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Changes in microfilament organization and surface topography upon transformation of chick embryo fibroblasts with Rous sarcoma virus

(actin/*src* gene product/membrane changes/indirect immunofluorescence)

EUGENIA WANG AND ALLAN R. GOLDBERG

The Rockefeller University, New York, N.Y. 10021

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ABSTRACT **A series of morphological changes occurred when chick embryo fibroblasts infected with the *NY*68 mutant of Rous sarcoma virus were shifted from nonpermissive temperature (41°) to permissive temperature (37°). We observed three distinct stages in cell morphology and surface topography that were correlated with a reduction in the organization and assembly of actin-containing microfilament bundles. Our observations suggest that control of microfilament organization and surface topography are responsive to the presence of a functioning transforming gene (*src*) product of Rous sarcoma virus.**

Transformation of chick embryo fibroblasts by Rous sarcoma virus is independent of virus replication (1, 2) and is under the influence of a viral gene that is called the *src* gene because it contains sequences specific for sarcomagenesis (3). Cells infected with virus mutants that have a temperature-sensitive lesion in the *src* gene product are transformed at a permissive temperature but not at a nonpermissive temperature. Thermal denaturation of the *src* gene product of some of these mutants is reversible and thus provides a useful system for studying changes in cell metabolism that are associated with transformation.

The presence of a functional *src* gene can be correlated with a broad array of alterations (2), many of which involve the membrane. Included in this category of membrane-associated phenomena in transformed cells are increased agglutinability with plant lectins, loss of surface proteins, changes in adhesion properties, changes in contact inhibition of growth and locomotion, changes in membrane transport, elevated levels of a protease that is a plasminogen activator, and changes in cell morphology. In particular, alteration in cell shape has been used as a convenient marker for assaying cellular transformation (4–6). Changes in the morphology and surface topography of mammalian cells are associated with the assembly and organization of cytoplasmic fibers such as microtubules, 100 Å filaments, and microfilament bundles (7–9).

The cytoskeleton of transformed cell lines seems to be altered (7, 9). Studies using fluorescent antibody directed against actin showed that the arrangement of actin filaments in cables is lost or much reduced in mouse and rat cells transformed by simian virus 40 (10–12). McNutt *et al.* (13, 14) had drawn a similar conclusion from their earlier results using transmission electron microscopy. In the present study we describe changes in cell shape, topography, and cytoplasmic fibers in chick cells infected with Rous sarcoma virus that are associated with the action, either direct or indirect, of the *src* gene product.

MATERIALS AND METHODS

Cells and Viruses. The growth and maintenance of normal and virus-infected chick embryo fibroblasts was as described (15). In all experiments, cultures were grown in Scherer's medium containing 10% tryptose phosphate broth and 5% calf serum. The temperature-sensitive transformation mutant *NY*68 of the Schmidt-Ruppin strain of Rous sarcoma virus was obtained from Drs. Kawai and Hanafusa. All temperature-shift experiments were done on cells seeded at a density of 0.5 to 1.0 $\times 10^6$ cells per 35 mm^2.

Scanning Electron Microscopy. Tertiary cultures of normal and virus-infected chick embryo fibroblasts were grown on Corning no. 1 glass coverslips. Samples were fixed with 1% glutaraldehyde, dehydrated with ethanol, and subjected to critical point drying before observation with an ETEC scanning electron microscope.

Transmission Electron Microscopy. Infected cells were fixed with 1% glutaraldehyde and postfixed in 1% OsO_4. Sections were cut parallel to the substratum and were collected on carbon-parlodion coated grids for staining with hot uranyl acetate (16) and lead citrate (17). They were examined with a Philips 300 electron microscope.

Fluorescence Microscopy. We have developed an indirect immunofluorescent method to detect the state of assembly and organization of actin-containing microfilament bundles using antibody against DNase I (18). The detailed preparation of antibody and the processing of samples for indirect immunofluorescence will be described in another report (Wang and Goldberg, in preparation). Briefly, we have taken advantage of the observation (19) that DNase I binds to actin to visualize actin-containing microfilaments. Formaldehyde-fixed and acetone-treated cells were incubated successively with DNase I and goat antibody against rabbit globulin coupled to fluorescein. Labeled cells were viewed in a fluorescence microscope.

