Proc. Natl. Acad. Sci. USA
Vol. 76, No. 7, pp. 3377–3381, July 1979
Cell Biology

Decrease in epidermal growth factor receptor levels and production of material enhancing epidermal growth factor binding accompany the temperature-dependent changes from normal to transformed phenotype

(cell culture/temperature-sensitive mutant)

PHYLLIS GUINIVAN AND ROGER L. LADDA*

Division of Genetics, Department of Pediatrics, College of Medicine, The Pennsylvania State University, Hershey, Pennsylvania 17033

Communicated by W. M. Krogman, April 12, 1979

ABSTRACT **Normal rat kidney (NRK) cells infected with a temperature-sensitive mutant of Kirsten sarcoma virus (Ts cells) exhibited normal monolayer morphology identical to that observed for uninfected cells (NRK cells) at the nonpermissive temperature, 39°C, but grew as multilayered foci resembling NRK cells transformed by the wild-type virus (KNRK cells) at 32°C, the permissive temperature. NRK cell division was stimulated by epidermal growth factor (EGF), and these cells showed high levels of EGF receptors, as determined by ^{125}I-labeled EGF binding. KNRK cells were unresponsive to EGF and no EGF receptors were detectable. Ts cells also were unresponsive to EGF at both temperatures, but exhibited just detectable EGF binding at 32°C and 10–15% of NRK cell binding at 39°C. Use of EGF added to the culture medium by these cells paralleled the receptor levels. Crossfeeding experiments among NRK, KNRK, and Ts cultures indicated that Ts cells at the permissive temperature and KNRK cells at both temperatures produced a heat-stable substance(s) which stimulated DNA synthesis in NRK cells independent of the presence of serum or of EGF. Conditioned medium from the transformed cultures also significantly enhanced EGF binding to NRK cells. These studies demonstrated a correlation between the transformed phenotype and the receptor levels of a potent cell mitogen, EGF, which was readily reversible in the Ts cultures. In addition, cultures expressing the transformed phenotype produced material that did not compete for the EGF receptor but did enhance EGF binding, in contrast to other reports involving sarcoma virus-transformed cells.**

Normal cells in culture require serum for the initiation of cell division and for the maintenance of viability during the stationary phase of the cell cycle (1–3). Viral or chemical transformation produces a permanent cell line often with a significantly reduced serum requirement and enhanced proliferative activity (4). These changes suggest that serum normally exerts some regulatory function on cells that is lost upon transformation. The growth-regulating components of serum remain poorly characterized, but with the isolation of small polypeptides with growth-promoting activity, experiments may now be designed to study the cellular events associated with the initiation of cell division in normal and transformed cells (5–9). Polypeptide growth factors apparently bind to specific receptors on the cell membrane and thereby initiate the events leading to the regulation of cell division (10, 11). The differing serum requirements of normal and transformed cells may be related to quantitative or qualitative alterations (or both) of their specific membrane receptors for growth factors (12), thus altering the response of the cells to serum factors.

In the following experiments, we investigated the growth-promoting activity and binding characteristics of epidermal growth factor (EGF) on normal rat kidney (NRK) cells and derivatives of these cells transformed by Kirsten murine sarcoma virus (KNRK cells) and a temperature-sensitive mutant of the virus (Ts cells). Whereas NRK cells readily bound EGF, we found that KNRK cells did not bind EGF and that EGF binding to the Ts cells was dependent on the normal phenotype at the nonpermissive temperature. Absent or lowered EGF binding by KNRK and Ts cells was accompanied by a lack of cellular responsiveness to the mitogenic effect of EGF. Furthermore, Ts cells at the permissive temperature and KNRK cells at both temperatures produced a heat-stable substance(s) which stimulated the initiation of DNA synthesis and enhanced EGF binding to normal cells. Thus, in this Ts cell system the specific modulation of EGF receptors reflects the changes accompanying transformation.

