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Inhibition of DNA synthesis in cultures of 3T3 cells by isolated surface membranes

(growth control/density-dependent inhibition of growth/cell surface membranes)

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ABSTRACT **When added to a sparse culture of 3T3 cells, a surface membrane-enriched fraction from 3T3 cells inhibited the rate of DNA synthesis in a time- and concentration-dependent manner. The membrane preparation had no effect on the rate of DNA synthesis of simian virus 40-transformed 3T3 cells. A similar membrane preparation from transformed cells had a lesser inhibitory effect on 3T3 cells and no effect on transformed cells. The inhibition by membranes was reversible. The data suggest that, when added to growing 3T3 cells, 3T3 surface membranes can mimic the effect of increasing cell density on DNA synthesis.**

The rate of cell growth, measured by initiation of DNA synthesis or increase in cell number, of a confluent monolayer of certain "normal" cells in culture is much lower than it is for the same cells at a sparse density (1–3). Loss of density-dependent inhibition of growth usually accompanies transformation by oncogenic viruses (4, 5), chemicals (6), or irradiation (6).

Several mechanisms have been proposed to explain how increased cell density exerts its regulatory effect. One theory is that limitation of growth at a confluent density results from depletion of soluble factors in the bulk medium (7) or at the cell surface (8) or possibly the release of inhibitors into the bulk medium (7). When a wound is made in a quiescent confluent 3T3 monolayer, cells that move into the wound begin to grow while cells in the same medium in the confluent layer remain quiescent (9). In addition, 3T3 cells are able to maintain a stable saturation density despite daily replacement of the medium (5). These results argue against changes in the bulk medium being responsible for density-dependent inhibition of growth.

A second theory proposes that direct cell–cell contact, perhaps involving interaction of complementary cell surface factors on adjacent cells, provides a signal for regulation of cell growth (9). Such cell surface factors might be similar to the factors that mediate cell–cell adhesion in various systems (10). In agreement with this notion are recent experiments which suggest that, by binding to cell surface receptors, succinylated concanavalin A can inhibit cell division (11).

If complementary cell surface factors do in fact mediate growth control, it seems reasonable to assume that they interact in a manner analogous to the way a hormone interacts with its receptor or a substrate with its enzyme. This assumption led us to ask whether factors present on isolated cell surface membranes could interact with intact cells in culture to mimic the effect of increased cell density on growth.

MATERIALS AND METHODS

3T3 and SV3T3 Cell Cultures. 3T3 (12) and simian virus 40-transformed (SV3T3) (5) cells were obtained from H. Green. Cultures were grown at 37° in a 10% CO_2/90% air atmosphere in Dulbecco's modified Eagle's medium (DME) (K. C. Biological) supplemented with 10% (vol/vol) heat-inactivated calf serum (K. C. Biological), penicillin (100 units/ml) and streptomycin (100 μg/ml) (Gibco), and L-glutamine (0.1 mg/ml). Medium was changed every 3 days. 3T3 were carried in Falcon T75 flasks by serial transfer of 1:1000 dilutions of trypsinized confluent cultures. SV3T3 were transferred every 3–4 days by plating 2.5×10^5 cells per Falcon T25 flask. Fresh 3T3 cultures were started from frozen stocks after six to eight serial transfers. Cells were shown to be free of mycoplasma by autoradiography or by Microbiological Associates.

