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Cell Biology

An 18,000 molecular weight polypeptide induces early events and stimulates DNA synthesis in cultured cells*

(growth control/uptake/insulin/albumin)

HENRY R. BOURNE† AND ENRIQUE ROZENGURT‡

Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, England

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ABSTRACT **Extracts of serum-free medium, conditioned by contact with SV40-transformed BHK cells, stimulate DNA synthesis in cultured fibroblasts. From this source, we have purified a homogeneous basic protein of 18,000 molecular weight, termed fibroblast derived growth factor. In submicrogram quantities, fibroblast derived growth factor stimulates DNA synthesis in mouse 3T3 cells, in the absence of added serum. Prior to the onset of DNA synthesis, both serum and fibroblast derived growth factor induce an array of nearly simultaneous biochemical changes in the membrane of 3T3 cells that include stimulation of the uptake of nucleosides, 2-deoxyglucose, and $^{86}Rb^+$. These results strongly suggest that the early events are integral components of the proliferative response, rather than coincidental effects of nonmitogenic molecules present in serum.**

Many mammalian cells stop growing when the culture medium becomes depleted of growth factors. Such cells, resting in the G_1/G_0 or A (1) state, are stimulated to synthesize DNA and proliferate when fresh serum is supplied (2–7). Although the serum-dependent proliferative response has been intensively investigated as an *in vitro* model of growth regulation, its biochemical basis remains obscure.

Prior to the onset of DNA synthesis, serum induces an array of "early events" (8–16) that do not require *de novo* protein synthesis. This array includes stimulation of the uptake of nucleosides (8–10), potassium (11), phosphate (8–10), and nutrients such as glucose (12, 13) as well as changes in cellular content of cyclic nucleotides (10, 14). These early events are potentially important both as links, in the biochemical chain of events, leading to proliferation, and as markers for investigating the interactions of proliferative stimuli with their cellular receptors (15, 16). However, the chemical complexity of serum leaves open the possibility that some of its early effects may be exerted by molecules that are unrelated to stimulation of DNA synthesis.

This possibility can only be resolved by the use of chemically pure growth promoting molecules (growth factors). Growth factors have been extracted from serum (17–20) and tissues (21–24), as well as medium conditioned by contact with cultured cells (25–28). From medium conditioned by simian virus transformed baby hamster kidney (BHK) fibroblasts, Bürk partially purified a protein that stimulates cell migration and growth, in the presence of exogenously added serum (29, 30). The substance was operationally termed migration factor.

The discovery that extracts of simian virus 40-transformed BHK cell-conditioned medium can efficiently stimulate DNA synthesis in a nutritionally competent serum-free medium led us to purify and characterize a protein that induces both early biochemical changes and DNA synthesis. Experiments with this factor clearly implicate these early events as components of the proliferative response. Because of its source and its multiple effects on cell functions, in addition to migration, we term this substance fibroblast derived growth factor (FDGF).

MATERIALS AND METHODS

Cells. Swiss mouse 3T3 cells (31), propagated as previously described (11), were subcultured into 30 mm Nunc dishes in 6% fetal calf serum (Flow); medium was changed at 3 days. All assays of factor activity were performed on confluent and quiescent cells 5–8 days later, after washing the monolayer twice with Dulbecco's modified Eagle's medium (D-MEM) to remove residual serum. Mouse embryo fibroblasts (9) and Swiss mouse 3T6 cells (31) were propagated as described. HE-33 cells are human lung embryo fibroblasts, kindly supplied by M. Franks; they were used at the ninth passage.

Assay of Active Fractions. All determinations of DNA synthesis were performed in a 50:50 (vol/vol) mixture of D-MEM with Waymouth medium (32), because preliminary experiments indicated that this combined medium effectively supported stimulation of DNA synthesis in the absence of serum, while D-MEM alone did not. In 3T6 cells, the enhancing activity of the Waymouth medium is due to the presence of vitamin B_{12} (K. Mierzejewski and E. Rozengurt, manuscript in preparation). For determinations of DNA synthesis, the medium contained either 0.2 μM (5 μCi/ml) or 3 μM (1 μCi/ml) of [^{3}H]thymidine, for autoradiography or incorporation into acid-precipitable DNA, respectively.

