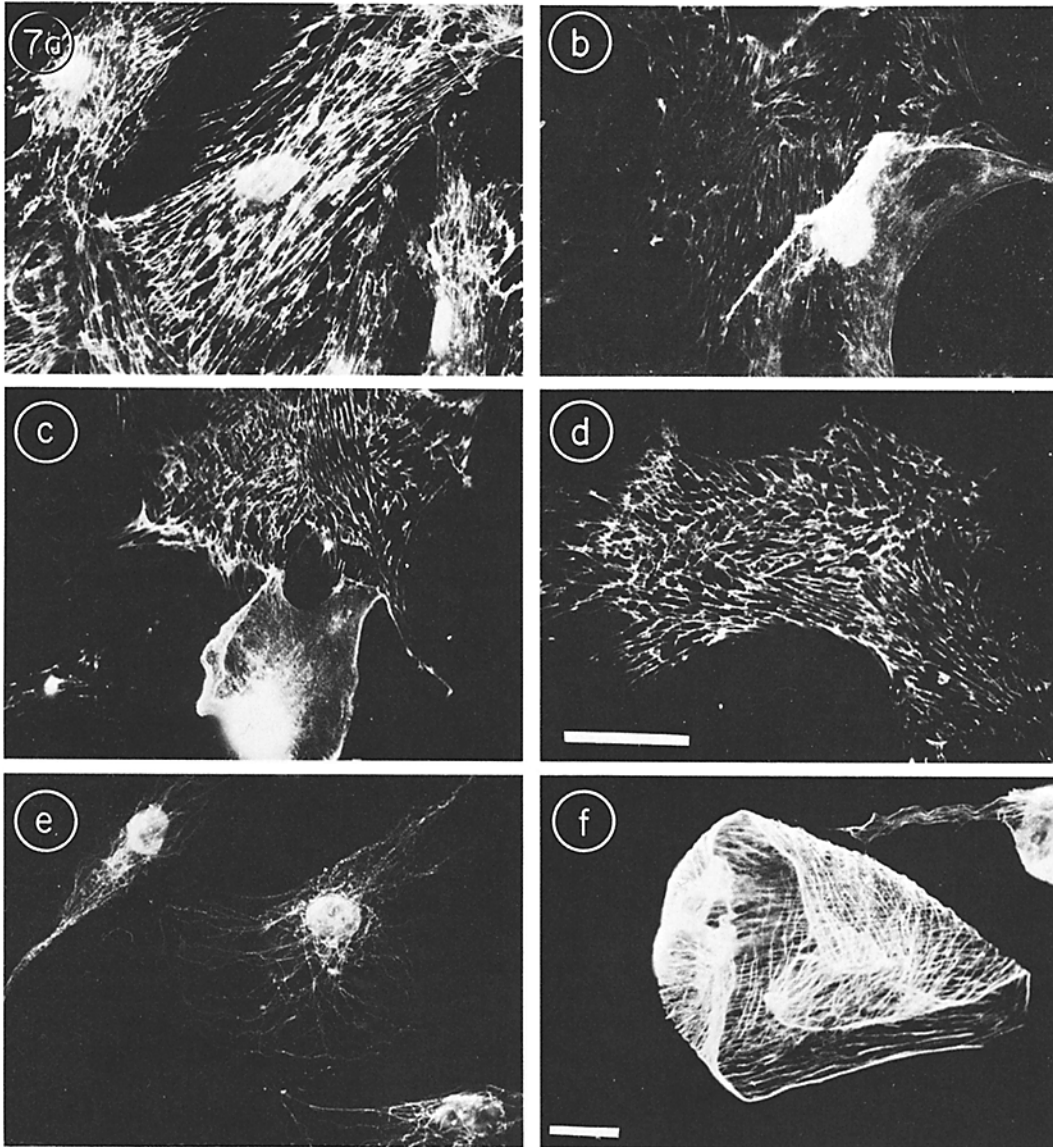
tracted "ghosts" began to peel off the substrate during the staining procedure. This was particularly common at 0.1% NP-40 when 60–70% of the cell protein had been extracted. Nonspecific staining of the nucleus was seen at this concentration with both preimmune and immune sera. The LETS staining was essentially unaltered, except



**FIGURE 6** LETS protein, actin, and tubulin immunofluorescence in NIL-HSV cultures. Cells were plated at  $2 \times 10^4/35$  mm dish in DME plus 5% FCS and fixed at 72 h. (*a* and *b*) LETS protein. Separate fields. Panel *a* is the more typical. Panel *b* shows a group of cells with small "stitches" of LETS staining. (*c*) Pre-immune serum. (*d*) Actin. (*e*) Tubulin. (*a-e*) 40  $\times$  objective. (*f*) Actin at higher magnification (63  $\times$ ). (*g*) Tubulin at higher magnification (63  $\times$ ). Bars, 50  $\mu$ m.

that where ghosts were peeling back, often one layer of the LETS was found partially detached from the substrate along with the cell ghost while the lower layer remained intact and attached to the substratum (Fig. 7*b* and *c*). In areas where the top layer and nucleus had peeled off completely, an intact lower network was seen in the absence of any other cellular material visible by phase contrast (Fig. 7*d*). Staining for actin re-

vealed loss of some cells. In the remaining cells it was found that most of the actin bundles apparently resisted extraction, but that there was much less background fluorescence, presumably because soluble or nonfilamentous actin had been removed. It was also remarkable that where the cell ghost peeled back, all of the actin peeled back, in contrast to the separation frequently seen for the LETS network (Fig. 7*f*).



**FIGURE 7** Effect of NP40 treatment on organization of LETS protein, actin, and tubulin. Cells were plated at  $3 \times 10^5/60$  mm plate in DME plus 0.3% FCS and used at 72-h. NP40 extraction was carried out at room temperature for 15 min and terminated by washing and formaldehyde fixation. (a and b) 0.1% NP40 in PBS plus 2 mM EDTA, 2 mM PMSF; LETS protein (EDTA and PMSF were added in some experiments to inhibit proteolysis; similar results were obtained without these additions). (c and d) 0.01% NP40 in PBS plus 2 mM EDTA, 2 mM PMSF; LETS protein. (e) 0.01% NP40 in PBS; tubulin. (f) 0.01% NP40 in PBS; actin. (a-e) Photographed with  $63 \times$  objective. (f) Photographed with  $40 \times$  objective. Panel f shows a cell peeling off the substratum showing the actin cables detaching with the cell ghost. Contrast with panels b and c where LETS protein on the substratum as cells peel off. Bars,  $25 \mu\text{m}$ .

