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Retroviruses Expressing Different Levels of the Normal Epidermal Growth Factor Receptor: Biological Properties and New Bioassay

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Two retroviral DNAs that encode the normal human epidermal growth factor (EGF) receptor hEGFR have been generated by inserting a h*EGFR* cDNA into two different retroviral vectors. One DNA (pCO11-EGFR-neo) also contained a linked selectable marker gene (neo^R). The other (pCO12-EGFR) only expresses h*EGFR*. When introduced into NIH3T3 cells, the two DNAs and the viruses derived from them induced a fully transformed phenotype, including focal transformation and growth in agar or low serum, but transformation depended entirely upon EGF being present in the growth medium. Compared with pCO11-EGFR-neo, pCO12-EGFR induced EGF-dependent transformation 2–5 times more efficiently and expressed higher numbers of receptors (4×10^5 vs. 1×10^5 EGF receptors per cell). The results indicate that transforming potential is directly related to the number of EGF receptors. In defined, serum-free medium that contained only very low concentrations of insulin (0.6 μg/ml) and transferrin (0.6 μg/ml), hEGFR-virus infected cells were able to grow with EGF as the only growth factor. Moreover, daily incubation of the cells with EGF for only 30 min was sufficient to induce growth. NR6 cells, which lack endogenous EGF receptors, were transformed as efficiently as NIH3T3 cells by the hEGFR virus. The dose-dependent growth response to EGF of infected NR6 cells grown in serum-free medium can be used as a highly sensitive bioassay for the quantitative assessment of EGF and transforming growth factor type α (TGFα). This bioassay is at least as sensitive as previously reported radioimmunoassays and can measure a much wider concentration range (10 pg–100 ng/ml). Uninfected NR6 cells or NR6 cells infected by helper virus alone can be used as controls for the EGF specificity of growth stimulation.

Key words: cell transformation, neoplastic, receptor, epidermal growth factor, transforming growth factor, oncogenes, genetic vectors, retrovirus, bioassay

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INTRODUCTION

Growth factors and their receptors have been hypothesized to serve an important physiologic role in normal growth and differentiation, as well as to contribute to the pathogenesis of some tumors [1]. The possible relationship between this class of proteins and oncogenesis has been underlined by the finding that several viral oncogenes (v-*onc*) of acute transforming retroviruses have been derived from genes that encode growth factors or growth factor receptors. These include the v-*sis* oncogene, which originated from the gene encoding the B chain of platelet-derived growth factor (PDGF) [2], v-*erbB* from the epidermal growth factor (EGF) receptor gene [3], and v-*fms* from the colony stimulating factor-1 (CSF-1) receptor gene [4].

Compared with their normal cellular homologs (c-*onc*), many oncogenes, including v-*erbB* and v-*fms*, have been found to possess deletions and/or mutations that significantly increase their transforming activity. By contrast, the most striking alteration of c-*erbB* in human tumors seems to be its overexpression. Elevated levels of apparently normal EGF receptors, secondary to amplification or overexpression of c-*erbB*, have been observed in several types of cancer, most of which are of epidermoid origin [5,6]. Amplification and overexpression have also been found in several tumor-derived human cell lines, including the A431 epidermoid carcinoma cell line [7–11]. These observations have fostered the belief that increased numbers of EGF receptors might contribute to tumor formation.

To test this hypothesis experimentally, we inserted the human c-*erbB* proto-oncogene (h*EGFR*) cloned from A431 cells into a retroviral vector [12]. NIH3T3 cells transfected with the h*EGFR* retroviral DNA or infected with the corresponding rescued h*EGFR* virus expressed high levels of normal human EGF receptors (4×10^5 receptors per cell) and developed a fully transformed phenotype in the presence of EGF. The cell transformation depended completely upon EGF in that no biological alterations were detected in its absence. Cells expressing high levels of EGF receptors formed tumors in male nude mice (which produce significant amount of endogenous EGF) [13], while control cells did not. Tumor development was much faster when exogenous EGF was administered to the mice [12]. Using a cDNA from A431 cells, Di Fiore et al. [14] confirmed the ability of an overexpressed normal EGF receptor to induce EGF-dependent NIH3T3 cell transformation.

To extend our EGF receptor studies, we have now placed the A431 h*EGFR* gene in a second retroviral vector and compared this second DNA and its recovered virus to the previously described DNA and virus. We have found that the transforming potential of the two h*EGFR*-expressing viruses correlated with their EGF receptor expression and that short daily incubations with EGF are sufficient to induce growth of cells expressing high EGF receptor levels. Using serum-free conditions, we have also demonstrated that the growth of h*EGFR*-expressing cells is sensitive to very low levels of EGF (10 pg/ml); this growth response can be used as a highly sensitive bioassay for the quantitative assessment of EGF and transforming growth factor type α (TGFα).

