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Immunocytochemical localization of tubulin, actin, and myosin in axonemes of ciliated cells from quail oviduct

(immunocytochemistry/electron microscopy/protein transfer/cilia)

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ABSTRACT **Tubulin, actin, and myosin have been localized in isolated demembranated ciliated cells from quail oviduct by immunocytochemistry in both light and electron microscopy by using purified antibodies. The peripheral doublets and the central tubules are stained by the antitubulin whereas the kinetosomes are poorly stained. Actin antibodies clearly stain the axonemes, but only on the proximal-half portion, whereas myosin antibodies stain a small area of the axonemes just above the ciliary neck region.**

Axonemes from cilia and eukaryotic flagella present the definite substructure of nine doublets surrounding two singlet central tubules. These microtubules are associated with different appendages that are necessary components for the movement of the organelle (1). It is well established that the movement of cilia and flagella is produced by the sliding of the doublets of the axonemes (1, 2) involving the tubulin–dynein interaction. In addition to tubulin and dynein, >170 minor polypeptides are involved in the structure of axonema (3). One of the minor polypeptides of isolated axonema from *Chlamydomonas* flagella has been identified as an actin-like protein (4). The association of actin and myosin with the basal bodies of cilia from trachea epithelium has also been suggested by immunocytochemistry by using immune sera (5).

Thus, we have searched for the presence of actin and myosin in cilia from the epithelium of the quail oviduct by using purified antibodies instead of sera. Tubulin was also localized as a positive control to which actin and myosin localization could be compared.

MATERIALS AND METHODS

The oviduct of laying japanese quail (*Coturnix coturnix japonica*) was removed and some magnum segments were quickly frozen and sectioned—without previous fixation—with a cryostat. The sections were 8 μm thick.

Demembranated ciliated cells were obtained by treating the magnum by one of two procedures described by Anderson (6) and Torres *et al.* (7). In procedure 1, the extraction medium (6) consists of 0.25 M sucrose, 20 mM Hepes buffer (pH 7.5), 1 mM EDTA, 25 mM KCl, and 0.05% Triton X-100 (6); with this procedure, the pellet contained mainly demembranated ciliated cells in which the ciliary cortex remained associated to the nucleus. In procedure 2, the extraction medium consists of 0.25 M sucrose, 20 mM Tris·HCl buffer (pH 8.8), 1 mM EDTA, 25 mM KCl, 0.05% Triton X-100, and 0.1% 2-mercaptoethanol (7); with this procedure, the pellet contained mainly demembranated ciliary cortices and isolated nuclei.

Antibodies. The antitubulin antiserum was prepared by immunization of a sheep with porcine brain tubulin prepared according to the method of Shelansky (8). Monospecific antibodies—which have been previously characterized (9)—were purified by immunoabsorption on a column of tubulin purified on phosphocellulose (10). The antiactin and antimyosin antisera were prepared by immunization of rabbits with either actin or myosin from frog skeletal muscle. Monospecific antibodies—which have been previously characterized (11)—were purified by immunoabsorption. Purified, monospecific rabbit anti-rat IgG antibodies—a gift from J. C. Antoine (Institut Pasteur, Paris)—were used in control experiments. Purified peroxidase-labeled sheep anti-rabbit IgG and rabbit anti-sheep IgG were purchased from Institut Pasteur. The antiactin antibody was also directly labeled with peroxidase (12).

Detection of Ciliary Cortex Antigens by Antibody Binding on Blots. Ciliary cortices extracted according to procedure 2 were analyzed on 10% $NaDodSO_4$/polyacrylamide gel electrophoresis (13). The proteins were then transferred onto nitrocellulose sheets by diffusion (14) and the detection of antigens on blots by antibodies was carried out according to Towbin *et al.* by using peroxidase-conjugated antibodies (15).

Immunocytochemistry. Pellets of demembranated cells or cortices were resuspended in a buffer containing 0.1 M KCl and 20 mM Tris·HCl (pH 7.2), centrifuged at 2,000 × *g* in observation chambers (11, 16), and, treated in the chamber for immunocytochemistry with or without prior fixation with 2% paraformaldehyde. Preparations were incubated with purified antibodies (8–30 μg/ml) for 1 hr at 20°C and washed three times in Tris/KCl. The peroxidase-labeled antibodies were then applied (12 μg/ml) for 1 hr. After three washes, the peroxidase activity was revealed (17).

