Cyclic AMP, the microtubule–microfilament system, and cancer
(cyclic nucleotides/reproductive control/cell cytoskeletal system/cell morphology/cell membrane macromolecules)

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ABSTRACT Additional evidence is presented for the previously proposed existence in normal fibroblasts of a cyclic AMP-dependent network of microtubules and microfilaments, which is connected with cell membrane elements on one end and with nuclear structures on the other and whose disorganization leads to malignant transformation. In the presence of cyclic AMP derivatives sufficient to promote integrity of this network, cell growth limitation in suspension, increased transport of ATP, & the relatively tranquilized membrane of the normal fibroblast are also inhibited. A pattern of distribution of actin and tubulin has been demonstrated showing aggregated actin deposits which are presumably responsible for the oscillatory knob activity of cells with the transformed habitus. Specific orientations of microtubular and filamentous elements with respect to the nucleus can be demonstrated. The hypothesis that the microtubular–microfilamentous structure conveys growth-regulatory information from the cell membrane to the nucleus and that its disorganization can lead to malignancy has been extended to explain various cellular manifestations.

The standard Chinese hamster ovary culture (CHO-K1) displays the classical stigmata of malignantantly transformed cells grown in vitro: it has a compact and pleomorphic structure with a surface studded with knobs (or blebs) instead of the smooth, highly elongated spindle-shape characteristic of normal fibroblasts; it grows in a random pattern so that colonies arising from single cells on a petri dish approximate a circular outline instead of the loops and whorls that characterize colonies of normal fibroblasts (1); it shows no contact inhibition of growth but readily grows in three dimensions; and it is typically sensitive to agglutination by low concentrations of lectins, which do not agglutinate normal fibroblasts. In 1971, we reported that dibutyryl cyclic AMP (Bt2cAMP) causes CHO-K1 cells to lose these transformation characteristics and to assume the morphological habitus approaching that of normal fibroblasts (2). Similar findings were simultaneously reported by Johnson et al. (3) in mouse and rat sarcoma cells.

This process, which we named reverse transformation (RT), was shown to be synergized by testosterone, testolactone, and various prostaglandins and does not require new protein synthesis (2, 4, 5). We also showed that reverse transformation could be prevented by Colcemid, which causes disorganization of cellular microtubules, or by cytochalasin B, which inhibits microfilaments (2, 6). Moreover, just as cAMP derivatives cause reverse transformation of transformed cells, Colcemid and cytochalasin B cause normal fibroblasts to develop knob activity and other characteristics of the transformed state (6). We further showed by electron microscopy that the microtubules of the transformed cells are sparse and randomly arranged, whereas after reverse transformation, they form a dense, orderly, parallel network traversing most of the length of the resulting fibroblast-like structure (7).

We, therefore, proposed that in the normal fibroblast, a common network consisting of an orderly array of both the rigid microtubular and the contractile microfilament elements is responsible for normal regulation of cell growth, that cAMP plays a necessary role in the formation and operation of this structure, and that disruption of either component disorganizes the overall structure. The microtubule–microfilament network (MT–MF) was postulated as carrying information from the cell surface to the genome, thus regulating cell reproduction. Transformed cells having lost this informational transfer, at least in some cases by disorganization of this MT–MF network, multiply in unregulated fashion, producing unlimited growth in vitro and tumors in vivo (2, 4, 6–12). The involvement of cell surface elements in this reaction was demonstrated not only by the change in lectin agglutination accompanying the reverse transformation change, but also by decreased ability of antisera to the CHO-K1 cell to produce agglutination and capping and decreased effects of both lectins and of such antisera to produce the typical conversion to a spherical shape that normally occurs when surface-attached CHO-K1 cells are treated with these agents (4, 8, 13). Reverse transformation also causes loss of lethal interaction of human and Chinese hamster cell surface antigens with specific antisera and complement. Finally, we demonstrated that the knobs characteristic of the transformed state are in violent and constant motion, whereas RT cells and normal fibroblasts exhibit smooth, relatively tranquil membranes (6).

Work of other laboratories has confirmed and extended aspects of these studies. The phenomenon of reverse transformation is exhibited by several different transformed cells when treated with Bt2cAMP or similar agents (3, 14). Electron microscopic studies (15) of 3T3 and L929 cells have confirmed that Bt2cAMP treatment alters the cellular distribution of the microfilaments and microtubules, leading to their alignment into parallel patterns, similar to that demonstrated for the MT–MF system of CHO-K1. Brinkley et al. confirmed the development of a patterned array of microtubular strands in cells treated with reverse transformation agents, using immunofluorescence techniques (14, 16). Similarly, as we had proposed (2, 4, 6, 12), an orderly pattern of microfibrils resembling that which we demonstrated with the microtubules was demonstrated to exist in normal but not in transformed cells (17–19), using immunofluorescence with antisera against actin. Edelman et al. (20, 21) have emphasized the role of specific cell membrane structures in the control of growth by components of the MT–MF system and have invoked the capping reaction as an element in the overall picture. Singer (22) has considered several molecular aspects of cell membranes, and Willingham has reviewed effects of cAMP (23).