Surface Membrane Preparation. 3T3 were grown to confluency and SV3T3 to multilayer density in Falcon 150-mm dishes. Each dish was washed once with 20 ml of Ca^{2+}- and Mg^{2+}-free Hanks' salt solution (Gibco) buffered with 0.02 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) at pH 7.4 (CMF) and incubated 10 min at 37° with 20 ml of 1 mM EDTA in CMF. Cells were freed by scraping with a rubber policeman, collected by centrifugation at 150 × *g* for 5 min, and resuspended at 4° in 50 ml of Hanks' solution buffered at pH 7.4 with 0.02 M Hepes and containing bovine serum albumin (5 mg/ml). All steps which follow were carried out at 4°. After total cell number was determined with a hemacytometer (typically 1 to 5×10^7 3T3 or 12 to 40×10^7 SV3T3 cells per preparation), the cells were centrifuged (150 × *g* for 5 min) and resuspended in 8 ml of homogenization buffer (0.25 M sucrose/10 mM Tris, pH 7.4/0.2 mM $MgCl_2$, bovine serum albumin, 5 mg/ml) containing 1 mg of DNase I (3T3) or 2 mg of DNase I (SV3T3) (Sigma DN100). The cells were broken in a Dounce homogenizer (Kontes) with a tight-fitting (type B) pestle until 95% of the cells were broken (determined by using a hemacytometer). The homogenate was centrifuged at 27,000 × *g* for 15 min and the pellet was resuspended in homogenization buffer with a Dounce homogenizer. This supension was layered on a discontinuous Ficoll gradient consisting of 1.4 ml of 35% Ficoll (wt/vol in homogenization buffer), 1.4 ml of 25% Ficoll, and 1.4 ml of 9% Ficoll. One gradient was used for the 3T3 suspension and two gradients were used for the SV3T3 suspension. The gradients were centrifuged at 104,000 × *g* for 8–9 hr in an SW 50.1 rotor. The visible band present at each interface and the pellet were collected, individually diluted with 10 ml of 10 mM Tris, pH 7.4/140 mM NaCl/bovine serum albumin, 5 mg/ml and centrifuged at 39,000 × *g* for 20 min. Fractions were individually resuspended in 2 ml of the same solution for determination of enzyme markers. For use in experiments with cells in culture, the band (B-1) on top of the 9% Ficoll was diluted by adding 8 ml of DME/10% calf serum, centrifuged as before, and resuspended with a vortex mixer in DME/10% calf serum/L-glutamine, 0.1 mg/ml to the desired membrane concentration. The membrane suspension and control medium were sterilized in Kimble 12 × 75 mm dis-

Abbreviations: SV3T3, simian virus 40-transformed 3T3 cells; DME, Dulbecco's modified Eagle's medium; CMF, Ca^{2+}- and Mg^{2+}-free Hanks' solution (Gibco) buffered with 0.02 M Hepes, at pH 7.4; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2'-ethanesulfonic acid; dThd, thymidine.

posable glass culture tubes by exposure to a 15-W General Electric (G1JT8) germicidal lamp at 30.5 cm for 5 min.

Protein in the cell fractions was determined by labeling the cells in culture with [^{3}H]leucine (0.5 μCi/ml, 57.4 Ci/mmol in the solution added to the medium) (New England Nuclear) beginning at sparse density until collection. Trichloroacetic acid-precipitable material was collected on 0.45-μm filters (Millipore HA) and its radioactivity was determined. Total phosphate in B-1 was determined as described (13). The following enzymes were determined as indicated: phosphodiesterase (EC 3.1.4.1) (14), NADH diaphorase (EC 1.6.99.x) (15), and cytochrome *c* oxidase (EC 1.9.3.1) (16). Acid phosphatase (EC 3.1.3.2) was assayed at pH 5.0 with *p*-nitrophenyl phosphate as substrate. The incubation mixture contained 0.15 M acetate, 2 mM $MgCl_2$, 0.1% (vol/vol) Triton-X 100, 1 mM *p*-nitrophenyl phosphate (Sigma), and enzyme in a total volume of 1.1 ml. The change in absorbance at 420 nm, recorded after the pH was adjusted to 10.7, was compared to a *p*-nitrophenol standard. β-Glucuronidase (EC 3.2.1.31) was assayed with *p*-nitrophenyl glucuronide (Sigma) as substrate and the conditions described for acid phosphatase. Ouabain-sensitive Na^+,K^+-ATPase (EC 3.6.1.3) was determined by using the conditions described by Schimmel *et al.* (17) with [γ^{32}-P]ATP (New England Nuclear) as substrate (3 mM ATP, 300,000 cpm per assay) and 3 mM $MgCl_2$. At the end of the reaction the radioactivity in $^{32}P_i$ was determined as described (18).

Measurement of DNA Synthesis. Experiments were performed in Linbro FB-16-24-TC (2 cm^2) dishes in duplicate. [^{3}H]Thymidine ([^{3}H]dThd) incorporation into trichloroacetic acid-insoluble counts was measured essentially as described by Pardee (19). Cells in each dish were washed once at 37° with DME/10% dialyzed calf serum and incubated with 1 μCi (2.2 Ci/mmol) of [^{3}H]dThd (New England Nuclear) in 1 ml of DME/10% dialyzed calf serum for 2 hr at 37° in a 10% CO_2/90% air atmosphere. Cells were then washed once with 2 ml of Hanks' solution with bovine serum albumin at 4°, 1 ml of 5% (wt/vol) trichloroacetic acid at 4° was added, and the cells were incubated for 30 min at 4°. The cells were then rinsed once with 5% trichloroacetic acid and treated as described by Pardee (19). Cell number was determined in duplicate dishes with a Coulter counter. In some experiments cells were labeled with ^{14}C-labeled protein hydrolysate (0.75 μCi/ml, 54 Ci/matom) (Amersham/Searle) as a way of determining relative cell protein per dish. The rate of [^{3}H]dThd incorporation into DNA is linear for at least 3 hr under these conditions.