For light microscopy, the bottom coverslip of the observation chambers was removed, mounted in glycerin jelly (Difco), and observed with a Zeiss Ultraphot microscope. For electron microscopy, the stained material was postfixed with 1% OsO_4 for 15 min, dehydrated in ethanol, and embedded in araldite. Thin sections were observed without conventional staining at 60 or 80 kV. Alternatively, frozen sections were incubated with the various antibodies (30 μg/ml). The peroxidase-conjugated antibodies were used at a concentration of 12 μg/ml.

RESULTS

The epithelium of laying quail oviduct, and especially the ciliated cells, has been described (18). It is composed of goblet cells and ciliated cells regularly interspersed. This luminal epithelium covers the tubular gland cells.

Tubulin Localization. On frozen sections observed by light microscopy, the tubulin antibody strongly stains the outer part of the ciliated cells (Fig. 1A *Top*). This staining, about 5 μm high, corresponds to the whole length of cilia. The body of the cell is poorly stained. In tangential sections, the staining is apparently arranged as in a chessboard, with each stained square corresponding to the ciliary apparatus of one cell. The tubulin

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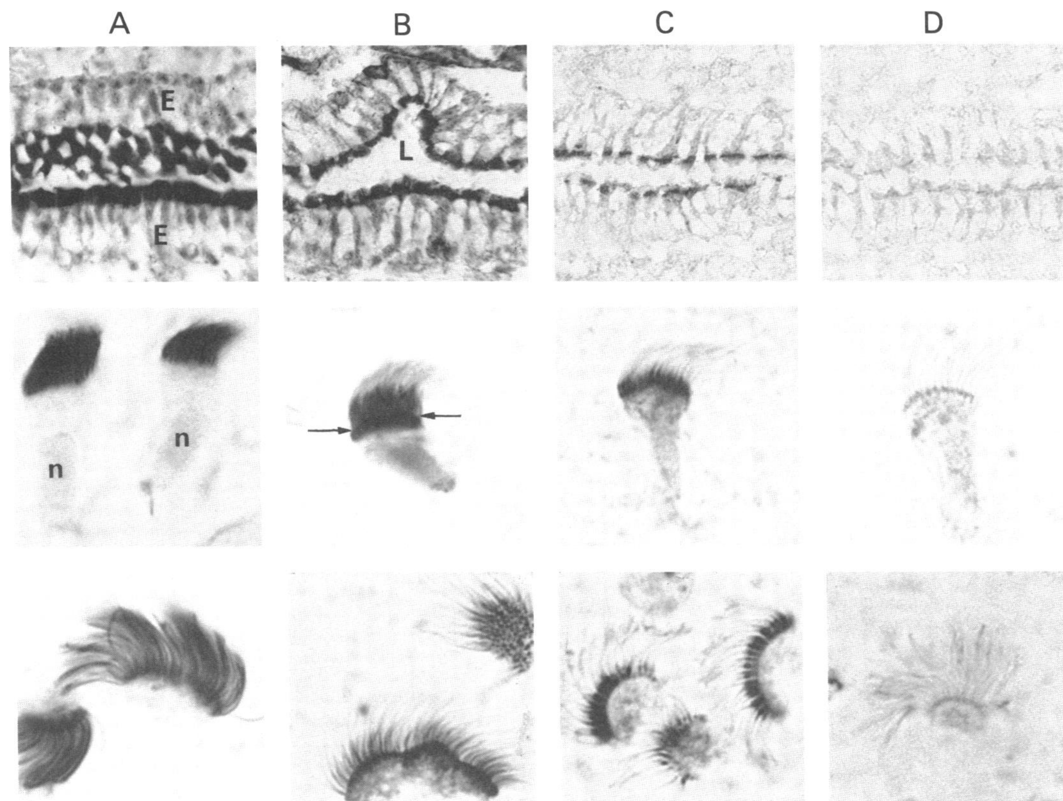

