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Tropomyosin synthesis accompanies formation of actin filaments in embryonal carcinoma cells induced to differentiate by hexamethylene bisacetamide

(cytoskeleton/immunofluorescence/*in vitro* differentiation/mouse teratocarcinoma/tropomyosin antibody)

D. PAULIN, J. PERREAU, H. JAKOB, F. JACOB, AND M. YANIV

Département de Biologie Moléculaire, Institut Pasteur, 25, rue du Dr. Roux, 75015 Paris, France

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ABSTRACT **Hexamethylene bisacetamide (HMBA) induces *in vitro* the cytodifferentiation of PCC3/A/1 mouse embryonal carcinoma (EC) cells. In EC cells, actin is associated with surface structures but microfilament bundles are not seen. After 2 days of HMBA treatment, rounded EC cells are converted to flat adhesive ones with a developed cytoskeleton containing actin and tropomyosin. The ratio of actin to total proteins is constant in EC cells and their HMBA derivatives; but a striking difference is observed for one of the newly synthesized proteins (M_r 34,000) identified as tropomyosin. Synthesis of tropomyosin is followed by its association with actin microfilament bundles, as revealed by indirect immunofluorescence microscopy with specific antibodies.**

Several embryonal carcinoma (EC) cell lines have been isolated from mouse teratocarcinoma. These cells resemble early embryonic cells and some of them can differentiate *in vivo* or *in vitro* into derivatives of the three germ layers (1–4). With one EC cell line, PCC3/A/1, spontaneous differentiation is obtained *in vitro* by growth to confluency or by formation of cell aggregates and results in the appearance of a mixture of cell types (5). The differentiated cells are generally nonmalignant and exhibit different surface antigens compared to EC cells (6).

Recently, it was shown that immunofluorescence studies with antibodies against actin provide a means to follow cellular differentiation (7). In EC cells, actin is not organized in stress fibers but is distributed diffusely in the cytoplasm or concentrated in surface structures reflecting the round shape and low adhesion to the substratum. Striking differences were observed during differentiation. Flat cells acquired the characteristic arrangement of well-developed actin cables. These changing patterns of actin organization occur without modification of the relative amount of actin. Information concerning effectors that govern the development of actin fibers will be helpful in understanding the mechanism controlling actin organization.

It was of interest to study a system in which EC cells are induced to cytodifferentiate in a homogeneous population developing actin cables after several hours of treatment that leads to anchorage-dependent cell growth.

Attempts were made to direct this process to unique pathways of differentiation. By using hexamethylene bisacetamide (HMBA), it was shown that multipotential mouse EC cells are induced to differentiate into a cell type that exhibits new adhesive properties (8). It is shown that, concomitantly with the morphological changes and cell surface modifications, a cytoskeleton network develops in all the treated EC cells. We found also that HMBA induction is accompanied by a change in the pattern of proteins synthesized by these cells. One striking difference is a large increase in the synthesis of tropomyosin. Immunological studies revealed that, during the differentiation process, there is acquisition of a cytoskeleton network of filaments containing actin and tropomyosin. These results are described for PCC3/A/1 cells. Other EC strains gave variable results.

MATERIALS AND METHODS

Cell Cultures. EC PCC3/A/1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum and plated (3×10^6) on plastic tissue culture dishes (Falcon) (5). Differentiation was induced with 5 mM HMBA in the same medium followed by changes of HMBA-containing medium every 2 days (8). Nonconfluent monolayers were labeled at 37°C by incubation for 20 hr with [^{35}S]methionine [20 μCi/ml, 1000 Ci/mM (1 Ci = 3.7×10^{10} becquerels); Amersham] in medium containing a total of 7 μg of methionine per ml. Cells were labeled with [^{14}C]proline or [^{14}C]glutamic acid (25 μCi/ml, 250 mCi/mM; New England Nuclear) for 20 hr.

