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Growth Control in Cultured Mouse Fibroblasts: Induction of the Pleiotypic and Mitogenic Responses by a Purified Growth Factor

(cellular growth state/fibroblast growth factor/hydrocortisone/DNA synthesis)

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ABSTRACT **Addition of serum to quiescent mouse fibroblasts induces a series of macromolecular changes (a pleiotypic response) followed by DNA synthesis and cell division. A new pituitary hormone, fibroblast growth factor, and hydrocortisone acting at physiological concentrations can completely replace exogenously added serum for the induction of these events in lines of BALB/c 3T3 cells. The induction of cell growth is specific for cultured fibroblasts; no stimulation is observed for mouse epithelial cells or virally transformed fibroblasts.**

Fibroblast cells in tissue culture normally exist in one of two reversible growth states, a state of rapid proliferation (growing) and a state of relative quiescence (1, 2). In nontransformed mouse fibroblasts, transition between these two states is predominantly regulated by growth-promoting substances in animal sera (3, 4). This transition from a resting to a growing state is considered to be accomplished by a sequential series of regulated steps; the accompanying detectable changes collectively form a "pleiotypic response" (5). These sequential changes include alterations in cyclic nucleotide levels (6, 7), stimulation of cellular transport systems (5), polyribosome formation (2), protein synthesis (5), ribosomal and tRNA synthesis (8), and eventually the induction of DNA synthesis followed by cell division (1, 9).

Recently a growth-promoting activity (3) for a line of BALB/c 3T3 cells has been purified from bovine pituitary extracts (10, 11). This homogeneous polypeptide hormone, termed fibroblast growth factor (FGF), in combination with the glucocorticoid, hydrocortisone, could induce DNA synthesis and cell division in quiescent cultures in the presence of serum exhausted for its growth-initiating ability (11). We now show that FGF and hydrocortisone can replace exogenously added serum for delivery of the full "pleiotypic" and mitogenic response in lines of BALB/c 3T3 cells and that FGF is indeed specific for growth activation of mouse fibroblast cultures; little or no growth initiation occurs in mouse epithelial or transformed cells.

MATERIALS AND METHODS

Materials. FGF was purified from bovine pituitary glands as described (11). It was greater than 95% pure on electrophoresis through Na dodecyl sulfate–polyacrylamide gels and had a molecular weight of 13,400 (11). Hydrocortisone (cortisol: Δ_4 Pregnen-11β,17α,21-triol-3,20-dione) was purchased from Calbiochem, and insulin (bovine pancreas) was the generous gift of Dr. N. Kaplan. Insulin had been further purified over commercial preparations by column chromatography. Bovine serum albumin and purified gelatin were obtained from Schwartz, and the synthetic polypeptides poly(Lys) and poly(Asp, Ser, Ala) (ratio 1:1:1) were kindly donated by Dr. T. Shier.

Growth and Handling of Cells. BALB/c 3T3a cells (10) were plated at 10^5 cells per 5-cm petri dish in 5 ml of Dulbecco's modified Eagle's medium and 10% calf serum (Colorado Co.) and grown at 37° in a 10% CO_2 atmosphere (v/v) until confluent. Six days after they were seeded, cell monolayers were washed twice with modified Eagle's medium + 250 μg/ml of bovine serum albumin. Then modified Eagle's medium containing 0.1 ng/ml of FGF, 0.5 μg/ml of hydrocortisone, and 250 μg/ml of bovine serum albumin was added and the cultures were incubated a further 3 days (final cell density: 4.5 to 5.0 $\times$ 10^5 per plate). Less than 0.5% of the cell nuclei became labeled with [^{3}H]thymidine during the subsequent 24-hr period. BALB/c 3T3b and c were sublines derived from the BALB/c line of Dr. S. Aaronson; Swiss 3T3-4A was a subline of the 3T3-4 line of Dr. H. Green; resting secondary mouse embryos were prepared in 0.5% calf serum; and primary baby mouse kidney cells were prepared in 10% horse serum (9). The primary and secondary cultures' medium was removed and replaced by serum-free medium 3 days before use, as described for the BALB/c 3T3a cultures.

