CONTACT-INHIBITED REVERTANT CELL LINES ISOLATED FROM SV40-TRANSFORMED CELLS

II. Ultrastructural Study

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ABSTRACT

The ultrastructural appearances of normal 3T3, SV40-transformed 3T3 (SV-3T3), and F1A revertant cell lines are compared. Both confluent and subconfluent cultures are described after *in situ* embedding of the cells for electron microscopy. There is striking nuclear pleomorphism in F1A revertant cells, with many cells having large nuclei compared to the less variable nuclear morphology of both normal 3T3 and SV-3T3 cells. Under the culture conditions used, deep infoldings of the nuclear envelope are prominent in growing cells, e.g., subconfluent normal 3T3 and confluent SV-3T3 cells. Such infoldings are infrequently seen in cultures which display contact inhibition of growth, e.g., normal 3T3 or F1A revertant cells grown just to confluence. In confluent cultures, the cytoplasmic organelles in revertant cells closely resemble those of normal 3T3 cells. In both normal and revertant cells in confluent culture, the peripheral cytoplasm (ectoplasm) has many 70 A filaments (alpha filaments), which are frequently aggregated into bundles. Alpha filaments are also abundant in the ectoplasm near regions of cell-to-cell apposition and in the motile cell processes (filopodia). The abundance and state of aggregation of alpha filaments correlates with contact inhibition of movement and growth in these cell lines since fewer bundles of alpha filaments are seen in growing cells than in contact-inhibited cells. This observation suggests that these filaments may be an important secondary component in the regulation of contact inhibition of movement and, possibly, of growth in normal and revertant cells.

INTRODUCTION

A comparison of the ultrastructure of normal and malignant cells in vivo by many investigators has revealed a variety of alterations in the nuclear and cytoplasmic structures of malignant cells (6, 7, 9, 10, 19, 37). Frequently, deep indentation of the nuclear envelope has been observed in malignant cells (6, 7, 9, 10); this finding has occasionally been interpreted as a characteristic feature of certain malignancies (11). In addition, alterations in mitochondria, endoplasmic reticulum, and structures at the cell periphery have been described in malignant cells (19, 34, 37, 55). However, there have been few tests to determine whether the observed alterations are causally related to the malignant state itself or only secondary to the metabolic conditions in vivo imposed by the nature of tumor growth. In vitro studies on cells transformed by tumor viruses allow the detailed examination of the phenomenon of contact inhibition of growth and movement and its relation to the state of the tumor virus in the cells. With the isolation of revertant cell lines from

SV40[1] transformed cells (17), it becomes possible to compare the ultrastructure of two types of 3T3 cells both containing the entire SV40 genome: the F1A revertant cells which exhibit contact inhibition of growth, and the parental SV40-transformed 3T3 cells which lack contact inhibition. Contact inhibition of growth is indicated by the tendency of the cells to grow as a monolayer and to achieve a growth plateau at low-saturation densities in culture, as shown in the companion paper (17).

In this study, the fine structure of the F1A revertant cell line (17) is described and compared to the structures of both normal 3T3 cells and 3T3 cells transformed by SV40 virus (SV-3T3 cells). Emphasis is placed on those structural features which may relate to the growth state of the cells as well as to the biochemical and virological characteristics of the same cell lines (17). Particular attention is given to (*a*) the configuration of the nuclear envelope, and (*b*) the population of ectoplasmic filaments, since in this study these structures appear to be related to the presence of contact inhibition of growth and movement.

MATERIALS AND METHODS

Cell Lines

The origin, history, and maintenance conditions of cultures of normal 3T3, SV-3T3, and F1A revertant cells have been described (17).

Electron Microscopy

Confluent and subconfluent cultures of normal 3T3, SV-3T3, and F1A revertant cells were prepared *in situ* for electron microscopy by a technique modified after that of Robbins and Gonatas (43). The cells were grown either for 2 days at low density for subconfluent cultures, or just to confluence on carbon-coated cover slips in Leighton tubes in Eagle's MEM × 4 (17). Confluent cultures contained cells which were in contact with many neighboring cells as observed by light microscopy. In contrast, subconfluent cultures contained cells which were actively growing; both cells in contact with each other and isolated cells were present in these cultures.

The cultured cells were fixed for 15 min in cold 1% paraformaldehyde, 2% glutaraldehyde in 0.08 M sodium cacodylate, pH 7.4, containing 5 mM $CaCl_2$, a dilution of Karnovsky's fixative (28). After a rinse in 0.1 M cacodylate buffer at pH 7.4, the cells were fixed in cold 1% osmium tetroxide in 0.1 M cacodylate buffer (5 mM in $CaCl_2$) for 1 hr. Several of the cultures were stained with 0.5 or 1.0% uranyl acetate in Veronal-acetate buffer, pH 5.0, for 30 min. The cultures were dehydrated in a graded series of ice-cold ethanol-water mixtures and embedded in Epon 812 (30). Thin sections were cut parallel to the plane of the glass (to gain maximum exposure of cytoplasmic detail) with diamond knives and a Porter Blum MT-2 microtome. The sections were examined on unsupported grids in a Siemens Elmiskop I electron microscope.

RESULTS

Light Microscopy

The growth characteristics and morphology of the revertant cell lines, as determined by light microscopy, have been described and compared to those of normal 3T3 and SV-3T3 cell lines (17). Of importance to the ultrastructure is the fact that the revertant cells are large polyhedral cells which generally have a single nucleus but exhibit a remarkable tendency to form both single- and multinucleated giant cells. However, the revertants show contact inhibition of growth in that saturation densities similar to those of normal 3T3 cells are achieved. SV-3T3 cells are smaller and do not spread out over the surface of the cover slip as much as normal or revertant cells. Also, the SV-3T3 cells pile on top of one another, lack orientation relative to one another, and continue to undergo mitosis in hyperconfluent cultures.

Electron Microscopy

For brevity, the results will be organized so that each of the major organelles is described separately. In each organelle category, the appearance of the revertant organelles will be presented and then compared to the corresponding organelles in both normal and transformed cells.

Nucleus

The nucleus of a typical mononucleated revertant cell (Fig. 1) is round to ovoid and is

[1] *Abbreviations used in this paper:* ATP, adenosine triphosphate; MEM × 4, Eagle's minimal essential medium supplemented with a fourfold concentration of vitamins and amino acids, 10% fetal calf serum, and antibiotics; Rer, rough-surfaced endoplasmic reticulum; Ser, smooth endoplasmic reticulum; SV-3T3, simian virus–transformed 3T3 cells; SV40, simian virus 40.