Gel Electrophoresis. Three gel electrophoresis systems were used. (*i*) Two-dimensional gel electrophoresis was performed according to O'Farrell (9) in the presence of rat muscle actomyosin as marker. Approximately 1×10^6 cpm corresponding to 3×10^5 cells was used for each electrophoresis. Gels were stained with Coomassie brilliant blue R, dried, and autoradiographed by exposure to Kodak x-ray films for 1 week. Spots were cut from the gels; the gel material was immersed in scintillation liquid (Koch–Light, Colnbrook, England) and the radioactivity was assayed. (*ii*) To follow the mobility shift of tropomyosin, 3.5 M urea was added to the 10% sodium dodecyl sulfate gels in the second dimension (10, 11). Cells were stained, dried, and autoradiographed as above. Purified rat muscle tropomyosin was used as standard in these gels. (*iii*) Monodimensional sodium dodecyl sulfate/15% polyacrylamide slab gel electrophoresis was performed as described by Laemmli, using the discontinuous Tris·HCl system (12). Densitometric scanning of the autoradiograph was carried out with a Vernon recording spectrophotometer.

Immunological Studies. The immunofluorescence staining was performed as described (7). Cells grown and treated on glass coverslips were fixed for 10 min with 3.7% formaldehyde at room temperature, followed by methanol and acetone.

The actin antibody was prepared against sodium dodecyl sulfate-denatured chicken actin as described by Lazarides and Weber (13) and was a gift of M. Osborn. The rat cardiac tropomyosin antibody was a gift of J. Leger and was prepared in a guinea pig. These antibodies were specific for tropomyosin

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Abbreviations: EC, embryonal carcinoma; HMBA, hexamethylene bisacetamide.

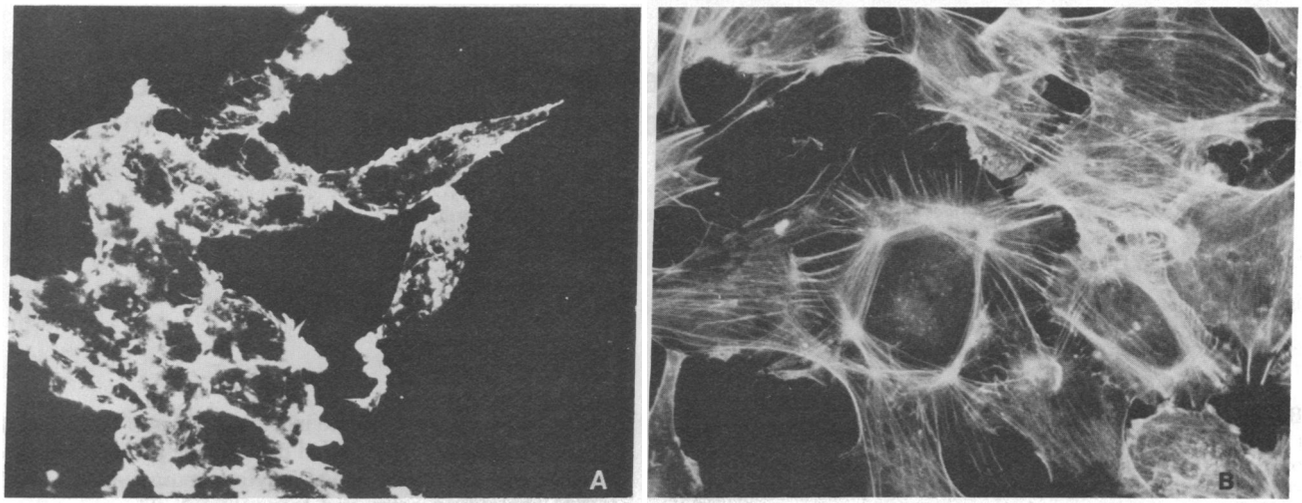

FIG. 1. Immunofluorescent staining of actin organization. (*A*) Exponentially growing PCC3/A/1. (*B*) PCC3/A/1 cells after 2 days of treatment with 5 mM HMBA. Note the different patterns of actin staining, showing ruffles and microvilli in *A* and typical skeleton in *B*. (×400.)

and not for other contractile proteins (14). Immunoabsorption with purified tropomyosin coupled to Sepharose was used as control experiments. Fluorescent rabbit IgG anti-guinea pig globulin (Miles) was used for tropomyosin detection and fluorescent sheep IgG anti-rabbit globulin (Miles) was used for actin detection. The coverslips were mounted and viewed with epifluorescent optics in a Zeiss photomicroscope III or a Leitz Diavert II.

