Journal of Cellular Biochemistry 21:29–38 (1983)
Evolution of Hormone-Receptor Systems 261–270

# Normal Mouse Serum Contains Peptides Which Induce Fibroblasts To Grow in Soft Agar

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The untransformed mouse fibroblast cells NIH/3T3, C3H/10T1/2, and rat NRK cells do not grow in soft agar in medium supplemented with 10% fetal calf serum. When fetal calf serum in the growth medium was supplemented with less than 1% of sera from mice or other vertebrates, however, these cells responded, forming large colonies. The morphology of soft agar colonies was a function of the treated cell type. In the presence of 10% serum from C57BL/6 mice, NRK cells grew to smooth-surfaced spherical colonies, while NIH/3T3 colonies showed individual round cells on their surface and C3H/10T1/2 cells grew as extended cells forming columns of end to end connected fibroblasts. Mus Musculus Castaneus-Epithelial (MMC- E) cells were not stimulated to grow in soft agar under these conditions. The major fibroblast colony-inducing factor (F-CIF) was partially purified from mouse serum by acid/ethanol-extraction, gel permeation chromatography, and reverse-phase high-pressure liquid chromatography. F-CIF is a polypeptide which does not compete for binding to epidermal growth factor (EGF) receptors, but stimulates normal fibroblasts to form small colonies in semisolid medium and very large colonies in the presence of added EGF (2 ng/ml). In contrast to unfractionated mouse serum, purified F-CIF did not induce C3H/10T1/2 cells to grow in soft agar, suggesting that serum contains additional cell type-specific agar growth-stimulating activities.

**Key words: peptides, fibroblasts, normal mouse serum, colony formation, epidermal growth factor**

Abbreviations: NRK, normal rat kidney; F-CIF, fibroblast colony inducing factor; EGF, epidermal growth factor; TGF, transforming growth factor; MSV, murine sarcoma virus; DMEM, Dulbecco's modified Eagle's medium; PBS, 0.15 M NaCl in 0.1 M sodium phosphate at pH 7.4; HPLC, high pressure liquid chromatography.

Received May 28, 1982; accepted November 18, 1982.

While testing hyperimmunesera raised against murine leukemia virus structural proteins, we observed that many of these sera phenotypically transform fibroblast cells in either monolayer culture or by inducing soft agar growth. This finding prompted us to also test normal sera from mice and other species for soft agar growth induction of untransformed fibroblast cells. All of the sera tested induced colony formation in semisolid medium.

A family of peptides with the ability to phenotypically transform rat fibroblast cells in culture, designated transforming growth factor (TGF), was originally obtained from medium conditioned by mouse sarcoma virus (MSV) transformed 3T3 cells [1]. Subsequently polypeptides with similar activity were found in medium conditioned by chemically transformed mouse cells [2] and by human carcinoma [3] and melanoma [4] cells. Intracellular TGF has also been isolated from neoplastic as well as some nonneoplastic tissues [5,6] including normal embryo [7,8]. A novel class of TGF which is dependent upon epidermal growth factor (EGF) for soft agar growth stimulation has been recently described [9,10]. We report here the partial purification and characterization of fibroblast colony inducing factor (F-CIF) from normal serum of C57BL/6 mice and conclude that F-CIF is related to the EGF-dependent TGF family of growth factors.

## MATERIALS AND METHODS

### Cell Culture Conditions

The 536–7 rat fibroblast cells [11] and NIH/3T3 [12] cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum. C3H/10T1/2 Cl 8 and MMC-E cells [13,15] were grown in Eagle's minimal essential medium with 10% heat inactivated fetal calf serum.

### Soft Agar Growth Assay

One milliliter of 0.3% Noble Agar (Difco) containing the test cells was layered onto the 2.5 ml of 0.5% basal agar in a 60-mm petri dish (Falcon). The top agar contained 200 $\mu$l of trypsinized test cells ($5 \times 10^5$ cells/ml, $1 \times 10^5$ cells/ml final concentration per plate) in DMEM supplemented with 10% fetal calf serum (FCS), an aliquot of the lyophilized sample to be tested in 300 $\mu$l of phosphate buffered saline (PBS); and 500 $\mu$l of 0.6% Noble Agar in DMEM with 10% FCS. EGF was added at 2 ng/ml (final concentration per plate) where indicated. The bottom agar contained 0.5% Nobel Agar in DMEM supplemented with 10% FCS. The cells were incubated at 37°C in a humidified 5% $CO_2$/95% air atmosphere. The assay was read unfixed and unstained at 7 days. Colony size was determined by use of a measuring insert to the ocular lens of a mounted phase microscope.