At concentrations of NP-40 of 1% and higher, few cells remain attached to the cover slip after the extensive washing involved in staining, and the most prevalent feature was single LETS pro-

tein networks marking the positions previously occupied by cells. LETS protein and actin staining of cells extracted with NP-40, but not fixed at all, resulted in the loss of more cells than under the

same extraction conditions followed by formaldehyde treatment; but, apart from this, general features were the same. Cells extracted with NP-40 and then stained with anti-tubulin showed loss of much of the material stained by this antibody (Fig. 7*e*). When extraction was carried out in the absence of calcium, fine discontinuous fibers were stained (Fig. 7*e*), whereas in the presence of calcium no fibers remained after NP-40 extraction.

#### *Extraction with Chelating Agents*

EDTA causes cells to round up, and is commonly used to remove cells from a substrate. To see whether, in the course of this process, the LETS network is left behind, we treated NIL cultures with EDTA (0.02%) or EGTA (2 mM) for various times at 37°C. Dense cultures that had been kept in 5% FCS were easily detached

from the substrate as a sheet of cells, and after 15 min only a very few cells remained. Very little LETS or actin was left behind on the substrate (Fig. 8*c* and *d*), and we therefore conclude that most of the LETS protein is detached at the same time as the cells. This finding is in agreement with biochemical results (our unpublished data). Small flecks which stain with antisera to LETS protein or actin were occasionally seen (Fig. 8*c*) perhaps accounting for the observation that small amounts of cell proteins, including LETS, can be detected in substrate-attached material left after EDTA removal of cells (7).

NIL cells maintained in 0.3% FCS behaved differently with regard to EDTA or EGTA removal; treatment for 15–30 min resulted in very little rounding up of cells, and more prolonged incubation (1–2 h) was required to achieve removal of cells from the substrate. As the cells

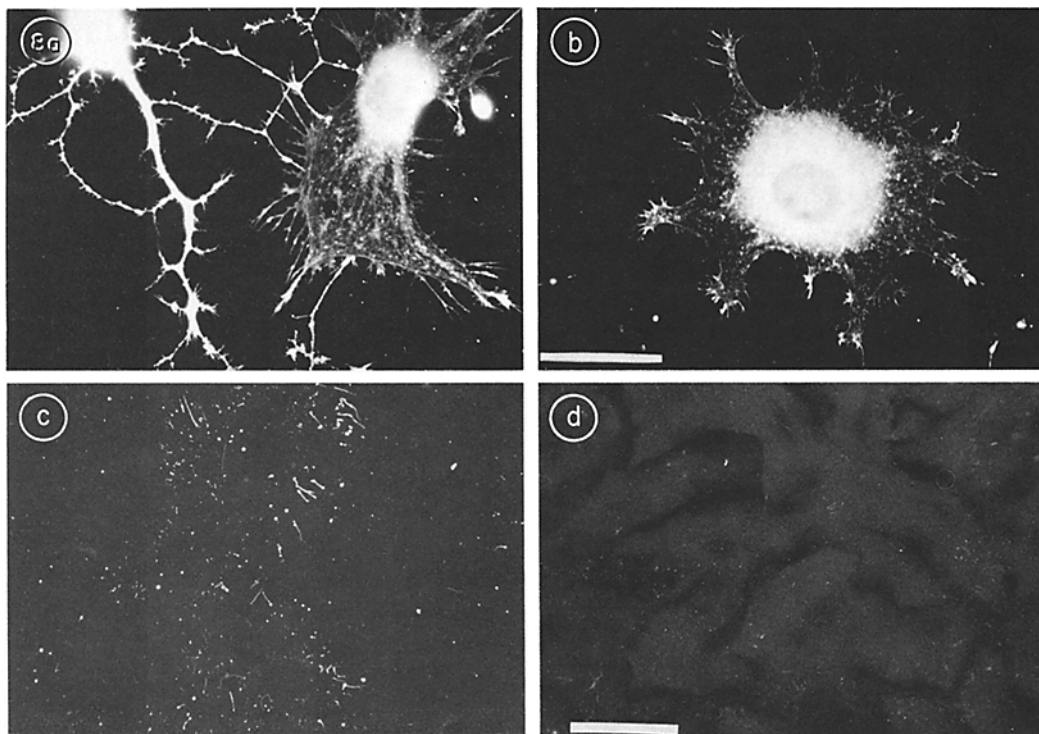
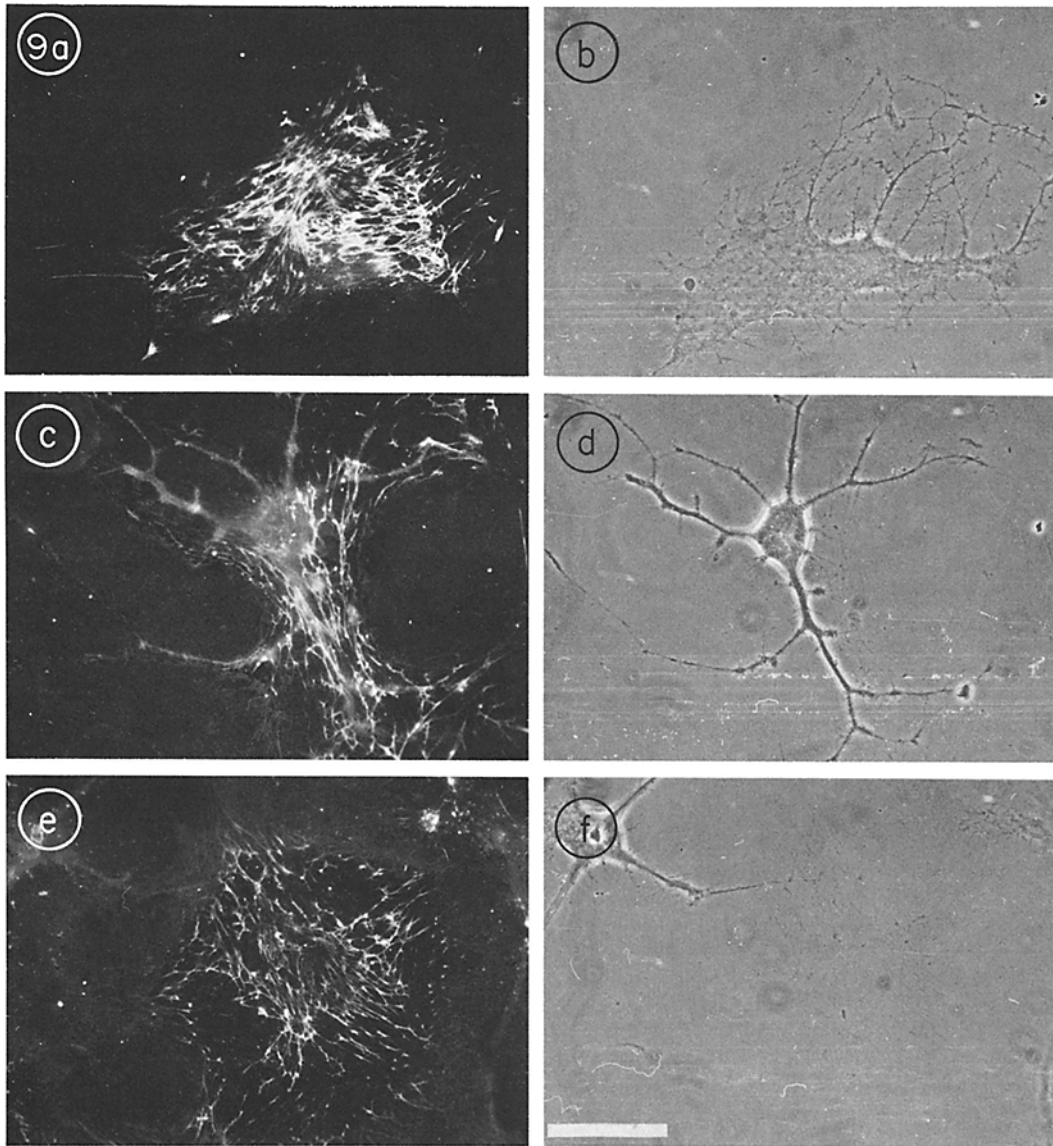


