Glucocorticoid Hormone Modulation of Both Cell Surface and Cytoskeleton Related to Growth Control of Rat Glioma Cells

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ABSTRACT   We have shown that glucocorticoids reversibly change the growth control of rat C_6 glioma cells from a transformed to a normal pattern. Here we report that the glucocorticoid hormone hydrocortisone (Hy) modulates structure and function of cell surface and cytoskeleton. The hormone is shown to cause: (a) increased flattening and adhesion to solid substrates and to fibrin layers, (b) inhibition of the cell shape change triggered by catecholamines and cAMP, (c) extensive fibronectin deposition on normally fibronectinless cells' surface, and (d) microtubule rearrangement. Comparison of Hy-hypersensitive and Hy-resistant variants showed that microtubule rearrangements correlate with the growth control change induced by Hy, whereas fibronectin deposition does not.

The pattern of proliferation control of C_6 rat glioma cells (1) changes under the action of the glucocorticoid hormone hydrocortisone (Hy). This hormone restores serum and anchorage dependence to the fully transformed and malignant cells and interferes with cell cycle control by affecting cell traversing rate exclusively at the G_1 phase of the cell cycle (2). Hy also renders C_6 cells dependent on high external Ca^{2+} concentrations for growth (3) and sensitive to a G_1 block by microtubule-disrupting agents (4, 5).

We show here that Hy induces cell flattening, increases cell adhesion to plastic surfaces and to fibrin layer and inhibits the characteristic cell shape change induced by catecholamines and cAMP in glial cells. The basis for this change was examined by analyzing the effects of the hormone on cell surface and cytoskeleton of Hy-hypersensitive and Hy-resistant C_6 variants. We show that the mechanisms by which Hy confers a new growth pattern to C_6 glial cells probably involve alterations of cytoskeletal architecture.

MATERIALS AND METHODS

Cells and Culture Conditions: Rat C_6 glioma cell line (CCL 107) was originally obtained from American Type Culture Collection (Rockville, MD). Isolation and characterization of C_6 variants are described in the accompanying paper (2) and in reference 6. For culture conditions, see accompanying paper (2).

Immunofluorescence Staining: Cells were grown on 0.81-cm^2 coverslips to different cell densities. For surface fibronectin staining, coverslip cultures were rinsed in phosphate-buffered saline (PBS) for 5 min in 3.7% formaldehyde-PBS and washed thoroughly before adding 12 μl of 1/100 dilution rabbit anti-bovine cold insoluble globulin (CIG) whole serum (generously

provided by Dr. D. Rifkin [New York University] and Dr. R. Hynes [Massachusetts Institute of Technology]). After incubating for 20 min at 37°C in humidified atmosphere, coverslips were extensively washed, 12 μl of a 1/25 dilution FITC-conjugated goat anti-rabbit IgG (Miles, Yeda Ltd., Rehovot, Israel) was added, and incubated for another 20 min at 37°C. Upon several washings in PBS, coverslips were rinsed once in distilled water, mounted in 90% glycerin–10% carbonate buffer, pH 9.3, and sealed with nail polish. Controls consisting of omitting the first antiserum or substituting rabbit anti-bovine CIG by nonimmune rabbit serum were routinely included in all experiments and were consistently negative under the above described conditions. For cytoskeleton staining, coverslip cultures were rapidly washed in Ca^{2+}-free PBS at room temperature before fixing in methanol for 6 min at −15°C, as suggested in reference 7. The methanol treatment was adopted as having significantly better results, after testing several other fixation and permeabilization procedures. After rehydration in Ca^{2+} free PBS, coverslips were covered with 12 μl of a 1/25 dilution rabbit-antitubulin antiserum (generously provided by Dr. D. Rifkin, New York University; and Drs. E. Meier and O. L. Jorgensen (University of Copenhagen, Denmark); see reference 8) or antiactin antiserum (generously provided by Dr. K. Burridge, Cold Spring Harbor Laboratories). In cases where cells were negative for anti-actin staining, lower dilutions (1/10 or 1/5) were also used. All reactions were run with internal positive controls (usually Swiss mouse 3T3 fibroblasts) and negative controls (as described for fibronectin staining). After incubation for 30 min at 37°C with the first antiserum, coverslips were washed, FITC-conjugated goat anti-rabbit serum (1/25 dilution) was added, and the preparation was incubated and mounted as described for fibronectin staining.

All coverslips were examined under a Zeiss Universal microscope equipped with 200 W HBO lamp and epifluorescence. Photomicrographs were taken on Kodak Tri-X 400 ASA film and for the same exposure time (usually 30 s for fibronectin and 45 s for cytoskeleton). All films were developed with Kodak D-76 developer for 8 min at 22°C.

