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Expression of Growth Factors and Oncogenes in Normal and Tumor-derived Human Mammary Epithelial Cells¹

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ABSTRACT

The expression of genes which may be involved in the regulation of human mammary epithelial cell growth [transforming growth factors α and β] and tumorigenesis [*c-myc*, *erbB2*, epidermal growth factor receptor (EGFR), *Ha-ras*, *pS2*] has been compared in similarly cultured normal cell strains and tumor cell lines. We have found that the normal breast cells produce high levels of EGFR mRNA, which are translated into nearly 10^5 low affinity epidermal growth factor-binding molecules/cell. In the estrogen receptor-negative lines examined, the EGFR gene was expressed at levels comparable to those in the normal cells. In contrast, EGFR and transforming growth factor α mRNAs were reduced in estrogen receptor-positive tumor lines compared to estrogen receptor-negative lines and normal cells. Steady state mRNA levels for transforming growth factor β , *erbB2*, *c-myc*, and *Ha-ras* in the normal cells were greater than or comparable to those in all of the breast tumor lines. Furthermore, in the absence of gene amplification, only one of the genes examined (*i.e.*, *pS2*) was overexpressed in a subset of the tumor cells compared to their normal counterparts. Several reports by other investigators have described overexpression of some of these genes in breast biopsies and in tumor lines in studies lacking normal controls. Thus, our results, in which the same genes were not overexpressed compared to normal cells unless amplified, underscore the importance of including appropriate normal controls in studies aimed at defining aberrant patterns of gene expression in tumor cells.

INTRODUCTION

A characteristic property of tumor cells is the unregulated expression of genes encoding growth factors, their receptors, and/or various intermediates in the complex network of cellular growth control. Aberrant gene expression due to amplification and/or rearrangement of particular protooncogenes has been associated with specific forms of cancer (1, 2).

With the availability of cloned genes to use as hybridization probes, it has become possible to analyze the expression and structure of particular genes by comparing tumor cells with their normal counterparts. We have taken this approach in initiating a study of human breast cancer. A unique feature of this analysis is the use of normal mammary epithelial cells grown in long-term cell culture as controls for comparison with a series of ER⁺³ and ER⁻ tumor cell lines (3). In previous studies, established breast carcinoma cell lines have been examined without such a comparison (4-8).

A second feature of this analysis has been the use of a new medium (9), which supports the growth of both normal and tumor cells. Previous efforts have been devoted to the devel-

opment of techniques and media which allow the growth of normal human mammary epithelial cells in culture (10-14). In 1984, the first serum-free medium for long-term culture of these cells was developed by Hammond *et al.* (15). However, since most tumor cell lines do not grow in this medium, we have made modifications which result in long-term growth of normal human mammary epithelial cells as well as the growth of tumor cell lines (9). In this medium (DFCI-1), normal breast epithelial cells from mammoplasties have been grown at population-doubling times of approximately 20 h for 15-20 passages before senescence. Unlike the immortal HBL-100 milk epithelial cell line frequently used as a normal control (16), these cells are not transformed. The tumor cell lines examined in this report were originally established in media with 10% FCS (Ref. 17; see references therein); they grow for several passages but not indefinitely in DFCI-1 medium. In this paper, we have compared normal cells grown in DFCI-1 medium with tumor cell lines grown in either α -MEM plus 10% FCS or DFCI-1 medium.

We have analyzed the expression of a series of genes known or suspected to be involved in mammary tumorigenesis. The EGF receptor gene was chosen because several studies have indicated an inverse relationship between the number of estrogen receptors and EGFR in breast tumor tissues (18-21). We have also assessed the expression of the estrogen-responsive *pS2* gene, which encodes a secreted polypeptide of unknown function (22), since high levels of its mRNA have been detected in approximately 90% of ER-positive breast tumor specimens, only rarely in ER-negative biopsies, and never in normal mammary tissue (23).

Other genes involved in growth regulation, including *erbB2* (24-27), *c-myc* (26, 28-30), and *Ha-ras* (31-34), are amplified and/or overexpressed in different subsets of breast tumor biopsies and have therefore been included in this study. In addition, we have examined expression of the genes for TGF α and TGF β , two peptide growth factors secreted by and probably involved in growth control in breast epithelium (6, 35-37).

MATERIALS AND METHODS

Cells and Culture Conditions. The three normal human mammary epithelial cell strains used in this study were derived from reduction mammoplasty specimens from different individuals. Strain 30N was developed in this laboratory following published procedures (38). The development and characterization of growth requirements for the 172 and 184 strains have been described (14, 39). For our experimental purposes, the three normal strains were grown in DFCI-1 medium, containing 1% FCS (9). Breast tumor cell lines T47D, MDA-MB231, BT-20, and ZR-75-1 [all from ATCC] and MCF-7 (Michigan Cancer Foundation) and Hs578T [H. Smith (40)] were cultured in α -MEM supplemented with 2 mM glutamine, 10 μ g/ml insulin, 1 mg/ml dextrose, 0.1 mM minimal essential amino acids, and 10% FCS unless otherwise indicated. Penicillin (100 units/ml) and streptomycin (100 μ g/ml) were present in both media. For gene expression studies in DFCI-1 medium, subconfluent tumor cell cultures growing in α -MEM + 10% FCS (above) were seeded (at 2×10^6 cells/p150 dish) in DFCI-

