

# Identification of a Membrane-Cytoskeletal Complex Containing the Cell Adhesion Molecule Uvomorulin (E-Cadherin), Ankyrin, and Fodrin in Madin-Darby Canine Kidney Epithelial Cells

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**Abstract.** Cell-cell contact is an important determinant in the formation of functionally distinct plasma membrane domains during the development of epithelial cell polarity. In cultures of Madin-Darby canine kidney (MDCK) epithelial cells, cell-cell contact induces the assembly and accumulation of the  $\text{Na}^+, \text{K}^+$ -ATPase and elements of the membrane-cytoskeleton (ankyrin and fodrin) at the regions of cell-cell contact. Epithelial cell-cell contact appears to be regulated by the cell adhesion molecule uvomorulin (E-cadherin) which also becomes localized at the lateral plasma membrane of polarized cells. We have sought to determine whether the colocalization of these proteins reflects direct molecular interactions which may play roles in coordinating cell-cell contact and the assembly of the basal-lateral domain of the plasma membrane. Recently, we identified a complex of proteins containing the  $\text{Na}^+, \text{K}^+$ -ATPase, ankyrin, and fodrin in extracts of whole MDCK cells (Nelson, W. J., and R. W. Hammerton, 1989. *J. Cell Biol.* 108:893-902). We have now examined cell extracts for protein complexes containing the cell adhesion molecule uvo-

morulin. Proteins were solubilized from whole MDCK cells and fractionated in sucrose gradients. The sedimentation profile of solubilized uvomorulin is well separated from the majority of cell surface proteins, suggesting that uvomorulin occurs in a protein complex. A distinct portion of uvomorulin (30%) cosediments with ankyrin and fodrin ( $\sim 10.5S$ ). Further fractionation of cosedimenting proteins in nondenaturing polyacrylamide gels reveals a discrete band of proteins that binds antibodies specific for uvomorulin,  $\text{Na}^+, \text{K}^+$ -ATPase, ankyrin, and fodrin. Significantly, ankyrin and fodrin, but not  $\text{Na}^+, \text{K}^+$ -ATPase, coimmunoprecipitate in a complex with uvomorulin using uvomorulin antibodies. This result indicates that separate complexes exist containing ankyrin and fodrin with either uvomorulin or  $\text{Na}^+, \text{K}^+$ -ATPase. These results are discussed in the context of the possible roles of uvomorulin-induced cell-cell contact in the assembly of the membrane-cytoskeleton and associated membrane proteins (e.g.,  $\text{Na}^+, \text{K}^+$ -ATPase) at the contact zone and in the development of cell polarity.

**T**HE plasma membrane of polarized epithelial cells is divided into two functionally and biochemically distinct domains, the apical and basal-lateral plasma membranes (for reviews see Simons and Fuller, 1985; Rodriguez-Boulan and Nelson, 1989). The mechanisms involved in the development and maintenance of the characteristic protein composition of these domains are not well understood (Matlin, 1986; Bartles and Hubbard, 1988; Rodriguez-Boulan and Nelson, 1989). However, detailed observations on the distributions of membrane proteins during the development of polarity in cultures of Madin-Darby canine kidney (MDCK) epithelial cells indicate that cell-cell contact plays an important role. For instance, marker proteins of the basal-lateral membrane domain, such as the  $\text{Na}^+, \text{K}^+$ -ATPase, are randomly distributed over the plasma membrane of single cells (Balcarova-Ständer et al., 1984; Nelson and Veshnock, 1986; Sztul et al., 1987; Vega-Salas et al., 1987; Hertzlinger and

Ojakian, 1984). Upon induction of cell-cell contact, these proteins gradually become localized to the basal-lateral membrane (Nelson and Veshnock, 1986; Balcarova-Ständer et al., 1984; Vega-Salas et al., 1987) and, at least in the case of the  $\text{Na}^+, \text{K}^+$ -ATPase (Nelson and Hammerton, 1989), appear to become part of an insoluble cytoskeletal complex (see also Salas et al., 1988). Initiation of cell-cell contact induces the reorganization and assembly of elements of the membrane-cytoskeleton, comprised of ankyrin and fodrin (Nelson and Lazarides, 1984; Bennett, 1985; Marchesi, 1985). Those proteins form a metabolically stable and Triton X-100-insoluble protein matrix (Nelson and Veshnock, 1986, 1987a) that is linked directly to integral membrane proteins (e.g.,  $\text{Na}^+, \text{K}^+$ -ATPase) on the basal-lateral membrane (Nelson and Veshnock, 1987b; Morrow et al., 1989; Nelson and Hammerton, 1989; Nelson, 1989).

Cell-cell contact in epithelial cells is mediated by the junc-

tional complex which includes five distinct components (Farquhar and Palade, 1963; Edelman, 1986; Takeichi, 1988): zonula occludens (tight junction); zonula adherens; desmosomes; gap junctions; and species-specific, but homologous,  $\text{Ca}^{++}$ -dependent adhesion molecules (termed uvomorulin, E-cadherin, L-CAM, CAM 120/80). Recent studies have indicated that uvomorulin mediates an early adhesion event between epithelial cells that appears to be a prerequisite for the assembly of other components of the junctional complex (Gumbiner et al., 1988). This early adhesion event involves homotypic interactions between uvomorulin molecules on adjacent cells that lead to cell aggregation (Nagafuchi et al., 1987). However, homotypic interactions between uvomorulin do not appear to be sufficient to induce cell aggregation: L cells transfected with a truncated uvomorulin cDNA lacking the coding sequence for part of the cytoplasmic domain do not aggregate (Nagafuchi and Takeichi, 1988), suggesting that interactions between the cytoplasmic domain of uvomorulin and cytoplasmic proteins are important for uvomorulin function in regulating cell-cell interactions (see also Ozawa et al., 1989).

Here we show that a portion of the uvomorulin pool in MDCK epithelial cells is present in a complex containing elements of the basal-lateral membrane-cytoskeleton. This complex, containing uvomorulin and the membrane-cytoskeletal components ankyrin and fodrin, occurs in whole cell detergent extracts that have been fractionated in sucrose gradients and then in nondenaturing polyacrylamide gels. The identification of this complex supports a model in which cell-cell contact, through uvomorulin, plays a role in inducing the assembly of the membrane-cytoskeleton and associated integral membrane proteins (e.g.,  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase; Nelson and Hammerton, 1989) at the contact zone between cells, leading to the formation of the basal-lateral domain of the plasma membrane.

## Materials and Methods

### Cells

A clone of MDCK cells (Nelson and Veshnock, 1986) was maintained at single cell density for 60 h by plating cells at a low cell density ( $\sim 1.5 \times 10^4$  cells/cm<sup>2</sup>) and then trypsinizing and replating at that cell density every 18–24 h. At the end of this preculture step, the "contact naive" cells were trypsinized and plated on hydrated collagen gels in 35-mm<sup>2</sup> petri dishes at confluent cell density ( $3 \times 10^5$  cells/cm<sup>2</sup>) in DME containing 5  $\mu\text{M}$   $\text{Ca}^{++}$  and 10% dialyzed FBS (low  $\text{Ca}^{++}$ -containing medium). Under these culture conditions there is little or no cell-cell contact and no assembly of elements of the junctional complex (Nelson and Veshnock, 1987a; Pasdar and Nelson, 1988). Cell-cell contact was induced in these cultures after 8–16 h by raising the  $\text{Ca}^{++}$  concentration of the growth medium to 1.8 mM. Cell surface-exposed proteins on MDCK cells were labeled with <sup>125</sup>I using the lactoperoxidase method (Antonicek et al., 1987) as described (Nelson and Hammerton, 1989).