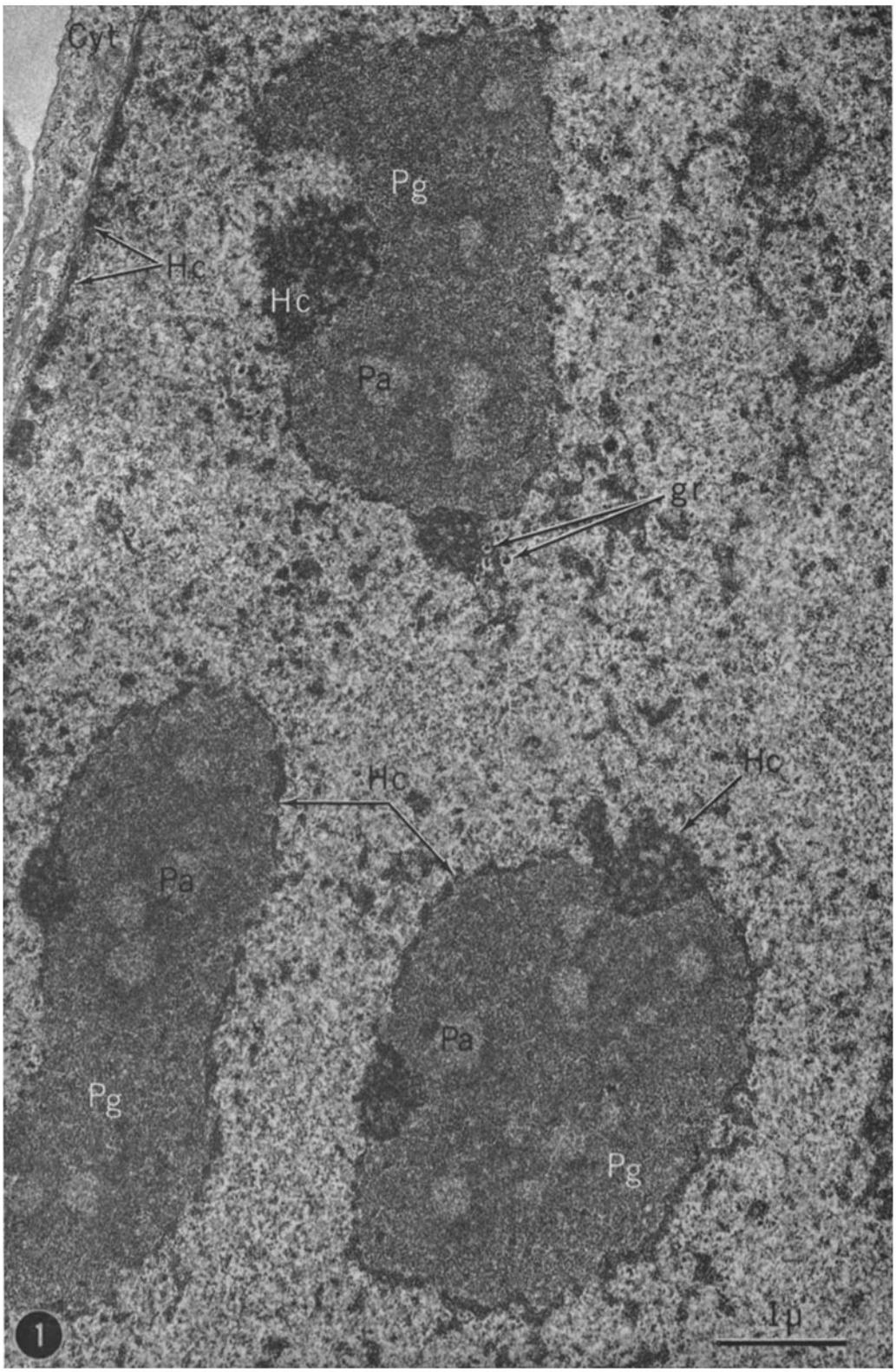


FIGURE 1 Electron micrograph at low magnification of a portion of the nucleus of an F1A revertant cell in confluent culture. The nucleoli are large and have prominent lucent areas, i.e. pars amorpha (*Pa*), and dense granular regions, i.e., pars granulosa (*Pg*). Each nucleolus has a rim of heterochromatin (*Hc*) which is focally thickened. Also, a thin layer of heterochromatin (*Hc*) lines the inner membrane of the nuclear envelope at upper left. Deep indentations of the nuclear membranes are infrequently seen in the typical revertant cells in confluent culture. Small perichromatin granules (*gr*) are present. A small rim of cytoplasm (*Cyt*) is present at upper left. × 20,000.

enclosed by a nuclear envelope consisting of a perinuclear cisterna delimited by inner and outer nuclear membranes. The nuclear envelope generally has a smooth outline, free of deep indentations in confluent cultures. Occasional revertant cells are exceptions to this general pattern and have a nuclear envelope in which both membranes are deeply infolded into the nucleoplasm. Usually, a thin layer of heterochromatin lines most of the inner membrane of the nuclear envelope of typical revertant cells.

There are often 5–10 nucleoli in typical revertant cells (Fig. 1). Giant nuclei may contain large pleomorphic nucleoli and many small nucleoli, occasionally achieving as many as 15–35 nucleoli per cell. The individual nucleoli in typical revertant cells are usually large with prominent pars amorpha and pars granulosa components (Fig. 1). The nucleoli are often completely surrounded by a layer of heterochromatin which varies in thickness. Dense perichromatin bodies, 600–1000 A in diameter, are frequently observed, and are similar to those described in other cells by Watson (56) and Bernhard and Granboulan (10). No viral inclusion bodies are identified.

Nuclei in normal 3T3 cells exhibit much less variation of morphology than those in the revertant cells although a few giant cells with single nuclei can be found in confluent normal 3T3 cultures. The normal 3T3 nuclei are round to ovoid, have a smooth outline in confluent cultures, but may exhibit deep indentations of the nuclear envelope in subconfluent cultures. Normal 3T3 nuclei are usually slightly smaller than typical revertant nuclei, and the nucleoli are often both fewer and smaller. Occasional large nucleoli morphologically similar to revertant nucleoli (Fig. 1) can be found in a few of the normal 3T3 nuclei.

Transformed SV-3T3 nuclei appear smaller than revertant nuclei and even slightly smaller than normal 3T3 nuclei. Deep infoldings of both membranes of the nuclear envelope into the nucleoplasm are observed more frequently in confluent SV-3T3 cultures than in confluent normal 3T3 or revertant cultures. There generally are no more nucleoli in SV-3T3 cells than in normal 3T3 cells. However, individual SV-3T3 nucleoli are often large and morphologically can resemble the nucleoli of revertant cells (Fig. 1). Perichromatin bodies may be found in both normal 3T3 and SV-3T3 cells and are similar in size to those found in revertant 3T3 cells.

Endoplasmic Reticulum

Revertant cells contain both cisternae of ribosome-studded or "rough-surfaced" endoplasmic reticulum (Rer) and tubules of "smooth" endoplasmic reticulum (Ser) (Fig. 2). The Rer and Ser are in continuity with each other (Fig. 2), a feature also noted in other cells (18, 27, 36).

The Rer cisternae of the revertant cells are focally dilated with a flocculent material which is not present in the lumen of the Ser in confluent cultures. The pattern of ribosomes on the Rer membranes tends to be orderly with few areas free of ribosomes except where continuity with the Ser is established or probable. The endoplasmic reticulum of the normal 3T3 cells closely resembles that of the revertant cells in confluent cultures. In SV-3T3 cells, the endoplasmic reticulum appears to be organized less frequently into a delicate branching and anastomosing system of cisternae and tubules. Also, the cisternae are dilated less frequently with flocculent material; however, occasional cells within the SV-3T3 cultures contain abundant cisternae dilated with granular material. On these dilated cisternae, small regions of the Rer membranes appear frequently to be free of ribosomes, but do not appear to connect rough to smooth endoplasmic reticulum.

Cell Periphery

The periphery of revertant cells is a specialized region, approximately 0.1–0.2 μ in thickness, which closely resembles the periphery of normal 3T3 cells in confluent cultures. Therefore the peripheries of both normal 3T3 and revertant cells will be described together for confluent cultures; comparisons will then be made to the transformed SV-3T3 cell periphery.

The components of the cell periphery of both normal 3T3 and revertant cells are the "unit" trilaminar plasma membrane, a specialized layer of subjacent cytoplasm called ectoplasm (Figs. 3, 4, 5, 6, 8), and a surface coat material which is preserved here only as focal plaques covering a small percentage of the cell surface (Figs. 10, 11, 12). The trilaminar plasma membrane is 75–90 A in thickness, is invaginated to form micropinocytotic vesicles, and protrudes to form small cyto-