Mutagenization of ST1 Cells with N-Methyl-Nitro-N-Nitrosoguanidine: 4.5×10^6 exponentially growing ST1 cells were treated with several different concentrations of freshly made *N*-methyl-nitro-*N*-nitrosoguan-

idine (MNNG) solutions. 1% survival (measured by relative plating efficiency) was observed for cultures treated with 1.2 mg/ml MNNG for 3.5 h. These cultures were allowed to grow for 8 d to recover from mutagenesis. Mutation rate, assayed by the frequency of thioguanine (TG) resistance, was 2×10^{-5}, estimated from cultures plated at 10^5 cells per 60-mm dish; no TG-resistant colonies were seen in nonmutagenized cultures even at 10^6 cells per 60-mm dish. Morphological variants were frequent among colonies originated from mutagenized cultures. The mutagenized population was screened for Hy-resistant variants in agarose suspension cultures, giving negative results.

Plasminogen Activator Assay: We prepared fibrin plates by adding 0.1 ml of a 1 mg/ml bovine thrombin solution to each petri dish (Nunc, 35 mm diameter), followed by addition of 2 ml of 0.5% fibrinogen solution in PBS (bovine fibrinogen I; ICN Nutritional Biochemicals, Cleveland, OH), rapidly mixing, and allowing to stand for gelification. All solutions were filter sterilized in 0.22-μm GS type Millipore filter. Thrombin (33% purity was prepared and generously provided by Dr. J. R. Giglio [Faculdade de Medicina de Ribeirao Preto, Universidade de Sao Paulo]). Cells were plated directly onto this fibrin layer at different cell densities, and lysis zones or growth (in case cells did not produce plasminogen activator) were monitored.

Fibrinolytic activity was quantitated using the method described by Unkeless et al. (9). Radioiodinated ^{125}I-fibrinogen coated Linbro 24-well plates were activated to fibrin immediately before use by incubation with 0.5 ml/well 2.5% FCS-DME for 1 h at 37°C. Cells were either plated directly onto the ^{125}I-fibrin coated wells or seeded in regular plates, allowed to reach exponential phase (usually 48 h), washed, and incubated with serum-free DME for 6 h. Radioactivity released to the supernatant by cells or by medium conditioned by the cells in serum-free medium was measured by applying aliquots to paper filters, drying, and counting in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, CA). Total radioactivity per well was determined by incubation with 0.5 ml/well 0.1% trypsin solution. Results were expressed as percent of total radioactivity released as a function of time. Less than 5% radioactivity was released in controls incubated with unconditioned serum-free medium or fresh 5% FCS-DME.

RESULTS

Alterations of Cell Morphology, Adhesion, and Cytoskeleton Functioning Caused by Hy

Hy causes flattening of C_6 glioma cells anchored to a solid substrate, leading to organized, nonoverlapping monolayers (Fig. 1, *A* and *B*). Also, upon trypsinization, hormone-treated cells take longer to detach from the substrate (3 and 15 min, respectively). These alterations of adhesion and morphology suggested that the hormone might be inducing modifications of cell surface composition and/or cytoskeleton. Another indication came from plating ST1 variant, at low cell density, on top of a fibrin layer (Fig. 2*A*). Single cells grew into three-dimensional colonies, resembling growth in agarose suspension. Addition of Hy to such cultures caused the cells to

acquire a stretched and elongated shape and to adhere firmly to fibrin; growth of these fibrin-attached cells was completely arrested (Fig. 2*B*). Hy renders the growth of ST1 cells anchorage dependent (2); however, it seems that fibrin does not provide an adequate substrate so as to allow growth in the presence of Hy.

Our experiments were made possible because these glial cells do not secrete plasminogen activator irrespective of the presence or absence of Hy. That is not the case with the majority of transformed cells (9–11). When 3T6 fibroblasts were plated onto fibrin layers, the cells digested the fibrin and grew on the bottom of the dish. The lack of plasminogen activator activity in ST1 cells was also confirmed by the ^{125}I fibrin digestion assay (carried out as described in Materials and Methods). The same assay applied to SV3T3 and 3T6 fibroblasts was strongly positive.

Cell flattening and increased adhesion to fibrin are late effects of Hy, taking up to 24 h to be clearly observed. An early effect of the hormone can also be shown to occur and to be dependent on cytoskeleton alterations, namely, inhibition of the morphological change caused by catecholamines and cAMP. Hydrocortisone inhibits the modification in cell shape brought about by cAMP in ST1 cells (Fig. 3*A*). This is an early hormonal effect that can be observed with the first 2 h of hormone treatment (Fig. 3*B*). Addition of theophylline (a phosphodiesterase inhibitor) along with cAMP or dibutyryl-cAMP did not alter these results. This hydrocortisone action seems to depend on protein synthesis since it is blocked by cycloheximide (Fig. 3*B*). Hy also inhibited the cell shape modification induced by isoproterenol (not shown).