### Cell Extraction and Fractionation of Solubilized Proteins on Sucrose Gradients

Confluent monolayers of MDCK cells in low  $\text{Ca}^{++}$ -containing medium were transferred to 4°C and rinsed twice with ice-cold buffer containing 15 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1 mM PMSF. After completely draining the petri dish of wash buffer, the cells were extracted for 5 min on a rocking platform in 300  $\mu\text{l}$  of extraction buffer containing 15 mM Tris-HCl, pH 7.5, 120 mM NaCl, 25 mM KCl, 2 mM EGTA, 2 mM EDTA, 0.1 mM DTT, 0.5 mM PMSF, and 0.5% (vol/vol) Triton X-100. The cells were scraped off the petri dish in extraction buffer, and the cell residue was

pelleted at 48,000 g for 10 min. The resulting supernatant was layered onto a 3.8-ml linear 5–20% (wt/wt) sucrose gradient buffered in 15 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.1 mM DTT. The gradients were centrifuged at 60,000 rpm for 5 h at 4°C in the SW60Ti rotor (Beckman Instruments, Inc., Fullerton, CA) of the L8-70M ultracentrifuge (Beckman Instruments, Inc.) then fractionated from the bottom to the top into 20 fractions of 200  $\mu\text{l}$  each. Fractions were either processed immediately or frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  (for further details see Nelson and Hammerton, 1989).

### Immunoprecipitation

5–10  $\mu\text{l}$  of affinity-purified uvomorulin antibodies or  $(\text{NH}_4)_2\text{SO}_4$ -precipitated nonimmune serum were added to 200  $\mu\text{l}$  of sucrose gradient fractions. After 1–2 h at 4°C, protein A-Sepharose CL-4B beads (Pharmacia Fine Chemicals, Piscataway, NJ) were added to the samples. After 45 min on a rocking platform, the beads were sedimented at 12,000 g for 1 min and washed three times in a buffer containing 15 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.1 mM DTT, and 0.5% (vol/vol) Triton X-100. The final pellet of beads was boiled in SDS sample buffer (Laemmli, 1970), and the proteins were processed for SDS 5–12.5% linear gradient PAGE.

### PAGE

Protein complexes separated on sucrose gradients were analyzed further by nondenaturing PAGE (Morrow and Haigh, 1983). Linear 2–4% polyacrylamide gels were cast without a stacking gel (Nelson and Hammerton, 1989). Fractions (80–120  $\mu\text{l}$ ) were applied directly from the sucrose gradients and electrophoresed at a constant 50 V for  $\sim 60$  h at 4°C; the running buffer was changed every 12 h. Alternatively, fractions from the sucrose gradient were diluted with 4 $\times$  SDS sample buffer and denatured (at 100°C for 5 min), and then the proteins were separated by electrophoresis in SDS 5 or 10% polyacrylamide gels (Laemmli, 1970).

### Antibodies

The uvomorulin antibody used in the biochemical analysis was raised in rabbits against purified extracellular domain (86 kD) of mouse uvomorulin. The specificity of the antibody has been characterized and reported previously (Vestweber et al., 1987; Boller et al., 1985). Affinity-purified antibodies were isolated using the extracellular domain of uvomorulin bound to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals). The morphological analysis of uvomorulin by immunofluorescence was performed using this antibody and a polyclonal rabbit antibody raised against the purified extracellular domain of uvomorulin from canine kidney. The antibody reacts specifically with uvomorulin (Shore, E. M., and W. J. Nelson, manuscript in preparation). Antibodies against ankyrin, fodrin, and  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase have been described and characterized previously (for details see Nelson and Veshnock, 1986, 1987b; Nelson and Hammerton, 1989).

### Immunoblotting

Proteins were transferred electrophoretically from either nondenaturing or SDS-polyacrylamide gels onto sheets of nitrocellulose in a buffer containing 15 mM Tris-acetate, pH 8.3, 0.1% (wt/vol) SDS, 20% isopropanol for 3 h at 250 mA (Towbin et al., 1979). Transferred proteins were visualized with India ink, and proteins were identified by immunoblotting with specific antisera. All whole antisera were precipitated at 50% saturation with  $(\text{NH}_4)_2\text{SO}_4$  and used at a dilution of 1:1,000. The affinity-purified uvomorulin antibodies were used at a 1:100 dilution. Antigen-antibody complexes were identified with <sup>125</sup>I-labeled protein A and autoradiography as described previously (Nelson and Veshnock, 1986). Protein distributions in sucrose gradient fractions were determined by scanning densitometry of the resulting autoradiograms using a spectrophotometer (DU-7; Beckman Instruments, Inc.) equipped for automatic integration.

### Immunofluorescence

The preparation of MDCK cells for cryosectioning and subsequent immunofluorescence microscopy will be reported in detail elsewhere (Wang et al., 1990). Briefly, cells were fixed in a freshly prepared solution containing 2% (wt/vol) paraformaldehyde, 75 mM L-lysine, and 10 mM sodium metaperiodate, pH 7.2, and embedded in agar blocks which were infused with sucrose and frozen in isopentane supercooled with liquid nitrogen.

1- $\mu$ m cryosections were cut from the frozen blocks at  $-25^{\circ}\text{C}$ , collected on polylysine-coated glass slides and processed for indirect immunofluorescence microscopy (Pasdar and Nelson, 1988). Cells grown on coverslips were processed for immunofluorescence as described previously (Nelson and Veshnock, 1986; Pasdar and Nelson, 1988). Cells and sections were viewed in a microscope (Axiophot; Carl Zeiss, Inc., Thornwood, NY) equipped with epifluorescence and photographed with Tri-X-Pan film (Eastman Kodak Co., Rochester, NY).

## Results

### Similar Distributions of Uvomorulin, Ankyrin, and Fodrin in Polarized MDCK Cells

Indirect immunofluorescence of confluent monolayers of polarized MDCK cells grown in the presence of cell-cell contact reveals extensive overlap in the distributions of uvomorulin and the membrane-cytoskeletal proteins ankyrin and fodrin (Fig. 1). En face views of cells stained with uvomorulin antibodies (Fig. 1 *B*) show prominent staining at the plasma membrane at the contact zone between adjacent cells (*arrow*), similar to that of fodrin and  $\text{Na}^+, \text{K}^+$ -ATPase (see Nelson and Veshnock, 1986). Staining of monolayers of polarized MDCK cells cryosectioned perpendicular to the substratum shows that uvomorulin is localized to the entire length of the lateral plasma membranes of adjacent cells (Fig. 1 *C*). Little or no staining is found on the apical membrane. Staining of similar cryosections with ankyrin or  $\alpha$ -fodrin (Fig. 1, *D* and *E*, respectively) antibodies shows a distinct overlap in the distributions of these components of the membrane-cytoskeleton with that of uvomorulin; all cells exhibit strong staining along the lateral membrane, with additional staining at the basal membrane (Fig. 1, *D* and *E*). The  $\text{Na}^+, \text{K}^+$ -ATPase, a marker protein of the basal-lateral membrane domain, is localized prominently to the lateral and basal membranes of polarized MDCK cells but is absent from the apical membrane (Fig. 1 *F*).

