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Growth Factor Requirements of Oncogene-Transformed NIH 3T3 and BALB/c 3T3 Cells Cultured in Defined Media

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We report conditions for the efficient growth of NIH 3T3 and BALB/c 3T3 cells cultured in a defined medium supplemented with either platelet-derived growth factor (PDGF) or pituitary-derived fibroblast growth factor (FGF). The oncogenes v-*mos*, v-*src*, v-*sis*, and c-H-$ras_{Val\ 12}$ can induce morphological transformation of these cells and can release them from the mitogen requirement for growth, while the oncogene v-*fos* cannot abrogate the PDGF-FGF requirement. The radically different behavior of normal and transformed NIH 3T3 cells in PDGF-FGF-free defined medium can form the basis of a sensitive new fibroblast transformation assay.

The growth of fibroblasts in culture is most easily achieved in basal medium supplemented with serum as a source of substratum attachment factors and polypeptide growth factors. The principal serum mitogen is platelet-derived growth factor (PDGF) (17), a heterodimeric protein encoded by the proto-oncogene c-*sis* and a related gene product (20, 31). Other identified serum mitogens are the insulinlike growth factors or somatomedins (28). Various oncogenes that can induce morphological transformation of fibroblasts also reduce the serum requirement for growth (22, 27); specifically, it is the PDGF requirement that is reduced (2, 15, 18, 22). In some cases, transformants secrete PDGF-like growth factors (4, 8), which may account for the reduced PDGF requirement. We report here the defined-medium components required for the growth of normal and oncogene-transformed murine fibroblast cell lines. One goal of this work has been to devise new fibroblast transformation assays based upon cell culture in media lacking sufficient growth factor(s) to support normal fibroblast growth. We have established culture conditions in medium lacking PDGF which can detect transformation by an assortment of oncogenes.

Our cell culture procedure is a modification of previously described methods (15, 18). Tissue culture dishes (diameter, 35 mm) were precoated at 37°C with 1 mg of polylysine per ml (average molecular weight of 70,000) for 2 h, then rinsed with phosphate-buffered saline, and incubated overnight in Dulbecco modified Eagle medium containing 15 μg of human fibronectin per ml. These attachment factor-coated dishes were used for plating freshly trypsinized fibroblasts in Dulbecco modified Eagle medium containing calf serum. After a 3-h cell attachment period, the cells were refed with a 3:1 mixture of Dulbecco modified Eagle medium and Ham F12 medium supplemented with 8 mM sodium bicarbonate, 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.4), 4×10^{-6}M manganese chloride, 3 mM histidine, 10^{-5}M ethanolamine, 10^{-7}M sodium selenite, 5 mg of transferrin per liter, 500 mg of bovine serum albumin-linoleic acid complex per liter, and 2×10^{-6}M hydrocortisone. The medium supplements and attachment factors were purchased from Sigma Chemical Co. and from Collaborative Research, Inc. The next day (day 0), cells were refed with the above medium supplemented with mitogens and were refed accordingly on a 3-day schedule. The medium change on day 0 also served to remove any residual serum components which might otherwise gratuitously stimulate cell growth. It is essential that cultures maintained for more than 3 days be on poly-D-lysine-coated plates (to prevent gradual cell detachment), while cultures harvested sooner be maintained on poly-L-lysine-coated dishes (to facilitate trypsinization).

PDGF and insulinlike growth factors are the serum factors which are mitogenic for certain fibroblast cell lines (15, 18, 28), but their expense makes them prohibitive for regular use. Pituitary-derived fibroblast growth factor (FGF) can effectively replace PDGF under certain culture conditions (11, 28), while hyperphysiological concentrations of insulin can substitute for insulinlike growth factors (28). We have used FGF and insulin (purchased from Collaborative Research) in most of our defined-medium studies because of economic considerations.

We first explored the growth of sparsely plated NIH 3T3 murine fibroblasts (13) in media variably supplemented with PDGF, FGF, and insulin. Cell counts at days 0 and 5 were used to compute the number of cell doublings in a 5-day period. NIH 3T3 cells grew in appropriate defined media virtually as well as in serum-containing medium (Table 1).

