the downregulation of TRH-R number by TRH is complex and involves at least two effects on TRH-R mRNA activity. TRH caused a rapid effect that appeared to involve post-transcriptional modification of TRH-R mRNA and a long-term effect that led to a new steady-state level of mRNA activity. This contrasts with reports in which homologous regulation of plasma membrane receptors for interleukin-2 (*13*) and epidermal growth factor (*14*) were associated with increased levels of receptor mRNA, even when receptor number was downregulated (*15*). At least two explanations for the mechanism of the TRH-induced decrease in TRH-R mRNA activity are possible. TRH may increase the rate of TRH-R mRNA degradation. This possibility cannot be assessed directly because a complementary DNA (cDNA) probe for TRH-R mRNA is not available. Alternatively, TRH may cause a modification of TRH-R mRNA so that its activity is decreased; the modification would most likely be covalent because it persists during the RNA preparation, which would almost certainly dissociate any noncovalently attached regulatory factor. The long-term effect of TRH on receptor mRNA activity may be mediated by a different mechanism and may involve transcriptional or post-transcriptional effects, or both.

These results show that receptor mRNA obtained from a defined and well-characterized cell line can be translated, and the receptor can then be incorporated into the oocyte plasma membrane and coupled to a measurable biological response. Although mRNA obtained from a variety of tissues (*16, 17*) or from cDNA vectors (*18*) have been expressed in the oocyte, our experiments demonstrate expression of a plasma membrane receptor mRNA from a permanent, cloned cell line. This approach may be extended to the study of regulation of mRNA for other cell-surface receptors in other cell lines when probes for receptor mRNA are not available.

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Epidermal Growth Factor–Dependent Transformation by a Human EGF Receptor Proto-Oncogene

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The epidermal growth factor (EGF) receptor gene *EGFR* has been placed in a retrovirus vector to examine the growth properties of cells that experimentally overproduce a full-length EGF receptor. NIH 3T3 cells transfected with the viral DNA or infected with the corresponding rescued retrovirus developed a fully transformed phenotype in vitro that required both functional *EGFR* expression and the presence of EGF in the growth medium. Cells expressing 4×10^5 EGF receptors formed tumors in nude mice, while control cells did not. Therefore, the *EGFR* retrovirus, which had a titer on NIH 3T3 cells that was greater than 10^7 focus-forming units per milliliter, can efficiently transfer and express this gene, and increased numbers of EGF receptors can contribute to the transformed phenotype.

MOST ONCOGENES ARE ALTERED versions of evolutionarily conserved cellular genes that apparently subserve important functions in normal growth and development (*1*). These normal genes are often called proto-oncogenes to emphasize their genetic relationship to oncogenes. Oncogenes typically induce cellular transformation in vitro with high efficiency, while the proto-oncogenes from which they were derived are usually much less active. Many studies have demonstrated the tumorigenic potential of oncogenes, but the possible role of proto-oncogenes in tumor formation has been less completely documented.

The epidermal growth factor (EGF) receptor, which is the product of a proto-oncogene, enables cells to undergo DNA synthesis in response to either of the known physiologic EGF receptor ligands, EGF and TGF-α (*2, 3*). The v-*erb*B oncogene of avian erythroblastosis virus encodes a truncated version of the EGF receptor (*4*). The oncogenic properties of v-*erb*B demonstrate that an altered EGF receptor can contribute directly to tumorigenesis. A potential role for normal EGF receptor in tumor formation has also been proposed, because increased numbers of apparently normal EGF receptors may be present in various human tumors and tumor cell lines, including the epidermoid carcinoma A431 cell line (*5*). However, the oncogenic potential of a full-length EGF receptor has not been demon-

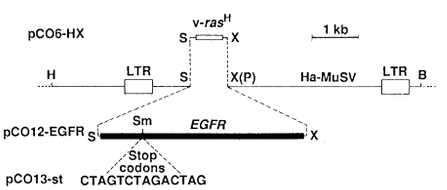

Fig. 1. Construction of the human EGF receptor retroviral genome. Restriction endonuclease sites: H, Hind III; S, Sac II; Sm, Sma I; P, Pst I; X, Xho I; and B, Bam HI. The *EGFR* retroviral vector (pCO12-EGFR) was derived from pCO6-HX, a construct in which the full-length Harvey murine sarcoma virus (Ha-MuSV) genome was cloned in pBR322 (*11*); the v-*ras*^{H} gene is flanked by the long terminal repeat (LTR) and by 5′ and 3′ untranslated regions. In pCO6-HX, the viral Pst I site at nucleotide 1759 (*20*) has been replaced with an Xho I linker (*21*). To insert the h*EGFR* cDNA, the entire coding region of p21 *ras* (Sac II–Xho I) was removed and replaced by the 4.2-kb fragment coding for the full-length hEGF receptor, derived from a pMMTV *EGFR* plasmid (*6*). The pCO13-st was derived from pCO12 by insertion of a universal translation terminator linker at a Sma I site at codon 220 in the *EGFR* cDNA; this construct therefore encodes a nonfunctional protein.

