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Control of DNA synthesis in growing BALB/c 3T3 mouse cells by a fibroblast growth regulatory factor

(cell surface component/glycoprotein/uridine diphosphate *N*-acetyl-D-glucosamine/quiescent fibroblasts/growth regulation)

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ABSTRACT **A cell surface component from quiescent BALB/c 3T3 mouse cells that inhibits DNA synthesis and cell division when added to a culture of growing 3T3 cells has been detected. The inhibition of DNA synthesis by this factor was dependent on concentration and time of incubation; a transient exposure of cells to the factor followed by incubation in its absence for 20 hr was sufficient to elicit its inhibitory effect. The active component appears to be protein in nature, as judged by heat inactivation and trypsin sensitivity. Extracts obtained in an identical manner from quiescent 3T3 cells that had been preincubated *in situ* with uridine diphosphate *N*-acetyl-D-glucosamine (UDP-GlcNAc) did not inhibit DNA synthesis. The effect was specific for UDP-GlcNAc: incubation with three other nucleotide sugars yielded active component. Incubation of the inactive component from UDP-GlcNAc-treated cells with purified *N*-acetyl-β-D-glucosaminidase *in vitro* restored its inhibitory property. Extracts from growing cells failed to inhibit DNA synthesis. These results suggest that reversible glycosylation with *N*-acetyl-D-glucosamine residues may serve as a regulatory signal for the conversion of the active factor to its inactive form. We propose that the onset of quiescence of 3T3 cells is due to a causal relationship between depletion of growth factors in the culture medium and the presence of the active regulatory factor on the cell surface that inhibits DNA synthesis; conversion of the regulatory factor to its inactive form under favorable nutritional status may be viewed as a switch that allows DNA synthesis to resume.**

For many cultured cell lines, serum is a necessary component for growth. When these cells are seeded in low serum concentration, growth ceases at sparse culture density and the cells, arrested primarily in the G_1/G_0 stage of the cell cycle, remain viable for some time (1, 2). Although the basic mechanism(s) by which cells achieve quiescence remains to be elucidated, results obtained by Holley *et al.* (cited in ref. 3) suggest that, at least in an epithelial cell line, certain physiological conditions such as depletion of serum factors and low molecular weight nutrients, accumulation of inhibitory substances, and a decrease in the number of available "receptors" for growth factors, either singly or in various combinations, might be responsible for the onset of quiescence. It has been suggested (2) that the primary sites of interaction on the cells of the factors described above are the various components of the cell surface. In an earlier report (4) we showed that quiescent BALB/c 3T3 mouse cells in conditioned medium exhibited low metabolic activity with respect to uptake of three different classes of metabolites, whereas cells preincubated *in situ* for 60–120 min with uridine diphosphate *N*-acetyl-D-glucosamine (UDP-GlcNAc) showed stimulation of uptake of these nutrients; the increased uptake potential was accompanied by the restoration of *N*-acetyl-D-glucosaminyl (GlcNAc) residues on the uncharged oligosaccharide chains of some cell surface acceptors. Because of the apparent general nature of the metabolic stimulation upon glycosylation of uncharged acceptors, we examined the effect of glycosylated and deglycosylated acceptors on DNA synthesis. We report here the existence of a cell surface macromolecular component in quiescent BALB/c 3T3 cells that inhibits DNA synthesis and cell division in growing 3T3 cells. We also provide evidence that glycosylation of cell surface acceptors with GlcNAc residues abolishes the inhibitory effect of this factor on DNA synthesis. In this communication we tentatively refer to this component as "fibroblast growth regulatory factor" (FGRF) because of its ability to control cell proliferation in growing fibroblasts. Recently, Whittenberger and Glaser (5) have shown that a surface membrane-enriched fraction from confluent 3T3 cells inhibits DNA synthesis in sparse culture of 3T3 cells.

MATERIALS AND METHODS

Cell Culture. BALB/c 3T3 cell line and the culture conditions were those described previously (4). Quiescent cultures were obtained by seeding 5×10^5 cells in 60-mm petri dishes in 4% calf serum and incubating them for 60–96 hr without medium change (4). Cell numbers were determined by a Coulter Counter after the cells were detached from the culture dishes with 0.025% trypsin (GIBCO) in phosphate-buffered saline (P_i/NaCl) without the divalent cations.

