# Immunoelectron Microscopic Studies of the Sites of Cell-Substratum and Cell-Cell Contacts in Cultured Fibroblasts

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ABSTRACT Our object was to obtain information about the molecular structures present at cell-substratum and cell-cell contact sites formed by cultured fibroblasts. We have carried out double immunoelectron-microscopic labeling experiments on ultrathin frozen sections cut through such contact sites to determine the absolute and relative dispositions of the three proteins fibronectin, vinculin, and α-actinin with respect to these sites.

(a) Three types of cell-substratum and cell-cell contact sites familiar from plastic sections could also be discriminated in the frozen sections by morphological criteria alone, i.e., the gap distances between the two surfaces, and the presence of submembranous densities. These types were: (i) focal adhesions (FA); (ii) close contacts (CC); and (iii) extracellular matrix contacts (ECM). This morphological typing of the contact sites allowed us to recognize and assign distinctive immunolabeling patterns for the three proteins to each type of site on the frozen sections.

(b) FA sites were immunolabeled intracellularly for vinculin and α-actinin, with vinculin labeling situated closer to the membrane than α-actinin. Fibronectin was not labeled in the narrow gap between the cell surface and the substratum, or between two cells, at FA sites. Control experiments showed that this could not be ascribed to inaccessibility of the FA narrow gap to the immunolabeling reagents but indicated an absence or severe depletion of fibronectin from these sites.

(c) CC sites were labeled intracellularly for α-actinin but not vinculin and were labeled extracellularly for fibronectin.

(d) ECM sites were characterized by large separations (often >100 nm) between the cell and substratum or between two cells, which were connected by long cables of extracellular matrix components, including fibronectin. In late (24–36 h) cultures, ECM contacts predominated over the other types. ECM sites appeared to be of two kinds, one labeled intracellularly for both α-actinin and vinculin, the other for α-actinin alone.

(e) From these and other results, a coherent but tentative scheme is proposed for the molecular ultrastructure of these contacts sites, and specific functional roles are suggested for fibronectin, vinculin, and α-actinin in cell adhesion and in the linkage of intracellular microfilaments to membranes at the different types of contact sites.

The nature and molecular structure of the sites of contact of fibroblastic cells with one another and with the substrata on which they grow are subjects of much recent interest. Three major types of investigation have been pursued in this area: (*a*) at the biochemical level, extracellular matrix molecules such as fibronectin (for review, see reference 42) and intracellular cytoskeletal molecules such as α-actinin (50, 55) and vinculin (12, 26) have been isolated and implicated in the formation of such contacts; (*b*) at the light microscopic level, interference reflection microscopy has been used to define different sites of contact of cells to substrata (20, 46, 47), and immunofluorescence microscopy to provide information at the resolution of the light microscope about some of the molecular species associated with those sites (4, 8, 19, 23, 26, 34, 45, 57, 69); and

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(*c*) at the electron microscopic level, the ultrastructural morphology of the contact sites has been investigated (2, 6, 11, 37–39, 54), and immunoelectron microscopic studies of the extracellular components of these sites have been initiated (13, 15, 24, 25, 40, 56). While much has been learned from these investigations, there is still considerable uncertainty about the molecular ultrastructure of different types of contact sites and particularly about their transmembrane relationships.

In this paper, we have concentrated on the electron microscopic level of investigation. In particular, we have performed double immunoelectron microscope labeling studies on cross-sections through fibroblastic contact sites, using antibodies directed to fibronectin, vinculin, and α-actinin to label simultaneously and at high resolution both extracellular and intracellular components associated with different types of contact sites. For this purpose techniques developed in our laboratory (15) for the immunolabeling of ultrathin frozen sections of cultured cells were used. These immunolabeling studies combined with morphological observations in transmission electron micrographs of both ultrathin frozen and plastic sections have been used to characterize more fully the molecular ultrastructure of three types of contact sites which have been recognized in earlier studies: focal adhesions, close contacts, and extracellular matrix contacts. Our evidence suggests that the same three types of contact sites occur both at cell-substratum and cell-cell interfaces. These results provide some new insights into the nature and molecular composition of fibroblastic contact sites, and suggest a resolution of some of the apparently conflicting data concerning the role of fibronectin in cell contacts. A preliminary report of this work has appeared (16).

## MATERIALS AND METHODS

### Cell Culture

Embryonic chick heart (ECH) fibroblasts were prepared from ventricle explants from 10-d-old chicken embryos by a sandwich method (18). After 4 d in sandwich culture, pulsating heart muscle cells in the center of the explants were removed by pipetting and the remaining fibroblast population was further selected with a differential attachment method (53). The selected ECH fibroblasts were then frozen and stored in liquid nitrogen, and cells between three and seven passages were used for our experiments. Cells were cultured in a 50/50 mixture of Dulbecco's modified Eagle medium (DME) and Coon's modified F-12 (Ham) medium, supplemented with 10% fetal calf serum (FCS), 10 μg/ml garamycin (Schering Corporation, Kenilworth, NJ), and 2 mM glutamine. Cells were seeded at an initial density of $5 \times 10^4$ cells per 35 $mm^2$ tissue culture dish.

### Immunolabeling Reagents

Rabbit and guinea pig antibodies to purified chicken plasma fibronectin (22) were affinity purified, and demonstrated to be monospecific for the cellular fibronectin from ECH fibroblasts, by methods described elsewhere (51). These antibodies also cross-reacted with calf serum fibronectin (see below). Rabbit and guinea pig antibodies to chicken gizzard α-actinin (27) and vinculin (26) were prepared and characterized as monospecific antibodies, as described. As secondary reagents, affinity-purified and cross-adsorbed goat antibodies against rabbit IgG and guinea pig IgG were prepared (30). Affinity-purified guinea pig antibodies to goat IgG were those previously used (15).

For the immunofluorescence double labeling studies, the affinity-purified goat antibodies were labeled with either dichlorotriazinyl-aminofluorescein (Research Organics) or rhodamine-lissamine sulfonylchloride according to Brandtzaeg (10). The modified antibodies were fractionated on a DEAE-cellulose column and fractions containing three to four fluorophore molecules per molecule of antibody were used. For double labeling immunoelectron microscopic studies, the secondary goat antibodies were conjugated with either ferritin (48) or Imposil as described (21, 30).

### Specimen Preparation for Immunoelectron Microscopy: The Gelatin-Roll Method

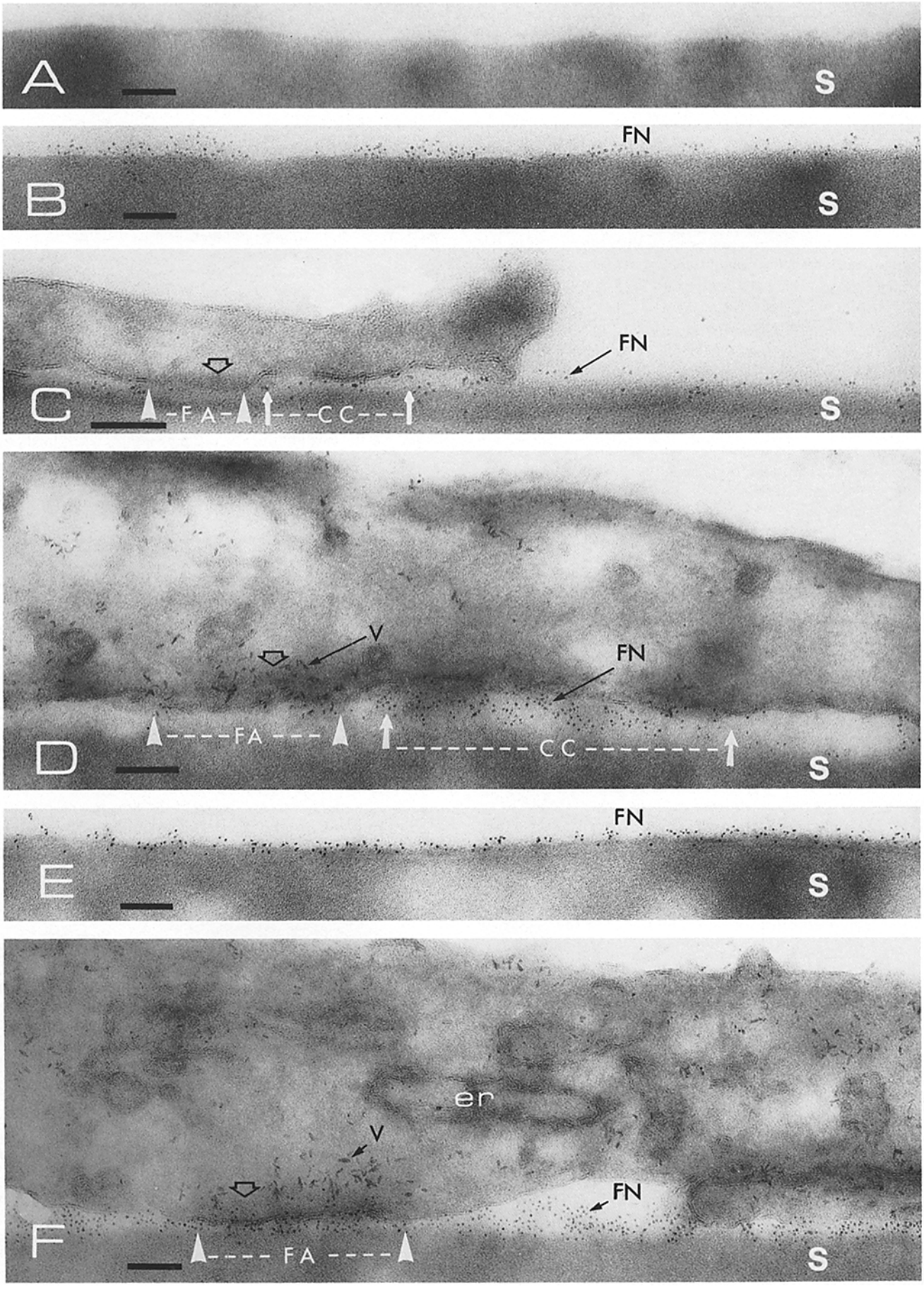
CROSS-LINKED GELATIN FILMS. To obtain sections that contain numerous monolayered cells with identical orientation with respect to the substratum for morphological and immunolabeling studies, ECH fibroblasts grown on a cross-linked gelatin film were packed into a roll for sectioning. The procedure, briefly described earlier (15), is given here in detail with improvements that we have since developed. The substratum film was prepared by coating the acid-washed glass cover slips with a thin layer of 5% (wt/vol) gelatin plus 5% (wt/vol) sucrose solution heated in a hot water bath (>90°C). Excess of solution on the glass cover slip was removed by pipette and the coated cover slip was then cooled and air dried at room temperature. For cross-linking of the film, the coated coverslips were first soaked with cold PBS (0.13 M NaCl in 0.02 M sodium phosphate buffer, pH 7.4) on ice for 10 min, followed by fixation with 4% (vol/vol) glutaraldehyde in PBS on ice for 5 min and then at room temperature for 30 min. This procedure results in insoluble fixed protein films of 4-μm average thickness, as judged by electron microscopy of vertical sections of the films. After five washes with PBS in a period of 20 min, the free aldehyde groups on the surface of the fixed film were conjugated either with other protein (see Fig. 1*E*) or quenched with 0.1 M glycine in PBS for 30 min, followed by five more washes.

Before culturing cells on this cross-linked gelatin film, the coated cover slips were transferred into a clean dish filled with DME supplemented with 10× antibiotics (100 μg/ml garamycin and 25 μg/ml Amphotericin-B) and then incubated at 37°C for 1 h for sterilization. After two washes with normal medium for 1 h, the substratum was ready for cell culture.

In this report, we studied two groups of ECH fibroblasts seeded on the cross-linked gelatin substratum, referred to as early cultures (6–12 h after seeding) and late cultures (24–36 h after seeding).