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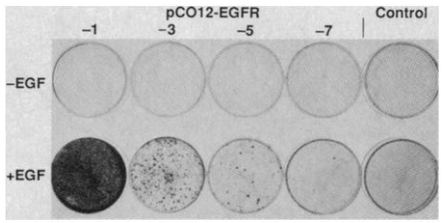

Fig. 2. Titration of h*EGFR* virus. NIH 3T3 cells were infected with 100-fold serial dilutions of an amphotropic pseudotyped virus preparation (from colony 25) and incubated in growth medium with 2% fetal calf serum in the presence or absence of EGF (20 ng/ml). Control cells were infected with amphotropic helper virus alone at 10^{-1} dilution. The dishes were stained with methylene blue and carbol fuchsin 2 weeks after infection. No foci were seen in dishes that did not receive EGF, although the cell monolayer became confluent. Similar results were obtained with cells grown in 10% fetal calf serum, but the denser background than with cells grown in 2% fetal calf serum made the individual foci more difficult to visualize.

strated directly. Efforts to induce the fully transformed phenotype by transfer of the EGF receptor proto-oncogene (*EGFR*) into established cells have thus far not been successful; the only biological difference from parental cells was that EGF-dependent DNA synthesis was achieved for serum-starved cells (*3*).

We have now placed the full-length human (h) *EGFR* gene in a retrovirus vector to examine the growth characteristics of cells that overproduce this growth factor receptor. Using this vector to introduce *EGFR* into established mouse cells either via gene transfer or via infection as a high titer retrovirus, we find that overproduction of these receptors confers a transformed phenotype that is dependent on the presence of EGF.

An h*EGFR* complementary DNA (cDNA), isolated from the A431 cell line (*6*), was placed in a Harvey murine sarcoma virus (Ha-MuSV) genome from which the viral oncogene v-ras^H has been deleted so that *EGFR* is the only functional gene (Fig. 1). This plasmid is designated pCO12-EGFR. We also generated a control plasmid, pCO13-st, which was identical to pCO12-EGFR except that a translation stop codon had been inserted within the amino terminal coding sequences of *EGFR* so that pCO13-st should not encode a functional hEGF receptor.

The two DNA clones were transfected onto mouse NIH 3T3 cells via calcium phosphate precipitation (*7*) and grown with or without EGF (20 ng/ml). In the absence of EGF, no obvious morphological differences from untransfected cells were noted for pCO12-EGFR or pCO13l-st. However, when grown in the presence of EGF, culture dishes that had received pCO12-EGFR formed multiple foci (400 foci per microgram of DNA) of morphologically transformed cells. Untransfected control cells or cells transfected with the premature termination mutant achieved a somewhat higher density with EGF than without EGF, but they did not display focal changes. To confirm that the foci contained cells that were expressing the receptor, an hEGF receptor–specific antibody was used in an immunofluorescence assay (*8*). The areas of focal transformation correlated closely with those cells that gave positive fluorescence. We conclude that hEGF receptor expression has led to ligand-dependent focal transformation of the NIH 3T3 cells.

To have a suitable group of cells from which a high titer h*EGFR* virus might be obtained, we cotransfected pCO12-EGFR with an antibiotic resistance gene (*9*) (neo^r) as a selectable marker to derive individual colonies of cells expressing hEGF receptors. As expected, when the transfected cells were grown with geneticin and EGF, many of the colonies derived from cells that had received pCO12-EGFR contained refractile cells that grew over each other, and about 10% of the colonies were composed principally of transformed cells. Approximately 50 to 90% of the cells from several transformed colonies fluoresced brightly with the antibody to the hEGF receptor, while a colony that was composed of nontransformed cells in the presence of EGF gave a negative result by fluorescence. Colonies derived from cells that received the premature termination mutant pCO13-st did not appear transformed and resembled cells transfected only with neo^r.