For immunoprecipitation studies, the technique described by Griffin *et al.* (15) was used with the following modifications. [^{35}S]Methionine cell extracts [20 mM Tris, pH 9/137 mM NaCl/1 mM $CaCl_2$/0.5 mM $MgCl_2$/10% (wt/vol) glycerol, 1% Nonidet P40/1 mM diisopropyl fluorophosphate] were incubated for 2 hr at 4°C in the presence of 1 M KCl and then centrifuged for 10 min at 10,000 × *g*. The supernatant was diluted twice before incubation with anti-tropomyosin antibody.

Tropomyosin and Actomyosin Purifications. Rat muscle tropomyosin was extracted according to the procedure described by Fine *et al.* (16). About 10^8 cells with or without HMBA treatment were labeled with [^{35}S]methionine, and tropomyosin was purified in the presence of 200 μg of muscle tropomyosin carrier. Muscle actomyosin was purified from glycerinated rat or mouse muscle according to Yang and Perdue (17).

RESULTS

Appearance of Actin Cables after HMBA Treatment. EC PCC3/A/1 cells grown in culture are rounded with little cytoplasm, and they adhere strongly to each other. Growth in the presence of 5 mM HMBA converted them into well-spread large cells. These morphological changes were irreversible after 2 days of treatment. Indirect immunofluorescence microscopy with antibodies to actin showed striking differences in the actin organization. In EC cells, actin-specific fluorescence was found predominantly in surface structures (ruffles, microvilli) but no filament bundles were revealed (Fig. 1*A*). After 2 days of HMBA treatment, a new actin pattern was apparent. More than 80% of the cells had developed actin cables with characteristic stress fibers described for other differentiated cells (18, 19). To quantitate the amount of actin, the spots were cut from the gels (see below) and the radioactivity present in them was determined. The ratio of actin to the total cell protein was nearly identical in the two cell types: about 5% of the total [^{35}S]methionine-labeled proteins applied to the gel.

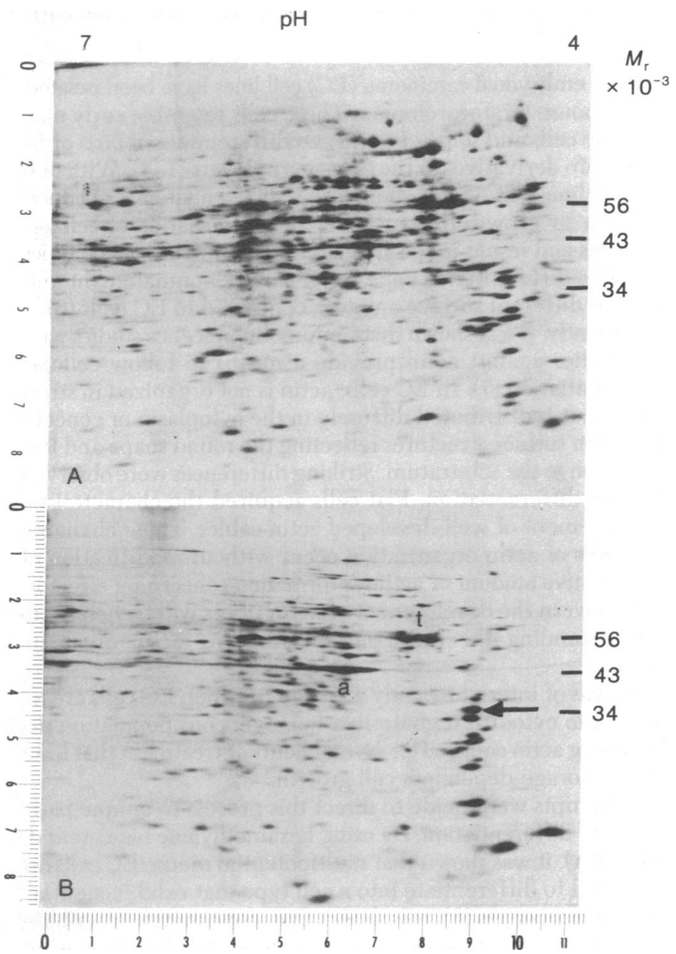

FIG. 2. Autoradiograms of two-dimensional gels of cell proteins labeled with [^{35}S]methionine. The first dimension (isoelectric focusing) was loaded with 5 × 10^5 cpm. The second dimension was performed with 15% acrylamide and 0.17% bisacrylamide. The autoradiographs were developed after 1 week of exposure. Positions of actin (a) and tubulin (t) were identified by running purified protein markers. (*A*) EC PCC3/A/1 cells without treatment. (*B*) PCC3/A/1 cells after 2 days of incubation with 5 mM HMBA. Arrow indicates the position of the polypeptide identified as tropomyosin.