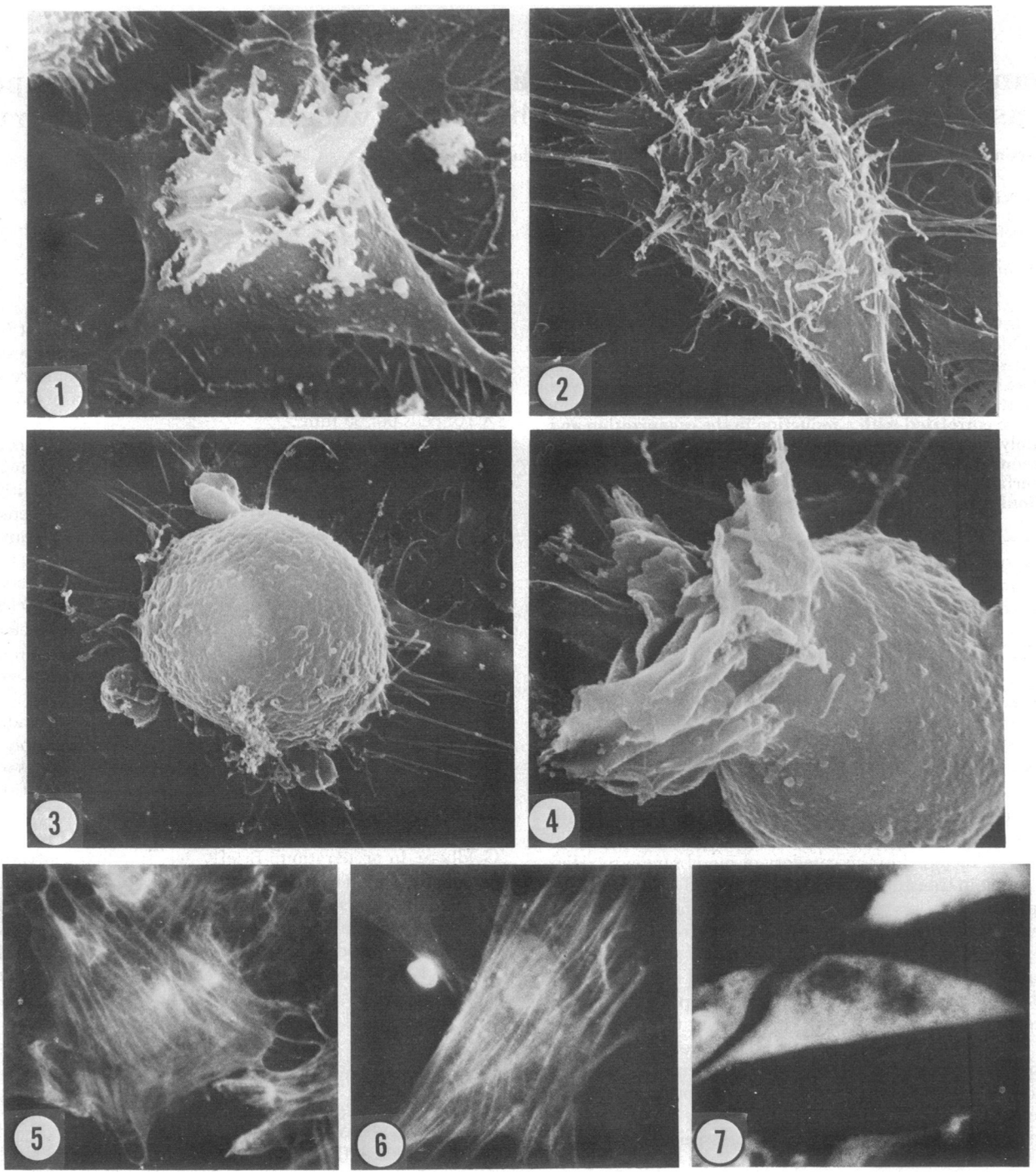
RESULTS

Light microscopic observations

Chick embryo fibroblasts infected with *NY*68 were transferred twice at permissive temperature (37°) to obtain complete transformation of the culture. The culture was subdivided; half was maintained at 37° and the other half at the nonpermissive temperature (41°). Cells growing at 41° appeared fibroblastic and showed parallel alignment in contrast with the round cells that were maintained at 37°. When cultures were shifted from 41° to 37°, approximately 50% of the cells converted to the rounded transformed morphology by 12–14 hr after temperature shift. About 80–90% of the cell population had assumed the transformed phenotype by 24 hr after shift. A variable portion of the population (up to 10–20%) never assumed the rounded morphology.

Changes in surface topography

We observed three stages in the change in surface topography when *NY*68-infected chick embryo fibroblasts were transferred from nonpermissive to permissive temperature. Stage 1 began



FIGS. 1–4. Scanning electron micrographs showing changes in cell morphology and surface topography during the process of cell transformation. Chick embryo fibroblasts infected with *NY*68 at different times after a shift from nonpermissive temperature (41°) to permissive temperature (37°). *Fig. 1.* 1 hr; ×2000. *Fig. 2.* 2 hr; ×2000. *Fig. 3.* 24 hr; ×2000. *Fig. 4.* 24 hr; ×4000.

FIGS. 5–7. Visualization of actin-containing microfilament bundles by indirect immunofluorescence. Fixation and staining of cells are described in *Materials and Methods*. *Fig. 5.* Normal chick embryo fibroblasts; ×1000. *Fig. 6. NY*68-infected chick embryo fibroblasts at stage 1, 1–2 hr after temperature shift; ×1000. *Fig. 7. NY*68-infected chick embryo fibroblasts at stage 3, 24 hr after temperature shift; ×1000.