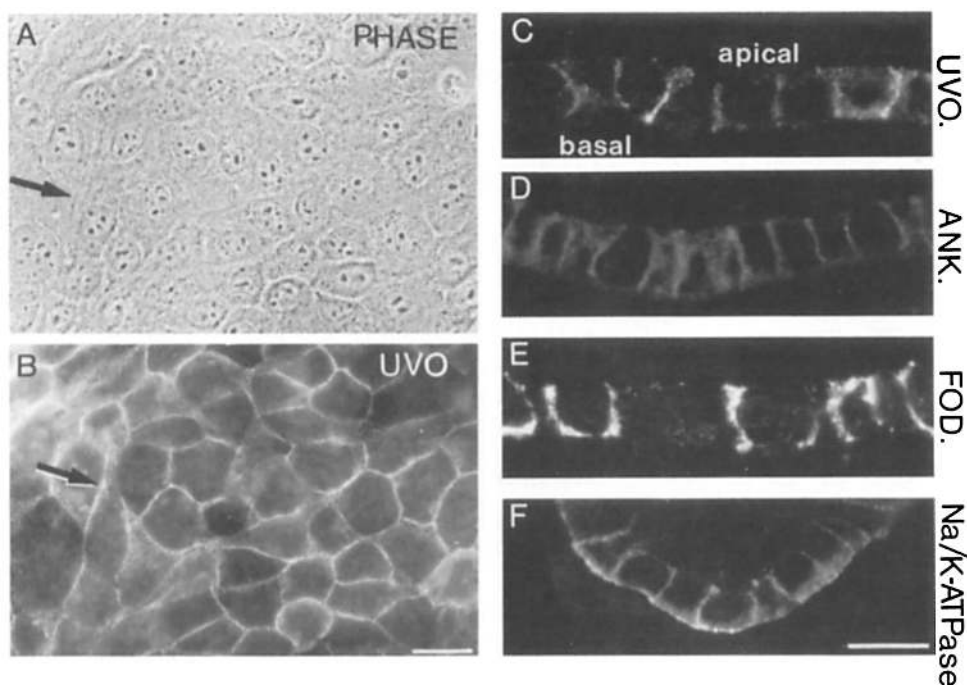
### Solubility Properties and Distribution of Uvomorulin in MDCK Cells Grown in the Absence or Presence of Extensive Cell-Cell Contact

In the presence of extensive cell-cell contact (Fig. 2 *a*; *HC-M*),  $\sim 65\%$  of uvomorulin remains insoluble after extraction with an isotonic buffer containing Triton X-100. Under identical culture conditions,  $>70\%$  of fodrin (Nelson and Veshnock, 1986) and  $>55\%$  of  $\text{Na}^+, \text{K}^+$ -ATPase (Nelson and Hammerton, 1989) are not extracted from these cells. Analysis of the distribution of the Triton X-100-insoluble pool of uvomorulin in these cells shows that it is localized predominantly to the contact zone between adjacent cells (Fig. 2 *b*). This distribution is identical to those of fodrin, ankyrin, and  $\text{Na}^+, \text{K}^+$ -ATPase (for examples see Fig. 1).

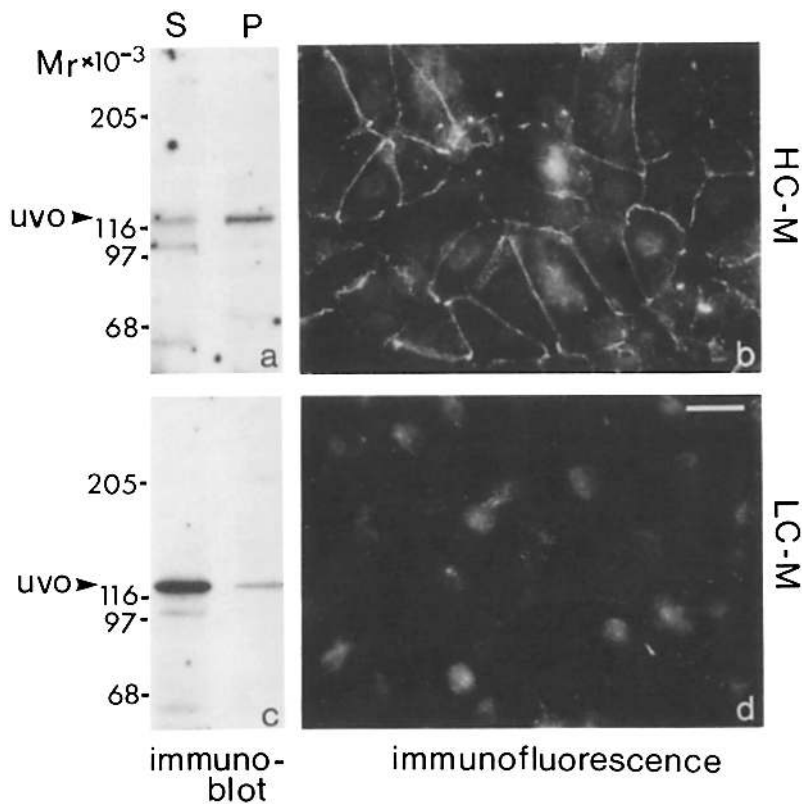
In the absence of extensive cell-cell contact (Fig. 2 *c*; *LC-M*),  $>90\%$  of uvomorulin is extracted from the cells in isotonic buffer containing Triton X-100. (Under these extraction conditions we have obtained similar results in the presence or absence of  $\text{Ca}^{++}$  in the extraction buffer; data not shown.) The large proportion of uvomorulin solubilized from MDCK cells in the absence of cell-cell contact is similar to the fractions of fodrin ( $>70\%$ ; Nelson and Veshnock, 1986) and  $\text{Na}^+, \text{K}^+$ -ATPase ( $\sim 90\%$ ; Nelson and Hammerton, 1989) extracted. Immunofluorescence analysis of MDCK cells grown in low  $\text{Ca}^{++}$ -containing medium and then extracted with isotonic buffer containing Triton X-100 before fixation reveals a reduced fluorescence signal compared with cells in extensive contact with each other. The uvomorulin staining pattern in these cells is relatively diffuse (Fig. 2 *d*). Previous studies have shown that fodrin and  $\text{Na}^+, \text{K}^+$ -ATPase also are diffusely distributed in MDCK cells grown in the absence of extensive cell-cell contacts (Nelson and Veshnock, 1986).

