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Steroid hormones mediate reversible phenotypic transition between transformed and untransformed states in mouse fibroblasts

(growth control/regulatory mutant/steroid hormone/tumorigenicity in *nude* mice)

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ABSTRACT **Hydrocortisone at physiological concentrations reversibly inhibits DNA synthesis in ST1 cells (a line of mouse fibroblasts possessing 40 chromosomes and derived from Swiss 3T3 cells). This inhibitory activity is a property of glucocorticoids, but the β-OH of C-11 of glucocorticoids is not essential for the inhibition. The steroid hormone restores to ST1 cells dependency on serum, density, and anchorage for growth. When injected into *nude* mice, ST1 cells generated malignant invasive fibrosarcoma. Injections of dexamethasone into tumor-bearing animals blocked tumor growth. The steroid hormone seems to induce a reversible transition between a transformed and a "normal" phenotype. ST1 cells treated or untreated with hydrocortisone are not responsive to fibroblast growth factor, epidermal growth factor, or prostaglandin $F_{2\alpha}$ whereas they are responsive to a factor that is a contaminant in bovine serum albumin.**

It has been shown (1) that growth of mouse fibroblasts is regulated by hormone-like factors [namely, epidermal growth factor (EGF) and fibroblast growth factor (FGF)] as well as by the classical hormone hydrocortisone. Other factors of similar nature may also be involved but we are still far from a definition of growth control systems (2). More insight could be gained if adequate growth regulatory variants or mutants of 3T3 cells could be isolated. A vast screening of Swiss mouse 3T3 fibroblasts aimed at obtaining variants that respond differently to hormones and growth factors was undertaken. The basic assumption was that these variants would retain partially active growth control systems, thus constituting good candidates for a class of "regulatory mutants" of the growth control mechanism. Such mutants would be useful not only for the identification of new growth factors but also for the characterization of growth control mechanisms. Furthermore, we assumed that the 3T3 cell line (3) would be a suitable cell system for this genetically oriented approach.

We have recently reported (4) on the isolation of ST1 cells, a line with only 40 chromosomes, derived from Swiss 3T3 fibroblasts. In this paper we present results showing that a single hormone, hydrocortisone, restores to ST1 cells their dependence on density, serum, and anchorage for growth. We also show that this hormone inhibits the growth of ST1-induced tumors in athymic *nude* mice. These findings suggest that the ST1 line is a useful "regulatory mutant" of the nature postulated above.

MATERIALS AND METHODS

Cells. Swiss 3T3 cells (3) were derived from a sample obtained from American Type Culture Collection. ST1 cells are a line of mouse fibroblasts derived in our laboratory (4) from Swiss 3T3 cells. Growth medium consisted of 90 vol of Dulbecco's modified Eagle's medium (DMEM) containing 1.2 g of sodium bicarbonate per liter, 5 vol of fetal calf serum, and 5 vol of calf serum. Screw-cap glass flasks, flushed with 5% CO_2/95% air and sealed, were used for stock cultures. Experiments were carried out in plastic tissue culture dishes (Falcon), glass dishes, or glass coverslips maintained in sealed plastic boxes with a humidified atmosphere and flushed with the 5% CO_2/95% air mixture. No difference in cell growth has been observed between plastic and glass surfaces.

Saturation Density, Growth Curve, and Plating Efficiency Determinations. For saturation density determinations, subconfluent cultures were seeded and growth was followed by cell counting in a Neubauer chamber after trypsinization. Doubling time and saturation density were estimated from the exponential phase and plateau level, respectively. For plating efficiency determinations, 300–500 cells were plated in 50-mm-diameter dishes and incubated for 7–10 days for colony development. After incubation the cultures were fixed and stained with crystal violet and the number of colonies was scored.

Suspension Cultures. Cells were plated in 4 ml of Methocel medium onto agar plates (5). Methocel medium consists of DMEM containing 1% methylcellulose and 10% fetal calf serum; the agar plates are made with 0.9% agar in DMEM/10% fetal calf serum. Plates were refed weekly by carefully removing the old medium and adding 5–10 ml of fresh Methocel medium. Because cells grow at the interface of the agar/Methocel medium, this procedure does not disturb the colony distribution or development. Colonies were counted by using a dark-field light box.