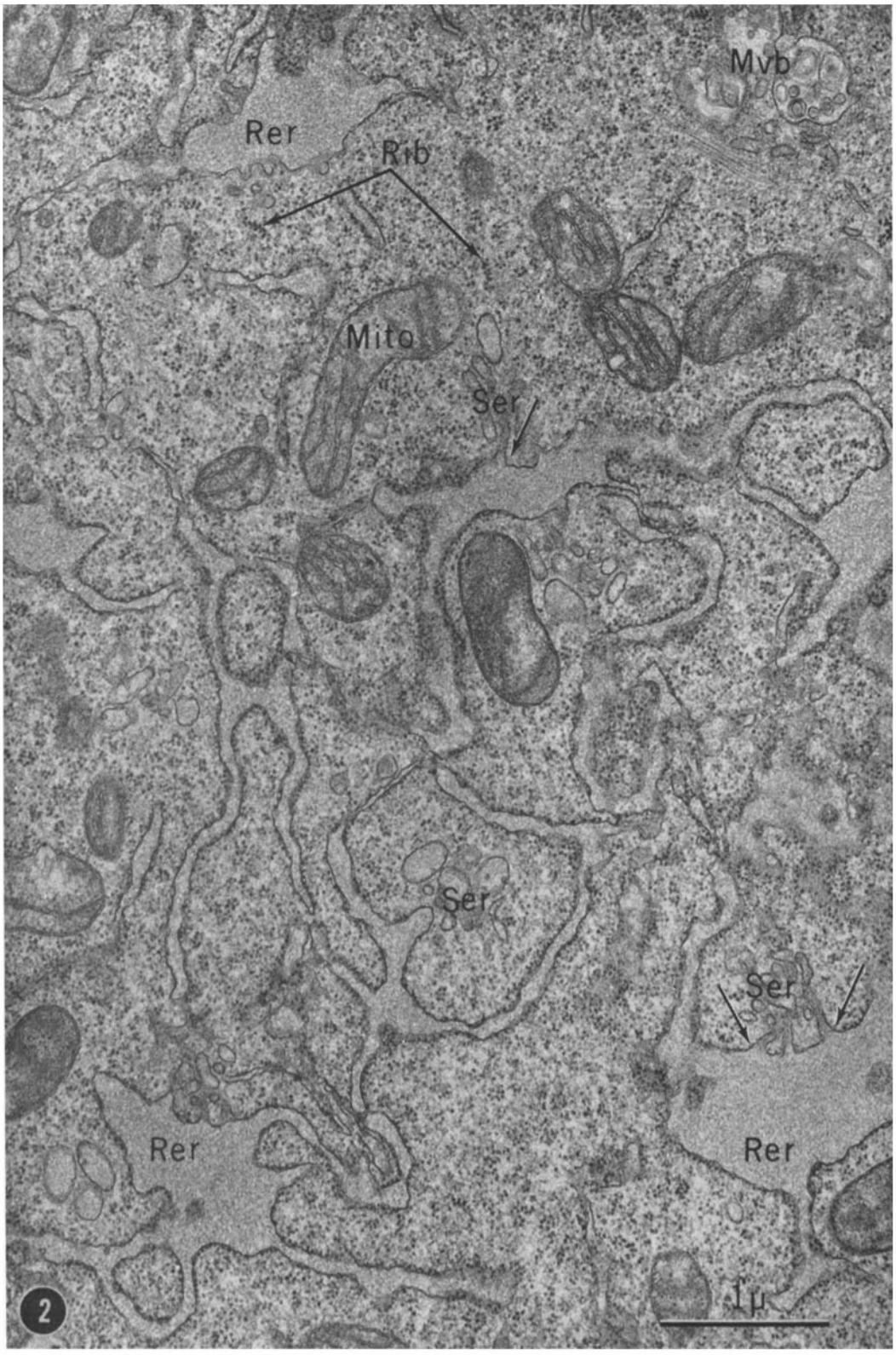


FIGURE 2 A portion of cytoplasm of an F1A revertant cell in confluent culture. The cytoplasm contains mitochondria (*Mito*) and an interlacing network of cisternae and tubules of endoplasmic reticulum. The cisternae of ribosome-studded endoplasmic reticulum (*Rer*) are focally dilated with finely granular material. A few tubules of smooth endoplasmic reticulum (*Ser*), lacking ribosomes, are seen occasionally in continuity with Rer cisternae (at arrows). Free ribosomes (*Rib*) are abundant. A multivesicular body (*Mvb*) is visible at upper right. × 26,000.

plasmic extensions and long finger-like microvilli or filopodia.

Ectoplasm of both normal 3T3 and revertant cells contains a high concentration of thin filaments and microtubules as well as a few ribosomes, tubules of smooth endoplasmic reticulum, and small vesicles. This ectoplasm is not sharply demarcated from the remainder of the cytoplasm (endoplasm) since its concentrated population of filaments and microtubules is continuous with a less concentrated population of filaments and microtubules lying in the endoplasm. In contrast, the tubules of Ser form a branching and anastomosing network throughout the cytoplasm but are in low concentration in the ectoplasm. Mitochondria, lysosomes, and cisternae of Rer are excluded from the ectoplasm. Occasionally, a few flattened tubules of Rer can be found within the ectoplasm. The ectoplasm fills the filopodia in which the filaments are oriented parallel to the long axes of the filopodia.

Ectoplasm of both normal and revertant cells has a prominent system of filaments which consists of two types of filaments, distinguished on the basis of diameter, state of aggregation, and association with the plasma membrane (Figs. 3, 4). One of the types of filaments is sometimes called "microfilaments" (49) but in this presentation will be called *alpha filaments*, which are 60–80 A in diameter (Fig. 3). These filaments are aggregated into felt-like meshworks in which the filaments often are approximately parallel. There are focal regions of dense staining in these meshworks. The length of individual alpha filaments is not evident since the filaments appear closely associated and occasionally appear to branch and aggregate side-to-side; however, such a feature could be produced by superimposition of filament images within the section thickness. The alpha filaments attach to the cytoplasmic surface of the plasma membrane in small dense regions which exclude pinocytotic vesicles (Fig. 5). Alpha filaments also form long slender bundles that course through the interior of the cytoplasm in a manner similar to what has been described for "stress fibers" (12). These alpha *filaments* resemble 70 A filaments described in a number of cell types in which they are believed to have a contractile function (26, 40).

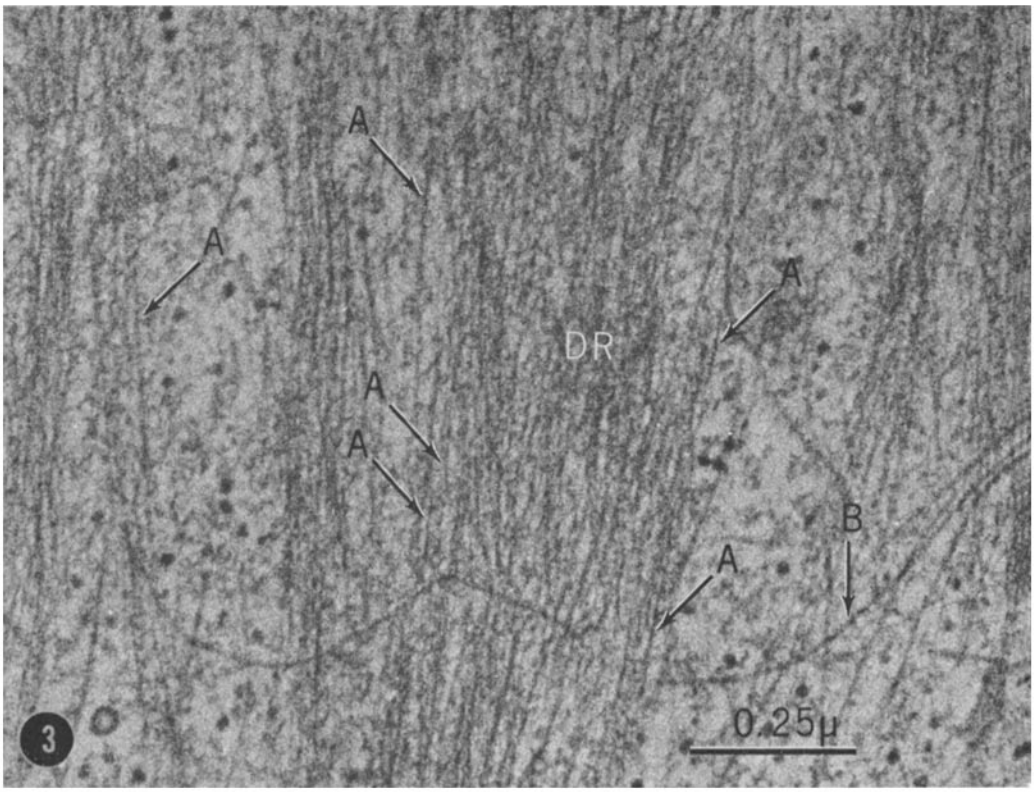
The other type of filaments will be called *beta filaments* (Fig. 4), which are 100 A in diameter and tend to run in loose fascicles. The beta filaments often course parallel to each other and can be followed for long distances in the cytoplasm since they are discrete and rarely branch or attach to each other. Occasionally, they run at a variety of angles relative to each other and form looping configurations. Beta filaments occur throughout the endoplasm and extend into the ectoplasm. They frequently surround organelles such as the smooth endoplasmic reticulum and are particularly prominent near the Golgi complex.

Microtubules, 250 A in diameter, are prominent in the ectoplasm of both normal 3T3 and revertant cells and are often just subjacent to regions where the alpha filaments are in high concentration. Microtubules are also present throughout the cytoplasm and are often concentrated near the Golgi complex.

Comparison of the filament distribution in the three cell types is facilitated by cutting sections parallel to the plasma membrane which, within a limited region, represent grazing sections through the ectoplasm. The population of alpha filaments appears similar in revertant cells (Fig. 5) compared to normal 3T3 cells in confluent cultures (Fig. 6). In both normal and revertant cells, alpha fila-

FIGURE 3 Alpha filaments (*A*) seen at high magnification of a thin section through the ectoplasm of a normal 3T3 cell in confluent culture. These 60–80 A filaments form a closely meshed network in the ectoplasm in which the individual filaments run roughly parallel but cannot usually be followed for long distances since they are either superimposed on one another or branch and aggregate side-to-side. Focal regions of dense staining are present where the alpha filaments attach to each other (*DR*). A few beta filaments (*B*) are visible but are slightly thicker, 90–110 A in diameter, have less tendency to aggregate, and can be followed for longer distances in the cytoplasm than alpha filaments. × 84,000.