Autoradiography was performed by labeling cells as above with [^{3}H]dThd (5 μCi/ml; 56.4 Ci/mmol) for 1 hr. Cells were rinsed once with 2 ml of phosphate-buffered saline (Gibco) at 4° and fixed with 3.7% formaldehyde in phosphate-buffered saline for at least 30 min. Cells were rinsed twice with 2 ml of water, dried, and layered with NTB-2 nuclear track emulsion (Kodak). After 3–5 days of exposure, the emulsion was developed with D-19 and fixed with Ektaflo (Kodak). The cells were stained with hematoxylin (Harleco). Dish bottoms were punched out and mounted on slides, and the percentage of nuclei that were labeled was determined in duplicate dishes by two observers using microscopy at ×400.

RESULTS

Cell Fractionation Analysis. The distribution and activities of protein and subcellular markers shown in Table 1 are representative of the fractionation achieved for 3T3 and SV3T3 cells. The specific activity of the surface membrane markers Na^+,K^+-ATPase and phosphodiesterase was enriched 6- to 13-fold in fraction B-1 relative to the initial homogenate. B-1 contained, to a much lesser extent, enzyme activities characteristic of other cellular organelles (20). Fraction B-1 therefore consisted mainly of cell surface membranes.

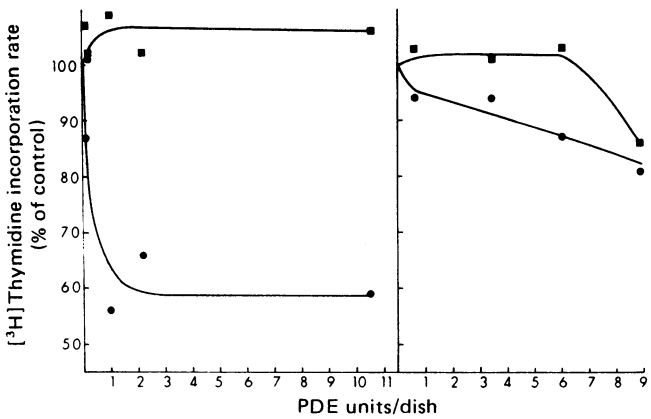

FIG. 1. [^{3}H]dThd incorporation rate by sparse 3T3 and SV3T3 as a function of membrane concentration. 3T3 and SV3T3 cells were plated in Linbro dishes at 0.05 × 10^4 and 0.03 × 10^4 cells per dish, respectively, in 1 ml of medium containing 0.75 μCi of ^{14}C-labeled protein hydrolysate (54 mCi/matom). At 54–60 hr later, the medium was changed to fresh medium without the isotope. About 72 hr after plating, a suspension of surface membranes (fraction B-1) from 3T3 or SV3T3 was added to dishes in 0.3 ml of medium at a membrane concentration expressed in units of phosphodiesterase (PDE) activity (1 unit of PDE activity hydrolyzes 10 nmol of substrate per hr). Controls received 0.3 ml of medium without membranes. The rate of [^{3}H]dThd incorporation into trichloroacetic acid-insoluble counts was determined 24 hr after addition of surface membranes. Values for [^{3}H]dThd dpm per dish have been normalized to [^{14}C]protein dpm in the same dish. The range of control values in the four experiments shown are as follows. Cell number per dish: 3T3, 0.63 to 1.66 × 10^4; SV3T3, 1.73 to 2.76 × 10^4. [^{14}C]Protein dpm per dish: 3T3, 560–900; SV3T3, 840–1700. [^{3}H]dpm/[^{14}C]dpm: 3T3, 19.0–21.9; SV3T3, 42.2–43.1. (*Left*) 3T3 surface membranes added to 3T3 cells (●) and SV3T3 cells (■) in two separate experiments. (*Right*) SV3T3 surface membranes added to 3T3 cells (●) and SV3T3 cells (■) in two separate experiments. Results are averages from duplicate dishes.