Assay of DNA Synthesis Stimulation. Subconfluent stock cultures were plated in DMEM/3% serum, at 2×10^4 cells per cm^2. After 24 hr the cultures were washed with phosphate-buffered saline and fresh DMEM/0.2% serum was added. After 24 hr of incubation, the cultures were considered to be serum-starved. Serum, hormones, or factors whose activities were to be assayed were added to these cultures. At 12 hr after additions, [^{3}H]thymidine (0.25 μCi/ml, 0.1 μM) was incorporated for 12 hr and [^{3}H]thymidine uptake into DNA was determined as described (1).

***Nude* Mice.** Athymic *nude* mice of the Swiss genetic background (obtained from G. Sato's laboratory, University of California, San Diego, CA) were raised by crossing *nu/nu* homozygotes. The average litter size was 12. Because the female *nude* mouse has a tendency to attack and eat the offspring, normal foster mothers of the A strain were used until the animals reached age 21 days. From this point on, the *nude* mice

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Abbreviations: FGF, fibroblast growth factor; EGF, epidermal growth factor; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; DMEM, Dulbecco's modified Eagle's medium.

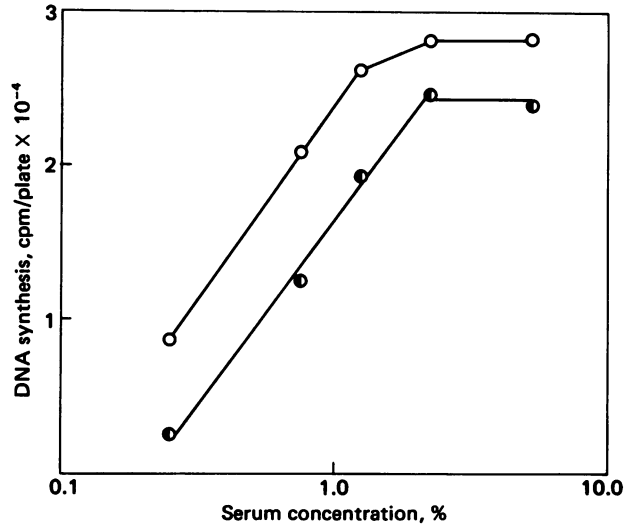

FIG. 1. Restimulation of DNA synthesis in ST1 cells. Serum-starved cells were restimulated by an increase in serum concentration and [^{3}H]thymidine uptake into DNA was determined. ○, Fetal calf serum; ◐, fetal calf serum plus hydrocortisone (300 ng/ml) added at the time of serum concentration increase. Each point is the average of two plates; deviation, <10%.

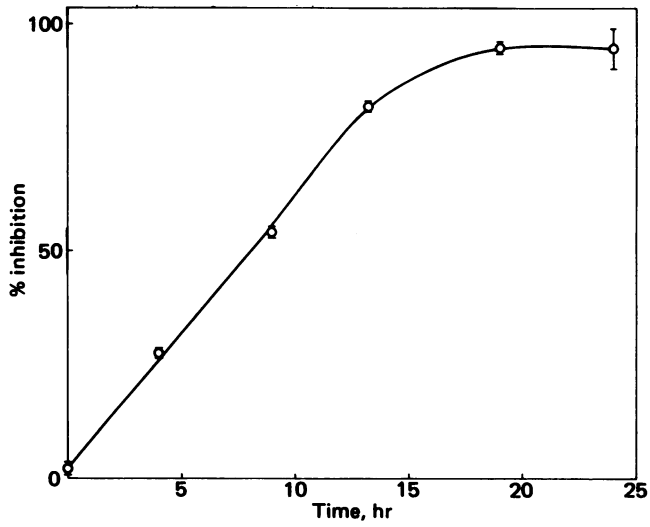

FIG. 2. Kinetics of inhibition of DNA synthesis by hydrocortisone in serum-starved ST1 cells. All cultures were given hydrocortisone (300 ng/ml) and incubated; at different times thereafter, cultures were pulsed (60 min) with [^{3}H]thymidine and incorporation into DNA was determined. Percentage inhibition was estimated by comparison of hormone-treated cultures and untreated control cultures and is plotted against time of incubation with hydrocortisone.

were kept in separate cages. Autoclaved cages provided with air filters as well as sterile water, food, and wood granulate bedding were routinely used. Handling of the animals was under strict aseptic conditions and the diet was supplemented with vitamins. Under these conditions and in spite of the less-than-optimal fertility standards, the animals had outstanding life-spans (approximately 400 days).