The present paper describes further experiments and studies on the roles of cAMP and the MT–MF system and some inferences about their role in cancer.

Abbreviations: cAMP, 3',5'-cyclic AMP; Bt2cAMP, dibutyryl cAMP; MT–MF network, microtubular–microfilamentous network; FCM, macromolecular component of fetal calf serum.
METHODS AND MATERIALS

Cells used were the Chinese hamster ovary cell (CHO-K1), which is a stable hypodiploid line derived by spontaneous transformation from a fibroblast culture (24) and a Chinese hamster ovary cell, with a normal fibroblastic habitus, CHO-III.

Methods of cell cultivation and single cell plating on surfaces (25) or in agar suspension (26) have been described. All cells were grown in F12, supplemented either with 5–10% fetal calf serum or its macromolecular fraction (FCM). Experiments were performed in multiplicate, and each cell plating at least in duplicate, with individual values agreeing within 5–10%.

cAMP can elicit a wide variety of cellular actions by mechanisms which in most cases are still obscure (27, 28). In the experiments described here, Colcemid and cytochalasin B were used in an attempt to reverse specific cAMP actions as an indication that such actions do indeed require participation of the MT–MF system. Use of these reagents involves certain disadvantages, such as their intrinsic ability to inhibit cell multiplication, and the tendency of Colcemid to accumulate cells in metaphase. For these reasons we report here only experiments with sufficiently high drug concentrations to produce effects within 1–3 hr.

Active transport of α-[14C]aminobutyrate was measured by inoculating 5 × 10⁶ cells in 1 ml of F12 FCM10 into each of a series of 35-mm plastic petri dishes which were incubated overnight. Identical dishes without cells served as controls. Test chemicals plus 0.15 ml of a 1:50 dilution of α-[14C]aminobutyrate (ICN, specific activity 26.9 mCi/mlliter) were added to each dish in a final volume of 1.25 ml and incubated 1.5 hr. The dishes were removed from the incubator and placed on a cool damp towel to achieve room temperature quickly. The supernatant liquid was carefully removed and the cell layer was washed four times in growth medium. Analysis of these washings showed their total cell counts and the radioactivity content of the last washing to be negligible when compared respectively to the cells remaining attached and their contained radioactivity. The cells adhering to the dishes were then completely removed by a 10-min incubation at 37° in 0.5% trypsin solution, a concentration 10-fold greater than that routinely used to remove attached cells. Examination of the plates revealed that more than 99% of the cells were removed. The radioactivity of an aliquot of the cell harvest equivalent to at least 10⁶ cells was measured in a scintillation and expressed as cpm/10⁶ cells.

Anti sera against tubulin and actin, respectively, were kindly furnished to us by B. R. Brinkley and E. Lazarides. The immunofluorescence procedures followed were those described by these investigators (16–18).

Various conditions can be used to achieve reverse transformation of CHO-K1 cells. All of these treatments furnish cAMP or a closely related derivative to the cells, inhibit the action of phosphodiesterase in the cells, synergize the action of cAMP by an undetermined mechanism, or use a combination of these actions. The most common condition used and adopted as standard utilized 0.5–10 mM Bt2cAMP plus 30 μM testolactone. However, similar effects can also be obtained with 1 mM 8-bromo-cAMP alone, 1 mM N6Bt2cAMP, or 1 mM cAMP plus 0.2 mM isobutylmethylxanthine. The period of exposure to the reagents varied from 6 days for certain growth experiments to 1.5 hr when cell growth was not a necessary part of the experimental observation. Higher concentrations of the reagents made possible more rapid responses and avoidance of more slowly developing side effects of the drugs. Photomicrograph

Table 1. Growth of CHO-K1 cells in the presence and absence of reverse transformation agents

<table>
<thead>
<tr>
<th>Medium</th>
<th>Plating efficiency, %</th>
</tr>
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<tbody>
<tr>
<td>On plastic</td>
<td>In agar</td>
</tr>
<tr>
<td>Growth medium alone</td>
<td>78.7</td>
</tr>
<tr>
<td>Reverse transformation agents added</td>
<td>72.3</td>
</tr>
</tbody>
</table>

In standard growth medium (F12 FCM5) single CHO-K1 cells grow readily into colonies either on the surface of plastic dishes or in agar suspension. Addition of 1 mM Bt2cAMP plus 30 μM testolactone has little effect on the surface growth but suppresses growth in agar suspension. Three hundred cells were inoculated in every case.