Determination of Uptake Rates and Macromolecular Synthesis was substantially as described (8) except that 20 μCi/ml of 10 μM [5-^{3}H]uridine or a mixture of 10 μCi/ml of [^{3}H]aminoacids (Amersham) was added, as indicated in the *legends*. Uptake of ^{3}H radioactivity was measured from the trichloroacetic acid-soluble cpm incorporated per 30 min in lysed cell suspensions. Incorporation of [^{3}H]uridine and [^{3}H]aminoacids into the cellular pool was approximately linear during this time (8) in lysed cell suspensions. *DNA synthesis* was measured exactly as described (8); the radioactive labeling times were from 7 to 30 hr after additions unless otherwise stated. The percentage of radioactively labeled nuclei in the cell population was the average of five fields, each containing about 200 cells. *Protein synthesis* during a 2-hr period was estimated directly from the incorporation of [^{3}H]aminoacids into trichloroacetic acid-precipitable material, as radioactive labeling times of 2 hr or more are sufficiently long for saturation of the intracellular amino-acid pool (8). *RNA synthesis*

Abbreviation: FGF, fibroblast growth factor.

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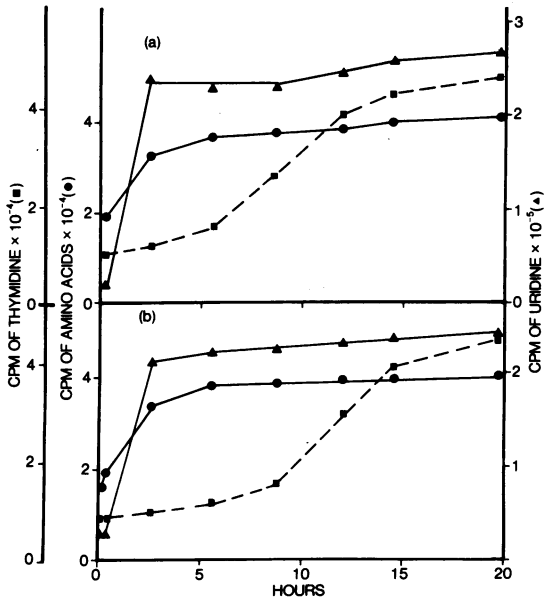

FIG. 1. Uptake changes in some macromolecular precursors. Cell cultures were prepared as described in *Methods*. Either (*a*) 20 ng/ml of FGF or (*b*) 10% dialyzed serum (6 mg/ml) was added to resting cultures (see Fig. 2 for radioautography data) at time zero. One of [^{3}H]aminoacids (●), 5 μCi/ml of [^{3}H]uridine at 2.5 μM (▲), or [^{3}H]thymidine (■) was added at various times and the cultures were isolated 30 min later. The cpm of each isotope incorporated into trichloroacetic acid-soluble material per 30 min/mg of cell protein is recorded. *Time-points* indicate the mid-point of the radioactive labeling time. Approximately 85–90% of the cells' nuclei became radioactively labeled with [^{3}H]thymidine after FGF or serum additions.