DNA Synthesis. For assays of DNA synthesis three separate test systems (see Tables 1 and 2) were employed. First, cells growing in 10% serum ($2–3 \times 10^5$ cells) were incubated with FGRF for 24 hr at 37°C (unless specified otherwise) in a CO_2 incubator and pulsed with [^{3}H]thymidine (New England Nuclear) at 1 μCi/ml for 2–4 hr in the same medium (1 Ci = 3.7×10^{10} becquerels). After the incubation fluid had been removed, the cells were washed with P_i/NaCl and precipitated with 10% trichloroacetic acid at 4°C for 30 min. The precipitate was scraped off, deposited on a Whatman GF/C filter paper, and washed thoroughly with 5% trichloroacetic acid solution. The radioactivity on dried filter papers was determined in 3a70B scintillation fluid (Research Products International). In the second test system the cells were grown for 72 hr in complete medium with 4% serum to achieve quiescence. At the start of the experiment, the medium was replaced with complete medium containing 10% serum and [^{3}H]thymidine at 0.5 μCi/ml. Extracts were added and the cells were incubated for 24 hr at 37°C in a CO_2 incubator. The cells were processed for acid-precipitable radioactivity as described above. This long incubation time was used to measure the total amount of DNA synthesis in order to eliminate possible artifacts due to variations in the rate of thymidine uptake. Control experiments indicated that acid-precipitable radioactivity was located in DNA as

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Abbreviations: UDP-GlcNAc, uridine diphosphate *N*-acetyl-D-glucosamine; GlcNAc, *N*-acetyl-D-glucosamine; FGRF, fibroblast growth regulatory factor; P_i/NaCl, phosphate-buffered saline.

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judged by the alkali stability and sensitivity toward deoxyribonuclease.

The number of cells actively synthesizing DNA was determined by autoradiography of labeled nuclei. Cells growing in complete medium with 10% serum were incubated with the extracts for 24 hr at 37°C in a CO_2 incubator and pulsed with [^{3}H]thymidine at 1 μCi/ml for 2 hr. The cells were fixed and prepared for autoradiography essentially as described (5).

Protein was determined by the procedure of Lowry *et al.* (6). Sources of reagents were as described previously (4).

RESULTS

Isolation of FGRF. The active growth regulatory factor (see below) was extracted by incubating P_i/NaCl-washed quiescent 3T3 cells *in situ* for 60 min at 37°C with serum-free medium containing 0.2 M freshly prepared urea (cf. ref. 7). Inclusion of 1 mM phenylmethanesulfonyl fluoride, a protease inhibitor, did not influence the activity or the stability of these preparations. The extract was centrifuged at 27,000 × *g* for 15 min and the supernatant fluid, after exhaustive dialysis at 4°C against glass-distilled deionized water, was lyophilized. The dry material was dissolved in a small volume of sterile P_i/NaCl and stored at 4°C. Alternatively, the extract after centrifugation was concentrated by vacuum dialysis, dialyzed against P_i/NaCl containing 0.2 M urea and 1 mM dithiothreitol, and fractionated through a Sephadex G-200 column (74 × 1.6 cm) equilibrated at 4°C with the P_i/NaCl urea solution containing dithiothreitol (Fig. 1). Only two fractions, Fraction A, eluting at the exclusion volume, and fraction B, eluting slightly ahead of β-galactosidase (molecular weight 540,000), showed significant inhibition of DNA synthesis. Upon sodium dodecyl sulfate/polyacrylamide gel electrophoresis, both fractions were found to contain a mixture of polypeptides (in the molecular weight range 20,000–150,000), indicating heterogeneity; because of the presence of denaturant, it was not possible to identify the active component(s).

To isolate growth regulatory factor from growing cells as well as from quiescent cells that had been preincubated with UDP-GlcNAc (designated "restored" cells; cf. ref. 4), the extracts were prepared in an identical manner as described for the quiescent cells. All manipulations except gel filtration were carried out under sterile conditions. Bacterial contamination was monitored by streaking solutions on nutrient broth/agar plates. Cell cultures incubated with or without extracts were judged to be free of mycoplasma by autoradiography.