### Fractionation of Solubilized Uvomorulin on Sucrose Gradients

The colocalization of uvomorulin, ankyrin, and fodrin at the



**Figure 1.** Localization of uvomorulin, ankyrin, fodrin, and  $\text{Na}^+, \text{K}^+$ -ATPase in confluent monolayers of MDCK cells. Monolayers of MDCK cells grown under conditions of cell-cell contact were processed for indirect immunofluorescence either as whole mount cultures grown on coverslips (*A* and *B*) or as 1- $\mu$ m cryosections cut perpendicular to the substratum (*C-F*). Cells were stained with antibodies specific for either uvomorulin (*B* and *C*; *A* is the phase-contrast image of the immunofluorescence image of *B*), ankyrin (*D*),  $\alpha$ -fodrin (*E*), or the  $\alpha$  subunit of  $\text{Na}^+, \text{K}^+$ -ATPase (*F*). Bars, 10  $\mu\text{m}$ .



**Figure 2.** Extraction of uvomorulin from MDCK cells and location of the Triton X-100-insoluble pool of uvomorulin. Confluent monolayers of MDCK cells grown in the presence (*HC-M*; *a* and *b*) or absence (*LC-M*; *c* and *d*) of cell-cell contact were extracted with an isotonic buffer containing 0.5% Triton X-100 as described in Materials and Methods. For immunoblotting (*a* and *c*), equivalent amounts of the resulting soluble (*S*) and insoluble (*P*) cell extracts were separated on a SDS 5–12.5% gradient polyacrylamide gel, and then the proteins were transferred electrophoretically to nitrocellulose and reacted with uvomorulin antibodies. A prominent band corresponding to uvomorulin (120,000  $M_r$ ) is identified in the immunoblots (arrowhead). Positions of molecular weight markers are shown: myosin (205,000  $M_r$ ),  $\beta$ -galactosidase (116,000  $M_r$ ), phosphorylase b (97,000  $M_r$ ), and BSA (68,000  $M_r$ ). For indirect immunofluorescence (*b* and *d*), cells were fixed with 1.75% (wt/vol) formaldehyde after extraction. Cells were incubated with antibodies specific for uvomorulin followed by goat anti-rabbit IgG conjugated to biotin and then streptavidin-Texas red. Representative fields of cells were photographed with similar exposures. Note extensive staining of cell-cell contacts in cells grown in high  $Ca^{++}$  containing medium (*b*), but reduced fluorescence signal and diffuse staining in cells grown in low  $Ca^{++}$  containing medium (*d*). Bar, 20  $\mu$ m.

plasma membrane of confluent monolayers of polarized MDCK cells raises the possibility that these proteins may interact. Our strategy to investigate the association of integral membrane proteins with the membrane-cytoskeleton is based on the analysis of protein complexes extracted gently from whole cells in an isotonic buffer containing 0.5% Triton X-100 (for details see Nelson and Hammerton, 1989). However, as shown in Fig. 2 *a*; uvomorulin is relatively resistant to extraction from confluent MDCK cells in which there is extensive cell-cell contact. We have reported previously that high concentrations of salts or chaotropic agents are required to extract this class of proteins but that this results in dissociation of integral membrane proteins from complexes containing ankyrin and fodrin (Nelson and Hammerton, 1989; see also Davis and Bennett, 1986). Consequently, we have sought to analyze the pool of proteins that can be gently extracted from MDCK cells grown in the absence of extensive cell-cell contacts before their assembly into the insoluble membrane-cytoskeleton. As shown in Fig. 2 *c*, >90% of uvomorulin is extracted from these MDCK cells, and previous studies have shown that fodrin is also relatively soluble (>70%) (Nelson and Veshnock, 1986). Significantly, we have presented evidence previously that this soluble pool of fodrin has characteristics expected of a precursor of the insoluble membrane-cytoskeleton (Nelson and Veshnock, 1987a).

Proteins were extracted in an isotonic buffer containing Triton X-100 from MDCK cells grown in the absence of cell-cell contact. Solubilized proteins were separated on linear 5–20% (wt/wt) sucrose gradients. Immunoblotting of proteins from sucrose gradient fractions with affinity-purified uvomorulin antibodies reveals that the distribution of

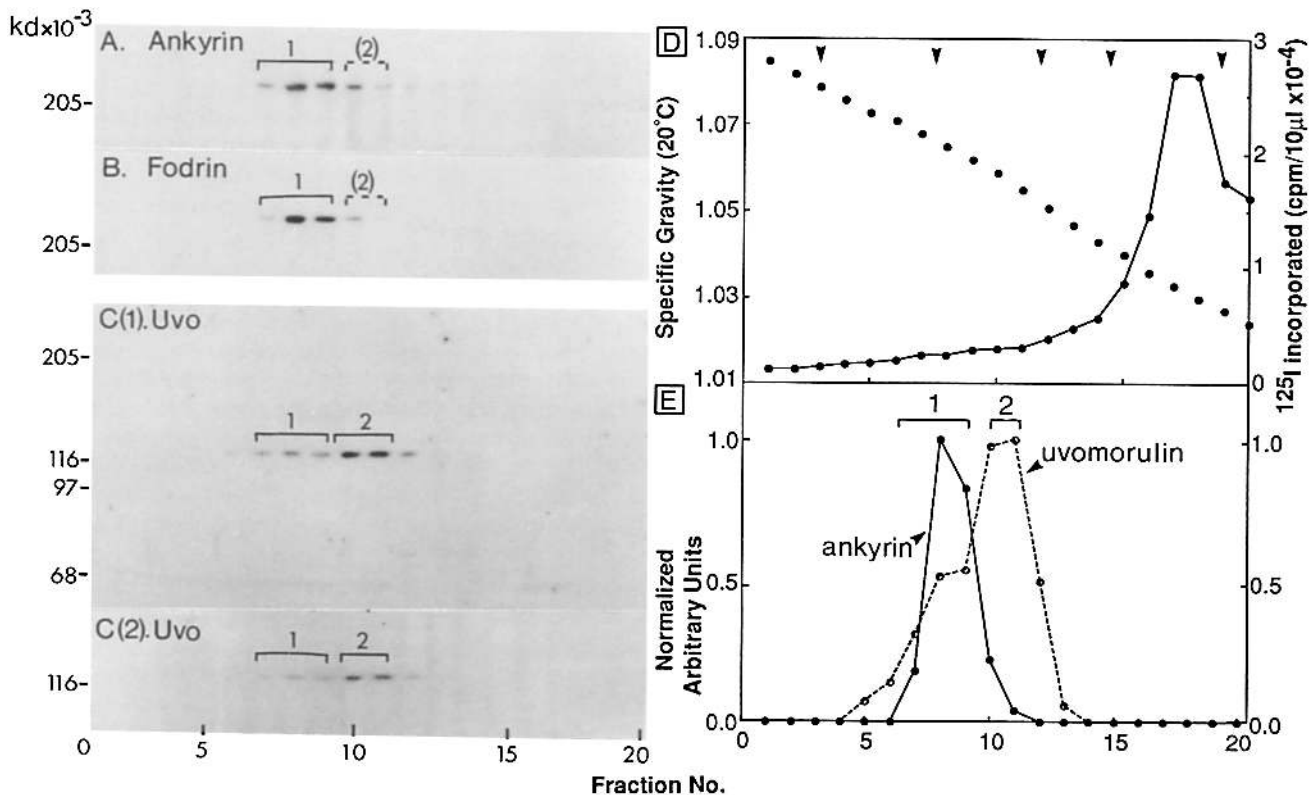
uvomorulin is well separated from that of the majority of proteins that had been labeled with  $^{125}I$  on the cell surface (Fig. 3, *D* and *E*). The distribution profile of uvomorulin is biphasic (Fig. 3, *C* and *E*) with peak 1 in fractions 7–9 ( $\sim 10.5S$ ) and peak 2 in fractions 10–12 ( $\sim 8S$ ). Regression analysis reveals that 30% of the uvomorulin is present in peak 1 and 70% in peak 2.<sup>1</sup> We have consistently detected a biphasic distribution of uvomorulin in these sucrose gradients in five independent experiments; immunoblots from two of these experiments are presented (Fig. 3 *C*). In addition, it is noteworthy that increasing or decreasing the time of centrifugation resulted in a commensurate shift in the biphasic sedimentation profile of uvomorulin (data not shown).

