

Effects of Calcium and Hormones on the Cytoskeleton and Cell Proliferation

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Transformation of cells in culture as a model system for the study of tumorigenesis relies on a variety of selective growth assays. The most commonly used assays are (1) the density assay, which takes advantage of the ability of transformed cells to grow to a high saturation density (Todaro et al. 1964); (2) the serum assay, which employs low concentrations of serum or depleted serum to inhibit the proliferation of normal cells (Smith et al. 1971); and (3) the anchorage assay, which deprives cells of a charged solid substrate for attachment and spreading (Stoker 1968). These assays have been productively employed in determining the precise contribution of viral genes to transformation. However, the mechanism underlying the ability of cells to respond to any of these restrictive culture conditions remains poorly understood. In this paper we discuss our recent work on this question, which has led us to examine the behavior of a set of cell lines in serum-free culture.

Four Classes of Transformants and Revertants

Transformants

Transformants can differ widely in phenotype, even among cell lines transformed by the same tumor virus (Risser and Pollack 1974); therefore, different selective assays must be used to fully characterize the transformed phenotype of a given cell line. For SV40 transformation of both mouse 3T3 cells and diploid rat embryo fibroblasts, extensive analysis has revealed that anchorage transformants are invariably serum-transformed, but that serum transformants often remain anchorage-dependent (Risser et al. 1975). Our interpretation of this result is that the loss of a high serum requirement represents an early event in SV40 transformation and that additional selective pressure is needed to generate anchorage transformants (Chen et al. 1981; Pollack 1981). Such ordering of the loss of growth requirements in vitro may or may not reflect the progressive development toward intractable malignancy in vivo (Foulds 1969),

but in any event it provides a strategy for resolving transformation into sub-component parts.

Although the requirements for serum growth factors and anchorage are separable, in some cases the anchorage requirement is nullified in the presence of additional growth factors. Growth factors secreted by tumor cells can stimulate normal postcrisis rat cells to grow without anchorage (DeLarco and Todaro 1978). High serum concentrations can stimulate normal hamster fibroblast lines to grow without anchorage (O'Neill et al. 1979). Establishment as an immortal cell line appears to be a prerequisite for responding to such exogenous factors in the anchorage assay, since transforming growth factors fail to elicit anchorage-independent growth in precrisis normal diploid fibroblasts, under conditions where they do promote the growth of normal established cell lines (Kaplan et al. 1981).

Revertants

Just as one applies selective pressure to aid in detecting transformed derivatives of normal cells, the reverse process aids in isolating phenotypically normal revertant cells from a transformed population (Pollack et al. 1968). Density-sensitive revertants of a fully transformed line concomitantly regain an anchorage requirement but need not regain a high serum requirement (Vogel et al. 1973). Direct selection of serum-sensitive revertants from a fully transformed line yields cells with the unusual combination of a high serum requirement and the ability to grow without anchorage (Vogel and Pollack 1973; Chen et al. 1981). This type of cell line (normal serum-dependence, anchorage-independent) completes the four potential phenotypes that are possible with these two restrictive assays (Table 1). In past years, we have used these four types of cells, all derived from one fully transformed cell line, to determine the manner in which a set of newly discovered phenotypic traits correlates with the loss of either the serum requirement or the anchorage requirement. For example, we have recently found that the presence of a novel variant of SV40 T antigen is specifically associated with the absence of the anchorage requirement within these four classes of cell lines (Chen et al. 1981).

Growth-factor Requirements of the Four Classes

The basis for the loss of the high serum requirement in transformation has been exceedingly difficult to analyze by serum fractionation (Holley and Kiernan 1968), but resolution now seems likely through analysis of specific hormone requirements for cell growth (Barnes and Sato 1980). In an early study, Temin (1968) showed that Rous sarcoma virus (RSV) reduced the requirement of chick embryo fibroblasts for the insulin or insulinlike growth factor. In the presence of plasma, which lacks platelet-derived growth factors (PDGFs) found in serum, the addition of PDGF markedly elevated the saturation density of normal fibroblasts (Ross and Vogel 1978). Transformed cells, including serum transformants that remain anchorage-dependent, grew to equally high saturation densities in the absence or presence of PDGF (Scher et al. 1978). Thus, a normal cell's PDGF requirement, like its requirement for high serum, may be lost as a stable intermediate event in the total transformation process.

