15. S. J. Singer and G. L. Nicholson, *Science* **175**, 720 (1972); M. S. Bretscher, *ibid.* **181**, 622 (1973); A. R. Oseroff, P. W. Robbins, M. M. Burger, *Annu. Rev. Biochem.* **42**, 835 (1973).
16. T. Borsos, R. R. Dourmashkin, J. H. Humphrey, *Nature (London)* **202**, 251 (1964); J. H. Humphrey and R. R. Dourmashkin, *Adv. Immunol.* **11**, 75 (1969); C. H. Packman, S. I. Rosenfeld, R. I. Weed, J. P. Leddy, *J. Immunol.* **117**, 1883 (1976).
17. J. K. Smith and E. L. Becker, *J. Immunol.* **100**, 459 (1968); P. J. Lachmann, E. A. Munn, G. Weissmann, *Immunology* **19**, 983 (1970); T. R. Hesketh, R. R. Dourmashkin, S. N. Payne, J. H. Humphrey, P. J. Lachmann, *Nature (London)* **233**, 620 (1971); E. B. Giavedoni and A. P. Dalmasso, *J. Immunol.* **116**, 1163 (1976); C. R. Alving *et al.*, *ibid.* **118**, 342 (1977).
18. VBS-gel is an isotonic vernal-buffered saline containing 0.1 percent gelatin, 0.001*M* Mg^{2+}, and 0.00015*M* Ca^{2+}, as described by H. J. Rapp and T. Borsos, *Molecular Basis of Complement Action* (Appleton-Century-Crofts, New York, 1970).
19. We thank T. Borsos for his invaluable support and advice.

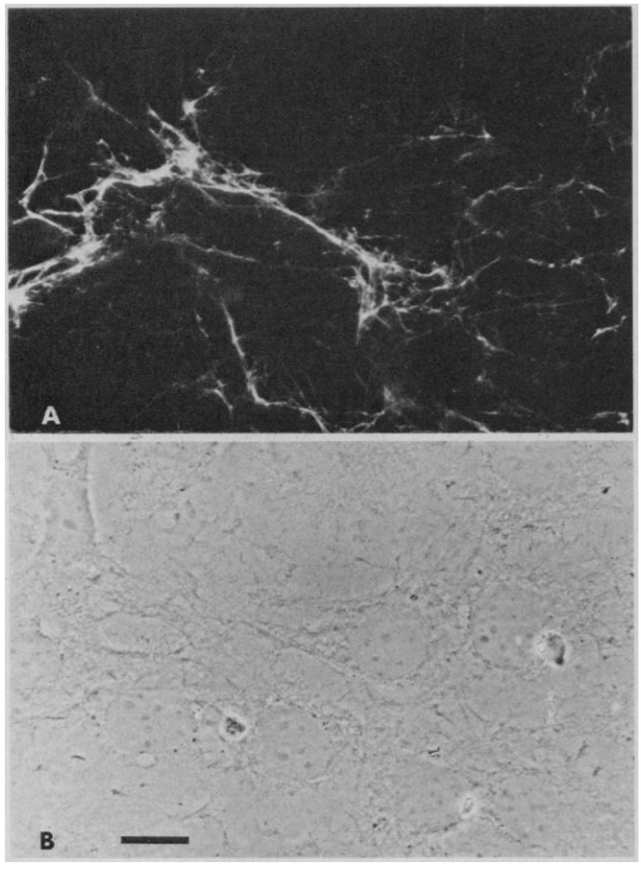
22 February 1977; revised 5 April 1977

Control of a Cell Surface Major Glycoprotein by Epidermal Growth Factor

Abstract. *When the serum concentration of the culture medium is below 0.7 percent, 3T3 mouse cells lose most of their large external transformation sensitive (LETS) protein at the cell surface. Subsequent addition of epidermal growth factor results in the reappearance of massive fibrillar LETS protein networks on the surface of confluent 3T3 cells. Thirteen other hormones tested do not have this effect. It appears that epidermal growth factor can control the expression of LETS protein.*