Magnifications can be estimated from the fact that average nuclear cell diameters are 20–25 μm.

EXPERIMENTAL RESULTS

Control of Cell Reproduction in Suspension. Normal fibroblasts grow on glass or plastic surfaces but not in suspension, whereas transformed cells usually grow well in either situation. This differential behavior indicates a difference in interaction between the cell surface and the environment which is important in growth regulation and which has been altered by transformation. Table 1 demonstrates that single cells of CHO-K1 in the native state grow equally well on plastic surfaces or in agar suspension. In the presence of reverse transformation conditions, however, excellent growth is still achieved on the plastic surface but no growth whatever occurs in suspension. The concentration of FCM is critical here, since higher concentrations can neutralize the effect of the cAMP derivatives used. This procedure would appear to be useful as a quantitative measure of reverse transformation and as a selective technique for isolating particular phenotypes. The intrinsic toxicity of Colcemid and cytochalasin B to reproduction made impossible a test of these drugs on this effect.

Effect of Reverse Transformation on Active Transport of α-[14C]Aminobutyric acid in CHO-K1 Cells. Active transport is a property of the cell surface membrane (29). Fig. 1 demonstrates that reverse transformation conditions increase the accumulation of α-[14C]Aminobutyric acid inside CHO-K1 cells. The curve has a particularly steep slope in the region where the change in morphology produced by cAMP is maximal, but

Table 2. Reverse transformation agents increase the accumulation of α-[14C]aminobutyric acid inside CHO-K1 cells

<table>
<thead>
<tr>
<th>Reagents added</th>
<th>cpm/10⁶ cells*</th>
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<tbody>
<tr>
<td>None</td>
<td>109 ± 6</td>
</tr>
<tr>
<td>1 mM Bt2cAMP + 30 μM testolactone</td>
<td>152 ± 12</td>
</tr>
<tr>
<td>13 μM Colcemid</td>
<td>55 ± 7</td>
</tr>
<tr>
<td>4 μM Cytochalasin B</td>
<td>61 ± 3</td>
</tr>
<tr>
<td>13 μM Colcemid + 4 μM cytochalasin B</td>
<td>53 ± 3</td>
</tr>
<tr>
<td>1 mM Bt2cAMP + 30 μM testolactone + 13 μM Colcemid</td>
<td>123 ± 5</td>
</tr>
<tr>
<td>1 mM Bt2cAMP + 30 μM testolactone + 4 μM cytochalasin B</td>
<td>130 ± 6</td>
</tr>
</tbody>
</table>

Colcemid, cytochalasin B, or their combination act in opposition to cAMP and decrease the transport of α-[14C]aminobutyrate produced by Bt2cAMP + testolactone. CHO-K1 cells (5.0 × 10⁶) were incubated overnight in a petri dish in growth medium. Thereafter, the reagents indicated were added to different dishes and incubation was continued for a total of 3 additional hours. α-[14C]Aminobutyric acid was added to all dishes for the last 1.5 hr of incubation. The cells were washed with nonradioactive medium and trypsinized, and their contained radioactivity was measured.

* ± SEM.
transport continues to increase until concentrations approximately 8 times higher are achieved. In this interval of increasing concentration of the reverse transformation reagents, further morphologic change also continues in the direction of even greater stretching of the resulting spindle-shaped cells. Experiments are shown in which the concentrations of the reagents are sufficiently high to complete the action within 1–3 hr to avoid complications like metaphase arrest or other time-dependent side effects of these drugs. Typical results are presented in Table 2. The data demonstrate: either Colcemid or cytochalasin B, by itself, lowers the amount of active transport of \( \alpha-[^{14}C]\)aminobutyrate; either agent also lowers the augmented transport produced by Bt2cAMP plus testolactone; and the maximum effect of both agents simultaneously is similar to that produced by either one alone, as is to be expected if both are contributing to the inactivation of the same mechanism.