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³ The abbreviations used are: ER⁻, estrogen receptor negative; ER⁺, estrogen receptor positive; cDNA, complementary DNA; EGFR, epidermal growth factor receptor; TGF α , transforming growth factor α ; TGF β , transforming growth factor β ; FCS, fetal calf serum; α -MEM, α -minimal essential medium; SDS, sodium dodecyl sulfate; SSC, standard saline-citrate (1 \times SSC = 150 mM NaCl-300 mM sodium citrate; 1 \times SSPE, 150 mM NaCl-10 mM NaH₂PO₄-1 mM EDTA, pH 7.4).

1 medium. Cells were harvested 3 days later at 60–70% confluence and RNA isolated as described below.

RNA Isolation and Analysis. Cell monolayers approximately 50% confluent were lysed in 4 M guanidinium isothiocyanate and purified by centrifugation through a 5.7 M CsCl cushion (41). Total RNA was denatured and analyzed on a 1.3% agarose-2.2 M formaldehyde gel, and the RNA was electroblotted to Zetabind nylon filters (Bio-Rad). Filters were prehybridized for 2 to 4 h at 42°C in 1 M NaCl, 50% formamide, 10% dextran sulfate, 1% SDS, and 250 µg/ml sonicated salmon sperm DNA. Hybridization with ³²P-labeled probes [2×10^9 dpm/µg DNA (42)] was performed for 24–36 h at 42°C; the filters were washed for 30 min in 2× SSC/0.2% SDS at 25°C and for 1–2 h in 2× SSC-1% SDS at 65°C. Dot blot analysis was performed by spotting 2-fold serial dilutions of total RNA (denatured in 3.75% formaldehyde-4× SSC at 65°C for 15 min) onto Zetaprobe nylon filters using a Schleicher & Schuell dot blot manifold. Hybridization conditions were identical to those for the Northern blot analysis.

DNA Isolation and Southern Blot Analysis. High molecular weight DNA was isolated from cultured cells (43). Five µg of DNA were digested with the restriction enzyme *Bam*HI, fractionated on 0.7% agarose gels, and blotted onto nylon filters by the alkaline transfer procedure (44). Hybridization to ³²P-labeled probes was for 12–24 h at 65°C in 1 M NaCl, 1% SDS, 10% dextran sulfate, and 250 µg/ml denatured salmon sperm DNA. Filters were washed at 65°C in 2× SSPE-1% SDS for 30 min and 0.5× SSPE-1% SDS for 30 min–1 h before autoradiography.

Source of Hybridization Probes. The DNA probes used in the analysis are as follows: the *Eco*RI 1.35-kilobase fragment from pSP6C17N3 (45) is specific for TFGα; the *Clal*/*Eco*RI 1.4-kilobase fragment from pmcB122 (46) corresponds to the 3rd exon of *c-myc*; the *Eco*RI 1.8-kilobase fragment from HER64-1 (47) contains the 5' part of the EGFR cDNA; the 1.6-kilobase *Eco*RI internal fragment from the cDNA (7) recognizes *erbB2*; the 1-kilobase *Eco*RI fragment from pβAS (48) is specific for TGFβ; the 600-base pair *Sma*I fragment from pEJ6.6 (49) recognizes Ha-*ras*; the 570-base pair *Eco*RI-*Sa*II fragment from p6a1 (50) detects N-*ras* mRNA; the 400-base pair *Pst*I A fragment is at the 5' end of the cDNA for *pS2* (51); the 2.0-kilobase *Pst*I fragment from pA1 bearing the chicken cDNA recognizes β-actin (52).

EGF Receptor Binding and Scatchard Analysis. For EGF binding experiments, cells were grown to confluence in 35-mm 6-well dishes ($1.0\text{--}1.5 \times 10^6$ cells/well), then serum and growth factor deprived for 20 h. After 2 washings with F-12/α-MEM (1:1) salts containing 1% (w/v) bovine serum albumin, cells were incubated for 2 h at 37°C in 0.5 ml of the same buffer containing 2.5 ng/ml ¹²⁵I-EGF (Amersham; 170 µCi/µg) in the presence and absence of 10^{-7} M unlabeled EGF (Sigma; receptor grade). Following this incubation, the cells were washed twice with ice cold 150 mM NaCl and lysed in 0.5 ml 0.2 N NaOH. Radioactivity was determined in a Minaxi gamma counter with a counting efficiency of 55%. Specific binding is the difference between total binding and the number of counts bound in the presence of excess unlabeled EGF. Under these conditions, nonspecific binding is not greater than 5% of the total. Scatchard analysis was performed under similar conditions except that increasing amounts of ¹²⁵I-labeled EGF (0.05–10 ng/ml) were added to the incubations.