FIGURE 4 Beta filaments (*B*) are prominent in this section which passes through the endoplasm of a normal 3T3 cell in confluent culture. This electron micrograph is at lower magnification than Fig. 3. The beta filaments (90–110 A in diameter) form loose fascicles and looping configurations. Tubules and vesicles of smooth endoplasmic reticulum (*Ser*) often are surrounded by beta filament fascicles. The chemical compositions of alpha and beta filaments are unknown. × 65,000.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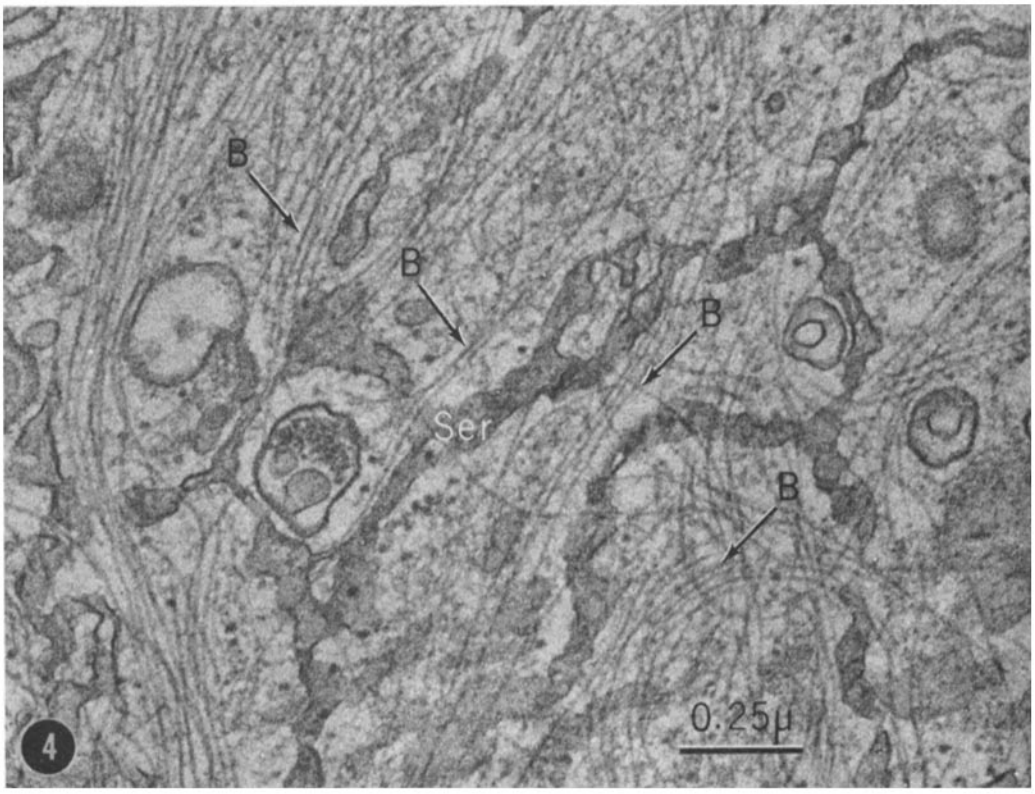
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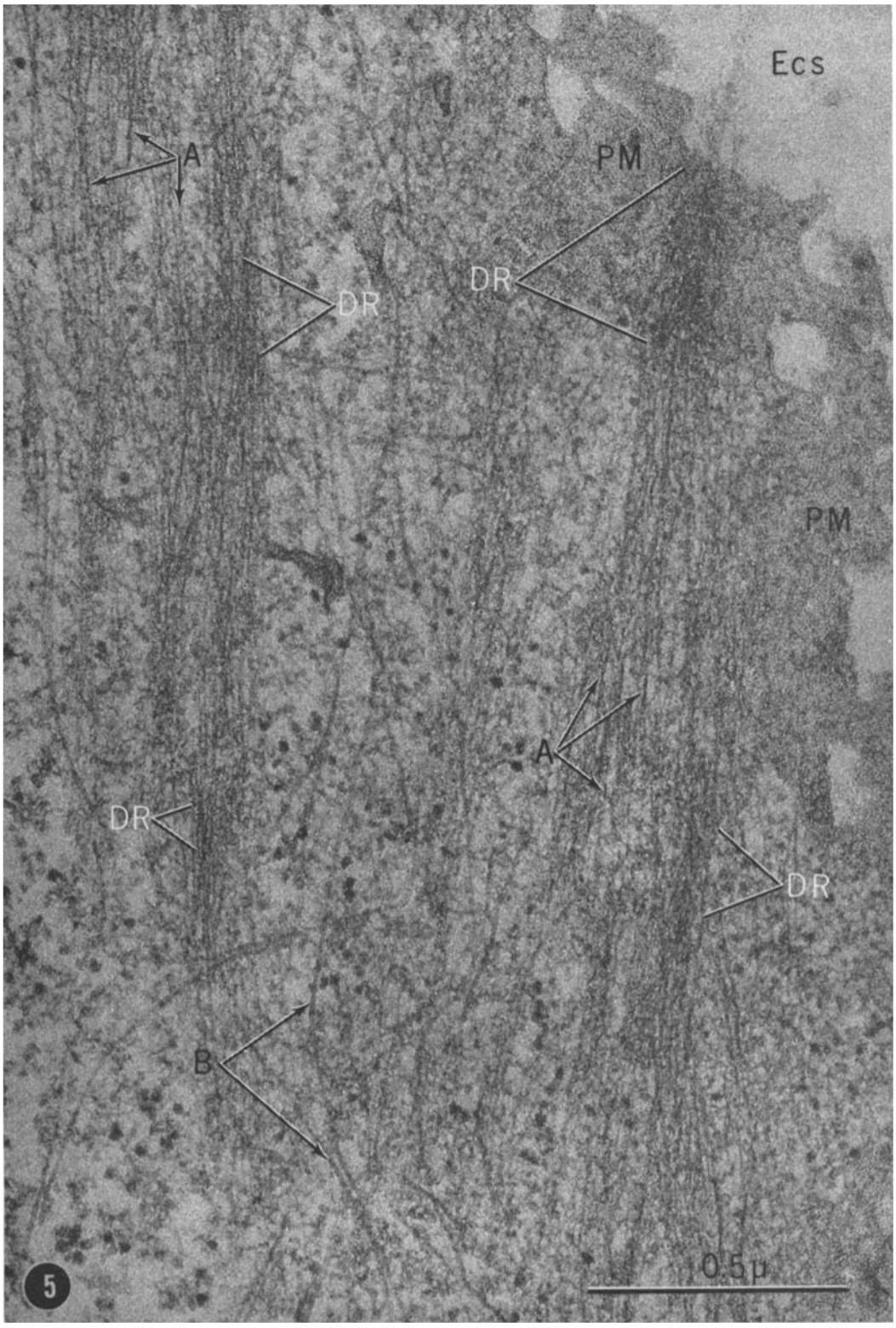


FIGURE 5 High magnification of an oblique section through the ectoplasm of a revertant cell. Groups of alpha filaments (*A*) are present and can attach to the plasma membrane (*PM*) in small dense regions (*DR*). Dense regions are also present in the bundles of alpha filaments at a distance from the plasma membrane. Since the plasma membrane is viewed obliquely, it is seen as a gray region at the interface between the cell and the extracellular space (*Ecs*). × 80,000.