An amphotropic helper virus (*10*) was used to "rescue" pCO12-EGFR as a virus (called h*EGFR* virus) from each of several colonies that had displayed a high proportion of hEGF receptor positive cells. When the culture fluids from different colonies were titrated on uninfected NIH 3T3 cells, the biological properties of each h*EGFR* virus preparation were identical to those shown in Fig. 2. As had been true with the gene transfer experiments, focal transformation of the infected cells depended on the presence of EGF in the medium (1 to 100 ng/ml). The titers ranged from 5×10^6 to 5×10^7 focus-forming units per milliliter, with excellent agreement between focus and fluorescence assays. This titer is less than one order of magnitude lower than wild-type Ha-MuSV and about two orders of magnitude higher than that of a c-ras^H cDNA proto-oncogene in the same vector (*11*).

When h*EGFR* virus–infected NIH 3T3 cells and transfected cells from which the virus was derived were tested for two additional characteristics of transformation, growth in agar (Fig. 3) and in low serum concentration (Fig. 4), they were virtually identical. As had been true for focus formation, they behaved as phenotypically transformed cells in agar or low serum only in the presence of EGF. With EGF, the transfected and infected cells formed large colonies in agar (Fig. 3). They achieved a rate of exponential growth in 1% serum that was about twice as great as that of control cells and grew to a much higher cell density (Fig. 4); without EGF, they grew poorly, as did control cells.

In addition to the ligand-dependent biological activity of hEGF receptors described above, two other criteria also suggested that hEGF receptors in the transfected and infected cells were normal. As expected from normal hEGF receptors, immunofluorescence indicated that the receptors were internalized in the presence of EGF. Immunoprecipitation of hEGF receptors (*12*) from transfected and infected cells precipitated an approximately 175-kD band that migrated similarly to hEGF-receptor in control human KB cells. Quantitative binding analysis with ^{125}I-labeled EGF indicated that the transfected and infected cells contained about 4×10^5 hEGF receptors, compared with about 10^4 mouse EGF receptors on control NIH 3T3 cells.

In limited tumorigenicity assays, male nude mice, which contain endogenous EGF (*13*), were inoculated with 10^7 pCO12-EGFR–transformed cells infected with amphotropic virus. The mice were then either given daily subcutaneous doses of EGF (5 μg/day) or no additional treatment. The pCO12-EGFR–transformed cells gave rise

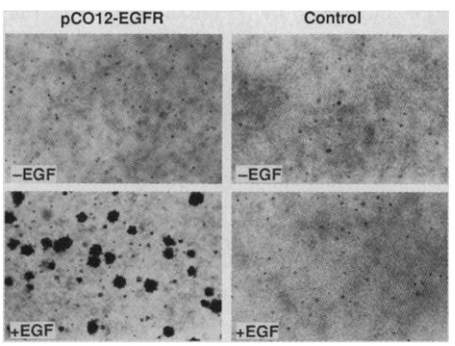

Fig. 3. EGF-induced growth in agar by h*EGFR* transformed cells. Cells from a colony (25) transfected with pCO12-EGFR were placed in agar in the presence or absence of EGF (20 ng/ml). Identical results were obtained with cells infected with h*EGFR* virus. Controls that failed to grow in agar, with or without EGF, were cells cotransfected with pCO13-st and neo^r selected for G418 resistance and parental NIH 3T3 cells. Photographs were taken after 2 weeks growth in agar.

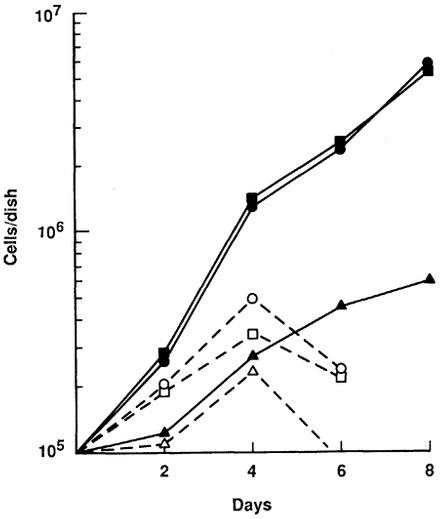

Fig. 4. Dependence of cell growth on h*EGFR* and EGF. Cells (10^5) were seeded (day 0) and grown in Dulbecco's minimal essential medium plus 10% fetal calf serum. Beginning the following day, the cells were grown in 1% fetal calf serum. Solid lines represent cells grown with EGF (20 ng/ml) supplementation throughout (from day 0). Interrupted lines represent cells not given EGF. Squares represent cells derived from a colony (25) transfected with pCO12-EGFR DNA and pNeo, circles represent NIH 3T3 cells infected with h*EGFR* virus, and triangles represent control NIH 3T3 cells infected with amphotropic helper virus. The small differences seen here in the growth of cells without EGF have not been noted in other assays.