Hydrocortisone Induction of Fibronectin Deposition on C_6 Cell's Surface

A highly conspicuous alteration of cell surface induced by Hy in ST1 cells is the deposition of fibronectin (FN) on the cell surface. This hormonal effect was demonstrated by indirect immunofluorescence using rabbit-anti-bovine CIG. No FN was found on the surface of ST1 cells (Fig. 4*A*). Physiological concentrations of Hy (250 ng/ml) caused progressive accumulation of an extensive FN meshwork (Fig. 4*B*). This is a relatively late hormonal effect, i.e., a meshwork throughout the whole culture could only be seen after 24 h of hormone treatment. It is important to note that FN deposition also
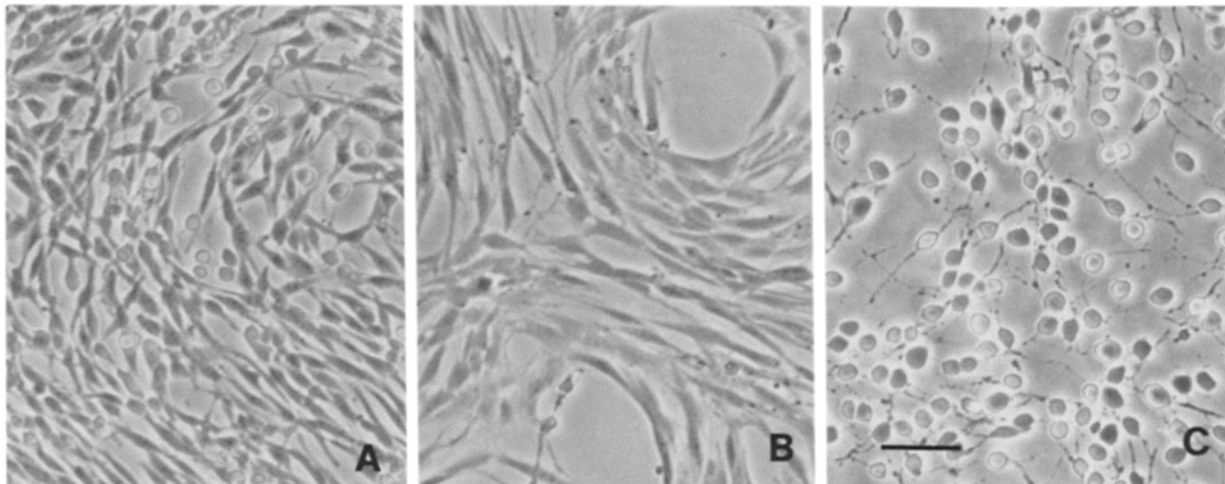

FIGURE 1   Morphological alterations caused in ST1 cells by Hy (flattening) and cAMP (rounding up). (*A*) Control, untreated cells. (*B*) Treated with 250 ng/ml Hy for 24 h. (*C*) Treated with 1 mM cAMP for 30 min. Bar, 30 μm.

occurred when cells were cultured in hormone-supplemented serum-free medium (not shown).

Induction of FN deposition probably contributes to the increased flattening and spreading to solid substrate and adhesion to fibrin. However, we have evidence indicating that adhesion and spreading of C_6 cells on solid substrate depend on components other than FN. Plating of C_6 cells (several variants) onto dishes coated with bovine CIG, in serum-free medium, did not result in cell spreading, irrespective of the presence of Hy in the hormonal supplement. Well-spread cells were obtained only when dishes were precoated with fetal calf serum. Serum components that are not displaceable by several washings with PBS are necessary for optimal cell adhesion and spreading.
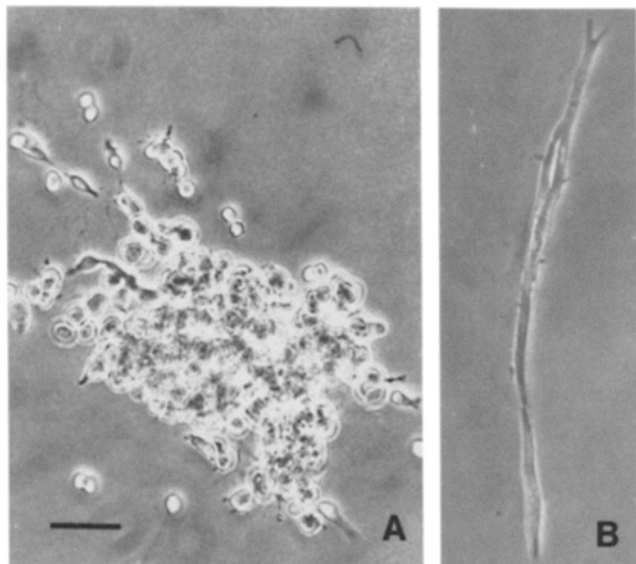

FIGURE 2 Effect of Hy on growth of ST1 cells on top of a fibrin layer. 10^3 cells were seeded on 35-mm fibrin coated dishes in 1 ml of 15% FCS-DME, in the absence (A) or presence (B) of Hy (250 ng/ml). Refeeding, by gentle aspiration of the old medium, every 3 d. Micrographs taken at 9 d after seeding. Bar, 30 μm.