1 hr after temperature shift and lasted for 2 hr. Unique, flower-like membrane ruffles appeared in the nuclear region (Fig. 1). The flower-like ruffle was formed by a direct upward extension of the free cell surface. During this period the cells generally retained their fibroblastic shape. Although the ruffling activity of the free cell surface was predominant at this time, we occasionally observed marginal ruffles in the cell periphery, especially in regions of cell-to-cell contact.

During stage 2, 3–12 hr after temperature shift, long cellular processes retracted to the nuclear region, leaving numerous retraction fibers around the cell periphery (Fig. 2). The nuclear region became elevated and numerous microvilli appeared on the cell surface. The retraction of the cell periphery often led to the detachment and disappearance of the long cellular pro-

cess. The increased degree of retraction finally resulted in the gradual loss of the asymmetrical fibroblastic cell shape. By the end of stage 2, the cell appeared spindle-shaped and showed many microspikes and retraction fibers.

Stage 3, 12–24 hr after the temperature shift, was characterized by a gradual conversion of the spindle shape to a completely round form (Fig. 3). By this time the cell was covered with small blebs, and the number of retraction fibers and microvilli had diminished markedly. Higher magnification of the same cell showed budding virus as hemispherical herniations on the cell surface. We observed virus budding from the main cell body, the surface of microvilli, and the retraction fibers. There appeared to be no preferential location on the surface for virus maturation. Occasionally, membrane ruffles similar to those seen in stage 1 were observed on these round cells. Fig. 4 shows these ruffles at higher magnification on a cell that was fixed 24 hr after the temperature shift. Interestingly, except for the small surface blebs of budding viruses, the surface topography of *NY*68-infected cells at this stage resembles that of mitotic cells. Both stage 3 cells and mitotic cells are round and have a smooth surface that is covered by a few microvilli, blebs, and microspikes.