Tumorigenicity Test. After trypsinization of subconfluent cultures, ST1 cells were resuspended in growth medium and 1–2 × 10^6 cells in 0.25 ml were injected subcutaneously into the scapular region of 4-week-old animals. Palpable tumors were detected within 10 days of the injection in 100% of the animals. Different inoculum sizes have not been tested. Tumor size was periodically estimated by palpation. At the end of an experiment, the tumors were carefully dissected, weighed, and fixed in 10% formaldehyde for histopathological studies.

Hormones, Serum, and Other Chemicals. Crystalline insulin and steroid hormones were obtained from Sigma Co. and Nutritional Biochemical Corp., respectively. Three batches of fetal calf serum were used: two from Flow Laboratories and one from GIBCO; only one batch of calf serum (from GIBCO) was used. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) trimethylamine salt was from The Upjohn Co. Epidermal growth factor (EGF) was a purified preparation of mouse EGF (6). Fibroblast growth factor (FGF) was a purified preparation obtained from frozen bovine pituitary gland by a process described elsewhere (7). Several batches of bovine serum albumin were obtained from Sigma Co. and British Drug Houses, Ltd. Partially purified somatomedin B and highly pure somatomedin C were kindly provided by J. Van Wyck.

RESULTS

Steroid Inhibition of DNA Synthesis. ST1 cells are only partially dependent on serum for growth: DNA synthesis was decreased when the serum concentration was decreased but a significant basal level of DNA synthesis remained (25% after 24-hr incubation at low serum concentration). When the serum content was increased, again, DNA synthesis was restimulated. Hydrocortisone did not prevent this DNA synthesis restimulation. Nonetheless, the serum dose-response curve was shifted toward higher concentrations when the steroid was added at the time of increasing the serum concentration (Fig. 1).

The residual DNA synthesis found in serum-starved cultures was essentially abolished by the addition of hydrocortisone to the medium. This inhibitory action of hydrocortisone increased progressively with time of incubation, reaching a maximum after 20 hr (Fig. 2). Serum-starved cells treated with hydrocortisone could be restimulated by increasing the serum concentration, indicating that the hydrocortisone effect is completely reversible. Thus, the addition of hydrocortisone to cultures restores serum-dependent growth and, when serum-starved in the presence of the steroid, ST1 cells accumulate in a G_0 state (4).

The inhibitory action of a number of steroids was measured in order to assess the structural features required for the inhibitory activity (Fig. 3; Table 1). The main implications of these results are as follows. (*i*) The configuration of C-20 and

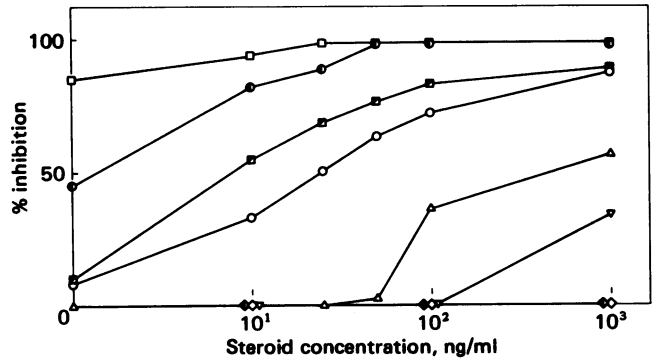

FIG. 3. Inhibition of DNA synthesis in serum-starved ST1 cells by different steroids. After 20 hr of incubation, [^{3}H]thymidine was incorporated for 4 hr and uptake into DNA was measured. Percentage inhibition was obtained by comparing treated with untreated cultures. □, Dexamethasone; ◐, hydrocortisone; ■, deoxycorticosterone; ○, cortisone; △, progesterone; ▽, 17β-estradiol; ◆, testosterone; ◇, dihydrotestosterone.