### EGF Competition Assays

Purified EGF [14] was radiolabeled with [Na$^{125}$I] as previously described [1,3]. Binding of EGF [1] was measured on subconfluent monolayers of formaldehyde-fixed A431 cells in 16-mm tissue culture wells (Linbro No. 76–033–05). Competitions were initiated by the addition of 0.2 ml of binding buffer (DMEM containing 1 mg/ml of bovine serum albumin and 50 mM 2-[bis(2-hydroxyethylamino]ethanesulfonic acid, pH 6.8) containing 2 ng/ml of $^{125}$I-EGF with or without an aliquot of the lyophilized sample to be tested. After a 1-hr incubation at 22°C, the binding buffer

was removed, cells were washed four times with binding buffer (1 ml), and the quantity of EGF specifically bound was determined in a Beckman gamma counter.

### Source of F-CIF

Pooled normal mouse serum was dialyzed for 60 hr against distilled water in Spectrapor 3 dialysis tubing (Spectrum Medical Industries). The retentate was lyophilized and extracted using a modified acid/ethanol procedure [5].

### Purification of F-CIF

**Extraction of F-CIF.** Lyophilized mouse serum (90 ml) was extracted with 180 ml of a solution containing 375 ml of 95% (v/v) ethanol and 7.5 ml of concentrated HCl, containing 33 mg of phenylmethylsulfonyl fluoride and 34 trypsin inhibitory units of aprotinin from bovine lung (Sigma Chemical Company, St Louis). Sixty milliliters of distilled water was added and the serum extracted for 24 hr at 4°C. Following centrifugation at 15,000 rpm for 15 min (Rotor 35, Beckman), the supernatant was adjusted to pH 5.2 with concentrated ammonium hydroxide. One milliliter of 2 M ammonium acetate buffer, pH 5.2, was added per 100 ml of extract supernatant. The resultant precipitate was removed by centrifugation at 25,000 rpm for 30 min (Rotor 35, Beckman). Two volumes of cold ethanol and 4 vol of ether were added and the mixture allowed to stand at −20°C for 48 hr. The precipitate was collected by centrifugation at 8,000 rpm for 30 min (Rotor 19, Beckman), reconstituted in 1 M acetic acid, and dialyzed against 0.1 M acetic acid. The supernatant after centrifugation was concentrated by lyophilization and reconstituted in 1 M acetic acid for further purification of F-CIF.

**Gel permeation chromatography.** The supernatant containing F-CIF activity was further purified by gel permeation chromatography on a column (2.5 × 85 cm) of Bio-Gel P-10 (200–400 mesh, Bio-Rad Laboratories). The column was equilibrated with 1 M acetic acid at 22°C and at a constant flow rate of 12 ml/hr. Fractions (4.9-ml) were collected. Aliquots were lyophilized for subsequent determinations of EGF-competing activity and growth promoting activity in soft agar.

**Reverse-phase high-pressure liquid chromatography (HPLC).** F-CIF was further purified by reverse phase HPLC, as previously published [4]. The separation was performed on a μBondapak $C_{18}$ column (10-μm particle size, 0.39 × 30 cm, Waters Associates) at a flow rate of 1 ml/min at 40°C. Lyophilized samples were reconstituted in saturated guanidine-HCl, adjusted to pH 2 with 10% (v/v) trifluoroacetic acid, and applied through the sample injector to the column which was equilibrated with 0.05% trifluoroacetic acid. The column was then eluted with a linear acetonitrile gradient in 0.045% trifluoroacetic acid. The column effluent was collected in 3.0-ml fractions. Aliquots were lyophilized for subsequent growth-stimulation assays.