Comparison of the biphasic distribution of uvomorulin in sucrose gradients with the distributions of ankyrin and fodrin (Fig. 3, *A–C*) by immunoblotting with specific antibodies shows that ankyrin and fodrin cosediment in a single symmetrical peak of proteins in fractions 7–9 at  $\sim 10.5S$  (see also Nelson and Hammerton, 1989), overlapping the small peak (peak 1) of uvomorulin (Fig. 3 *E*). We detect little or no ankyrin or fodrin in fractions 10–12 that contain the remaining 70% of uvomorulin (peak 2) (Figure 3E).

#### **Ankyrin, Fodrin, and a Fraction of Uvomorulin Comigrate in Nondenaturing Polyacrylamide Gels**

Sucrose gradient fractions 6–13 were further analyzed by electrophoresis through nondenaturing 2–4% polyacryl-

1. Data were fitted to a mixture of two gaussian curves and to a single gaussian curve using a nonlinear least squares routine. A likelihood ratio test (chi-squared = 24.1; 2 degrees of freedom) was significant at the  $p < 0.001$  level, rejecting the single gaussian fit.



**Figure 3.** Sedimentation of solubilized uvomorulin, ankyrin, and fodrin in sucrose gradients. Confluent monolayers of MDCK cells grown in low  $\text{Ca}^{++}$ -containing medium were extracted with an isotonic buffer containing Triton X-100. The solubilized proteins were sedimented on 5–20% sucrose gradients in an isotonic buffer. Gradients were fractionated (20 fractions), and the distribution of proteins was determined by immunoblotting with specific antibodies to ankyrin (A),  $\alpha$ -fodrin (B), or uvomorulin (C, 1 and 2; these represent the results of two independent experiments). The autoradiograms from the Western blots (A and C) were quantified by scanning densitometry (E); the values obtained were normalized to the highest value (peak fraction), which was assigned a value of 1. Note that two peaks of uvomorulin are resolved: peak 1 ( $\sim 10.5S$ ) and peak 2 ( $\sim 8S$ ). (D) Distribution on the sucrose gradients of  $^{125}\text{I}$ -labeled surface proteins extracted from MDCK cells (●—●); arrowheads correspond to the sedimentation of standard proteins of known Svedberg unit of sedimentation coefficient values (apoferritin, 17.2S; catalase, 11.35S; aldolase, 7.35S; BSA, 4.6S; cytochrome C, 1.7S).

amide gels (Fig. 4). Proteins were transferred electrophoretically to nitrocellulose, detected by staining with India ink (Fig. 4 A), and reacted with affinity-purified uvomorulin antibodies (Fig. 4 B). India ink staining reveals two major bands of proteins in fractions 7–11 (*open and solid arrowheads*). Immunoblotting shows that uvomorulin antibodies react with the slower migrating of these two bands of proteins (*open arrowheads*) in fractions 8–10 but have little or no reaction with the faster migrating band of proteins (*solid arrowhead*). In addition, a second protein band in fractions 11–13, that has a faster electrophoretic mobility than either of the two major India ink-stained bands in fractions 7–10, also reacts with the uvomorulin antibodies (Fig. 4 B, *solid circle*).

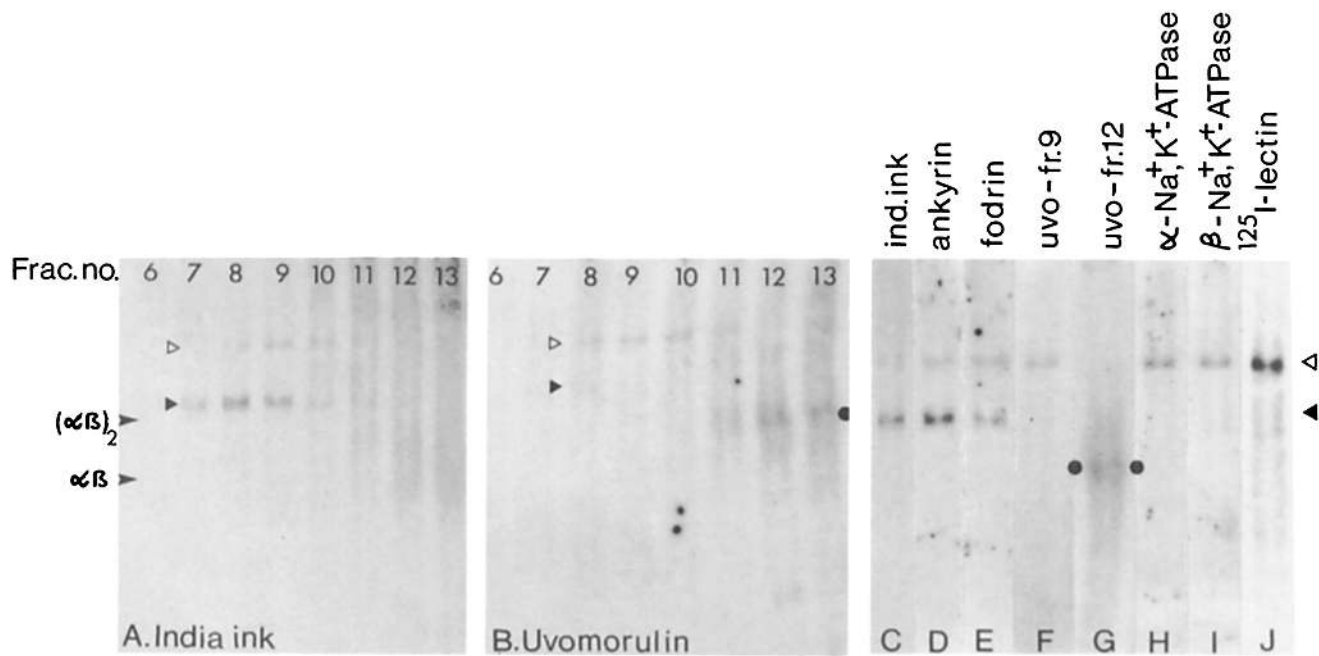
Immunoblotting with ankyrin (Fig. 4 D) or fodrin (Fig. 4 E) antibodies shows that both of the major India ink-stained protein bands in fractions 7–10 react with these antibodies, as shown in detail previously (Nelson and Hammerton, 1989). Both of these bands of proteins have electrophoretic mobilities slower than those of purified spectrin heterotetramers and dimers. The slower migrating band of ankyrin and fodrin (*open arrowhead*) has an electrophoretic mobility identical to that of the slower migrating protein band that binds uvomorulin antibodies (compare Fig. 4, D–F). The

second protein band (*solid circle*) that reacts with uvomorulin antibodies (from fractions 11–13) does not react with either ankyrin or fodrin antibodies (compare Fig. 4, D–G).