MATERIALS AND METHODS

Retroviral Vector Construction

The 12-kb plasmid pCO11-EGFR-neo was derived from pGV16, which is a murine retroviral based shuttle vector that contains a selectable genetic marker (neo^R)

(Fig. 1) [15]. Initially, the v-*ras*^{H} 2.1-kb BamHI-EcoRI fragment from the Harvey murine sarcoma virus (Ha-MuSV) genome was inserted between the 5′ long terminal repeat (LTR) and the *neo*^{R} gene, giving rise to plasmid pBW1160 [16]. The PstI site present downstream from the *ras* coding sequences had been replaced with XhoI. The original h*EGFR* clone was a 4.2- kb XbaI-NcoI cDNA fragment, derived from pMMTV-EGFR [17], that contained the complete EGF-receptor coding sequences; the fragment was made SacII-XhoI compatible by linker ligation. To generate pCO11-EGFR-neo, the 0.8-kb SacII-XhoI fragment of pBW1160, containing the entire coding region of v-*ras*^{H}, was removed and replaced by the 4.2-kb SacII-XhoI h*EGFR* fragment. In pCO11-EGFR-neo, h*EGFR* is inserted within 1.3-kb of noncoding sequences (BamHI-EcoRI) derived from Ha-MuSV DNA, and its expression is under the control of the pGV16 LTR, which was derived from the Moloney murine sarcoma virus (Mo-MuSV).

Plasmids pCO12-EGFR and pCO13-st have been described previously [12]. The 15 kb pCO12-EGFR was derived from a full-length Ha-MuSV DNA cloned in pBR322 (pCO6-HX) in which h*EGFR* has been inserted in place of the *ras* gene (Fig. 1). Plasmid pCO13-st is identical with pCO12-EGFR, except that a universal translation terminator linker was inserted at the SmaI site at codon 220 in *hEGFR* so that no functional hEGF receptor is transcribed.

Cell Culture and Transfection Assays

NIH3T3 cells [18], NR6 [19], and KB cells (American Type Culture Collection, Rockville, Maryland) were maintained in Dulbecco's modified Eagle (DME) medium

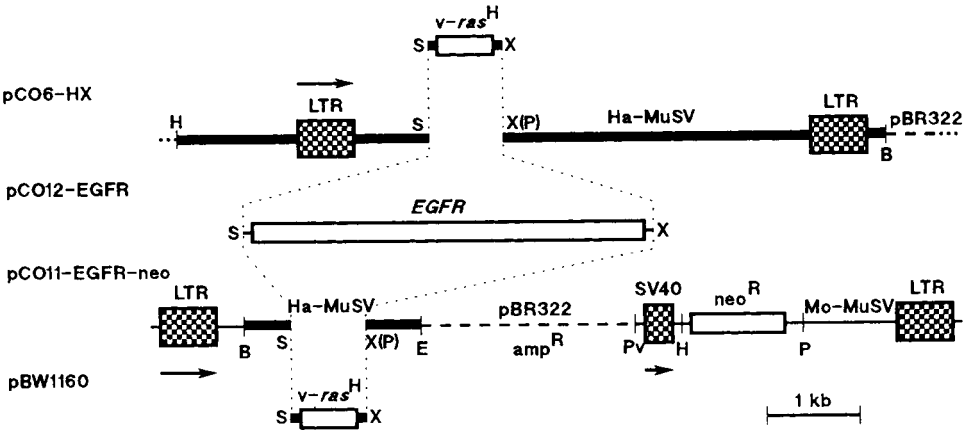

Fig. 1. Construction of the normal hEGF receptor retroviral genomes. Plasmid pCO12-EGFR was derived from the Harvey murine sarcoma virus (Ha-MuSV) cloned in pBR322 (pCO6-HX). pCO11-EGFR-neo was constructed from pGV16 [15] in which p21 v-*ras*^{H} with flanking sequences had been cloned into the BamHI and EcoRI sites (pBW1160) [16]. In both pCO6-HX and pBW1160, the PstI site of Ha-MuSV at nucleotide 1759 [46] has been replaced by an XhoI linker [16]. In both cases, the entire coding region of v-*ras*^{H} (SacII-XhoI) was removed and replaced by the 4.2-kb fragment coding for the full-length h*EGFR* cDNA (XbaI-NcoI) [17], which was made compatible by linker ligation (SacII-XhoI). Ha-MuSV sequences are shown in bold lines; pBR322, in broken lines. Restriction endonucleases sites: H, HindIII; S, SacII; P, PstI; X, XhoI; B, BamHI; and Pv, PvuII. Open boxes: coding sequences of v-*ras*^{H}, *EGFR*, and neo^{R} genes; cross-hatched boxes, Ha-MuSV and Mo-MuSV LTRs, and SV40 promotor; arrows, direction of transcription.

supplemented with 50 1U/ml penicillin, 50 μg/ml streptomycin, and 10% heat inactivated fetal calf serum (FCS).