MATERIALS AND METHODS

Cells. NRK, KNRK, and Ts mutant cultures were developed by Scolnick and coworkers (13, 14); the Ts mutant originally was designated Ts 371-clone 5. Each cell type was routinely cultured in plastic culture dishes or flasks in Dulbecco's modified Eagle's enriched medium (GIBCO) with 10% fetal calf serum at 32°C and 39°C, the permissive and nonpermissive temperatures, respectively, for Ts cells transformed by the temperature-sensitive mutant. Initial Ts cultures showed heterogeneous morphology and growth characteristics at the nonpermissive temperature. Ts cells were repeatedly cloned at both temperatures to select for specific characteristics: multilayered focal growth pattern at 32°C and "normal" monolayer growth pattern at 39°C. Five Ts clones with stable characteristics were selected from over 100 surveyed; two clones were studied in detail and have demonstrated stable features on transfer between 32°C ⇌ 39°C over the past 4 years. Cell numbers and cell size were determined with a Coulter Counter equipped with a Coulter Channelizer.

Epidermal Growth Factor. EGF was isolated from the submaxillary glands of 25- to 40-g male Swiss mice (Charles River Breeding Laboratories) by the method of Savage and Cohen (15). The purity and identity of the isolated EGF was characterized by biological assays (16), amino acid analysis, and the presence of a single band after electrophoresis on sodium dodecyl sulfate/polyacrylamide gels. ^{125}I-Labeled EGF was prepared by a modification of the chloramine-T method of Greenwood *et al.* (17). Specific activities ranged from 28,000

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: EGF, epidermal growth factor; NRK cells, normal rat kidney cells; KNRK, NRK cells transformed by Kirsten sarcoma virus; Ts, temperature sensitive.

*To whom correspondence should be addressed.

to 36,000 cpm per ng of EGF. ^{125}I-labeled EGF was diluted in 50 mM phosphate buffer (pH 7.5), containing 0.5 mg of bovine serum albumin (Miles) per ml and stored in 1-ml aliquots at −20°C. In these experiments, ^{125}I-labeled EGF was used within 7–14 days after preparation.

DNA Synthesis. Stimulation of DNA synthesis was evaluated by growing cells in serum-free medium or in low serum medium (0.25%) for 4 days and then changing to test medium containing EGF followed by 22 hr of further culture; [^{3}H]-thymidine (1 μCi/ml) was added for 3 hr. Culture dishes were rinsed three times with Hanks' balanced salt solution and trypsinized to remove cells. Cells were transferred to 0.45-μm Gelman filters on a suction apparatus and rinsed twice with cold 5% trichloroacetic acid. Filters were dried overnight and radioactivity was measured in a Beckman scintillation counter by placing filters in 10 ml of nonaqueous scintillation cocktail (Amersham).

Binding Experiments. All bindings were performed in 60-mm plastic petri dishes. Initially cells were plated at 5×10^5 cells per dish and assayed at confluence. For time course studies, a constant low dosage of ^{125}I-labeled EGF (4 ng; 150,000 cpm/dish) was added directly to the cells and incubated at 32°C or 39°C for 1, 3, 5, 15, 30, 90, and 180 min; the final volume of reaction mixture was 1.5 ml. Cells were rinsed four times with cold Hanks' solution (pH 6.8), containing 1 mg of bovine serum albumin per ml; 0.5 ml of 0.25% trypsin (GIBCO) was added to remove cells and plates were rinsed twice with 0.5 ml of lysing buffer containing 50 mM Tris·HCl (pH 7.2), 0.5% sodium dodecyl sulfate, and 1 mM EDTA. The cell lysate was transferred to plastic vials and radioactivity was measured in a Beckman Biogamma II counter.

Saturation binding was performed as above except that increasing amounts of ^{125}I-labeled EGF were added to culture dishes and incubation was carried out for 55 min. Nonspecific binding was determined in the presence of 7.5 μg of unlabeled EGF and was consistently less than 2% of total binding. Scatchard plots (18) were used to estimate the number of EGF receptors per cell and per μm^2 of cell surface area. A radioreceptor assay for EGF was used to determine the concentration of EGF in culture medium derived from NRK, KNRK, and Ts cells at various times during a single passage. Details of the assay are described elsewhere (19). Fibroblastic growth factor, multiplication stimulating activity, dexamethasone, and KNRK and Ts cell conditioned media were evaluated for possible competition for EGF receptors (see Fig. 4).