FIXATION AND ULTRATHIN FROZEN SECTIONING. After appropriate times of culture, cells were fixed by a two-stage procedure (66) first with a mixture of 60 mM ethylacetimidate and 3% paraformaldehyde in PBS (pH 7.8) plus 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 60 mM sucrose for 5 min at room temperature,

FIGURE 1 Control experiments concerning the accessibility of cell-substratum contact sites to immunolabeling reagents, on vertical ultrathin frozen sections of ECH fibroblasts. In *A–D*, a glycine-quenched cross-linked gelatin substratum was used; In *E–F*, a fibronectin-coupled cross-linked gelatin substratum was used. The substrata (*S*) were either incubated in various media for 10 h (*A, B, E*) or were seeded with cells for 10 h (*C, D, F*). Focal adhesions (*FA*) are indicated between two arrowheads; cytoplasmic dense plaques at FA sites are designated by thick open arrows; close contacts (*CC*) are demarcated between two thin arrows. *FN* indicates immunolabeling (with ferritin-antibody) for fibronectin; *V* (with Imposil-antibody) for vinculin; *er* is a portion of endoplasmic reticulum. Bars, 100 nm. (*A*) Glycine-quenched substratum, incubated in medium containing no serum, single immunolabeled for fibronectin. Without serum or cells there is no fibronectin labeling. (*B*) Same as *A*, except incubated in medium containing 10% FCS. Uniform labeling of the surface of the substratum is due to noncovalent binding of serum fibronectin. (*C*) Same as *B*, except seeded with cells in the serum-containing medium. *FA* shows no fibronectin labeling, whereas *CC* region adjacent to it does show labeling. (*D*) Same as *C*, except that double immunolabeling for fibronectin and vinculin was done. *FA*, showing intense labeling for vinculin near the membrane in the region of the dense plaque, again shows no labeling for fibronectin. *CC* shows intense fibronectin labeling. (*E*) Fibronectin-coupled substratum, incubated in medium without serum, doubly immunolabeled for fibronectin and vinculin. There is a uniform surface labeling of covalently bound fibronectin on the substratum; the absence of labeling for vinculin serves as a control for the specificity of vinculin labeling. (*F*) Same as *E*, except seeded with cells. Note that *FA*, exhibiting labeling for vinculin near the membrane in the region of the dense plaque, is now labeled for fibronectin in the gap between the cell and substratum, in contrast to the case in *D*.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followed by a mixture of 2% glutaraldehyde and 3% paraformaldehyde in the same buffer, but at pH 7.4, for 30 min. After rinsing with buffer, fixed cells were infused with 0.6 M sucrose and 0.02% $NaN_3$ in PBS for 15 min. The specimens were either immediately processed for sectioning or stored at 4°C up to 2 wk for future use. In the presence of 0.6 M sucrose infusion solution, the gelatin film with cells was mechanically peeled off the cover slip and rolled up into a cylindrical roll. The roll was cut into 2-mm segments which were oriented and mounted longitudinally on a copper specimen holder. The segments were rapidly frozen in Freon (refrigerant 12, Virginia Chemicals Inc., Dallas, TX) at its melting temperature. To obtain cross-sections through the cell-substratum interface, the frozen segment was set perpendicularly to the plane of sectioning and ultrathin sections were cut in the frozen state (−84°C) using a glass knife according to the methods described previously (63, 65). In other studies not shown in this report, the frozen segment of the gelatin roll was set parallel to the plane of sectioning to obtain sections parallel to the plane of the substratum. The frozen-sectioning was performed with a DuPont-Sorvall Ultramicrotome MT-2 with a cryoattachment (DuPont Instruments, Inc., Newtown, CT).

## Double Indirect Immunolabeling for Electron Microscopy

To label pairwise two antigens (vinculin, α-actinin, or fibronectin) on the same ultrathin fixed frozen section, the procedures described by Geiger et al. (30) were used. The double labeling experiments for ConA-binding proteins (CBP) and fibronectin were carried out as previously described (15). After the immunolabeling, the sections were positively stained by an absorption method essentially as described (64). Specimens were examined in a Philips EM-300 electron microscope operated at 60 kV.

Several types of control experiments were performed in double immunolabeling procedures: (*a*) an excess of a glutaraldehyde-cross-linked insoluble antigen (α-actinin or fibronectin) or of formaldehyde-fixed vinculin at a concentration of 0.5 mg/ml was added together with the primary antibody mixtures to compete specifically for the labeling of that antigen (Fig. 6 *B*): (*b*) nonimmune rabbit or guinea pig IgG was added to replace one primary antibody and thus remove the specific labeling by that antibody; (*c*) In case of labeling for CBP, 0.1 M methyl α-D-mannopyranoside was added together with ConA during the double labeling procedure (Fig. 2 *B*). In each of these cases, the controls showed negligible degrees of labeling. In addition, experiments were carried out in which the primary rabbit and guinea pig antibodies to a pair of antigens were reversed, but the secondary antibody conjugates remained the same, to show that the labeling pattern of the two antigens were specific (as shown in Figs. 3 and 4).

## Plastic Sectioning Electron Microscopy

To examine the ultrastructure of cell surface attachment sites in cross sections in plastic embedded specimens, cells were cultured on cross-linked gelatin films as described in detail above. Early and late cultures of ECH fibroblasts were fixed at various times after seeding. The medium was replaced with 2% glutaraldehyde, 3% paraformaldehyde, 0.02% tannic acid in PBS plus 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 60 mM sucrose at pH 7.3 and left at room temperature for 10 min. After thorough rinsing in the same buffer the film with cells was mechanically peeled off the cover slip and postfixed with 1% osmium tetroxide in the above buffer at pH 6 and 4°C for 10 min. The fixed film sheets with attached cells were rinsed five times with water, stained *en bloc* with 2% uranyl acetate for 30 min, dehydrated through acetone, and infused in Epon 812. Since the osmicated films were fragile, more than 10 cell-film sheets were piled up in a small drop of Epon. The polymerized Epon drops were then reembedded and oriented in the Epon block. Thin sections were made and stained with uranyl acetate and lead citrate.

## Double Immunofluorescent Labeling and Light Microscopy

ECH fibroblasts on glass cover slips were cultured for 10 h and fixed in 3% paraformaldehyde in PBS plus 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 60 mM sucrose for 10 min at room temperature. Cells were permeabilized with 0.5% Triton X-100 for 4 min. Double indirect immunolabeling was carried out essentially as described (30). Final mounting of the labeled cells in PBS was essential for simultaneous immunofluorescent and interference reflection microscopic (IRM) observations of the same cells; otherwise 90% glycerol and 10% Tris-HCl buffered at pH 8 produced sharper fluorescence images.

Labeled cells were observed with a Zeiss Photomicroscope III, modified for IRM according to the method of Bereiter-Hahn et al. (6). Fluorescence microscopy was performed as previously described (26).

# RESULTS

## Classification of Contact Sites in Cross-section by Morphological Observations of Ultrathin Frozen Sections

The main body of this work deals with the immunoelectron microscopic labeling of ultrathin frozen sections of ECH fibroblasts grown on a cross-linked gelatin substratum, and the assignment of immunolabeling patterns to contact sites that are recognized by morphological criteria. In such ultrathin frozen sections, the appearance of contact sites is different from that of comparable plastic sections for a number of reasons, a major one being the low degree of positive staining of the section that is required to detect the ferritin and Imposil labels. The plasma membrane is often clearly discernible in frozen cross-sections, but filamentous structures associated with the cytoplasmic and extracellular faces of the membrane are not as clearly delineated as in plastic sections; such filamentous structures are instead recognized by their localized diffuse densities. By the criteria of the spacing between the ventral cell surface membrane and the substratum, and the appearance of sub-membranous and/or extracellular densities, three types of contact sites that have been described in earlier studies of fibroblast cultures (see Discussion) were recognized in cross-section in ultrathin frozen sections.

(*a*) Focal Adhesions (FA): These were characterized by a spacing of 10–20 nm between the bilayer membrane and the substratum and an electron-dense cytoplasmic zone extending into the cell up to 60 nm from the membrane (open large arrows in Fig. 1 *C*, *D*, and *F*). FA are frequently observed just posterior to the leading edge of a cell in the early cultures as has been described (2).

(*b*) Close Contacts (CC): These had a 30–50 nm spacing between the cell surface and substratum, and generally exhibited submembranous densities parallel to the membrane (Fig. 1 *C*, *D*; Fig. 2 *A*, *B*). CC often occupied a larger linear dimension in the section than FA, corresponding to their greater surface areas (1, 6, 37, 46).

(*c*) Extracellular Matrix Contacts (ECM): In this type of contact, the ventral cell surface of the fibroblast was generally far removed from the substratum (>100-nm spacing), but was sporadically connected to the substratum by large and cable-like filamentous aggregates of extracellular matrix components. Examples of these three types of cell-substratum contacts in frozen sections are shown in Figs. 1 and 3–6.

By the same criteria of gap spacing and submembranous and extracellular densities, three types of cell-cell contacts corresponding to the FA, CC, and ECM cell-substratum contacts could be discriminated in the ECH frozen sections, particularly in the later subconfluent cultures. However, in FA and CC cell-cell contacts, the submembranous densities within the two cell domains were often not symmetrical in appearance. Examples of these types of intercellular contact sites are shown in Figs. 2, 3, 5, and 6.

To correlate these observations in frozen sections with those from more conventional plastic sections, and in view of the use of an unusual substratum (cross-linked gelatin) instead of glass, some experiments were performed with similarly grown ECH fibroblasts embedded in plastic. All three types of cell-substratum and cell-cell contacts discerned in the frozen sections were recognized in the plastic sections (Fig. 7), but in addition FA and CC showed filamentous material in the gaps between the cell surface and the cross-linked gelatin substratum (Fig. 7 *A*,