Also, FN deposition is neither necessary nor sufficient for the modification in growth control induced by Hy. The evidence for this comes from analysis of Hy-resistant C_6 variants and of ST1 variant clones isolated from MNNG-mutagenized cultures.

We examined the effect of Hy on FN deposition in C_6 variants. The results of Fig. 5 show that: (a) occasional FN fibers were found in some of the variant clones in the absence of any hormone treatment; and (b) treatment with Hy resulted in extensive deposition of FN in all clones under conditions responsive to Hy (3D and P_{11}) or Hy-resistant (P_5 and P_7) in a dense meshwork of short (P_5 and P_7) or long (P_{11} and 3D) fibers. These results indicated that the induction of FN deposition by Hy was not sufficient to allow alterations of cell growth control.

Clones of different morphological types were also isolated from ST1 cells mutagenized with MNNG (as described in Materials and Methods). In one of these clones (E_5-C_2), Hy treatment rapidly and effectively caused both the growth control change and inhibition of the change in cell shape caused by cAMP; however, no FN was detected up to 72 h of hormone treatment. FN could clearly be seen by 120 h of Hy treatment but never reached the levels observed with parental (ST1) cells. These results suggest that FN deposition is not necessary for Hy to change growth control in ST1 cells.

Microtubules' Rearrangements Caused by Hy in Hormone-sensitive Cells

We probed the effect of Hy treatment on C_6 cells' cytoskeleton by indirect immunofluorescence using antitubulin and antiactin antisera.

ST1 cells stained with antitubulin presented a "dispersed" pattern in which cytoplasmic fluorescence is seen but clearly a defined microtubule network is seldom displayed (Fig. 6A). Hy-treated ST1 cells presented a "network" pattern, i.e., 100% of the cells showed a clearly defined microtubule network distributing radially from a bright perinuclear region (Fig. 6B). This network (as well as that displayed by normal 3T3
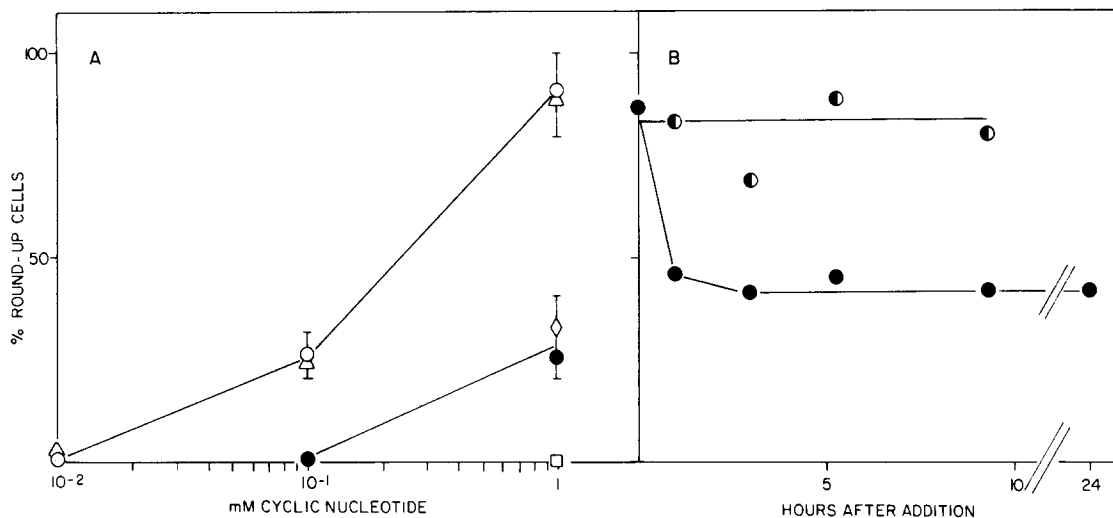