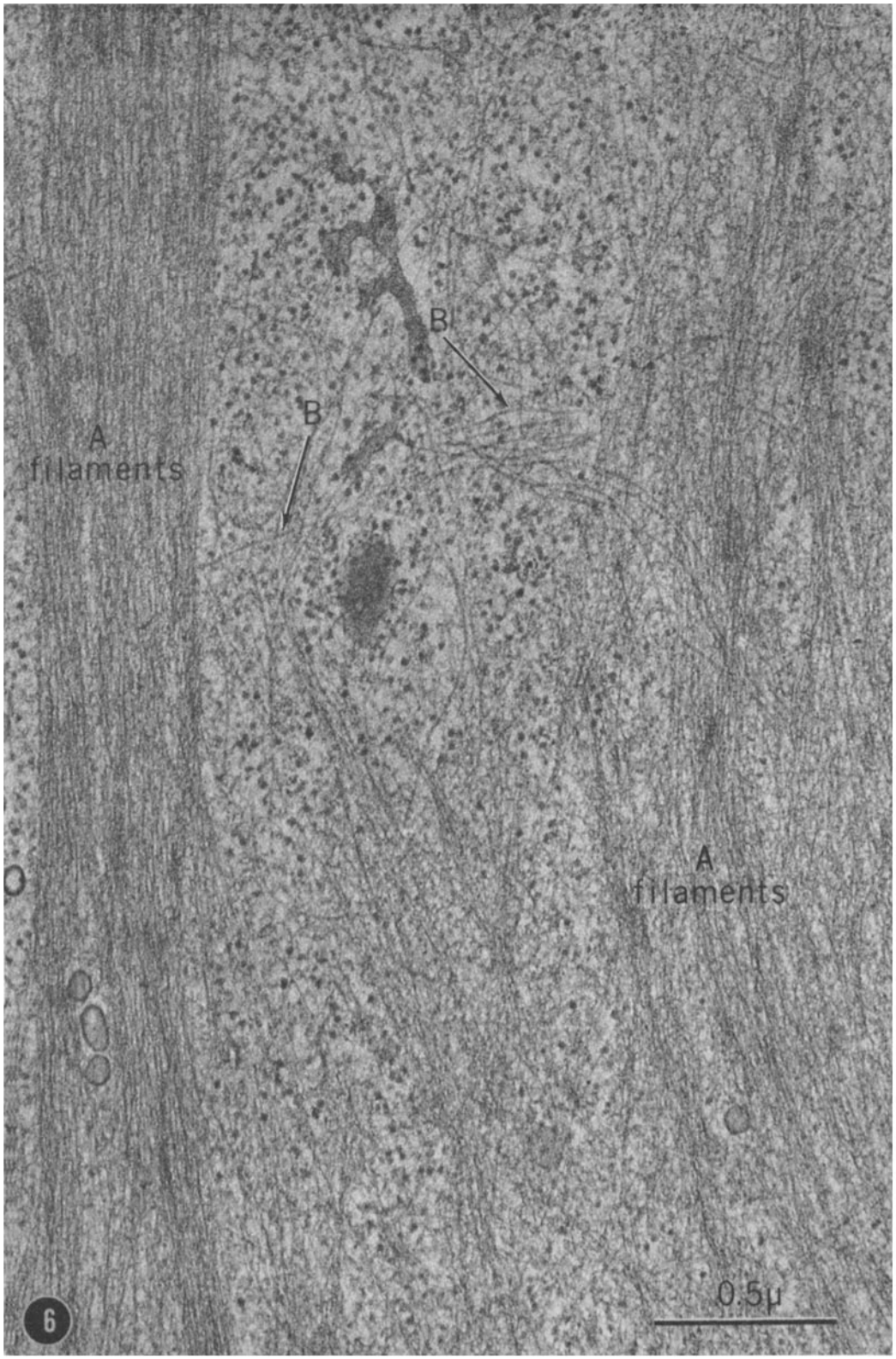


FIGURE 6 Electron micrograph of a grazing section through the ectoplasm of a normal 3T3 cell showing the concentration of alpha filaments (*A filaments*) for comparison to those in Fig. 7. The alpha filaments may be aggregated into broad sheets (at right) or into dense bundles (at left), which may also course deep in the cytoplasm. A few beta (*B*) filaments are also visible. × 56,000.

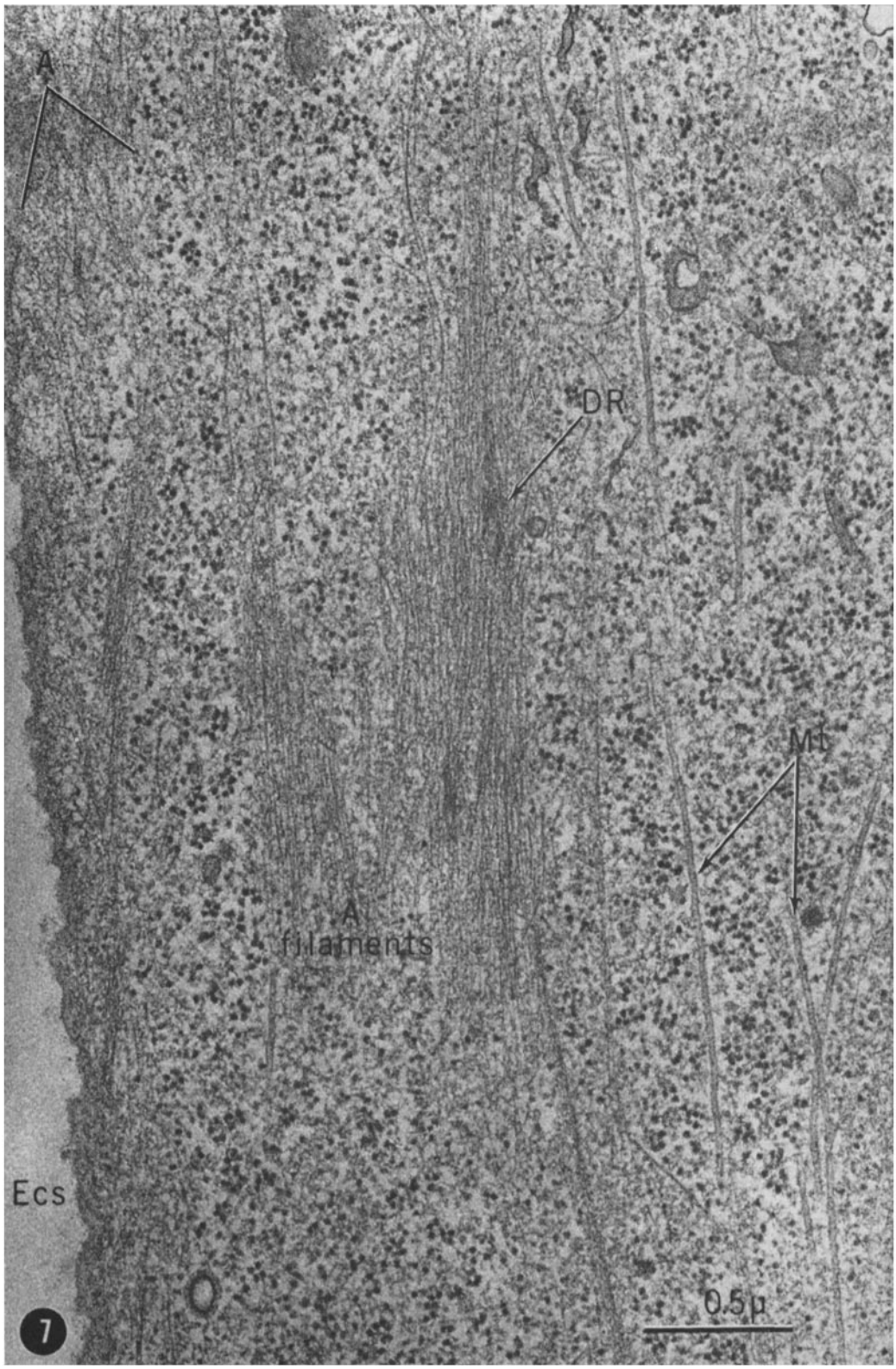


FIGURE 7 Electron micrograph of a grazing section through the ectoplasm of a SV-3T3 cell in a region which demonstrates alpha filaments (*A*) which are aggregated into bundles. In general, strikingly fewer alpha filament bundles are present in SV-3T3 cells than in normal 3T3 cells at confluence. Focal dense regions (*DR*) are stained in the mass of alpha filaments. Microtubules (*Mt*), 250 A in diameter, are prominent in this section. × 46,000.

ments are less prominent in the ectoplasm of cells in subconfluent cultures than in the ectoplasm of cells in confluent cultures. Alpha filaments have a similar distribution in cells in subconfluent and confluent culture in that they are present in filopodia, are in long bundles adjacent to the plasma membrane along a relatively straight free border of the cell, are concentrated at regions of cell-to-cell contact (Fig. 8), and may be near cell-to-substratum contacts.

During mitosis, the alpha filaments in normal and revertant cells are organized into a layer which, in sections, appears as a loose filamentous meshwork in the ectoplasm that conforms to the spherical shape of the cell. In this filamentous meshwork the alpha filaments are not parallel but are interwoven with each other. The alpha filaments are also present in the filopodia but not in the small cytoplasmic blebs at the surface of the mitotic revertant cell.

SV-3T3 cells have a relatively poorly developed system of ectoplasmic filaments (Figs. 7 and 9). Although alpha filaments can be found in the cytoplasm, they are not as frequently concentrated into dense meshworks in the ectoplasm as in normal 3T3 or F1A revertant cells in confluent cultures. Frequently, regions of ectoplasm appear free of aggregates of alpha filaments and beta filaments in SV-3T3 cells (Figs. 9 and 13). Such regions are infrequently observed in normal cells in confluent culture (Fig. 11) but are commonly found in subconfluent normal cultures.

Beta filaments are usually prominent within the endoplasm and also are more abundant in contact-inhibited cells than growing cells. They do not appear to converge or be concentrated in regions of individual cell-to-cell contact in the cells described in this paper.