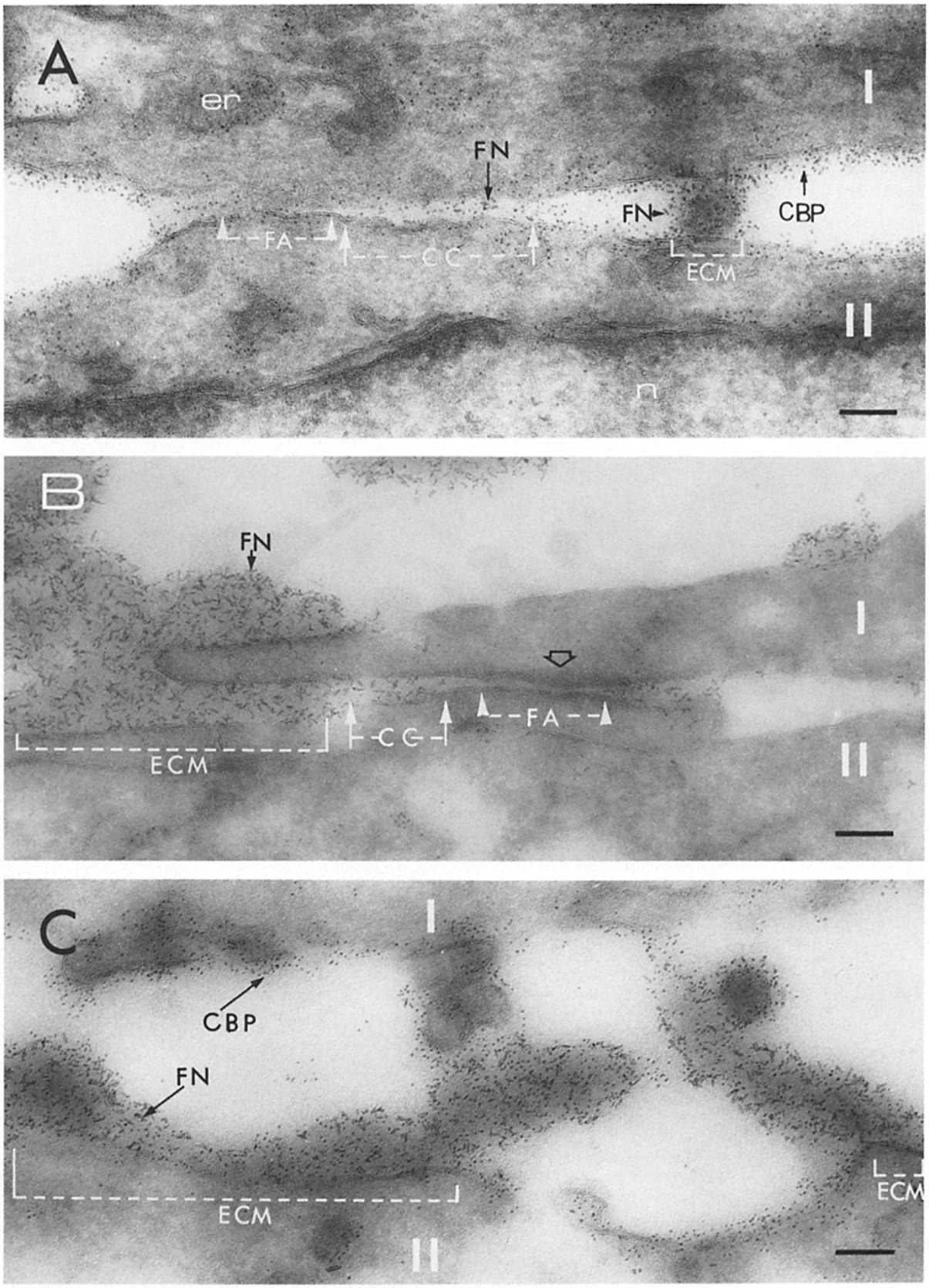


FIGURE 2 Control experiments concerning the accessibility of cell-cell contact sites to immunolabeling reagents on vertical ultrathin frozen sections of 30-h cultures of ECH fibroblasts. Indirect double immunolabeling was done for fibronectin (*FN*) with Imposil-antibody and for concanavalin A-binding proteins (*CBP*) with ferritin-antibody. The two cells making contact are designated *I* and *II*, and in addition to *FA* and *CC* contacts, extracellular matrix contacts (*ECM*) are demarcated. Open thick arrow designates cytoplasmic dense plaque at *FA* site; *er*, a portion of endoplasmic reticulum; *n*, the nucleus. Bars, 100 nm. (*A*) and (*C*) are adjacent regions of the same specimen. In *A*, an *FA* site is labeled for *CBP* but not for fibronectin, while *CC* and *ECM* sites are labeled for both. In *C*, *ECM* cables are large, elongated structures connecting two cells where their surfaces are far apart. (*B*) Control experiment in which 0.1 M methyl α-D-mannopyranoside was added together with the concanavalin A to compete away the ferritin labeling of *CBP*. Only Imposil labeling for fibronectin is observed, and is absent from the *FA* site.

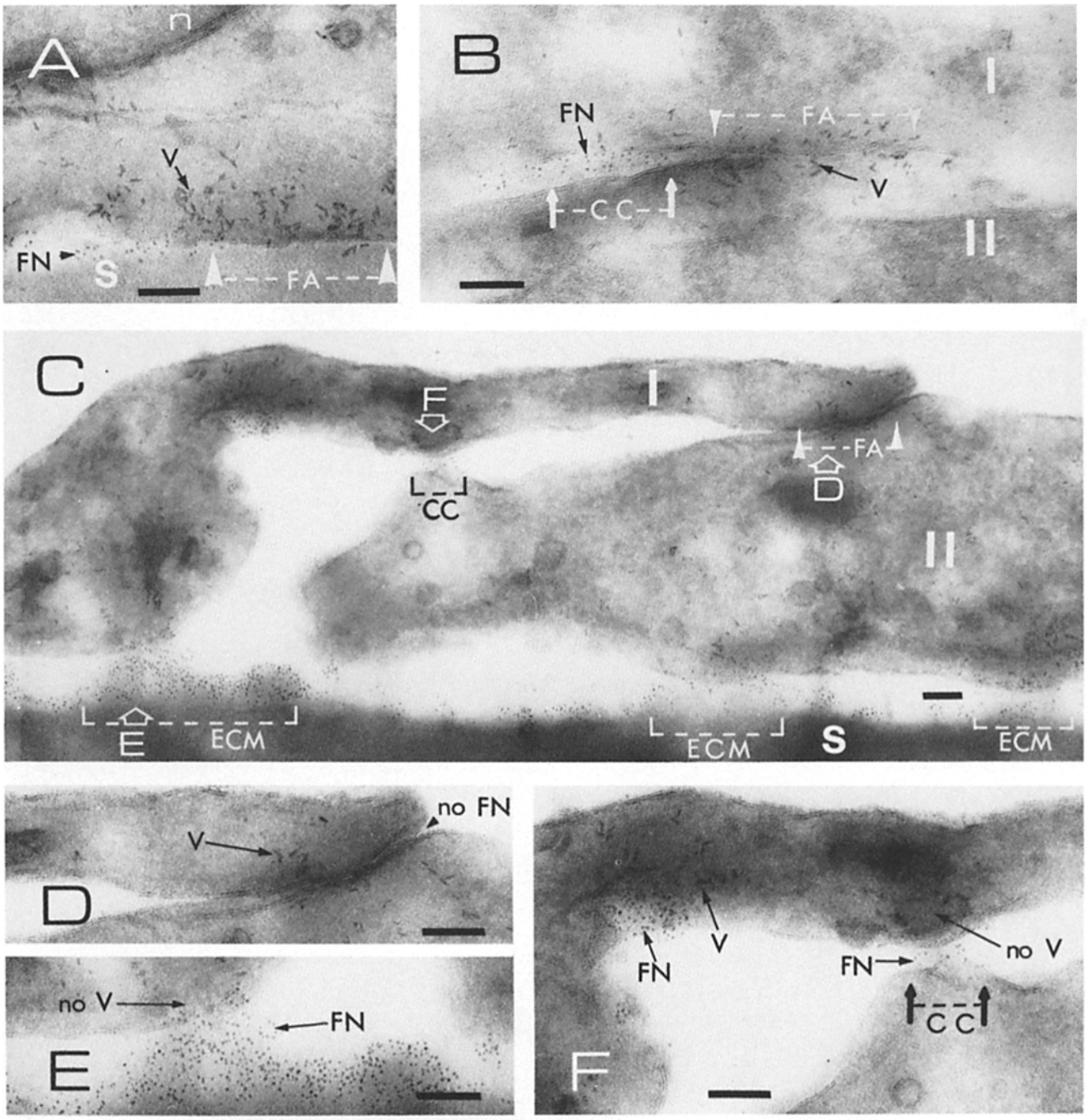


FIGURE 3 Indirect double immunoelectron microscopic labeling for vinculin (Imposil-antibody) and fibronectin (ferritin-antibody) on vertical ultrathin frozen sections of ECH fibroblasts in *A* early (6-h) culture and in *B–F* late (36-h) culture. Bars, 100 nm. Other symbols as in Figs. 1 and 2. (*A*) Cell-substratum contact region. An *FA* site is densely labeled for vinculin (*V*). Fibronectin (*FN*) labeling is absent in the *FA* site although it is intense immediately adjacent to *FA*. (*B*) Cell-cell contact region. An *FA* site is seen which is labeled for vinculin in both cells *I* and *II*, but shows no labeling for fibronectin. The latter is strongly labeled in the adjacent *CC* site. (*C*) A low-magnification field of a cell-cell contact region; the portions labeled with open thick arrows and the letters *D*, *E*, and *F* are shown enlarged in the panels *D*, *E*, and *F*, respectively. (*D*) An *FA* site labeled for vinculin but not fibronectin. (*E*) An *ECM* site labeled for fibronectin but not vinculin. (*F*) A *CC* site labeled for fibronectin but not vinculin.

*B*, and *D*) that were not clearly delineated in the frozen sections. There were also interesting details in the ECM-type of contacts in plastic sections which are presented in a later section.

## *Relative Distributions of Contact Sites in Cultures of Different Ages*

There were remarkable changes in the distribution of cell-substratum contacts between ECH cells 6–12 h after plating (early cultures) and cells 24–36 h after plating (late cultures) on the cross-linked gelatin substratum, accompanying the conversion from sparse to nearly confluent cell distributions. In the early cultures, there were undulations in the ventral cell surface, but the distance of separation of the surface from the substratum rarely exceeded 100 nm (Figs. 1 *C*, *D*; 3 *A*; and 5 *A*, *B*). In the later cultures, however, the average distance of separation markedly increased, with distances often exceeding 300 nm (Figs. 3 and 4). There were corresponding changes in

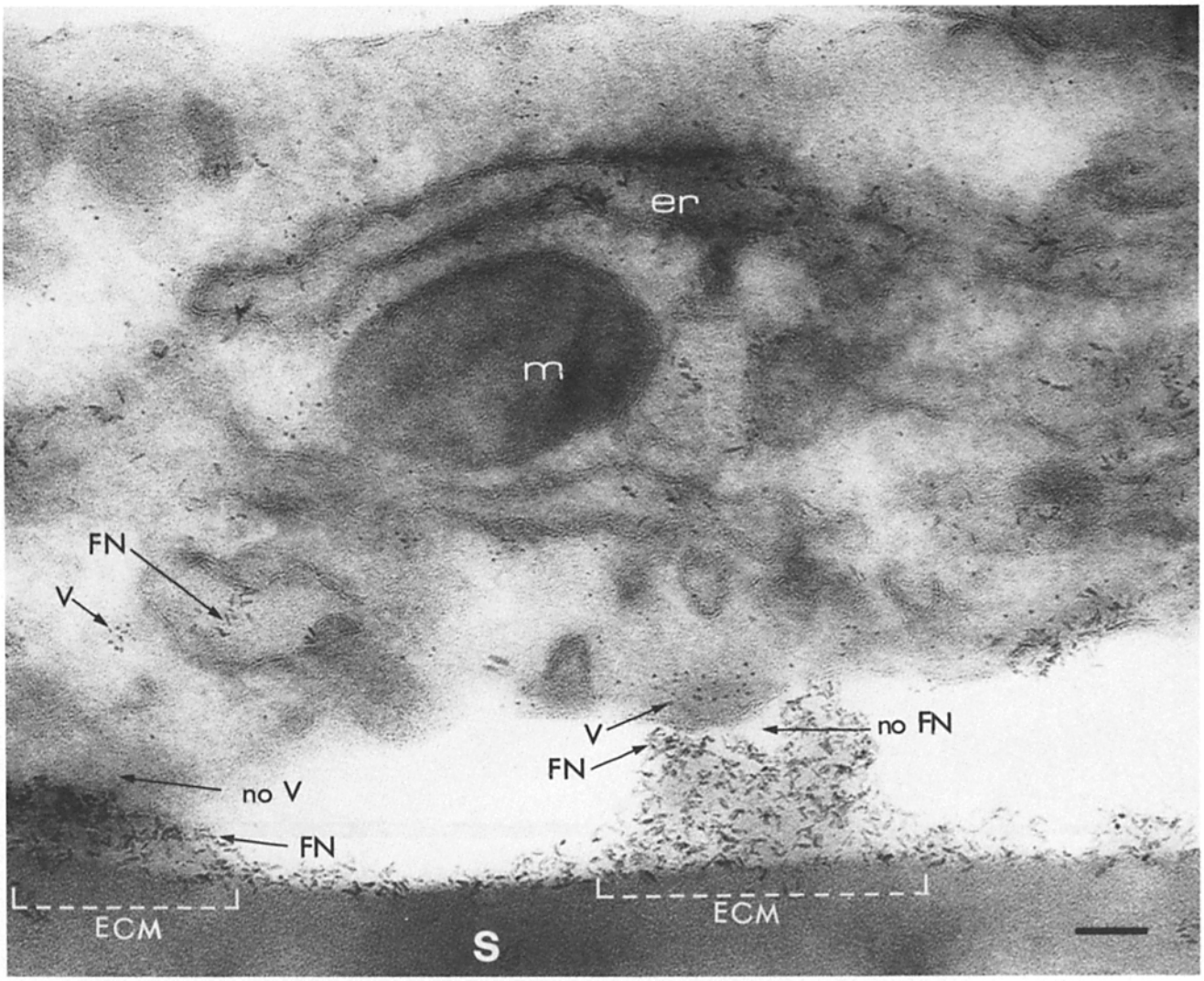


FIGURE 4 Indirect immunolabeling for vinculin and fibronectin of a vertical ultrathin frozen section through a cell-substratum interface of a 36-h ECH fibroblast culture. The immunolabeling procedure was interchanged from that of Fig. 3: the primary antibodies were reversed; guinea pig antibodies to vinculin were used with rabbit antibodies to fibronectin, followed by the same secondary antibody reagents as used in the experiments in Fig. 3. This resulted in labeling for vinculin with ferritin-antibody and for fibronectin with Imposil-antibody. The field shown is of a portion of the ventral surface well-removed from the cell periphery, and exhibiting predominantly *ECM* cell-substratum contact sites. The *ECM* at the right exhibits a translucent region devoid of fibronectin labeling at the outer surface of the membrane, and intense localized vinculin labeling near the membrane in the region of a dense plaque. The *ECM* at the left shows no translucent region and no vinculin labeling. Cytoplasmic labeling for both proteins is specific, often in nonoverlapping clusters. One fibronectin cluster (arrow) appears within a membrane-bounded vesicular body. Symbols as in previous figures. *m*: mitochondrion. Bar, 100 nm.

the distribution of the cell-substratum contact sites from predominantly FA and CC sites in early cultures (Table I, Column 3) to predominantly (>80%) ECM sites in later cultures (Table I, Column 10).