FIGURE 3 ST1 cells rounding-up caused by cAMP: effects of Hy and cycloheximide. (A) Cyclic nucleotide dose-response curve. Cells were treated with Hy (250 ng/ml) for 24 h before being submitted to the indicated concentrations of cAMP (●) and cAMP +1 mM theophylline (◊). 60 min after cyclic nucleotides addition, cultures were scored for rounded-up cells. Hormone-untreated cultures (same density as Hy-treated ones) were also subjected to cAMP (O), dibutyryl cAMP (△), and cGMP (□). (B) Hy treatment as a function of time. 250 ng/ml Hy alone (●) or with 1.5 mM cycloheximide (◉) were added to the culture medium at zero time. At the indicated times, cultures were submitted to 1 mM cAMP and, 60 min later, scored for rounded-up cells.
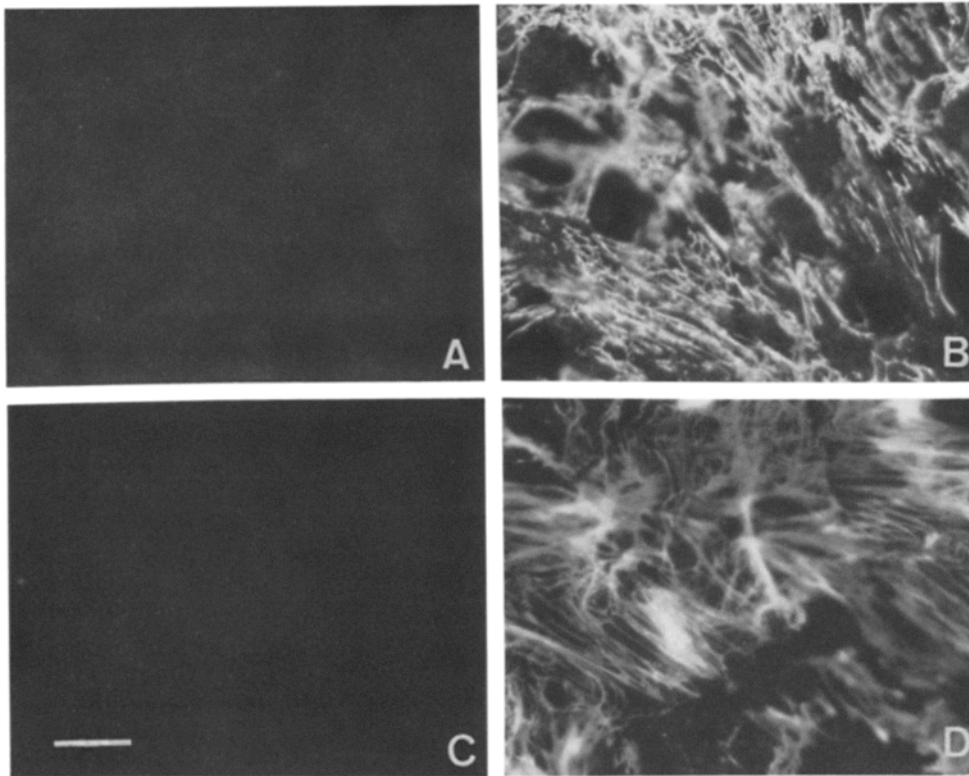
FIGURE 4 Immunofluorescence staining for fibronectin: effect of Hy treatment on ST1 cells. Cells were cultured in the absence (A and D) or presence (B and C) of Hy (250 ng/ml) for 72 h prior to staining for fibronectin as described in Materials and Methods. A, B, and C: ST1 cells. D: Swiss 3T3 fibroblasts. C: control, nonimmune rabbit serum. A, B, and D: anti-CIG rabbit serum. Bar, 6 μm.

fibroblasts used as controls) was totally disrupted by colchicine treatment (0.4 μg/ml for 1 h). Hy treatment induced the same change in microtubule patterns in clones P_2 and P_{11} which are Hy-resistant in suspension but Hy-sensitive when anchored to solid substrates.

The same fluorescence pattern shown in Fig. 6A was also seen for cells kept in 0.2% FCS-medium or 5% FCS-medium plus 2.5 mg/ml polybrene for 24 h. This polycation causes extensive cell flattening without interfering with growth rate (13). Therefore reduced growth rate and flattening were not effective to lead to the network pattern observed upon Hy treatment.

Antitubulin staining of the Hy-resistant variant, clone P_7, presented the "dispersed" fluorescence pattern irrespective of Hy treatment (Fig. 6, C and D). The anchorage-dependent variant clone 3D, on the other hand, displayed the "network" pattern even in the absence of Hy. These results show a strong correlation between microtubules' rearrangement and the growth control change induced by Hy.

Staining of microfilaments with antiactin was carried out with C_6 and its variant clones. No actin cables were ever found in clones ST1, P_2, P_5, P_7, or P_{11} despite the use of culture conditions that optimize visualization of these fibers (low serum medium, coated surfaces, etc.). Under Hy treatment, no cables were found in hormone-sensitive or -resistant variants. In fibroblasts, the fibronectin network on the outer surface is found to correlate with actin cables underneath the membrane (14, 15). In the glial cells studied, the fibronectin meshwork induced by Hy was not accompanied by the appearance of actin cables. The only variant in which characteristic cables were found was the anchorage-dependent clone 3D, irrespective of Hy treatment (not shown).