## RESULTS

Normal sera were tested for the presence of soft agar growth-stimulating activity using the clonal NRK rat fibroblast cell line, which had been selected for soft agar growth stimulation by TGF. As shown in Table I, three out of three mouse sera and three out of four sera from other mammals, including human, were positive for colony-inducing activity. The measured response of tested sera varied five- to six-

**TABLE I. Presence of Soft Agar Growth-Stimulating Activity in Normal Sera***

| Serum supplement | Soft agar colonies > 60 μm No./60-mm petri dish |
|---|---|
| Human | 8,700 |
| Rabbit | 34,000 |
| Rat | 8,800 |
| Guinea pig | 9,100 |
| Calf | 60 |
| Goat | 22,000 |
| Mouse C57BL/6 | 27,000 |
| Mouse BALB/c | 16,000 |
| Mouse NFS/N | 6,000 |
| None | 12 |

*Anchorage-independent growth of untransformed rat fibroblast cells induced by normal sera from different species. A suspension of $10^5$ 536-7 cells in 1 ml of 0.3% top agar was seeded on 2 ml bottom agar in a 60-mm petri dish. The growth medium was DMEM supplemented with 7% fetal calf serum and 10% of the indicated test serum. Colony growth was monitored after 7 days.

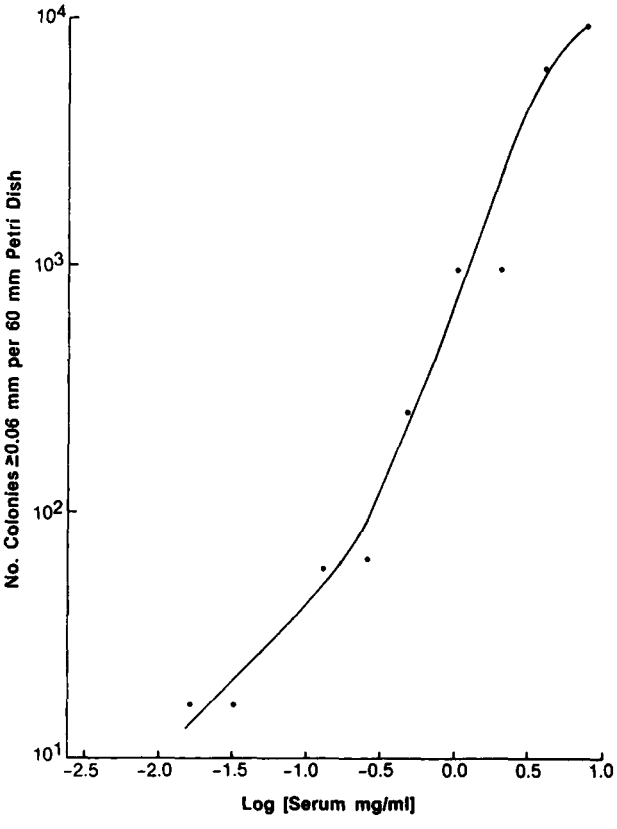


Fig. 1. Soft agar growth-stimulating activity in normal mouse serum. C57BL/6 serum was serially diluted in twofold steps, added to 0.3% top agar (Noble) containing 536-7 rat fibroblasts at $10^5$ cells/ml and scored at 7 days for colony formation.

fold between species. Repeated testing of different pools of sera from the same species showed a two- to four-fold variation in the response (data not shown). The soft agar growth-stimulating activity in sera from mice is generally higher than in sera from human, rat, guinea pig, and calf, and could be readily detected over two logs of dilution of unconcentrated serum (Fig. 1). The anchorage-independent growth of fibroblasts was dependent on the continuous presence of F-CIF.

The colony-inducing activity in normal sera was also tested with other nonneoplastic cells. Figure 2 shows the response of 536–7 rat fibroblast (panel B), NIH/3T3 clone 7 (panel C), and C3H/10T1/2 clone 8 (panel D) cells to normal C57BL/6 mouse serum. All fibroblast cells tested responded in the presence of mouse serum with anchorage-independent growth; however, the colony morphology differed for each cell type. The 536–7 cells formed spherical colonies with a smooth surface, whereas serum-induced colonies of NIH/3T3 cells showed individual round cells on their surface. The C3H/10T1/2 cells formed rather unusual colonies with cells extending in longitudinal arrays. An epithelial rodent cell line MMC-E did not grow in agar in response to treatment with serum. The latter cells do respond with morphological transformation to treatment with crude TGF [16].