RESULTS

Normal Breast Cells Produce Higher Levels of EGF Receptor RNA than ER+ Breast Cancer Cell Lines. The expression of the EGF receptor gene was investigated by quantitating steady state levels of its RNA by Northern blot analysis of total cellular RNA. Fig. 1A (top) shows the gel electrophoretic profiles of normal (172 and 184, Lanes 1 and 2) and tumor (MCF-7, Lane 3) cell RNAs, hybridized with the indicated probes. The normal cells synthesize a 10.5-kilobase transcript of the EGF receptor gene which is not detected in the MCF-7 cells. Hybridization of the same filter to a chicken β-actin probe confirms the presence of equivalent RNA amounts in all lanes (Fig. 1A, bottom).

The analysis of other preparations of MCF-7 RNA using dot blots (Fig. 1B, Lane 4) or Northern hybridization (Table 1) shows that the MCF-7 as well as the ZR-75-1 (Lane 5) and T47D (Lane 7) cell lines, contain quantities of EGF receptor mRNA approximately 16-fold lower than those found in the three normal strains (Lanes 1–3). By contrast, the ER- MDA-MB231, Hs578T, and BT-20 cell lines (Fig. 1B, Lane 6; Table 1) produced much higher amounts of EGFR mRNA. The highest levels were found in BT-20 cells (Table 1), in which the gene is amplified (53, 54).

High EGFR mRNA Expression Is Not Due to Gross Alteration or Amplification of the Gene in Normal Cells. To determine whether high EGFR mRNA expression in the normal cells was a result of gene amplification, the organization and copy number of the EGF receptor gene in the normal breast cells was compared with MCF-7, BT-20, and A431 [adenocarcinoma cell line, where amplification of the EGFR gene was first described (47)]. No gross genomic rearrangements were observed by analysis of *Eco*RI, *Msp*I- [data not shown], or *Bam*HI-restricted total DNA [Fig. 2] using cDNA probes corresponding to 5' and 3' [data not shown] segments of the mRNA [pHER-A64-1 and A64-2, respectively (47)]. A *Bam*HI restriction fragment length polymorphism (≈3.9 kilobases) particular to the 184 normal strain was, however, noted (Fig. 2). The EGFR gene dosage in the normal cells is comparable to that detected in the MCF-7 line. The gene is amplified in the BT-20 and A431 lines approximately 7- and 12-fold, respectively, when compared to the 184 strain in DNA dot blot analyses (data not shown), in agreement with other studies (47, 54). Thus, the elevated expression of EGFR mRNA in the normal cells is not the result of a detectable change at the genomic level.

Increase of EGF-binding Capacity of the Normal Cells Relative to the Tumorigenic MCF-7 Cells. To confirm that the high levels of mRNA observed in the normal cells were translated into an active protein, we compared the EGF-binding capacity of the 184 strain with that of the MCF-7 cells, which express $1\text{--}3 \times 10^3$ receptors/cell (4, 5). As indicated in the two experiments shown in Table 2, EGF binding to normal 184 cells is 20- to 40-fold higher than to MCF-7 cells, irrespective of the final cell densities. These measurements were performed under conditions where maximal, saturable binding occurred for both cell types (i.e., 2 h at 37°C) with the EGF concentration determined by saturation binding studies (Fig. 3).

Scatchard analysis of the EGF binding data for the normal breast epithelial cells demonstrates the existence of $\approx 5.8 \times 10^4$ EGF receptor molecules/cell with a K_d of 0.96 nM ($r^2 = 0.97$) (Fig. 3, inset). Thus, the high mRNA expression of the EGFR gene in the normal mammary epithelial cells translates into a high level of EGF-binding proteins in these cells. This receptor number is comparable to those reported for some tumor lines which lack estrogen receptors (e.g., BT-20, MDA-MB231) (4, 5).

Equal Expression of *erbB2* by Normal and Tumor Cells. The steady state level of *erbB2* in the normal cell strains was not significantly different from that in the breast tumor cell lines examined here (Table 1). This contrasts with the observations of Kraus *et al.* (7) who reported *erbB2* overexpression in the ZR-75-1 cell line. In the SK-BR-3 tumor cell line, however, where the gene is amplified approximately 4- to 8-fold, we detect at least 50-fold higher *erbB2* mRNA levels than in the normal cell strains⁴ in agreement with Kraus *et al.* (7).

pS2 mRNA Is Detectable Only in ER-positive Tumor Cell

⁴ Unpublished results.