In early cultures, which were relatively sparse, cell-cell contacts were rarely detected. These were observed in later cultures, along with cell-substratum contacts recorded in the same frozen sections. In contrast to the high incidence of ECM sites at the cell-substratum interfaces in such late cultures FA and CC sites together made up the majority of cell-cell attachments (Table II, Column 3).

## Controls for Immunolabeling of Contact Sites

One of the main features of our experiments is the potentiality for immunoelectron microscopic labeling of components within the narrow gaps in FA and CC types of cell-substratum and cell-cell contacts. The question arises whether these narrow gaps in the ultrathin frozen sections are accessible to the labeling reagents. One type of experiment to test this point was reported in our previous paper (15). A different set of control experiments is shown in Fig. 1. When medium containing fetal calf serum was applied to, and later washed off, the surface of the glycine-quenched, cross-linked gelatin substratum in the absence of any cells, fibronectin from the serum became noncovalently attached to the surface of the substratum. Frozen sections of such serum-treated substrata showed a specific (compare Fig. 1 *A* and *B*) immunolabeling of fibronectin on the surface in a more-or-less uniform distribution, using a single indirect ferritin-antibody procedure. (This demonstrates that the antibodies to chicken plasma fibronectin used in this investigation cross-react with fetal calf serum fibronectin as well as the fibronectin produced by the ECH fibroblasts; see below). When cells were grown on the cross-linked gelatin

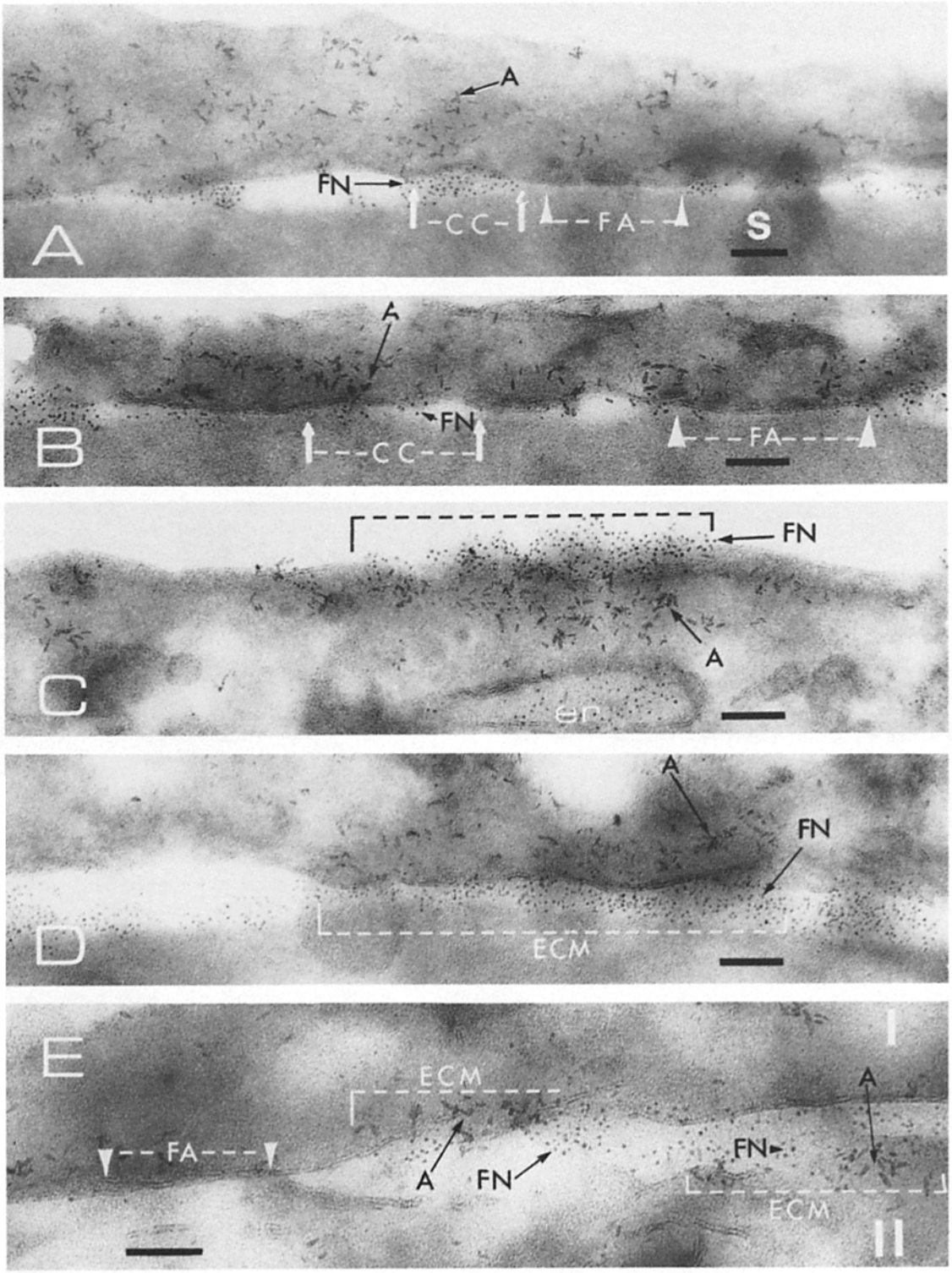


FIGURE 5 Indirect double immunoelectron microscopic labeling for α-actinin (black symbol *A*) (with Imposil-antibody) and for fibronectin (with ferritin-antibody) on vertical ultrathin frozen sections through contact sites in 10-h (*A*, *B*) and 30-h (*C*, *D*, *E*) cultures. Symbols are as in previous figures. Bars, 100 nm. (*A*) At *FA* site, there is no labeling for fibronectin, and labeling for α-actinin is relatively sparse and removed from the membrane. At *CC* site, labeling for both proteins is intense. (*B*) At *FA* site, there is no labeling for fibronectin and little for α-actinin, but along the membrane immediately to either side of the *FA*, labeling for both proteins is found. At *CC* site, both proteins are labeled. (*C*) With the late culture cell, the dorsal surface of the cell shows codistribution of extracellular fibronectin and intracellular α-actinin. Fibronectin labeling is also seen within the *er*. (*D*) An *ECM* cell-substratum contact region, showing submembranous density and α-actinin labeling close to the membrane. (*E*) A cell-cell *FA* site with no fibronectin and little α-actinin labeling is present. Two *ECM* sites that appear asymmetric are seen, the one in the center of the figure intensely labeled for α-actinin in cell *I* but not cell *II*, the other at the right of the figure labeled for α-actinin in cell *II* but not cell *I*.

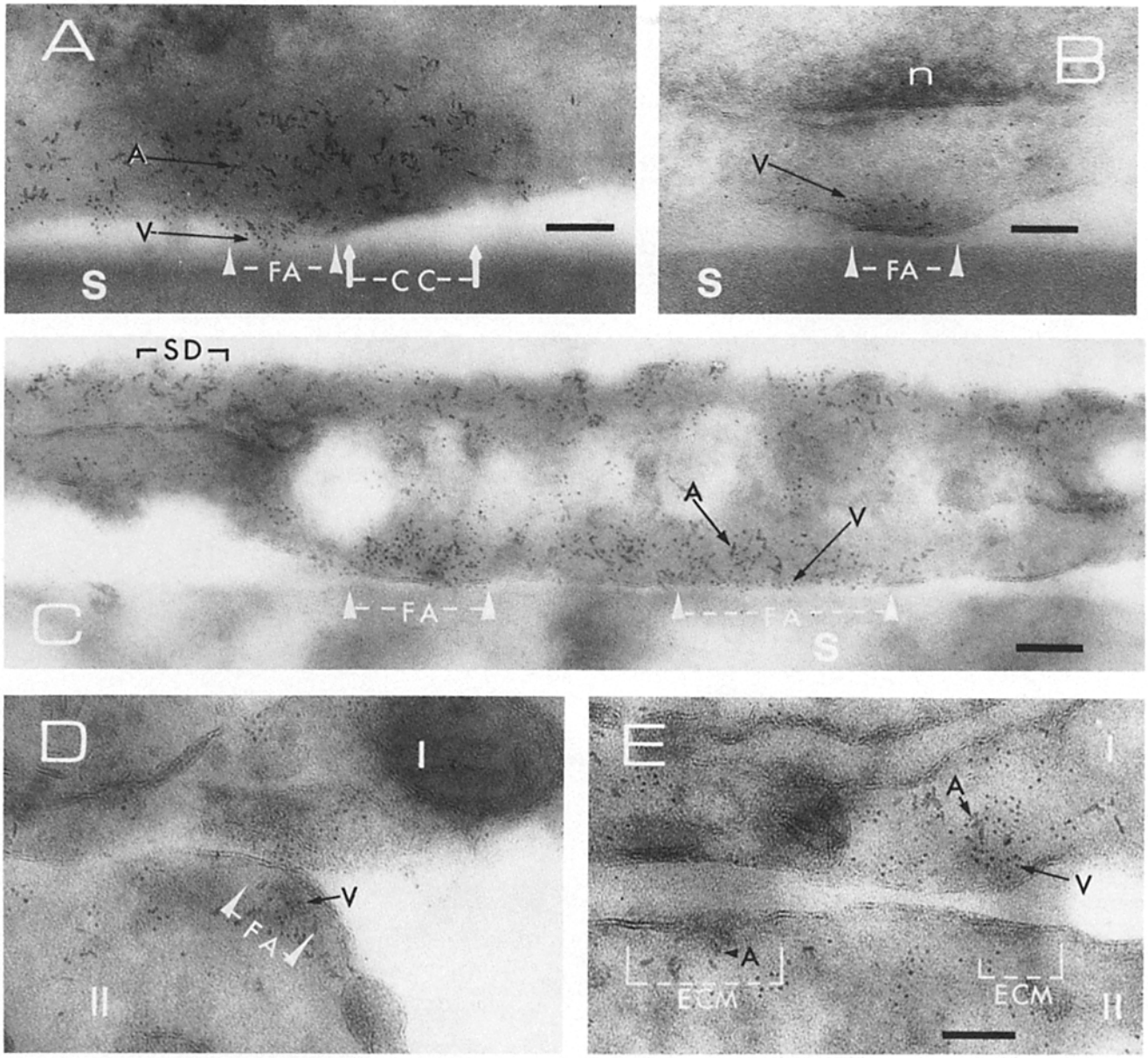


FIGURE 6 Indirect double immunoelectron microscopic labeling for vinculin (ferritin-antibody) and α-actinin (Imposil-antibody) on vertical ultrathin frozen sections through contact sites in 6-h (*A*, *B*, *C*) and 30 h (*D*, *E*) culture. Symbols as in earlier figures. Bars, 100 nm. (*A*) and (*C*) *FA* sites show both vinculin and α-actinin labeling, with the former generally closer to the membrane than the latter. The vinculin labeling is also more sharply confined to the region subtending the membrane at *FA* sites than is α-actinin labeling. At the *CC* site in *A*, there is little or no vinculin but intense α-actinin labeling. (*B*) A control experiment in which α-actinin labeling was competed away using cross-linked α-actinin (see Materials and Methods). Only ferritin labels for vinculin are observed confined to a submembranous density associated with an *FA* site. (*D*) A cell-cell *FA* site shows vinculin labeling close to the membrane. (*E*) Two *ECM* sites with different immunolabeling patterns are seen. The one at the left shows α-actinin labeling but little vinculin labeling, while the one at the right shows considerable vinculin labeling close to the membrane in cell *I*, and α-actinin labeling further removed from the membrane.