FIGURE 8 Treatment of NIL8 culture with chelating agents. Cells were plated at  $1 \times 10^5/35$  mm dish in DME plus 5% or 0.3% FCS and treated at 72 h with 2 mM EGTA or 0.02% EDTA in PBS as indicated. (*a* and *b*) Cells in 0.3% FCS treated for 2 h with 2 mM EGTA and stained for actin.  $63 \times$  objective. Note retraction fibers. (*c*) Cells in 5% FCS treated for 15 min with 0.02% EDTA and stained for actin. Note specks of fluorescence outlining position previously occupied by cell. (*d*) As panel *c* stained for LETS protein. Note "light shadow" appearance of places previously occupied by cells and occasional flecks of fluorescence.  $40 \times$  objective. Bars,  $50 \mu\text{m}$ .

retracted from the substratum, they formed a rounded central cell body with long retraction fibers. The retraction fibers stained strongly with antibody to actin (Fig. 8*a* and *b*). During the retraction, some of the LETS protein was left on the substratum in the form of fibrillar networks (Fig. 9). Eventually when all cells had detached, only a field of fibrillar "footprints" remained.

These stained with antiserum to LETS protein but did not stain for actin and tubulin.

This difference in behavior between well-spread serum-starved cells and density-arrested cultures is consistent with the difference in distribution of LETS protein between these two culture conditions. In the well-spread cells a large proportion of the LETS protein network is beneath the cells



**FIGURE 9** Treatment of sparse, low serum-arrested cells with EGTA. Cells in 0.3% FCS were treated for 2 h with 2 mM EGTA and stained for LETS protein. (*a*, *c*, and *e*) Immunofluorescence for LETS protein. (*b*, *d*, and *f*) Phase-contrast illumination of same fields. Note retraction of cell but integrity of LETS network, which remains substrate attached. 63 × objective. Bar, 50 μm.

and is left behind when the cells detach. In the less-well spread dense cultures, the network of LETS fibrils surrounds the cells and detaches with them.

### *Trypsin Treatment*

LETS protein can be completely removed from the cell surface by brief trypsin treatment (19). Conditions are similar to those reported to cause loss of actin microfilament bundles (34) and to be sufficient to initiate mitosis in other cell systems (6, 39, 4, 51, 30). In an attempt to correlate the role of LETS protein in cell attachment with the presence of actin bundles, well-spread cells were subjected to mild tryptic hydrolysis, and fixed at intervals to examine the surface for LETS protein and the interior for actin distribution. Such a series is illustrated in Fig. 10.

Treatment of semi-confluent, low-serum-blocked cultures with 1  $\mu\text{g}/\text{ml}$  trypsin for 2 min left the cell shape essentially unaltered; very few cells showed any signs of rounding up, and all the well-spread cells had apparently intact LETS and actin structures. It is remarkable, however, that this mild treatment resulted in loss of the black shadows seen in control cultures, and most cells stained positively for LETS protein on their lower surface (not shown). By 5 min, most cells showed clear signs of some rounding up; there were retraction fibers that stained with anti-actin antibody, and more substrate was visible between cells (Fig. 10*d*). Where actin microfilament bundles remained, they stained less brightly than in controls and tended to be located at the periphery of the cell. There was much less LETS protein staining on the surface of such rounded cells, and it was often fragmented in nature (Fig. 10*c*).

After 10 min of trypsin treatment, some cells had detached completely from the cover slips. In those remaining, there was a range of appearances, from a few cells that still looked reasonably well spread with actin structures still evident or a substantial LETS network on the surface, to a majority of almost entirely rounded up cells with exceedingly long retraction fibers and no discernible LETS structure. At 30 min, it was still possible to find small areas of attached cells lacking LETS protein and actin bundles, but the vast majority of the cells were well rounded or had detached during the fixation and staining procedures.

It is not possible from this series, nor from

others with even closer time-points or lower trypsin doses, to decide whether in general the LETS protein is more or less susceptible to trypsin than are the actin microfilament bundles. It is evident that even within an essentially synchronized culture there is sufficient heterogeneity to render such a generalization meaningless, and double staining of individual cells may be required to answer this specific question.

### *Dithiothreitol Treatment*

LETS protein at the surface of fibroblasts is cross-linked by disulfide bonds (22). We have examined the LETS protein distribution after DTT treatment to see whether the integrity of the LETS network is affected by disruption of these disulfide bonds. Cells that had been plated in 0.3% or 5% FCS were treated for periods up to 2 h with 1 or 10 mM DTT in PBS or growth medium. Only 10 mM DTT for long periods of time (over 1 h) caused any discernible change in the LETS pattern and even then the change was rather slight (Fig. 11). This was true whether or not the cells were fixed before staining. Therefore, maintenance of the distribution of LETS protein fibers is not solely due to disulfide bonding.