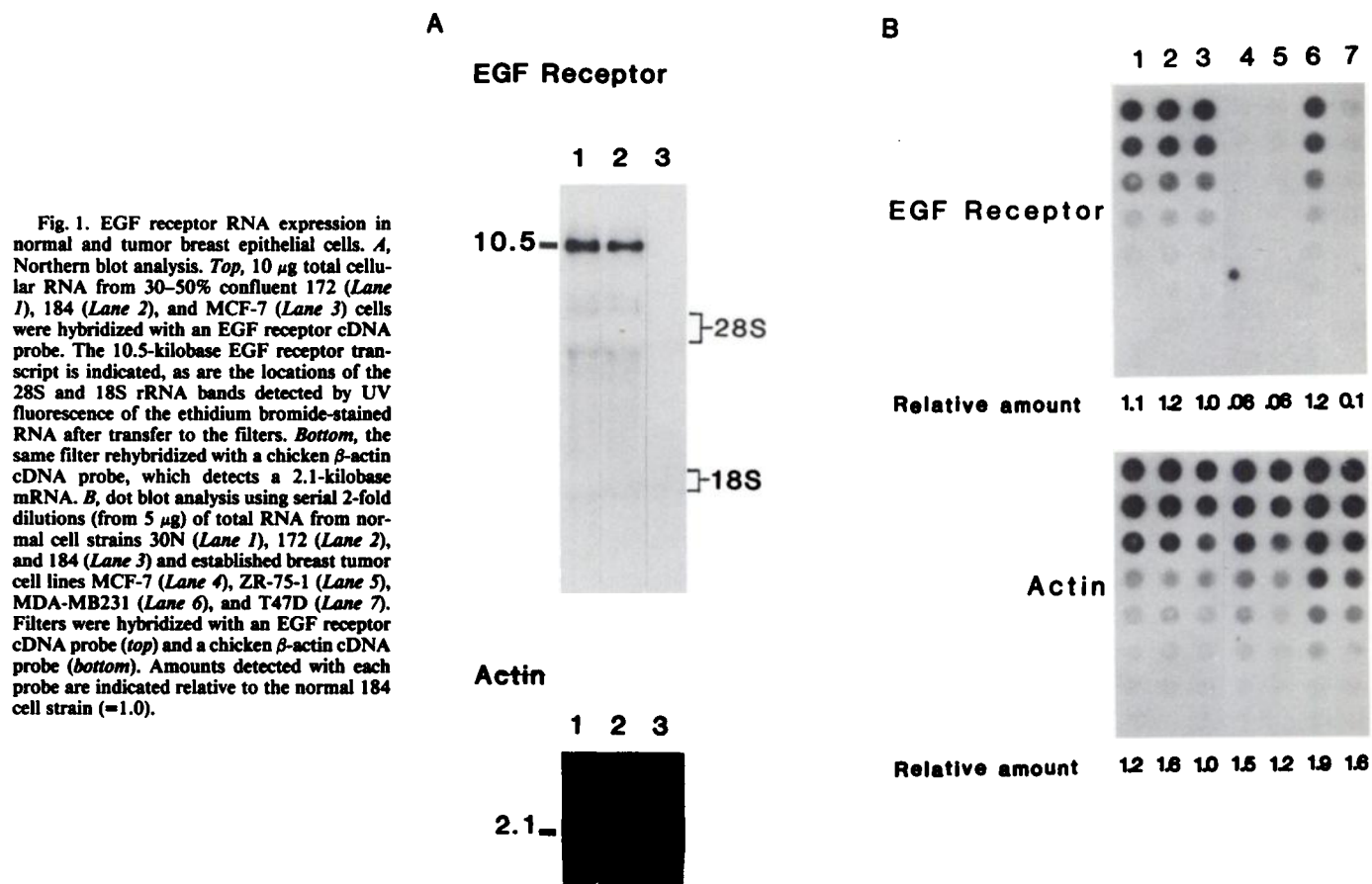


Fig. 1. EGF receptor RNA expression in normal and tumor breast epithelial cells. *A*, Northern blot analysis. *Top*, 10 μ g total cellular RNA from 30–50% confluent 172 (Lane 1), 184 (Lane 2), and MCF-7 (Lane 3) cells were hybridized with an EGF receptor cDNA probe. The 10.5-kilobase EGF receptor transcript is indicated, as are the locations of the 28S and 18S rRNA bands detected by UV fluorescence of the ethidium bromide-stained RNA after transfer to the filters. *Bottom*, the same filter rehybridized with a chicken β -actin cDNA probe, which detects a 2.1-kilobase mRNA. *B*, dot blot analysis using serial 2-fold dilutions (from 5 μ g) of total RNA from normal cell strains 30N (Lane 1), 172 (Lane 2), and 184 (Lane 3) and established breast tumor cell lines MCF-7 (Lane 4), ZR-75-1 (Lane 5), MDA-MB231 (Lane 6), and T47D (Lane 7). Filters were hybridized with an EGF receptor cDNA probe (*top*) and a chicken β -actin cDNA probe (*bottom*). Amounts detected with each probe are indicated relative to the normal 184 cell strain (=1.0).