films in the media containing the calf serum, cross-sections of cell-substratum interfaces showed an absence of fibronectin labeling within FA sites, although intense fibronectin labeling in immediately adjacent CC sites was observed (Fig. 1 *C* and *D*). If, however, a low concentration of fibronectin was first covalently bound to the surface of the cross-linked gelatin film (Fig. 1 *E*) before the cells were plated on the substratum, fibronectin could then be detected in the gaps at FA sites (Fig. 1 *F*). (The gaps at the FA sites were not noticeably increased by the presence of the small amount of covalently bound fibronectin). The FA sites were additionally identified in Fig. 1 *F* by their intense submembranous labeling for vinculin in these double immunolabeling experiments (see below). These results provide an additional demonstration that fibronectin could be detected in the gaps of cell-substratum FA sites if that protein was constrained to be present there, and therefore the absence of immunolabeling for fibronectin in the usual cell-substratum FA sites (15; Fig. 1 *C* and *D*; and below) indicates that fibronectin is in fact absent or severely depleted from those sites. Indeed, fibronectin is probably actively removed from

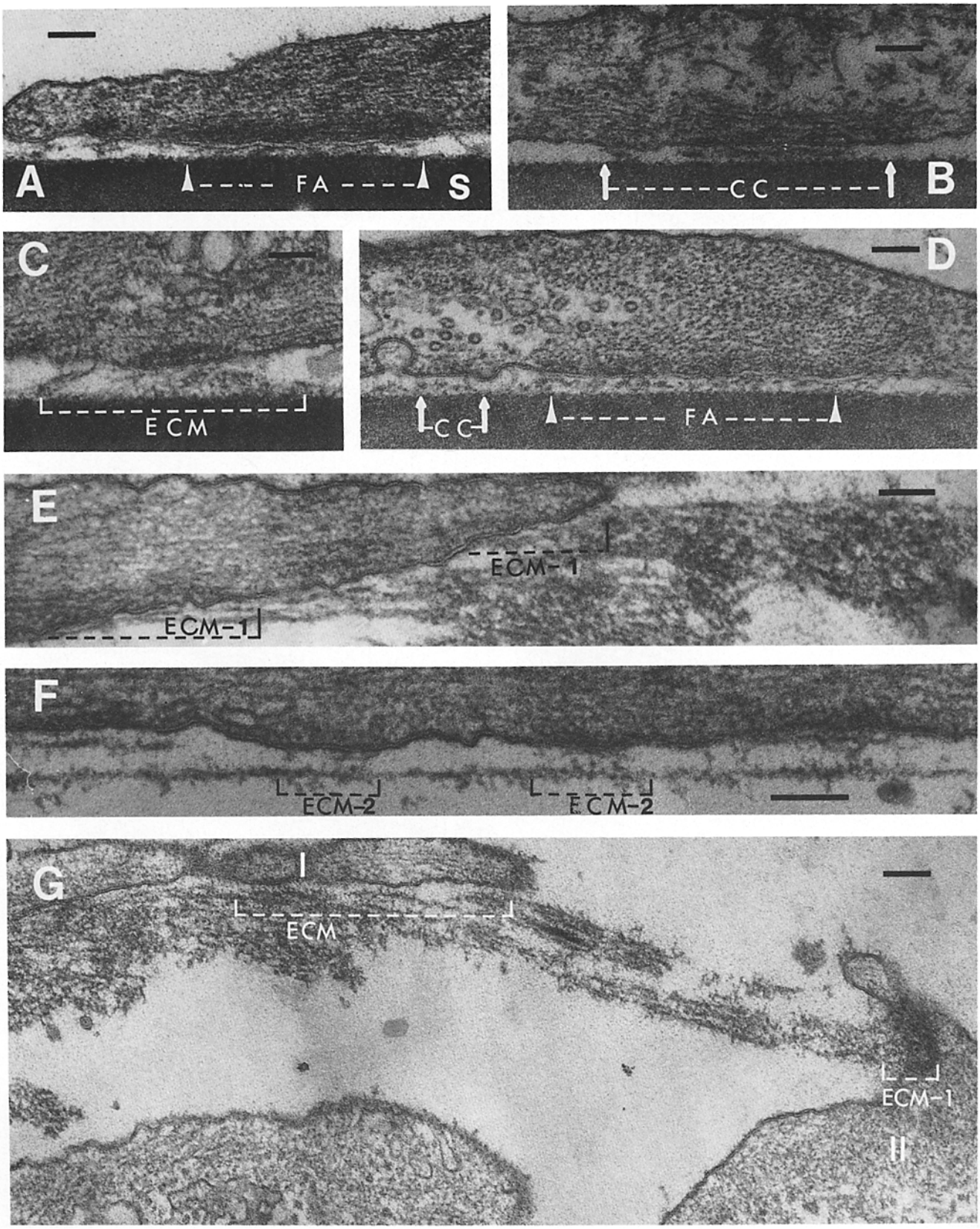


FIGURE 7 Vertical sections through cell-substratum and cell-cell contact regions of plastic-embedded specimens of ECH fibroblasts grown on cross-linked gelatin substrata. (*A*) and (*B*) are from 8-h cultures, *C–G* from 30-h cultures. In *A* and *D*, cell-substratum *FA* sites, near the cell periphery show dense submembranous filamentous plaques and fibrous material in the gaps between the cell surface and substratum. In *B*, a *CC* site shows a broader lateral array of submembranous filaments, as well as a parallel array of filaments within the cell-substratum gap. In *C*, and *E–G*, *ECM* contact sites are shown, which in some cases are distinguished as *ECM-1* or *ECM-2*. At *ECM-1* sites, filaments of extracellular matrix material appear to terminate at the outer membrane surface, to be complemented by intracellular microfilaments terminating at the inner membrane surface. In *G*, an *ECM-1* site at the right in cell *II* shows a dense plaque under the membrane. In *F*, *ECM-2* sites show lateral, rather than end-on, connections of extracellular matrix filaments to the membrane, complemented by lateral arrays of microfilaments on the other side of the membrane. Other *ECM* sites in *C* and *G* are not numbered because the morphological distinctions are not clear. Other symbols as in Figs. 1 and 2. Bars, 100 nm.

TABLE I

*Summary of Morphology and Double Immunolabeling Results for Attachment Sites Formed Between the Cell and Substratum*

| | | Early cultures | | | | | | | Late cultures | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Contact sites | Spacing | Total contacts* | Fibronectin | | Vinculin | | α-Actinin | | Total contacts‡ | Fibronectin | | Vinculin | | α-Actinin | |
| | *nm* | % | | | | | | | % | | | | | | |
| Focal adhesions | 10–20 | 22 | 0§/60 | −‖ | 59/60 | + | 48/60 | + | 6 | 0/8 | − | 8/8 | + | 6/8 | + |
| Close contacts | 30–50 | 50 | 85/133 | + | 8/133 | − | 74/133 | + | 10 | 12/13 | + | 2/13 | − | 10/13 | + |
| Extracellular matrix contacts | >50 | 28 | 68/68 | + | 4/68 | − | 50/68 | + | 84 | 112/112 | + | 22/112 | ±¶ | 61/112 | + |

* Percentage of total attachment sites identified. Total attachment sites observed was 400.

‡ Total attachment sites observed was 200.

§ The numerator is the number of times the indicated type of sites were positively labeled for the protein in question, while the denominator is the total number of such sites for which the protein in question was one of the pair of proteins that was immunolabeled.

‖ Attachment sites that were labeled with >60% frequency for a given protein were considered positively labeled for that protein (designated: +); if labeled with <20% frequency, were considered negative for that protein (designated: −).

¶ The low frequency of definitely positive labeling for vinculin in ECM sites is attributed to the existence of two kinds of ECM sites, one positive and one negative for vinculin (see text).

TABLE II

*Summary of Morphology and Double Immunolabeling Results for the Attachment Sites Formed Between Two Cells*

| Contact sites | Spacing | Total contacts* | Fibronectin | | Vinculin | | α-Actinin | |
|---|---|---|---|---|---|---|---|---|
| | *nm* | % | | | | | | |
| Focal adhesions | 10–20 | 28 | 0‡/37 | −§ | 37/37 | + | 27/37 | + |
| Close contacts | 30–50 | 40 | 29/54 | + | 5/54 | − | 38/54 | + |
| Extracellular matrix contacts | >50 | 32 | 36/36 | + | 5/36 | ±‖ | 25/36 | + |

* Percentage of total attachment sites identified. Total attachment sites observed was 200.

‡ The numerator is the number of times the indicated type of sites were positively labeled for the protein in question, while the denominator is the total number of such sites for which the protein in question was one of the pair of proteins that was immunolabeled.

§ Attachment sites that were labeled with >60% frequency for a given protein were considered positively labeled for that protein (designated: +); if labeled with <20% frequency, were considered negative for that protein (designated: −).

‖ The low frequency of definitely positive labeling for vinculin in ECM sites is attributed to the existence of two kinds of ECM sites, one positive and one negative for vinculin (see text).

those sites (compare Fig. 1 *B* and *C*) upon cell spreading, as previously suggested (4).

This latter type of control experiment can not readily be used to determine the accessibility of immunolabeling reagents to the gaps in FA and CC sites at cell-cell interfaces. Therefore, the control experiment described in our first paper (15) was used. This involved treating the frozen section of ECH fibroblasts with ConA, followed by antibodies to ConA, and finally ferritin- or Imposil conjugates of secondary antibodies directed against the anti-ConA antibodies. As shown in Fig. 2 *A* and *B*, labeling for fibronectin (with Imposil conjugates) was absent from intercellular FA sites although it was intense in nearby CC sites, whereas labeling for CBP (with ferritin conjugates) was intense within both FA and CC sites. Therefore, the absence of fibronectin labeling within the intercellular FA sites cannot be ascribed to an inaccessibility of the labeling reagents to the narrow gaps in those sites, a conclusion similar to that reached for cell-substratum FA sites (15).

## *Double Immunoelectron Microscopic Labeling of Ultrathin Frozen Sections*

The three proteins fibronectin, vinculin, and α-actinin were examined pairwise in double indirect immunolabeling experiments, using affinity-purified guinea pig and rabbit antibodies as primary reagents, and affinity-purified and cross-absorbed goat antibodies to guinea pig and rabbit IgG, after coupling to either ferritin or Imposil, as secondary reagents. In the experiments to be described next, 20 pairwise double immunolabeling experiments were done, resulting in 258 useful electron micrographs within which ~800 cell surface attachment sites were examined for their immunolabeling characteristics. These results are summarized for cell-substratum attachment sites in Table I and for cell-cell attachment sites in Table II, and representative micrographs are shown in Figs. 1–6.

## *Double Immunolabeling of Vinculin and Fibronectin*

CELL-SUBSTRATUM CONTACT SITES. At sites discriminated as FA by the morphological criteria discussed above, vinculin labeling (black letter *V*) was found close to the membrane in the dense submembranous plaques, but fibronectin labeling was absent (black letters *FN*, Figs. 1 *D* and 3 *A*). At CC sites, which were often adjacent to the FA sites, the reverse was the case: vinculin labeling was absent or low, at a density not greater than in scattered regions in the cytoplasm, while fibronectin labeling was intense in the extracellular space (Fig.