There is considerable interest in the large external transformation sensitive (LETS) protein, which is also known as cell surface protein (CSP), fibronectin, or galactoprotein a (*1*). These proteins are the only class of cell surface glycoprotein consistently reduced or absent in numerous transformed cell lines (*2*). There seems to be an inverse correlation between increase in tumorigenicity and decrease in the percentage of cells expressing LETS protein (*3*), and among 50 tumorigenic lines tested, 45 show significant reductions in LETS protein as detected by indirect immunofluorescence (*4*). Furthermore, the addition of LETS protein can partially restore to transformed cells normal morphology, adhesion, and contact inhibition of movement (*5*). Finally, the observation that LETS protein on the cell surface is altered during myogenesis led to the recognition of the involvement of LETS protein in myoblast fusion (*6*).

In attempts to understand the control of the expression of LETS protein at the cell surface, previous investigators showed that cell density (*7*) and cell-cell contact (*3*) are the prime events mediating its expression. It is only at the stage of extensive cell contact that massive fibrillar networks of LETS protein are formed (*3*, *8*). In most of these experiments, 5 to 10 percent serum was present in the culture medium, and it was not generally realized that the serum might have affected the expression of LETS protein. While studying the effect of



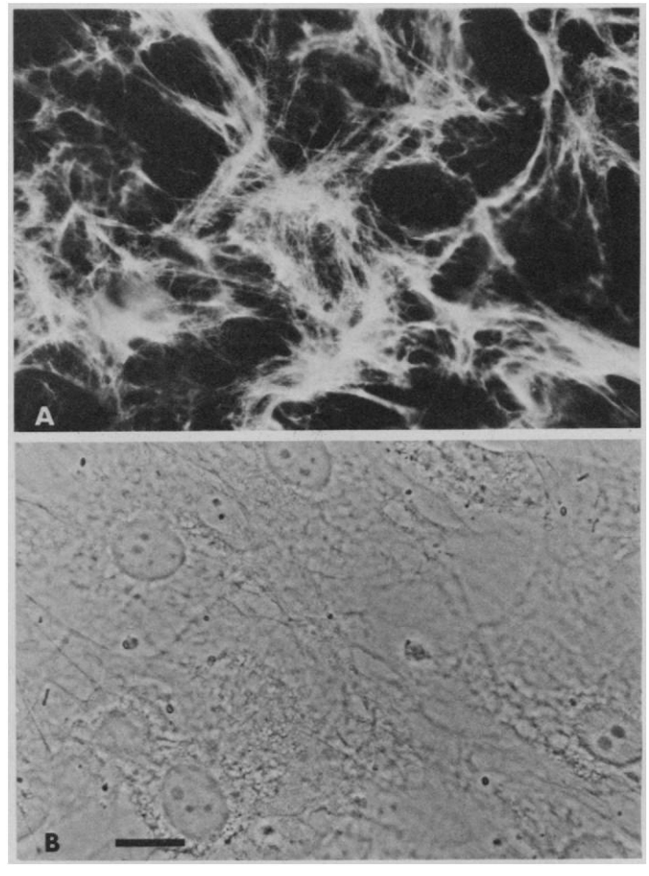


Fig. 1 (left). (A) Indirect immunofluorescence of 3T3 cells with LETS protein antiserum. The procedures for immunofluorescence staining and the specificity of the antiserum were described previously (*3*, *6*). 3T3 cells at confluency were incubated in Dulbecco's modified Eagle's medium containing 0.7 percent calf serum for 2 days. Most of the cells in culture lost LETS protein on the cell surface under these conditions. The photograph is of a field where LETS protein is most prominent. (B) Phase contrast micrograph of the same field. (Scale bar, 15 μm) Fig. 2 (right). (A) A culture similar to that in Fig. 1, but photographed 30 hours after the addition of EGF (1 ng/ml). (B) Phase contrast micrograph of the same field. (Scale bar, 15 μm)

thrombin on LETS protein of mammalian cells, we noticed that the serum-free control culture frequently lost the fibrillar network compared with cultures in 10 percent serum. This prompted us to investigate the relation between hormones and the expression of surface LETS protein. Here we report that among 13 hormones and growth factors tested, expression of LETS protein in 3T3 mouse cells is specifically influenced by Cohen's epidermal growth factor (EGF) (*9*).