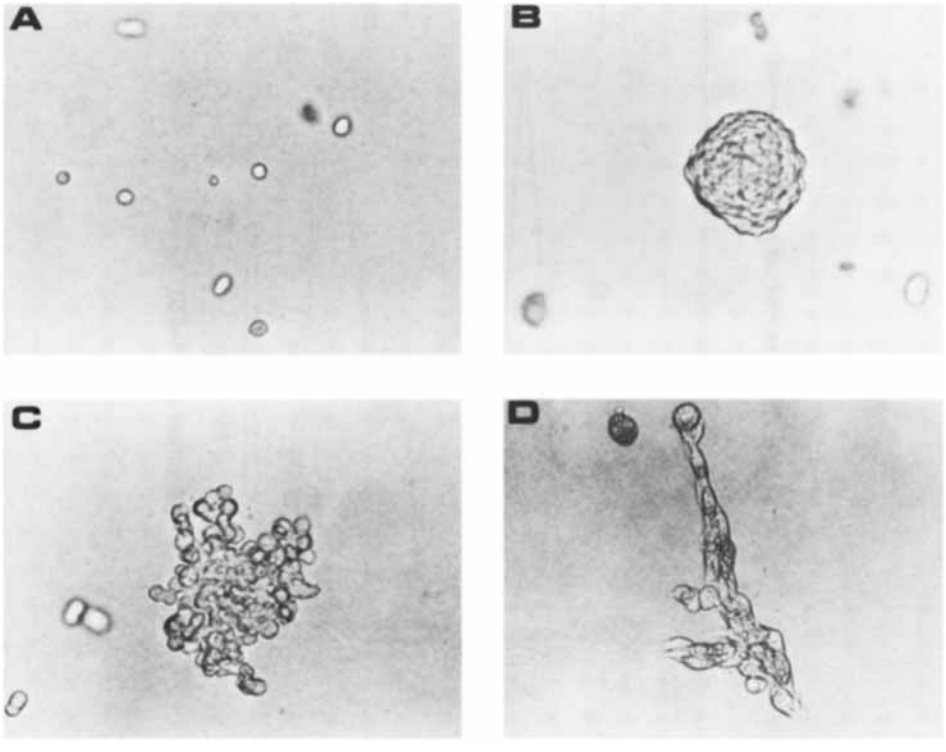


Fig. 2. Cell-specific colony morphology in the presence of C57BL/6 serum. Cells were seeded at $10^5$/ml in 0.3% top agar and plated on a layer of 2-ml 0.5% bottom agar in 60-mm plastic petri dishes. Agar was supplemented with normal fetal calf and C57BL/6 serum to final concentrations of 7% and 10%, respectively. A) Cell growth in the absence of the test serum; 536-7 cells used here as a representative control. B, C, and D) Cell growth in the presence of the mouse serum supplement. B) 536-7. C) NIH 3T3 clone 7 [13]. D) C3H/10T1/2 [14].

F-CIF was isolated and partially purified from normal mouse serum. To remove contaminating proteins, serum was extracted with acid/ethanol, and acid/ethanol-soluble partially purified F-CIF was subjected to gel permeation chromatography on Bio-Gel P-10. A representative chromatogram is illustrated in Figure 3. The bulk of contaminating protein was eluted in the exclusion volume of the column (data not shown) and was well separated from the F-CIF activity and EGF-competing activity. Two peaks of activity were found to be well resolved from each other. Fractions with F-CIF activity (P-10-A) had an apparent molecular weight of 8,000 to 11,000. Fractions having EGF-competing activity (P-10-B) were eluted in the total column volume. The size of soft agar colonies induced by fractionated serum was noticeably smaller (colonies 10–20 $\mu$m) than those induced by acid/ethanol-extracted serum at similar dilutions. However, testing column fractions in the presence of 2 ng/ml of EGF induced the formation of large colonies (120–360 $\mu$m). The fraction of cells responding with colony growth under these conditions was >80%.