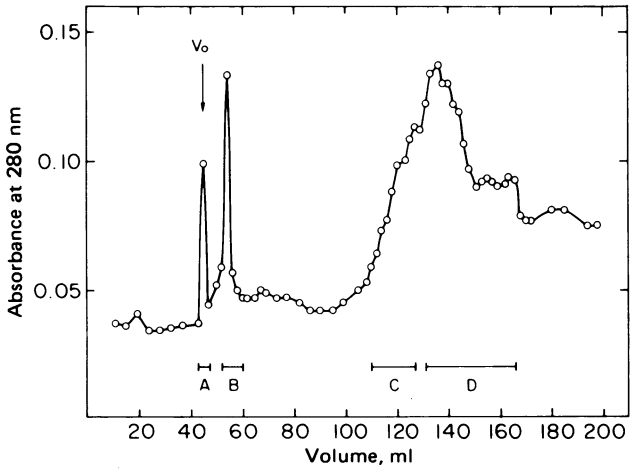

FIG. 1. Sephadex G-200 elution profile of extract from quiescent cells. The amount of protein loaded on the column was 3.6 mg (in 1.5 ml), and 3.5 mg was recovered. Approximately 2-ml fractions were collected at a flow rate of about 10 ml/hr. Fractions designated by letters were pooled, concentrated, and assayed for inhibition of DNA synthesis. The protein concentrations and percent inhibition of DNA synthesis by these fractions as compared to control were as follows: Fraction A (3 μg/ml), 42 ± 2%; fraction B (3.5 μg/ml), 38 ± 5%; fraction C (3.5 μg/ml), 12 ± 5%; fraction D (3.5 μg/ml), 5 ± 2%. Crude extract layered on the column inhibited DNA synthesis by 41 ± 6% (at 25 μg/ml). V_o, void volume.

The regulatory factor appears to be protein in nature. It was inactivated by heating at 75°C for 15 min. Further, treatment with trypsin also abolished its inhibitory activity; for example, after incubation of extract with crystalline trypsin at 50 μg/ml for 30 min at 37°C, the inhibition of DNA synthesis was less than 5%, whereas, untreated extract or extract incubated with trypsin plus soybean trypsin inhibitor showed about 50% inhibition. A control experiment revealed that trypsin plus trypsin inhibitor in the absence of extract did not inhibit DNA synthesis.

Inhibition of DNA Synthesis. In two separate test systems, unfractionated extracts from quiescent fibroblasts inhibited DNA synthesis (as measured by [^{3}H]thymidine incorporation) in growing 3T3 cells (Table 1). Although some variations were seen from extracts prepared from separate batches of cells over a period of 1 month, in all cases significant inhibition of DNA synthesis was observed. The inhibition was more drastic in test

Table 1. Inhibition of DNA synthesis by cell extracts

Source of extract*	Protein, μg/ml	Incubation, hr	DNA synthesis relative to 10% serum, %
Test system I: Growing cells†			
Quiescent cells	40	18	46
	40	20	64
	16	20	50
	20	21	50
	11	24	66
"Restored" cells‡	40	18	106
	40	20	74
	16	20	105
	20	21	100
	11	24	101
Growing cells	40	20	114
Test system II: Serum-stimulated quiescent cells§			
Quiescent cells	40	24	13
	20	24	45
	20	24	12
Growing cells	20	24	88

* Extracts were prepared as described in the text from separate batches of cells over a period of 1 month; each experiment represents a different extract.

† Cells (1 × 10^5) were seeded in complete medium with 10% serum and after 30–40 hr of growth the medium was replaced with fresh medium containing 10% serum. Extracts, prepared in P_i/NaCl, were added to the cells in a small volume and incubated at 37°C in a CO_2 incubator for the times indicated. DNA synthesis was measured by 2- or 4-hr pulse with [^{3}H]thymidine and determining radioactivity in the acid-precipitable fraction.

‡ "Restored" cells are quiescent fibroblasts preincubated at 37°C with 1 mM UDP-GlcNAc in P_i/NaCl for 60–120 min.