For transfection experiments, 3×10^5 NIH3T3 cells were plated in 35-mm dishes. Twenty-four hours later, cells were transfected with 1 μg of the h*EGFR* containing plasmid and 5 μg of carrier NIH3T3 DNA using the calcium phosphate method [20]. The cells were treated as previously described, except that dimethyl sulfoxide was not used [18]. As a selectable marker, 100 ng of a 6-kb plasmid carrying the neo^R gene under the control of an SV40 promoter (pSV2neo) [21] was cotransfected with pCO12-EGFR and pCO13-st. Cells that were not treated with G418 received EGF (Collaborative Research, Inc., Bedford, Massachusetts) 2 days after the transfection (20 ng/ml final concentration). Foci were counted after 3 weeks. When G418 (Geneticin, GIBCO, Grand Island, New York) was used for selection (500 μg/ml final concentration), it was added 2 days after the transfection. EGF was added 5 days later to some G418 treated dishes. G418-resistant colonies were counted after 10 days in G418. When cultivated without EGF, the colonies were grown to confluency for studies on mass cultures. If grown with EGF, individual colonies were selected on the basis of their transformed phenotype to obtain a suitable group of cells from which a high-titer virus that expressed h*EGFR* could be rescued by infection with a helper virus.

Growth in semisolid media was studied by plating a layer of 0.4% soft agar (DIFCO, Detroit, Michigan) containing 10^5 cells in suspension on the top of a firm basal layer of 0.6% agar. A feeder layer of 0.4% agar was applied weekly. The agar was prepared either in DME medium containing 10% FCS or in the serum-free medium which is defined below. In some plates, EGF was added at a final concentration of 100 ng/ml.

Generation of hEGER Expressing Viruses

G418-resistant colonies which acquired the transformed phenotype upon the addition of EGF were previously found to be the ones expressing high levels of EGF receptors [12]. To obtain high-titer viruses that expressed h*EGFR*, these colonies were superinfected with a helper virus, amphotropic murine leukemia virus (MuLV) [22], or Moloney MuLV, at a multiplicity of infection of about 0.1. Adsorption was performed for 1 h at 37°C in the presence of 4 μg/ml polybrene (Sigma Chemical Co., St. Louis, Missouri). Culture fluid from infected cells was harvested 10 days later, stored at −70°C, and used as virus stocks. Virus titrations were performed, after filtering stocks through a cellulose acetate filter (0.45 μm), by testing serial dilutions for their ability to induce EGF dependent focal tranformation of NIH3T3 cells.

Experiments in Serum-Free Medium

A modification of previously described conditions [23] was used. Sixty-millimeter dishes were precoated for 2 h at 37°C with 150 μg poly-D-lysine/ml water (molecular weight: 540,000; 1 ml/plate), rinsed with phosphate-buffered saline (PBS), and then coated overnight at 37°C with 40 μg human fibronectin/ml of DME medium (2 ml/plate). After washing the plates with PBS, 10^5 cells were plated in DME medium containing 10% FCS to allow attachment. Three hours later, the cells were washed and refed with defined, serum-free medium consisting of a 1:1 mixture of DME and Ham F12 medium (GIBCO) supplemented with 20 mM HEPES (pH 7.3),

42 μg/ml histidine, and 10 ml/liter ITS+ (Collaborative Research, Inc., Bedford, Massachusetts), resulting in a final concentration of 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenium, 1.25 mg/ml bovine serum albumin (BSA), and 5.35 μg/ml linoleic acid. In some experiments, only 1 ml/liter ITS+ was added so that the final concentration of the ITS+ components was decreased by a factor of 10. These conditions are called "low" defined medium. In some experiments, platelet derived growth factor (PDGF, 5 U/ml) and basic fibroblast growth factor (bFGF, 2 ng/ml) were used at saturating concentrations. HEPES and histidine were from Sigma Chemical Co. (St. Louis, Missouri). All other reagents were obtained from Collaborative Research, Inc. (Bedford, Massachusetts).

Thymidine Incorporation

DNA synthesis was assessed by ^{3}H-thymidine (25 Ci/mmol, Amersham, Arlington Heights, Illinois) incorporation. Cells were incubated overnight with ^{3}H-thymidine (1 μCi/ml) in defined medium containing different concentrations of EGF. The cells were then washed three times with PBS containing 0.1% cold thymidine, frozen at −20°C, washed again three times with ice-cold 0.2 N perchloric acid (PCA), and solubilized in 1 M NaOH. PCA-insoluble material was frozen in dry ice, thawed in ice water, washed with 0.2 N PCA, resuspended in 0.5 N PCA, and hydrolyzed at 90°C for 20 min. Incorporated ^{3}H-thymidine was assayed by counting the supernatant in a scintillation counter.