### *Colchicine Treatment*

Cells were treated with drugs that disrupt microtubules and microfilaments to determine whether these agents have a concomitant disruptive effect on the distribution of LETS protein on the cell surface.

NIL cells maintained in 0.3% FCS were treated with  $5 \times 10^{-5}$ – $10^{-3}$  M colchicine for 1 or 2 h at 37°C, then fixed and stained for LETS, actin, or tubulin (Fig. 12). After 2 h the cell shape had not altered at all, and there was essentially no alteration in the staining for LETS or actin (Fig. 11*d* and *f*). However, within 1 h there was a marked reduction in the appearance of filamentous tubulin staining in comparison with control cultures and by 2 h virtually no cells retained an intact microtubular array (Fig. 12*b*), although a few still displayed vestigial fragments that stained. These were predominantly around the hook-shaped body as reported by others (5, 13, 44).

HSV-NIL cells grown in 5% FCS and stained for tubulin did not show the bright fluorescent array of filaments characteristic of well spread NIL cells, but instead a fine mesh of fibers could be discerned (see Fig. 6*e* and *g*), and on colchicine



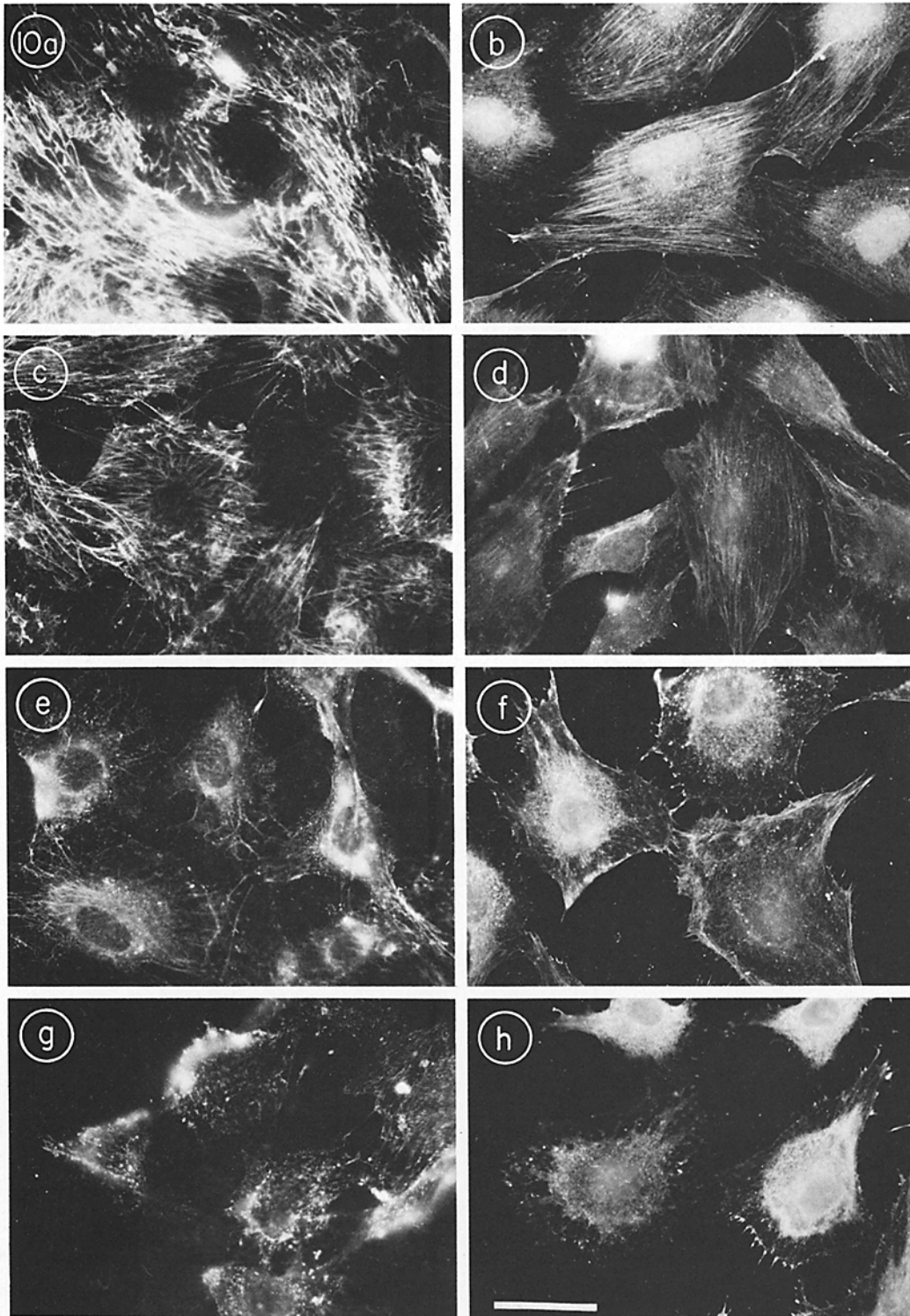


FIGURE 10 Time-course of mild trypsinization of NIL8 cells. Cells were plated at  $3 \times 10^5$ /60 mm dish in DME plus 0.3% FCS. At 72 h the cells were rinsed twice in PBS and incubated in trypsin ( $1 \mu\text{g}/\text{ml}$  in PBS) for the indicated times. Trypsinization was stopped by transfer of the cover slips to soybean trypsin inhibitor ( $10 \mu\text{g}/\text{ml}$  in PBS) for 10 min. (a) Control: cells were incubated for 30 min in trypsin that had previously been inactivated with soybean trypsin inhibitor. Stained for LETS protein. (b) As in panel a stained for actin. (c and d) 5 min; stained for LETS protein and actin, respectively. (e and f) 10 min. (g and h) 30 min.  $63 \times$  objective. Bar,  $50 \mu\text{m}$ .