DISCUSSION

Our results show that glucocorticoid hormone Hy is capable of modulating surface composition and cytoskeleton organi-

zation and functioning of C_6 glioma cells. Comparison of several C_6 growth variants show that only the effects of Hy on cytoskeletal elements correlate with the change in growth control induced by the hormone in these cells.

The effects of Hy on cell flattening and adhesion to solid substrates and to fibrin (Figs. 1 and 2) led us to consider possible hormonal effects on cell surface, membrane and cytoskeleton underlying the growth control change.

A striking effect of Hy on cell surface composition was detected as an extensive fibronectin deposition (Fig. 4). In the case of the Hy-hypersensitive variant ST1, no fibronectin was detected in the absence of the hormone including cells that had been permeabilized with detergents or organic solvents. Upon Hy treatment, fibronectin could be found both underlying isolated cells in sparse cultures and as a dense meshwork in confluent cultures. Glucocorticoids have been reported to cause modifications on the cell surface of hepatoma cells (16). In primary cultures of rat mammary epithelial cells, classical targets for glucocorticoids, Hy has been shown to stimulate growth by increasing type IV collagen content in the extracellular matrix (17). Glucocorticoids have also been reported to induce fibronectin deposition on human fibroblasts (18) and rat hepatocytes (19). Most likely, fibronectin is contributing to glial cells' flattening and increased