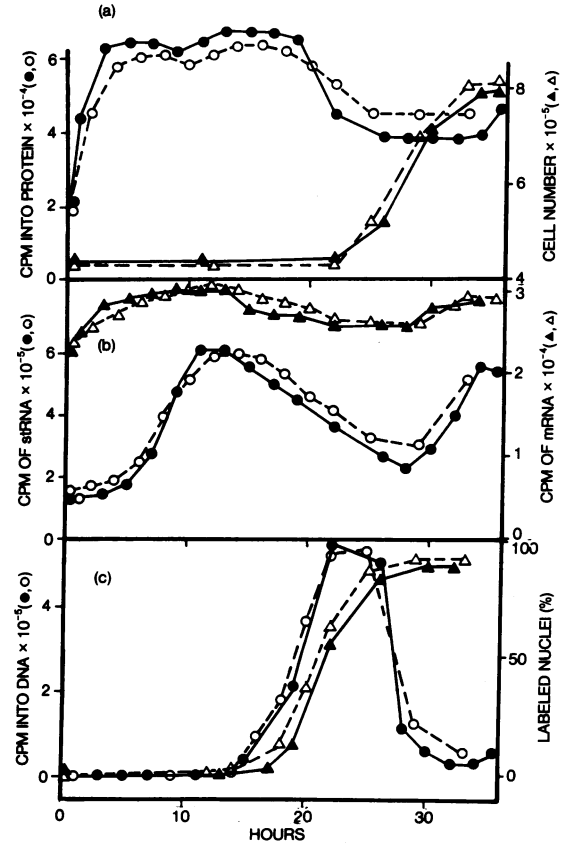

FIG. 2. Sequentially induced macromolecular changes. Cells were prepared as described in *Methods*. Either 25 ng/ml of FGF (*solid line*) or 10% dialyzed serum (6 mg/ml) (*broken line*) was added. Parallel cultures were then pulse-labeled for 2 hr with (*a*) [^{3}H]aminoacids for determination of protein synthesis (●, ○); or (*b*) [^{3}H]uridine for stable RNA (*stRNA* = rRNA and tRNA) synthesis (●, ○) or messenger RNA (*mRNA*) synthesis (▲, △); or (*c*) [^{3}H]thymidine for DNA synthesis (●, ○). Results are expressed in corrected cpm of isotope incorporated per 2 hr/mg of cell protein. *Time-points* indicate the mid-point of the pulse-label, except at zero time when cultures were radioactively labeled for 2 hr and then isolated immediately before the additions. In addition, (*a*) cell numbers (▲, △) and (*c*) the percentage (%) of [^{3}H]thymidine-labeled cell nuclei from cultures labeled from zero time until their isolation time (▲, △) are shown.

during a 2-hr period was corrected for alterations in the specific activity of the intracellular precursor with cellular growth state as described (8); results are expressed in terms of the RNA precursor pool when the precursor pool became saturated with radioactivity. Messenger RNA (mRNA) synthesis was measured by the amount of [^{3}H]RNA bound to poly(U) filters (12); nonmessenger RNA comprising ribosomal RNA (about 90%) and tRNA (about 10%) was obtained by deduction of the amount of mRNA from the total RNA values. *Cell numbers* were analyzed 45–48 hr after additions (after the first round and before the second round of cell division) in a Coulter Counter. *Polysomes* were prepared as before (8) on an 11-ml 0–40% (w/w) exponential, concave sucrose gradient in hypotonic buffers. Fractions from cells labeled with [^{3}H]aminoacids were precipitated with trichloroacetic acid, while [^{3}H]RNA was analyzed for its capacity to bind to poly(U) filters. The peak fractions of the polysomal profile (Fig. 3) correspond to 5 or 6 polyribosomes calibrated against a reticulocyte lysate.

RESULTS

Uptake and Macromolecular Changes. Confluent BALB/c 3T3 cells could not be maintained in a quiescent state with bovine serum albumin alone in the absence of serum, but addition of 0.1 ng/ml of FGF and hydrocortisone to the culture medium prevented any cell degeneration for at least 7 days. Resting cultures so prepared in medium free of exogenously added serum contained less than 0.5% of radioactively labeled nuclei after a 24-hr exposure to [^{3}H]thymidine (7). One of the earliest events after growth initiation by serum is the increased uptake of macromolecular precursors. This was followed for uridine, total amino acids, and thymidine uptake after addition of 10% serum or saturating amounts of FGF (25 ng/ml) to quiescent cultures. The rate of increase in the net incorporation into the cell was almost identical for both FGF and serum addition, there being no appreciable lag for increases in amino-acid uptake, a 15-min lag for uridine (13), and approximately a 3- to 5-hr lag for thymidine uptake (Fig. 1). Increased synthesis of protein (about 300% within 5 hr), mRNA (about 15–20% within 5 hr), structural RNA (90% ribosomal RNA) (about 350–400% within 12 hr), and DNA (starting at 15 hr and reaching a maximum at 23–24 hr of 60- to 100-fold) was almost identical for cultures activated with FGF or serum, both in the kinetics and extent of the increases (Fig. 2). Finally, cell division occurred from about 25 to 32 hr in both cases (Fig. 3*a*). The dose responses of