Table 1 Expression of protooncogenes and growth factors in normal and tumor-derived breast epithelial cells

Northern blots were hybridized with the indicated probes (see Figs. 1 and 2) and specific bands were quantitated by densitometric scanning. Values are expressed relative to the amounts detected in normal 184 cells (=10) and are the average of results from 3 or more RNA preparations. Data for DFCI-1 medium growth conditions, given in parentheses, are averages from at least two separate experiments. Equivalent RNA loading was verified by reprobing the blots with β -actin and rare corrections (less than 2-fold) are reflected in the values given. Standard errors were within 10–20%.

| | EGFR | TGF α | TGF β | c-myc | erbB2 ^a | pS2 | Ha-ras ^b | N-ras |
|---------------|----------------------|--------------|-------------|---------|--------------------|-----------------|---------------------|---------------------|
| Normal | | | | | | | | |
| 184 | 10 | 10 | 10 | 10 | 10 | UD ^c | 10 | 10 |
| 172 | 12 | 14 | 14 | 10 | 11 | UD | 9 | 13 |
| Tumor | | | | | | | | |
| ER- | | | | | | | | |
| MDA-MB231 | 6 | 9 | 9 | 3 | 10 | UD | 18 | 25 |
| Hs578T | 6 | UD | 11 | 9 | 5 | UD | 30 | 25 |
| BT-20 | 40 (30) ^d | 3 (3) | 6 (5) | 4 (13) | 16 | UD (UD) | 9 (7) | 15 |
| ER+ | | | | | | | | |
| MCF-7 | <0.5 (0.5) | 1–2 (2) | 6 (6) | 2–3 (5) | 12 | + (+) | 8 (4) | 90–150 ^d |
| ZR-75-1 | <0.5 (<0.5) | 2 (3) | 8–9 | 3 (5) | 19 (15) | + (+) | 10 | 60–100 ^d |
| T47D | <0.5 | 2 | UD | 2 | 10 | UD | 11 | 7 |

^a 4.6-kilobase transcript on Northern blots.

^b 1.2-kilobase transcript on Northern blots.

^c UD, undetectable.

^d Amplified gene.

Lines. Analysis of the normal and tumor cell lines for expression of the estrogen-inducible pS2 gene revealed hybridizable mRNA in only two cell lines: MCF-7 cells, where its presence was initially detected (55); and in ZR-75-1 cells. None of the other established lines, including the ER+ T47D cells, or the normal epithelial strains produced this mRNA (Fig. 4, pS2 probe).

mRNAs for Transforming Growth Factors α and β Are Produced by Normal Mammary Epithelial Cell Strains. TGF α mediates its growth-promoting effects through the EGF receptor (56). Its production by breast carcinoma cell lines has been documented (6) and led us to investigate the relative expression

of the gene in normal and tumor cells. In the MDA-MB231 cells (Fig. 4, TGF α probe, Lane 7; Table 1) mRNA levels were close to those in the three normal strains (Lanes 1–3), whereas in Hs578T cells (Lane 8) no TGF α mRNA was detected. Lower amounts of TGF α mRNA were quantitated in the ER+ tumor lines, MCF-7, ZR-75-1, and T47D (Lanes 4–6).

In contrast to the results with TGF α , no significant differences in TGF β mRNA expression were observed between the normal cell strains and most of the tumor lines (Fig. 4, TGF β probe; Table 1). The sole exception is the T47D line, which does not produce detectable amounts of TGF β mRNA (Lane

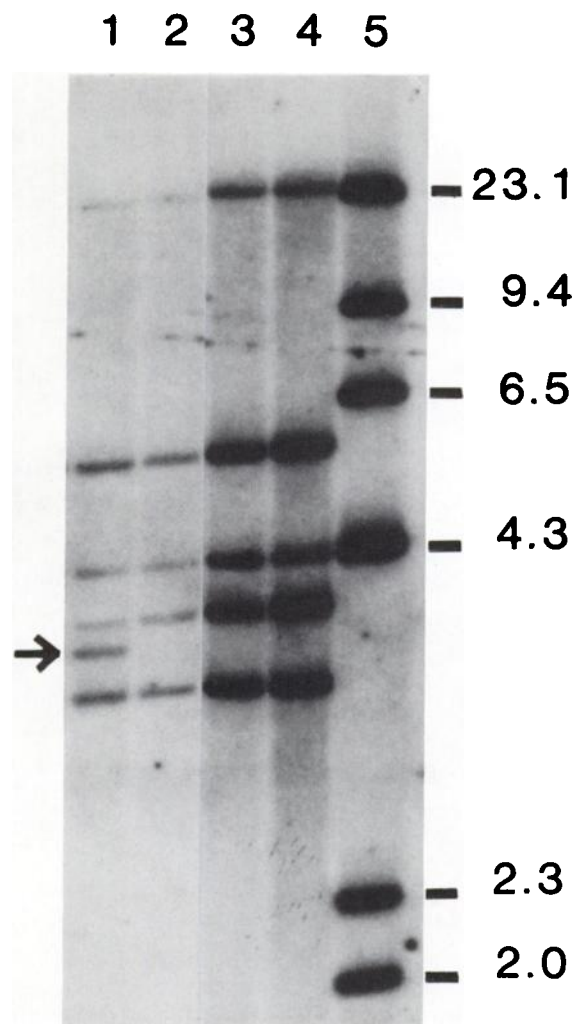


Fig. 2. Southern analysis of the EGFR gene in normal and tumor breast epithelial cells. Five μg of DNA from normal 184 (Lane 1) and tumor MCF-7 (Lane 2), BT-20 (Lane 3), and A431 (Lane 4) cells were digested with *Bam*HI and analyzed by Southern blotting as described ("Materials and Methods"). Hybridization with the EGFR cDNA probe is shown. Markers are a ^{32}P -labeled λ HindIII digest (Lane 5). Arrow, *Bam*HI restriction fragment length polymorphism at ≈ 3.9 kilobases observed in the 184 strain.