adhesion. However, flattening and increased adhesion per se do not seem to be sufficient to change the cell growth pattern, for the following reasons: (a) analysis of several C_6 variants (Fig. 5) indicates that fibronectin deposition is neither necessary nor sufficient for the change in growth control induced by Hy; (b) elsewhere we reported (13) that polycations (polylysine and polybrene) cause flattening and increased adhesion of ST1 cells to solid substrates but do not interfere with the mode of cell proliferation. However, when polycations are applied to cells that have been pretreated with Hy, not only are flattening and adhesion enhanced, but growth is completely blocked. Therefore, depending on Hy treatment, the cells' interaction with
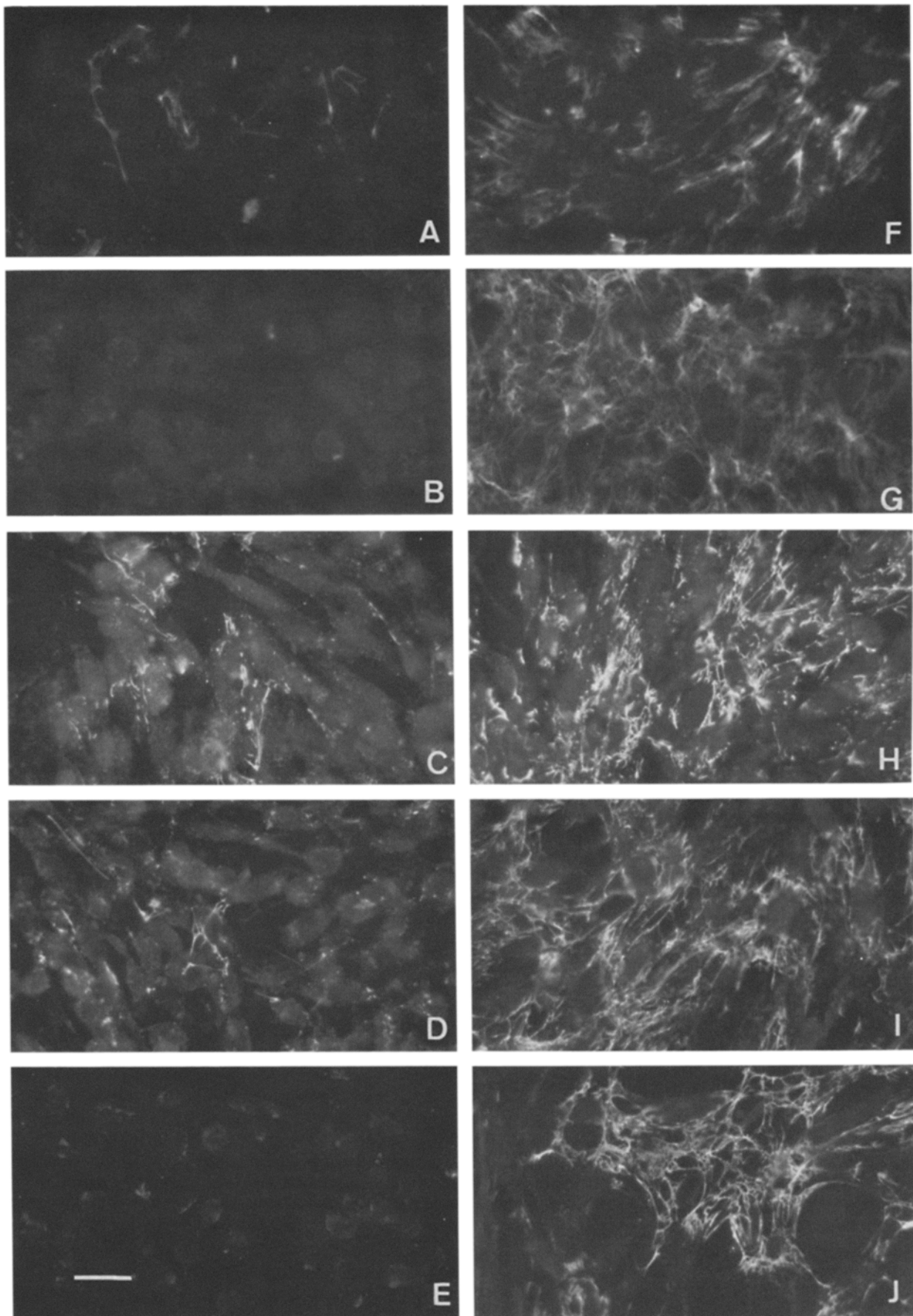
FIGURE 5   Immunofluorescence staining for fibronectin: effects of Hy treatment on C_6 cell variants. Untreated (*A*, *B*, *C*, *D* and *E*) and Hy-treated (250 µg/ml, 72 h) (*F*, *G*, *H*, *I*, and *J*) cultures were stained for fibronectin, as described in Materials and Methods. Clone 3D: *E* and *F*. Clone P_2: *B* and *G*. Clone P_5: *C* and *H*. Clone P_7: *D* and *I*; Clone P_{11}: *E* and *J*. Bar, 12 µm.
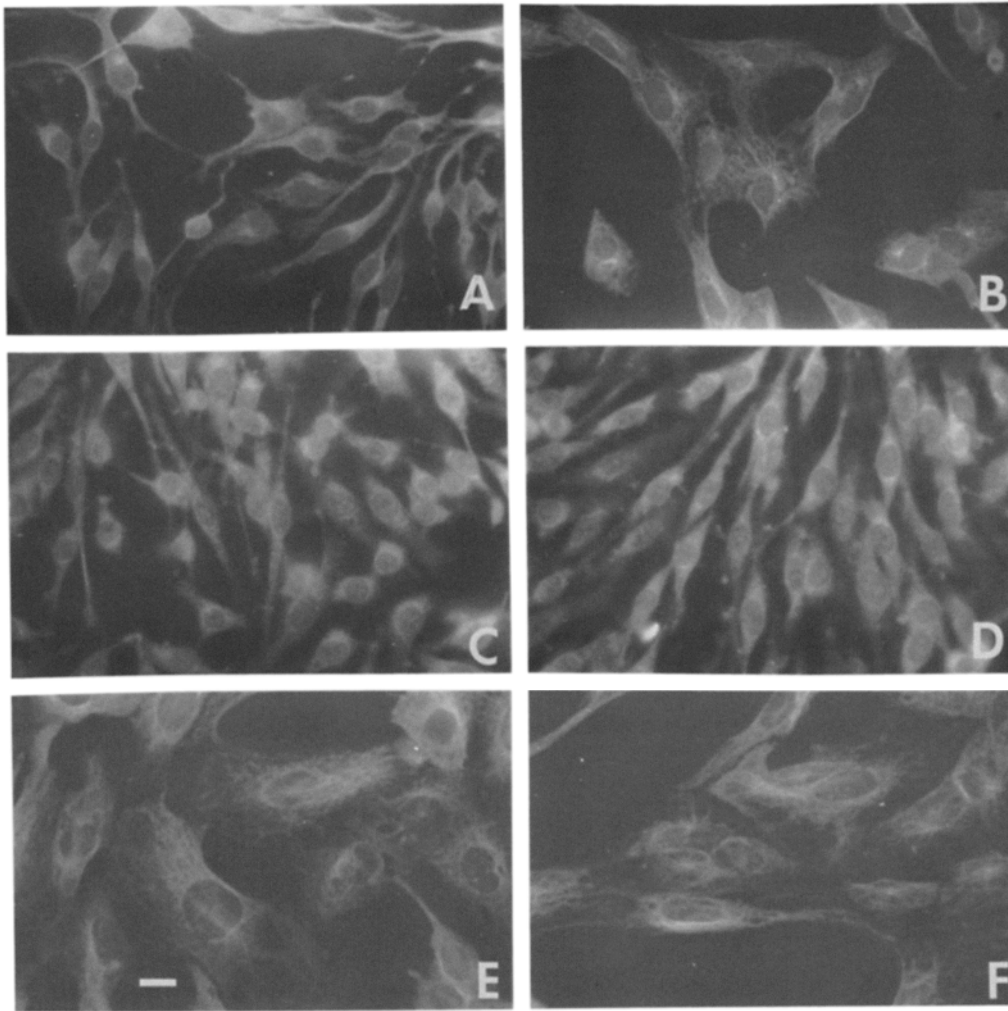
FIGURE 6 Immunofluorescence staining for tubulin: effect of Hy treatment. Clones ST1 (A and B). P₇ (C and D) and 3D (E and F) were cultured in the absence (A, C, and E) or presence (B, D, and F) of Hy (250 ng/ml for 48 h) before staining for tubulin as described in Materials and Methods. Bar, 6 μm.