Indirect immunofluorescence

We also have examined the changes in the organization and assembly of actin-like cytoplasmic fibers during the first 24 hr after temperature shift. Cell cultures were fixed and stained for visualization by indirect immunofluorescence microscopy (see *Materials and Methods*). The immunofluorescent staining pattern of normal chick embryo fibroblasts is shown in Fig. 5. By phase contrast optics the cells showed numerous arrays of stress fibers which stained strongly with antibody against DNase. These fibers generally were aligned parallel to the long axis of the fibroblasts in a pattern corresponding to the distribution of microfilament bundles.

The parallel alignment of actin-containing fibers, visualized by indirect immunofluorescence, was observed in *NY*68-infected cells at stage 1 (Fig. 6). We could not distinguish major differences in the staining patterns between normal fibroblasts and *NY*68-infected fibroblasts at this stage. However, as the process of transformation progressed into stage 2, the long, filamentous actin fibers gradually shortened and the number of fibers became reduced. Fig. 7 shows that a *NY*68-infected fibroblast at the permissive temperature has no distinct fluorescent fibers. This image is similar to the diffuse fluorescent pattern that was observed in 3T3 cells transformed by simian virus 40 that had been stained with antibodies against myosin, actin, or tubulin (20–22).

Changes in organization of microfilament bundles during transformation

Serial thin sections cut parallel to the substrate of flat embedded cells were examined by transmission electron microscopy. We observed bundles of oriented 40–70 Å microfilaments in fully spread chick embryo fibroblasts (Fig. 8). These filamentous bundles were localized primarily beneath the plasma membrane and were present predominantly in regions of cell-substrate contact. The parallel arrangement of the submembranous microfilament bundles coincides with the distribution and location of stress fibers in living cells and appears to be identical to that of the fluorescent actin-containing fibers seen in Figs. 5 and 6. In addition, microtubules and 100 Å filaments were seen distributed in linear arrays along the long axis of the fibroblasts.

Our observations showed that *NY*68-infected fibroblasts at stage 1 after the temperature shift possessed a parallel distribution of all three types of cytoplasmic fibers similar to that seen in their uninfected normal counterparts. However, examination of round cells at stage 3 demonstrated a considerable reduction in the number of microfilament bundles (Fig. 9). We observed only a few microfilament bundles throughout the cytoplasm. Moreover, they did not exhibit parallel distribution. A conspicuous increase in microfilaments in disorganized meshworks (23) or networks (24) was observed in the cortical region of these fully transformed cells (Figs. 10 and 11). Some of the microfilaments appeared to be intercalated with the plasma membrane and some of them formed bundles that filled entire microvilli. Microtubules and 100 Å filaments also were observed with reduced frequency and were randomly scattered throughout the cytoplasm.

DISCUSSION

The morphological changes associated with oncogenic transformation after a shift of *NY*68-infected chick embryo fibroblasts from nonpermissive temperature occurred in three sequential stages: (*i*) ruffling of the free cell surface similar to that described by Ambros *et al.* (5) appeared during the initial 3 hr after the temperature shift; (*ii*) peripheral cytoplasm and long fibroblastic processes retracted; and finally (*iii*) the fibroblasts became round. While these events were occurring the cell surface morphology first changed from a flat surface to one containing abundant surface structures such as microvilli, blebs, and microspikes. These surface structures, seen in the intermediate stages, were less apparent on cells in the later stages of transformation. Concomitant with changes in cell shape and surface complexity, the parallel alignment of the submembranous microfilament bundles was replaced by a meshwork (23) or network (24) configuration. The intracellular changes in microfilament configuration coincided with the changes in gross morphology and surface topography.

The morphological changes that we observed to occur after a shift of *NY*68-infected cells from nonpermissive to permissive temperature resemble the cell rounding and disappearance of microfilament bundles that take place in the G_2-phase of the normal cell cycle, just prior to mitosis. Several workers have previously commented on the similarity between transformed cells and cells progressing through mitosis (24, 25).