The first use of serum-free medium supplemented with hormones to compare the hormone requirements of normal and fully transformed cells detected

Table 1
Growth Properties of Four Classes of Cells
Derived from Mouse Fibroblasts

Class	<i>Sensitivity to growth control</i>		Cell line	Derived from
	<i>serum</i>	<i>anchorage</i>		
1	N	N	Swiss-3T3 BALB-3T3 LS ₂	Swiss MEF BALB MEF SV101
2	N	Tx	A γ 4 KiSV-A γ 4	SV101 A γ 4
3	Tx	N	SVR57 3T6 FL ₂ , BU ₂	3T3 Swiss MEF SV101
4	Tx	Tx	SV101, 884D KA31T Sarcoma 180	Swiss-3T3 BALB-3T3 mouse sarcoma

N refers to the behavior of normal cells, and Tx refers to the behavior of transformed cells. Typically, a serum transformant grows readily ($T_D < 35$ hr) in 1% serum, and an anchorage transformant forms colonies in semi-solid medium at an efficiency greater than 1%. SV101 is a standard SV40-transformed derivative of Swiss-3T3. 884D is a 3T3 clone transformed by the deletion mutant of SV40, *d*1884. KA31T is a Kirsten sarcoma virus transformant of BALB-3T3. LS₂ and A γ 4 are serum revertants of SV101. BU₂ and FL₂ are density revertants of SV101. SVR57 is a 3T3 clone isolated after infection with SV40 that grows in low serum but does not express viral antigens (Risser and Pollack 1974).

an epidermal growth factor (EGF) requirement that was lost upon transformation to anchorage independence and tumorigenicity (Cherington et al. 1979). In the same system, nontumorigenic serum transformants did not lose their EGF requirement, although some, but not all, of them had a reduced insulin requirement (Sager et al., this volume). Thus, the loss of the EGF requirement seems to be related to further stages in the transformation process.

We have developed a serum-free medium supplemented with hormones for the mouse cell line 3T3 in order to determine the hormone requirements of the four classes of cell lines listed in Table 1.

Calcium Requirement in Transformation

Calcium ions in culture medium are essential for cell viability. Physiological calcium levels are necessary for fibroblast motility and substrate adhesion (Takeichi and Okada 1972). Calcium ions are actively sequestered from the cytoplasm by mitochondria and the endoplasmic reticulum and are actively transported out of the cell. The remaining cytoplasmic calcium is "buffered" by a set of calcium-binding proteins such as calmodulin (Cheung 1980). The resulting low concentration of free calcium in the cytoplasm enables small changes in calcium flux to dramatically alter the activity of calcium-dependent proteins. It is therefore not surprising that variations of the calcium concentration can profoundly affect cell growth, as many investigators have found. For example, the presence of physiological levels of calcium was found to be neces-

sary for serum stimulation of cell growth, and in calcium-deficient medium, 3T3 cells were blocked in G₁ (Boynton et al. 1974). Elevation of calcium to 15 mM stimulated quiescent fibroblasts to proliferate, suggesting a direct mitogenic role for calcium ions (Dulbecco and Elkington 1975).

Some transformed fibroblast lines have a reduced calcium requirement for growth. Adenovirus-transformed rat fibroblasts form foci in low (0.1 mM) calcium (Freeman et al. 1967). RSV-infected chick cells can grow in low-calcium medium, whereas uninfected chick cells are reversibly blocked (Balk et al. 1973). SV40 produces transformants of human fibroblasts and mouse fibroblasts which can grow in low-calcium medium (Boynton and Whitfield 1976; Boynton et al. 1977). Finally, chemically transformed mouse fibroblasts have a greatly reduced calcium requirement (Paul and Ristow 1979).

One possible explanation for the quiescence induced by lowered extracellular calcium in normal cells is that cytoplasmic calcium levels track ambient calcium, resulting in decreases either in free cytoplasmic calcium, bound cytoplasmic calcium, or both. The calcium-receptor calmodulin mediates the effect of increased calcium concentration in many cellular events (Cheung 1980); therefore, calcium bound to calmodulin may be critical in cell growth. Interestingly, some transformed cells have been reported to contain two to three times the normal amount of calmodulin (Chafouleas et al. 1981). Thus, if free cytoplasmic calcium did track reduced ambient calcium, increased calmodulin might be expected to maintain a constant amount of calcium-calmodulin complex. On the other hand, if the concentration of free cytoplasmic calcium must itself be directly maintained at some fixed concentration for cell proliferation, calcium transformation would have to be accompanied by diminished export of cytoplasmic calcium necessary to maintain this proper amount of cytoplasmic free calcium in low-calcium medium.