RESULTS

Cell Growth and Morphology. NRK cultures grew as a monolayer, and cells were flattened and polygonal at both 32°C and 39°C. In the transformed wild-type derivative KNRK, cells were rounded and spindle-shaped, with apparently much less surface adherent to the culture dish. These cells no longer formed a monolayer, but rather exhibited a focal multilayered morphology at both temperatures. The Ts cells developed the multilayered focal morphology at 32°C, the permissive temperature, but resembled NRK cells at 39°C, the nonpermissive temperature. Morphological transition of Ts cells occurred when cultures were cycled between 32°C and 39°C. For cells adapted to 32°C and transferred to 39°C, the transformed growth pattern started to revert to the monolayer form as early as 4 hr and was completed by 24 hr. Morphological transition from 39°C to 32°C required 3 days. The morphology of NRK and KNRK cells was not affected by temperature.

In 10% fetal calf serum growth medium, NRK, KNRK, and Ts cells had similar rapid growth rates and achieved higher cell densities at 39°C than at 32°C (data not shown). Fig. 1 shows

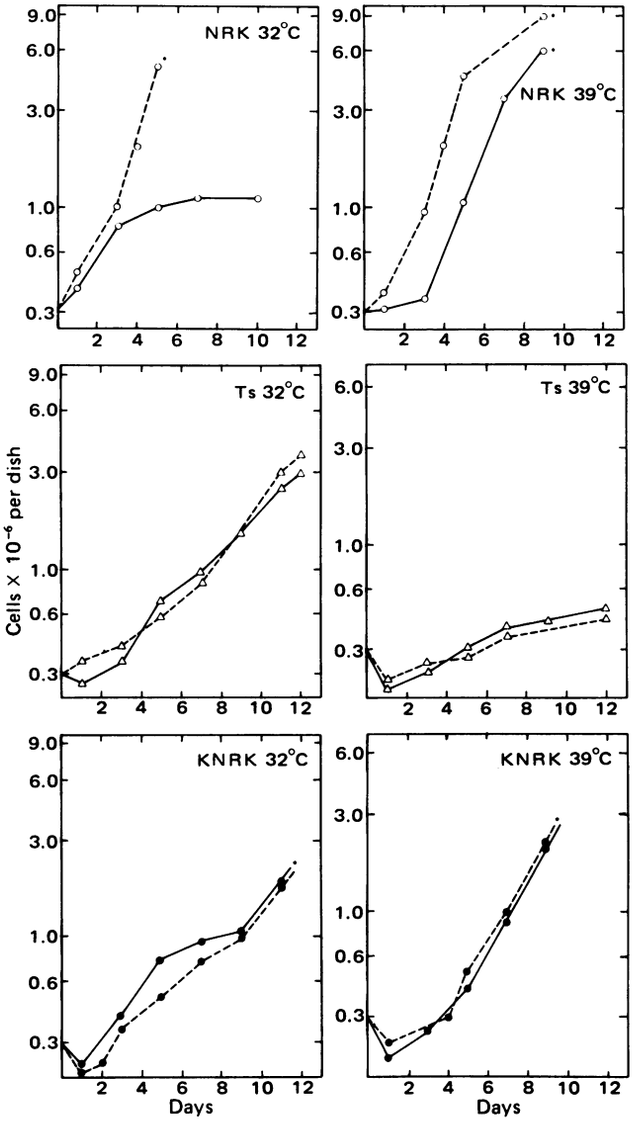

FIG. 1. Effect of EGF on growth rate of NRK, KNRK, and Ts cultures in low serum medium. Cells were plated in medium containing 0.25% fetal calf serum, which sustained viability but limited the growth rate of the cultures as compared to 10% fetal calf serum. After 12 hr, culture medium was replaced with medium containing 0.25% fetal calf serum with (- - -) or without (—) 25 ng of EGF per ml. Cell counts were obtained every other day and the medium was changed every 3 days. Similar growth patterns were observed in four separate experiments.

the growth patterns of NRK, KNRK, and Ts cells at 32°C and 39°C grown in medium with 0.25% fetal calf serum, with or without 25 ng of EGF per ml. NRK cells had a more rapid rate of growth at 39°C and achieved maximum cell density.