Our experiments indicate that the *src* gene product in transformed cells, either directly or indirectly, is able to alter both the assembly and organization of microfilament bundles. As we have shown in Figs. 8 and 12, microfilaments are abundant in regions of cell-to-cell apposition or areas of contact with the substratum where they form dense plaques (Fig. 12) and are associated with the plasma membrane (13, 14, 27). In untransformed nonmuscle cells, actin-containing microfilaments have been implicated in a number of cellular functions such as cytokinesis, endocytosis and exocytosis, cell adhesion to a substratum, cell locomotion, membrane ruffling, and maintenance of cell shape (7, 27–31).

Several possible sites of action for the *src* gene product should be considered. It is conceivable that the *src* gene product can directly attack microfilament bundles and cause disassembly and possibly depolymerization of actin-containing filaments. Alternatively, the *src* gene product might directly or indirectly attack, by specific proteolytic cleavage, a protein on the inner surface of the plasma membrane to which the microfilament bundles are anchored. However, the scission of microfilament bundles from the membrane might also be the result of proteolysis of a transmembrane protein or complex of transmem-

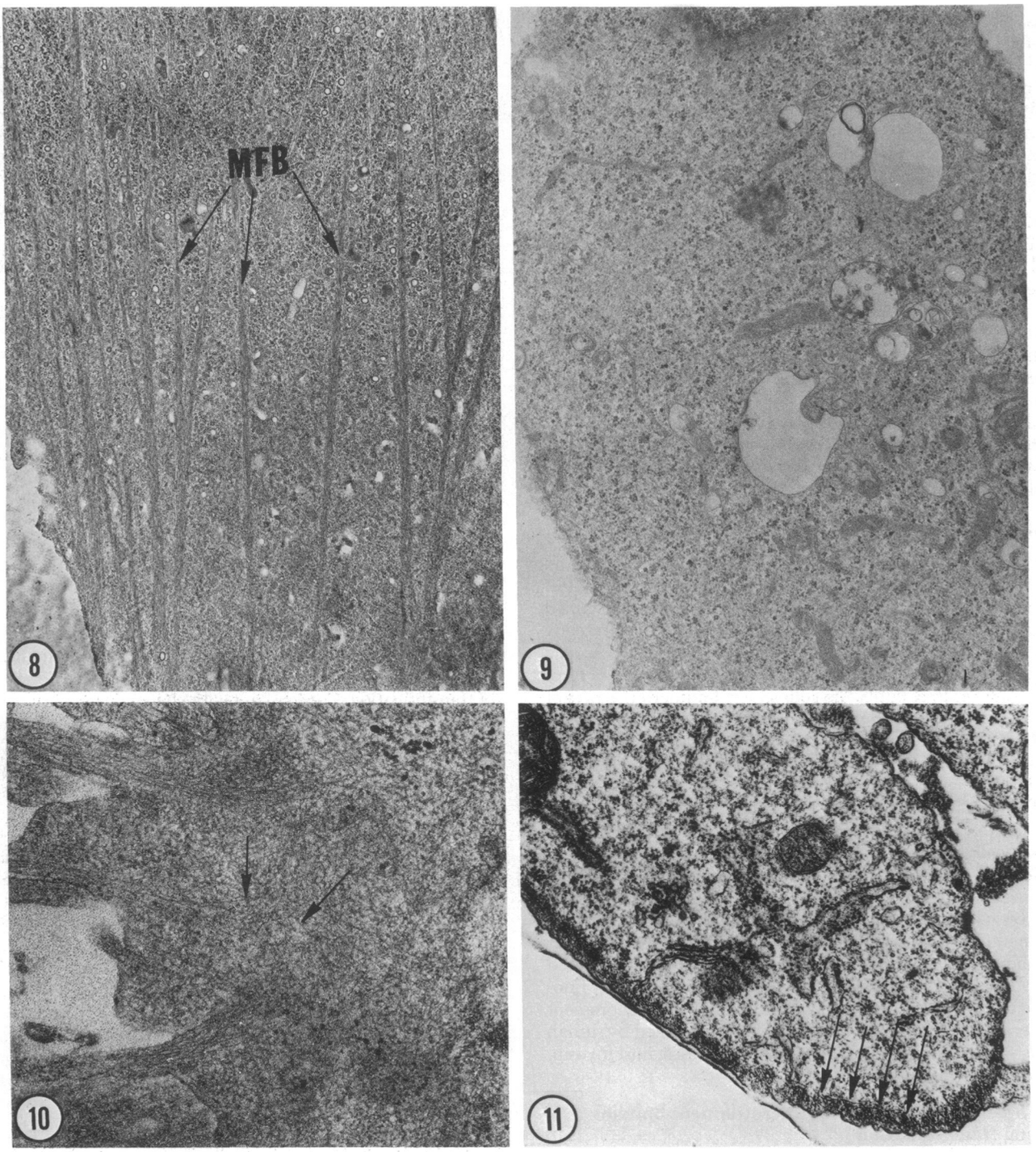


FIG. 8. Transmission electron micrograph of normal chick embryo fibroblast showing microfilament bundles (MFB); ×8300.

FIGS. 9–11. Transmission electron micrographs of *NY68*-infected chick embryo fibroblasts at stage 3, 24 hr after temperature shift. *Fig. 9.* Microfilament bundles are not evident in the cytoplasm; ×16,600. *Fig. 10.* Arrows indicate the microfilament meshwork that was observed at high magnification; ×41,600. *Fig. 11.* Cross section showing a layer of microfilament meshwork (arrows). The black line at the bottom of the cell is presumably the protein film to which the cell adhered; ×29,170.

brane proteins to which the actin-containing microfilaments might be bound.