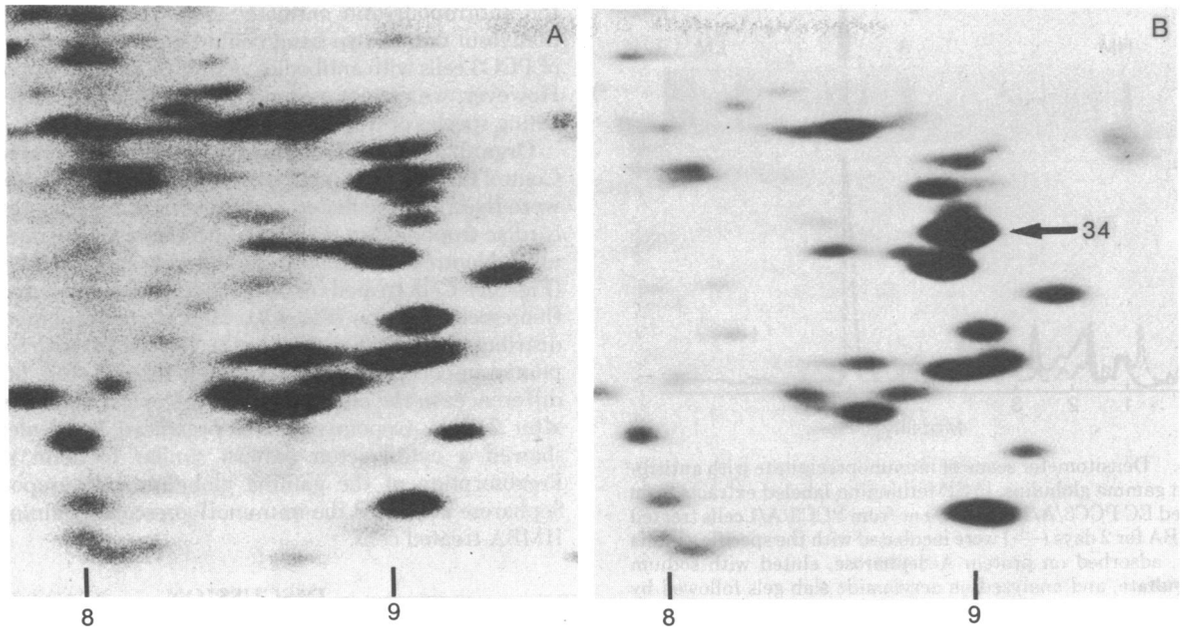

FIG. 3. Part of autoradiograms of two-dimensional gel electrophoresis shown in Fig. 2. (*A*) EC cells. (*B*) HMBA-treated EC cells. The arrow indicates the spot that comigrates with the α subunit of muscle tropomyosin marker and appears after HMBA treatment of EC cells PCC3/A/1.

Variation in Pattern of Proteins Synthesized after HMBA Treatment. Immunofluorescence studies demonstrated large modifications in the morphology of the cells grown in HMBA. To determine if these variations are accompanied by analogous changes in the nature of the proteins synthesized by these cells, PCC3/A/1 cells were incubated in the presence of 5 mM HMBA for periods of up to 6 days. During the last 20 hr of each incubation period, [^{35}S]methionine was added to the culture medium in order to label the newly synthesized proteins. Approximately 3×10^5 cells were recovered from each preparation and their proteins were analyzed by high-resolution two-dimensional gel electrophoresis. For comparison, the protein pattern of untreated but similarly labeled EC cells was analyzed concurrently. Differences were noted in the protein pattern when cells were incubated for 2 days with HMBA (Fig. 2). Further growth in HMBA for up to 6 days did not change the protein pattern. About 400 spots are resolved on the O'Farrell two-dimensional system under our experimental conditions. Careful comparison of a series of such gels from PCC3/A/1 cells before and after HMBA treatment revealed changes in about 40 positions: 25 peptide spots present in nontreated cells decreased in intensity, and about 15 new spots appeared.