Effect of 3T3 and SV3T3 Surface Membranes on Rates of DNA Synthesis by 3T3 and SV3T3 Cells. The rate of [^{3}H]dThd incorporation into trichloroacetic acid-insoluble counts, which is taken here as a measure of DNA synthesis, decreased sharply as 3T3 cell density increased, reaching the lowest value at saturation density (5 to 10 × 10^4 cells per cm^2). SV3T3 cells showed a much smaller decline in incorporation rate as cell density increased. At a confluent cell density, at which 3T3 incorporation rate was 5–10% of maximum, SV3T3 incorporation rate was 80–90% of maximum.

Fig. 1 shows the effect of addition, for 24 hr, of a suspension of isolated surface membranes (B-1) on rates of [^{3}H]dThd incorporation into trichloroacetic acid-insoluble counts as a function of membrane concentration. Because phosphodiesterase is located predominately in the surface membrane, the level of its activity provides a measure of surface membrane mass. The addition of 3T3 surface membranes decreased the rate of incorporation of [^{3}H]dThd into DNA by sparse 3T3 cells in a concentration-dependent manner, leveling off at about 60% of control. One unit of phosphodiesterase activity, corresponding to about five cell equivalents of surface membrane per cell, decreased the rate of [^{3}H]dThd incorporation to 70% of control. At a membrane concentration such that the rate of DNA synthesis by 3T3 cells was at a minimum, the rate of DNA synthesis in SV3T3 cells was similar to the control.

At membrane concentrations at which 3T3 surface membranes were nearly fully inhibitory, SV3T3 surface membranes

Table 1. Distribution and activities of protein and subcellular markers in the fractionation of 3T3 and SV3T3 cells

Fraction	Exp. 1 Protein	Phosphodiesterase	Acid phosphatase	NADH diaphorase	Cytochrome c oxidase	Exp. 2 Protein	Na^+,K^+-ATPase
3T3							
Homogenate							
27,000 × *g*:	(100)*	0.76	11.82	2.66	20.8	(100)†	0.24
Supernatant	(57.0)	0.25(24.4)	4.45(21.4)	1.24(26.6)	n.d.‡	(64.5)	n.d.‡
B-1	(4.0)	6.44(34.6)	18.31(6.3)	4.39(6.6)	4.8(0.9)	(3.9)	3.22(52.4)
B-2	(6.3)	1.58(13.3)	10.55(5.7)	2.14(5.1)	6.7(2.0)	(4.8)	1.80(35.8)
B-3	(1.2)	0.31(5.0)	38.80(4.0)	1.17(5.3)	277.8(5.7)	(10.8)	0.43(19.3)
Pellet	(4.0)	1.10(5.9)	32.65(11.2)	9.96(15.1)	149.7(29.0)	(1.6)	n.d.‡
SV3T3							
Homogenate							
27,000 × *g*:	(100)§	0.23	4.04	0.88	11.0	(100)¶	0.04
Supernatant	(66.1)	0.18(51.8)	2.82(46.11)	0.15(11.6)	n.d.‡	(78.3)	n.d.‡
B-1	(3.4)	1.41(20.7)	8.87(7.4)	1.71(6.5)	2.4(0.7)	(4.5)	0.25(29.4)
B-2	(3.9)	0.93(15.8)	8.03(7.8)	2.38(10.6)	9.8(3.5)	(7.5)	0.15(29.4)
B-3	(0.4)	0.39(0.7)	4.39(0.4)	1.75(0.8)	3.2(0.1)	(8.6)	0.15(32.6)
Pellet	(13.9)	0.38(22.5)	3.70(12.7)	1.12(17.6)	40.9(51.3)	(18.5)	0.02(9.5)

3T3 and SV3T3 cells fractionated as described in *Materials and Methods*. B-1 was on top of the 9% Ficoll, B-2 was between 9% and 25% Ficoll, B-3 was between 25% and 35% Ficoll, and the pellet was at the bottom of the gradient tube. Because the membrane fraction was prepared in the presence of serum albumin (5 mg/ml), the relative enzyme specific activities were determined by using cells uniformly labeled with [^{3}H]leucine and are expressed as μmol/hr per 10^6 dpm [^{3}H]leucine counts for ATPase, phosphodiesterase, and acid phosphatase, μmol/min per 10^6 dpm for NADH diaphorase, and nmol/min per 10^6 dpm for cytochrome *c* oxidase. Numbers in parentheses are the percent recovery of activity relative to the homogenate taken as 100%. The specific activities (in μmol/hr per 10^6 dpm) for β-glucuronidase in Exp. 1 for 3T3 cells were 1.09 in the homogenate and 0.25 in B-1; for SV3T3 cells the specific activities were 0.33 in the homogenate and 0.13 in B-1. The specific activity of phosphodiesterase in B-1 from Exp. 2 was increased over the value in the homogenate 8-fold for 3T3 and 6-fold for SV3T3.