Confluent 3T3 cells in Dulbecco's modified Eagle's medium supplemented with 10 percent calf serum had massive fibrillar networks of LETS protein as detected by indirect immunofluorescence. The pattern was similar to the patterns previously reported (*8*). When the medium containing 10 percent serum was changed to 0.7 percent serum for 2 days, most of the LETS protein disappeared. In certain areas of the culture LETS protein could be detected, but in a seemingly deteriorated form as shown in Fig. 1. When such cultures were supplemented with epidermal growth factor (1 ng/ml for 30 hours) the distribution of LETS protein was as shown in Fig. 2 (*10*). Thus, it appears that the massive network of fine fibers of LETS protein can be induced by the addition of EGF. This effect is completely blocked by the presence of a 1:200 dilution of antiserum to EGF (*10*).

Epidermal growth factor can stimulate the growth of 3T3 cells (*11*). We therefore investigated whether the EGF-mediated induction of cell growth and expression of LETS protein are associated. Table 1 shows that the induction of LETS protein by EGF occurred even at high doses of the hormone, when no significant cell growth had been stimulated. Thus, our evidence suggests that the stimulation of cell growth by EGF is dissociable from the enhancement of LETS protein expression by the same hormone. At the highest dose of EGF (50 ng/ml) the morphology of 3T3 cells was altered to a spindle shape, although the organization of fibrillar LETS protein remained similar.

To determine whether the induction of LETS protein is specifically stimulated by EGF, we tested a series of other hormones and serum components. Table 2 shows that the following hormones are unable to induce the expression of LETS protein: insulin, hydrocortisone, growth hormone, gonadotropin, thrombin, calcitonin, somatomedin B, somatomedin C, triiodothyronine, adrenocorticotropic hormone (ACTH), α-melanocyte–stimulating hormone, thyroid-stimulating hormone, and parathyroid hormone (*12*). Thus, among the hormones we tested, only EGF appeared to be specific for the induction of LETS protein in 3T3 cells.

For EGF to have an effect on LETS protein expression in 3T3 cells, the presence of 0.7 percent serum was essential. Further reduction in the serum concentration invariably induced a high rate of cell death and morphological abnormality even when EGF was present. Attempts to substitute 0.7 percent serum with the following hormones alone or in combination were unsuccessful: insulin (50 ng/ml to 1 μg/ml), hydrocortisone (10 ng/ml to 100 ng/ml), somatomedin B (10 ng/ml to 1 μg/ml), somatomedin C (10 ng/ml to 1 μg/ml), thrombin (50 ng/ml to 2 μg/ml), gonadotropin (1 μg/ml). Possibly, there is involvement of the so-called survival factor (*13*) in this phenomenon. The nature of such a factor, however, is unknown.

Table 1. Relation between LETS protein expression and cell growth. Confluent 3T3 cells grown on 12-mm coverslips in 60-mm dishes were incubated in medium containing 0.7 percent calf serum for 2 days. After the addition of EGF, 40 μl of [^{3}H]thymidine (0.2 mc/ml in 50 percent ethanol, 6.7 c/mmol, New England Nuclear) was added to the 60-mm dishes and labeling was allowed to occur between 16 and 18 hours; the radioactivity of trichloroacetic acid insoluble materials was then counted (*16*). Coverslips were fixed 24 hours after the addition of EGF and processed for indirect immunofluorescence with LETS protein antiserum.