On the surface of the revertant cells is a focal coating of loose filamentous material (Fig. 12) which occupies only a small amount of the total cell surface in the type of specimen preparation used in this study. Such focal collections of material presumably represent part of the protein-polysaccharide layer coating a wide variety of cell surfaces (8, 42) and commonly referred to as glycocalyx. These focal plaques of filamentous surface coat form two layers: One layer is 400–500 A in thickness, and is formed by a condensation of fine filaments. This layer is separated from the trilaminar plasma membrane proper by the second layer which is approximately 200 A in

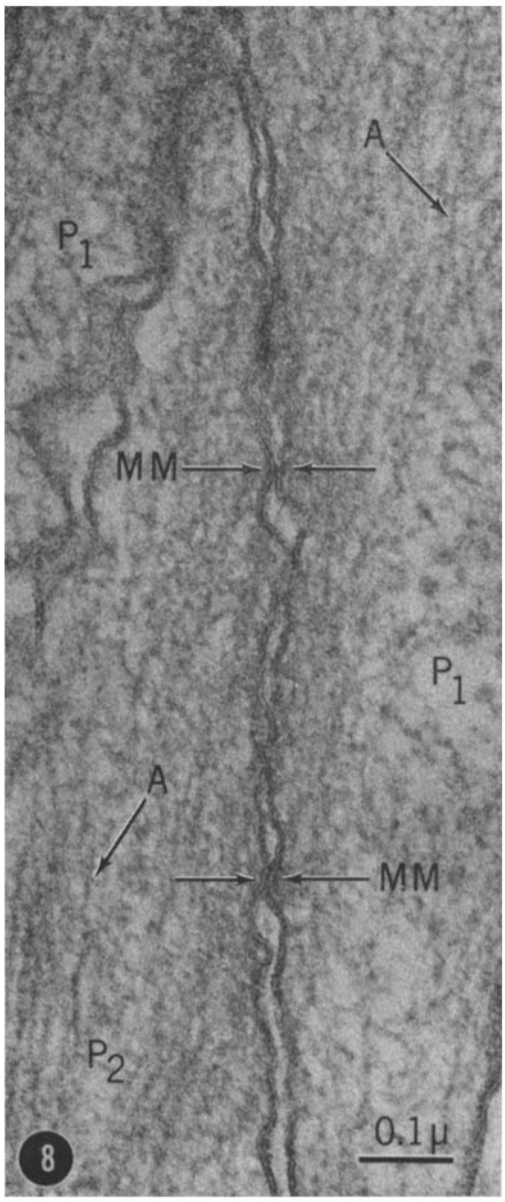


FIGURE 8 High magnification micrograph of a section through the region of contact between the processes (P_1 and P_2) of two normal 3T3 cells in confluent culture, stained *en bloc* with uranyl acetate and on sections with lead citrate. The trilaminar plasma membrane is visible in this preparation. Cell junctions formed by membrane-to-membrane contact (*MM*) are infrequently found in all of the 3T3 cell types studied and will require the use of special techniques for exact characterization. The ectoplasm near these regions of contact contains abundant alpha filaments (*A*). × 120,000.

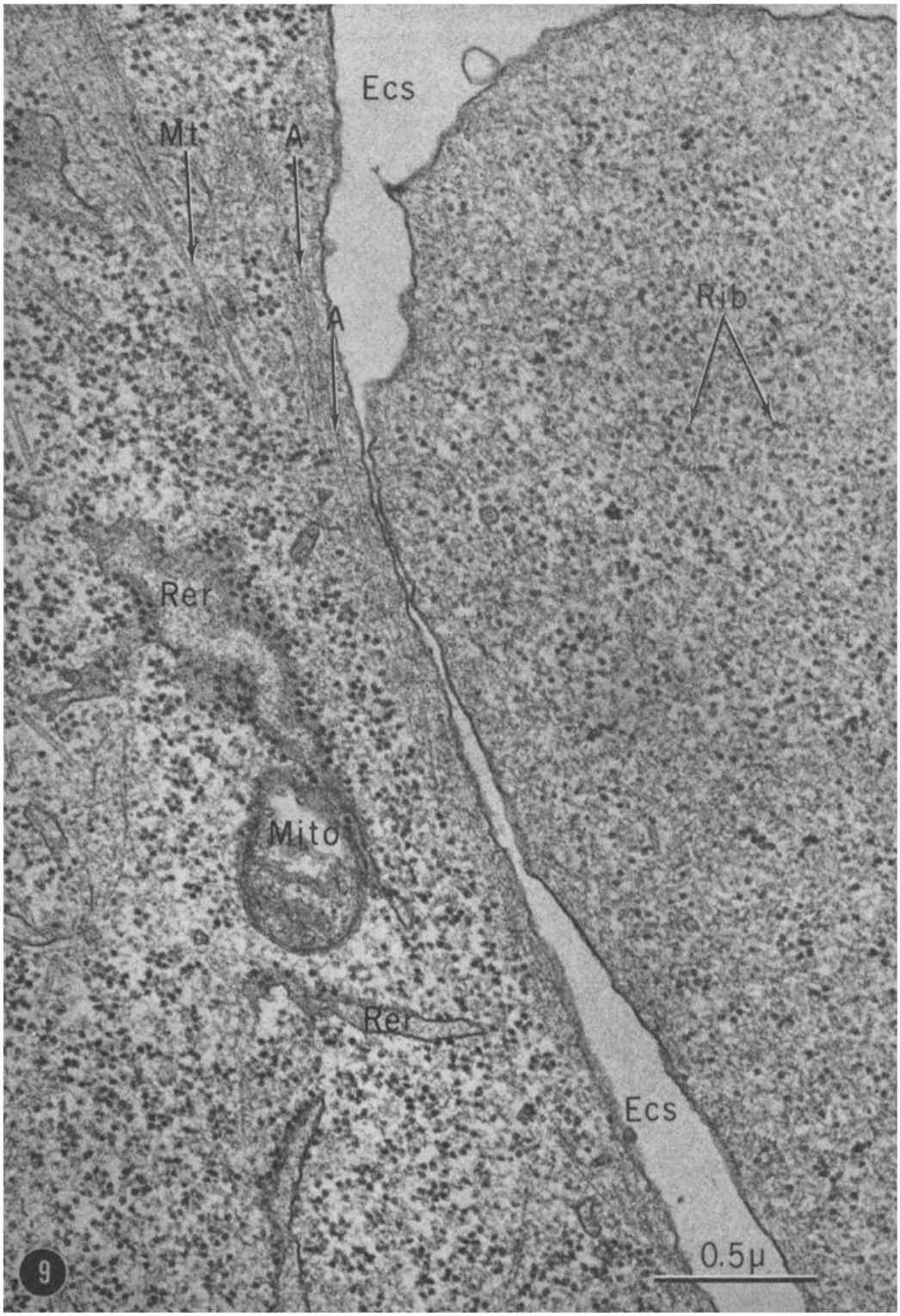


FIGURE 9 Intermediate magnification of a region of contact between two SV-3T3 cells in confluent culture. Only a few alpha filaments (*A*) are present in the ectoplasm near the region of contact between the cells. The plasma membranes are closely approximated but are separated by a 50–100 A interspace continuous with the extracellular space (*Ecs*). Free ribosomes (*Rib*), tubules of rough endoplasmic reticulum (*Rer*) and a mitochondrion (*Mito*) are present. *Mt*, microtubules. × 50,000.

thickness and is electron lucent. The layered appearance of the surface coat is reminiscent of the lamina densa and lamina lucida of the basal lamina of many epithelial cells (21) and the external lamina of muscle cells (21). Normal 3T3 cells (Figs. 10 and 11) have more plaques of filamentous surface coat than revertant cells in confluent cultures. The surface plaques frequently cover the membrane where bands of alpha filaments appear to attach to the cytoplasmic surface of the membrane thereby resembling half-formed cell junctions of the adherens (26) type (McNutt, et al. in preparation). No plaques of surface coat are identified on the surface of SV-3T3 cells (Fig. 13).

Extracellular Space

The extracellular space is represented by that material which adheres to the surface of the cover slip and cells during initial fixation in a relatively large quantity of dilute aldehyde solution (Fig. 14). In the revertant cultures, there are only traces of amorphous material on the cover slip. Normal 3T3 cells in subconfluent culture also are associated with very little of this fibrillar material. Confluent cultures of normal 3T3 cells, which have been confluent for approximately 1 day, have abundant strands of fibrillar material (Fig. 14); these strands lack periodicity and resemble fibrillar material in cultures of 3T6 cells (a mouse fibroblast cell line) producing hydroxyproline-containing protein, as described by Goldberg and Green (24). This fibrillar material occasionally appears attached to the focal plaques of surface coat material. No such fibrillar material is visualized in SV-3T3 cultures.[2]

Cell-to-Cell Junctions

The plasma membranes of adjacent revertant cells in confluent cultures frequently are closely apposed but separated by a 200 A interspace with little apparent specialization. In addition, cell borders which are not closely apposed can be observed. Ectoplasmic alpha filaments are abundant near the regions of cell-to-cell contact in subconfluent and confluent normal 3T3 and revertant cells (Fig. 8). Very few subjacent alpha filaments are present at regions of cell-to-cell contact in SV-3T3 cultures (Fig. 9). Occasionally, in SV-3T3 cells, alpha filaments are aggregated at regions of cell-to-cell apposition but this is observed much less frequently than in normal or revertant cells. Specialized intercellular junctions are encountered infrequently and appear to involve a small percentage of the total cell surface. The exact characterization and comparison of the few membrane-to-membrane contacts and very close appositions which are observed in the normal 3T3 (Fig. 8), revertant, and SV-3T3 cultures requires use of special techniques (37). This will be the subject of a separate paper (McNutt et al., in preparation).