Immunoprecipitation

Cells were seeded at 2 × 10^{6}/100 mm dish in complete medium. Eight hours later, cells were radiolabeled for 12 h with 250 μCi/ml ^{35}S-methionine (1,000 Ci/mmol, Amersham, Arlington Heights, Illinois), in 4 ml of methionine-free Eagle's medium containing 2% FCS. Cells were harvested by scraping into 3 ml of ice-cold STE buffer (20 mM TRIS HCl pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1% aprotinin), centrifuged at 500*g* for 5 min, and solubilized in 1 ml of lysis buffer (20 mM HEPES pH 7.4, 1% Triton X-100, 10% glycerol, 1% aprotinin). Cell lysates were clarified at 100,000*g* for 60 min. Immunoprecipitation was performed as previously described [24], using a rabbit polyclonal anti-EGF receptor antibody, Ab 2913 [25], or an anti-hEGF receptor monoclonal antibody (EGFR1, Amersham, Arlington Heights, Illinois), and proteins were analyzed by SDS-PAGE on 8.5% gels [26]. Gels were fluorographed [27] and subjected to autoradiography. The amount of EGF receptor was quantitated by excising the immunoprecipitated band. Counts in the gel slices were extracted with 2 ml of 30% H_2O_2, 1% NH_4OH for 24 h and then counted by liquid scintillation after addition of 10 ml Aquasol.

RESULTS

We have recently reported development of a high-titer retrovirus that expressed hEGFR under the control of the Ha-MuSV LTR; the viral DNA was called pCO12-EGFR and the resulting virus hEGFR (Fig. 1) [12]. To obtain a second virus that expressed lower levels of h*EGFR* and contained a selectable marker unrelated to cell transformation, we introduced the h*EGFR* cDNA into another retroviral vector. This latter construction, called pCO11-EGFR-neo, has the coding sequences of the EGF receptor under the control of the Mo-MuSV LTR and contains the neo^{R} marker gene

under the control of a SV40 promoter (Fig. 1). As had been true of pCO12-EGFR, transfection of pCO11-EGFR-neo onto NIH3T3 cells induced multiple foci of transformed cells only when EGF was added to the medium (Table I). The transforming efficiency was somewhat lower and the foci smaller for pCO11-EGFR-neo than for pCO12-EGFR, suggesting that the latter expressed higher levels of hEGF receptor. No transforming activity was found in the absence of EGF, nor for the premature termination mutant pCO13-st with or without EGF.

Previous experiments had shown that high-titer virus could be obtained from cells cotransfected with pCO12-EGFR and pSV2neo by growing them with EGF and G418 and selecting individual morphologically transformed colonies. We therefore selected cells for G418 resistance, after transfection of pCO11-EGFR-neo by itself and cotransfection of pCO12-EGFR with pSV_2neo (Table I). When the cells transfected with these two constructions were grown in the presence of EGF, many of the G418-resistant colonies that developed were composed of morphologically transformed cells that were refractile and spindle shaped and grew over each other. Approximatively 20% of the pCO11-EGFR-neo transfected colonies were morphologically transformed, while almost 50% of the colonies that arose from cotransfection of pCO12-EGFR and pSV2neo were transformed (Table I). These results again suggested that pCO11-EGFR-neo might express lower levels of hEGF receptors than pCO12-EGFR. As expected, no transformed colonies were seen in the absence of EGF, nor in pCO13-st transfectants even with EGF. Immunofluorescence studies, using an hEGFR-specific antibody (EGFR1), indicated that foci and transformed G418-resistant colonies expressed easily detectable levels of hEGF receptors, with an excellent correlation between the high expression of EGF receptors and the transformed phenotype (data not shown).

Viruses able to transmit an EGF-dependent transformed phenotype were obtained using a helper virus to superinfect G418-resistant, morphologically transformed colonies expressing hEGF receptors. The viruses derived from pCO11-EGFR-neo

TABLE I. h*EGFR* Transforming Activity on NIH3T3 Cells

	pCO11-EGFR-neo		pCO12-EGFR		pCO13-st	
	+EGF	−EGF	+EGF	−EGF	+EGF	−EGF
Foci per μg DNA[a]	250	0	800	0	0	0
Transformed/total G418 colonies per μg DNA (%)	260/1,420 (18%)	0/1,380 (0%)	550/1,140 (48%)	0/1,220 (0%)	0/1,310 (0%)	0/1,120 (0%)
Growth in agar[c]	2.6%	0	8.8%	0	0	0
Titer of h*EGFR* virus/ml[d]	10^4		10^7		N.D.[b]	
EGF receptors/cell (type)[e]	1×10^5 (human)		4×10^5 (human)		10^4 (mouse)	

[a]Results are expressed per μg of h*EGFR* plasmid DNA.

[b]N.D., not determined.

[c]To be able to compare the EGF-induced growth in soft agar for each construction, cells for this experiment came from G418-selected mass cultures that had not been treated with EGF before being placed in agar. 10^5 cells were seeded in suspension. Results are expressed as the % of suspended cells that formed colonies in agar.

[d]h*EGFR* viruses were derived from G418 colonies expressing high levels of hEGF receptors.

[e]Receptor determinations were made on morphologically transformed cells that had been selected in EGF and G418 after transfection of the pCO11-EGFR-neo and pCO12-EGFR DNAs. Similar results were obtained for cells infected by hEGFR or by hEGFR-neo viruses that were derived from these transfected cells.

and pCO12-EGFR have been designated hEGFR-neo and hEGFR viruses, respectively. When titrated on NIH3T3 cells, similar results were obtained when the viruses were assayed by indirect immunofluorescence or by EGF dependent foci. The titer of the hEGFR-neo virus was found to be about three orders of magnitude lower (10^4 focus-forming units [ffu]/ml) than for the hEGFR virus (10^7 ffu/ml) (Table I).