Table 2. EGF binding to 184 normal and MCF-7 tumor cells

Determination of EGF binding to 184 and MCF-7 cells at the indicated final cell densities was performed by incubation for 2 h at 37°C with ^{125}I -EGF concentrations of 12.5 ng/ml (Experiment 1) or 7.5 ng/ml (Experiment 2) in the presence and absence of 10^{-7} M unlabeled EGF. The difference between triplicate assays for total and nonspecific binding is given.

| Experiment | Cell type | Cells/35 mm | Specific ^{125}I -EGF binding (fmol/ 10^6 cells) |
|------------|-----------|-------------------|---|
| 1 | 184 | 1.6×10^6 | 99.7 |
| | MCF-7 | 1.2×10^6 | 2.7 |
| 2 | 184 | 5.6×10^5 | 95.6 |
| | MCF-7 | 3.4×10^5 | 2.4 |
| | MCF-7 | 1.2×10^6 | 2.4 |
| | MCF-7 | 1.8×10^6 | 5.0 |

6). These results are not surprising in view of reports demonstrating that the regulation of TGF β activity is posttranslational (37, 57).

N-ras Is Overproduced by Established Tumor Cell Lines When Amplified. The *N-ras* gene is overexpressed in the MCF-7 and ZR-75-1 lines compared to the normal strains (Fig. 4, *N-ras* probe; Table 1). Amplification of the *N-ras* gene approximately 10-fold in the MCF-7 (Ref. 58 and data not shown) and ZR-75-1 (data not shown) lines accounts for these increased levels.

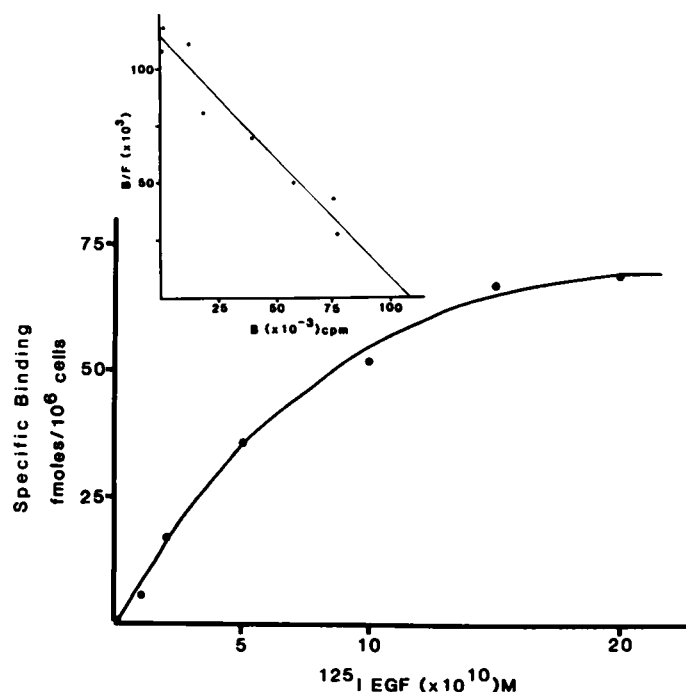


Fig. 3. Analysis of EGF binding in normal 184 cells. Cells were grown to confluency (1×10^6 /35-mm well) and incubated with increasing concentrations of ^{125}I -EGF (0.05–10 ng/ml) for 2 h at 37°C as described in "Materials and Methods." Specific binding is the difference (duplicate determinations) between total ^{125}I -EGF binding in the presence and absence of 10^{-7} M radioinert EGF. Inset, Scatchard plot of the data.

In contrast, *Ha-ras* mRNA levels are nearly identical in the normal strains and in the tumor lines (Table 1).

Expression of *c-myc* Is Lower in Tumor Cell Lines than in Normal Cells. Fig. 4 (*myc* probe) shows that *c-myc* steady state mRNA levels are somewhat higher in the normal strains (Lanes 1–3) than in the tumor lines (Lanes 4–8), and a similar result is shown quantitatively in Table 1. Thus, contrary to the overexpression of *c-myc* that is associated with gene amplification in biopsy samples (26, 28, 29), we have found that *c-myc* mRNA levels are generally lower than normal in the tumor lines examined here.

Effect of Different Growth Media on Gene Expression. In order to ascertain whether the differential gene expression observed in the comparisons of normal and tumor cells was due simply to the different culture media in which the cells were grown, mRNA levels were examined in cells grown in the low serum-containing DFCI-1 medium. The MCF-7 cell line can be successfully cultured in DFCI-1 medium for periods of 1 to 2 weeks at population-doubling times (≈ 25 h) similar to those in the standard medium containing 10% FCS (9). The differential expression of EGF receptor, TGF α , and *pS2* genes observed when MCF-7 cells were cultured in the standard medium was maintained when these tumor cells were grown for 2–3 population doublings in DFCI-1 medium (Table 1). Thus, constituents in the DFCI-1 medium did not alter the expression of these genes under short-term culture conditions. Similar results were obtained in comparisons of the growth of the ZR-75-1, BT-20, and MDA-MB231 lines in DFCI-1 medium (Table 1). However, growth in DFCI-1 medium increased the amount of EGF receptor mRNA in the MCF-7 cells to detectable levels and augmented *c-myc* expression in BT-20 cells. The components of DFCI-1 medium responsible for this effect are being investigated.