Mitochondria, Golgi Complex, Lysosomes, and Coated Vesicles

In confluent cultures of the three cell lines studied, there were few striking or consistent differences noted in these organelles, although no special study was performed.

DISCUSSION

This study provides evidence that two morphologic features of the three cell lines studied appear to be related to the growth conditions of the culture and may be important factors in the regulation of contact inhibition of movement and growth. First, under the conditions of culture, deep indentations of the nuclear envelope are present in 3T3 cells lacking contact inhibition of growth, i.e., normal 3T3 cells in subconfluent culture and SV-3T3 cells in confluent culture. In contrast, indentations of the nuclear envelope are relatively rare in contact-inhibited cultures, i.e., confluent cultures of either normal 3T3 cells or the typical revertant cells. These findings indicate that, within this system, marked indentation of the nuclear envelope correlates with an active state of growth in culture rather than the transformed state per se. Deep indentations of the nuclear envelope (6, 7, 9, 10, 11) have been described in malignant tumors in vivo and occasionally have been considered characteristic of certain types of malignant cells (11). The results of the present study suggest that nuclear indentations in cells in vivo should be interpreted with caution since they may be a reflection of active growth or possibly contraction in some cells (33). However, the presence of a morphological change in the configuration of the

[2] Surface replica experiments, similar to those of R. D. Goldman and E. A. C. Follett (1969. *Exp. Cell Res.* **57**:263), show that a small amount of fibrillar surface coat material is produced by SV-3T3 cells in hyperconfluent culture but this amount is much less than is produced by either normal or revertant cells.

nuclear membranes associated with normal 3T3 growth is of interest because of the known relationship of DNA synthesis with membrane in some cells (15, 29, 46). Nuclear indentations often appear to contact the nucleolus, an observation also made by Bernhard and Granboulan (10). The specific relationship of nuclear indentations and either DNA or RNA synthesis in the cell lines studied is not known; however, the observed correlation of indentations with growth state does suggest that some such relationship may exist.

The second morphologic feature correlated with the growth state of the culture is the presence of abundant 70 A alpha filaments in the ectoplasm of both normal 3T3 and revertant cells in confluent cultures. A special emphasis is placed on alpha filaments since they are abundant near regions of individual cell-to-cell contact in normal 3T3 subconfluent cultures. The impression in the present study that alpha filaments are related to contact inhibition is based on the observation that cells from cultures exhibiting contact inhibition often have a dense concentration of alpha filaments in bundles in the ectoplasm, whereas either cells from growing cultures or cells lacking contact inhibition have fewer bundles of alpha filaments. In cells from cultures lacking contact inhibition, the few bundles of alpha filaments which are present vary in location but may be near regions of individual cell-to-cell contact or in the ectoplasm distant from such contacts. Comings and Okada (16) similarly noted that confluent fibroblastic cells contain more cytoplasmic filaments than subconfluent cells when trypsinized, pelleted cells are compared. However, they did not distinguish between 70 A (alpha) and 100 A (beta) filaments as subpopulations in the complex array of filaments seen in the cytoplasm of cultured cells.

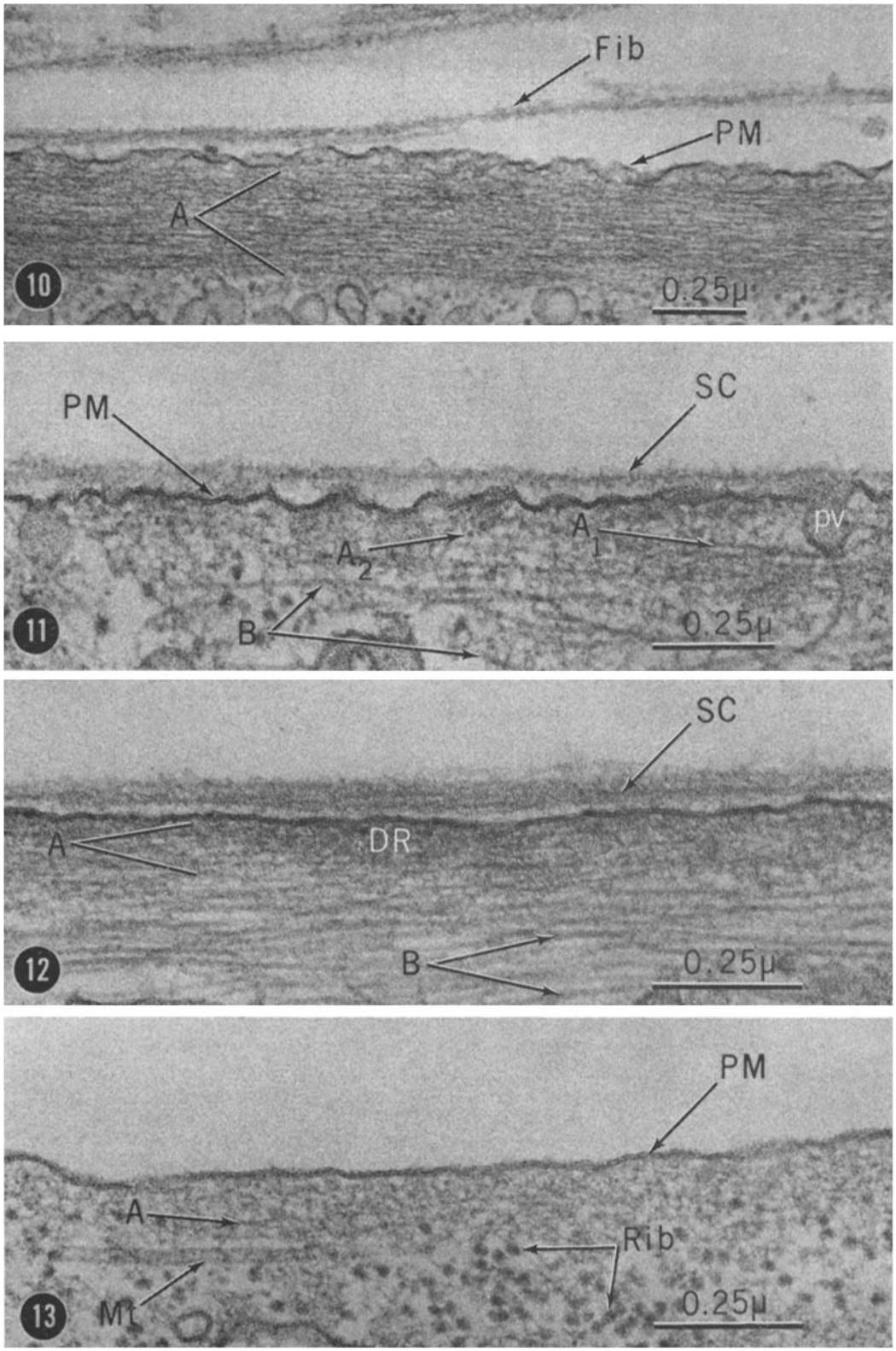
It is possible that the abundance and state of aggregation of the alpha filaments may be important factors in regulating contact inhibition of movement (1, 2, 3, 4, 5) and, possibly, of growth as well (51, 52). In this study the correlation between the presence of alpha filaments and contact inhibition of growth may actually be a reflection of the high correlation of contact inhibition of growth in confluent cultures with the contact inhibition of movement in the individual cells (1, 14, 51). Several functions have been proposed for the small 50–80 A filament population observed frequently in cultured cells. Functions which might be important for contact inhibition of movement, as described by Abercrombie and Ambrose (2, 3), would include the participation of 70 A filaments in the control of cytoplasmic viscosity (12, 40), in a possible contractile mechanism (26, 39, 40, 41), and their participation in cell-to-cell junctions (26). During contact inhibition of

FIGURE 10 Intermediate magnification of the periphery of a normal 3T3 cell in confluent culture showing a bundle of alpha filaments (*A*) in the ectoplasm. Pinocytotic vesicles are absent in such a region of plasma membrane (*PM*). The extracellular space contains loose fibrillar (*Fib*) material which adheres to the cell surface at left. × 58,000.

FIGURE 11 High magnification of the periphery of normal 3T3 cell. There are focal regions where alpha filaments are decreased in number and can be found in both oblique (A_1) and cross-section (A_2). These regions are seen more frequently in subconfluent than confluent cultures. Also, focal plaques of surface coat material (*SC*) are present on normal cell surfaces in confluent and subconfluent cultures. This material is often separated from the plasma membrane (*PM*) by an electron-lucent zone. The surface coat material resembles the fibrillar strands in the extracellular space (see Figs. 11 and 14). A portion of a pinocytotic vesicle (*pv*) is at right. *B*, beta filaments. × 77,000.