§ Cells were grown for 72 hr in complete medium with 4% serum to achieve quiescence. At the beginning of the experiment the medium was replaced with complete medium containing 10% serum and [^{3}H]thymidine at 0.5 μCi/ml. Extracts were added and the cells were incubated for 24 hr at 37°C in a CO_2 incubator. DNA synthesis was measured by determining radioactivity in the acid-precipitable fraction.

system II because under these conditions cells were released from the quiescent state by serum, resulting in synchronized growth, and total DNA synthesis (instead of rate) was measured. The results presented in Table 1 also show that extracts prepared from restored cells or cells growing actively in 10% serum had little or no effect on DNA synthesis. It should be mentioned that extracts prepared from restored cells gradually became inhibitory with storage at 4°C, possibly due to action of glycosidases present as contaminants (see below); for example, a freshly prepared extract that showed no inhibition on day 1 inhibited DNA synthesis by about 30% when tested after 7 days.

The kinetics of [^{3}H]thymidine incorporation (Fig. 2) after incubation for 24 hr with the regulatory factors from quiescent and restored cells showed that, in all cases, after an initial lag of about 20 min (presumably because of equilibration of the thymidine pool), the rates of incorporation were linear for 4 hr; a 50% reduction in rate was seen with cells incubated with FGRF from quiescent cells, whereas cells incubated with an equivalent fraction from restored cells showed a slight stimulation over control with 10% serum only. It should be emphasized that, as was the case with unfractionated extract, none of the fractions pooled after passage of restored cell extract through a Sephadex G-200 column showed inhibition of DNA synthesis.

The inhibitory effect of the regulatory factor obtained from quiescent cells after gel filtration was also monitored by autoradiography, which provides a true indication of the number of cells synthesizing DNA after entering S phase and is independent of the specific activity of the thymidine pool inside the cell. The results presented in Table 2 show that in two separate experiments the labeling index was reduced by an average value of 55% when the cells were incubated with the factor isolated from quiescent cells; the factor isolated from restored cells showed the same labeling index as that seen in 10% serum only.

Measurement of cell number after a 48-hr growth period in complete medium with 10% serum in the presence of (*i*) unfractionated extract from quiescent cells (40 μg of protein per ml) and (*ii*) fraction B isolated after gel filtration (4 μg of protein per ml) revealed significant reduction in cell number (42% and 57%, respectively) as compared to control in complete medium with 10% serum only.

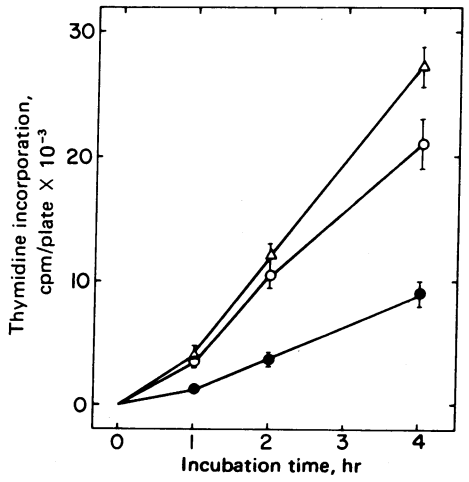

FIG. 2. Kinetics of [^{3}H]thymidine incorporation. Cells were seeded in complete medium with 10% serum, and after 42 hr of growth the medium was replaced with fresh medium containing 10% serum. The kinetics of thymidine incorporation was measured after 24 hr of incubation at 37°C in a CO_2 incubator in the presence or absence of fraction B (4 μg of protein per ml each) isolated from quiescent cells (●) or restored cells (△). ○, 10% serum only.

Table 2. Inhibition of DNA synthesis as revealed by autoradiography*

Source of extract	% nuclei labeled Exp. 1	Exp. 2
No extract	67	51
Quiescent cells	33	32
Restored cells†	70	54

* Cells growing in complete medium with 10% serum were incubated with fraction B (4 μg of protein per ml) for 24 hr at 37°C in a CO_2 incubator and pulsed with [^{3}H]thymidine for 2 hr. The cells were fixed and prepared for autoradiography. The values are averages of the number of nuclei counted from at least 10 separate fields, each containing about 30 cells.

† Quiescent cells were preincubated for 2 hr with 1 mM UDP-GlcNAc prior to preparation of extract.

The cumulative results presented thus far clearly show that a macromolecular component isolated from quiescent 3T3 cells inhibited DNA synthesis and cell division in growing fibroblasts.