The above-mentioned hypothetical mechanisms for disruption of microfilament bundles all would result from intracellular changes. Another possible mode of action for the *src* gene product might involve a proteolytic effect at the outer surface of the plasma membrane. Such an effect could be on a transmembrane protein to which microfilament bundles are anchored. In particular, the proteins or glycoproteins composing the adhesion plaques might be likely candidates for such action of the *src* gene product. Interestingly, Pollack and Rifkin (32) have reported that addition of plasmin or trypsin at levels that are insufficient to detach cells from the growth surface can cause the dissolution of actin cables in fully spread cells. This

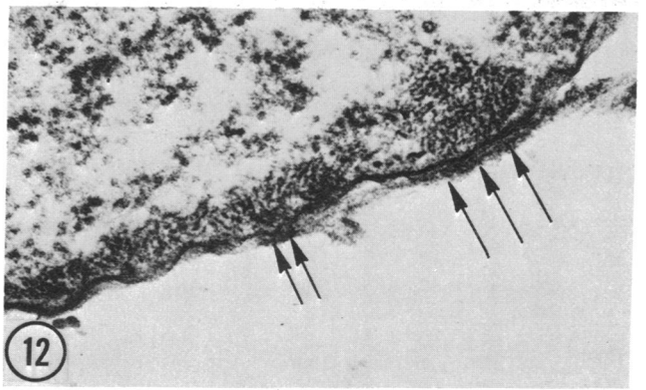


FIG. 12. Transmission electron micrograph of normal chick embryo fibroblast. Cross section perpendicular to the substrate (arrows) showing microfilament bundles and their possible attachment to adhesion plaques; ×70,830.

hypothesis would predict that the *src* gene product is either a protease or can activate a protease that can attack surface proteins.

All of these speculations have in common the idea that *src* gene product causes disruption of actin-containing microfilament bundles. We cannot exclude the possibility that the *src* gene product may directly or indirectly alter microtubules and 100 Å filaments, which are also elements of the cytoskeleton. It seems plausible that changes in the organization of the cytoskeleton can affect cell shape and thereby alter the cell's transverse through its normal cell cycle and its ability to interact with neighboring cells.

After this work was completed Edelman and Yahara (33), using an antibody directed against actin, reported changes in microfilament bundle organization when NY68-infected chick embryo fibroblasts were shifted from nonpermissive temperature to permissive temperature.

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