Table 1. Stimulation of DNA synthesis in 3T3 cells and inhibition of DNA synthesis in ST1 cells by different steroids

Steroid	Stimulation of "normal" 3T3 cells: Conc. for half-maximal stimulation, ng/ml	Stimulation of "normal" 3T3 cells: Relative maximal stimulation, %	Inhibition of "variant" ST1 cells: Conc. for half-maximal inhibition, ng/ml	Inhibition of "variant" ST1 cells: Relative maximal inhibition, %
Dexamethasone	1	100	1	100
Hydrocortisone	6	100	2	100
Deoxycorticosterone	40	13	7	88
Cortisone	—	Inactive	20	86
Progesterone	—	Inactive	100	56
Estradiol	—	Inactive	—	30*
Testosterone	—	Inactive	—	Inactive
Dihydrotestosterone	—	—	—	Inactive

The values (mean of three experiments) for ST1 cells were estimated from dose-response curves obtained from experiments such as the one in Fig. 3. Stimulation of DNA synthesis by 3T3 cells was assayed by addition of steroids to serum-starved cultures; values were estimated from dose-response curves. Inactive means no effect at concentrations up to 10^3 ng/ml.
* At 10^3 ng/ml (no significant inhibition below this concentration).

C-21 of hydrocortisone (common to all glucocorticoids) is necessary because the substitution of $-CH_2OH$ (in hydrocortisone) for $-CH_3$ (in progesterone) caused a sharp decrease in activity and the sex steroids, which lack C-20 and C-21, were completely inactive. (*ii*) The OH of C-11, which is essential for glucocorticoid activity (8), is not necessary for the inhibitory activity on ST1 cells because deoxycorticosterone (without OH at C-11) and cortisone (with O=C at C-11) were quite active. Therefore, the structural features required for the inhibitory activity of steroids on ST1 cells do not exactly follow those required for glucocorticoid activity. On the other hand, the stimulatory activity of steroids on "normal" 3T3 cells perfectly follows their glucocorticoid activity (9).

By plating efficiency experiments, we were able to show that steroid inhibition of DNA synthesis was not due to cell killing. At 12 hr after seeding 500 cells per 60-mm plate in DMEM/10% serum, the medium was replaced by DMEM/0.2% serum with or without hydrocortisone (300 ng/ml). After 96 hr of incubation, the medium was changed back to fresh DMEM/10% serum without hydrocortisone and the plates were again incubated for colony development. The mean (±SEM) plating efficiencies determined after fixing and staining were 52.0 ± 6.0% and 52.7 ± 4.8% for hydrocortisone-treated and untreated cultures, respectively.

Effect of Hydrocortisone on Morphology, Saturation Density, and Growth Rate. Addition of hydrocortisone to cells growing in high serum medium (5–10%) caused a rapid change in their appearance. In the absence of the steroid, the cells were not organized, exhibited abundant crisscrossing, and showed a tendency to pile up. The hormone caused the cells to become flat and strictly organized in parallel without overlapping, suggesting a high level of contact inhibition of movement. In colonies grown in the absence of hydrocortisone, the cells were loosely packed, not organized, and poorly attached to each other. By autoradiography we determined that DNA synthesis occurred throughout the colony. In the presence of hydrocortisone, cells from the center of the colony were isometric and tightly attached to neighboring cells, with labeled nuclei occurring exclusively in the periphery of the colony where cells were organized in parallel bundles.

Another consequence of hydrocortisone treatment was a change in saturation densities attained by the cells. Table 2 shows that ST1 cells grow as well in calf serum as in fetal calf serum, reaching very high densities with cells packed in multilayers. The presence of hydrocortisone decreased saturation density to one-third in fetal calf serum and to one-seventh in calf serum; in both cases, the cells did not go beyond a strict monolayer without overlapping. Thus, in the presence of hydrocortisone, fetal calf serum supports growth of ST1 cells better than does calf serum. The same difference in growth-promoting activities between fetal calf and calf serum was observed in assays of DNA synthesis stimulation in serum-starved ST1 cells (see Table 4).