Factor Purification. Initial steps of purification were as described by Bürk (29, 30). Briefly, 6 liter of D-MEM containing 10% tryptose phosphate broth [conditioned for 4 days by approximately 16×10^9 simian virus 40-BHK cells (subline SV28)], were subjected to protein precipitation by 66% ammonium sulfate. The precipitate was resuspended, dialyzed, and applied at neutral pH to a Dowex AG-50W X2 column, and activity eluted with tri-sodium orthophosphate at pH 12. Active fractions, after dialysis and lyophilization, were chromatographed on a Sephadex G-75 column under dissociating conditions (0.02 M HCl, 0.14 M NaCl). Activity appeared in fractions corresponding to a molecular weight between 15,000 and 35,000.

In the final step not previously described, active fractions were pooled, neutralized, dialyzed against 5 mM sodium phosphate at pH 7.5, and applied to a 1×25 cm carboxymethylcellulose column (Whatman CM-52) previously equilibrated with the same buffer. After washing with 25 ml of column buffer, activity was eluted with a 140 ml linear gradient of 0–600 mM NaCl. In most preparations two peaks of activity were seen; the second and larger peak (eluting at 200–300 mM

Abbreviations: BHK cells, baby hamster kidney cells; FDGF, fibroblast derived growth factor; D-MEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin.

* With the technical assistance of Margaret Grimaldi.

† Present address: University of California, San Francisco, Calif. 94143.

‡ To whom reprint requests should be addressed.

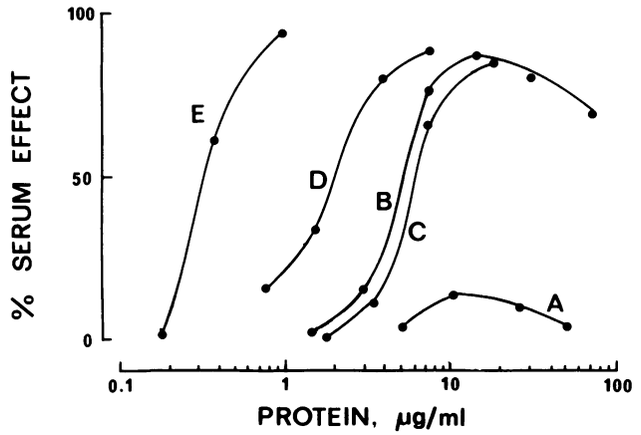

FIG. 1. DNA synthesis in 3T3 cells in response to FDGF at different stages of purification from simian virus 40-transformed BHK-conditioned medium. Extracts, in a volume of 5–50 μl, were added to confluent and quiescent 3T3 cells in 2 ml of D-MEM-Waymouth medium containing 0.125% BSA and [^{3}H]thymidine (see *Materials and Methods*). Incorporation of radioactivity into acid-insoluble material was determined 40 hr later. Results are expressed as a percentage of the stimulation (1.2×10^6 cpm per dish) produced by 10% fetal calf serum. In the presence of BSA alone, the cells incorporated 7.6×10^3 cpm per dish. Purification steps are described in *Materials and Methods*: (A) conditioned medium; (B) ammonium sulfate precipitate; (C, D, and E) represent, respectively, pooled active fractions from Dowex-50, Sephadex G-75, and carboxymethylcellulose columns.

NaCl) was used in the experiments reported here. Overall, a typical purification (see *Results*, Fig. 1) resulted in a 20-fold purification and 6% recovery of FDGF activity, with respect to the ammonium sulfate precipitate. Growth promoting activity can not be extracted from D-MEM containing 10% tryptose phosphate broth unless the medium was conditioned by the cells.