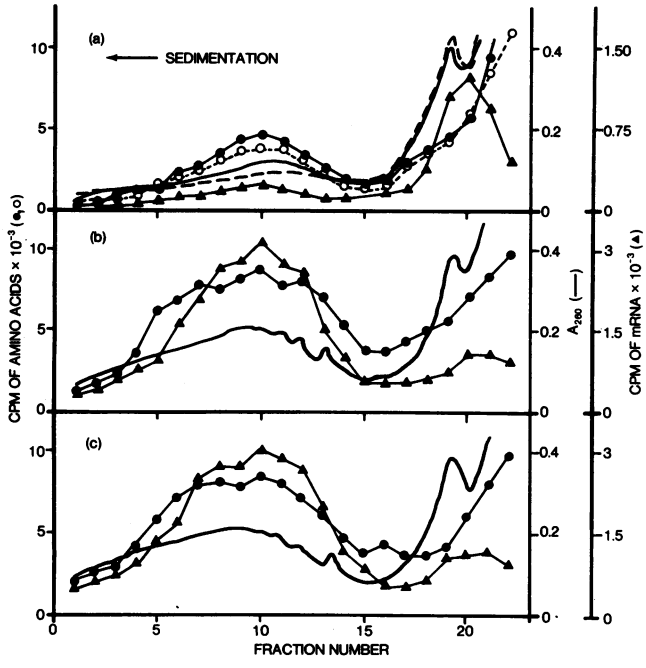

FIG. 3. Formation of polyribosomes. Sucrose gradients of polyribosomes isolated for (*a*) resting cell cultures without hydrocortisone (*broken line*) and with hydrocortisone (*solid line*); (*b*) cells stimulated by FGF; or (*c*) cells stimulated by serum. Incorporation of [^{3}H]aminoacids into nascent peptides (●, ○), A_{260} (*solid line*), and mRNA (▲) are indicated. Cells were grown in 9-cm petri dishes in 10 ml of medium and allowed to become quiescent in 0.1 ng/ml of FGF and 250 μg/ml of bovine serum albumin with 0.5 μg/ml of hydrocortisone (*solid line*) or without (*broken line*) hydrocortisone. After 3 days, polysomes were isolated from cultures 6 hr after (*a*) no additions, (*b*) addition of 25 ng/ml of FGF, or (*c*) addition of 10% dialyzed serum (6 mg/ml). Cultures were radioactively labeled for 2 min with 250 μCi/ml of [^{3}H]aminoacids in modified Eagle's medium containing one-fifth the normal concentration of amino acids (●, ○) or for 6 hr immediately before isolation with [^{3}H]uridine. Two petri dish cultures were isolated for each isotope and four (4×10^6 cells) for nonradioactive cultures.