1 *D*). Cell-substratum ECM sites were always intensely labeled for fibronectin, but labeling for vinculin was of two kinds (Fig. 3 *C*): the majority of the sites showed only the low and irregular density characteristic of the cytosol (Figs. 3 *E* and 4, lower left), but ~20% of the ECM showed significant submembranous labeling for vinculin within a dense plaque (Fig. 4, lower right of center). The latter type of ECM often exhibited an electron lucent region between the extracellular matrix cable and the cell surface (Fig. 4, lower right of center) which was not labeled for fibronectin and which was not seen with the majority type of ECM sites. In addition Fig. 4 shows intracellular labeling for fibronectin within what appear to be vesicular bodies scattered through the cytoplasm, which may represent vesicle-bound fibronectin on its secretory pathway through the cell.

CELL-CELL CONTACT SITES: There was a strong similarity in the double immunolabeling patterns for vinculin and fibronectin at cell-cell as at cell-substratum contact sites. In sites designated as FA by their small intercellular gap, a submembranous dense plaque, and a location near the leading edge of at least one of the two cell partners (Fig. 3 *B*, *C*, and *D*), vinculin labeling was generally marked and localized where there was a submembranous dense plaque, but fibronectin labeling was absent. The vinculin labeling on the two sides of the intercellular FA was often not equally intense (Fig. 3 *D*). At CC sites (Fig. 3 *B*, *C*, and *F*) there was only slight labeling for vinculin, but there was substantial labeling for extracellular fibronectin. At ECM sites, which were always densely labeled extracellularly for fibronectin, there was usually little or no labeling for vinculin (Fig. 3 *C* and *E*), although occasionally at submembranous plaques associated with a cell-cell ECM site, significant vinculin labeling was observed (ECM in lower far-right corner of Fig. 3 *C*).

## Double Immunolabeling of α-Actinin and Fibronectin

CELL-SUBSTRATUM CONTACT SITES: At FA sites, fibronectin labeling was absent, and intracellular α-actinin labeling (black letter *A*) was present but was generally removed some distance from the membrane, at the outer regions of the submembranous dense plaque (Fig. 5 *A* and *B*). At CC sites, extracellular fibronectin labeling was intense, and labeling for α-actinin was generally more intense and closer to the membrane than at FA sites (Fig. 5 *A* and *B*). At regions of the cell-substratum interface where FA and CC sites were adjacent to one another, as in Fig. 5 *A*, these distinctions in relative densities of immunolabeling were most clearly apparent. In late cultures, a codistribution of extracellular fibronectin and intracellular α-actinin labeling was sometimes evident at discrete sites of submembranous density (SD) on the dorsal surfaces of the cells (Fig. 5 *C*), and was often seen at ECM sites on the ventral surface (Fig. 5 *D*).

CELL-CELL CONTACT SITES: The double immunolabeling results for α-actinin and fibronectin were closely similar with cell-cell as with cell-substratum contact sites. At FA sites, no fibronectin labeling was detected and labeling for α-actinin, when present, was removed from the central region of the submembranous dense plaque (Fig. 5 *E*). At CC sites (not shown), significant fibronectin labeling was observed within the intercellular gap, and α-actinin labeling was present near the membrane in at least one of the two contacting cells. At ECM sites, along with the intense labeling for extracellular fibronectin, α-actinin labeling was often concentrated close to the membrane (Fig. 5 *E*).

## Double Immunolabeling of Vinculin and α-Actinin

CELL-SUBSTRATUM CONTACT SITES: Simultaneous labeling for both vinculin and α-actinin was observed at FA sites, but their relative distributions at these sites was sometimes difficult to determine in frozen sections. The uncertain orientation and disposition of the membrane associated microfilaments with respect to the plane of the section added to the difficulties. However, in a significant number of instances (Fig. 6 *A* and *C*) FA sites clearly showed a higher density of vinculin than α-actinin labeling closer to the membrane. The vinculin labeling in the FA was often also more sharply localized near the membrane than was the more dispersed α-actinin labeling (Fig. 6 *A*). At CC sites, vinculin labeling was generally no more extensive than in the cytosol, whereas α-actinin labeling was intense (Fig. 6 *A*). At the dorsal surfaces of the cell, vinculin and α-actinin labeling were both observed, but at some dorsal regions (*SD* in Fig. 6 *C*) concentrated labeling for α-actinin with relatively sparse vinculin labeling was found. Within the interior cytoplasm specific vinculin labeling appeared to be more densely and diffusely distributed than α-actinin (Fig. 6 *C*).

CELL-CELL CONTACT SITES: FA sites as defined morphologically generally showed more intense vinculin than α-actinin labeling close to the membrane (Fig. 6 *D*), but often not symmetrically in the regions of the two cells contributing to an FA site. At CC sites, by contrast α-actinin labeling was of high density and vinculin labeling was low (not shown). ECM sites appeared to be of at least two different types with respect to vinculin/α-actinin double labeling: one type being positive for α-actinin but essentially negative for vinculin (Fig. 6 *E*, left, cell *II*) and the other positive for both, with vinculin labeling closer to the membrane than α-actinin (Fig. 6 *E*, right, cell *I*).

## ECM Sites in Plastic-Embedded Sections

The suggestion from the double immunolabeling experiments with ultrathin frozen sections that ECM sites might be of at least two types, one exhibiting vinculin labeling at submembranous densities and the other not, was further examined morphologically in plastic sections cut through cell-substratum and cell-cell interfaces, particularly in the late cultures. Detailed examination of the attachment between the cables of extracellular matrix and the cell membrane, and of intracellular microfilaments and the cell membrane at the same ECM sites, suggested that two types could be distinguished, labeled ECM-1 and ECM-2 in Fig. 7 *E*, *F*, and *G*. ECM-1, accounting for ~20% of ECM sites, showed simultaneously an end-on approach of the extracellular matrix fibrils to the membrane and an end-on attachment of intracellular bundles of microfilaments to the cytoplasmic surface of the same membrane. ECM-2, the majority of ECM sites, were characterized by extensive lateral associations both of extracellular matrix fibrils and of intracellular microfilaments to the membrane. The relative abundance of these two types of ECM sites as recognized morphologically in plastic sections fits with the suggestion that ECM-1 corresponds to the ECM sites in frozen sections that showed significant submembranous concentrations of vinculin, while EMC-2 corresponds to the ECM sites in which vinculin labeling was absent or severely depleted.

### *Light Microscope Observations of ECH Monolayers*

To compare our electron microscopy analyses of cell-substratum contact sites with several different kinds of light microscope observations that have been reported of these sites (see Discussion), a few experiments were performed with ECH monolayer cultures grown directly on glass cover slips. (For the interference reflection measurements discussed below, the cells could not be grown on the cross-linked gelatin substratum.) Four kinds of light microscope observations were made on the same cells: Nomarski differential interference contrast (DIC), interference reflection microscopy (IRM), and double indirect immunofluorescent labeling for vinculin and fibronectin. Two examples of these results are reproduced in Fig. 8.

IRM of living cells has been used to identify two types of cell-substratum contact sites (1, 6, 46, 47), the focal contacts or focal adhesions (FA), which appear as punctate dark areas around the cell periphery, and close contacts (CC), which appear as broader gray areas often surrounding or adjacent to the FA. By comparing the IRM patterns of living ECH fibroblasts with their counterparts that were fixed, permeabilized, and immunolabeled, we first found that the gray areas corresponding to CC were no longer visible with the treated cells. However, dark punctate areas near the leading edge or the trailing edge of a cell (double arrowheads in Fig. 8 *B* and *F*, respectively) could be found that were immunolabeled for vinculin (Fig. 8 *C* and *G*), but not for fibronectin (Fig. 8 *D* and *H*). These areas corresponded to the FA. Related observations have been made by other investigators (4, 8, 23, 34, 57).

In addition to such IRM images, however, we also observed dark punctate areas (double arrow, Fig. 8 *F*) and dark streaks (double arrow, Fig. 8 *B*) outside the perimeter of the cell on the adjacent cell-free substratum; these dark areas and dark

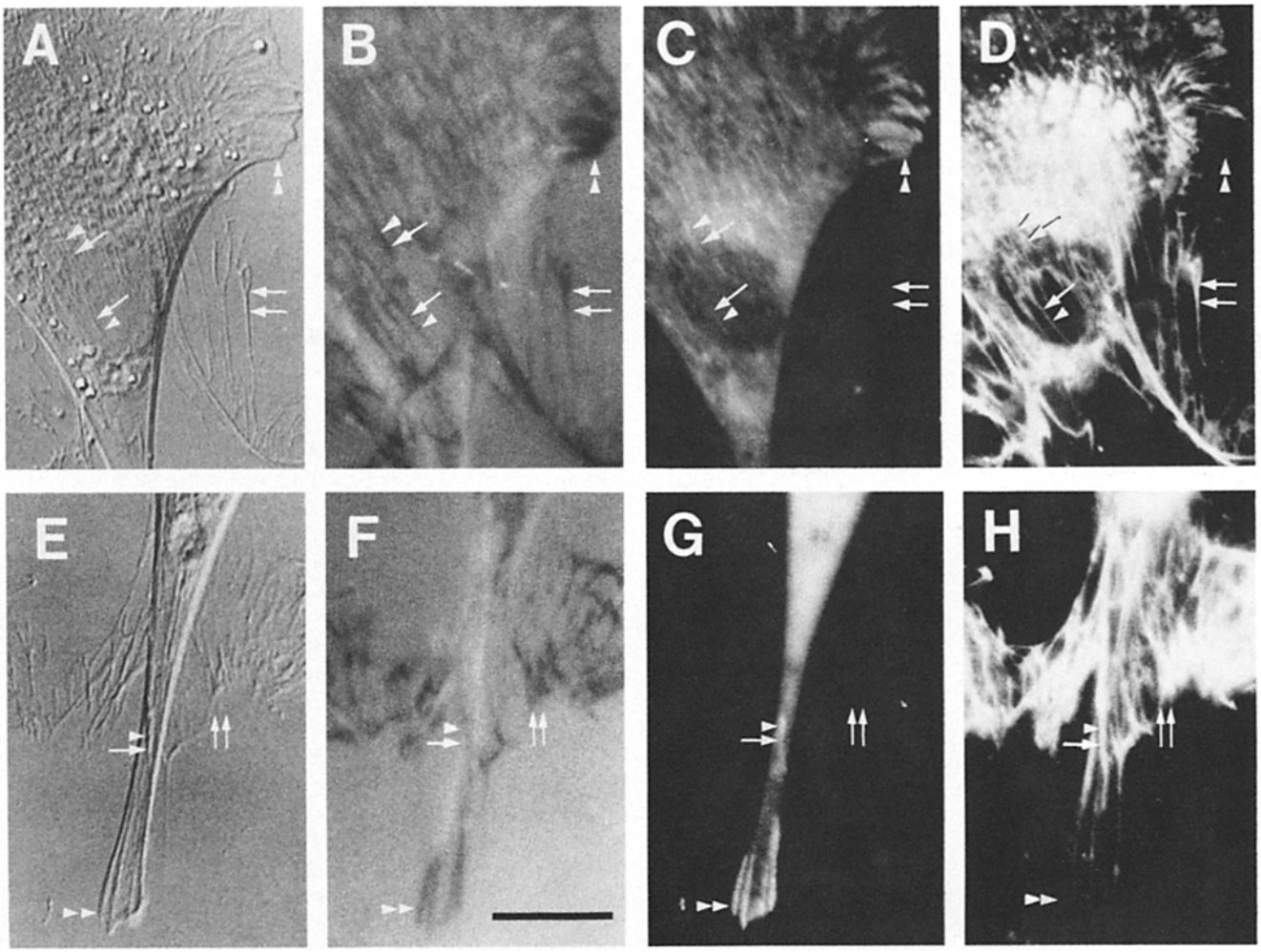


FIGURE 8 Light microscopic observations of ECH fibroblasts on glass substrata after 10-h culture. *A–D* are of the same cell including its leading lamellum, while E–H include another cell's trailing edge. In all cases, focus is at the cell-substratum interface. All figures are at the same magnification. (Bar (in *F*), 15 μm). *A* and *E* are Nomarski differential interference contrast images (DIC); *B* and *F* are interference reflection microscopic images (IRM); *C* and *G* show immunofluorescent labeling for vinculin; and *D* and *H* show immunofluorescent labeling for fibronectin. The identically positioned double white arrowheads in *A–D*, and in *E–H*, designate sites at the cell periphery that are recognized as *FA* by their dark images in IRM, their fluorescent labeling for vinculin, and the absence of labeling for fibronectin. The identically positioned tandem double arrows in *A–D* and in *E–H* point to extracellular cables in the DIC images that are far removed from the cell body, that give dark images in IRM, and do not label for vinculin but label intensely for fibronectin. The identically positioned tandem arrow-arrowhead pairs in *A–D* and in *E–H* point to the cable-like structures in the DIC images that lie under the ventral surfaces of the cells, give dark IRM images, and show coincident immunolabeling for both vinculin and fibronectin. See text for further details and interpretation.

streaks were associated with cable-like structures in the corresponding DIC images (double arrows in Fig. 8 *A* and *E*), and were immunolabeled for fibronectin (Fig. 8 *D* and *H*) but not for vinculin (Eg. 8 *C* and *G*). These particular dark images in IRM are therefore clearly not attributable to focal adhesions, but are instead associated with fibronectin-containing structures (cables) attached to the substratum.