* 1.6×10^6 dpm for 1.3×10^7 cells.

† 5.2×10^6 dpm from 4.0×10^7 cells.

‡ No detectable activity.

§ 2.2×10^7 dpm from 11.0×10^7 cells.

¶ 10.5×10^7 dpm from 12.8×10^7 cells.

produced only slight inhibition of the [^{3}H]dThd incorporation rate of 3T3 cells. The specific activity of phosphodiesterase in B-1 from a series of experiments averaged 45 nmol/hr per μg of total phosphorus for 3T3 cells and 30 nmol/hr per μg of total phosphorus for SV3T3 cells. Because the specific activity of the phosphodiesterase was similar in 3T3 and SV3T3, the phosphodiesterase units in the *left* panel provide a measure of surface membrane mass comparable to the units in the *right* panel. The ranges of total phosphodiesterase activity in the homogenates of 3T3 and SV3T3 were consistent with the fact that 3T3 has a cell surface area two to three times that of SV3T3 (21).

The fraction of 3T3 nuclei that is autoradiographically labeled with [^{3}H]dThd decreases with increasing 3T3 surface membrane concentration (Fig. 2). 3T3 surface membranes did not decrease the fraction of SV3T3 nuclei labeled. Autoradiographic labeling of nuclei with [^{3}H]dThd gives a true indication of the fraction of cells that have initiated DNA synthesis (entered S phase) because it is independent of the specific activity of the DNA precursor pool.

Table 2 shows the results of a larger number of experiments identical in design to those in Fig. 1. [^{3}H]dThd incorporation has been normalized to both cell number and ^{14}C-labeled protein, and the results are similar, indicating that the apparent decrease in DNA synthesis is not an artifact of cell counting. The apparent rate of DNA synthesis by 3T3 cells decreased significantly in the presence of SV3T3 surface membranes. However, the effect of SV3T3 membranes always was less than that of 3T3 membranes. In some cases there was a decrease in average cell number, as a percentage of control, in the cultures to which membranes had been added that was greater than that expected from inhibition of mitosis. These decreases in cell number in the presence of a surface membrane suspension appeared to be random and not large enough to account for the decrease in incorporation rate even if one were to assume that cells in S phase are selectively lost from these cultures.

Table 3 shows that [^{3}H]dThd incorporation rate by 3T3 cells

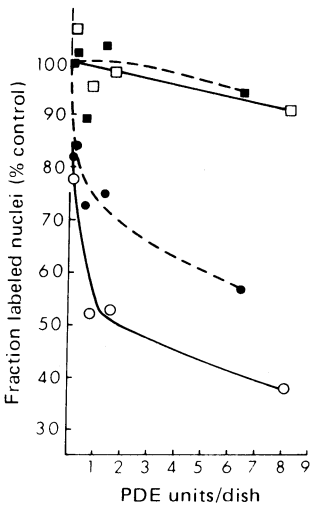

FIG. 2. Fraction of labeled nuclei in sparse 3T3 and SV3T3 cells incubated with 3T3 surface membranes. Cells plated and treated as in Fig. 1 except no ^{14}C-labeled protein hydrolysate was added. Cells were pulsed with [^{3}H]dThd 24 hr after addition of the surface membrane suspension and were prepared for autoradiography as described in *Materials and Methods*. (○, ●), 3T3; (□, ■), SV3T3; (○, □), Exp. 1; (●, ■), Exp. 2. Controls: 3T3, 52.2–55.7% labeled nuclei; SV3T3, 68.4–74.4% labeled nuclei.