Effects of FDGF on Cell Function. Uptake of [^{3}H]uridine (10), 2-[^{3}H]deoxyglucose (13), and $^{86}Rb^+$ (11) into acid-soluble cellular pools was measured as previously described. Migration of cells into a wound on the monolayer was also measured as previously reported (29, 30).

RESULTS

Purification. Fig. 1 depicts the potency of FDGF, as assessed by stimulation of [^{3}H]thymidine incorporation into DNA of 3T3 cells, at successive steps of the purification procedure (described in *Materials and Methods*). Ammonium sulfate precipitation concentrated the activity and removed substances that inhibit responsiveness of 3T3 cells (Fig. 1). The ammonium sulfate precipitate contained 13 discrete protein bands on sodium dodecyl sulfate/polyacrylamide electrophoresis. Dowex-50 chromatography and Sephadex gel filtration together produced about 4 to 5-fold purification of the activity.

The final step, salt gradient elution of FDGF activity adsorbed to carboxymethylcellulose, provided a 5-fold further purification (Fig. 1), and produced a substance that migrated as a single band on sodium dodecyl sulfate/gel electrophoresis (extrapolated molecular weight of 18,000) (Fig. 2). This preparation also revealed a single protein band when subjected to disc-gel electrophoresis at acid pH (Fig. 2). Unless otherwise noted, all experiments reported here were performed with this pure material.

Pure FDGF is likely to be a glycoprotein, because its activity was more than 95% destroyed by pepsin (0.3 mg/ml for 6 hr at 37°), heating for 30 min at 80°, or exposure to 50 mM sodium periodate (24 hr at pH 5 and 4°).

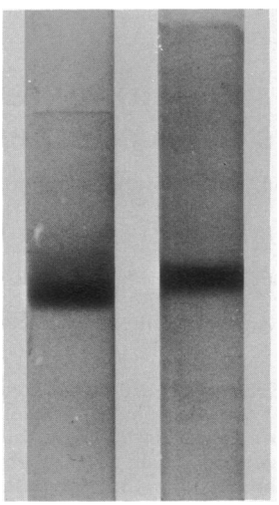

FIG. 2. Pure FDGF, stained with Coomassie brilliant blue, in polyacrylamide gels. Ten micrograms of protein was applied to a 10% gel at pH 4.5 (right) or a 15% gel containing 0.1% sodium dodecyl sulfate at pH 8.3 (left), and electrophoresis performed exactly as described (33).

DNA Synthesis. FDGF alone produced a time- and concentration-dependent stimulation of DNA synthesis in 3T3 cells, assessed by autoradiography of labeled nuclei (Fig. 3). The extent of stimulation by high concentrations of FDGF was nearly equal to that produced by 10% serum.

The effect of FDGF on DNA synthesis was potentiated by addition of either bovine serum albumin (BSA) or insulin (Figs. 3 and 4). Studies of certain other purified peptide factors have routinely used medium supplemented with BSA (22), ostensibly to prevent denaturation or nonspecific adsorption of low concentrations of active peptide. The potentiating effect of BSA was more prominent with low FDGF concentrations and longer periods of incubation (Fig. 3). Further studies will be necessary to determine what proportion of this effect of BSA is mediated via an action on cells, rather than by preserving the FDGF molecule. The BSA effect is not simply due to the presence of nonspecific protein, since comparable concentrations of ovalbumin fail to potentiate the action of FDGF on DNA synthesis (Fig. 4).