A third possibility exists for calcium regulation of growth in addition to either inefficient pumping of calcium or a lowered amount of free cytoplasmic calcium coupled to an increased amount of calcium receptors such as calmodulin. Reduced extracellular calcium may prevent normal cell proliferation by impairing such cell-surface events as hormone binding and proper substrate attachment, rather than by perturbing cytoplasmic calcium-dependent events. In that case, transformed cells would escape from calcium control as a secondary consequence of their reduced requirements for extracellular signals such as hormones and attachment.

Although considerable support exists for the involvement of calcium as a mediator in at least some aspects of cell growth, more work is required in this area to permit definitive conclusions. We have begun to examine the relationship of the calcium requirement for growth and the serum and anchorage requirements using the four cell classes listed in Table 1.

Anchorage Requirement and Cytoskeletal Organization

When a cell can grow without anchorage, it has lost the need for both attachment and cell spreading (Folkman and Moscona 1978; Ben Ze'ev et al. 1980). Both cell attachment and spreading require reorganization of the cytoskeleton. The major cytoskeletal protein of both normal and transformed cells is actin (Pollard and Weihing 1974). The actin of a normal spread cell is partitioned into unpolymerized G-actin and a set of filamentous arrays (Spudich et al.

1977). These arrays are found in the cell as a submembranous gel matrix along with other cytoskeletal components, as part of a matrix of single microfilaments that fills the cytoplasm interacting with other cytoskeletal filaments, and as part of the large microfilament cables located at the adherent side of cells directly under the surface (Heuser and Kirscher 1980). The cables end at the site of cell-surface attachment points to the substrate, which contain vinculin and are interdigitated with external fibronectin (Geiger 1979).

Normal cells that are induced to round up lose large cables, regaining them upon attachment and spreading (Bragina et al. 1976). In tumorigenic anchorage-transformed cells, the actin is permanently repartitioned among the different macromolecular arrays; more of the actin is found in the microfilament gel matrix and less of it is found in the cables (Heuser and Kirschner 1980). As a result, for fibroblasts, there is a consistent loss of cables upon anchorage transformation (Pollack et al. 1975; Verderame et al. 1980).

Because of the reduced calcium requirement and increased calmodulin concentration of some transformed cells, we have examined the possible role of calmodulin in maintaining the ordered cytoskeleton of the normal fibroblast. To do this we have employed the drug chlorpromazine and its derivatives. In binding to calmodulin, this drug blocks reactions that require the active calcium-calmodulin complex (Cheung 1980). These agents prevented cell spreading and migration (Connor et al. 1981). We have found that the phenothiazines directly perturb the actin cytoskeleton at concentrations that are well below those necessary to detach the cells from their substrate.

EXPERIMENTAL METHODS

Cell Culture

Our standard cell-culture techniques have been described previously (Verderame et al. 1980, Chen et al. 1981). In the serum-free growth experiments reported below, cells to be grown in serum-free medium were first plated in medium containing 10% serum; 12 to 24 hours later, they were washed twice with serum-free Dulbecco's modified Eagle's medium (DME) before addition of experimental media. Alternatively, the dishes were pretreated with 10% serum as described previously (Barnes and Sato 1980), and cells were plated directly into serum-free medium. In both cases, cells to be plated were suspended by a wash with 2 mM EDTA in PBS and allowed to detach in the incubator in a small volume of PBS. Triple-distilled H₂O was prepared as described previously (Barnes and Sato 1980). MCDB 402 medium was made according to the formula developed by Ham's laboratory (Shipley and Ham 1981), modified for use with 10% CO₂ (3.7 g/liter of sodium bicarbonate; 4.6 g/liter of sodium chloride). We added penicillin (100 units/ml) and streptomycin (100 µg/ml) as well to all media.

All growth-factor supplements were stored at -20°C as 100×-1000× stock solutions in PBS. Human transferrin was obtained from Sigma. Insulin, fibroblast growth factor (FGF), and EGF were obtained from Collaborative Research. Gimmel factor was prepared as described by Barnes and Sato (1980) from female rat submaxillary glands (Pel-Freeze), as the 35% ammonium sulfate precipitate of a crude extract.