Table 2. [^{3}H]Thymidine incorporation rate in sparse 3T3 and SV3T3 cells incubated with 3T3 or SV3T3 surface membranes

Phosphodiesterase activity, units/dish	Cell type	dThd [^{3}H] incorporation rate (% control) normalized to: Cell no.	dThd [^{3}H] incorporation rate (% control) normalized to: [^{14}C]Protein	Cell recovery, % of control
		3T3 Surface Membranes		
0.1–0.3	3T3	101(3)	94(2)	98
	SV3T3	109(3)	105(2)	103
1–3	3T3	69(4)	67(3)	93
	SV3T3	110(3)	106(2)	98
10–11	3T3	68(2)	64(2)	68
	SV3T3	112(1)	106(1)	86
22–28	3T3	52(2)	55(2)	86
	SV3T3	93(2)	106(1)	81
		SV3T3 Surface Membranes		
0.3–0.6	3T3	95(3)	91(2)	95
	SV3T3	110(2)	103(1)	99
3–4	3T3	76(3)	80(2)	100
	SV3T3	98(2)	101(1)	106
5–9	3T3	76(3)	81(3)	105
	SV3T3	103(3)	100(3)	102
20–30	3T3	66(2)	74(1)	96
	SV3T3	93(2)	92(1)	92

[^{3}H]dThd incorporation rate into trichloroacetic acid-insoluble counts was determined 24 hr after addition of surface membranes as described in *Materials and Methods*. Cells were plated and treated as in Fig. 1. [^{3}H]dThd incorporation rate is given for a particular phosphodiesterase activity range and has been normalized to cell number and to ^{14}C-labeled protein determined in the same experiment. The numbers in parentheses are the number of experiments in a particular range. The average initial cell concentrations were: 3T3, 1.3×10^4 per dish; SV3T3, 2.15×10^4 per dish.

decreased as a function of time after addition of 3T3 surface membranes, reaching 18.2% of control at 60 hr. The degree of inhibition of DNA synthesis by a suspension of surface membranes added to sparse 3T3 cells approaches the degree of inhibition of DNA synthesis observed in a confluent monolayer of 3T3 cells in the absence of surface membranes.

The inhibition of DNA synthesis observed with membranes was reversible, as measured by comparing the efficiency of plating of trypsinized 3T3 cells 48 hr after receiving surface membranes to that of cells which did not receive membranes (Table 4). The average [^{3}H]dThd incorporation rate by cells receiving membranes from the three experiments was 39% of control whereas the average efficiency of plating was 95.5% of control. The percent recovery of trypsinized cells exposed to membranes was the same as that of control cells.

Table 5 shows that inhibition of DNA synthesis in 3T3 cells is not the result of removal, by 3T3 surface membranes, of a soluble component from the bulk medium that is required for DNA synthesis. Incorporation rate by 3T3 cells in the presence of medium with surface membranes (medium + membranes) was decreased to 57–58.5% of control at the higher membrane concentrations. When the same medium was added to fresh cultures of 3T3 cells after removal of the surface membranes

Table 3. [^{3}H]Thymidine incorporation rate of 3T3 cells as a function of time after addition of 3T3 surface membranes

Cells plated, no./dish	Time after addition of membranes, hr	[^{3}H] dThd incorporation rate, % of control
	Exp. 1	
700	6	96.4
500	24	58.1
250	48	36.3
	Exp. 2	
500	12	56.7
380	36	32.7
250	60	18.2

Cells were treated as in Fig. 1 except that no ^{14}C-labeled protein hydrolysate was added. Exp. 1, 6.2 phosphodiesterase units per dish. Exp. 2, 12.6 phosphodiesterase units per dish. Cells were pulsed with [^{3}H]dThd at the indicated time after addition of surface membranes. Cell densities in control dishes ranged between 3900/dish and 17,600/dish in Exp. 1 and between 7400/dish and 20,100/dish in Exp. 2. Control incorporation rate from individual experiments ranged between 1.87 and 2.99 dpm/cell. [^{3}H]dThd incorporation was normalized to cell number and is expressed as a percentage of the incorporation rate at the indicated times in control dishes not receiving membranes. Values are means from duplicate dishes.

by centrifugation (preincubated medium), rates of incorporation were similar to control.

The ability of isolated 3T3 surface membranes (washed free of bovine serum albumin with CMF) to inhibit DNA synthesis was significantly decreased by treatment of the membranes with trypsin. Nearly total inactivation of inhibitory activity occurred after treatment of the membranes with 0.01 mg of trypsin per mg of membrane protein at 37° for 10 min.