FIG. 1. Tubulin (*A*), actin (*B*), and myosin (*C*) localization by indirect immunoperoxidase method (except *B Bottom*) in light microscopy. (*D*) Control. (*Top*) Frozen sections of oviduct without fixation. (×450.) Antitubulin (*A*), antiactin (*B*), antimyosin (*C*), and anti-rat IgG (*D*) antibodies, each at 30 μg/ml. (*A*) Only the ciliary apparatus of the epithelial cells (E) is stained. (*B*) Only the apical part of the epithelial cells is stained. L, lumen. (*C*) A strong staining is observed on the proximal part of the ciliary apparatus. (*Middle*) Demembranated ciliated cells (*A*, *B*, *C*, *D*). Paraformaldehyde fixation (*A*, *D*) or no fixation (*B*, *C*). (×1,600.) (*A*) Antitubulin antibody, 8 μg/ml. Only the ciliary apparatus is stained; n, nucleus. (*B*) Antiactin antibody, 16 μg/ml. The proximal half of cilia is stained as well as some apical material corresponding to the terminal web. Arrows indicate the beginning of the cilia. (*C*) Antimyosin antibody, 16 μg/ml. (*D*) Anti-rat IgG antibody, 30 μg/ml. (*Bottom*) Isolated ciliary cortices. Paraformaldehyde fixation (*A*, *B*, *C*) or no fixation (*D*). (×1,600.) (*A*) Antitubulin antibody, 16 μg/ml. (*B*) Peroxidase antiactin antibody conjugate, 30 μg/ml. (*C*) Antimyosin antibody, 30 μg/ml. A well-defined staining is observed on the proximal part of cilia. (*D*) Peroxidase-labeled sheep anti-rabbit IgG antibody, 12 μg/ml.

antibody stains almost exclusively the ciliary apparatus of isolated demembranated ciliated cells and ciliary cortices observed by light microscopy (Fig. 1*A Middle* and *Bottom*). However, in ciliary cortices the cilia are not packed together as closely as in demembranated cells, allowing the visualization of individual cilia.

By electron microscopy, it appears that demembranated cells are almost exclusively stained along the whole length of their cilia (Fig. 2 *Upper Right*). Basal bodies are poorly stained and no staining can be found around the basal bodies, on microvilli, in the cytoplasmic material, or in the nuclei. Transverse sections observed at high magnification show that the peripheral doublets as well as the central tubules are decorated by the tubulin antibodies (Fig. 3 *Upper Left*). The radial spokes and the central sheath appear rather electron dense, as if they were stained. However, it is difficult to decide if this corresponds to a true staining or to a diffusion of the diaminobenzidine precipitate. All transverse sections of cilia are stained. This corresponds to the fact that cilia are stained from the proximal part to the tip as seen in longitudinal sections.

Actin Localization. On frozen sections, the actin antibody stains the outer part of the ciliated cells (Fig. 1*B Top*). The staining is weaker than that obtained with antitubulin antibodies and seems to be restricted to the proximal part of the cilia. In demembranated cells and ciliary cortices, the actin antibody clearly stains the proximal-half part of the ciliary apparatus (Fig. 1*B Middle* and *Bottom*). The cytoplasm just beneath the cilia is also strongly stained, whereas it is not stained by the tubulin antibody. Nuclei are faintly stained.

By electron microscopy, the staining also appears to be located on the proximal half of the cilia (Fig. 2 *Lower Left*), on microvilli, and on a cytoplasmic material surrounding the basal bodies. All of this material is bound to the terminal web that is also decorated by this antibody. Observation of transverse sections of cilia reveals the presence of a reaction mainly associated with the peripheral doublets (Fig. 3 *Top Right* and *Middle Right*). The central tubules also seem to be faintly stained. Dynein arms but not the radial spokes may be stained. The dark dots of staining visible around the cilia probably correspond to microvilli. Some axonemal sections are stained and others are not, depending on the level of the section in the axonema.

Myosin Localization. On frozen sections, the myosin antibody stains the proximal part of the ciliary apparatus, whereas the cytoplasm of the cells appears to be quite unstained (Fig. 1*C Top*). In demembranated cells and ciliary cortices, the myosin antibody stains 1.3 μm of the proximal part of the cilia (Fig. 1*C Middle* and *Bottom*).