#### **Uvomorulin and $\text{Na}^+, \text{K}^+$ -ATPase Are in Separate Complexes with Ankyrin and Fodrin**

Comparison of the electrophoretic mobilities of uvomorulin with the  $\alpha$  and  $\beta$  subunits of  $\text{Na}^+, \text{K}^+$ -ATPase in nondenaturing polyacrylamide gels reveals that uvomorulin and  $\text{Na}^+, \text{K}^+$ -ATPase comigrate in the same India ink-stained protein band together with ankyrin and fodrin (Fig. 4, D–F, H and I). This result raises the possibility that this protein band comprises a large complex of proteins. To investigate this, we immunoprecipitated proteins from sucrose gradient fractions with affinity-purified uvomorulin antibodies and then analyzed the immunoprecipitates for the presence of ankyrin, fodrin, and  $\text{Na}^+, \text{K}^+$ -ATPase (Fig. 5).

Fig. 5 A shows a Western blot of uvomorulin immunoprecipitates that were reacted with  $\alpha$ -fodrin antibodies. Fodrin is detected in fractions 7–10 ( $\sim 10.5S$ ); ankyrin had an identical distribution (data not shown). This distribution is identical to that of fodrin in the sucrose gradient (Fig. 3 B) and to that of the small peak of uvomorulin (peak 1,  $\sim 10.5S$ ; Fig. 3, C and E). Note that a nonimmune serum did not immuno-



**Figure 4.** Nondenaturing PAGE of protein complexes containing uvomorulin, ankyrin, fodrin, and  $\text{Na}^+, \text{K}^+$ -ATPase. Solubilized MDCK cell proteins in sucrose gradient fractions (6–13) were separated in nondenaturing 2–4% gradient polyacrylamide gels. Proteins were transferred electrophoretically to nitrocellulose, stained with India ink (A and C), and immunoblotted with antibodies specific for uvomorulin (B, F, and G), ankyrin (D),  $\alpha$ -fodrin (E), the  $\alpha$  or  $\beta$  subunits of  $\text{Na}^+, \text{K}^+$ -ATPase (H and I, respectively), or  $^{125}\text{I}$ -labeled lectins (J; a mixture of wheat germ agglutinin, concanavilin A, and *Helix pomatia*). A and B show the immunoblots of fractions 6–13 from the sucrose gradients; C–E and H–J represent pooled sucrose gradient fractions 7–10; F and G represent fractions 9 and 12, respectively. Three major bands of proteins are detected; one protein band (open arrowhead) binds antibodies to uvomorulin (B and F), ankyrin (D),  $\alpha$ -fodrin (E), and the  $\alpha$  and  $\beta$  subunits of  $\text{Na}^+, \text{K}^+$ -ATPase (H and I, respectively); a second protein band (solid arrowhead) only binds antibodies to ankyrin (B) and  $\alpha$ -fodrin (E); a third protein band (solid circle) reacts with uvomorulin antibodies in fractions 11–13 (B and G). The electrophoretic mobilities of purified spectrin heterotetramers ( $[\alpha\beta]_2$ ) and dimers ( $[\alpha\beta]$ ) (purified by methods described in Bennett, 1983) are shown for comparison with the protein complexes containing ankyrin, fodrin, and integral membrane proteins.

precipitate fodrin from fraction 9 (Fig. 5 B), supporting the specificity of the coimmunoprecipitation of fodrin and uvomorulin with uvomorulin-specific antibodies.

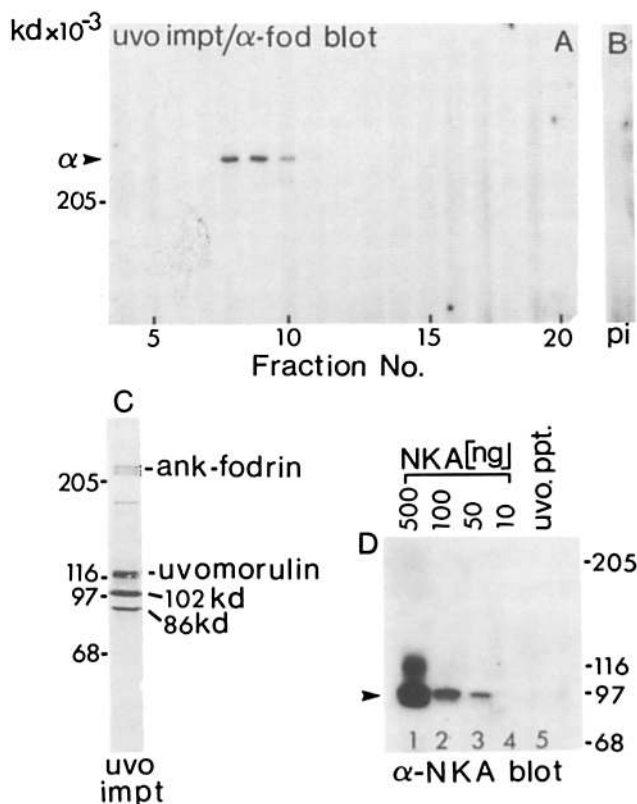
To visualize other proteins coimmunoprecipitated with uvomorulin, we analyzed proteins extracted from cells that had been metabolically labeled with  $^{35}\text{S}$ methionine. Sucrose gradient fractions 7–13, which include the total distribution of uvomorulin (Fig. 3, C and E), were quantitatively immunoprecipitated with uvomorulin antibodies. Fig. 5 C shows the resulting autoradiogram. Coimmunoprecipitated with uvomorulin (120,000 *M*) are a discrete number of proteins with molecular weights of  $\sim 240,000$ , 230,000, 215,000, 102,000, and 86,000. The group of high molecular mass proteins (240,000, 230,000, and 215,000 D) have electrophoretic mobilities in the SDS-polyacrylamide gel similar to those of fodrin subunits and ankyrin, respectively (see Fig. 5, A and C). The presence of the 102,000- and 86,000-D proteins in immunoprecipitates of uvomorulin has been described previously (Peyrieras et al., 1985; Ozawa et al., 1989); the identity and function of these proteins is at present unknown. Note that an 0.5 M KCl wash of the immunoprecipitates dissociates the complex of ankyrin, fodrin, and uvomorulin (data not shown; see also Nelson and Hammerton, 1989). The stoichiometry of the proteins coimmunoprecipitated with uvomorulin is complex. It should be noted that the fractions of the sucrose gradient (7–13) used in the immunoprecipitation contain the complete sedimentation profile of uvomorulin, of which  $\sim 30\%$  comigrates with ankyrin and

fodrin (Fig. 3). Since ankyrin and fodrin are bound also to other integral membrane proteins in these fractions (e.g.,  $\text{Na}^+, \text{K}^+$ -ATPase; Nelson and Hammerton, 1989), the exact stoichiometry of the proteins in these complexes must await characterization of all components.