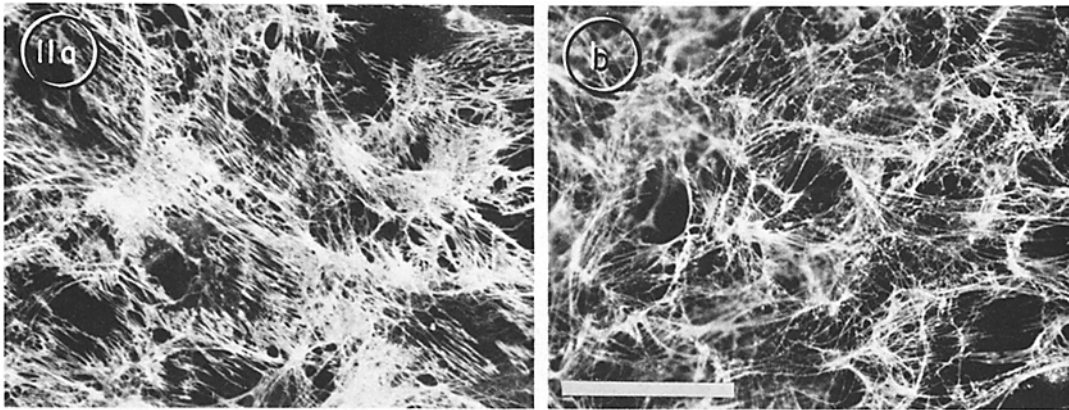


FIGURE 11 Treatment of NIL8 cultures with the sulfhydryl reagent DTT. Cells were plated at  $3 \times 10^4$  35 mm dish in DME plus 5% FCS and kept for 6 days without medium change. DTT at  $100 \times$  concentration in  $H_2O$  was added directly to the dish to give a final concentration of 10 mM. The cells were incubated at  $37^\circ C$  for 2 h in a 5%  $CO_2$  incubator and the treatment terminated by thorough washing in PBS. Cells were stained without fixation. (a) Control: cells incubated for 2 h without medium change. Stained for LETS protein. (b) 10 mM DTT for 2 h. Stained for LETS. Note some diminution of staining as compared with controls but that remaining LETS protein is still fibrillar, i.e., it does not patch or cap even after reduction. Bar, 50  $\mu m$ .

treatment they too were removed, leaving an amorphous fluorescence throughout the cell (not shown).

#### Cytochalasin Treatment

Cytochalasin B at concentrations of 5–10  $\mu g/ml$  had a dramatic effect on cell shape; NIL cells grown in 0.3% serum rounded up rapidly leaving “arborized” extensions adhering to the substrate. The whole cell stained brightly for actin with concentrations at the nodes of the processes; no stress fibers could be seen (Fig. 13b). LETS protein was also associated with the cell and its processes (Fig. 13c and d), and some was left behind as footprints on the substrate as was the case after EDTA and EDTA treatment. Again, the results differed for cells grown in 5% FCS. The cells arborized in much the same way, but the effects on the LETS network differed. The dense array of fibers was perturbed and holes appeared in the network, often above cells (Fig. 13f). The general appearance suggested that the cells retracted away from the LETS protein fibrillar network, leaving it as a separate extracellular matrix above the cells.

#### Procaine Treatment

Procaine is a local anesthetic that is thought to affect cytoskeletal organization (29, 35). Cells in 0.3% FCS treated with 1 mM procaine for up to

2 h showed only minimal disturbance of actin or LETS protein organization. No marked effect was seen until 10 mM procaine was used, and then actin cables were not so frequently seen, cells rounded up, and LETS networks were left attached to the substrate. The black shadow appearance persisted beneath rounding cells (Fig. 14).