EGF (ng/ml)	[^{3}H]thymidine incorporation into DNA (count/min)	Massive LETS protein networks
0	600	−
0.1	1,800	−
0.5	9,400	−
1	12,600	+
5	10,500	+
20	9,800	+
50	2,200	+

Table 2. Effect of hormones on the induction of massive LETS protein networks. Serum-starved, LETS protein-deficient 3T3 cells were treated with 0.7 percent calf serum and various hormones (*12*). Twenty-four hours later, cells on coverslips were processed for indirect immunofluorescence with LETS protein antiserum.

Hormones	LETS protein network
Epidermal growth factor (1 ng/ml)	+
Insulin, bovine (50 ng/ml)	−
Insulin, bovine (1 μg/ml)	−
Hydrocortisone, 50 ng	−
Growth hormone, human (1 ng/ml)	−
Growth hormone, human (1 μg/ml)	−
Gonadotropin, horse (2 units)	−
Gonadotropin, horse (10 units)	−
Calcitonin, porcine (0.1 ng/ml)	−
Calcitonin, porcine (1 ng/ml)	−
Somatomedin B, human (5 ng/ml)	−
Somatomedin B, human (1 μg/ml)	−
Somatomedin C, bovine (5 ng/ml)	−
Somatomedin C, bovine (1 μg/ml)	−
Triiodothyronine (5 μg/ml)	−
ACTH (5 μunit/ml)	−
α-Melanocyte–stimulating hormone (1 μg/ml)	−
Thyroid-stimulating hormone, ovine (1 μg/ml)	−
Parathyroid hormone, bovine (0.5 ng/ml)	−
Thrombin, human (1 μg/ml)	−

To investigate whether serum dependence of the expression of surface LETS protein is a general phenomenon of all cultured cells, we tested the following cell types for their response to serum. Initially, the derivative lines of 3T3 mouse cells, SV3T3, Py3T3, and SV-Py-3T3 were examined. Although these lines expressed little LETS protein in sparse culture, confluent 1-week-old cultures of SV3T3, Py3T3, and SV-Py-3T3 expressed a significant amount of LETS protein networks in medium containing 5 percent calf serum. When the serum concentration was reduced to 0.03 percent for 2 days, the fibrillar structures of LETS protein were less altered than in the case of the parental 3T3 cells. Thus the serum dependency of LETS protein expression is almost 25-fold less in transformed 3T3 cells than in untransformed 3T3 cells. Attempts to reduce the serum concentration further to minimize LETS protein expression in these transformed cells, while maintaining a high survival rate, were not successful. Therefore, we could not determine whether EGF also stimulates LETS protein expression in SV-3T3, Py-3T3, and SV-Py-3T3 cells. For example, we were unable to use 0.01 percent serum as the basal level supplemented with EGF to maintain LETS protein networks in these cells. However, for other types of normal cells, hormone-dependent expression of LETS protein could readily be demonstrated. Secondary fibroblasts of the mouse, rat, and hamster, and cells of the established lines NiL-8, and BHK had almost undetectable amounts of LETS protein when cultured with 0.02 percent serum for 3 days, but after the addition of EGF (2 ng/ml) for 30 hours the expression of LETS protein was restored to the level found in cells growing in 10 percent serum. However, an important exception was noted, in that the fibrillar networks of LETS protein of chick embryo fibroblasts remained unaffected even when they were cultured in serum-free medium for 2 days.

Cell culture systems seem to be most promising for the development of a total

understanding of cell-hormone interaction (*14*). Without cells in culture it would have been difficult to establish that the expression of a cell surface protein can be controlled by EGF, and we can now investigate the number of events that precede the appearance of LETS protein networks when EGF has bound to the cell surface. Since LETS protein is a differentiated product of fibroblasts, myoblasts, and some epithelial cells (*15*), the present findings support the notion that probably some of the differentiated functions of a given cell type are controlled by hormones. It will be of interest to investigate whether the production of collagen, glycosaminoglycan, and other differentiated products of fibroblasts are also controlled by EGF.