In the presence of EGF and 0.25% fetal calf serum, NRK cells showed an enhanced growth rate, particularly at 32°C, and the final cell density was comparable to NRK cells grown in medium containing 10% fetal calf serum at 32°C and 39°C. NRK cells exposed to EGF reached saturation densities of 3×10^6 cells per 60-mm culture dish within 5 days of plating, and the cell monolayer would then slough off of the dish. In the absence of EGF, cell proliferation reached a plateau at 4.5 days at a much lower cell density, and the monolayer remained attached to the culture dish for up to 14 days at 32°C.

Ts cells grew more slowly at 39°C compared to NRK and KNRK cells, but at 32°C, Ts cells showed growth comparable

Table 1. EGF concentration in conditioned medium during a single passage at 39°C

Day	NRK	KNRK	Ts
1	25.4	26.1	25.1
2	—	—	—
3	21.0	—	—
4	—	24.7	23.4
6	10.5	23.1	18.0
8	8.1	—	—

An EGF radioreceptor assay (19) was used to determine the concentration of EGF in culture medium at various times during a single passage. The medium was originally prepared with 25 ng of EGF per ml. Values are ng of EGF per ml of medium and are the average of determinations on three separate dishes.

to KNRK cells. KNRK and Ts cells exhibited no apparent response to EGF at either temperature.

EGF Use by Cells in Culture. The disappearance of EGF added to the culture medium by the three cultures was measured over an 8-day period (Table 1). NRK cell medium showed a striking reduction in EGF. In contrast, KNRK cell medium showed little evidence of EGF degradation even at high cell densities, but Ts cell medium showed about a 28% depletion of EGF at 39°C and essentially none at 32°C.

EGF Binding. Time course studies showed that maximum EGF binding to NRK cells was reached between 50 and 60 min and indicated the remarkable differences between NRK, Ts, and KNRK cells at both 32°C and 39°C (Fig. 2). KNRK cells showed no evidence of EGF binding. Ts cells bound little EGF at 32°C, but at 39°C, EGF binding was considerably increased.

The effect of EGF concentration on EGF binding is shown in Fig. 3 and Table 2. NRK cells exhibited a large EGF binding capacity at both temperatures, but consistently bound a greater amount of EGF at 32°C than at 39°C. KNRK cells showed no detectable binding at 32°C or 39°C. Ts cells showed some EGF binding at 32°C, which was estimated to be less than 1% of NRK cells at 32°C. At 39°C, Ts cells exhibited a greater EGF binding capacity and was estimated to be about 10–15% of NRK cell binding at 39°C.

Scatchard plots of the binding data were used to estimate the number of EGF receptors per cell (Fig. 3, *insets*). NRK cells had about 3.5×10^5 and 1.5×10^5 receptors per cell at 32°C and 39°C, respectively. The dissociation constant was about 3–5 × 10^{-9} M for NRK cells with essentially no difference between 32°C and 39°C. Ts cells at 39°C were estimated to have about 20,000 EGF receptors per cell, with a dissociation constant essentially the same as NRK. In contrast, Ts cells at 32°C showed little binding and a significant decrease in the affinity of the receptors for EGF.

Switching NRK cells from 32°C to 39°C produced a detectable change in EGF binding within 36 hr. With Ts cells, the transition from 32°C to 39°C was accompanied by enhanced EGF binding within 24 hr; when Ts cells were switched from 39°C to 32°C, EGF binding decreased gradually over 72 hr.