Experiments in which calcium concentration was varied employed chelex

(Bio-Rad)-treated fetal calf serum (FCS) prepared as described previously (Brennan et al. 1975) and added to calcium-free DME to a concentration of 10% (v/v). Calcium chloride was added to various final concentrations (see Results and Discussion). The cells were plated in DME with 10% FCS, and 12 to 24 hours later (day 1) the medium was changed to calcium-free DME plus 10% chelex-treated FCS and supplemented with CaCl_2 in the range of 0.01 mM to 2.0 mM. Duplicate dishes were counted on day 3 and day 6 with a model ZB Coulter counter.

Cytoskeletal Assays

In studies on the effects of chlorpromazine, 10^5 early-passage mouse embryo fibroblasts were plated onto 60-mm dishes in 10% FCS. Two days later the medium was removed and replaced with medium containing chlorpromazine. Tests of adhesion were done by rinsing plates with PBS, detaching adherent cells with trypsinization, and counting them with a model ZB Coulter counter.

Chlorpromazine hydrochloride (Sigma) was dissolved in water and stored at -20°C . Chlorpromazine sulfoxide was a gift from Smith, Kline, and French.

With fluorescent phalloidin, which has a high affinity for filamentous actin, we used a Leitz epifluorescence microscope to score cells by one of four classes of actin-cable organization. These classes ranged from cells containing very large cables throughout the cytoplasm (I) to cells showing no discernible cables (IV) (Verderame et al. 1980).

RESULTS AND DISCUSSION

Reduced Insulin Requirement

We used the four classes of 3T3-derived cells (Table 1) to determine whether transformed cells that have reduced requirements for serum, anchorage, or both might also have reduced requirements for insulin. Investigators in G. Sato's laboratory devised a hormone mix for the 3T3 subclone 3T3-L1 (Serrero et al. 1979). We have confirmed their result that hormones can replace serum for 3T3-L1, and we have used this hormone mix to develop a serum-free medium in which 3T3 and its descendants grow at colonial densities with high plating efficiency (Fig. 1). We used the MCDB 402 medium developed in R. Ham's laboratory specifically for mouse fibroblasts (Shipley and Ham 1981). Recently, workers in Ham's laboratory combined the use of hormones and an optimal basal medium to successfully grow human fibroblasts at clonal density (Bettger et al. 1981). For 3T3-L1, the MCDB 402 medium plus hormones worked as well as 10% serum for supporting exponential growth. Without any hormonal supplement, essentially no growth occurred (Fig. 2).

The hormone mix we used for testing Swiss-3T3 and its derivatives consisted of 10 $\mu\text{g}/\text{ml}$ of insulin, 10 ng/ml of FGF, 5 $\mu\text{g}/\text{ml}$ of transferrin, and 5 $\mu\text{g}/\text{ml}$ of Gimmel factor. Gimmel factor contained contaminating EGF, so that 5 $\mu\text{g}/\text{ml}$ of Gimmel factor provided the medium with approximately 1 ng/ml of EGF, as judged by radioreceptor assay (data not shown).

For Swiss-3T3, insulin appeared to be the most stringently required hormone. For instance, the rate of growth without insulin was considerably lower than the rate of growth without FGF (Table 2). This requirement was confirmed in the colony-forming assay. 3T3 cells showed a very strong insulin dependence for colonial growth (Fig. 1; Table 2). Insulin dependence was lost