LAN BO CHEN
ROBERT C. GUDOR
Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724
TUNG-TIEN SUN
Department of Biology, Massachusetts Institute of Technology, Cambridge 02139
ANTHONY B. CHEN
MICHAEL W. MOSESSON
Department of Medicine, State University of New York, Down State Medical Center, Brooklyn 11203

References and Notes

1. R. O. Hynes, *Biochim. Biophys. Acta.* **458**, 73 (1976); K. M. Yamada, S. S. Yamada, I. Pastan, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3158 (1975); A. Vaheri, in *Virus-Transformed Cell Membranes*, C. Nicolan, Ed. (Academic Press, New York, in press); C. G. Gahmberg, S. Hakomori, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3329 (1973).
2. R. O. Hynes, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2208 (19 → N. M. Hogg, *ibid.* **71**, 489 (1974); G. G. Wickus, P. E. Branton, P. W. Robbins, in *Control of Proliferation in Animal Cells*, B. Clarkson and R. Baserga, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1974), p. 541.
3. M. W. Mosesson and R. A. Umfleet, *J. Biol. Chem.* **245**, 5728 (1970); L. B. Chen, P. H. Gallimore, J. K. McDougall, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3570 (1976).
4. L. B. Chen, J. K. McDougall, P. H. Gallimore, unpublished results.
5. K. M. Yamada, S. S. Yamada, I. Pastan, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1217 (1976).
→ L. B. Chen, *Cell* **10**, 393 (1977).
7. R. O. Hynes and J. M. Bye, *ibid.* **3**, 113 (1974); C. G. Gahmberg, D. Kiehn, S. Hakomori, *Nature (London)* **248**, 413 (1974); E. S. Pearlstein and M. D. Waterfield, *Biochim. Biophys. Acta* **362**, 1 (1974).
8. J. Wartiovaara, E. Linder, E. Ruoslahti, A. Vaheri, *J. Exp. Med.* **140**, 1522 (1974).
9. S. Cohen, *J. Biol. Chem.* **237**, 1555 (1962).
10. The epidermal growth factor (EGF) used in the preliminary experiment was kindly provided by S. Cohen. For the subsequent experiments we used EGF from mouse submaxillary glands purified according to Taylor *et al.* [J. M. Taylor, W. M. Mitchell, S. Cohen, *J. Biol. Chem.* **247**, 5928 (1972)]. Antiserum against EGF was raised in rabbits. A 1:1000 dilution of our antiserum preparation completely blocked the stimulation of DNA synthesis in confluent 3T3 cells by 1 ng of EGF per milliliter.
11. H. A. Armelin, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2702 (1973).
12. The sources of the hormones were as follows: insulin, hydrocortisone, growth hormone, and gonadotropin were from Sigma; calcitonin, triiodothyronine, ACTH, α-melanocyte-stimulating hormone, thyroid-stimulating hormone, and parathyroid hormone were from Calbiochem; somatomedin B and somatomedin C were from Kabi; and highly purified human thrombin was generously provided by J. W. Fenton. The 3T3 cells were a gift of H. Green.
13. D. Paul, A. Lipton, I. Klinger, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 645 (1971).
14. I. Hayashi and G. H. Sato, *Nature (London)* **259**, 132 (1976); J. Pindyck, M. W. Mosesson, M. W. Roomi, R. D. Levere, *Biochem. Med.* **12**, 22 (1975).
15. L. B. Chen, N. Maitland, P. H. Gallimore, J. K. McDougall, *Exp. Cell Res.*, **106**, 39 (1977).
16. L. B. Chen and J. M. Buchanan, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 131 (1975).
17. We thank Dr. J. D. Watson and H. Green for support and advice. We also thank G. Albrecht-Buehler and J. K. McDougall for critical comments, S. Chait for photographic work, and M. Szadkowski for manuscript preparation. L.B.C. is supported by a fellowship from the Helen Hay Whitney Foundation. This work is supported by the American Heart Association (Nassau Chapter, New York) to L.B.C. and a grant from NIH to M.W.M.