TABLE 1. Growth of NIH 3T3 cells in defined media[a]

FGF (ng/ml), PDGF (ng/ml), or serum	No. of cell doublings in 5-day assay supplemented with insulin (μg/ml):		
	0	10	30
None	<0[b]	<0[b]	<0[b]
FGF (10)	2.4	3.6	3.5
FGF (30)	3.4	4.6	4.6
FGF (100)	4.4	5.6	5.5
PDGF (5)	3.2	3.9	3.8
PDGF (15)	4.3	5.1	5.0
PDGF (50)	4.3	5.0	5.1
10% calf serum	6.3		

[a] NIH 3T3 cells were sparsely plated on fibronectin-coated dishes as described in the text. On day 0, the cells from two plates were trypsinized and counted: $(9.4 \pm 0.4) \times 10^3$ cells per 35-mm dish. Duplicate cultures were refed with basal defined media supplemented with different concentrations of insulin, FGF, or PDGF. Two other cultures were fed with serum-containing medium. All cultures were appropriately refed on day 3, and cells were trypsinized and counted on day 5. Cell counts from duplicate cultures always varied <6%. Cell doublings were calculated as $\log_2$ (day 5 cell number divided by day 0 cell number).

[b] Extensive cell death had occurred by day 5.

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TABLE 2. Growth of BALB/c 3T3 cells in defined media[a]

FGF (ng/ml)	No. of cell doublings in 6-day assay supplemented with insulin (μg/ml):			
	0	10	30	100
0	0.0	0.9	1.2	1.3
2	0.1	1.8	2.2	2.3
7	0.7	3.7	4.0	4.1
25	3.0	5.4	5.4	5.5
100	5.0	6.2	6.3	6.5

[a] Equal numbers of BALB/c 3T3 cells were sparsely seeded onto 35-mm precoated dishes, and the cell number on day 0 was $(7.8 \pm 0.6) \times 10^3$ per dish. Duplicate cultures were fed with different concentrations of FGF and insulin, refed on day 3, and counted on day 6.

PDGF or FGF was essential for growth, and in their absence, the cells slowly lost viability and detached from the plate with mortality visibly evident after 3 days in a mitogen-free medium. Insulin was not essential for growth, but 10 μg of insulin per ml potentiated the activity of PDGF or FGF three- to fourfold.

BALB/c 3T3 murine fibroblasts (1) also required FGF for growth (Table 2), although FGF-deprived senescent cells retained viability. Insulin (30 μg/ml) potentiated FGF mitogenicity between 5- and 10-fold. Epidermal growth factor in conjunction with FGF or insulin did not influence the growth of either NIH 3T3 or BALB/c 3T3 cells (data not shown), despite the documented effects of epidermal growth factor on these cells in other culture systems (5, 28).

We next examined the effects of fibroblast transformation on mitogen requirements for cell growth. We transformed NIH 3T3 and BALB/c 3T3 cells with four retroviral oncogenes (v-*src*, v-*mos*, v-*sis*, and v-*fos*) and one cellular oncogene (c-H-$ras_{Val\ 12}$) chosen for the functional diversity of their encoded proteins. Retroviral oncogenes are transduced and, most often, mutated cellular oncogenes under the transcriptional control of strong retrovirus promoters. v-*src* is one of a family of retroviral oncogenes which encode tyrosine-specific protein kinases (12). The v-*mos* gene product bears sequence resemblance to protein kinases (12), although a biochemical activity of the *mos* protein has yet to be demonstrated. The v-*sis* protein is mitogenic and is closely related to the beta chain of PDGF (20, 31). v-*fos* encodes a nuclear protein of unknown function (6). c-H-*ras* is one of at least three *ras* genes (9, 26) which encode membrane-associated guanine nucleotide-binding proteins (23). The transforming potential of c-H-$ras_{Val\ 12}$ isolated from a human bladder tumor cell line is due to a missense mutation in amino acid codon 12 (29). We transfected NIH 3T3 and BALB/c 3T3 cells by standard protocol (32) with plasmids containing these oncogenes (see Table 3, footnote *a*). In some cases, the oncogene-bearing plasmid also contained a gene (neo^r) conferring resistance to the neomycin analog G418; in other cases, a second plasmid containing neo^r was cotransferred with the oncogene-bearing plasmid. A clone of each resultant G418-resistant, morphologically transformed cell type was expanded for analysis of growth in defined medium with and without FGF and insulin.

Random clones of NIH 3T3 cells transformed with *mos*, *ras*, *src*, or *sis* could grow in the absence of FGF (Table 3). With respect to the growth rate in the presence of FGF, the percentage of cell doublings in medium without FGF ranged from 71% for *src* to 100% for *mos*. Insulin still enhanced the growth of these transformed cells. The v-*fos* transformant, by contrast, failed to grow appreciably in FGF-free medium. We examined the levels of v-*fos* RNA in these cells in the presence and absence of FGF and found no appreciable difference (data not shown). Furthermore, when pools of v-*fos*-transfected cells were cultured in defined medium lacking FGF, no colonies developed (data not shown). It is therefore likely that the *fos* gene product does not abrogate the FGF growth requirement.