By electron microscopy, the staining appears to be restricted to the proximal part of cilia (Fig. 2 *Lower Right*). No staining can be found around the basal bodies in the transitional region between basal bodies and cilia or in the cytoplasmic material.

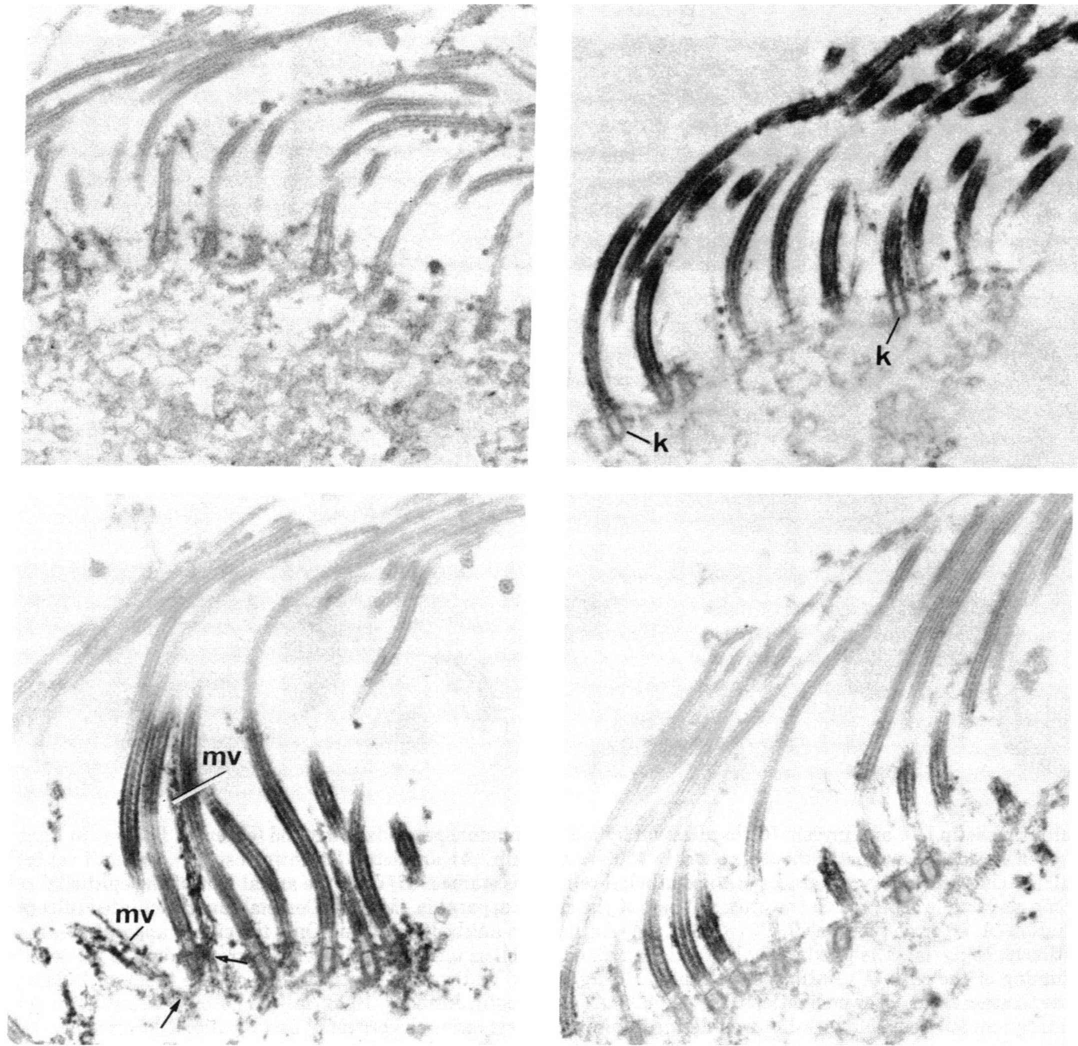

FIG. 2. Ultrastructural immunocytochemistry by indirect immunoperoxidase method. (×15,000.) (*Upper Left*) Control; demembranated ciliated cell; paraformaldehyde fixation. Anti-rat IgG antibody, 30 μg/ml. (*Upper Right*) Tubulin localization; demembranated ciliated cell; paraformaldehyde fixation. Antitubulin antibody, 16 μg/ml. Axonemes are strongly stained whereas kinetosomes (k) are poorly labeled. (*Lower Left*) Actin localization; isolated ciliary cortex; no aldehyde fixation. Antiactin antibody, 8 μg/ml. The proximal half of the axonemes is stained as well as the microvilli (mv). The material that surrounds the kinetosomes is in continuity with the microfilaments of the microvilli (arrows). (*Lower Right*) Myosin localization; isolated ciliary cortex; no aldehyde fixation. Antimyosin antibody, 8 μg/ml. The proximal 1.3 μm of the axonemes is stained.

Although rootlets are occasionally stained, the microvilli are not stained. Transverse sections show that the staining is localized mainly on the external part of the peripheral doublets (Fig. 3 *Lower Left*). It seems that some projections from the doublets to the plasma membrane are stained. Only the cilia cut in their proximal region display a staining in transverse sections.

DISCUSSION

Our observations suggest that in addition to tubulin—a protein expected to be found in cilia—actin and myosin are also associated with the axonema.