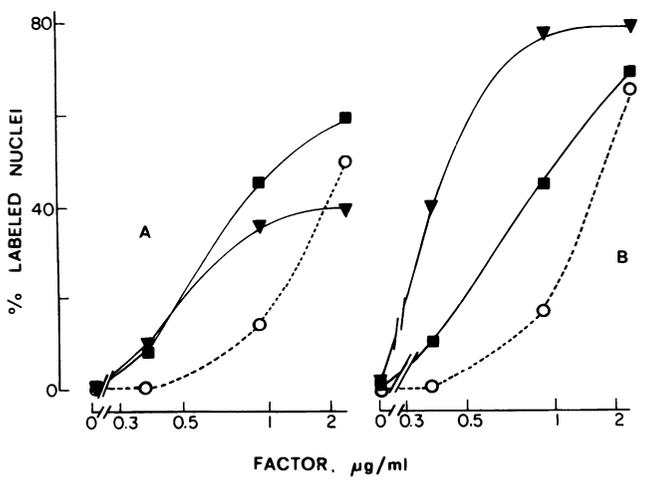

FIG. 3. DNA synthesis in 3T3 cells, assessed by autoradiography 20 hr (A) and 40 hr (B) after continuous exposure to [^{3}H]thymidine and various concentrations of FDGF alone (○), FDGF plus 0.125% BSA (▼), or FDGF plus 100 ng/ml of insulin (■).

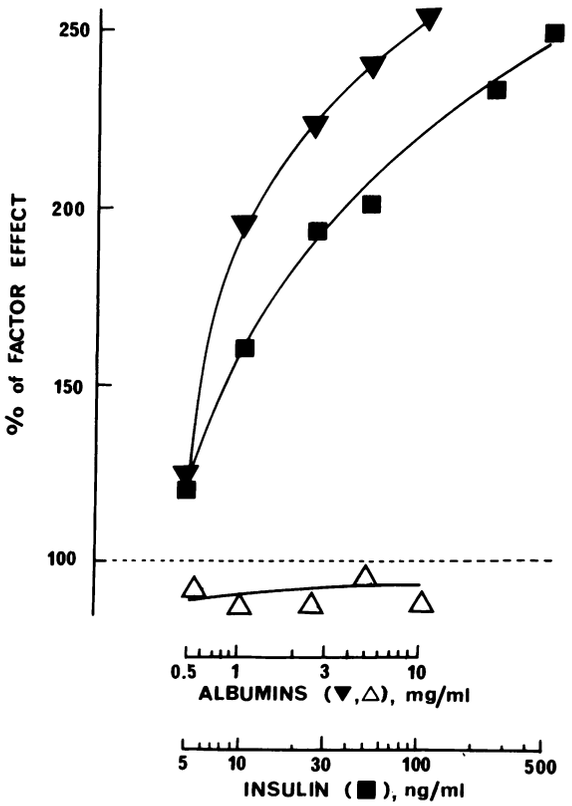

FIG. 4. Effect of various concentrations of insulin (■), BSA (▼), and ovalbumin (△) on stimulation of [^{3}H]thymidine incorporation by 3T3 cells exposed for 40 hr to 0.75 μg/ml of FDGF. Results are expressed as a percentage of the stimulation produced by FDGF alone (275 × 10^3 cpm per dish). In the absence of FDGF, [^{3}H]thymidine incorporation was less than 6 × 10^3 cpm per dish at all concentration of the other proteins tested. In this experiment, fetal calf serum produced [^{3}H]thymidine incorporation of 890 × 10^3 cpm per dish.

Insulin potentiated the effect of FDGF at physiologically relevant concentrations (5–50 ng/ml), although the insulin effect was more prominent at higher concentrations (Fig. 4). Hydrocortisone (0.01–10 μg/ml) did not increase the response of 3T3 cells to FDGF (results not shown).

Fig. 5 compares the effects of FDGF, and FDGF plus insulin, on DNA synthesis in two other murine cell lines and a human cell line. Each line, blocked in G_1/G_0 by serum depletion, responded to fresh serum with an increased rate of entry into DNA synthesis (Fig. 5, legend). FDGF alone stimulated DNA synthesis in mouse embryo fibroblasts and mouse 3T6 cells, although less effectively (on a μg basis) than in 3T3 cells (compare Fig. 5 with Fig. 3); insulin increased the response to FDGF in both mouse lines. In human embryonic cells (HE 33), addition of insulin was necessary for detection of a response to FDGF. HE 33 cells also responded less well to serum than did the mouse cells.