The rate of growth, measured by time of duplication (T_D) of the cell population, also changed with hydrocortisone. For ST1 cells growing at their maximal rate, T_D = 15 hr. When hydrocortisone was added to an exponentially growing culture, growth slowed and a new steady state was reached with T_D = 20 hr (Fig. 4). The change in rate of growth of an exponentially growing culture occurred at about 13 hr after hydrocortisone addition.

Effect of Steroids on Anchorage Dependence and Tumorigenicity. When plated in methylcellulose suspension, ST1 cells developed macroscopic colonies (up to 3 mm) in 21 days. The plating efficiency observed in several experiments ranged between 1 and 5%. These results were only observed with medium containing 10% serum; with 0.2–5% serum, the efficiency of plating was consistently zero. Under the same conditions, "normal" 3T3 cells never developed colonies; rare microcolonies of four to eight cells could be found but most cells remained isolated and alive. When ST1 cells were plated in Methocel medium containing 10% serum plus hydrocortisone, the cells behaved like "normal" 3T3 cells and did not develop into colonies. The inhibition of colony formation was not due to cell

Table 2. Effect of hydrocortisone on saturation density of ST1 cells

Serum	Medium change	Saturation density, cells per $cm^2 \times 10^{-5}$: With hydrocortisone	Saturation density, cells per $cm^2 \times 10^{-5}$: Without hydrocortisone
Fetal calf	+	1.6	4.6
	−	0.7	1.9
Calf	+	0.6	4.2
	−	0.3	2.2

Cells were plated at 10^4 cells per cm^2 in 10% fetal calf serum or 10% calf serum; 20 hr later, hydrocortisone (300 ng/ml) was added to half of the cultures. The medium of some plates was changed every other day. Cells were counted daily.

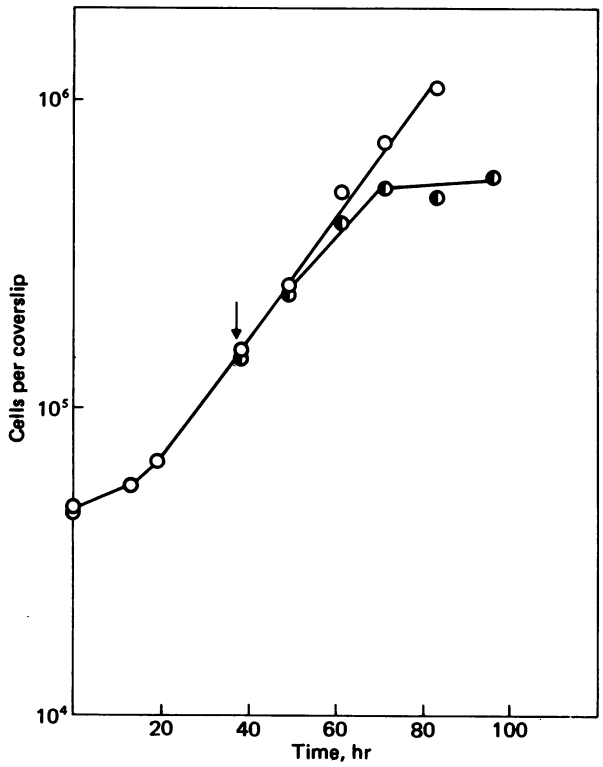

FIG. 4. Effect of hydrocortisone on ST1 cell doubling time and saturation density. Cells were plated on 3.2 cm^2 glass coverslips in DMEM/5% fetal calf serum. At the time indicated by the arrow, some cultures received hydrocortisone (300 ng/ml) (◐) and others were kept as controls (no additions) (○). The number of cells per coverslip was determined periodically; each point in the graph is the mean of three coverslips.

death. ST1 cells were plated in Methocel medium/10% serum in the presence and absence of hydrocortisone. By 21 days later, plates without hormone had developed macrocolonies (about 3 mm in diameter) with plating efficiencies between 1.5 and 2%, whereas plates with hydrocortisone showed no colonies. At this point, the medium in the latter plates was changed to fresh Methocel medium/10% serum without hydrocortisone and replaced weekly with medium containing no hydrocortisone. After 25 days, macrocolonies were visible at a plating efficiency between 1.5 and 2%. Therefore, the steroid caused a complete growth arrest in suspension but this arrest was totally reversible.