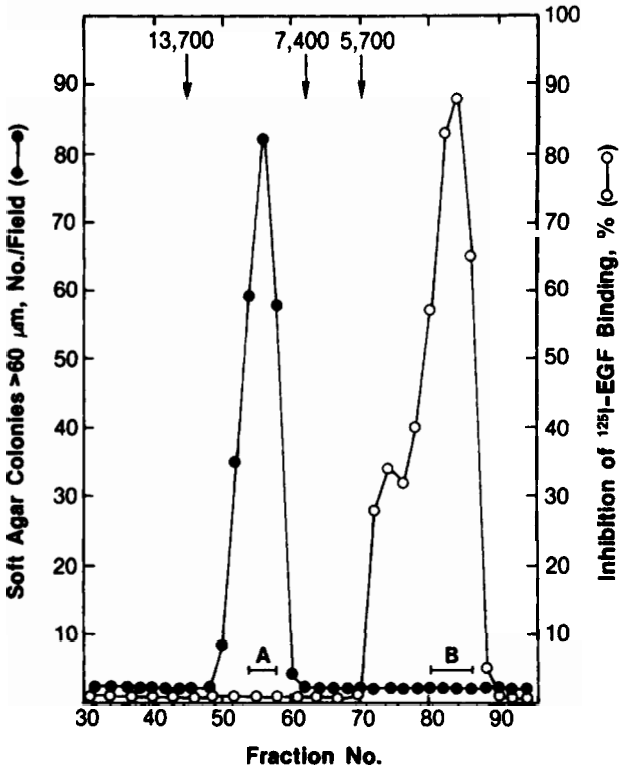


Fig. 3. Gel permeation chromatography of acid/ethanol extracted mouse serum on Bio-Gel P-10. Elution pattern of acid-soluble protein from 90 ml of normal mouse serum. The elution was performed with 1 M acetic acid at 22°C at a flow rate of 12 ml/hr. 4.9-ml fractions were collected. Aliquots of the indicated fractions were lyphilized and assayed for (1) EGF-competing activity with 1-$\mu$l aliquots (○—○); soft agar growth with 10-$\mu$l aliquots in the presence of 2 ng/ml EGF (●—●). For reference, the elution volumes of ribonuclease A ($M_r$ = 13,700); MSA ($M_r$ = 7,400) [22], and insulin ($M_r$ = 5,700) were determined.

F-CIF was further purified by reverse phase HPLC. Pool P-10-A, after gel permeation chromatography of the acid/ethanol-soluble growth-promoting activity of mouse serum, was chromatographed on a μBondapak C18 column. A typical elution pattern is shown in Figure 4. F-CIF activity of individual fractions was determined in the soft agar growth assay without or with added EGF. Two distinct peaks of soft agar growth-promoting activity were found. F-CIF in fractions 18 and 26 induced the formation of small colonies (10–20 μm). Very large colonies (120–360 μm) were formed in the presence of added EGF. Reassaying column fractions at tenfold lower concentrations, detected colony inducing activity in fraction 18 only, suggesting that this fraction represented approximately 90% of the total F-CIF activity present in mouse serum. No EGF-competing activity was detected in fraction 18. The major F-