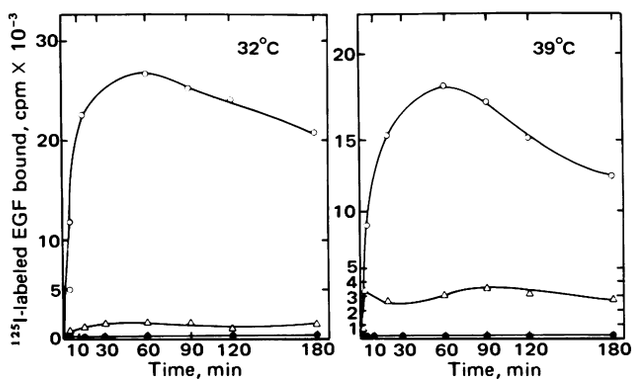

FIG. 2. Time course of ^{125}I-labeled EGF binding to NRK, Ts, and KNRK cells at permissive (32°C) and nonpermissive (39°C) temperatures. Medium (1.5 ml) containing 4 ng of ^{125}I-labeled EGF was added to each confluent 60-mm culture dish. At the end of each time interval, cells were harvested and specific binding was determined. ○, NRK cells; △, Ts cells; ●, KNRK cells.

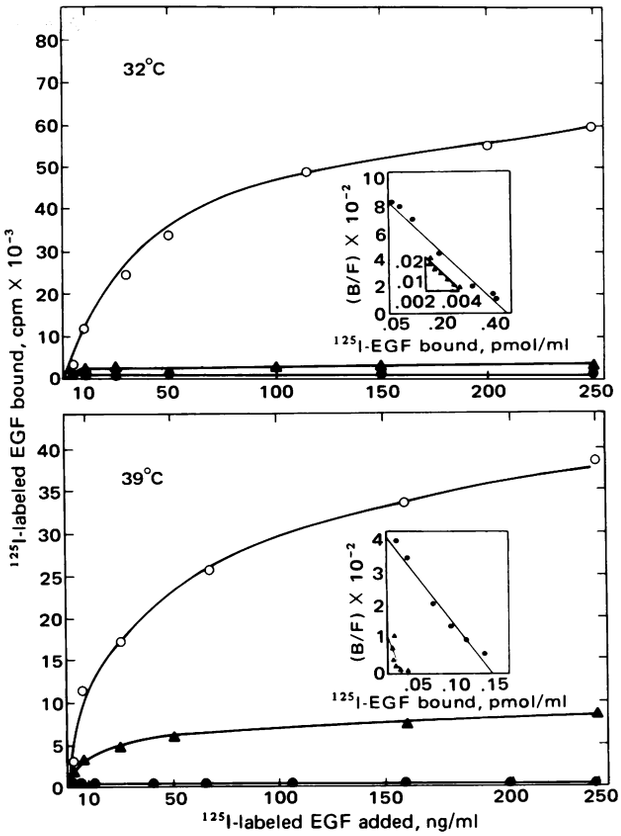

FIG. 3. Effect of EGF concentration on the binding of ^{125}I-labeled EGF to NRK, Ts, and KNRK cells at permissive (32°C) and nonpermissive (39°C) temperatures. Medium (1.5 ml) containing 1–250 ng of ^{125}I-labeled EGF was added to each confluent 60-mm culture dish for 55 min. Cells were then harvested and specific binding was determined. (*Insets*) Scatchard plots of data in the main figure. (B/F, bound/free.) These relationships were consistently observed in five or more separate binding experiments. ○, NRK; ▲, Ts; ●, KNRK.

Table 2. Number of EGF receptors per cell estimated by Scatchard analyses

Cell		Receptors per cell	Receptors per μm^2 surface area	K_d, M
NRK:	32°C	350,000	650	4.5×10^{-9}
	39°C	150,000	260	3.8×10^{-9}
Ts:	32°C	2,000–3,000	2–4	1.5×10^{-8}
	39°C	20,000	32	1.9×10^{-9}
KNRK:	32°C	None	—	—
	39°C	None	—	—

Values were calculated from the data in Fig. 3.