Tropomyosin Is One of the New Proteins. The region of the two-dimensional gels corresponding to coordinates 7.5–10.0 in the first dimension and 3.5–5.5 in the second dimension is enlarged in Fig. 3 to demonstrate the drastic increase in the relative rate of synthesis of a polypeptide (indicated by an arrow) upon HMBA treatment. The amount of radioactivity in this position normalized to the total counts applied to the gel increased about 20-fold during the growth in HMBA (from 0.016% to 0.3% of total labeled proteins). The polypeptide present in this position before HMBA treatment may be identical to the newly synthesized one or another protein that migrates in the same position.

Cellular proteins known to migrate in this region (32,000–34,000; pI, 4.2–4.5) are tropomyosins (10, 20, 21). To ascertain the nature of the polypeptide, two types of biochemical experiments were performed. Tropomyosin isolated from purified actomyosin complexes of skeletal muscle was used as a marker in the two-dimensional separation. The smaller α component (34,000) migrated at the same position as the HMBA-induced radioactive spot. No radioactivity was detected at the position of the heavy β subunit (36,000) (10, 22). To characterize the putative HMBA-induced tropomyosin species further, we made use of the fact that all known tropomyosins lack proline whereas they are rich in glutamic acid (23). After 2 days of HMBA treatment, cells were divided into two groups: one was incubated for 20 hr with [^{14}C]glutamic acid and the other, with [^{14}C]proline. Fig. 4 compares the protein patterns obtained from these two labelings. One of the spots labeled with glutamic acid (arrow) comigrated with nonradioactive α-tropomyosin as revealed by comparison of the stained gel with the autoradiogram. No trace of proline incorporation was detected at this position. Several other spots in the same region of the gel (30,000–36,000; pI, 4.2) lack proline. However, the other polypeptides did not react with the antitropomyosin serum (as shown below). In another experiment, purified α-tropomyosin and [^{35}S]methionine-labeled tropomyosin extracted from HMBA-treated cells were submitted to the two-dimensional electrophoresis but, in the second dimension, we added 3.5 M

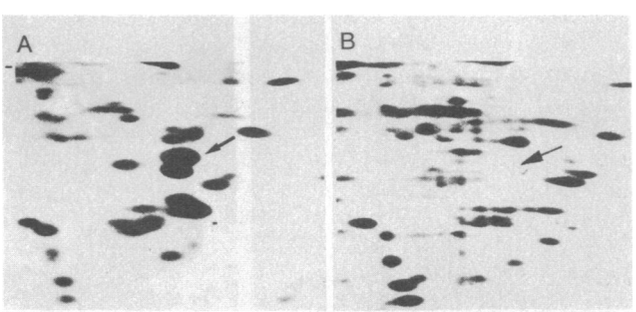

FIG. 4. Part of autoradiograms of two-dimensional gel electrophoresis of HMBA-treated cells grown in a medium containing [^{14}C]glutamic acid (*A*) or [^{14}C]proline (*B*) at 25 μCi/ml. Arrows indicate the position of 34,000 spot that comigrates with α tropomyosin. It is present with [^{14}C]glutamic acid labeling but absent with [^{14}C]-proline labeling.