10 January 1977; revised 4 March 1977

Dopamine and Adenosine 3′,5′-Monophosphate Responses of Single Mammalian Sympathetic Neurons

Abstract. *Acetylcholine (ACh), dopamine, and dibutyryl-adenosine 3′,5′-monophosphate (dbcAMP) were applied iontophoretically to the rabbit superior cervical ganglion cells from triple-barreled micropipettes, and the response was recorded by intracellular techniques. All ganglion cells tested responded to the depolarizing action of ACh, whereas less than half of the cells that responded to ACh were hyperpolarized by dopamine. This effect was blocked by low concentrations of haloperidol. None of the cells examined responded to dbcAMP applied by iontophoresis. Hence, the present result is not consistent with the concept that a cyclic AMP mechanism underlies the hyperpolarizing effect of dopamine.*

Dopamine (DA) has been identified as the neurotransmitter, released on preganglionic stimulation from the small intensely fluorescent (SIF) cells, and responsible for the generation of the slow inhibitory postsynaptic potential (slow IPSP) of the rabbit superior cervical ganglion cell (*1*). It has been suggested that the action of DA depends on the intracellular cyclic adenosine 3′,5′-monophosphate (AMP) system that alters the membrane permeability by protein kinase–mediated phosphorylation; indeed, it was demonstrated that cyclic AMP is generated by presynaptic stimulation of, or by the application of DA to, the ganglion (*2, 3*). In support of this concept, it was reported that, as evaluated by the sucrose-gap method, monobutyryl AMP as well as the dibutyryl derivative (dbcAMP) caused a membrane hyperpolarization similar to that elicited by DA (*3*). However, results obtained in our laboratory with similar techniques failed to confirm these findings (*4*). Accordingly, our investigation was undertaken to evaluate, by microelectrode techniques, the effects of DA and dbcAMP on single sympathetic neurons. The intracellular recording techniques alleviate some of the shortcomings and ambiguity often associated with the sucrose-gap method, which records global activities of cell populations.

Acetylcholine (ACh), DA, and dbcAMP were applied by iontophoresis from triple-barreled micropipettes positioned close to the ganglion cells, and the response was recorded by intracellular techniques; a simplified experimental arrangement is illustrated in the inset of Fig. 1 (*5*). To exclude the possibility that the absence of responses of dbcAMP or DA (or both) could be due to excessive distance between the micropipette and the ganglion cell, the proximity of the micropipette was estimated in terms of the rise time of the ACh potential. In our study, only those cells that exhibited a rise time of ACh potentials of less than 30 msec were included; in many instances, neuronal spikes could be elicited by a strong current pulse delivered through the ACh micropipette.

The ACh potential was elicited by single current pulses of brief duration; the duration of current pulses varied from 5 to 10 msec in different experiments. Altogether, 31 cells (18 ganglia) showed a satisfactory ACh potential. The mean amplitude of ACh potentials was 8.7 ± 3.1 mv (mean ± S.D.), with a mean rise time of 27.9 ± 5.3 msec and a mean half-decay time of 46.0 ± 14.7 msec. The amplitude of ACh potentials remained relatively constant provided that the ejection current was unchanged. Of the 31 cells that exhibited a satisfactory ACh potential, 11 cells responded to iontophoresis of DA by a definite membrane hyperpolarization (Fig. 1A). In order to obtain DA responses, tetanic current pulses had to be used (30 hertz, 30 msec/pulse, for 4 to 5 seconds). The amplitude of DA hyperpolarization ranged from 0.5 to 4 mv, with a mean of 1.8 mv, and its duration varied from 4 to 12 seconds. Haloperidol (0.1 to 1 μM), a DA