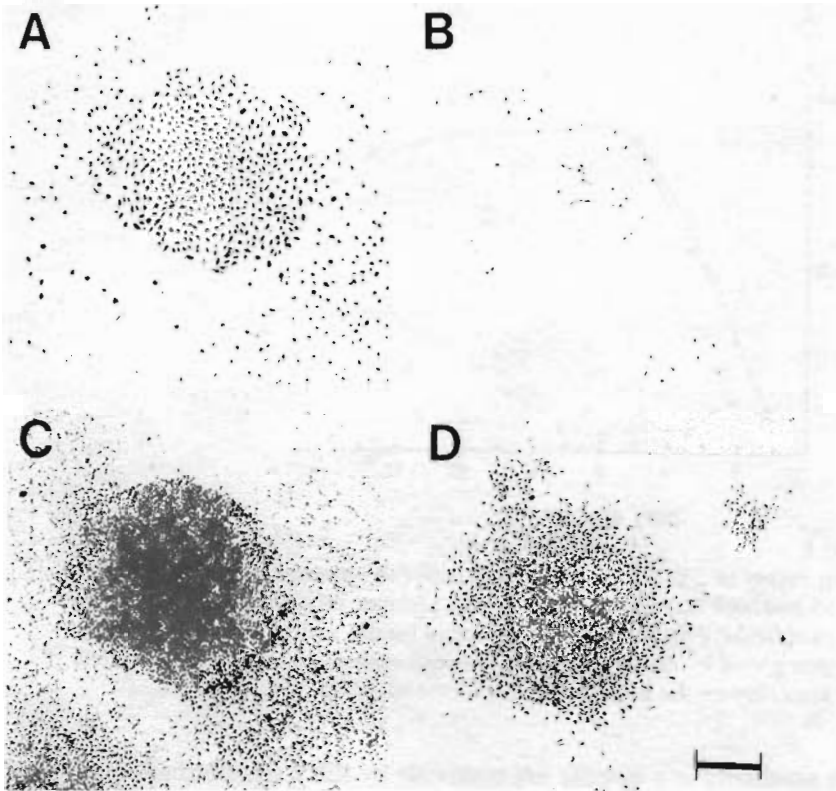


Figure 1

Typical colonies of 3T3 (A,B) and SV101 (C,D) growing in serum-free MCDB 402 with insulin (A,C) and without insulin (B,D). Bar represents 0.5 mm.

upon transformation by SV40, as SV101 formed colonies efficiently in medium without insulin (Fig. 1; Table 3). The colonies tended to be somewhat smaller or less dense than colonies in medium containing insulin; however, insulin deprivation inhibited the growth of normal 3T3 cells much more dramatically than it inhibited the growth of SV101 (Fig. 3).

A γ 4 is a revertant of SV101 that has regained sensitivity to the serum assay but remained competent in the ability to grow in semisolid medium (Table 1). A γ 4 has a strong insulin dependence, similar to that of 3T3 (Table 3). This cell line clearly separated the anchorage requirement from the insulin requirement for growth. Similarly, 3T6 grew well without insulin but did not grow without anchorage (Tables 3 and 1).

We next examined the insulin requirement of mouse cells transformed by other agents. To date, all but one transformed cell line that we have examined showed a reduced insulin requirement, including RNA transformants and tumor-derived cell lines (Table 3). The Kirsten sarcoma virus (KiSV)-transformed serum revertant, KiSV-A γ 4, is a notable exception. Although KiSV caused A γ 4 cells to round up and increased their ability to grow without anchorage (Vogel and Pollack 1974), it failed to induce A γ 4 to grow without insulin (Table 3). We have recently obtained evidence that many SV101-derived revertants have lost considerable amounts of integrated SV40 DNA (G. Blanck et al., in prep.). It is interesting that A γ 4, which has reverted to

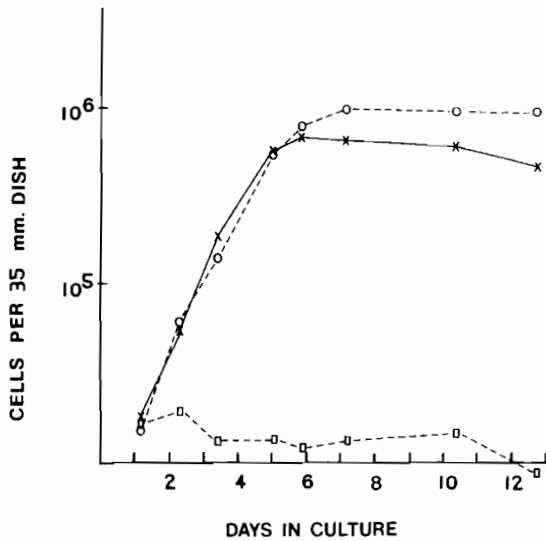


Figure 2

Growth curves of 3T3-L1 in MCDB 402 with full hormone mix (○), with 10% FCS (x), and with no supplement (□). Full hormone mix consisted of 2.5 $\mu\text{g}/\text{ml}$ of Gimmel factor, 2 ng/ml of FGF, 1 $\mu\text{g}/\text{ml}$ of insulin, and 5 $\mu\text{g}/\text{ml}$ of transferrin. Cells were plated in 10% FCS and washed with serum-free DME approximately 12 hr later, before the addition of experimental media.

serum sensitivity in a manner not reversible by KiSV transformation, also has retained all of the SV40 sequences found in the parental line, SV101. Both of these observations are consistent with the hypothesis that A γ 4 arose from SV101 by a second cellular mutation, rather than by a true reversion affecting SV40 DNA (G. Blanck et al., in prep.).