Nature of Inhibition. The inhibition of DNA synthesis by the regulatory factor from quiescent cells was concentration dependent (Fig. 3); about 70% inhibition was observed at a concentration of 10 μg of protein per ml.

The results displayed in Fig. 4 show that the extent of inhibition of DNA synthesis was also time dependent. With increasing time of incubation with the regulatory factor, a progressively greater inhibition was seen. Control experiments showed that the factor isolated from restored cells did not have any significant effect on DNA synthesis.

An interesting aspect of the inhibition phenomenon is depicted in Table 3: a transient exposure of cells to the regulatory factor, even for a short period of 60 min, followed by incubation for 24 hr in complete medium with 10% serum *without* the factor was sufficient to elicit the inhibitory effect on DNA synthesis. These results also appear to indicate that the inhibitory effect cannot be attributed to the removal from the medium by direct interaction with the factor of soluble serum components present in small quantity that are required for DNA synthesis.

Interconversion of Active and Inactive Forms. In experiments described above the extracts prepared from restored cells (i.e., quiescent 3T3 cells preincubated with UDP-GlcNAc *in situ*) employed as controls did not inhibit DNA synthesis. Because our previous experiments have shown that incubation of quiescent 3T3 cells with UDP-GlcNAc under the conditions

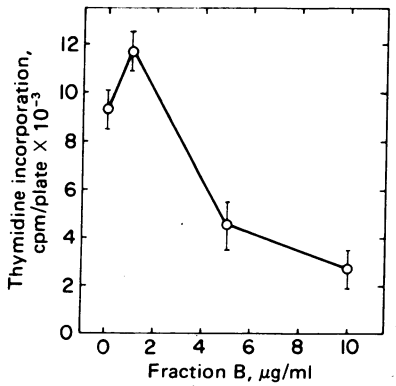

FIG. 3. Inhibition of [^{3}H]thymidine incorporation as a function of protein concentration. The experimental protocol was the same as described in the legend of Fig. 1, except various concentrations of fraction B from quiescent cells were used. The cells were pulsed with [^{3}H]thymidine for 2 hr at 37°C.

Table 3. Effect of preincubation time on inhibition of DNA synthesis*

Preincubation, hr	[^{3}H]Thymidine incorp., cpm/plate	% incorp.†
1	9,600 ± 400	45 ± 2
2	10,700	50
2.5	9,960 ± 660	47 ± 3
4	13,820 ± 500	65 ± 2
6	11,760 ± 1460	55 ± 7

* Cells growing in 10% serum were preincubated with fraction B (4 μg of protein per ml) for the time periods indicated. After incubation, the medium was removed and fresh medium containing 10% serum was added. After a period of 24 hr at 37°C in a CO_2 incubator, thymidine incorporation was measured by a 2-hr pulse.

† Relative to the control value of thymidine incorporation (21,300 cpm) by cells growing in complete medium with 10% serum only. The data are shown as mean ± range.

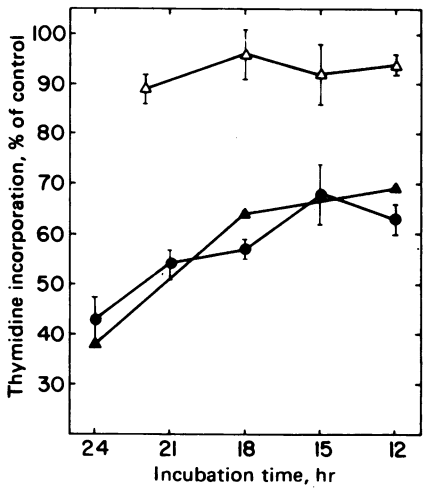

FIG. 4. Effect of incubation time on inhibition of DNA synthesis. Cells seeded in complete medium with 10% serum were grown for 36 hr and the medium was changed to fresh medium containing 10% serum. At various times after the medium was changed, unfractionated extracts (20 μg of protein per ml) were added. At the end of 24 hr, DNA synthesis was measured by a 2-hr pulse with [^{3}H]thymidine at 1 μCi/ml. The value of 100 equals 17,000 cpm/plate. The incubation time refers to the number of hours cells were exposed to extracts immediately prior to thymidine addition. ● and ▲, Extract from quiescent cells; △, extract from restored cells.