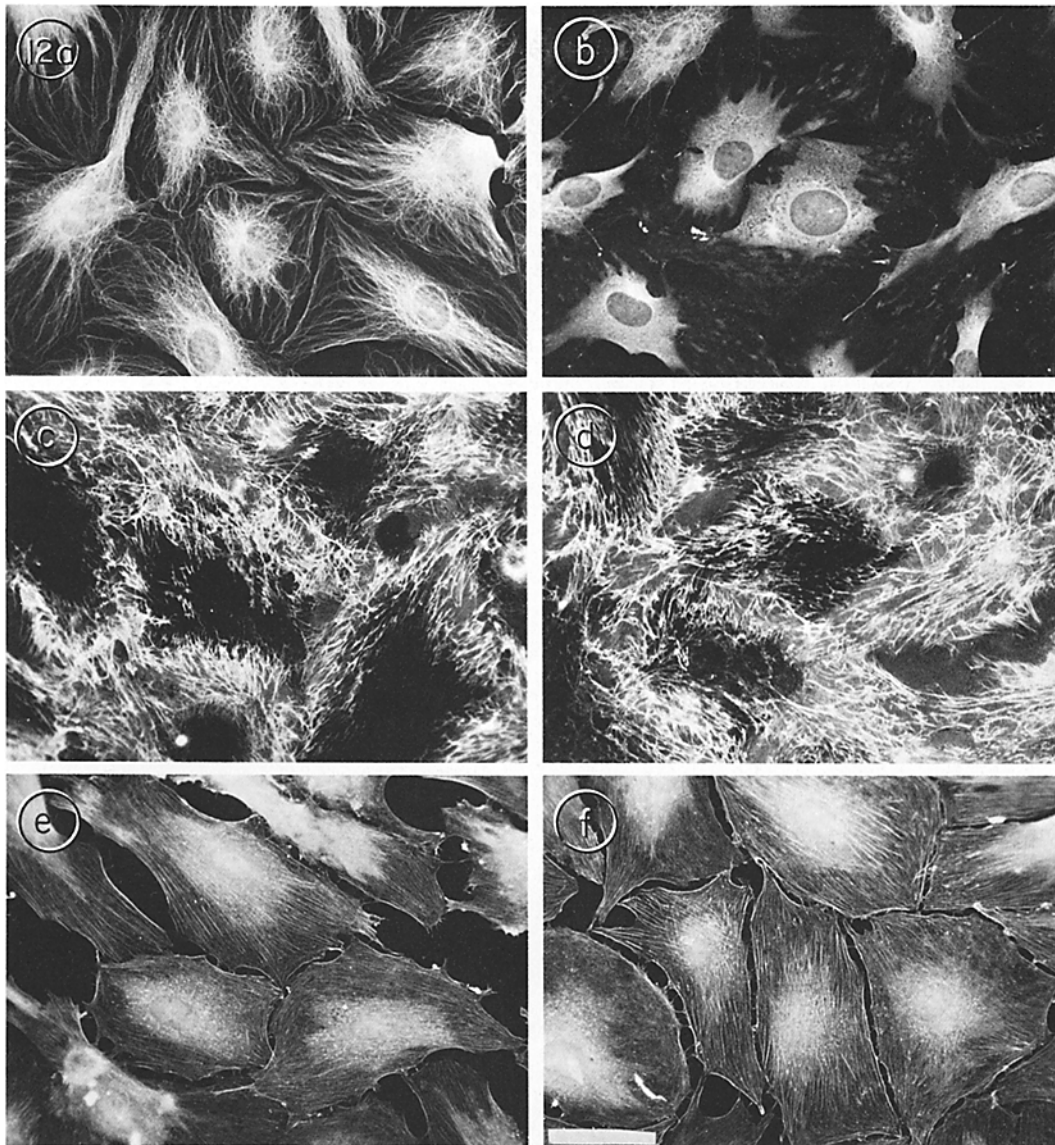
#### DISCUSSION

The objective of these experiments was to investigate the location of LETS protein in normal and transformed fibroblasts in culture and to ask whether we could find evidence for any association between the extracellular organization of LETS protein and the cytoskeleton within the cell. This was of interest because of the correlations that have been demonstrated between transformation and alterations in cytoskeleton, and because LETS protein also is transformation sensitive.

#### Distribution of LETS Protein

We have established that LETS protein is organized on the outer surface of NIL cells in an elaborate network, in agreement with reports on other cells (43, 7). However, the precise distribution of the network varies with the culture conditions.

The observation that a considerable amount of the LETS protein is located underneath the cells



**FIGURE 12** Effect of colchicine treatment on tubulin, LETS protein, and actin organization. Cells were plated at  $10^5/35$  mm dish in DME plus 0.3% FCS and kept for 48 h before addition of colchicine to a final concentration of 1 mM. Incubation was terminated by formaldehyde fixation after rapid washing in warm PBS. Similar results were obtained at  $5 \cdot 10^{-5}$  M. (*a*, *c*, and *e*) control cultures stained for tubulin, LETS protein, and actin, respectively. (*b*, *d*, and *f*) 2 h incubation in 1 mM colchicine. Stained for tubulin, LETS protein, and actin, respectively.  $40 \times$  objective. Bar,  $50 \mu\text{m}$ .

is of particular interest. As early as 4–5 h after trypsinization and replating, LETS protein fibrils are seen underneath cells, and the amount increases with time in culture (Fig. 5). When NIL cells are plated in low serum, they do not continue to divide, but spread out and adhere strongly to the substratum. There is then a very extensive

network of fibrils beneath the cells. The network under the cells is frequently difficult to stain, in live preparations or after fixation with formaldehyde. That the network is indeed under the cells can be readily demonstrated by several procedures. (*a*) After treatment of cells with acetone (Fig. 4*c*) or NP-40 (Fig. 7), antibody penetrates

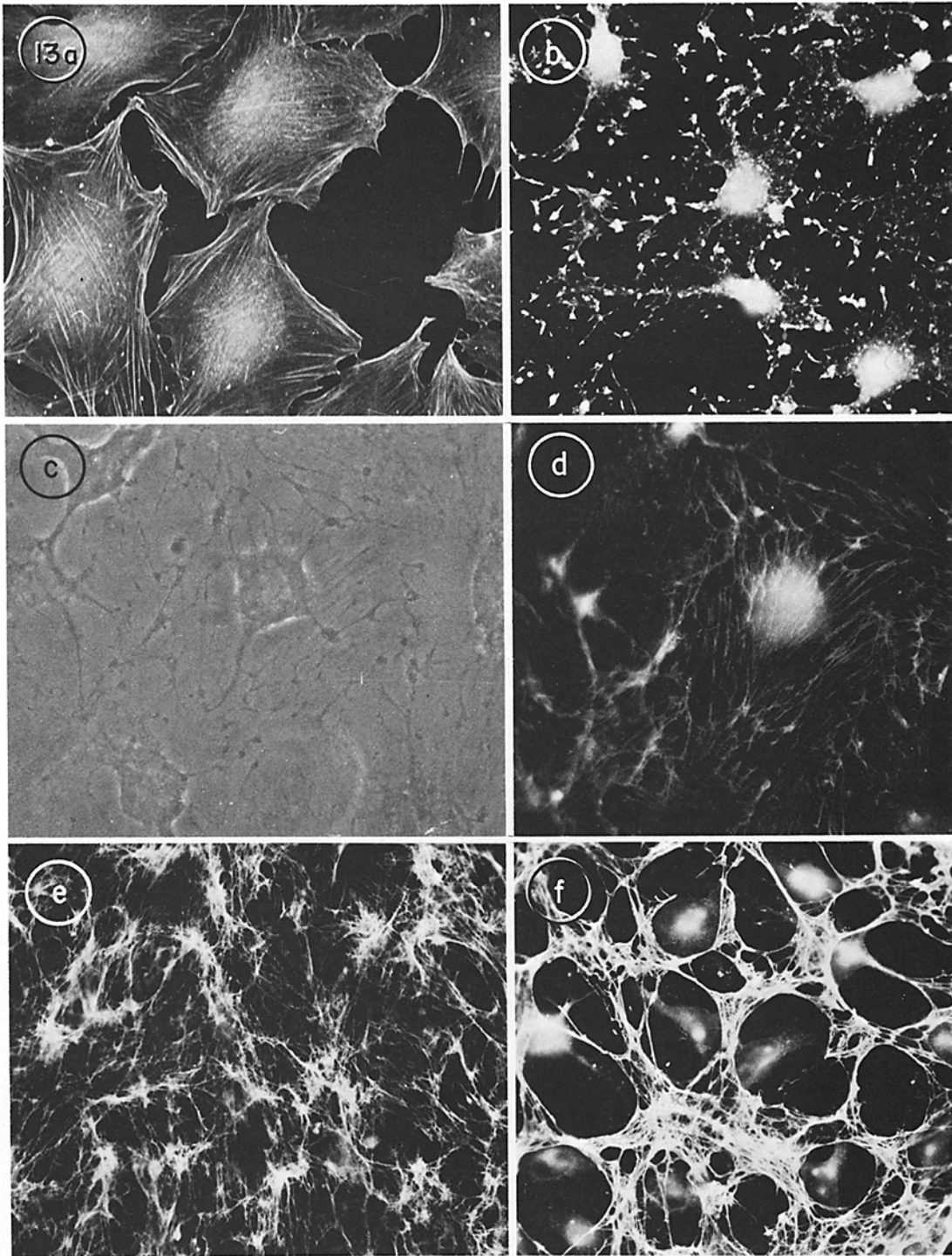


FIGURE 13 Effects of cytochalasin B treatment on NIL8 cells. (a and b) Cells cultured in 0.3% FCS and stained for actin. (a) Control. (b) 5  $\mu\text{g/ml}$  cytochalasin B, 1 h. Note retraction of cells in (b) and formation of "crystals" of actin. (c and d) Cells cultured in 0.3% FCS and treated with 5  $\mu\text{g/ml}$  cytochalasin B for 1 h. Phase (c) and anti-LETS fluorescence (d) of same field. Note retracting cells leaving fibrillar network of LETS protein on substratum. (e and f) Cells cultured in 5% FCS and stained for LETS protein. (e) Control. (f) 5  $\mu\text{g/ml}$  cytochalasin B, 2 h. Note retraction of cells leaving fibrillar network of LETS protein which also retracts into cords and clumps. 40  $\times$  objective.