Table 2

Two Different Methodologies for Measuring Hormone Requirements: Growth Rate and Plating Efficiency

Hormones added	Doublings in 7 days	Plate efficiency after 10 days (%)
Full mix	3.9 \pm 0.3	16 \pm 1
-Insulin	0.4 \pm 0.2	0.6 \pm 0.2
-FGF	2.6 \pm 0.1	12 \pm 1

The data show the effects of insulin and FGF on the growth of Swiss-3T3 cells. Hormone requirements were examined by inoculating cells directly into serum-free MCDB 402 with hormones, at densities of 10^4 per 60-mm dish for doublings or 10^3 per 60-mm dish for plating efficiency. The hormone mix consisted of 10 $\mu\text{g}/\text{ml}$ of insulin, 1 $\mu\text{g}/\text{ml}$ of transferrin, 10 ng/ml of FGF, and 5 $\mu\text{g}/\text{ml}$ of Gimmel factor. Doublings were computed as $(\log N_D/N_0)/\log 2$, where N_D is the number of cells at day D . Plating efficiencies were determined as $(100 \times \text{colonies})/1000$ cells plated. The criterion for colony formation was a colony with a diameter greater than 0.5 mm (\sim 50 cells). Values are the averages \pm s.d. obtained with triplicate plates for each condition.

Table 3
Insulin Growth Requirement of Normal and Transformed Mouse Cells

Cell line	Class	Plating efficiency ^a (%)		R.P.E. (-Ins/+Ins) ^b	Relative growth rate ^c
		10% S	SFM + H		
BALB-3T3	1	10	9	.02 ± .04	n.d.
Swiss-3T3	1	13	8	.05 ± .02	.34 ± .01
KiSV-Aγ4	2	11	6	.02 ± .02	n.d.
Aγ4	2	14	10	.03 ± .02	.39 ± .03
SVR57	3	15	8	.12 ± .05	n.d.
BU ₂	3	15	7	.12 ± .03	.48 ± .09
3T6	3	32	24	.33 ± .02	.67 ± .05
884D	4	32	26	.20 ± .05	.57 ± .05
Sarcoma 180	4	24	24	.38 ± .04	.82 ± .02
SV101	4	15	11	.47 ± .11	.87 ± .02
KA31T	4	15	16	.49 ± .07	n.d.

Insulin growth requirements were determined as described in Table 2.
^aValues are the averages of duplicate plates. S indicates serum; SFM + H indicates serum-free medium plus hormones.
^bPlating efficiency without insulin divided by the plating efficiency with insulin.
^cDoublings in 4 days without insulin divided by doublings in 4 days with insulin. Values are the averages ± S.D. obtained with duplicate plates for each condition; n.d. indicates not determined.

The loss of the insulin requirement correlated with the loss of a high serum requirement (Table 3); however, this correlation was only qualitative. Anchorage-dependent serum transformants of 3T3 have a threefold reduction in their insulin requirement compared with that of normal 3T3 (Table 3), but this is still fourfold higher than the insulin requirement of the anchorage-independent

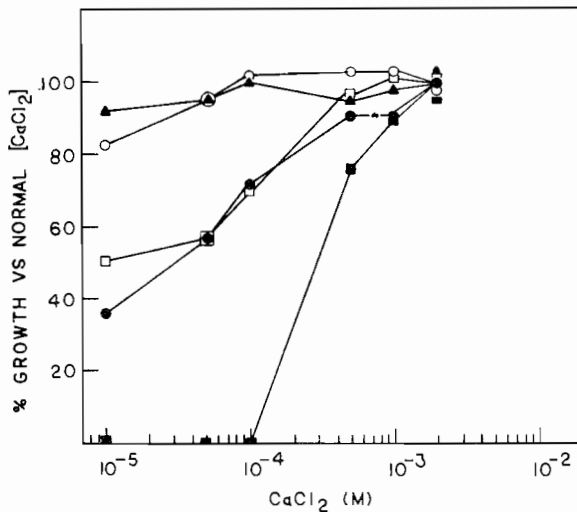


Figure 3
Calcium requirement of normal, transformed, and revertant cell lines. For a given cell line, the growth rate at the indicated calcium concentration relative to the growth rate in 2 mM calcium is displayed. (●) 3T3; (○) SV101; (□) LS₂; (■) Aγ4; (▲) FL₂.

transformants such as SV101. Apparently, there is a spectrum of reduced insulin requirements among the serum transformants.