used here resulted in the glycosylation of uncharged cell surface acceptors with GlcNAc residues (4), it seemed plausible that the lack of inhibitory effect on DNA synthesis may be a consequence of glycosylation of the regulatory factor in restored cells. Results presented in Table 4 show that only extracts obtained from quiescent cells preincubated with UDP-GlcNAc had less of the active component; cells incubated with three other nucleotide sugars yielded extracts that inhibited DNA synthesis to an extent similar to that seen with extract from quiescent cells. The results of Table 4 also show that inhibition of DNA synthesis, or lack thereof, after incubation for 24 hr with various extracts in complete medium with 10% serum was not an artifact due to sloughing off of cells from petri dishes, because the number of [^{14}C]leucine-labeled cells recovered at the end of 24 hr was almost the same under all incubation conditions.

A separate experiment to convert the inactive regulatory factor to its active form was carried out *in vitro*. The inactive regulatory factor isolated from UDP-GlcNAc-pretreated cells after passage through a Sephadex G-200 column was incubated with 0.05 unit (unit as defined by supplier, Boehringer) of purified beef kidney *N*-acetyl-β-D-glucosaminidase per ml for 30 min at 37°C; the mixture was again passed through a Sephadex G-200 column to separate the enzyme from the regulatory factor. Assays for DNA synthesis with growing 3T3 cells in test system I (see Table 1) showed that the glucosaminidase-treated factor (at 3 μg of protein per ml) inhibited DNA synthesis by 35 ± 7% (average of four separate assays), whereas untreated factor from restored cells at the same protein concentration inhibited DNA synthesis by less than 5% (average of six separate assays). An important control included with this experiment was incubation of the *active* FGRF from quiescent cells with glucosaminidase under identical conditions: no significant difference in inhibition of DNA synthesis was seen before and after enzyme treatment. Further, extracts obtained from quiescent cells preincubated *in situ* with and without glucosaminidase exhibited similar extent of inhibition (43 and 47%, respectively). These results appear to rule out the possibility that contaminating glycosidase activities, if any, in glucosaminidase preparation were able to modify the activity of FGRF by removing sugar residues other than GlcNAc residues. It should be noted that trypsin abolished the inhibitory effect of the active FGRF but did not convert the inactive form to its active form (as revealed by no inhibition by extracts from restored cells before and after treatment with crystalline trypsin).

Table 4. Specific restoration of inhibition of DNA synthesis by a factor obtained from quiescent cells preincubated with UDP-GlcNAc*

Source of extract		Incorporation, cpm/plate		
		[^{14}C]Leucine,	[^{3}H]Thymidine	
Cells	Preincubation	Exp. 1	Exp. 1	Exp. 2
No extract	—	2550 ± 50	21,200 ± 1800 (100)	24,600 ± 1000 (100)
Quiescent	—	2500 ± 100	11,800 ± 900 (56)	13,900 ± 700 (57)
Quiescent	GDP-Man	2450 ± 50	12,100 ± 200 (57)	15,200 ± 300 (62)
Quiescent	UDP-Glc	2300 ± 150	10,100 ± 100 (48)	16,200 ± 100 (66)
Quiescent	UDP-Gal	2400 ± 200	11,700 ± 1000 (55)	13,600 ± 500 (55)
Quiescent	UDP-GlcNAc	2450 ± 100	18,900 ± 200 (89)	20,300 ± 800 (82)

* Exp. 1: For labeling with leucine, cells were plated in 10% serum with [^{14}C]leucine at 0.5 μCi/ml for 24 hr and chased with 1 mM nonradioactive leucine for 12 hr in complete medium with 10% serum. The medium was replaced with fresh medium containing 10% serum and various extracts (20 μg of protein per ml) were added. After an incubation period of 24 hr at 37°C in a CO_2 incubator, the rate of DNA synthesis was measured during a 2-hr pulse with [^{3}H]thymidine at 1 μCi/ml. Exp. 2: The protocol was identical to that of Exp. 1 except that no leucine was used to label cell proteins. All values are means of duplicate measurements ± range. Numbers in parentheses are percent of no-extract controls.