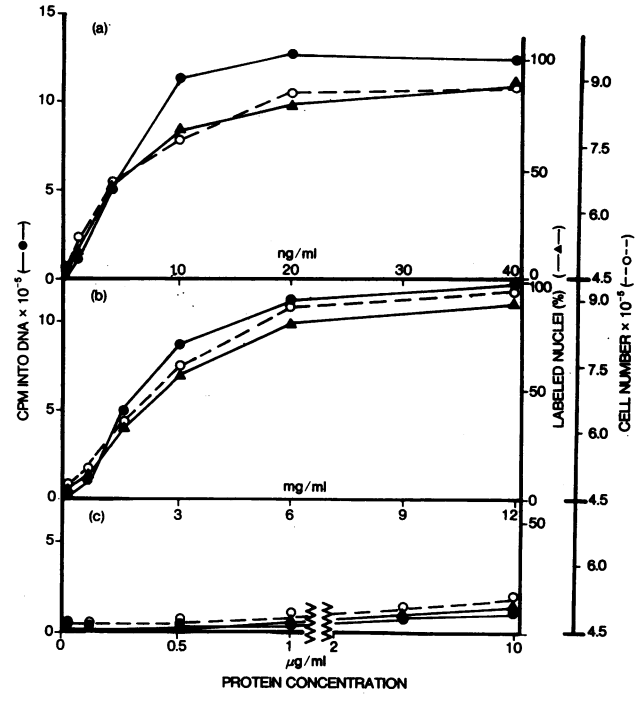

FIG. 4. Induction of DNA synthesis and cell multiplication. Cultures were prepared as described in *Methods*. Increasing concentrations of (*a*) FGF in ng/ml, or (*b*) calf serum in mg/ml, or (*c*) insulin in μg/ml was added. The cpm of [^{3}H]thymidine incorporated into DNA (●), the percentage (%) of cells with radioactively labeled nuclei (▲), and the cell numbers (○) per 5-cm dish are shown. Cultures could also be maintained for several days when 10 ng/ml of insulin replaced FGF in the above medium; increased insulin concentrations added thereafter gave the same results as in (*c*).

varying concentrations of FGF for the stimulation of protein synthesis at 6 hr, ribosomal RNA synthesis at 14 hr, and the eventual induction of DNA synthesis were roughly parallel; maximal stimulations were achieved at 10–20 ng/ml in all cases (not shown). Finally, the ability of FGF or serum to induce polysome reformation measured 6 hr after additions was also virtually identical (Fig. 3). The mRNA location is also shown in resting cultures; approximately 28% is in polysomes and 72% in the 30S–80S messenger ribonucleoprotein complex previously reported (8). Additions of either FGF or serum results in greater than 85% of the mRNA being located in the polysomal region of the sucrose gradient. Addition of hydrocortisone alone to quiescent cultures in the absence of serum induces no detectable changes compared with control cultures except for a small increase in polyribosome formation (Fig. 3*a*). The extent of the increase would probably be too small (about 10%) to detect a net increase in overall protein synthesis. Without hydrocortisone and with only FGF present, all uptake and macromolecular synthetic rates studied were approximately 40–50% and 20–25%, respectively, of the values measured with hydrocortisone present (not shown).

Induction of DNA Synthesis and Cell Division. The activation of the macromolecular machinery of the cell prior to DNA synthesis appeared to be identical for FGF and hydrocortisone or serum addition to quiescent BALB/c 3T3 cells. Addition of increasing concentrations of FGF to resting cell cultures with hydrocortisone also caused concomitant increases in the amount of [^{3}H]thymidine incorporated into DNA, the percentage of cells with radioactively labeled nuclei, and in cell number (Fig. 4*a*). Stimulations were achieved in the range 1–10 ng/ml (10^{-10}–10^{-9} M), and the same maximal stimulations as those obtained with 20% serum were achieved with 10–20 ng/ml of FGF (Fig. 4*b*). Insulin has also been reported to stimulate the growth of fibroblasts at relatively high, nonphysiological concentrations (10^{-7}–10^{-6} M) (14), although in our systems 10^{-6} M insulin stimulated only about 10% of the cells to synthesize DNA. Hydrocortisone alone failed to initiate DNA synthesis in the absence of serum, in agreement with Armelin (10) (Table 1), while FGF without hydrocortisone only initiated about 20% of the cell population to synthesize DNA and divide (Table 1). To obviate the possibility that addition of FGF and hydrocortisone was helping or potentiating the growth-promoting ability of trace amounts of other growth hormones bound to the bovine serum albumin, we replaced this carrier protein in the culture medium by gelatin or completely synthetic polypeptides with the same results (Table 1).