Two sources of artifacts could be suspected: (*i*) the antibodies recognize proteins other than actin or myosin in the cilia, and (*ii*) actin and myosin are not localized on cilia *in vivo* but redistribute during cell extraction. Concerning the first point, the antibodies used have been isolated on immunoabsorbants that contained either purified actin or myosin and were tested on myofibrils and fibroblasts (11). Moreover, we have tested the specificity of the purified actin and myosin antibodies by their binding patterns on blots of ciliary cortex proteins. Most of the cortex proteins are transferred from the $NaDodSO_4$ slab gel onto the nitrocellulose sheet (data not shown). The actin antibody stains only one band that comigrates with actin (Fig. 4). Therefore, the staining of axonemes is due to the presence of actin. The myosin antibody does not stain any protein on the blots—probably due to the fact that myosin is present in low amounts in the cortices and is, in addition, poorly transferred. Although this experiment cannot demonstrate that the myosin of ciliary cortices is indeed recognized by the antibody, it shows that no protein other than myosin is recognized. Because we have obtained a strong staining of cilia at antibody concentrations as low as 8 μg/ml, it is quite probable that the staining of cilia is due to the presence of myosin.

It is worth mentioning that this antibody is directed against frog skeletal muscle myosin (11). Although it has been reported that there are few—if any—crossreactions between skeletal and smooth muscle myosins (19), our antibody seems to indeed crossreact with both myosins because it stains, even at low concentration, stress fibers of mouse fibroblasts (11) and the smooth muscles that are present in the frozen sections of quail oviducts.