To rule out the possibility that the viruses obtained from the morphologically transformed cells displayed the EGF-dependent transformed phenotype as a result of the selective pressure imposed by incubation with EGF prior to superinfection, we also tested culture fluid from a mass culture of NIH3T3 cells that had been cotransfected with pCO12-EGFR and pSV_2neo, selected with G418 in the absence of EGF, and superinfected with a helper virus. Although the titer (10^3 ffu/ml) was significantly lower than the hEGFR virus derived from pCO12-EGFR transfected cells selected with EGF, this virus readily induced EGF dependent transformation of NIH 3T3 cells.

The ability of pCO11-EGFR-neo-transfected and hEGFR-neo-virus-infected cells to confer anchorage-independent growth in semisolid medium was also studied. As was true of pCO12-EGFR cells [12], pCO11-EGFR-neo cells grew in agar in an EGF-dependent manner when the growth medium was 10% FCS or a defined, serum-free formulation. The number and the average size of the colonies appeared to be larger with pCO12-EGFR cells than with pC011-EGFR-neo cells. No growth was seen in the absence of EGF, nor in control cells in the presence or absence of EGF (Table I).

To study the integrity of the hEGF receptor, its function, and its relative abundance in pC011-EGFR-neo and pCO12-EGFR cells, we performed EGF binding, immunoprecipitation, immunofluorescence, and in vitro phosphorylation studies. Quantitative binding with ^{125}I-labeled EGF of pCO11-EGFR-neo- and pCO12-EGFR cells produced results that correlated with the biological data; pCO11-EGFR-neo cells contained about $1–2 \times 10^5$ human EGF receptors per cell, while pCOl2-EGFR contained about $3–5 \times 10^5$ (Table I). Parental NIH3T3 and pCO13-st cells expressed fewer than 1×10^4 mouse EGF receptors per cell. In immunoprecipiation assays, we found that the EGF receptors from both constructions migrated as an approximatively 175-kd band (Fig. 2). These bands comigrated with hEGF receptors from KB cells (data not shown), which express about 1.5×10^5 normal receptors per cell [24,28,29]. The content of ^{35}S-methionine in the EGF receptor bands confirmed the quantitative binding studies; pCO12-EGFR cells contained about four times more receptors than pCO11-EGFR-neo cells, and 20–50-fold more than control NIH3T3 cells (data not shown, obtained using a rabbit polyclonal antibody, Ab 2913, that recognized mouse and human EGF receptors). Furthermore, the estimated number of EGF receptors found in KB cells resembled that of pCO11-EGFR-neo cells, confirming again the level of EGF receptor expression in these cells. Immunofluorescent studies indicated that the hEGF receptors of pCO11-EGFR-neo and pCO12-EGFR cells were normally internalized upon the addition of EGF (Beguinot and Pastan, unpublished observations). Finally, the kinase activity of the hEGF receptors, as measured by autophosphorylation and phosphorylation of exogenous substrates, correlated with the amount of receptors expressed by the various clones and was EGF stimulated (Beguinot and Pastan, unpublished observations). These results suggest the hEGF receptors encoded by the cDNA are structurally and functionally normal.

We also tested the ability of pCO12-EGFR-transfected NIH3T3 cells to grow in low serum (1%), in serum-free defined medium, and in "low" defined medium, as

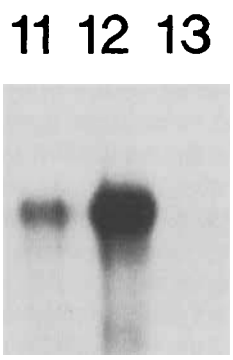

Fig. 2. Immunoprecipitation of the hEGF receptor. ^{35}S-labeled cell extracts were prepared and immunoprecipitated with an anti-hEGFR monoclonal antibody (EGFR1, Amersham) from a mass culture of NIH3T3 cells that had been selected with G418 after transfection of the DNAs (**11**, pCO11-EGFR-neo; **12**, pCO12-EGFR; **13**, pCO13-st). No EGF was added to the medium to avoid the selection of cells carrying high levels of EGF receptors and to be able to compare the relative EGF receptor expression. pCO12-EGFR cells contained about four times more EGF receptors than pCO11-EGFR-neo cells. The hEGF-receptors migrated as a ~ 175-kd band.