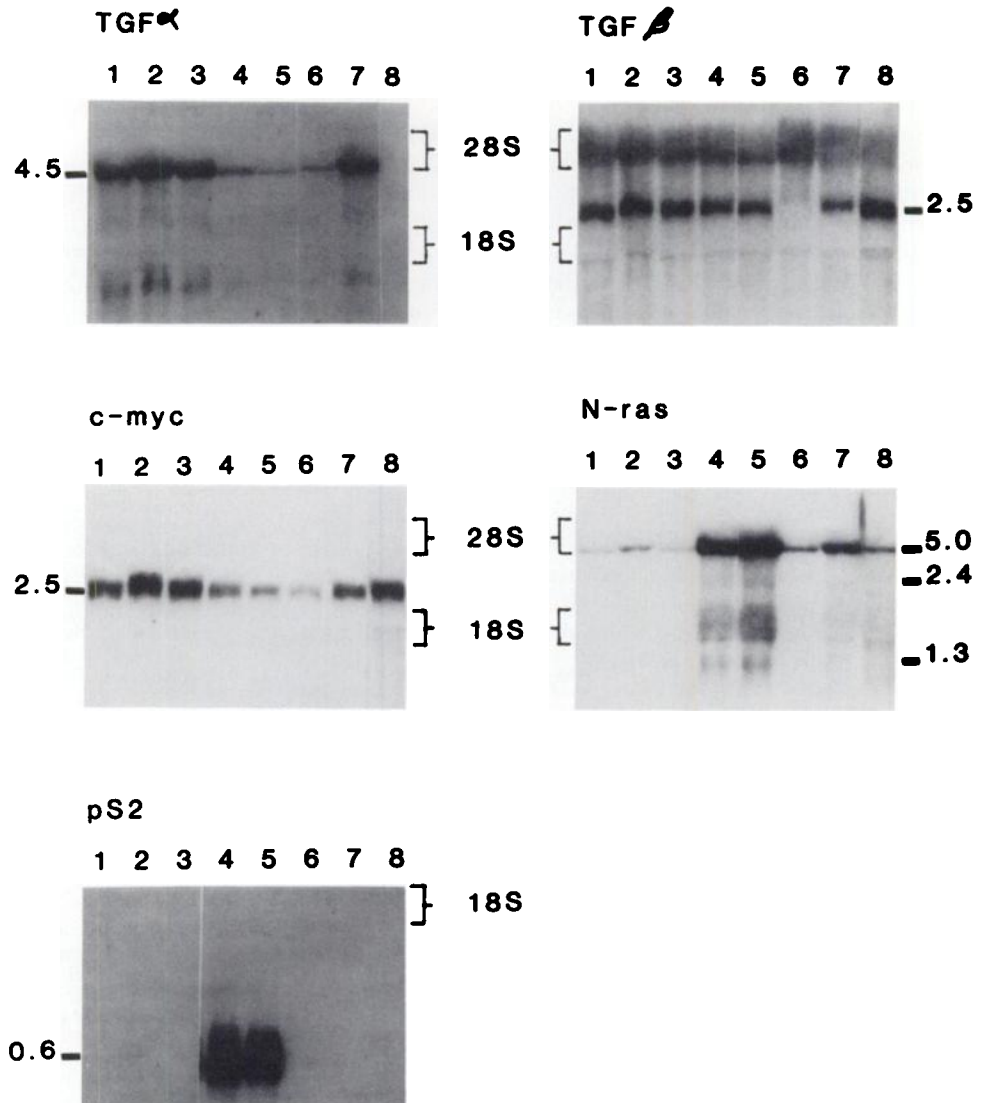


Fig. 4. Expression of TGF α , TGF β , c-myc, N-ras, and pS2 in normal and tumor-derived breast epithelial cells. Thirty μ g total cellular RNA from normal cell strains 30N (Lane 1), 172 (Lane 2), 184 (Lane 3), and established breast tumor cell lines MCF-7 (Lane 4), ZR-75-1 (Lane 5), T47D (Lane 6), MDA-MB231 (Lane 7), and Hs578T (Lane 8) were analyzed by Northern blotting with the indicated probes. Specific band sizes are indicated in the panels for each of the transcripts analyzed. Some cross-hybridization to either the 28S or 18S rRNA was observed for the TGF β and N-ras probes, respectively.

DISCUSSION

We have compared normal and tumor-derived human breast epithelial cells for their expression of several genes, whose altered structure or expression has been associated with the initiation and/or progression of breast cancer. Although some studies of gene expression in tumor cell lines have already been reported (8, 59–61), this is the first in which ER+ and ER– breast tumor lines have been compared with normal breast epithelial cells growing in long-term cell culture and under the same culture conditions.