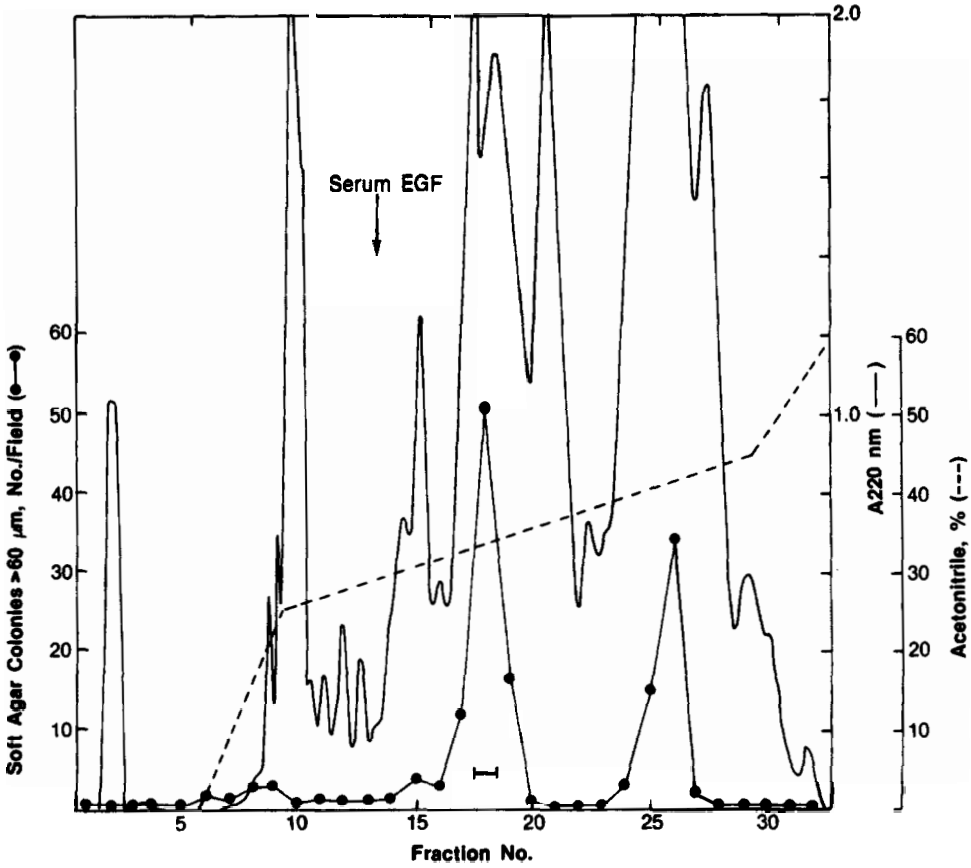


Fig. 4. Reverse-phase high-pressure liquid chromatography of F-CIF. Elution pattern of peptide from P-10-A (Fig. 3) on a μBondapak $C_{18}$ column in 0.05% trifluoroacetic acid. Elution was achieved with a linear 10-min gradient of 0-25% acetonitrile in 0.045% trifluoroacetic acid, followed by a linear 1-hr gradient of 25–45% acetonitrile in 0.045% trifluoroacetic acid. 3.0 ml fractions were collected. 10-μl aliquots of the indicated fractions were lyophilized and assayed for soft agar growth in the presence of 2 ng/ml EGF (●——●). For reference, the retention time of "serum-EGF" (pool P-10-B, Fig. 3) was determined (indicated by an arrow).

**TABLE II. Sensitivity of F-CIF to Hydrolytic Enzymes***

| Treatment | Incubation mixture | Addition after incubation | Soft agar colonies > 60 μm No./60-mm petri dish |
|---|---|---|---|
| Control | F-CIF[a] | PBS | 5,200 |
| | PBS | F-CIF | 4,900 |
| | PBS | PBS | 0 |
| RNAase | F-CIF | PBS | 4,800 |
| | PBS | F-CIF | 5,400 |
| | PBS | PBS | 0 |
| Trypsin | F-CIF | PBS | 240 |
| | PBS | F-CIF | 5,300 |
| | PBS | PBS | 0 |

*Sensitivity of mouse serum-derived F-CIF to proteolytic cleavage. Partially purified F-CIF (pool P-10-A, Fig. 3) were lyophilized and reconstituted at 1 mg/ml of protein [21] in PBS. 500 μg of protein was incubated with the indicated enzymes bound to beaded agarose (Sigma). As a control for the effects of incubation, F-CIF and PBS were also incubated without the addition of enzyme. Following incubation, all F-CIF samples were mixed with an equal volume of PBS containing 2 ng/ml EGF (final concentration) and assayed with 536-7 rat fibroblast cells at 50 μg/ml. Incubated PBS samples were either mixed with an equal volume of PBS or with untreated, unincubated F-CIF and assayed as described above.
[a]F-CIF was partially purified by acid/ethanol extraction and gel permeation chromatography (pool P-10-A, Fig. 3).

CIF activity eluted from a μBondapak C18 column at 34% acetonitrile, whereas serum-derived EGF (P-10-B) eluted at 29% acetonitrile using identical conditions for the elution of peptides.

The chemical nature of partially purified F-CIF was determined by treatment with various hydrolytic enzymes (Table II). Digestion of F-CIF with trypsin completely abolished its ability to induce agar growth. Removal of trypsin and subsequent addition of F-CIF to the enzymatic digest caused test cells to grow in soft agar. RNAase treatment of F-CIF did not affect its soft agar growth stimulating property. Thus, the colony-inducing activity of F-CIF is protein.