To determine whether  $\text{Na}^+, \text{K}^+$ -ATPase ( $\alpha$  subunit; 100,000 *M*) occurs in a complex with uvomorulin, ankyrin, and fodrin, we analyzed uvomorulin immunoprecipitates by immunoblotting with  $\alpha$  subunit  $\text{Na}^+, \text{K}^+$ -ATPase antibodies. We do not detect  $\text{Na}^+, \text{K}^+$ -ATPase in the uvomorulin immunoprecipitate (Fig. 5 D, lane 5); coelectrophoresis and blotting of different quantities of purified  $\text{Na}^+, \text{K}^+$ -ATPase show that the limit of  $\alpha$  subunit  $\text{Na}^+, \text{K}^+$ -ATPase detection is  $\sim 5$  ng of protein (Fig. 5 D, lanes 1–4).

## Discussion

An understanding of mechanisms involved in the spatial organization of proteins in cells is central to the problem of how cells become structurally and functionally polarized. Several lines of evidence suggest an important role of cell–cell contact in the development and maintenance of epithelial cell polarity (see Introduction for references). In the absence of cell–cell contact, many epithelial cell types exhibit little or no polarity in the distributions of plasma membrane and cytoplasmic proteins. However, upon induction of extensive cell–cell contact there is a gradual reorganization of proteins and the development of cell polarity.



**Figure 5.** Coimmunoprecipitation of ankyrin and fodrin in a complex with uvomorulin. (A) Solubilized proteins from MDCK cells were fractionated on sucrose gradients (see Materials and Methods and Fig. 3), immunoprecipitated with uvomorulin antibodies, and processed for immunoblotting with  $\alpha$ -fodrin antibodies. A single protein band is detected (240,000  $M_r$ ; arrowhead). (B) A preimmune rabbit serum (*pi*) was used to immunoprecipitate proteins from fraction 9 of the sucrose gradient. Western blotting with fodrin antibodies showed that no fodrin was immunoprecipitated. (C) Confluent monolayers of MDCK cells were metabolically labeled for 1 h with [ $^{35}$ S]methionine (Nelson and Veshnock, 1986) and extracted with isotonic buffer containing Triton X-100. Solubilized proteins were fractionated on sucrose gradients. Fractions 7-13 (containing the complete sedimentation profile of uvomorulin; see Fig. 3) were combined, immunoprecipitated with uvomorulin antibodies, separated on an SDS 10% polyacrylamide gel, and processed for fluorography. (D)  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase was purified from canine kidney outer medulla (Jørgensen, 1974), and various quantities of protein (10-500 ng) were electrophoresed in an SDS 5-10% linear gradient polyacrylamide gel (lanes 1-4). Fractions 7-13 from a sucrose gradient of solubilized MDCK cell proteins were combined, then immunoprecipitated with uvomorulin antibodies, and electrophoresed (lane 5) in the same gel as the purified  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase. Proteins were transferred electrophoretically to nitrocellulose and processed for immunoblotting with antibodies specific for the  $\alpha$  subunit  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase (100,000,  $M_r$ ; arrowhead). The reactive protein band with an electrophoretic mobility slower than that of the  $\alpha$  subunit  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase is a nonreduced complex of  $\alpha\beta$   $\text{Na}^+$ ,  $\text{K}^+$ -ATPase (e.g., see Nelson and Veshnock, 1987b). Note that overexposed autoradiograms revealed the presence of a faint band of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase  $\alpha$  subunit in the lane containing 10 ng purified enzyme. Since the protomeric complex of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase is  $\alpha:\beta$ , the limit of  $\alpha$  subunit detection is  $\sim 5$  ng of protein. No  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase band was detected in the uvomorulin immunoprecipitate in overexposed autoradiograms (lane 5).

Detailed analyses of the molecular mechanisms involved in cell-cell interaction have shown that a family of cell surface proteins plays a critical role in regulating cell-cell recognition and adhesion (Edelman, 1986; Takeichi, 1988). Neutralization of the activity of an appropriate cell adhesion molecule with a specific antibody blocks cell-cell interaction, leads to perturbation of cellular morphogenesis and cell polarity, and results in the disaggregation of cells and tissues (Nagafuchi et al., 1987; Damsky et al., 1983; Hyafil et al., 1981; Behrens et al., 1985; Gallin et al., 1986; Vestweber et al., 1987; Volk et al., 1987; Hirai et al., 1989a,b; for reviews see Edelman, 1986; Takeichi, 1988). However, it is unknown how interactions between cell adhesion molecules on the plasma membranes of adjacent cells are translated by the cells into the spatial reorganization of proteins and the development of cell polarity. It has been suggested that interactions between uvomorulin and cytoplasmic proteins may be important in these events (Hirano et al., 1987; Ozawa et al., 1989; Takeichi, 1988).

### A Membrane-Cytoskeletal Complex Containing Uvomorulin, Ankyrin, and Fodrin

In polarized MDCK cells, uvomorulin occurs over the entire length of the lateral plasma membranes of adjacent cells (Fig. 1; see also Gumbiner and Simons, 1986; Behrens et al., 1985). At the resolution of the light microscope, there appears to be little or no concentration of uvomorulin in the apical zone of the lateral plasma membrane where the zonula adherens occurs in some other cell types (see below). The distribution of uvomorulin overlaps with those of ankyrin and fodrin, which also are localized to the lateral and basal plasma membranes, with none of these proteins detected at the apical membrane (Fig. 1; see also Morrow et al., 1989).

The distribution of uvomorulin in MDCK cells appears to be different from that observed in some other epithelial cell types (Boller et al., 1985; see also Takeichi, 1988). For instance, in intestinal epithelial cells, uvomorulin is localized along the entire lateral membrane, but higher concentrations are localized at the apical aspect of the lateral membrane in the region of the membrane containing the zonula adherens. Significantly, fodrin also has a different distribution in these cells, occurring at the apical membrane as well as being localized to the basal and lateral plasma membranes as in MDCK cells (reviewed by Mooseker, 1985). The fraction of fodrin at the apical membrane is associated with the terminal web of the brush border. Unlike intestinal epithelial cells, MDCK cells have a poorly defined brush border. Thus, differences in the distributions of uvomorulin and fodrin between MDCK cells and intestinal epithelial cells may reflect specializations of the plasma membrane and cytoskeleton in the region of the apical membrane that are associated with the formation of a brush border in the intestinal cells.