The second source of possible artifact—the redistribution of

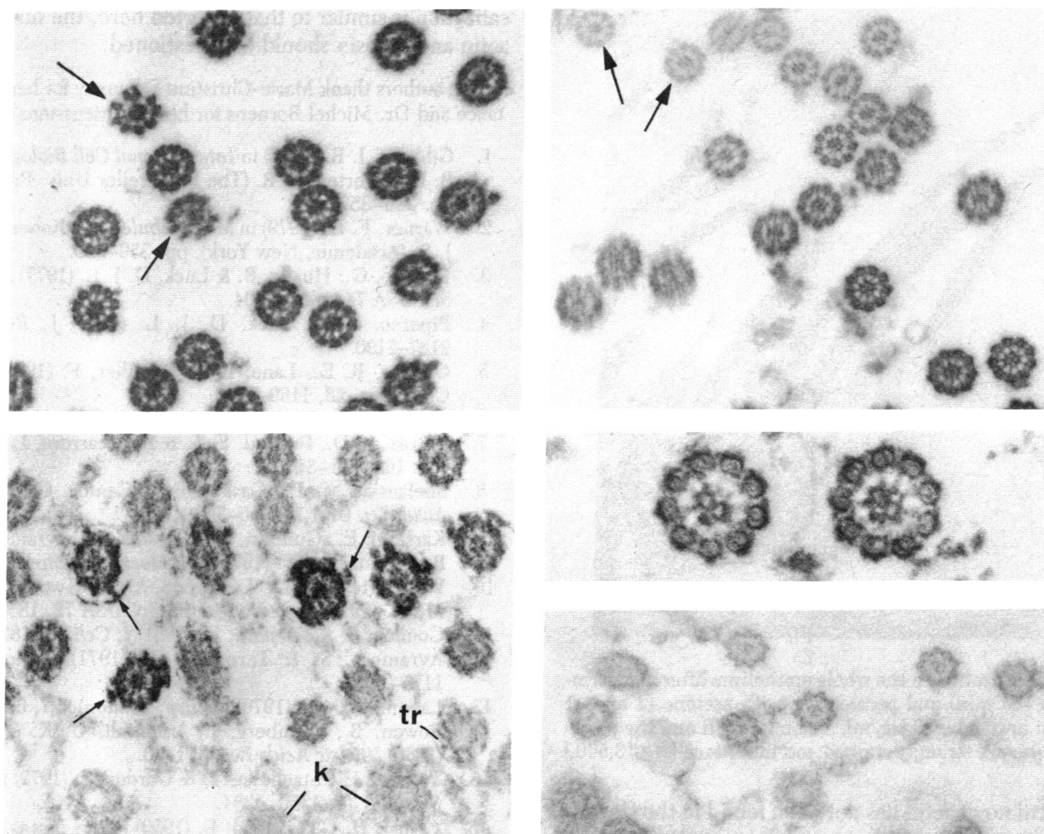

FIG. 3. Ultrastructural immunocytochemistry by indirect immunoperoxidase method (except *Middle Right*). Cross section. (*Upper Left*) Tubulin localization; no aldehyde fixation. Antitubulin antibody, 12 μg/ml. The apical parts of cilia characterized by peripheral singlets are stained (arrows). (×40,000.) (*Top Right*) Actin localization; paraformaldehyde fixation. Antiactin antibody, 16 μg/ml. Some axonemal sections are stained; other sections are not stained (arrows). (×40,000.) (*Middle Right*) Actin localization; paraformaldehyde fixation. Peroxidase-labeled antiactin antibody, 30 μg/ml. (×82,000.) (*Lower Left*) Myosin localization; no aldehyde fixation. Antimyosin antibody, 8 μg/ml. Few axonemal sections are stained. The sections of kinetosomes (k) or transitional region (tr) are unstained. Some projections from the doublets toward the ciliary membrane are stained (arrows). (×40,000.) (*Bottom Right*) Control; no aldehyde fixation. Anti-rat IgG antibody, 30 μg/ml. (×40,000.)

actin during preparation of demembranated cells or ciliary cortices—can be ruled out because (*i*) the staining observed with both actin and myosin antibodies is restricted to a specific region of cilia, and (*ii*) the same localization was reproducibly found in demembranated cells, isolated ciliary cortices with or without aldehyde fixation, and on frozen sections. Moreover, similar results were obtained by using the whole epithelium fixed with paraformaldehyde, which was then permeated with cold acetone (Fig. 5). In this last method, redistribution of actin cannot be invoked.

The staining of the cilia obtained with the tubulin antibody confirms the results of Gordon *et al.* (20) and is not surprising. However, some comments should be made. (*i*) In both isolated cortices and demembranated cells, the whole cytoplasm is devoid of staining. This may be due to an extraction of labile microtubules during preparation of the cells. (*ii*) The basal bodies are poorly stained. Such a lack of staining of centrioles has been reported (21, 22). This phenomenon could be due to a difference between tubulin of the axonema and tubulin of the basal bodies and centrioles. However, the antibody used in this work stains partially purified centrioles from human cultured lymphocytes (23). Centrioles are usually surrounded by a dense fibrillar matrix that contains—among other proteins—actin (5, 24). Depending upon the level of compaction of this matrix, tubulin antibody may or may not be able to reach the centriolar microtubules.