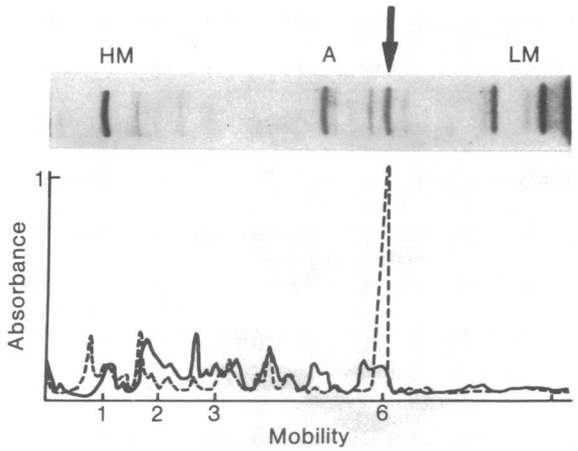

FIG. 5. Densitometer scans of immunoprecipitate with antitropomyosin gamma globulins. [^{35}S]Methionine-labeled extracts from nontreated EC PCC3/A/1 cells (—) or from PCC3/A/1 cells treated with HMBA for 2 days (- - -) were incubated with the specific gamma globulins, adsorbed on protein A-Sepharose, eluted with sodium dodecyl sulfate, and analyzed on acrylamide slab gels followed by autoradiography. The arrow indicates the position of the band comigrating with pure α tropomyosin. Molecular weight markers: HM, heavy myosin, 240,000; A, actin, 43,000; LM, myosin light chains, 25,000, 17,000, and 15,000.

urea to the gel (10, 11). Under these conditions, the radioactive and the marker tropomyosin comigrated at the same position with an apparent molecular weight of 45,000 (results not shown).

Antibodies against Rat Cardiac Tropomyosin Crossreact with HMBA-Induced 34,000 Polypeptide. To obtain additional evidence for the induction of tropomyosin synthesis by HMBA, we tried to immunoprecipitate the 34,000 protein with antitropomyosin antiserum. Methionine-labeled cell extracts from control or HMBA-treated EC cells were incubated overnight with antitropomyosin gamma globulins and subsequently adsorbed on protein A-Sepharose. The complexes were eluted and analyzed by polyacrylamide gel electrophoresis. A radioactive band comigrating with α-tropomysin was precipitated from the differentiated cell extracts whereas EC cells did not show any 34,000 protein component immunoprecipitated by the antitropomyosin antibody (Fig. 5). No significant tropomyosin radioactive band could be precipitated in extracts of PCC3 cells with antibodies against rat cardiac tropomyosin. However, we cannot exclude the presence of a different reacting species of tropomyosin (14, 24, 25).

Organization of Tropomyosin in HMBA-Treated Cells. Control EC cells or EC cells treated for up to 5 days with HMBA were fixed and incubated with specific antibodies against rat cardiac tropomyosin and then with fluorescent gamma globulins. Nontreated EC cells showed only low diffuse staining (Fig. 6*A*). Cells treated for 30 hr with HMBA showed a positive fluorescent reaction (Fig. 6*B*). Most of the tropomyosin was distributed in patches all over the cytoplasm, with some tropomyosin-containing fibers crossing the cell. Fig. 6*C* shows differences in the organization of the cytoskeletal structures: after 2 days, tropomyosin was organized in filaments and showed a cytoskeleton pattern similar to actin staining. Preabsorption of the gamma globulins with tropomyosin-Sepharose abolished the immunofluorescence staining of the HMBA-treated cells.

DISCUSSION

HMBA has been shown to be a powerful agent for the *in vitro* induction of differentiation and hemoglobin synthesis in mouse erythroid cells transformed by the Friend virus (26, 27). Malignant mesenchymal cell lines from human glioblastoma are induced to express a new differentiated phenotype and to increase the synthesis of procollagen (28). Terminal differentiation of mouse neuroblastoma cells is also induced by growth in HMBA (29).

Growth of EC PCC3/A/1 cells in the presence of 5 mM HMBA for 48 hr induced a drastic morphological change in these cells. The rounded EC cells gave rise to large cells that possessed enlarged cytoplasm with projections or pseudopods. The new cellular type lost the following embryonic markers: F9 antigen(s), alkaline phosphatase (8), and DNase (30). Properties characteristic of differentiated derivatives appeared such as the presence of plasminogen activator and the loss of tumorigenicity (8). In addition, the HMBA-induced cells harbored fibronectin which is not expressed in PCC3 cells before treatment (unpublished results). The cell type obtained after HMBA treatment is not yet identified.