Normal Cells Synthesize High Levels of the mRNAs for the EGF Receptor and TGF α . We have found that the expression of the mRNA for the EGFR is much lower in the ER+ tumor cell lines than in ER– tumor cells. This result is consistent with previous EGF-binding studies which quantitated the number of EGF receptors in breast tumor cell lines (4, 5). In addition, our data show that the production of EGFR mRNA and its encoded protein in normal cells is quantitatively similar to that in ER– tumor lines. Scatchard analysis of EGF binding in the normal cells indicated the presence of approximately 6×10^4 receptors/cell, with a dissociation constant of 1 nM. This is the lower of the two affinity forms of the EGF receptor measurable in breast tumor epithelial cells (4, 5, 62). According to the report of Roos

et al. (62), this form is generally observed in cells with high total receptor number (*e.g.*, MDA-MB231, BT-20); therefore its predominance in the normal cells may be correlated with the high total binding in these cells. Since the normal cells studied here do not have detectable quantities of estrogen receptors,⁴ our results suggest that high EGFR expression in the absence of gene amplification may be more related to ER–negative status than to tumorigenicity.

The tumor growth factor TGF α is produced by normal (63) as well as tumor cell populations (64) and acts by binding to the EGF receptor (56). The normal breast cells analyzed here produce higher levels of TGF α mRNA than the tumor cell lines. Activity measurements on some of the same tumor cells by Dickson *et al.* (6) correlate well with the relative amounts of TGF α mRNA detected in our study, with the exception of estrogen-stimulated MCF-7 cells, where the activity was comparable to that found in the MDA-MB231 line (6). In agreement with our results, Peres *et al.* (8) have recently shown that levels of TGF α mRNA in the MDA-MB231 cell line are greater than those detected in the MCF-7, T47D, and ZR-75-1 cells. Our results that normal breast cells produce higher levels of TGF α mRNA than most of the tumor lines analyzed, regardless of ER status suggest that no direct relationship exists between TGF α production and tumorigenicity. Similar conclusions have

been drawn by Perroteau *et al.* (65) who quantitated the levels of immunoreactive TGF α present in breast tissue extracts and found no significant correlation with the pathological state of the tissue.

ER+ Tumor Cell Lines Express *pS2* but Do Not Overproduce *Ha-ras* mRNAs. The estrogen-responsive *pS2* gene expression in cultured cells correlates well with the results from immunocytochemical analyses, which demonstrated *pS2* expression in 88% of ER+ breast tumors and failed to detect the *pS2* product in normal breast tissue (23). As predicted from these studies, we have found that *pS2* mRNA is absent in the normal as well as in the hormone-independent tumor cell lines analyzed but is very high in two of the estrogen-dependent lines.

Contrary to the indications of De Bortoli *et al.* (33), who quantitated 10-fold higher amounts of Ha-ras p21 protein in breast tumors (70% were ER+) than in normal breast tissue, we have observed no significant differences in the mRNA levels produced by normal and ER+ tumor cells in culture. Only slight increases in Ha-ras expression in the ER- tumor lines were detected. Our results are consistent with those of Ohuchi *et al.* (66), who concluded that enhanced *ras* expression is probably not involved in the maintenance of the transformed phenotype of breast tumor tissues.

Overexpression of EGFR, *erbB2*, and N-ras Occurs Concomitant with Gene Amplification in These Tumor Cell Lines. Increased EGFR gene expression relative to the normal cell strains was observed only in the BT-20 tumor cell line, in which the gene copy number is 7-fold amplified. Similarly, overexpression of the N-ras gene correlates with its overexpression in two of the ER+ tumor lines studied here. However, the significance of N-ras amplification for the tumorigenic progression of breast cancer has been questioned, since no tumor biopsy specimens have yet been reported with amplified N-ras sequences (34, 58).

A role for *erbB2* in breast cancer was proposed in view of studies that correlated *erbB2* gene amplification (25) and high protein levels (27) with a poor clinical prognosis. Expression of the *erbB2*/HER-2/*neu* oncogene is elevated in approximately 30% of breast cancer biopsies (24, 26-28). In cell culture, we have seen that normal cells express as much *erbB2* mRNA as the tumor cell lines (Table 1), none of which have an amplified *erbB2* gene (7).⁴ Thus, the tumorigenic phenotype of the cell lines analyzed here is not dependent upon high *erbB2* expression.

This comparison of normal and tumor-derived mammary epithelial cells grown in culture has revealed no consistent differences between the EGFR, TGF α , TGF β , *erbB2*, and N-ras mRNA levels of ER- tumorigenic and normal cells except when the corresponding genes were amplified. In the ER+ tumor lines, however, expression of EGF receptor and TGF α mRNAs was markedly lower than in the normal cells, and *pS2* was expressed. The molecular basis for differences between ER+ and ER- cells is a fundamental problem in breast cancer research. This study emphasizes the importance of comparative studies of normal and tumor cells under similar growth conditions for future investigations. Ideally such analyses should be carried out with primary tumor cells grown in culture. Work directed toward this goal is in progress in our laboratory.

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