TABLE II. EGF-Dependent Growth

	No. cell doublings in 8-day assay[a]					
	pCO12-EGFR NIH3T3 cells			Control NIH3T3 cells		
	1% FCS[b]	"Low" DM[b]	DM[b]	1% FCS[b]	"Low" DM[b]	DM[b]
No EGF	1.2	2.0	3.3	1.3	1.9	3.1
+EGF (30 min/day)[c]	N.D.[d]	N.D.[d]	5.1	N.D.[d]	N.D.[d]	3.2
+EGF (continuous)[c]	3.9	5.8	6.4	1.5	2.1	3.5

[a] 10^5 cells, previously grown in the absence of EGF, were plated in 60-mm dishes. Results are expressed as No. of cell doublings after 8 days of culture, and are the mean of two independent experiments. Cell counts were performed on duplicate plates for each experiment.
[b] Growth conditions in 1% FCS, in defined medium (DM), and in "low" DM are described in Materials and Methods.
[c] EGF was added to the medium at 20 ng/ml, either continuously, or 30 min per day. All plates were washed twice (at the end of the 30 min incubation) and media were changed daily.
[d] N.D., not determined.

described in the Materials and Methods. Table II summarizes the results, which are expressed as the number of cell doublings after 8 days of growth. In the absence of EGF, pCO12-EGFR cells grew similarly to control NIH3T3 cells. When grown with EGF, pCO12-EGFR cells grew 2–3 times faster than control cells and achieved a higher final cell density. The EGF dependent difference was greater in "low" defined medium than in regular defined medium, showing again that the growth advantage of pCO12-EGFR cells was highly dependent upon the presence of EGF and that it persisted even in very poor growth conditions. Moreover, the EGF-dependent growth of pCO12-EGFR cells did not require the continuous presence of EGF. Daily incubation of the cells with EGF for 30 min only, although not as efficient as the continuous presence of EGF, was sufficient to induce EGF-dependent growth. Similar results were obtained with hEGFR virus (Fig. 3). After 10 days of continuous EGF treatment, hEGFR-infected cells were still growing (1.2×10^7/60-mm dish), while

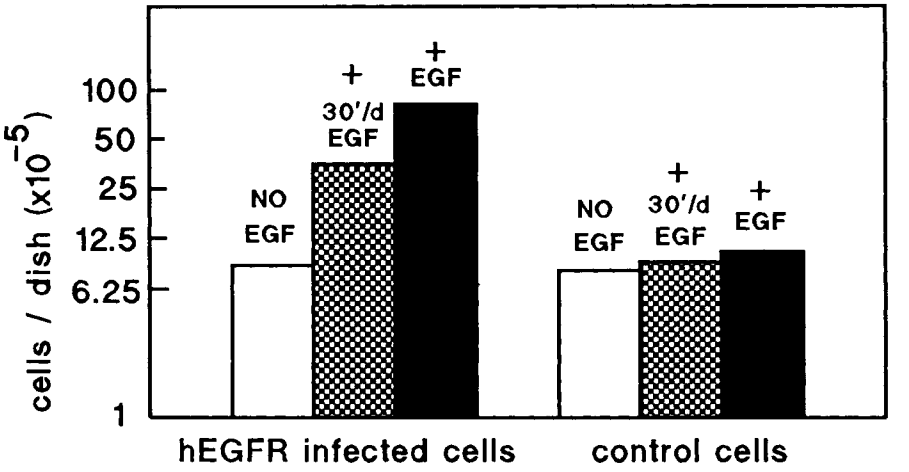

Fig. 3. Cell growth dependence on hEGFR and EGF. 1×10^5 NIH3T3 cells infected by pCO12-EGFR virus or by the amphotropic helper virus as control were seeded in 60-mm dishes coated with poly-D-lysine and fibronectin and kept in DME medium supplemented with 10% FCS to allow cell attachment. After 4 h, cells were washed and refed with serum-free defined medium. Black columns: cells in defined medium containing 20 ng/ml EGF; checkered columns: cells incubated daily for 30 min with medium containing 20 ng/ml EGF, then washed twice and refed with defined medium. The other plates were also washed twice every day, and refed with their respective medium. Results are given as number of cells/60-mm dish, counted after 8 days of culture, and are the mean of duplicate samples in two different experiments.

infected cells cultured without EGF were dying, as were control cells grown in the presence or absence of EGF.

NR6 cells, a variant of the Swiss 3T3 cells which do not express endogenous EGF receptors [19], were also infected with hEGFR virus. The virus conferred EGF-dependent focal transformation on NR6, with a titer similar to that obtained on NIH3T3 cells. When the hEGFR-virus-infected NR6 cells (NR6ER cells) were cultured in serum-free medium, EGF was found to induce dose-dependent cell growth, as assessed by ^{3}H-thymidine incorporation into DNA (Fig. 4). In this assay, the NR6ER cells displayed a detectable growth response to extremely low concentrations of EGF (10 pg/ml). Maximal growth response to EGF was seen at 100–1,000 ng/ml; no attenuation was noted even at the highest concentration tested (1,000 ng/ml). Thymidine incorporation measured in NIH3T3 cells infected by hEGFR virus yielded similar results (data not shown). As expected, parental NR6 cells or NR6 cells infected with the helper virus alone (NR6c cells) did not show a growth response to EGF. In contrast to NR6 cells, control NIH3T3 cells did respond somewhat to EGF, since they express about 10^4 endogenous mouse EGF receptors (data not shown). The sensitivity to EGF of NIH3T3 cells infected with the helper virus alone (or parental NIH3T3 cells) was equivalent to that of hEGFR-infected NIH3T3 cells, but saturation occurred at 1 ng/ml.