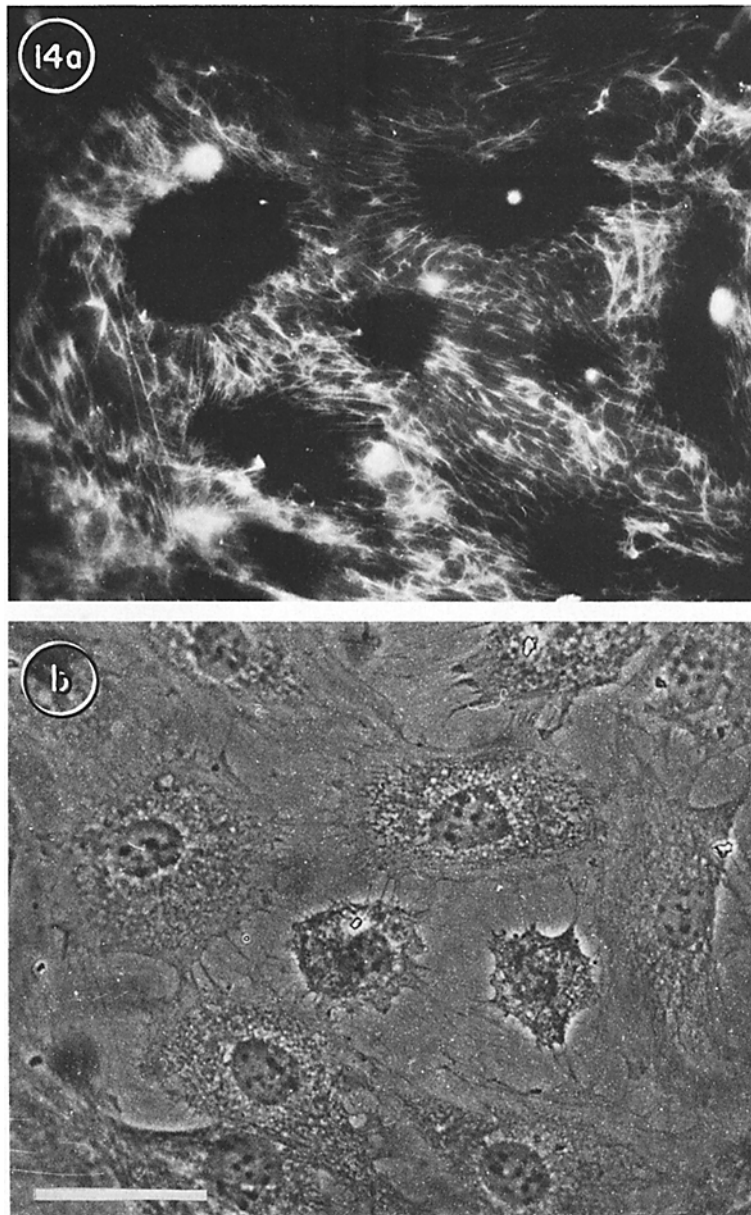


FIGURE 14 Effect of the local anesthetic procaine on LETS protein staining. Cells were plated at  $1.5 \times 10^5/60$  mm dish and kept for 48 h in DME plus 0.3% FCS. Procaine was added to give a final concentration of 10 mM. (a) Procaine; 10 mM for 1 h. Stained for LETS protein. Note diminished size of black shadows but retention of LETS fibrils on substrate. (b) Phase-contrast micrograph of same field. Note rounding up cells with retraction fibers. 40  $\times$  objective. Bar, 50  $\mu$ m.

readily and reveals fibrils beneath the cells. (b) Mild trypsinization results in loss of the "black shadow" appearance and permits staining of the lower cell surface, perhaps by reducing cell substrate adhesion. (c) Partial or complete detach-

ment of cells with chelating agents, NP-40, cytochalasin B, or procaine, reveals fibrillar networks under the cells. At intermediate stages, partial residual blocking of lower surface staining by partially detached cells can sometimes be seen

(e.g., Fig. 14). When cells have been completely detached under appropriate conditions, LETS footprints remain on the substrate (e.g., Figs. 7 and 9).

The finding that LETS protein is left in footprints on the substratum is consistent with biochemical measurements (9, 31). Culp (9) found LETS protein among the components of substrate-attached material (SAM) left after detachment of 3T3 cells with EGTA, and Noonan et al. (31) found an enrichment of LETS protein in a membrane fraction thought to derive from the lower surface of the cell.

LETS footprints are seen only when cells that have been kept in low serum (0.3% FCS) are removed from their substrate. In contrast, cells grown in 5% FCS retain their LETS fibrils when they are detached from the dish, e.g., with EGTA or EDTA (see Fig. 8). Additionally, "black shadows" are only found in cultures kept in low serum. There may be a correlation between increased adhesivity of cells in low serum and the formation of black shadows apparently protected from antibody staining. Whether LETS protein plays a crucial role in that increased adhesivity is a matter for speculation at this time, but is consistent with the observations reported here and elsewhere (3, 50).

A second major location of LETS protein fibrils is between cells where they contact each other. Fibrils often extend from one cell to another apparently without interruption. As cells become denser, this intracellular network becomes more extensive, and in confluent cultures of NIL cells, where some overlapping occurs, the LETS protein fibrils surround the cells in a continuous network. The same is true for cultures of primary and secondary chick embryo fibroblasts (43). When dense cultures are treated with chelating agents, the cell sheet with the LETS fibrillar network detaches almost intact, leaving very little material behind on the substratum (Fig. 8). The small speckles which stain for LETS protein or actin may be analogous to the footpads described by Revel et al. (36). These speckles are often arranged in a ring suggesting that they define the previous position of a cell. These results suggest either that the LETS protein fibrils under dense cells are not as well attached to the substrate as those under low-serum arrested cells or that, on attaining confluence, the cells alter the distribution of LETS protein so that it is no longer predominantly under the cells in a network. We

cannot at present decide which of these possibilities pertains.