DISCUSSION

In this paper we have shown that suspensions of a 3T3 cellular fraction derived mainly from surface membranes inhibit ini-

Table 4. Reversibility of inhibition by surface membranes

	PDE, units/dish	[^{3}H]dThd incorporation rate (% control) at 48 hr	EOP Fraction	EOP % of control
Exp. 1				
Control	0	100.0	24/40	100
+ Membranes	12.0	34.1	17/40	71.6
Exp. 2				
Control	0	100.0	147/400	100
+ Membranes	7.9	24.3	147/400	100
Exp. 3				
Control	0	100.0	44/108	100
+ Membranes	11.6	58.6	50/108	115

3T3 cells were plated at 200/dish and treated as in Table 3. [^{3}H]dThd incorporation rate into trichloroacetic acid-insoluble counts was determined at 48 hr after addition of 3T3 surface membranes. Duplicate dishes were trypsinized (0.02% trypsin in CMF for 5 min at 37°; eight dishes per sample) and the cells were plated at 40, 400, or 108 cells per T25 flask in each of three flasks. At 7–9 days later, cell colonies were fixed with 3.7% formaldehyde, stained with Giemsa, and counted. PDE, phosphodiesterase; EOP, efficiency of plating expressed as total colonies per flask divided by the number of cells plated.

Table 5. Effect of medium preincubated with surface membranes and cells on [^{3}H]thymidine incorporation rate

PDE, units/dish	[^{3}H]dThd incorporation rate, % of control: Medium + membranes	Preincubated medium
	Exp. 1	
1.7	60.0	99.5
18.0	58.5	94.5
	Exp. 2	
0.4	73.0	93.5
4.0	57.0	96.0

3T3 cells were plated and treated as in Fig. 1. At 24 hr after addition of surface membranes, [^{3}H]dThd incorporation rate into trichloroacetic acid-insoluble counts was determined (medium + membranes). The medium from these dishes was removed and centrifuged at 172,000 × g for 30 min. The supernatant from control medium or medium with membranes was sterilized with UV light and added to fresh cultures plated and treated as above. Then, 24 hr later [^{3}H]dThd incorporation rate was determined (preincubated medium). Values are the average of incorporation normalized to cell number and ^{14}C-labeled protein counts. PDE, phosphodiesterase.

tiation of DNA synthesis when added to sparsely growing 3T3 cells. Our experimental results are consistent with the interpretation that the surface membrane fraction inhibits initiation of DNA synthesis by the same mechanism that increased cell density exerts its effect, by increasing at or near a cell's surface the concentration of an inhibitory surface membrane component normally provided by adjacent cells at confluency.

We cannot rule out with certainty the possibility that the inhibitory activity is provided by some contaminating organelle other than the surface membrane. Inhibition of DNA synthesis by surface membranes shows a specificity that is consistent with the growth control of both the cell type from which they were isolated and the cell type to which they are added.

The results suggest that SV3T3 cells are defective in the ability to receive or integrate growth regulatory signals provided by interaction with normal cell surfaces. In addition, DNA synthesis of 3T3 cells is inhibited more strongly by 3T3 surface membranes than by SV3T3 surface membranes. This suggests that the cell surface of SV3T3 is relatively deficient in the ability to send a growth regulatory signal compared to the cell surface of 3T3 cells. Inhibition of DNA synthesis by surface membranes is reversible and therefore is not the result of a general toxic effect on the cells. Inhibition by surface membranes does not appear to be the result of removal of soluble factors from the bulk medium. This result suggests that a direct interaction between a cell's surface or its immediate environment and surface membranes is required.

Inhibition of DNA synthesis increases with time after addition of surface membranes. Cell density appears to regulate progression of cells through G1 into S phase (22). We assume, but have not shown, that surface membranes act in a similar manner. It is possible that, during each cell cycle, a given fraction of the growing cells escape inhibition by surface membranes so that in subsequent cycles the percentage of total cells arrested in G1 increases. 3T3 cells, but not SV3T3 cells, have an anchorage requirement for growth (23). It remains to be shown that surface membranes do not alter anchorage to the substratum in some subtle manner, thereby inhibiting growth.

Isolated surface membranes could exert their inhibitory effect on growth by producing a lateral diffusion barrier if they locally inactivated or removed soluble components required for DNA synthesis. Surface membranes could also exert their inhibitory effect by interacting with a complementary component on a cell's surface. Although we favor the latter possibility, our data cannot be used to exclude the possibility that the membranes locally reduce the concentration of a growth factor. Our assay system potentially provides a means of isolating the active components present in surface membranes.

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