Although the occurrence of actin has been reported in *Chlamydomonas* axonema on the basis of biochemical results (4), the staining obtained here was a surprise for us because the localization is restricted to the lower part of the cilia. In fact, actin seems to be associated mainly with the external part of the peripheral doublets. The weaker staining that is observed in the other part of the axonemes (Fig. 3 *Middle Right*) could be due to a local diffusion of the diaminobenzidine precipitate. The peripheral actin often also seems to be in continuity with the actin that surrounds basal bodies and with microvilli (Fig. 2

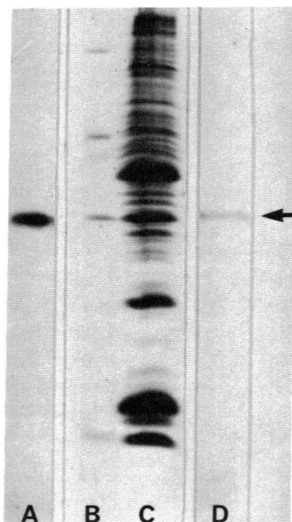

FIG. 4. Detection of actin from ciliary cortices by antibody binding on blots. The proteins from cortices prepared according to procedure 2 (see *Materials and Methods*) were fractionated on a 10% polyacrylamide slab gel. The proteins were transferred onto a nitrocellulose sheet, which was then incubated with the purified actin antibody as described. Lanes: A, blot of calf skeletal muscle actin stained with the purified antiactin; B, molecular weight markers stained with Coomassie blue. Calf myosin (220,000), human transferrin (85,000), human albumin (69,000), calf actin (43,000), and horse cytochrome *c* (12,000); C, ciliary cortex proteins stained with Coomassie blue; D, blot of ciliary cortex proteins stained with antiactin.

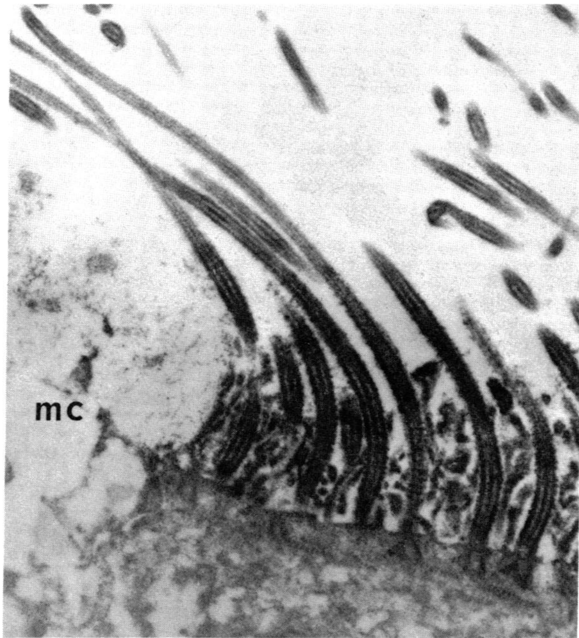

FIG. 5. Actin localization in the whole epithelium after paraformaldehyde fixation (30 min) and permeation with acetone (2 min at −20°C). Antiactin antibody, 30 μg/ml. The microvilli and the proximal-half part of cilia are strongly stained; mc, mucous cell. (×13,500.)

Lower Left). Until now, actin has not been found in the flagella of spermatozoa except around the centriolar complex (24, 25).

The well-defined localization of myosin at the proximal part of cilia, just above the transitional region, is still more perplexing. It seems to be involved in the interaction between axonema and plasma membrane because we often observed structures stained by the myosin antibodies that formed projections from the peripheral doublets toward the ciliary membrane. The presence of α-actinin was also suggested by immunocytochemistry in the proximal part of cilia from trachea (5).

It has been reported previously that intramembranous particles were present only in the proximal part of cilia from the quail oviduct (26). Moreover, thin sections revealed the presence of numerous links between doublets and the ciliary membrane only in this proximal region. Actin and myosin may well be two of the molecular components of such links. If the presence of actin and myosin is verified in other cilia, and if its localization is similar to that reported here, the functional role of actin and myosin should be questioned.

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