to progressively growing tumors at the site of inoculation in each of the five animals in each group. However, the latent period was almost twice as long in the uninjected mice when compared with the EGF injected animals (55 days versus 33 days, respectively). The tumor cells expressed hEGF receptors by RNA slot blot analysis and by immunofluorescence, and the h*EGFR* virus isolated from tumors displayed the EGF-dependent phenotype noted above. No tumors developed in any of ten animals inoculated with 10^7 control cells, although five received daily EGF injections, and the mice were observed for 90 days.

Our experiments indicate that high expression of full length *EGFR*, via gene transfer or via infection as a retrovirus, can confer a conditionally transformed phenotype to established mouse cells that normally express a low number of the analogous murine receptor. Cells with more than 10^5 hEGF receptors displayed a fully transformed phenotype in the presence of EGF; EGF induced only marginal changes in the growth of control cells, which contained less than one-tenth as many (rodent) receptors. It has been reported that transfection of h*EGFR* into established cells, resulting in the expression of more than 10^5 receptors per cell, led to ligand-dependent induction of cell DNA synthesis, but it did not confer any other properties of cell transformation (*3*). Because the other studies differed from ours in cell culture protocol, vector, and cDNA, we cannot predict what accounts for the greater biological activity seen in our EGF receptor experiments.

In the absence of ligand, the growth properties of our cells carrying about 4×10^5 hEGF receptors were similar to those of parental cells. These results demonstrate that normal EGF receptors are under stringent regulatory control, with an extremely low basal activity in the absence of EGF. The virtually complete dependence on ligand for biological activity of hEGF receptor is qualitatively different from the ligand-independent transformation seen with grossly altered receptors, such as the products of v-*erb*B (*2*), v-*fms* (*14*), and recombinants between *EGFR* and either the interleukin-2 receptor gene (*15*) or the v-*abl* oncogene of Abelson leukemia virus (*3*).

Our observations provide direct evidence that the number of EGF receptors can markedly influence the growth properties of tumor cells, as suggested by results obtained with human tumor lines that carry more than 10^6 EGF receptors; in these lines, revertant cells, selected by their lack of response to EGF, displayed fewer EGF receptors and had reduced tumorigenicity (*16*). Our in vitro data are formally analogous to those reported for c-*fms*, which probably encodes the colony-stimulating factor–1 (CSF-1) receptor (*14*). Those studies found that cellular transformation of NIH 3T3 cells by c-*fms* depended on the presence of CSF-1. In apparent contrast to our in vivo results with the hEGF receptor, Roussel *et al.* (*14*) obtained tumors only with cells transfected with both c-*fms* and CSF-1.

Our results also complement studies on cellular transformation via autocrine stimulation, which is inherently less stringent than extracellular stimulation because the autocrine mechanism requires that the ligand be made by a tumor cell that bears the appropriate receptor. Rosenthal *et al.* (*17*) and Stern *et al.* (*18*) have used gene transfer to express molecularly cloned TGF-α and a synthetic form of EGF, respectively, in cells that undergo cellular transformation in the presence of exogenous ligand. We have also found that h*EGFR* virus infection of NIH 3T3 cells transfected with a TGF-α expression plasmid induced EGF-independent cell transformation (*19*).

The development of a high titer h*EGFR* retrovirus has several implications. The inherent efficiency of virus infection, which is several orders of magnitude greater than DNA transfection, makes it possible, in a single step, to induce high EGF receptor expression in the majority of recipient cells in a mass culture. The analysis of such cells should preclude any theoretical selective bias that might be imposed by transfection, which even under optimal conditions induces stable transfectants in less than 1% of cells. The virus permits in vivo studies and random mutagenesis to be carried out more efficiently than with DNA. Also, cells with poor plating efficiencies, such as primary epithelial cells, can be subjected to manipulation much more easily by infection of a majority of the cells than by procedures that might induce phenotypic changes in only a small proportion of the recipients.

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