BALB/c cells transformed with *mos*, *ras*, and *sis* were also able to grow in FGF-free medium with cell doublings ranging from 53 to 86% of those in FGF-supplemented medium (Table 3). Insulin still potentiated the growth of these cells.

Based upon the growth properties of normal and transformed NIH 3T3 cells (Tables 1 and 3), we were optimistic that maintenance of NIH 3T3 cells in FGF-free medium following treatment with transforming agents (e.g., oncogenic DNA, oncogenic viruses, and chemical carcinogens) could constitute a new transformation assay. A new assay might have a different scope of sensitivity than existing transformation assays, such as the morphological transformation assay (focus assay) (14, 16, 19, 25) or tumorigenesis assays (3, 10). A prerequisite for the feasibility of defined

TABLE 3. Growth of oncogene-transformed cells in defined media[a]

Expt no. (days of growth)	Cell line	No. of cell doublings			
		Without FGF, without insulin	Without FGF, with insulin	With FGF, with insulin	Without FGF/with FGF ratio
1 (5)	NIH v-*sis*	2.9	5.1	6.1	0.84
	NIH c-H-*ras*	3.9	5.2	5.4	0.95
2 (8)	NIH v-*src*	2.4	4.1	5.8	0.71
	NIH v-*mos*	1.5	6.1	6.0	1.02
3 (5)	NIH v-*fos*	<0	0.4	4.3	0.10
4 (6)	BALB c-H-*ras*	5.1	6.2	7.8	0.81
	BALB v-*sis*	4.1	4.8	7.1	0.68
	BALB v-*mos*	2.7	3.4	6.5	0.53

[a] Transformed cell lines derive from G418-resistant, morphologically transformed colonies following gene transfer (32) of oncogene-bearing plasmids into NIH 3T3 or BALB/c 3T3 cells. NIH v-*src* cells are NIH 3T3 cells containing plasmids pKOneo (pBR322 vector with a simian virus 40 (SV40) early promoter-Tn*5* Neo^r fusion gene [D. Hanahan, unpublished data]) and pSarc-11 (bearing a portion of Rous sarcoma provirus including v-*src*) (24). NIH v-*mos* and BALB v-*mos* contain pKOneo and pM-1 (bearing v-*mos* as part of complete Moloney sarcoma provirus) (30). NIH v-*fos* contains pKOneo-fos (bearing the SV40 Neo^r gene and the FBJ osteosarcoma provirus) (7; D. Birnbaum, personal communication). NIH v-*sis* and BALB v-*sis* contain pKOneo-sis (bearing SV40 Neo^r and the Simian sarcoma provirus) (21; D. Birnbaum, personal communication). NIH c-H-*ras* and BALB c-H-*ras* contain pKOneo-T24 (bearing SV40 Neo^r and the mutant, transforming human c-H-*ras* gene) (29; Birnbaum, personal communication). Cells from each cell line were equally seeded onto precoated 35-mm dishes. On day 0, two plates of each cell line were counted. The other cultures were refed with unsupplemented basal medium, medium containing 30 μg of insulin and 25 ng of FGF per ml, or medium containing only insulin. Cultures were refed on days 3 and 6, and cells were trypsinized and counted after the number of days indicated in the table.