ST1 cells generated tumors when injected into athymic *nude* mice. Histopathological analysis indicated that these tumors were fibrosarcomas, with conspicuous anaplasia, high mitotic index, and invasive properties. Steroids inhibited tumor growth in the animals. A group of animals was injected with ST1 cells. When tumor size was between 0.5 and 1 cm, some of the animals were given periodic injections of hydrocortisone or dexamethasone. At 18 days after the beginning of steroid injection, the tumors of treated animals had a mass one-eighth that of control animals (Table 3). Histopathological examination of the tumors from the treated animals revealed arrested growth (virtually no mitosis) and, by morphological criteria, a much lower degree of malignancy. These results indicate that steroids block growth of ST1 cells also in the animal host.

Stimulation of DNA Synthesis in Serum-Starved ST1 Cells. In order to verify whether factors that stimulate growth of "normal" 3T3 cells would also stimulate ST1 cells, assays of [^{3}H]thymidine uptake into DNA were carried out with 3T3 and

Table 3. Effect of dexamethasone on tumor growth in *nude* mice

Animal*	Dexamethasone injection	Animal weight, g	Tumor weight, mg
1	−	22.1	194
2	−	23.7	202
4	−	20.7	242
5	−	18.5	86
6	+	15.3	22
7	+	15.0	29

Cells (1.5×10^6) were injected into seven 4-week-old animals of the same litter. After 20 days all animals had palpable tumors (0.5–1.0 cm); at this time, some animals were given dexamethasone injections (10 μg per animal) every other day; tumor growth was followed by palpation. At 18 days after the beginning of hormone injections, the animals were sacrificed and the tumors were dissected, fixed in 10% formaldehyde, and prepared for histopathological analysis.
* Animal 3 died before the end of the experiment.

ST1 cells in the presence and absence of hydrocortisone (Table 4). All of the factors known to stimulate 3T3 cells were inactive with ST1 cells except for insulin which had a modest activity in the absence of hydrocortisone. The most interesting finding was that commercial preparations of bovine serum albumin showed a strong activity both in the presence and in the absence of hydrocortisone. Moreover, a striking synergistic effect between insulin and albumin was observed in the presence of

Table 4. Growth factor activities for 3T3 and ST1 cells

Factor, hormone, or serum	Stimulation of DNA synthesis, %		
		ST1 cells	
	3T3 cells	Without hydrocortisone	With hydrocortisone
EGF	38	0	0
Insulin	65[a]; 4[b]	15[a]	0[a]
Hydrocortisone	15	—	—
FGF	44	0	0
Somatomedin:			
B	1.3	0	0
C	0.9	0	0
$PGF_{2\alpha}$	5	0	0
Bovine serum albumin	105	65	24
FGF + hydrocortisone	85	—	—
EGF + hydrocortisone	55	—	—
FGF + EGF	70	0	0
FGF + insulin	105[a]	11	0
EGF + insulin	103[a]	NT	NT
Albumin + insulin	106	70[a]	96[a]
$PGF_{2\alpha}$ + insulin	25[b]	NT	0[a]
Calf serum	100	100	100
Fetal calf serum	110	108	178

Activities were assayed by stimulation of DNA synthesis in serum-starved cells. Saturating concentrations were used for all factors: EGF, 100 ng/ml; insulin, a 2.5 μg/ml and b 50 ng/ml; hydrocortisone, 300 ng/ml; FGF, 1 μg/ml; somatomedin B, 1 μg/ml; somatomedin C, 30 ng/ml; $PGF_{2\alpha}$, 500 ng/ml; bovine serum albumin, 10 mg/ml; calf serum, 5%; fetal calf serum, 5%. Values are the means of three experiments. Activities were normalized by taking calf serum as reference. From five different batches of commercial crystalline bovine serum albumin, two had high activities (results in the table), two had low activities, and one had no activity; none of them had been defatted [albumin defatted by charcoal extraction (10) retains 100% of the activity]. Commercial ovalbumin had no activity. NT, not tested.