Reduced Calcium Requirement and Transformation

We have begun to examine cell lines of the four classes for their calcium growth requirement. Figure 3 shows that the lines tested differed in their abilities to grow in low calcium. In these experiments, growth was determined as the growth rate and presented as the fraction of the growth rate in normal 2 mM calcium.

Cell lines 3T3 and LS₂ belong to class 1 (Table 1). These lines were moderately sensitive to decreased calcium (Fig. 3). Cell lines belonging to class 3 (FL₂) and class 4 (SV101) were insensitive to decreased calcium and grew equally well in normal calcium and in medium with as little as 0.01 mM calcium (Fig. 3). The line belonging to class 2 (A γ 4), which was also strongly insulin-dependent, was extremely sensitive to decreased calcium. At calcium concentrations below 0.1 mM, the cells ceased growing and detached from the dish (Fig. 3). These data dissociate the growth requirement for calcium from the anchorage requirement and link the calcium and serum requirements. Serum factors decreased the requirement for calcium in both normal and transformed fibroblasts (McKeehan and McKeehan 1979). We do not yet know whether the requirement for calcium observed here is direct or whether it is an indirect consequence of a change in other ionic requirements, such as the requirement for magnesium (Rubin et al. 1979).

Hormones and Calmodulin as Regulators of Cytoskeletal Organization

We examined the response of normal precrisis diploid mouse fibroblasts to elevated concentrations of serum and found that such cells were not induced to grow in agarose (Table 4). In contrast, postcrisis 3T3 cells did respond to high serum concentrations by growing in suspension (Table 4). This latter response, although inefficient, is in agreement with earlier reports that the established hamster cell line BHK grew in suspension in high serum concentrations (O'Neill et al. 1979). Apparently, the transition from precrisis diploid strain to postcrisis aneuploid line is prerequisite to the serum modulation of anchorage independence.

The existence of a coupled anchorage and serum requirement in a normal

Table 4

Effect of Increased Serum Concentration on the Anchorage-independent Growth of Normal and Transformed Mouse Cells

Cell line	Serum concentration			
	10%	20%	30%	50%
MEF	< 0.001	< 0.001	< 0.001	< 0.001
3T3	< 0.001	0.1	0.3	< 0.001
SV101	5	6	8	< 0.001

Cells were plated into agarose at various serum concentrations. Colonies greater than 0.2 mm in diameter were scored 3 weeks later. Values are the percentages of cells plated that formed colonies.

cell suggests that cell shape may modulate the capacity of a cell to respond to the growth signal delivered in a defined medium by calcium and growth factors. Cell shape is generally agreed to be determined in large part by the cytoskeleton. We scored cells for cytoskeletal actin organization using fluorescent phalloidin (Verderame et al. 1980). The presence of large cables in mouse embryo fibroblast (MEF) cells and 3T3 cells was dependent on both serum and hormones. 3T3 cells in hormone-supplemented medium had fewer cables than 3T3 cells in 10% FCS or in hormone-free DME (Table 5, experiments 1 and 2). Cables were retained in 3T3 cells kept in serum-free medium lacking at least one hormone. EGF and insulin were especially critical; both are required for growth, and in the presence of both, cables were disorganized (Table 5, experiment 2). MEF cells incubated in serum-free medium for 48 hours contained several large cables (Table 5, experiment 7). When hormones, or 1% serum or 10% serum was added, the fraction with cables fell (Table 5, experiments 4, 6, and 8).

Chlorpromazine reduced the adhesion and the organization of actin in MEF cells, as did the addition of hormones or serum. We first examined the effects of chlorpromazine in the presence of serum. In DME with 10% FCS, cells detached from culture dishes within several hours at chlorpromazine concentrations greater than 50 μM . When we examined actin organization in these cells after 48 hours, we found that 10–15 μM chlorpromazine still caused significant detachment and also disrupted the cables in cells that remained attached. In the presence of serum, chlorpromazine at 3–5 μM neither effected adherence nor altered the cytoskeleton (Table 5, experiment 4).

Chlorpromazine is known to interact with serum proteins that may alter the effective drug concentration seen by the cell. When we dropped the serum concentration to 1%, or removed serum entirely, chlorpromazine at 3–10 μM caused extensive actin disorganization with only slight cell loss (Table 5, experiments 6 and 7). In serum-free medium with the full hormone mix, chlorpromazine had only a slight additional effect on the already disorganized cytoskeletons of most cells (Table 5, experiment 8). The SV40-transformed 3T3 line (SV101), which had no large cables, showed no change upon chlorpromazine treatment (Table 5, experiment 3).