Other dark streaks in IRM were observed underneath the cell body (arrow-arrowhead pairs in Fig. 8 *B* and *F*) which also were associated with cable-like structures in DIC images (Fig. 8 *A* and *E*), and were generally immunolabeled both for vinculin (Fig. 8 *C* and *G*) and for fibronectin (Fig. 8 *D* and *H*). From the similarities of these dark IRM images under cells to those on the cell-free adjacent substratum described in the previous paragraph, we suggest in the Discussion that such dark IRM images which are immunofluorescently labeled for both vinculin and fibronectin are not, or at least not necessarily, attributable to typical focal adhesions between the cell and substratum, as has sometimes been implied, but may rather be due to some type of ECM contact (ECM-1) such as we have observed in electron microscopy.

## DISCUSSION

### *Methodology of the Immunoelectron Microscopic Labeling Studies*

We have used techniques of high resolution immunoelectron microscopy developed in our laboratory to probe the molecular composition and structure of the contact sites formed by cultured fibroblasts with their substrata and with one another. The immunolabeling of ultrathin frozen sections of appropriately fixed specimens allows two important objectives to be achieved: (*a*) maximal retention of the cellular ultrastructure, by avoiding the use of detergents or other treatments to permeabilize the cells; and (*b*) direct access of the antibody reagents to their respective antigens whatever their sites of localization in a cell, by exposing those sites at the surface of the section. These techniques have previously been applied successfully to localize specific proteins in a variety of intact tissues (28–30, 59, 65, 67) and in cells in suspension culture (7). Their application to cells in monolayer culture initially described in a preliminary account of this work (15), is described extensively for the first time in this paper. A major obstacle to the study of monolayer cultures is the need to examine a large number of cells in similar defined orientations in a single electron microscope specimen. This obstacle was overcome in the present studies by the gelatin-roll method of specimen preparation. Other critical aspects of the technique are discussed elsewhere in detail (16, 30, 58, 65).

### *Morphology of Contact Sites*

A number of investigators have previously discriminated three types of cell-substratum contacts by electron microscope observations in plastic sections. Focal adhesions (FA, also called focal contacts), often found at the perimeter of cells, are discrete sites characterized by narrow gaps (10–20 nm) between the ventral cell surface and the substratum (2, 6, 11). Although they occupy only a small fraction of the interface they are the sites of strongest cell-substratum adhesion (1, 14, 36, 47). On the cytoplasmic surface of the plasma membrane at FA sites, microfilament bundles terminate (2, 37) with all filaments in the same orientation (5, 31, 60). Close contacts (CC), broader areas often found surrounding FA sites, are characterized by larger gaps (30–50 nm) between the cell surface and substratum (6, 19, 46, 47). A meshwork of microfilaments is often seen (19, 37) subtending the membrane at these sites. Extracellular matrix contacts (ECM) have previously been observed with cultured myoblasts (24) and fibroblasts (13, 25, 40, 56), as sites where strands and cables of extracellular matrix material including fibronectin connect the ventral cell surface to the substratum where they are separated by large distances (>100 nm).

The gelatin-roll method of specimen preparation involved growing cells on an unusual substratum, a thin film of glutaraldehyde cross-linked gelatin on glass. All of our evidence (15, Fig. 7) indicates that the morphological and adhesive properties of the fibroblasts cultured on cross-linked gelatin on glass are very similar to those of cells grown directly on plastic or glass. For example, FA, CC, and ECM contact sites were recognized in plastic sections of fibroblasts grown on the cross-linked gelatin substratum (Fig. 7). In ultrathin frozen sections cut through contact sites in a direction perpendicular to the plane of the cross-linked gelatin substratum, the same three types of contact sites could be recognized by morphological criteria alone, despite the relatively low contrast in the sections. (The contrast was maintained low so as to allow visualization of the ferritin and Imposil labels on the sections.) The criteria were the size of the gap between the cell surface and substratum, and the character of the not-well-resolved submembranous densities.

It was possible to recognize in the frozen sections a similar set of contacts at sites where the surfaces of two cells were apposed (38). Although sometimes rendered more difficult by the oblique orientations of the two cell surfaces with respect to the plane of the section, the morphological discrimination of FA and CC cell-cell contact sites could generally be made in cross-section on the basis of gap distances and submembranous densities as with the corresponding cell-substratum sites. In addition to these contact sites, gap junctions have also been recognized at fibroblast cell-cell interfaces (52). These are often flat parallel contacts with a narrow intercellular gap (4 nm) that are distinct from the FA and CC sites.

Whereas FA and CC contact sites have been discriminated and investigated in many previous studies, ECM contacts, although observed many times before (13, 24, 25, 40, 55), have not received the attention we believe they warrant. It was found that ECM-type contacts constituted ~30% of all cell-substratum and cell-cell contact sites in our early cultures (6–12 h), and that this increased to ~85% for cell-substratum, and >30% for cell-cell, contact sites in the late cultures (24–36 h) (Columns 3 and 10 in Table I, and Column 3 in Table II). In the late cultures, the small residual numbers of FA and CC sites between the cell and substratum were largely confined to the cell perimeter. The more interior regions of the ventral surface of the cell were largely lifted off the substratum (Fig. 4), and connected to it mainly by the sporadic ECM cable-like contacts. These morphological results are consistent with the well-known fact that the production and secretion of fibronectin (13, 43), and probably other extracellular matrix components, increases markedly with increasing cell density in normal fibroblast cultures. It is possible that such ECM contacts in culture are of greater relevance to the kinds of contacts fibroblasts make in vivo with their surrounding extracellular matrix and with neighboring cells than are the FA and CC contacts made to glass or similar artificial substrata.

The differentiation of these ECM contacts into two kinds, and their importance in the interpretation of light microscopic studies of fibroblast contact sites, are considered below.

## Immunolabeling Patterns of Contact Sites

A principal result of this study is the localization by pairwise double immunoelectron microscopic labeling methods of the proteins fibronectin, vinculin, and α-actinin with respect to the three types of morphologically recognized contact sites at cell-substratum and cell-cell interfaces. The three types showed characteristic immunolabeling patterns, as follows.

(*a*) Focal adhesions: These were always negative for fibronectin labeling, and generally positive for vinculin and α-actinin (Tables I and II). In double immunolabeling experiments in which fibronectin was one of the two components labeled, 68 cell-substratum (60 in early and 8 in late cultures) and 37 cell-cell FA sites were observed in cross-section and none of them showed any significant labeling for fibronectin. (Our antibodies would have detected either the calf serum fibronectin or the endogenous chicken cell fibronectin if either were present in these sites.) In the case of cell-substratum FA sites (15) and cell-cell FA sites (Fig. 2), Con-A binding proteins present within the narrow gaps at these sites could be immunolabeled while fibronectin was not. (Similar conclusions were derived by Fox et al. [23] from their immunofluorescence experiments.) In addition, if a thin layer of fibronectin was covalently bound to the substratum before cell culture, the still narrow gaps within cell-substratum FA sites could be immunolabeled for fibronectin (Fig. 1 *F*). These control experiments clearly demonstrate that if fibronectin had been present in the narrow gaps at FA sites, the immunolabeling reagents would have had access to it in these cross-sectioned specimens. Therefore, the absence of immunolabeling for fibronectin in our experiments signifies an absence or severe depletion of fibronectin from FA sites that are morphologically recognized as such in cross-section in electron microscopy. It should further be noted that in plastic-embedded specimens grown on the cross-linked gelatin substratum, FA sites exhibited extracellular strands in the gap between the cell surface and substratum (Fig. 7 *A* and *D*). It would appear that under our culture conditions there were one or more fibrous components present in the FA gaps but fibronectin was not one of them. These components may include glycosaminoglycans (61).

In general, at FA sites the labeling for vinculin was closer to the membrane than was the labeling for α-actinin. This was particularly evident when the two proteins were simultaneously double labeled (Fig. 6 *A* and *C*). These results considerably extend the original immunofluorescence observations of Geiger (26), who found that vinculin and α-actinin were both in part associated with cell-substratum FA sites. This relative disposition of vinculin and α-actinin is closely similar to the results of other immunoelectron microscopic labeling experiments with a variety of sites in different tissues where microfilaments or microfilament bundles terminate at cell membranes: the membrane-associated dense plaques in smooth muscle (30); the zonula adherens in the junctional complex of the intestinal epithelial brush border (30); and the fascia adherens of the intercalated disk membrane of cardiac muscle (67). At each of these sites, both vinculin and α-actinin are present, with vinculin labeling situated closer to the membrane than α-actinin. This suggests that a common ultrastructural assembly is involved at these different microfilament-membrane attachment sites, and that vinculin may serve as a peripheral protein linking the termini of the microfilament bundles to the membrane at these sites, as is discussed more fully elsewhere (29, 30), and as is schematically represented below (Fig. 9). Experiments on the in vitro binding of vinculin to actin filaments are consistent with such a role for vinculin (70).

(*b*) Close Contacts: These were generally positive for fibronectin and α-actinin, and essentially negative for vinculin (Tables I and II). A CC site densely labeled for fibronectin was often found immediately next to an FA site that showed no fibronectin labeling (cf. Fig. 5 *A*). The labeling for α-actinin was generally closer to the membrane at CC sites than it was at FA sites.

(*c*) ECM Contacts: The connecting cables at these sites always became labeled for fibronectin. While this indicates the cables contained fibronectin, it of course does not mean that fibronectin was their only constituent (9, 51, 61, 68). In submembranous regions of ECM sites, α-actinin labeling was generally present, but vinculin labeling occurred less frequently. On the other hand, in such instances where vinculin labeling was found at ECM sites (cf. Fig. 4, center) it was often as dense as at FA sites, whereas other ECM sites nearby (Fig. 4, left) showed no vinculin labeling at all, suggesting that the ECM sites might be of two kinds. In plastic sections that were not immunolabeled, morphological observations of the cables and fibrous strands at ECM sites (Fig. 7) suggested that the connections of these extracellular elements to the cell surface were of two different kinds, one lateral to the surface (Fig. 7 *F*) and the other end-on (Fig. 7 *E*, *G*). The end-on type often exhibited a marked submembranous density (Fig. 7 *G*, lower right; see also Fig. 2 *D* in reference 56). These two independent sets of observations have led us to propose, therefore, that ECM contacts may be of two kinds that show some analogies to the FA and CC sites. We suggest that one kind, referred to as *ECM-1* (Fig. 7), is characterized by submembranous densities and distributions of vinculin and α-actinin resembling FA sites, while the other kind, (*ECM-2* in Fig. 7), more frequent, has α-actinin and no vinculin present underneath the membrane, similar to CC sites.