Growth Factor(s). Conditioned medium from KNRK and Ts cultures (grown at the permissive temperature) enhanced EGF binding to confluent NRK cells by about 25–34% (Table 3). The KNRK cell medium was active to dilutions of 1:12 and Ts cell medium to dilutions of 1:2. Conditioned medium from Ts cultures grown at the nonpermissive temperature did not enhance EGF binding. The effect of KNRK cell medium was insensitive to repeated freezing and thawing and heat inactivation (100°C for 60 min). Protease inhibitors (*N*-α-tosyl-L-lysylchloromethane and *N*-α-tosyl-L-phenylalanylchloromethane) were added to the conditioned medium, but the stimulation of EGF binding and [^{3}H]thymidine incorporation remained the same. KNRK cell conditioned medium enhanced initiation of DNA synthesis in NRK cells (Table 4), but had no effect on quiescent Ts or KNRK cell cultures in refeeding experiments. Conditioned media from KNRK and Ts cultures at both temperatures were tested for EGF by a highly specific radioreceptor assay (Fig. 4). No EGF was detected in the samples tested.

DISCUSSION

The addition of EGF to NRK cell cultures significantly increased the rate of cell division above control cultures at both temperatures, but at 32°C, EGF increased the final density of NRK cells 2- to 3-fold greater than control cultures. KNRK and Ts cells showed no apparent growth response to EGF at either the permissive or nonpermissive temperatures. In similar studies, Rudland *et al.* (20) found that normal 3T3 cells and a chemically transformed temperature-sensitive mutant cell line of 3T3 responded to fibroblastic growth factor at the nonpermissive temperature by initiation of DNA synthesis and cell multiplication, but that the mutant showed no response to fibroblastic growth factor at the permissive temperature. The transformed mutant showed "normal" morphology at the nonpermissive temperature. These studies implied that the temperature-sensitive lesion in the mutant cells involved membrane alterations (i.e., either a lack of growth factor membrane receptors or defective receptors), but binding studies were not performed to test these alternatives.

Todaro *et al.* (21, 22) have shown that transformation of murine cells by RNA sarcoma viruses resulted in the loss of cellular receptors for EGF and multiplication stimulating activity. We have confirmed the loss of EGF binding in rat kidney cells transformed by Kirsten sarcoma virus and have shown, using cells transformed by a temperature-sensitive mutant virus, that the loss of EGF binding is dependent on the transformed phenotype characteristic of cells grown at the permissive temperature. At the nonpermissive temperature, Ts cells grew as a monolayer and exhibited consistent binding of ^{125}I-labeled EGF. Scatchard analysis demonstrated that these cells had a single type of membrane receptor with the same affinity as that found for the NRK cells. On the transfer of the Ts cells to the permissive temperature, a progressive change in cell morphology and growth pattern accompanied the reduction of ^{125}I-labeled EGF binding to just detectable levels. EGF receptor sites were estimated to be less than 10% of those detected at the nonpermissive temperature and, in addition, the apparent affinity of the receptor was nearly 10% of that at the nonpermissive temperature. Our findings suggest that the loss of ^{125}I-labeled EGF binding associated with the transformed phenotype may be due to an absolute decrease in the number of membrane receptors for EGF. However, a temperature-sensitive defect in the processing of the receptor in Ts cells is also possible and would account for the residual EGF binding

Table 3. EGF binding to NRK cells in presence of KNRK and Ts cell conditioned media

Test medium	cpm × 10^{-3}
Nonspecific binding (conditioned NRK medium + EGF, 6 g/ml	0.9
NRK conditioned medium	29.3
KNRK conditioned medium	37.5
Dilution 1:4	38.4
Dilution 1:12	37.6
Dilution 1:24	28.5
Ts conditioned medium (32 °C)	39.4
Dilution 1:2	36.4
Dilution 1:4	27.7
Ts conditioned medium (39°C)	28.5

KNRK and Ts conditioned medium was collected from cultures after 4 days of cell growth. Confluent NRK cultures were rinsed three times with Hanks' solution, refed with the conditioned medium, and incubated for 24 hr. Then 10 ng of ^{125}I-labeled EGF (350,000 cpm) was added to culture dishes and binding was determined. Values listed are total binding and are the average of determinations on two separate dishes. Four experiments confirmed these findings.