For other cultured mouse cells (Table 2), 25–100 ng/ml of FGF with hydrocortisone was sufficient to induce DNA syn-

TABLE 1. *Summary of mitogenic action of different components*

Additions	[^{3}H]DNA (cpm × 10^{-5})	Labeled nuclei (%)	Cell no. (× 10^{-6})
BSA	0.2	0.8	0.50
HC + BSA	0.5	1.6	0.53
FGF + BSA	2.3	18	0.68
FGF + HC + BSA	11.6	90	0.92
FGF + HC + gelatin	11.9	95	—
FGF + HC + polymers	11.8	89	—
Serum	12.4	95	1.00
FGF + HC + BSA + serum	12.6	94	0.95

Cultures contained 0.1 ng/ml of FGF and 250 μg/ml of bovine serum albumin (*BSA*) or 250 μg/ml of gelatin or a mixture of synthetic polypeptides comprising 250 μg/ml of poly(Asp, Ser, Ala) and 8 μg/ml of poly(Lys). After 3 days, 0.5 μg/ml of hydrocortisone (*HC*), 25 ng/ml of FGF, and 20% calf serum were added as indicated. Cells were labeled with [^{3}H]thymidine, and the cpm incorporated into DNA per culture, the percentage of cells with radioactively labeled nuclei, and the cell numbers per 5-cm dish are recorded. Quiescent cultures containing gelatin or synthetic polypeptides in place of bovine serum albumin gave identical results to those with bovine serum albumin alone.

FGF and hydrocortisone could restore the maximal induction time to that achieved with serum. (D. Gospodarowicz, unpublished results). In baby mouse kidney cultures there are two readily identifiable cell types, the fibroblasts and the epithelial parenchymal kidney cells. FGF and hydrocortisone induced DNA synthesis only in the fibroblasts and not in the epithelial cells. Finally, FGF was unable to increase DNA synthesis or cell division in polyoma-transformed (Table 2) or simian virus 40-transformed 3T3 cells (Table 2). This latter result is in agreement with the inability of pituitary extracts (10) or fractions from serum containing 3T3 growth-promoting ability (15) to enhance the growth rate of SV3T3 cells.

DISCUSSION

Previous reports have suggested that epidermal growth factor at low, physiological concentrations (10^{-10} M) could replace serum for partial induction of DNA synthesis in human fibroblasts (16), although the kinetics of increased polynucleotide synthesis were, however, slower than with serum. Insulin at high, nonphysiological concentrations can also induce DNA synthesis and cell division in quiescent fibroblasts in the presence of serum (14). In our systems, purified insulin at 10^{-6} M induces only marginal increases in DNA synthesis when added to cultures resting in serum-free medium for 3 days or more. If insulin at 10^{-8} M is added at 12, 24, 36, and 48 hr after addition of fresh serum-free medium, bovine serum albumin, and hydrocortisone to resting BALB/c 3T3 cultures, then approximately 39%, 24%, 11%, and 6% of the cell population, respectively, are induced to synthesize DNA (unpublished results). FGF, however, yields complete induction of DNA synthesis at any time. The removal of medium from resting cultures and its replacement by fresh serum-free medium is sufficient to initiate DNA synthesis in a small, but significant, fraction of the cell population. Thus, insulin may maintain those cells that are already initiated to grow rather than initiate DNA synthesis *per se* in resting cultures. Interestingly, for regenerating mammary cells in organ culture, insulin also exerts a permissive effect for the growth-initiating

TABLE 2. *FGF and different mouse cells*

Cell type	DNA synthesis			Labeled nuclei		Cell number	
	—*	+ FGF	+ Serum	—*	+ FGF	—*	+ FGF
BALB/c 3T3a	0.2	13	14	0.4	95	3.9	7.4
BALB/c 3T3b	0.1	12	13	0.5	100	7.0	13
BALB/c 3T3c	0.1	8.2	12	0.3	65	6.8	11
Swiss 3T3-4A	0.8	4.9	7.6	0.5	64	7.5	12
2nd embryos	0.1	4.9	5.1	0.4	81	16	28
BMK: fibroblasts	0.3	3.5	4.5	0.4	89	—	—
BMK: epithelial				0.6	1.0	—	—
Py3T3	3.2	2.9	7.4	—	—	10	9.0
SV3T3	2.1	1.8	8.4	30	26	5.1	4.2