FIGURE 12 High magnification of a small area of cell periphery of a revertant cell in confluent culture. Alpha filaments (*A*) may be abundant in the ectoplasm but are usually less prominent than in normal 3T3 cells. A dense region (*DR*) where alpha filaments appear to attach to the plasma membrane is also visible. Focal plaques of surface coat material (*SC*) can be found but they are seen less frequently than on normal 3T3 cells. *B*, beta filaments. × 92,000.

FIGURE 13 High magnification of a typical region of SV-3T3 cell periphery. The plasma membrane (*PM*) has a subjacent web of material in which a few alpha filaments (*A*) are recognizable. A few microtubules (*Mt*) are present. Free ribosomes (*Rib*) are abundant. Focal plaques of surface coat have not been observed. × 91,000.


Fib
PM
A
10
0.25μ
PM
SC
A1
pv
A2
B
11
0.25μ
SC
A
DR
12
B
0.25μ
PM
A
Rib
13
Mt
0.25μ

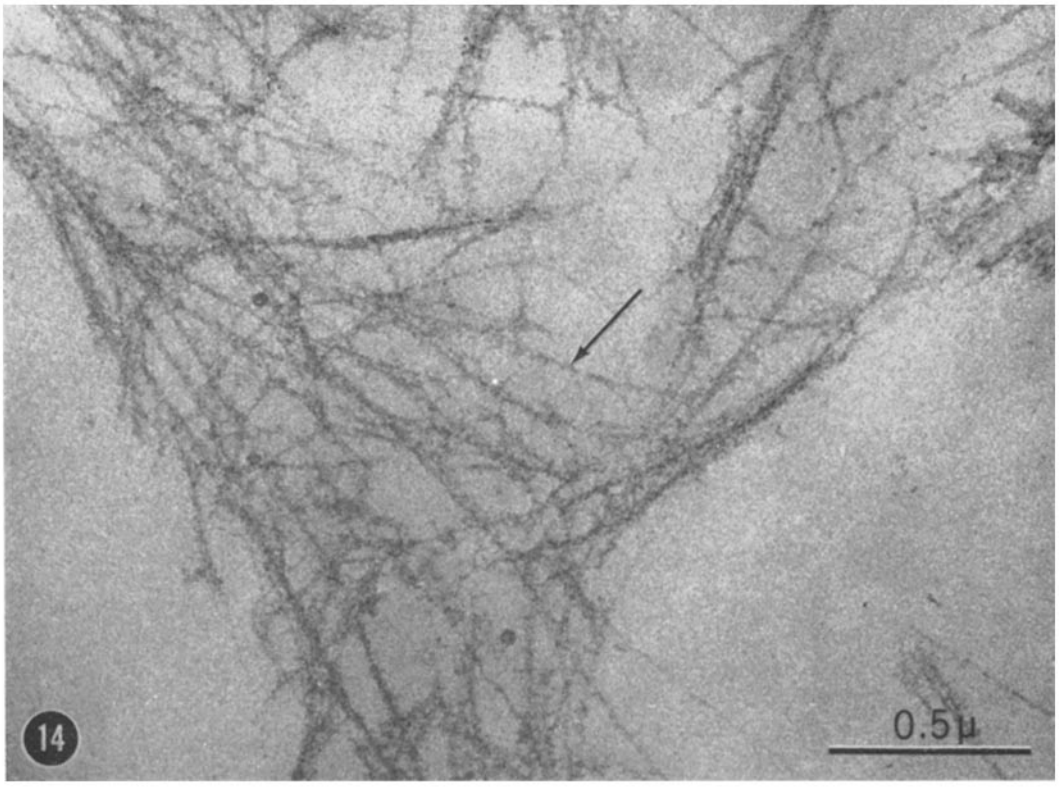


FIGURE 14 Fibrillar material in the extracellular space of a normal 3T3 confluent culture. Such material (for example, at arrow) is seen frequently; the amount appears to correlate with hydroxyproline-containing protein production of the culture. × 52,000.

movement, a change in the state of the 70 A filaments may be secondary to a cell-to-cell "recognition" step, which might involve surface charges from a molecule such as sialic acid (17) and/or membrane-to-membrane junctions (22, 35). After the recognition step, polymerization of alpha filaments parallel to the plasma membrane would tend to increase the local cytoplasmic viscosity because of the elongate shape and condensation of the protein molecules. Polymerization of similar 70 A filaments can occur in amorphous cytoplasmic extracts from *Amoeba proteus* upon the addition of adenosine triphosphate (ATP) (40). If the alpha filaments then participate in a general cytoplasmic contractile mechanism, they might generate tension and influence the cell to extend a pseudopod from another, less viscous location on the cell periphery. The regulation of cell mobility is probably quite complex but such an interplay of filaments might participate in the regulation of the direction of movement of the cell. A contractile function for alpha filaments in contact inhibition is suggested by the work of Ishikawa et al. (26) and others (39, 41), who found that 70 A cytoplasmic filaments in certain nonmuscle cells bind heavy meromyosin to form arrowhead complexes. This reaction indicates a relationship of these 70 A filaments in nonmuscular cells to the F-actin found in muscle cells (c.f. 50).

Bundles of alpha filaments do not specifically occur only in association with contact inhibition of movement since they have been shown to be present in cells lacking contact inhibition, e.g., macrophages (20), human ascites tumor cells (23), and SV-3T3 cells in this study. In the present study, emphasis is placed on the observation of a decreased concentration of bundles of alpha filaments in cells lacking contact inhibition when compared to cells exhibiting contact inhibition. These observations suggest a role for 70 A alpha filaments in a general mechanism for cytoplasmic viscosity and motility control. Unfortunately,

biochemical and morphological techniques do not yet allow an accurate quantitative comparison of the filament population of these cell types. The organization of the alpha filaments might also be related to the degree of spreading of the cells on the substrate since both normal and revertant cells are more spread than transformed cells. Such a relation of alpha filaments to the shape of the cell does not preclude a relationship to cytoplasmic viscosity and motility as well. Another important consideration is the fact that the filament system found in the cytoplasm of cultured 3T3 cells is not prominent in the relatively quiescent fibroblasts of adult tissues in vivo. In contrast, a prominent filament system can be seen in fibroblasts in fetal tissues (44, 45). Filaments, 50–80 A in diameter, are present in motile filopodia of early embryonic tissues undergoing morphogenetic movements, and may be aggregated near regions of cell-to-cell contact (53). Recently, Majno et al. have found that, at the time of wound closure, fibroblasts contain a prominent filament system which might play a role in wound contraction (33). Thus, this filamentous viscosity-motility system which is prominent in actively growing fibroblasts in vivo normally may be repressed in quiescent adult tissues. The abundance of alpha filaments in 3T3 cells cultured in vitro might represent an accentuation of the filament system as a result of the conditions of cell culture. However, the observed correlation between contact inhibition and presence of bundles of alpha filaments does suggest that such filaments could be a common basis for the changes in cell shape and motility which occur during in vitro transformation as studied in cultured cells.

The functional role of beta filaments in these cells is unknown. An increase in number of the 100 A beta filaments associated with contact inhibition has been observed in this study and in others (16). They may have an influence on cytoplasmic viscosity (12). Some workers have reported (25) that, in the cytoplasm of some cultured cells, the distribution of filaments, similar to beta filaments, correlates with the degree of spreading of these cells on the substrate. It is not certain that beta filaments are involved in contact inhibition of movement since they do not appear to be concentrated at regions of individual cell-to-cell contact in the 3T3 cells. Also, beta filaments may not participate in cytoplasmic contractility since the 100 A filaments of nonmuscle cells do not bind heavy meromyosin (26) and thus are not similar to F-actin.

Hydroxyproline-containing protein, presumably collagen, is detected in whole-culture digests only after the cells reach confluence (17), a fact which correlates with the appearance of the rather amorphous strands of extracellular material in the culture. A similar observation was made by Goldberg and Green (24) on 3T6 cells. The amorphous strands lack periodicity at high magnification and may be similar to basement membrane material which is known to contain collagen-like protein polymerized in a noncrossbanded form (48).

With the techniques utilized in this study, the focal nature of the surface coat material is not easily related to the uniform layer of glycoprotein covering the surface of many cell types (8, 42). The focal plaques of coat material are possibly part of this glycoprotein layer or glycocalyx. Frequently, the glycocalyx is demonstrated only by special techniques such as ruthenium red staining (31). A uniform coating of material causing ruthenium red-osmium deposition has been shown on normal cultured cells (35, 38, 54), and has been found to be of the same thickness on some transformed cells (38, 54) and increased in thickness on other types of transformed cells (35). Since the nature and stoichiometry of the ruthenium red reaction are unknown (32), the use of this reaction for quantitative work must be viewed with caution. In this study the focal plaques of surface coat material are decreased in SV-3T3 cells compared to normal 3T3 or revertant cells. Further work will be necessary to determine whether this focal surface coat material is related to the change in the cell surface architecture which has been observed by others in transformed cells (13, 47).

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