Early Events. Like serum and insulin (8–10), FDGF stimulates nucleoside uptake (Fig. 6, left): Both FDGF and 10% serum produced a 3-fold stimulation of the rate of [^{3}H]uridine accumulation; effects of both agents exhibited a well-defined temporal lag. A recent kinetic study (34) demonstrated that the concentration of stimulating agent (serum or insulin) predominantly affects the duration of the lag phase, rather than the final rate of nucleoside uptake. The effect of FDGF follows the same kinetics of stimulation (Fig. 6): The lag preceding maximal stimulation of uridine uptake by 2 μg/ml of FDGF was 10 min, while a 10-fold lower concentration exhibited a 20

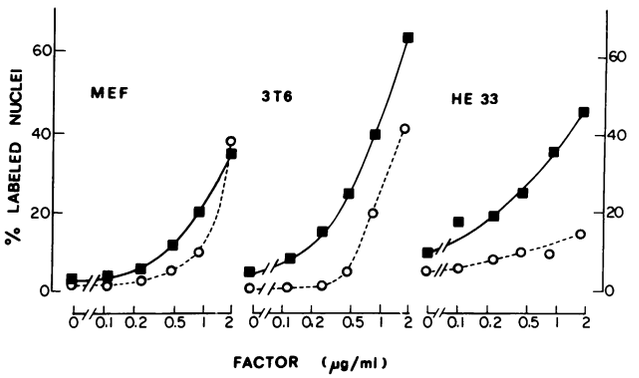

FIG. 5. DNA synthesis, assessed by autoradiography, in three cell lines exposed for 40 hr to various concentrations FDGF alone (○) or plus 100 ng/ml of insulin (■). In the same experiment, 10% fetal calf serum produced at 40 hr the following percentage of labeled nuclei: secondary mouse embryo fibroblasts (MEF), 98; 3T6 cells, 95; human lung embryo cells (HE-33), 73.

min lag; the final rate of [^{3}H]uridine uptake was identical for serum and both concentrations of FDGF. As is the case with serum (35), FDGF stimulates uptake of radioactive uridine, cytosine, and guanosine, but not adenosine (results not shown).

Like serum (12, 13) FDGF stimulates uptake of 2-[^{3}H] deoxyglucose (Fig. 6, right). In contrast with nucleoside uptake, no lag phase in stimulation was observed with either FDGF or serum. The stimulation occurred at FDGF concentrations effective in stimulating DNA synthesis, whether measured at 30 min (Fig. 6, insert) or 3 hr (not shown).

Serum also stimulates the uptake of $^{86}Rb^+$ (11), a convenient marker of potassium transport. This stimulation is blocked by ouabain, and is mediated by a membrane Na-K-ATPase. A maximally effective concentration of FDGF (2 μg/ml) consistently produced about one third of the stimulation of $^{86}Rb^+$ uptake induced by 10% serum (Fig. 7). This effect of FDGF, like that of serum, was prevented by 1.0 mM ouabain (not