C3H/10T1/2 Cl 8 fibroblast cells did not respond in the agar growth assay to treatment with HPLC-purified F-CIF from fraction 18 in the presence or absence of EGF. The unusual pattern of soft agar growth induced with serum (Fig. 1) therefore was either due to another serum factor or required collaboration between F-CIF and other serum components.

## DISCUSSION

We have shown here that normal serum from various vertebrate species induced growth of untransformed cells in soft agar. Cells responding to F-CIF included fibroblast lines of mouse and rat origin. Epithelial MMC-E cells did not form colonies in the presence of F-CIF. The pattern of colony growth in agar was a function of the cell type. C3H/10T1/2 Cl 8 cells grew in soft agar without alteration in its shape, ie, as extended cells. However, partially purified F-CIF did not induce these cells to grow in agar, suggesting the presence of multiple distinct peptides in serum which differ in their target-cell specificity. Differences were observed in the activity present

in sera from different species. Most notably, bovine calf serum, ie, the serum used in the establishment of these untransformed cell lines, was least effective in promoting soft agar growth. For mouse serum we have shown that (1) a supplement of less than 1% of serum was sufficient for soft agar colony formation, (2) the factor(s) responsible for soft agar growth stimulation are peptide(s), (3) partially purified F-CIF, free of serum-derived EGF, promotes soft agar growth but requires EGF to form large colonies in the soft agar growth assay.

The ability of polypeptides to reversibly induce nonneoplastic fibroblast [1] and epithelial [15] cells to grow in soft agar is a property which was first described for transforming growth factors (TGF) produced and released into the conditioned medium of MSV-transformed 3T3 cells [1]. These polypeptide growth factors are antigenically distinct but structurally related to EGF and induce anchorage-independent growth of fibroblasts in the absence of added EGF. A novel class of intracellular transforming growth factors, isolated from neoplastic as well as nonneoplastic cells, was recently described [9,10]. These polypeptide growth factors do not compete with EGF for receptor binding and require EGF for soft agar growth stimulation [10,17]. F-CIF, isolated and partially purified from serum, induces small colonies in the soft agar assay but requires the presence of added EGF to induce normal fibroblasts to form large colonies. HPLC-purified F-CIF did not compete for binding to EGF receptors and elutes at a higher retention time relative to serum-derived EGF. Thus, F-CIF appears to be related to the EGF-dependent TGF family of growth factors. For soft agar growth-stimulating assays a supplement of bovine calf or fetal bovine serum has always been used. Based on our results, a final assessment of the intrinsic soft agar groth-stimulating activity of highly purified F-CIFs and TGFs will require chemically defined media in soft agar growth assays.

There have been previous reports on the effect of serum on anchorage-independent growth of established cell lines [18–20]. In most cases, fetal calf or calf serum was used for these experiments. For example Peehl [18] found that nonmalignant human fibroblast strains could be reversibly induced to grow in soft agar when the serum level was raised to 20%. These findings were used to argue that the ability to grow anchorage-independent was intrinsic to normal as well as transformed cells. In addition to serum, hydrocortisone was required for optimal activity, and mouse 3T3 cells did not respond with agar growth under these conditions. Our findings suggest that the use of homologous human serum in place of fetal calf serum might have shown agar growth induction of human fibroblast cells at much lower levels of serum substitution. The lack of response of mouse 3T3 cells is in good agreement with our findings with calf or fetal calf serum. No attempt was made by these authors to identify the molecular nature of their serum factors.

In summary, our finding that normal animals contain in their serum F-CIF and additional cell type-specific agar growth-stimulating activities suggest that these polypeptides serve a normal function in the maintenance of tissues. F-CIF activity is also present in whole plasma (unpublished). Our findings raise the possibility that factors with F-CIF activity may be part of a control system for tissue homeostasis with positive and negative effector elements. Fractionation of normal serum and the sorting out of its components by assay for stimulation or blocking of soft agar growth with a variety of specific test cell lines may thus help us to identify physiological mechanisms of growth control.

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