Table 4. Incorporation of [^{3}H]thymidine by NRK cells after exposure to control and KNRK conditioned medium

Test medium	cpm × 10^{-5}
Serum-free DME	0.32
DME + 0.25% FCS	0.81
DME + 4% FCS	1.44
DME + 0.25% FCS + EGF (25 ng/ml)	1.27
DME + 4% FCS + EGF (25 ng/ml)	1.85
Serum-free DME + EGF (25 ng/ml)	0.40
Serum-depleted NRK medium*	0.70
Serum-depleted KNRK medium	1.72
Conditioned serum-free KNRK medium	0.92

NRK cells were plated at 1 × 10^6 cells in DME with 0.25% fetal calf serum. After 4 days, cultures were relatively quiescent and subconfluent. Various media were added to culture dishes and incubation was carried out for 22 hr. [^{3}H]Thymidine was added to medium to a final concentration of 1 μCi/ml and cultures were incubated. Values are the average of determinations on two separate dishes. Similar relationships were found in three separate experiments. DME, Dulbecco's modified Eagle's medium; FCS, fetal calf serum.

* Medium was gently aspirated from the culture dishes and immediately replaced.

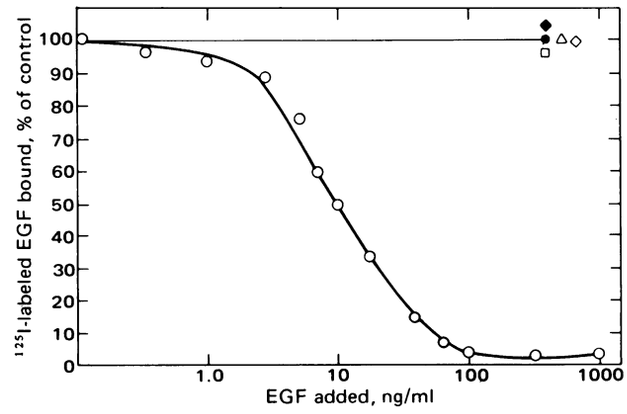

FIG. 4. Competition of ^{125}I-labeled EGF and unlabeled EGF for specific EGF receptors of NRK. FGF (100 ng/ml) (◆), multiplication stimulating activity (100 ng/ml) (◇), dexamethasone (1 μg/ml) (□), KNRK cell medium at 32°C and 39°C (●), and Ts cell medium at 32°C and 39°C (△) showed no evidence of competition for EGF receptors.

and the affinity change of the receptors at the permissive temperature. Since no biological response to EGF by Ts cells was observed at the permissive or nonpermissive temperature in spite of the significant difference in numbers of EGF receptors, it is possible that either the level of EGF receptors of Ts cells is still too low to stimulate growth or the receptors may be nonfunctional. The disappearance of exogenous EGF from the medium of NRK, KNRK, and Ts cells was found to be a direct reflection of the relative levels of receptors in the different cell lines. NRK cell medium exhibited a striking reduction in EGF concentration, whereas KNRK cell medium showed little change. Ts cells at 39°C, however, showed a 28% depletion of EGF from the culture medium. The depletion of EGF was similar to that observed in NRK cells, yet cell division was not stimulated. Presumably the Ts cell EGF–receptor complexes at 39°C are internalized, but perhaps these cell surface events are no longer coupled to the process of cell division (23, 24).

Modulation of EGF receptors was not peculiar to the temperature-sensitive mutant. NRK cells showed significantly greater numbers of EGF receptors at 32°C than at 39°C. Interestingly, this change was opposite to that demonstrated by the temperature-sensitive mutant. The growth responses of NRK cells to EGF in reduced serum were essentially the same at both temperatures. Thus, the total number of EGF receptors exhibited by NRK cells did not appear to affect the mitogenic response of cultures to EGF. This may be due to a difference in the relative turnover of the EGF–receptor complex as a function of temperature.