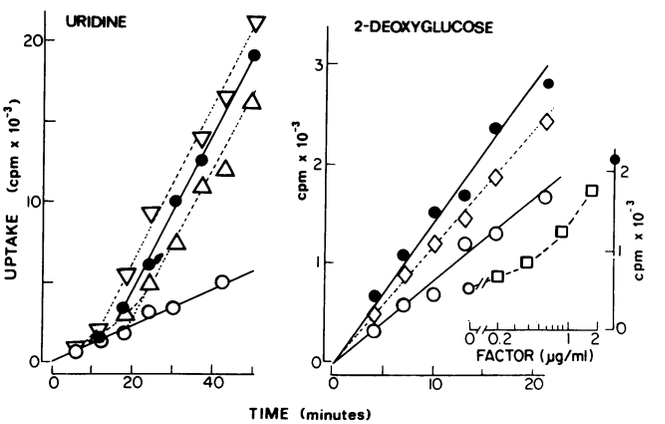

FIG. 6. Time course of accumulation of [^{3}H]uridine (left) and 2-[^{3}H]deoxyglucose (right) in 3T3 cells exposed to FDGF (▽,△,◇), 10% fetal calf serum (●), or D-MEM alone (○). At zero time, confluent and quiescent cells were washed with D-MEM and 1 ml of medium containing [^{3}H]uridine (1 μM, 1 μCi/ml) or 2-[^{3}H]deoxyglucose (0.05 mM, 2.5 μCi/ml), with or without FDGF or serum, was added. For 2-deoxyglucose uptake, cells were preincubated 30 min in glucose-free D-MEM. Acid-soluble radioactivity in the cells was determined as described (5, 8). FDGF concentrations (μg/ml) were 1.9 (▽), 0.19 (△), or 1.5 (◇). Insert (right): accumulation of 2-[^{3}H]deoxyglucose, in a separate experiment, after 30 min exposure to various concentrations of FDGF (□) or 10% fetal calf serum (●).

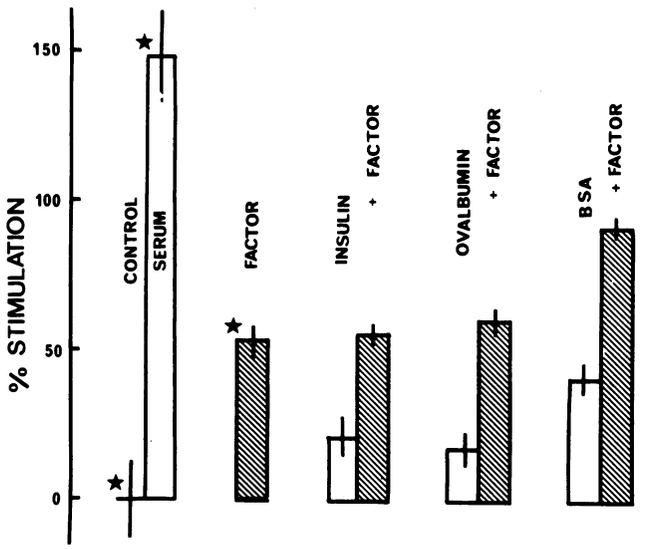

FIG. 7. Stimulation of ^{86}Rb transport by 10% fetal calf serum (Serum), FDGF (Factor, 2 μg/ml) alone, and with insulin (100 ng/ml), ovalbumin (0.125%), or bovine serum albumin (0.125%), alone or in combination with FDGF. Results are expressed as the percentage increase in ^{86}Rb uptake during a 30 min incubation with the various agents, as compared with uptake in serum- and protein-free D-MEM, (mean ± SD was 1270 ± 74 cpm/dish). The medium contained 5 mM K^+ and 2.5 μCi/ml of ^{86}Rb. Uptake during this period was linear with time, as previously described (11). Variation bars indicate either the range of duplicate determinations or the mean ±2 standard errors of the mean of six determinations (indicated by *).

shown). Because the maximal effect of FDGF was small in relation to that of serum, we examined the effects of two substances that augment FDGF's stimulation of DNA synthesis (Fig. 7): As previously reported (11), insulin produced a modest increase in $^{86}Rb^+$ uptake, but did not augment the effect of FDGF. In contrast, 0.125% BSA produced a larger stimulation of $^{86}Rb^+$ uptake in several experiments; this stimulation was additive to that produced by FDGF alone. The same concentration of ovalbumin failed to augment FDGF's effect.

In three preliminary experiments, 3T3 cells exposed for 20 min to a partially purified (through the gel filtration step) FDGF preparation showed a 25–30% decrease in adenosine 3′:5′-cyclic monophosphate (cyclic AMP), measured as described previously (10). In the same experiments, 10% serum caused a 40–50% decrease in cyclic AMP at 20 min.

Other Late Effects of FDGF. FDGF (1–2 μg/ml) and 10% serum stimulated equivalent migration of 3T3 cells into a wound on the monolayer (results not shown). Because we did not monitor migration-stimulating activity throughout the purification procedure, it is not clear whether all of the migration factor activity described by Bürk (29, 30) can be attributed to the more completely purified protein we term FDGF.