the same component of the extracellular milieu (polycations) can lead to cell proliferation block or not. This suggests that alterations other than those of cell surface composition are caused by Hy treatment of C₆ cells.

Others have found evidence suggesting that fibronectin is not directly related to growth control mechanisms (20, 21).

As described by others (12), catecholamines and cAMP cause a cell shape change in C₆ cells (Fig. 1 C). Hy inhibited this morphological change via a process that is dependent on RNA and protein synthesis (Fig. 3). This cell shape change is dependent on microtubules' functioning as indicated by colchicine- and nocodazole-blocking effects. We hypothesized that Hy inhibits the cAMP-induced cell shape change by promoting alterations on microtubule organization via transcription regulation. In favor of this idea is the change in the distribution of concanavalin A (Con A) receptors caused by Hy treatment in ST1 cells (J. Garrido, M. C. S. Armelin, and H. A. Armelin, unpublished results). Through the Con A-peroxidase technique (22) we observed that the distribution of Con A receptors changes from a discontinuous and clustered pattern (characteristic of transformed and mitotic cells) to an evenly distributed one (characteristic of normal cells [23, 24]). The hormone treatment also prevents the intense endocytosis observed as abundant peroxidase-positive vesicles in the cytoplasm. These observations suggest that Hy can affect the role that cytoskeletal elements play in mobility control of membrane receptors in ST1 cells.

A direct examination of microtubule organization by indirect immunofluorescence revealed two distinct patterns in Hy-sensitive clones which we classified as "dispersed" and "network" for control and Hy-treated cells, respectively. That the "network" pattern induced by Hy was not secondary to cell flattening was suggested by the fact that polycations do not change the "dispersed" pattern presented by hormone-untreated cells, even though extensive cell flattening is caused. Osborn and Weber (7) have stressed that the diffuse cytoplasmic fluorescence displayed by transformed fibroblasts should not be taken to mean that these cells lack well-formed microtubules; in fact, microtubule fibrils could be seen in all preparations of Hy-untreated cells. The fact that the Hy-resistant variant P₇ displays a "dispersed" microtubules distribution irrespective of hormone treatment (Fig. 6, C and D) suggests that rearrangements in microtubule organization are part of the mechanism(s) by which Hy changes growth control in these cells. Further support comes from experiments showing that Hy renders the ST1 variant susceptible to a G₁ phase block by colchicine or nocodazole (microtubule-disrupting agents) ~4 h before S phase (4, 5). Thus, Hy treatment makes microtubule integrity necessary for G₁ phase traversing. This role for microtubules has been suggested before (25–30). However, the effects of microtubule disrupters on G₁ phase cells seem to be complex (31–33) and have yet to be understood.

A correlation between microtubules' rearrangement and

the growth control change induced by Hy was shown to exist in cultures grown attached to solid substrates. The fact that clones P_2 and P_{11} are sensitive to Hy in solid substrate but resistant in agarose suspension does not necessarily imply that the growth inhibition caused by this hormone in suspension is independent of cytoskeletal rearrangements. Technical limitations have not allowed us to probe into the effects of Hy in suspension cultures by immunofluorescence. Other approaches are obviously needed to clarify the role of cytoskeleton in the growth control change induced by Hy.

Clone 3D was the only anchorage-dependent C_6 variant found and also the only one that failed to round up upon treatment with catecholamines and cAMP. Its cytoskeleton was also exceptional, since actin cables and organized microtubule networks were displayed by these cells in the absence of any hormone treatment. This cytoskeleton organization, so different from that of most C_6 cells, may, in fact, explain why 3D cells failed to present the morphological response to catecholamines and cAMP. As in normal versus transformed fibroblasts (34), the inability of 3D cells to grow in agarose suspension correlated with more the easily discernible microtubule network and the presence of actin cables.

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