DISCUSSION

The results documented above reveal the existence of a cell surface macromolecular component in quiescent BALB/c 3T3 cells, tentatively designated as fibroblast growth regulatory factor (FGRF), which inhibited DNA synthesis and cell division when added to a culture of growing 3T3 cells. A novel finding pertaining to this was that, unlike preparations obtained from quiescent cells, extracts prepared in an identical manner from quiescent cells preincubated with UDP-GlcNAc, which resulted in the glycosylation of some cell surface acceptors with GlcNAc residues (4), were inactive in inhibiting DNA synthesis; incubation of the inactive preparations isolated from UDP-GlcNAc-pretreated cells with purified *N*-acetyl-β-D-glucosaminidase *in vitro* restored the inhibitory effect on DNA synthesis. Further, extracts prepared from actively growing cells failed to show inhibition of DNA synthesis. We interpret these data to mean that the state of quiescence of 3T3 cells due to depletion of growth factors in the culture medium and the presence of active FGRF on the cell surface may be causally related; it should be emphasized, however, that the exact temporal relationship between the appearance of active FGRF on the cell surface and the onset of quiescence is not known as yet. It is reasonable to assume that, with the impending depletion of serum factors, cells might trigger a "signal" that leads to a gradual accumulation of this component to progressively slow down DNA synthesis; conversion of the active FGRF to its inactive form under favorable nutritional status may be viewed as a switch to turn off the regulatory factor and allow DNA synthesis to resume. Although it appears highly likely, we cannot claim that direct incorporation of GlcNAc residues onto the active FGRF is involved in the conversion to its inactive form. It may be recalled in this context that cell surface acceptors of quiescent 3T3 cells appear to be "underglycosylated" in terms of GlcNAc residues and, when these cells were incubated with UDP-GlcNAc, the uncharged acceptors were reglycosylated with concomitant stimulation of uptake of metabolites of three different classes, 2-deoxyglucose, uridine, and α-aminoisobutyric acid (4).

The precise nature of FGRF (in terms of number of components, molecular size, and carbohydrate composition) and its mechanism of action are not known at present. The results obtained thus far indicate that the regulatory factor is a protein and, in the absence of a protein denaturant, shows aggregation characteristics. The factor is easily extracted from intact cells by incubation in serum-free medium containing low concentration of urea without damaging cell integrity and decreasing cell viability (cf. ref. 7), indicating that the component is loosely attached to the cell surface. This notion is strengthened by the observation that the active FGRF may be converted to its inactive form by incubating *intact cells in situ* with UDP-GlcNAc; as shown previously (4), 60–80% of radiolabeled GlcNAc residues (from cells incubated *in situ* with UDP-[^{3}H]GlcNAc) were released under the same mild extraction procedure employed here. It is not known whether FGRF is a serum component that becomes adsorbed on the cell surface or is synthesized by the cell during nutritional stress. Recently, Pouyssegur and Yamada (8) have isolated a major membrane glycoprotein from confluent mouse 3T3 cells (designated glucose/glycosylation-regulated protein) that appears to exist in glycosylated form and as its nonglycosylated precursor, but whose function is yet to be elucidated. Because of the apparent similarity between this membrane glycoprotein and the two forms of FGRF described here, it would be interesting to examine the properties of these cell surface components to establish whether they are somehow related.

The inhibition of DNA synthesis by the active FGRF but not its inactive form raises an important question as to the mode of action of this factor. The results show that the inhibition was time- and concentration-dependent, and a transient exposure of cells to the factor was sufficient to elicit its inhibitory effect, although the maximum extent of inhibition was seen only after about 20 hr of incubation in complete medium with 10% serum after removal of unbound FGRF from the culture medium. Thus, the inhibitory effect of the factor may not be due to removal from the medium of some necessary growth factor or because of a general toxic effect resulting in the detachment of cells from the petri dishes. Although alterations in the membrane environment to influence the uptake of necessary nutrients is a possible mechanism (cf. ref. 4), nevertheless, alternate explanations such as masking of "receptors" for growth factors or direct interaction of the factor with some component of the DNA-synthesizing machinery inside the cell may not be ruled out. Whatever the exact mechanism may be, the involvement of a macromolecular component in the control of cell proliferation is a subject of considerable importance.

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