The EGF dose-response curve of NR6ER cells, as measured by ^{3}H-thymidine incorporation, was used as a highly sensitive bioassay for the quantitative measurement of the ligands that stimulate the EGF receptor: EGF and TGFα. The specificity of the bioassay can be assessed by testing the sample on NR6c cells. When the culture fluid of normal rat kidney (NRK) cells that expressed a synthetic TGFα gene (1/28

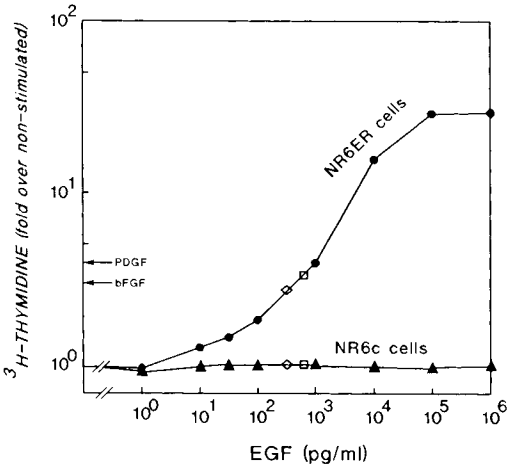

Fig. 4. Dose-dependent growth response to EGF of NR6 cells expressing (or not) high levels EGF receptors. NR6 cells (1×10^5) infected by pCO 12-EGFR virus (NR6ER cells, **solid circles**) or by helper virus alone (NR6c cells, **solid triangles**) were plated on coated dishes. Cells were grown for 48 h in DME medium with 10% FCS. Then they were placed in "low" defined medium (see Materials and Methods) without or with different concentrations of EGF (range: $1-1 \times 10^6$ pg/ml). The medium was changed daily. Forty-eight hours later, ^{3}H-thymidine was added at a final concentration of 1 μCi/ml, in the presence of the different concentrations of EGF. After overnight incubation, ^{3}H-thymidine incorporation was measured as described in Materials and Methods. The supernatant of a cell line producing TGFα (1/28 Cl.3 NRK cells) [30] and two growth factors, PDGF and bFGF, were also tested. The TGFα producing cells were grown in DME medium with 10% FCS to subconfluence, washed twice, and refed with "low" defined medium. After an overnight incubation and then filtration, the supernatant was tested directly (**open squares**) or diluted 1:1 with fresh "low" defined medium (**open diamonds**), on NR6ER and NR6c cells. The levels of growth stimulation induced by PDGF (5 U/ml) and bFGF (2 ng/ml) are indicated by an arrow and were quite identical for either NR6ER or NR6c cells. Results are from a single experiment. Two other experiments gave similar results.

Cl.3 NRK cells; see Fig. 4, legend) [30] was tested by the NR6ER assay, it was found to contain an activity that was equal to 0.7 ng/ml EGF. This result is similar to the amount of TGFα determined by a different assay [30]. The specificity of the activity was demonstrated by the failure of the culture fluid of this cell line to stimulate NR6c cells. Culture fluid from parental NRK cells also failed to stimulate NR6ER and NR6c cells (data not shown). As further specificity controls, two other growth factors (PDGF and bFGF), used at saturating conditions, were found to stimulate NR6ER and NR6c cells equally (Fig. 4).

DISCUSSION

The use of retroviruses to study the biological and biochemical properties of a protein encoded by a cloned gene has some advantages over DNA transfection. The efficiency of viral infection is several orders of magnitude higher than DNA transfection, which means that infection with high titer virus can, in a single step, induce high expression of the protein in the majority of cells. In contrast, the inefficiency of DNA-mediated gene transfer means that no more than 1% of the recipient cells will stably express the transfected gene. Consequently, infection can preclude any theoretical selective bias that might be imposed by DNA transfection and the isolation of

cells that express high level of the gene. Infection can also be used in some cell types that poorly support stable transfection of DNA.

These advantages led us to study the normal human EGF receptor by placing it in retroviral vectors so that expression of the gene could be studied via infection, as well as by DNA transfection [12]. In an initial study, an h*EGFR* cDNA was inserted in a vector derived from Ha-MuSV, yielding pCO12-EGFR. To obtain a high-titer EGFR virus, we found it useful to select individual colonies of morphologically transformed cells by cotransfecting pCO12-EGFR with a selectable marker (neo^R) and growing the transfectants in G418 and EGF. In the current study, clone pCO11-EGFR-neo was constructed by placing the h*EGFR* cDNA in a retroviral vector that contained the selectable marker neo^R. h*EGFR*-neo virus was obtained by growing pCO11-EGFR-neo-transfected cells in G418 and EGF, selecting morphologically transformed colonies, and superinfecting the cells with a helper virus. The EGF receptors expressed by the two constructions were found to be structurally and functionally normal by several criteria, including their size, their ability to bind to EGF and be internalized in response to this ligand, their kinase activity, and their EGF-dependent biologic activity.