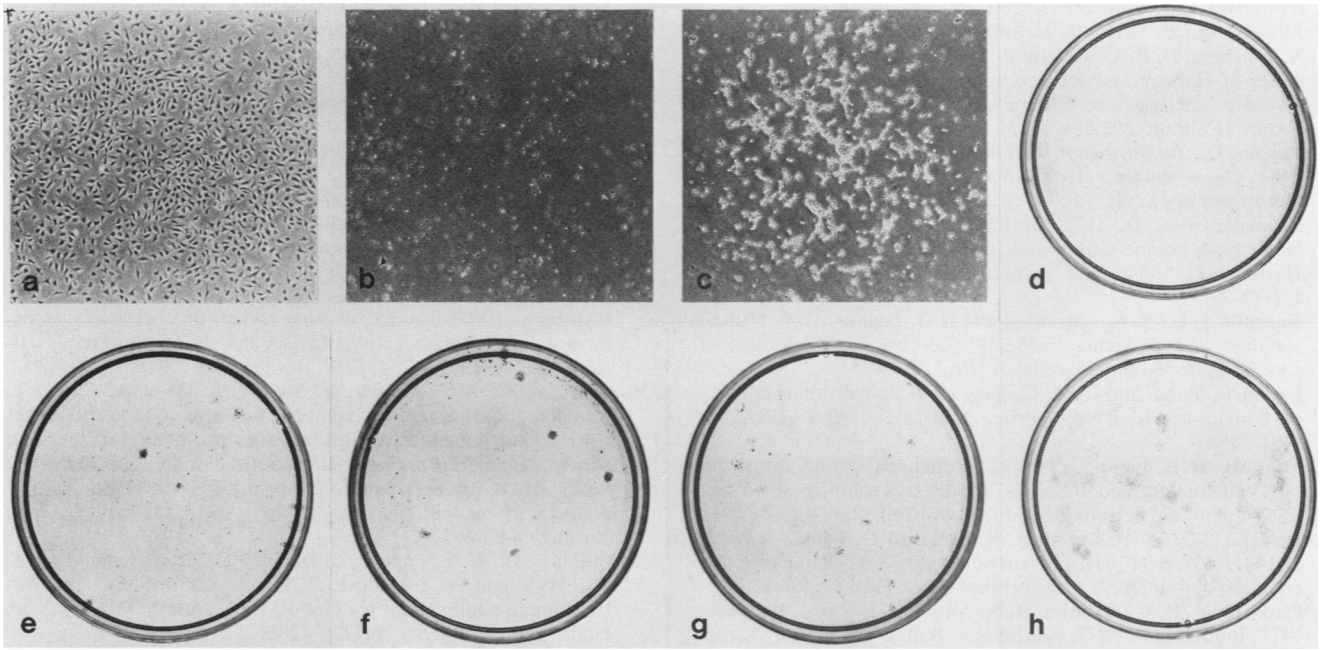

FIG. 1. Colony formation from mixed cultures of normal and transformed NIH 3T3 cells. NIH 3T3 cells (3×10^5) together with small numbers of transformed NIH cells were seeded onto precoated 60-mm dishes. Cultures were subsequently maintained in insulin-supplemented basal medium. (a) Phase-contrast micrograph (×80) of a mixed culture the day after plating. (b) Similar micrograph of a typical field 9 days after plating. (c) Transformed colony (×80) after 9 days from a mixed culture seeded with 100 NIH v-*src* cells. (d to h) Fixed and Giemsa-stained plates of 9-day mixed cultures. NIH 3T3 cells with no transformed cells (d), with 25 NIH v-*mos* cells (e), with 50 NIH c-H-*ras* cells (f), with 50 NIH v-*sis* cells (g), and with 100 NIH v-*src* cells (h). Some transformed colonies arising from the mixed cultures were too small or too diffuse to stain detectably.

media culture as a transformation assay is the ability to select for the outgrowth of small numbers of transformed cells among a vast excess of normal 3T3 cells. We tested whether *ras*-, *src*-, *sis*-, and *mos*-transformed NIH 3T3 cells can be easily detected when plated with a 10^3- to 10^4-fold excess of normal NIH 3T3 cells and maintained in defined medium containing insulin, but lacking FGF or PDGF.

Figure 1 illustrates the results of such reconstruction experiments. When mixed cell populations were plated on fibronectin-coated plates, they formed a near-confluent culture on day 0 (Fig. 1a). By day 8 in FGF-PDGF-free medium, most cells had died and detached (Fig. 1b), but the few transformed cells have developed into colonies (Fig. 1c). Colonies are observed on plates originally seeded with small numbers of *ras*-, *src*-, *mos*-, or *sis*-transformed cells in addition to normal 3T3 cells, but not with 3T3 cells alone (Fig. 1d to h). We are successfully exploiting such culture conditions as a new means for detecting oncogenes in cellular DNA from tumor cells following gene transfer into 3T3 cells (manuscript in preparation).

We showed that oncogene-induced morphological transformation of fibroblasts is often, but not always, accompanied by their capacity to grow in mitogen-free medium. The failure of *fos*-transformed 3T3 cells to grow without mitogens indicates that v-*fos* induces transformation by a fundamentally different mechanism than do several other oncogenes. We are currently seeking ways to compare the mechanisms by which *src*, *ras*, and *mos* oncogenes can release fibroblasts from the PDGF-FGF growth requirement.

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