The pharmacologically inactive chlorpromazine analog, chlorpromazine sulfide, which has a reduced binding affinity for calmodulin (Weiss and Levin 1978), also had a greatly reduced effect both on the adhesion and on cytoskeletal organization (Table 5, experiment 5). In summary, these results suggest that both adhesion and a well-ordered cytoskeleton are dependent on the availability of active cytoplasmic calmodulin.

CONCLUSION

A reduced calcium requirement and an altered cytoskeleton are both phenotypic markers of transformation whose generality is well established. Our results indicate that a reduced insulin requirement, originally discovered by Temin (1968) with RSV-infected chicken fibroblasts, is an additional phenotypic marker of fibroblasts transformed by a variety of agents. At the present time, the relationship of the loss of the insulin requirement and tumorigenesis is not clear. Whereas most tumorigenic cells we have examined have a reduced insulin requirement, one cell line, A γ 4, which requires insulin to the same

Table 5
Effect of Hormones and Phenothiazines on Adhesion and Actin Cytoskeleton

Exp. no.	Cell line	Medium	Serum	Growth factors	Drug	Drug amount (μM)	Adhesion (%)	Cables		
								I+II	I+II+III	
1	3T3	DME	none	none	none	0	100	47	100	
					CPZ	3	98	19	100	
					CPZ	9	44	0	0	
2	3T3	MCDB	none	full mix	0	n.d.	25	n.d.		
				-insulin	0	n.d.	66	n.d.		
				-FGF	0	n.d.	39	n.d.		
				-EGF	0	n.d.	58	n.d.		
3	SV101	DME	none	none	0	100	0	7		
				CPZ	3	68	0	11		
				CPZ	9	19	0	0		
4	MEF(4°)	DME	10%	none	0	100	46	97		
			10%	none	3	93	41	98		
			10%	none	9	57	13	98		

5	MEF(4°)	DME	10%	none	none	none	0	100	42	100
			10%	none	CPZ-S	3	93	48	100	
			10%	none	CPZ-S	9	96	33	98	
			10%	none	CPZ-S	27	95	42	99	
			10%	none	CPZ-S	81	76	12	88	
6	MEF(4°)	DME	1%	none	none	0	100	64	100	
			1%	none	CPZ	3	67	55	98	
			1%	none	CPZ	9	35	44	90	
7	MEF(4°)	DME	none	none	none	0	100	84	98	
			none	none	CPZ	3	70	34	94	
			none	none	CPZ	9	40	1	30	
8	MEF(4°)	DME	none	full mix	none	0	100	45	100	
			none	full mix	CPZ	3	87	34	99	
			none	full mix	CPZ	9	35	11	49	

Adhesion refers to the percentage of cells plated that adhered to the culture dish after various treatments, as described in Experimental Methods. Cables refers to the percentage of adherent cells that fell into the various categories of cytoskeletal organization (see Experimental Methods). CPZ indicates chlorpromazine; CPZ-S indicates chlorpromazine sulfoxide; n.d. indicates not determined.

degree as normal cells, forms tumors that later regress completely (Shin et al. 1975).

Both insulin deprivation and calcium deprivation affected class-1 (normal cells) and class-2 cells (anchorage-transformed, serum-normal) more dramatically than they affected the other two classes. This suggests that the insulin-sensitive and calcium-sensitive regulatory mechanisms may be closely linked. One possible common step might be a calcium-dependent reorganization of the cytoskeleton during the insulin-induced clustering of membrane receptors (Pollack 1981). We have found that the actin cytoskeleton in normal cells is sensitive to perturbation by a drug that binds calmodulin and thereby prevents calcium from exerting its effects through calmodulin. Thus, the same calcium-sensitive regulatory mechanism may be operating in all three of these assays.

We have recently succeeded in isolating transformants on the basis of their ability to grow at colonial densities without insulin and without physiological levels of calcium. The combined use of refined selective assays should aid in determining the molecular nature of growth regulatory mechanisms.

Note Added in Proof

We have recently been able to grow 3T3 and its derivatives in a totally serum-free medium by employing poly-D-lysine plus fibronectin-coated dishes, with EGF, insulin, and transferrin. Gimmel factor is not necessary under these conditions.

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