When the biologic properties of the two DNAs and the two viruses recovered by helper virus infection were tested, all were able to induce the fully transformed phenotype on NIH3T3 cells, including focal transformation, growth in low serum, and growth in agar. These alterations in the growth properties of the cells occurred only in the presence of EGF. The transfected or infected cells did not display any detectable biologic changes in the absence of EGF. Moreover the continuous presence of the growth factor was not required to stimulate growth. Incubation of the cells with EGF for only 30 min per day (the shortest time tested) was sufficient to induce a significant increase in growth. This result parallels previous in vivo data obtained with pCO12-EGFR transfected NIH3T3 cells [12]. When injected into male mice, these cells formed tumors more rapidly in animals given daily subcutaneous injections of EGF, which would have increased circulating EGF levels only for a short time. We also noted that EGF growth stimulation of NIH3T3 or NR6 cells infected with EGFR virus was not inhibited by continuous exposure to EGF concentrations as high as 1 μg/ml. Other cells carrying higher numbers of EGF receptors, such as human epidermoid carcinoma A431 cells (2×10^6 receptors per cell), have been reported to display growth inhibition at high concentrations of EGF [31,32].

The titer of EGFR virus was about three orders of magnitude higher than that of hEGFR-neo virus. We believe that two important differences between the viruses account, at least in part, for the much higher titer found in hEGFR virus. First, the very large predicted size (12 kb) of the hEGFR-neo RNA implies that it is probably packaged into virions much less efficiently than the 9-kb hEGFR viral RNA [33]. Second, our quantitative assessment of the average number of hEGF receptors per cell indicated that pCO12-EGFR-transfected cells and hEGFR-virus-infected cells expressed about four times as many receptors per cell as pCO11-h*EGFR*-neo cells or cells infected with hEGFR-neo virus.

An important biologic difference between the two constructions was that pCO11-EGFR-neo induced EGF-dependent cell transformation less efficiently than pCO12-EGFR. This result correlated with the latter DNA expressing more hEGF receptors than the former, which strongly suggests that the transforming potential of the EGF receptor depends upon the level of its expression, as well as on ligand concentration.

Similar, but indirect, observations have been reported previously by Santon et al. [34], who observed that the degree of gene amplification and of EGF receptor expression in different A431 clonal variants correlated with the growth of these tumor cells in vivo and in vitro.

The lack of detectable biologic activity from overexpression of h*EGFR* in the absence of ligand suggests that the receptor is stringently regulated so that it will be virtually inactive without ligand. These observations are qualitatively similar to those reported on c-*fms*, which encodes the colony stimulating factor-1 (CSF-1) receptor [35]. Overexpression of the c-*fms* gene induced transformation of NIH3T3 cells that depended upon CSF-1.

Our studies provide direct experimental support for the hypothesis that increased numbers of EGF receptors can contribute to oncogenicity. Several clinical studies of malignancies, especially cancers of the breast and lung, have suggested that EGF receptor overexpression is associated with increased malignancy, as defined by tumor spread, recurrence, and poor prognosis [36–39]. Similar data have recently been reported with the *HER-2/neu* oncogene, which displays sequence homology with *EGFR*, although a putative ligand has not yet been identified for its encoded protein. Amplification and/or overexpression of this gene in human tumors, especially in breast cancers, has been found to correlate with short survival and time to relapse [40]. In constrast to *EGFR*, the ability of overexpressed *HER-2/neu* to induce morphologic transformation of NIH3T3 cells does not appear to depend upon the exogenous addition of a hypothetical ligand [41].

The observation that biologic activity of EGF receptors, even when expressed at more than 10^5 receptors per cell, is entirely ligand dependent, suggests that this receptor contributes to the growth of tumor cells principally via an autocrine or paracrine mechanism. Indeed, increased production of TGFα by different types of cancers and tumor cell lines has been found to be frequently associated with increased levels of EGF receptor [42,43]. Elevated levels of TGFα have also been found in the urine of patients with disseminated breast cancer [44]. The accessibility of the EGF receptor on the cell membrane and the dependence of activity upon the binding of its ligand make it a potentially attractive target for therapy.

The dose-response relationship between EGF concentration and cell proliferation of NR6 cells infected with EGFR virus, as assessed by ^{3}H-thymidine incorporation, can be an extremely sensitive bioassay for the quantitative determination of EGF and TGFα. When the culture fluid of a cell line producing TGFα was tested in this assay, the observed EGF-like activity correlated with the concentration obtained previously with other assays [30]. The detection limit of the bioassay is 10 pg/ml, which is at least as sensitive as that reported for radioimmunoassays (RIA) [45]. The bioassay can measure EGF and TGFα concentrations that vary by four orders of magnitude (10 pg–100 ng/ml = 10^{-12}–10^{-8} M), which is much wider than by RIA. NR6 cells infected by helper virus alone (or parental NR6 cells) can be used to determine the specificity of growth stimulation. NR6 cells are superior to NIH3T3 cells for this assay. NIH3T3 cells contain low numbers of endogenous EGF receptors so that EGF and TGFα will induce a growth response, while the lack of endogenous EGF receptors on parental NR6 cells means that they will not respond to EGF and TGFα.

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