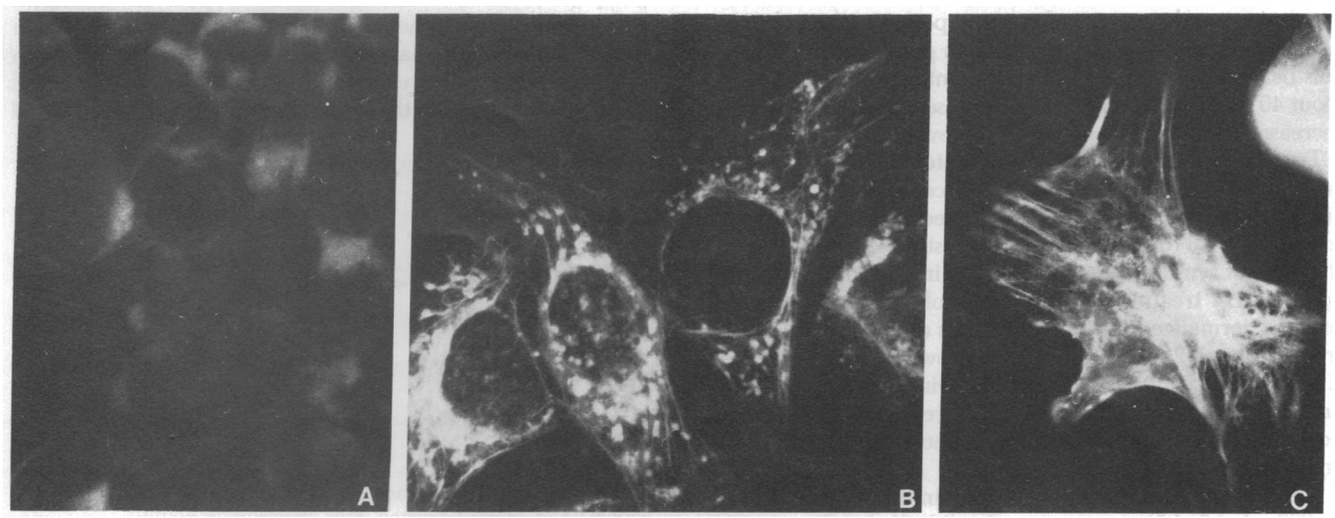

FIG. 6. Immunofluorescence staining with antitropomyosin antibodies. (*A*) Nontreated EC PCC3/A/1 cells. (*B*) After 30 hr of HMBA treatment. (*C*) After 2 days of HMBA treatment. Nonimmune guinea pig gamma globulin did not show any immunofluorescent staining. Note the absence of staining in EC cells and the different patterns of tropomyosin-detected patchy structures after 30 hr of HMBA treatment and tropomyosin fibers after 2 days of treatment. (×400.)

In EC cells, the majority of actin is nonfilamentous (7). By immunofluorescence studies, we could not detect any stress fibers typical of the cytoskeleton in differentiated cells. Our studies have shown that 80% of EC actin in PCC3 cells is present in nonpolymerized form (F. Markey, L. Sariano, and D. Paulin, unpublished results). These results imply the presence of one or more proteins that interact with actin to prevent its polymerization, such as profilin which has been purified from several tissues (31) and alkaline DNase which has been isolated from EC cells (30).

Alternatively, one to several proteins necessary for cytoskeleton formation may be absent from EC cells. HMBA-induced differentiation of EC cells was accompanied by appearance of actin-containing microfilaments without changes in the concentrations of intracellular actin during this process.

In an attempt to correlate the morphological and functional changes observed in HMBA-treated cells, we studied the protein pattern by two-dimensional gel electrophoresis. One of the major changes observed is the drastic increase in the synthesis of a 34,000 protein. According to several criteria, this polypeptide is a tropomyosin chain. (*i*) It comigrates with rat skeletal α-tropomyosin on the two-dimensional gels. (*ii*) It contains glutamic acid but lacks proline, a property common to all the tropomyosin chains studied. (*iii*) It is immunoprecipitated specifically with antibodies against rat cardiac tropomyosin. (*iv*) The mobility of this polypeptide in sodium dodecyl sulfate gels is shifted in the presence of urea. This tropomyosin is found associated with the cytoskeletal network as revealed by immunofluorescence studies with antitropomyosin antiserum. This result is in agreement with previous studies showing that eukaryotic nonmuscle cell cytoplasmic filaments contain both actin and tropomyosin (18, 32). It is possible that the induction of a new species of tropomyosin in EC cells regulates the formation of microfilament bundles in these cells.

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