Finally, LETS protein is found in complex branched fibrils extending over the upper surfaces of cells (Figs. 3 and 4). This is a relatively minor fraction of the LETS protein in sparse or low serum-arrested cultures but becomes the major fraction in dense cultures.

One further point concerns the possible location of LETS protein in relation to the plasma membrane. Since staining of live and fixed cells gives similar patterns, it is unlikely that LETS protein moves laterally in the plane of the membrane in response to antibody binding, i.e., patching and capping are not observed (cf. references 41, 11, and 12). Furthermore, treatment with DTT does not affect the overall distribution of LETS protein fibrils, showing that it is not merely the extensive disulfide bonding of LETS protein (22) which prevents lateral movement. The failure to solubilize LETS protein with non-ionic detergent or other mild extractants (23) argues against its being a membrane protein in the usual sense (17, 40). Its properties are more those of a structural or fibrous protein: ordered arrangement in fibrils, insolubility except in ionic detergents or chaotropic agents (23), separation from plasma membrane markers on subcellular fractionation (16), and lack of perturbation by treatment with antibodies.

In transformed cell cultures (NIL-HSV) the pattern of LETS protein we observe consists predominantly of intercellular bridging fibers. These are very short and not present on most cells; there is some clonal variation within the culture. Chen et al. (7) examined a series of adenovirus-transformed rat cell lines and found variations in the pattern of immunofluorescence staining for LETS protein, ranging from no staining, through short intercellular stitches as described here, to more extensive fibrillar networks. The extent of staining for LETS protein showed an inverse correlation with tumorigenicity.

#### *Relationship of LETS Protein Organization to Cytoskeletal Fibers*

Earlier work has shown correlations between LETS protein and organized actin microfilaments (see introductory paragraph). Some of the results presented here support this correlation, but others do not.

Both actin microfilaments and microtubules ap-

pear better ordered in well-spread cells, whether growing or serum arrested (Figs. 3 and 5). Confluent cultures, in which the cells are less well-spread, show less clearly organized actin microfilaments and microtubules. In contrast, LETS protein fibrils are more abundant in dense cultures than in growing ones (Figs. 2 and 3). Secondly, although sparse, growth-arrested cultures of normal cells have well-developed arrays of all three types of fiber (actin, tubulin, and LETS protein), the patterns are strikingly different (Fig. 3). These observations argue against a direct interaction, but they are not conclusive evidence against it.

There are several populations of LETS fibrils which are present in different proportions under different conditions and which may be distinct in their properties. Similarly, actin is seen in stress fibers or cables, as staining around the cell edge, presumably as a submembranous sheath (15, 27, 28), and as diffuse cytoplasmic fluorescence which could be soluble actin or actin arranged in structures with dimensions below the resolution of the immunofluorescence technique. It is conceivable that subsets of the various populations could be interrelated.

In order to investigate the possibility of more subtle interactions of this sort, we sought to perturb selectively the different structures and examine the effects on others. The LETS protein network was disrupted using mild trypsin treatment (Fig. 10). As described by Pollack and Rifkin (34) trypsin also disrupted the arrangement of actin. Although there was an overall correlation between the loss of surface LETS protein, of actin microfilament bundles, and rounding up of cells, we were unable to decide whether one network disappeared precisely in parallel with the other, owing to heterogeneity in the response of the cells.

An example of separation between actin structures and LETS network is found in the NP-40 extraction data; while NP-40 extracts much of the cell protein, it leaves both LETS and actin structures intact. However, the LETS remains at least in part associated with the substrate while the actin peels away from the substrate. Thus, each substructure can maintain its own integrity without direct association with the other. This does not address the question of whether they are interdependent at the formative stages, or are cross-linked to each other either directly or indirectly before NP-40 extraction. We can say, however, that NP-40 extraction, whether or not followed

by fixation, permits some separation of these two entities without disruption of either one.

Treatment of cells with cytochalasin B leads to more rapid turnover of LETS protein from cells<sup>1</sup> and blocks its regeneration after trypsin treatment. Furthermore, addition of LETS protein to transformed cells leads to reappearance of actin cables (3, 47). These results are consistent with a relationship between actin and LETS protein structures, but the contribution of indirect effects, for instance on cell morphology, must be considered. The immunofluorescence results on the effects of the drugs cytochalasin B and procaine (Figs. 13 and 14) show gross alterations in cell morphology as well as disruption of actin microfilaments.

Disassembly of microtubules with colchicine (Fig. 12) had no effect on cell shape, LETS protein fibrils or actin cables. This finding is confirmed by biochemical measurements.<sup>1</sup> Furthermore, the continued presence of microtubules in NIL-HSV cells in the absence of LETS protein argues against a close dependence of microtubules on LETS protein. Fuller et al. (14) and Edelman and Yahara (10) found transformation sensitivity of microtubules in SV3T3 and RSV-CEF, but this does not appear to be the case with NIL-HSV. Although NIL-HSV cells cannot be made to spread on the substrate by growth in low serum and therefore microtubular arrays characteristic of well spread cells cannot be observed, the type of staining seen in NIL-HSV cells closely resembles that of dense NIL cultures and is not greatly affected by addition of LETS protein (3).

It seems highly probable, therefore, that microtubules and LETS protein fibrils are independent and unrelated structures. The independence of actin microfilaments and LETS protein is less clearly established. While our results do not argue for a simple one-to-one relationship between LETS protein fibrils and actin-containing filaments, more subtle interactions between LETS protein and actin are still conceivable and would be consistent with many of the findings we report here. Earlier reports of the association of actin microfilament bundles with adhesion "plaques" at areas of close cell-substrate contact (2) represent an example of one such possible relationship.

<sup>1</sup> I. U. Ali and R. O. Hynes. 1977. Effects of cytochalasin B and colchicine on attachment of a major surface protein of fibroblasts. *Biochim. Biophys. Acta*. In press.

How these characteristics of fibroblasts maintained in tissue culture relate to the *in vivo* properties of such cells remains to be explored. Given its apparent involvement in adhesion, it is tempting to speculate that LETS protein may play a part in restraining cells in a particular site and that loss of LETS protein leads to reduced cell-cell and cell-substrate adhesion allowing cells to move from their normal location.

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