## On the Transmembrane Ultrastructure of Cell-Substratum Contact Sites

On the basis of the results presented in this paper and work of other investigators, we would like to propose a tentative scheme for the molecular composition and ultrastructure near the membrane at various types of cell-substratum contact sites. This scheme is an elaboration of a version previously presented (15). It is represented in highly diagrammatic form in Fig. 9. In part *A* of that figure, FA and CC sites are depicted, and in part *B*, the two kinds of ECM sites. The structural features incorporated in this scheme include the following:

(*a*) It is suggested that there are at least two kinds of association of intracellular actin-microfilaments with the cytoplasmic surface of the cell membrane: in addition to the well-established end-on attachments at FA sites, we propose that microfilaments can be attached along their lengths (laterally) to the membrane at CC sites. Each of these microfilament-membrane attachments is mediated by a different peripheral protein-integral protein system. The close localization of α-actinin at CC sites (Figs. 5 *A*, *B*; 6 *A*) suggests that protein as a candidate for a peripheral protein at CC sites, as depicted. Similarly, vinculin may be a peripheral protein at FA sites. Different integral membrane proteins, β at CC sites, and α at

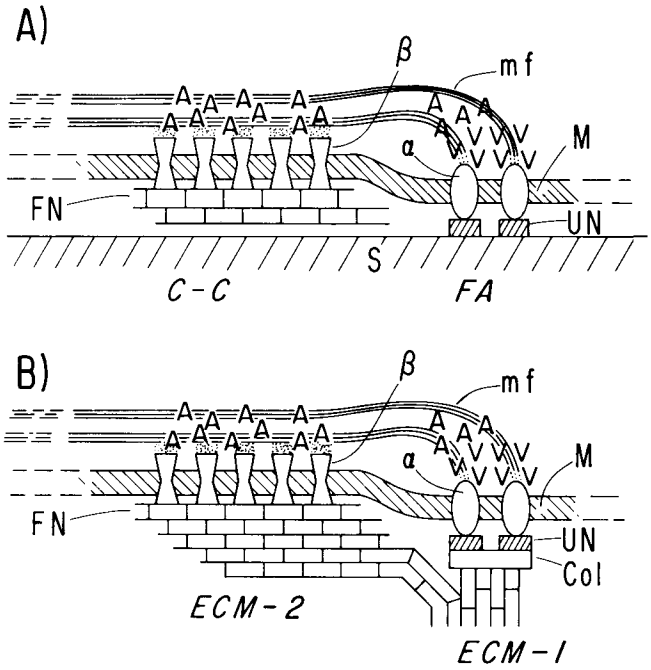


FIGURE 9 Highly schematic representations of the molecular ultrastructure and transmembrane interactions involved at fibroblast-substratum contact sites: (*A*) *FA* and *CC* sites; and (*B*) *ECM-1* and *ECM-2* sites. Similar representations apply to cell-cell contact sites (not shown). *M* designates the ventral surface membrane of the cell; *S*, the substratum; *mf*, microfilaments; *A*, α-actinin, *V*, vinculin; *FN*, extracellular fibronectin; *UN*, unknown factors thought to play a role in cell-substratum adhesion; *Col*, collagen; α and β, two distinct transmembrane integral membrane proteins. At *FA* sites microfilament bundles are known to terminate at the membrane, and vinculin may mediate that attachment by way of the α-proteins. Fibronectin is not involved in *FA* sites. *ECM-1* sites are suggested to be analogous to *FA* sites: fibronectin is pictured in the vicinity, but not directly involved in *ECM-1* attachment to the extracellular matrix. At *CC* sites, it is proposed that microfilaments are laterally associated with the cytoplasmic face of the membrane, perhaps linked to the β integral proteins by α-actinin; fibronectin filaments are pictured as linked to the β-proteins on the outer surface of the membrane. It is proposed that *ECM-2* sites are structurally analogous to *CC* sites. See text for further details.

FA sites, are depicted as spanning the membrane, and at their cytoplasmic domains, to provide specific sites of attachment for their respective peripheral proteins.

(*b*) On the extracellular surface, fibronectin is absent from FA sites, but is linked to the protruding domains of the β proteins at CC sites. (Such an attachment could be indirect, involving still another extracellular peripheral protein). In this manner, parallel arrays of fibronectin filaments on the exterior cell surface and microfilaments on the interior would be linked together. This could provide one way (ECM-2 sites providing another, see below) to account for immunofluorescent (41, 44) and immunoelectron microscopic (56) observations of colinear distributions of fibronectin and actin bundles lying parallel to fibroblast membranes, at either the ventral or dorsal surfaces. On the exterior cell surface at FA sites, some unknown serum factor (62) and/or some non-fibronectin fibrous component (61) (Fig. 7*A*) may be present. It is designated *UN* in Fig. 9, and is depicted as binding to the α integral protein.

(*c*) A conceptual simplification results from the suggestion that ECM contact sites, instead of being entirely different in nature, are related in their molecular ultrastructure to the FA and CC sites as just described. This is schematically represented in Fig. 9 B. The two types of ECM sites, ECM-1 and ECM-2 discussed above, are diagramed as structurally analogous to FA and CC sites, respectively. Fibronectin-containing strands and cables are associated with the membrane at both kinds of ECM sites, but it is proposed that the molecular nature of this membrane association is different at the two ECM sites, direct at ECM-2 and indirect at ECM-1. To be specific about this, but with no direct evidence of our own to support it, we have depicted collagen molecules as mediating the cable connections to the membrane at ECM-1. (Other investigators [cf. 35] have obtained evidence for membrane-collagen connections.) This at least has the virtue of providing a chemical and structural analogy between a membrane-collagen connection in ECM-1 sites and a membrane-gelatin substratum connection in FA sites. A specific prediction of the structural scheme for ECM contact sites depicted in Fig. 9 *B* is that the distinct integral membrane proteins α and β present in ECM-1 and ECM-2 sites, respectively, are the same as those in FA and CC sites, respectively.

It is also presumed that these speculations about the ultrastructures of cell-substratum contact sites apply to the analogous cell-cell sites. An issue that arises with FA and CC cell-cell contact sites, however, is whether their local ultrastructure is symmetrical across the two cell surfaces making the contact (38). For such structures to be symmetrical requires that an axis or a plane of symmetry run through the gaps within the FA and CC sites; i.e., whatever the substances present within the gaps (pictured as *UN* for FA sites and fibronectin for CC sites in Fig. 9 *A*), they must be part of a structure that possesses such symmetry properties.

We accept as obvious that these schematic representations may be oversimplified and incorrect in details, subject to extensive modification as additional information about the ultrastructure of fibroblast contact sites is obtained. They provide, however, a working hypothesis that correlates reasonably well the information that is currently available, and that can be tested by further experiments. The picture in Fig. 9, for example, suggests ways that fibronectin could play its demonstrated important roles in cell shape determination and in cell adhesion (3, 42, 71, 72) without directly participating in FA sites. Extended attachments of the CC type between long parallel arays of fibronectin strands and microfilaments across the membrane could impart rigor to the membrane and thus promote the flattening of the cell on the substratum (3, 71). In addition, fibronectin, by tethering the microfilaments laterally to the membrane as well as tethering the membrane to the substratum at CC sites, could promote the formation nearby of attachments of the ends of the same microfilaments to the membrane (i.e., promote the formation of nearby FA sites) and hence firm cell-substratum adhesions. Such a function for fibronectin might be related to the finding that CC sites containing fibronectin strands are often immediately adjacent to FA sites (cf. Fig. 2*A*, *B*).

Finally, the suggestion that there are distinct α and β integral membrane proteins at FA and CC sites can be explored experimentally. We have, for example, obtained evidence that a particular monoclonal antibody, JG22 (32, 33), prepared against chick embryo muscle cells, is directed to a distinct 140,000-dalton (140-kd) glycoprotein at the cell surface (see also, reference 49), and is concentrated at CC sites of ECH

fibroblast-substratum interfaces, but is absent from FA sites (17; to be published). This glycoprotein is a candidate for a $\beta$ integral protein that is depicted in Fig. 9.

## Localization of Fibronectin

It has been suggested (45, 57) that fibronectin might be present in FA sites, mediating the attachment of the cell to the substratum at those sites. Our immunoelectron microscopy results, however, indicate otherwise: fibronectin was not found in FA sites but was present in CC as well as ECM sites. On the other hand, using primarily light microscopy methods, a number of investigators have sought correlations between the immunofluorescent localization of fibronectin and that of some other protein such as actin or vinculin, or between fibronectin labeling and the interference reflection patterns of the same cells. From such experiments it has been suggested that while fibronectin is not found in all FA sites (and particularly not in those FA which are present at the cell periphery) it is found in some FA sites internal to the cell periphery (4, 45), as well as under unusual conditions of cell culture (57). These conclusions and inferences rely on criteria for the independent identification of focal adhesions by light microscopy methods, for example, as sites where immunofluorescent labeling for vinculin at the ventral surfaces of cells is found (4, 45, 57), or where dark patterns are observed by IRM (4). We believe, however, that these light microscopy methods are ambiguous and cannot lead to definitive conclusions about the relation between fibronectin and focal adhesions for the following reasons.

In the first place, we have observed in our immunoelectron microscopy experiments that vinculin labeling is associated not only with FA sites at the cell periphery, but also with certain ECM contacts well interior to the cell periphery, which we have designated ECM-1 (Fig. 4 *C*, center; Fig. 7). In immunofluorescence experiments, such ECM contacts would be readily accessible to the immunolabeling reagents for fibronectin, because the cell surface is lifted well above the substratum at such sites. Coincident immunofluorescent labeling patterns for vinculin and fibronectin, particularly in cultures that secrete large amounts of fibronectin and can form many ECM contacts, may therefore be due to ECM-1 sites and not FA.

Secondly, dark plaques and streaks observed by IRM cannot unambiguously be used to designate FA sites. Without going into the theoretical details of the IRM method (6, 46), we have demonstrated experimentally that extracellular matrix-produced plaques (Fig. 8 *E–H*, double arrows) and cables (Fig. 8 *A–D*, double arrows) even in the absence of overlying cells can show up as dark features in IRM which are difficult to distinguish from presumably true FA sites (Fig. 8, double arrowheads). Therefore, from a coincidence of immunofluorescence labeling sites for fibronectin with dark patterns in IRM it cannot be concluded that fibronectin is present in FA sites. It seems to us much more likely that such coincidences are due to ECM contact sites.

By micromanipulation studies, Harris (36) has shown that the sites of strong adhesion of a cell to its substratum are largely confined to the cell periphery, and that the rest of the internal ventral surface of a cell is not strongly attached to the substratum. It has also been observed (14), using IRM, that the detachment of the cell periphery from the substratum at the trailing edge of a fibroblast caused a profound and rapid retraction of the cell body which stopped only at another FA elsewhere at the cell margin. Some of the dark IRM streaks under the ventral cell surface were overrun and lost during this retraction. This information is consistent with the suggestion that true FA contact sites are largely confined to the cell periphery, and the internal contact sites that become immunolabeled for fibronectin might instead by mainly of the ECM variety.

Finally, even if the sources of ambiguity just described did not exist, there is the problem of resolution of FA sites in immunofluorescence microscopy. It is known from *en face* studies of FA sites in electron microscopy (37) that the bundles of microfilaments that terminate at the cell membrane often branch out into discrete subplaques that are dispersed within a surrounding close contact area. Such discrete subplaques are of a size generally below the resolution of the light microscope, but probably together constitute the plaque visualized by IRM or vinculin immunofluorescence. It is therefore always possible that a component that is present in CC sites (such as fibronectin) might appear by immunofluorescence to be localized to the FA sites, but actually be confined to the CC areas surrounding the FA sub-plaques.

To summarize: the facts and discussion presented in this section indicate that IRM and immunofluorescence labeling methods in the light microscope suffer from serious limitations in defining FA sites and therefore in establishing an association of fibronectin with such sites. Our immunoelectron microscopic observations, that indicate that fibronectin is not detectable within FA sites, circumvent these limitations. This does not mean that there are no circumstances where fibronectin can be associated with FA sites, but we would suggest that the burden of the proof rests with those who would claim so on the basis of light microscopy observations only.

It might then be suggested that a direct association of fibronectin does exist, if not with FA sites themselves, then with the FA-like sites at ECM-1 type contacts. However, ECM cables such as are involved at these sites most likely contain other fibrous components besides fibronectin, such as glycosaminoglycans (61) and collagen (9, 51, 68), which may be more directly bound to the external surface of the cell at those sites; the involvement of fibronectin at these ECM-1 sites could therefore be totally indirect (see above, Fig. 9).

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