In the presence of a low serum concentration (0.5%), by itself incapable of supporting cell growth, 2 μg/ml of FDGF stimulated a 4-fold increase in cell number in 5 days of culture (results not shown). In a limited number of experiments, the same concentration of FDGF alone or in combination with insulin (100 ng/ml) or BSA (0.125%) did not consistently support the continuous growth of 3T3 cells. Others (36) have noted that serum contains factors required for survival of 3T3 cells.

DISCUSSION

Many experimental attempts to elucidate the biochemical processes critical for the transition of cells from $G_0/G_1/A$ into the proliferative cell cycle used serum as the proliferative stimulus. In mouse 3T3 cells, such studies (8–16) identified a number of biochemical events that rapidly and reproducibly result from application of fresh serum, and precede the onset of DNA synthesis by several hours. These early events appear to result from changes in the activity of carriers or enzymes associated with the plasma membrane, and are independent of protein synthesis.

Which, if any, of these early events are causally related to the proliferative response? Drugs provide suggestive evidence. Thus, ouabain and synthetic analogs of cyclic AMP block serum's stimulation of DNA synthesis, and each also counteracts a serum-induced early event: activation of membrane Na-K-ATPase (11) and a fall in cellular cyclic AMP (10, 14), respectively.

The effects of purified growth factors are potentially more specific than those of serum, a complex mixture of molecules. FDGF, a homogeneous protein, is some 3000-fold more potent (on a mg basis) than fetal calf serum in stimulating DNA synthesis (Figs. 1, 3, and 5). Thus, the demonstration that FDGF stimulates the uptake of nucleosides, glucose, and $^{86}Rb^+$ (Figs. 6 and 7), as well as the preliminary observation that it induces a fall in cyclic AMP, strongly implicates each of these early events as an integral part of the proliferative response.

These implications are strengthened by more detailed comparison of some of the effects of serum and FDGF. The striking similarities between serum and FDGF, in the kinetics of the stimulation of [^3H]uridine uptake and specificity for other nucleosides, make it highly likely that the two stimuli regulate this function in the same way.

In contrast to nucleoside and glucose uptake, the stimulation of ouabain-sensitive ^{86}Rb uptake by FDGF alone, even at high concentrations, was considerably less than that induced by serum (Fig. 7). However, the addition of serum albumin, at a concentration similar to that supplied by 10% serum, greatly augmented both FDGF's stimulation of ^{86}Rb uptake (Fig. 7) and its ability to induce DNA synthesis (Figs. 3 and 4). Serum albumin should not be considered an inert molecule; rather it may actively support proliferative responses in lymphocytes (37), in 3T6 cells (K. Mierzejewski and E. Rozengurt, manuscript in preparation), and in 3T3 cells, by augmenting certain membrane responses, exemplified by stimulation of the Na-K pump. Our experiments do not rule out an active contaminant in the BSA preparation used, but the results are not simply due to nonspecific protein, because ovalbumin did not reproduce the effects of BSA (Figs. 4 and 7). The biochemical basis of insulin's ability to potentiate FDGF's stimulation of DNA synthesis (Figs. 3–5) is not known. The ultimate rate of DNA synthesis could be determined by complementary early biochemical changes induced by different growth factors. If so, the complementary event produced by insulin has not been identified.

How does a single molecule, like FDGF, interact with cells to stimulate a variety of nearly simultaneous events in the plasma membrane? How do these changes at the cell surface control the internal metabolism of the growth-stimulated cell that results in coordinated synthesis of protein, RNA, and DNA, and eventually in cell division? Chemically defined growth factors provide simpler experimental systems to test biochemical models (15, 16, 38) proposed to answer these questions. Demonstration that a pure mitogenic protein can induce an array of early membrane events emphasizes the idea (15, 16)

that the concerted nature of these events is more critical for the proliferative response than each individual change, considered alone.

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