**Nature of Membranous Knob Activity in the Transformed State.** In previous publications, we have described how the knobs that characterize the transformed cell are in constant, violent movement, imparting to the membrane oscillatory activities in which the individual knob-like structures protrude into the extracellular space to about \( \frac{1}{10} \) of the cell diameter and return within a time period of 15–30 sec. Disappearance of these knobs and the concomitant tranquilization of the membrane is the first manifestation of the cAMP-induced reverse transformation reaction. We propose that disintegration of the MT–MF system causes the actin-containing contractile elements to dissociate from the organized network and to collect at discrete points along the membrane. In these regions the contractile protein, no longer dynamically coupled to the rigid tubular structures, is free to contract with little or no load. The result then is a series of continuously oscillating foci at points along the cell membrane. Both the moderate knob activity, which occurs in transformed cells like CHO-K1, and the more intense knob activity that can be elicited by addition of sufficient cytochalasin B or Colcemid to these cells or even to normal fibroblasts are thus explained. The action of these drugs in producing the oscillating knob activity is specific (6).

This model is amenable to experimental test. It demands that actin be localized in a pattern similar to that of the knobs in transformed cells and even in normal cells treated with Colcemid or cytochalasin B. In a typical experiment normal Chi-

![Figure 1](image1.png)

**FIG. 1.** Increased transport of \( \alpha-[^{14}C]\)aminobutyrate caused by addition of 1 mM Bt2cAMP + 30 \( \mu \)M testolactone, in increasing multiples. Both agents were increased by a constant factor in the different experimental vessels.

![Figure 2](image2.png)

**FIG. 2.** Knob formation is accompanied by a redistribution of the actin-containing material from the long fibrous pattern, characteristic of the normal fibroblastic and reverse transformed cells, to clumped aggregates in the knobbed cell. Cells like CHO-K1 are naturally knobbed, while normal fibroblasts like CHO-III develop knobs in response to treatment with Colcemid or cytochalasin B. (A) Normal fibroblastic CHO-III cells treated with rabbit antiseraum against actin followed by fluorescent goat antiserum against rabbit gamma globulin. The bright regions indicate the presence of actin-containing bodies which form a parallel fibrous structure extending throughout the cell. Treatment of these cells with Bt2cAMP plus testolactone produces no change in this pattern. (B) The same cells as in A, but treated with 2 \( \mu \)M cytochalasin B for 4 hr before addition of the antiseraum against actin. The actin-containing material has been aggregated into clumps which take on the typical knob-like pattern of such cells. (C) CHO-K1 cells treated with cytochalasin B as in B and tested for immunofluorescence with anti-tubulin serum. Some of the tubulin appears in the typical knobbed pattern.
We have proposed that in the normal fibroblast the nucleus, and the production of perinuclear deposits with rays extending into or around the nucleus. These different patterns may well be associated with particular regions of the interphase life cycle. In any case, the results suggest specific patterns of association and interaction of actin and tubulin with the interphase nucleus.

DISCUSSION

Elucidation, principally by Porter (30), of the existence and structure of the cellular cytoskeletal system has made possible these as well as many other studies of its role in the cellular economy. We have used the term MT-MF network or system throughout our work to emphasize that there may not be complete correspondence between the growth-controlling elements and the entire cytoskeletal system of the cell.

In this and previous papers we have demonstrated the following: (a) A transformed cell can be changed back to the habitus of a normal fibroblast by increasing the cellular cAMP concentration. (b) This process of “reverse transformation” involves organization of a patterned network of microtubules and microfibrils throughout the cell cytoplasm. (c) Disruption of either component of the network by Colcemid or cytochalasin B, respectively, causes normal fibroblasts or reverse transformed cells to acquire or re-acquire transformation stigmata. (d) cAMP causes cell surface antigens to change their activity with respect to rounding by specific antisera or lectins and with respect to killing in the presence of complement by specific antisera. (e) The pulsating knobs responsible for membrane hyperactivity in the transformed state are associated with aggregated actin-containing deposits near the membrane, which disappear when cAMP reorganizes the MT-MF structure and restores a large measure of membrane tranquilization. (f) The transformed state causes significantly reduced adhesion of cells to each other and to plastic substrates compared with the fibroblastic state (12, 36, 37). (g) The organized MT-MF system appears to involve the interphase nucleus in complex patterned arrangements which may alter with the state of the reproductive cell cycle. (h) Active transport of α-[3H]aminobutyrate by CHO-K1 cells is markedly augmented by reverse transformation conditions. Wherever their use is feasible, cytochalasin B and Colcemid counteract these morphological, biochemical, and immunochemical actions of cAMP. Obviously, not every cell with an altered MT-MF network is necessarily malignant, nor need all malignant cells have suffered the same kind or degree of change in the MT-MF system. In fact, since a chain of physical and chemical events is probably involved in the growth regulatory process, a defect arising after the steps that involve the cAMP-dependent organization of the MT-MF system might result in malignancy without visible disruption of this network.