In addition to the observation that uvomorulin, ankyrin, and fodrin are colocalized in polarized MDCK cells, we have obtained three lines of biochemical evidence for the existence of a complex containing these proteins in MDCK cells. This complex was identified from a Triton X-100-soluble component of MDCK cells grown under conditions in which there is little or no extensive cell-cell contact and uvomorulin, ankyrin, and fodrin are relatively soluble. First, we showed that the sedimentation profile of solubilized uvomorulin in sucrose gradients is biphasic and that a reproducible portion ( $\sim 30\%$ ) of the profile overlaps the single peak

of cosedimenting ankyrin and fodrin at  $\sim 10.5S$  (Fig. 3). These sedimentation properties of ankyrin and fodrin are distinctly different from those of purified ankyrin or fodrin alone (see Nelson and Hammerton, 1989). Second, analysis of the electrophoretic mobility of the  $10.5S$  peak of cosedimenting proteins in nondenaturing polyacrylamide gels showed that uvomorulin, ankyrin, and fodrin comigrate in a single protein band (Fig. 4). The electrophoretic mobility of this apparent complex of proteins is distinctly slower than that of purified ankyrin, spectrin heterotetramers, or a fraction of uvomorulin ( $8S$ ) not in a complex with ankyrin and fodrin (see below). Third, a complex containing ankyrin, fodrin, and uvomorulin was coimmunoprecipitated with uvomorulin-specific antibodies from sucrose gradient fractions (Fig. 5).

In the sucrose gradients, we identified an  $\sim 8S$  peak of uvomorulin that contains  $\sim 70\%$  of the extracted protein. This fraction of uvomorulin has an electrophoretic mobility in nondenaturing polyacrylamide gels that is distinctly faster than that  $\sim 10.5S$  fraction of uvomorulin that appears to be in a complex containing ankyrin and fodrin. Significantly, the protein band containing the  $8S$  uvomorulin in the nondenaturing polyacrylamide gel does not react with ankyrin or fodrin antibodies. At present we do not know whether the  $\sim 8S$  peak of uvomorulin represents a portion of the protein that dissociated from complexes of ankyrin-fodrin during protein extraction and fractionation or whether it indicates the existence of a pool of uvomorulin in the cell that is not in a complex with these cytoskeletal components. The latter possibility is supported by previous immunofluorescence studies that have shown that uvomorulin also colocalizes at sites of cell-cell contact with other cytoskeletal proteins that include actin, vinculin, and  $\alpha$ -actinin (Hirano et al., 1987; Volk et al., 1987). Those cytoskeletal proteins may be candidates for binding to the  $8S$  fraction of uvomorulin. Such interactions might lead to the localization of a (major) fraction of uvomorulin at specialized membrane sites such as the zonula adherens (e.g., in intestinal epithelial cells) where vinculin,  $\alpha$ -actinin, and actin are concentrated (see Takeichi, 1988). In addition, two cytoplasmic proteins of 102,000 and 86,000  $M_r$  have been shown to coimmunoprecipitate in a complex with uvomorulin (Peyrieras et al., 1985; Ozawa et al., 1989). However, their function(s) remains unknown.

In nondenaturing polyacrylamide gels, the protein band that contains uvomorulin, ankyrin, and fodrin also appears to contain  $\text{Na}^+, \text{K}^+$ -ATPase (Fig. 4). We therefore sought to determine whether uvomorulin occurs in a complex that includes  $\text{Na}^+, \text{K}^+$ -ATPase in addition to ankyrin and fodrin. However, our results showed clearly that ankyrin and fodrin, but not  $\text{Na}^+, \text{K}^+$ -ATPase, were coimmunoprecipitated with uvomorulin in the presence of uvomorulin-specific antibodies. This result indicates the presence of at least two protein complexes containing ankyrin and fodrin heterotetramers: one that contains  $\text{Na}^+, \text{K}^+$ -ATPase and one that contains uvomorulin. It should be noted that the nondenaturing polyacrylamide gel system used here would not be expected to resolve separate complexes—for example, between ankyrin, fodrin, and  $\text{Na}^+, \text{K}^+$ -ATPase and between ankyrin, fodrin, and uvomorulin—into two bands. Since an ankyrin-fodrin heterotetramer has a combined molecular weight of  $\sim 1.3 \times 10^6$ , the addition of either  $\text{Na}^+, \text{K}^+$ -ATPase ( $\sim 140,000$  D) or uvomorulin (120,000 D) is a difference of  $<1.5\%$  in the total molecular mass of the complex and would not be detected.

It will be important in future studies to distinguish other membrane proteins in this complex and to determine their molecular organization.

Unlike the complex containing  $\text{Na}^+, \text{K}^+$ -ATPase, ankyrin, and fodrin that we identified previously (Nelson and Veshnock, 1987b; Nelson and Hammerton, 1989), we do not yet have independent evidence of the direct binding of uvomorulin to ankyrin or fodrin. However, previous studies have reported the binding of fodrin to N-CAM 180, a neural-specific cell adhesion molecule related to uvomorulin (Pollerberg et al., 1987). It also is possible that interactions between ankyrin, fodrin, and uvomorulin may be regulated by other proteins, such as the 102-kD or 86-kD proteins coimmunoprecipitated with ankyrin, fodrin, and uvomorulin (see Fig. 5 C; Ozawa et al., 1989). Alternatively, interactions may be regulated by adducin or protein 4.1, which modulate the formation of complexes between integral membrane proteins, ankyrin, spectrin, and actin in erythrocytes (Bennett et al., 1988; Ungewickell et al., 1979). Recently, adducin has been reported to be localized to the plasma membrane at the contact zone between epithelial cells (Kaiser et al., 1989).

### *Role of Cell-Cell Contact in the Biogenesis of Epithelial Cell Polarity*

Previous studies have shown that there is a close temporal and spatial interrelationship between cell-cell contact and the generation of epithelial cell polarity (see Rodriguez-Boulan and Nelson, 1989). It is possible that the generation of cell polarity may be initiated by homotypic interactions between uvomorulin molecules on the plasma membranes of adjacent cells. We suggest that this may induce the clustering of membrane proteins and components of the membrane-associated cytoskeleton at the contact zone, as has been suggested for the patching and capping of cell surface receptors in lymphocytes (Bourguignon and Bourguignon, 1984; Nelson et al., 1983). Cytoplasmic structural proteins of the membrane-cytoskeleton may function to facilitate clustering of membrane proteins by forming an interlinked protein matrix (Morrow and Marchesi, 1981). The observed recruitment and increased insolubility of uvomorulin, ankyrin, and fodrin at sites of cell-cell contact in MDCK cells upon cell-cell contact (see Fig. 2; Nelson and Veshnock, 1987a) would be consistent with this process. The gradual assembly of ankyrin-fodrin-based matrix at sites of uvomorulin-induced cell-cell contact may also involve the recruitment of complexes of ankyrin and fodrin that are linked to other integral membrane proteins, for example, the  $\text{Na}^+, \text{K}^+$ -ATPase (Nelson and Veshnock, 1987b; Nelson and Hammerton, 1989; see also Morrow et al., 1989), thus resulting in the development of a polarized distribution of specific proteins at the contact zone and the gradual formation of the basal-lateral membrane domain.

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