Abbreviations: *BALB/c 3T3a, 3T3b, 3T3c* sublines of BALB/c 3T3 (*Methods*); *2nd embryos*, secondary mouse embryos; *BMK*, baby mouse kidney cultures containing both fibroblast and epithelial cells; *Py3T3*, polyoma-transformed BALB/c 3T3b; *SV3T3*, simian virus 40-transformed 3T3-4A. Cultures were "resting" for 3 days (or 2 days for SV3T3) in serum-free medium as described in *Methods* before additions of saturating amounts of FGF (50 ng/ml except for 2nd embryos, which was 100 ng/ml). 0.5% or less of all cultures were radioactively labeled with [^{3}H]thymidine during the subsequent 24 hr except 30% for SV or Py3T3. DNA synthesis is expressed as the cpm of [^{3}H]thymidine incorporated into DNA per 10 cells; the fraction of cells with [^{3}H]thymidine-labeled nuclei as %; and the cell numbers (× 10^{-5}) per 5-cm petri dish (*Methods*).

* Controls, no additions.

thesis and cell division at comparable levels to those found with 20% serum. These mouse cells included other BALB/c 3T3 lines and secondary mouse-embryo fibroblasts. In a few other BALB/c 3T3 lines (e.g., BALB/c 3T3c) and the Swiss 3T3 cells, the induction of DNA synthesis was only about 60–70% of that compared with serum for cultures radioactively labeled from 7 until 30 hr after additions. In some of these cultures (3T3-4A), however, the time for maximal induction of DNA synthesis was delayed by 3–5 hr over the time attained with serum. If cultures were radioactively labeled for longer times, then the induction of DNA synthesis was comparable to that of serum. Additions of insulin with

pituitary hormone, prolactin (17). The potentiation of the action of FGF by hydrocortisone appears to convert a subclass of cells that are refractory to growth-activation by FGF into another resting state where they are sensitive to FGF addition (Table 1). Hydrocortisone fails to alter membrane-bound cyclase activities in cell extracts or to cause early changes in intracellular cyclic nucleotides (submitted for publication) and, therefore, most likely potentiates the effect of FGF via the internal protein-synthetic machinery of the cell, possibly by inducing synthesis or alterations in its surface receptors (11). Similar permissive effects of steroid hormones upon the growth of either a transformed ovarian cell line (18) or mammary cells (17) either in culture or in the animal have previously been reported. It is therefore interesting that hydrocortisone addition to resting fibroblasts causes a small increase in polyribosome formation even without FGF, suggestive of possible intracellular changes.

We still cannot completely exclude the possibility that trace amounts of other growth-stimulating hormones adhere to the cell surface even after extensive washings (19). However, cultures initiated to divide once in serum-free medium with FGF and hydrocortisone can be fully induced to initiate DNA synthesis again on further addition of FGF (unpublished results). This reduces by half the concentration of unknown components bound to the cell surface prior to the second initiation step, without any noticeable effects. With this one proviso, Dulbecco's modified Eagle's medium and only two defined macromolecules in this system are required at physiological concentrations for cell survival, increased uptake and macromolecular synthesis, induction of DNA synthesis, mitosis, cell division, and probably cell movement. It is interesting that high, nonphysiological concentrations of cyclic GMP can also increase uptake rates and macromolecular synthesis (P. S. Rudland and W. E. Seifert, unpublished results) and induce DNA synthesis in mouse fibroblasts (7), cell division in rat thymic lymphoblasts (20), and cell movement of leukocytes (21). It is possible that some mouse fibroblast lines require additional hormones for full mitogenic response (e.g., Table 2) similar to the permissive role of insulin in mouse mammary cultures (17). However, FGF and hydrocortisone initiate substantial cell growth in the absence of exogenously added serum in all mouse fibroblasts tested, but fail to stimulate growth in mouse epithelial cells or transformed fibroblasts. This type of growth-factor selectivity may represent the basis for differential regulation of the growth of various tissues or organs in the intact animal.

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