These considerations offer an explanation of other phenomena. Cells in different states of differentiation have different membrane macromolecules, presumably attached directly or indirectly to the MT-MF system. Attachment to such sites of molecules on other cells, molecules from the medium, or structures from a solid substrate could alter the mode or degree of attachment of the MT-MF units and, therefore, change the reproductive status of the cell. Herein may lie the explanation for the effect of lectins in inducing lymphocyte reproduction and the need for an appropriate solid surface for growth of normal fibroblasts. In the same way, contact inhibition of growth would appear explainable, as well as the induction of specific enzyme synthesis in cells of embryonic mouse brain only when they have aggregated with each other and sorted themselves out so that like cells are collected together (33). We have proposed that in the normal fibroblast the

special relationship of the MT-MF network with the nucleus. Representative pictures are shown in Fig. 3. Other investigators have published similar pictures (14, 16).

A variety of patterns are discernible in the distribution both of actin and of tubulin in or around the nucleus of interphase cells grown in vitro. These patterns include fiber-like deposits encircling the nucleus, a set of 2–12 discrete local concentrations of these proteins within or directly contiguous to

FIG. 3. Representative patterns of distribution of actin and tubulin in cells which suggest special structural and functional relationships with the nucleus. (A) Immuno-fluorescence of untreated CHO-K1 cell exposed to antiserum against actin. A heavy anti-actin precipitate surrounds the nucleus and numerous discrete regions are visible in or on the nucleus. (B) Immuno-fluorescence of CHO-K1 cell treated for 4 hr with 0.3 mM Bt2cAMP + 30 μM testolactone and then exposed to antiserum against tubulin. The great majority of the tubulin deposit is present as discrete bodies in or on the nucleus. (C) Immuno-fluorescence of CHO-K1 cells treated with 0.3 mM Bt2cAMP plus 30 μM testolactone for 4 hr and tested for immuno-fluorescence with antiserum against tubulin. An intense perinuclear deposit is seen with rays extending into the cytoplasm, and around and possibly into the nucleus.
MT–MF system is attached, directly or indirectly, to specific regions of the cell surface and ultimately to specific regions of the genome structure (5–12). In response to environmental signals delivered to the cell surface, different regions of the genome are exposed or sequestered to control cell division and perhaps other metabolic processes as well. Demonstration of a direct or indirect connection between the MT–MF network and the nucleus still requires substantiation. However, there is suggestive evidence: the existence of definite and specific patterns of association of actin and tubulin with the nucleus, and the well-known fact that treatment of cells with cytochalasin B causes expulsion of the nucleus while leaving a reasonably intact cytoplasm (34). The latter is difficult to explain without an intimate association between the microfibrils and the nucleus.

A malignant cell exhibits two principal alterations in behavior. Control of cell reproduction is lost so that growth proceeds without regard to the needs of the organism. In addition, the cell exhibits a propensity to break away from its original anchorage and set up foci of growth elsewhere in the body. Both of these characteristics are associated with acquisition of the transformation habitus in which the normal MT–MF network has become disorganized. Thus, loss of growth regulation and formation of oscillating aggregates of contractile material that aids dislodgement of the cell from its anchorage both could result from MT–MF disorganization. Reich and his coworkers have proposed that metastasizing cells acquire the ability to activate proteolytic enzymes that promote their detachment and invasion of other tissues (35). Relationships between this and the processes discussed here might explain why different malignancies have different invasive patterns. The transformation of oncogenic viruses may be due at least in part to the suppression or covering up of normal cell surface elements or creation of new ones, as a result of which distortion occurs in the normal MT–MF connections to the cell membrane. The fact that cells attached to and stretched out on solid surfaces assume a spherical shape under the action of lectins and antibodies on the one hand and proteases on the other implies that at least an important fraction of the surface structures to which elements of the MT–MF system connect are antigenic and involve protein and carbohydrate moieties.

Transformed cells frequently exhibit instability in chromosome number even in clonal populations. Defects in the MT–MF constitution could well be reflected in corresponding defects in the spindle resulting in errors of chromosomal distribution among the cell progeny, in a fashion similar to that resulting from extremely low concentrations of Colcemid (36). Finally, this formulation proposes that interphase microtubules are involved with the regulation of cell reproduction which proceeds unchecked when the microtubules are disorganized. It would follow, then, that whereas Colcemid blocks mitosis completely by preventing spindle formation, it should have no effect on the passage of cells through most of the interphase part of the reproductive life cycle. Such is the case, as has been discussed earlier (9).

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