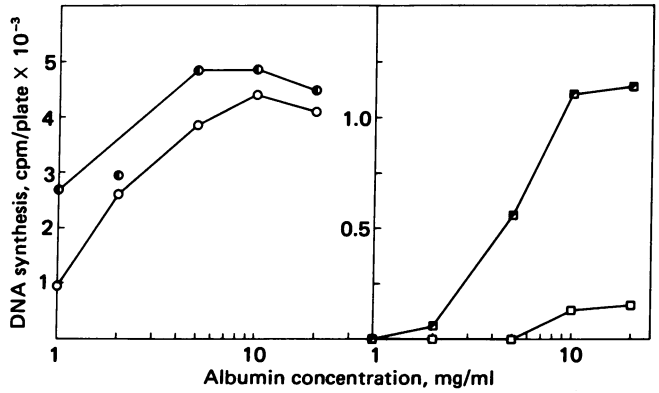

FIG. 5. Stimulation of DNA synthesis in ST1 cells by bovine serum albumin and insulin, in *Materials and Methods*. Insulin concentration was constant at 2.5 μg/ml. (*Left*) Untreated cultures. ○, Albumin; ◐, albumin plus insulin. (*Right*) Hydrocortisone-treated cultures (hormone added at the time of shift to low serum concentration). □, Albumin; ◪, albumin plus insulin.

hydrocortisone (Fig. 5). The activity of the albumin was due to a contaminant that is not dialyzable and is different from EGF, FGF, insulin, somatomedins B and C, and $PGF_{2\alpha}$. This active substance could be one of already described factors (11) but could also be a different serum factor.

We tested proteases for stimulation of DNA synthesis in ST1 cells. The results were negative; neither trypsin nor thrombin was active, irrespective of the presence or absence of hydrocortisone.

DISCUSSION

Glucocorticoids stimulate the growth of "normal" 3T3 cells (1, 12), a somewhat intriguing fact because normal fibroblasts are inhibited by glucocorticoids (13, 14). Therefore it is not surprising that ST1 cells are inhibited by glucocorticoids although the inhibitory activity could not be perfectly correlated with glucocorticoid activity.

The effect of a steroid hormone presents unique features. Addition of a single hormone to ST1 cultures reversibly changes the cell phenotype from a transformed to a "normal" pattern. However, it is still questionable how normalcy criteria, postulated in culture, correlate with *in vivo* physiology (15, 16). In this paper we present results showing that steroid injections into ST1 tumor-bearing animals caused two remarkable effects. First, the hormone blocked the growth of the tumor. Second, whereas tumors of untreated animals could be identified as fully malignant, invasive fibrosarcomas, tumors of hydrocortisone-treated animals showed arrested growth and a much lower degree of malignancy.

It is interesting to consider that hydrocortisone changes the morphologic features and the adhesion properties of ST1 cells (unpublished data). These changes must be due to alterations of the cell surface. Membrane proteins (17), sulfated mucopolysaccharides (18), or cytoskeleton (19) alterations could be responsible for both changes in growth control in culture and tumorigenicity in animals. Such alterations could be the primary effect of the hormone.

ST1 cells are not responsive to factors that stimulate growth of fibroblasts—namely, EGF, FGF (1), and $PGF_{2\alpha}$ (20). Hydrocortisone renders ST1 cells more like normal 3T3 cells as far as growth behavior goes. Nonetheless, this change does not lead to restoration of response to EGF, FGF, or $PGF_{2\alpha}$. Such observations indicate that density, serum, and anchorage dependency do not necessarily involve responsiveness to these factors. ST1 cells seem to have a phenotype that includes a partially active growth control system. This kind of cell might be a favorable cell system for identifying new growth factors such as the contaminant in albumin that we report in this paper. Furthermore, isolation of cells of this kind can be useful to resolve the components of the growth control mechanisms.

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