In cross-feeding experiments among NRK, KNRK, and Ts cells, we found that KNRK at both temperatures and Ts cells at the permissive temperature secreted material capable of stimulating the initiation of DNA synthesis and cell division in normal human diploid fibroblasts (unpublished observations) and in NRK cell cultures. DeLarco and Todaro (25) recently described the production of a growth factor by sarcoma virus-transformed 3T3 cells. The factor enhanced DNA synthesis and enabled untransformed cells to grow in agar, but competed with ^{125}I-labeled EGF for cell receptors. However, in contrast to the competition for EGF receptors reported by DeLarco and Todaro (25) by the sarcoma growth factor, the material isolated from KNRK and Ts cell conditioned media *enhanced* ^{125}I-labeled EGF binding to NRK cells. Preliminary studies have indicated that the active material(s) are polypeptide(s) with an estimated molecular weight range of 14,000–20,000.

The loss of EGF receptors and the production of growth-promoting material appear to be characteristic of cells transformed by murine sarcoma virus. In the cloned Ts cell system we have described, these features may be readily manipulated to provide a model for the analysis of the expression of the transformed phenotype.

We thank Drs. Edward Scolnick and Charles Scher for providing the normal and transformed cells for our study and Drs. Wayne Bardin and George Keller for helpful discussions and suggestions. This work was supported by a grant from the National Institute on Aging (AG 00421). R.L.L. is the recipient of a Research Career Development Award (K04 AG 00006) from the National Institute on Aging. P.G. is a predoctoral candidate in the Department of Anatomy.

1. Temin, H. (1972) in *Growth Nutrition and Metabolism of Cells*, eds. Rothblat, G. & Cristofalo, V. (Academic, New York), pp. 50–81.
2. Temin, H. (1971) *J. Cell Physiol* **78**, 161–170.
3. Stoker, M. (1973) *Nature (London)* **246**, 200–203.
4. Holley, R. & Kiernan, J. (1971) *Proc. Natl. Acad. Sci. USA* **60**, 300–304.
5. Dulbecco, R. (1970) *Nature (London)* **227**, 802–806.
6. Paul, D., Lipton, A. & Klinger, I. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 645–648.
7. Gospodarowicz, D. (1974) *Nature (London)* **240**, 123–127.
8. Seifert, W. & Rudland, P. (1974) *Nature (London)* **248**, 138–140.
9. Gospodarowicz, D. & Moran, S. (1975) *J. Cell Biol.* **66**, 451–457.
10. Hollenberg, M. & Cuatrecasas, P. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 2964–2968.
11. Cohen, S. & Carpenter, G. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 1317–1321.
12. Pardee, A. (1975) *Biochim. Biophys. Acta* **417**, 153–172.
13. Carchman, R., Johnson, G., Pastan, I. & Scolnick, E. (1974) *Cell* **1**, 59–64.
14. Scolnick, E., Stephenson, J. & Aaronson, S. (1972) *J. Virol.* **10**, 653–657.
15. Savage, C. & Cohen, S. (1972) *J. Biol. Chem.* **247**, 7609–7611.
16. Cohen, S. (1962) *J. Biol. Chem.* **237**, 1555–1562.
17. Greenwood, F., Hunter, W. & Glover, J. (1963) *Biochem. J.* **89**, 114–123.
18. Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.* **51**, 660–672.
19. Ladda, R., Bullock, L., Gianopoulos, T. & McCormick, L. (1979) *Anal. Biochem.* **93**, 286–294.
20. Rudland, P., Eckhart, W., Gospodarowicz, D. & Seifert, W. (1974) *Nature (London)* **250**, 337–339.
21. Todaro, G., DeLarco, J. & Cohen, S. (1976) *Nature (London)* **264**, 26–31.
22. Todaro, G., DeLarco, J., Nissley, S. & Rechler, M. (1977) *Nature (London)* **267**, 526–528.
23. Carpenter, G. & Cohen, S. (1976) *J. Cell Biol.* **71**, 159–171.
24. Das, M., Miyakawa, T., Fox, C., Pruss, R., Aharonov, A. & Herschman, H. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 2790–2794.
25. DeLarco, J. & Todaro, G. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4001–4005.