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Overexpression of the EGF receptor-related proto-oncogene *erb*B-2 in human mammary tumor cell lines by different molecular mechanisms

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Amplification of the *erb*B/EGF receptor and a structurally related gene, designated *erb*B-2, have previously been detected in a variety of human tumors. In a series of human mammary tumor cell lines, analysis of transcripts of these genes revealed elevated levels of one or the other in more than 60% of tumors analyzed. Eight cell lines demonstrated *erb*B-2 mRNA levels ranging from 4- to 128-fold above those of normal controls. *erb*B-2 expression was evaluated in comparison to the expression level of actin observed in these cell lines. There was no evidence of an aberrantly sized *erb*B-2 transcript in any of these lines. Immunoblot analysis indicated elevation in levels of the 185-kd product of the *erb*B-2 gene expressed by these cells. In four lines *erb*B-2 gene amplification in the absence of an apparent gene rearrangement was demonstrated. In a representative cell line of this type, SK-BR-3, the amplified *erb*B-2 gene copies were located in an aberrant chromosomal location. Four additional cell lines, which demonstrated 4- to 8-fold overexpression of *erb*B-2 mRNA, did not exhibit gene amplification. In a representative cell line of this type ZR-75-1, an apparently normal chromosomal location was found for the *erb*B-2 gene. Our findings indicate that overexpression of the *erb*B-2 gene in mammary tumor cell lines is frequent and associated with different genetic abnormalities.

Key words: *erb*B-2/gene amplification/growth factor receptor/mammary neoplasia/overexpression

Introduction

Cellular genes encoding effector molecules of growth regulation have been linked to the neoplastic process based on their homology to retroviral oncogenes. The c-*sis* proto-oncogene encodes a chain of platelet-derived growth factor (PDGF) (Doolittle *et al.*, 1983; Waterfield *et al.*, 1983), the v-*erb*B oncogene has been shown to encode a truncated form of the epidermal growth factor (EGF) receptor (Downward *et al.*, 1984), and the c-*fms* proto-oncogene product is related to the receptor for mononuclear phagocyte growth factor ($CSF\text{-}1_R$) (Sherr *et al.*, 1985). More recently, we and others (King *et al.*, 1985a; Schechter *et al.*, 1985; Semba *et al.*, 1985; Coussens *et al.*, 1985) have identified a second cellular analogue of v-*erb*B in the human genome. This gene, designated *erb*B-2, is related to but distinct from the gene encoding the *erb*B/EGF receptor (EGFR). The predicted amino acid sequence of the *erb*B-2 gene reveals the structural features of a growth factor receptor molecule with close similarity to the EGF receptor (Coussens *et al.*, 1985; Yamamoto *et al.*, 1986a), including a cysteine-rich extracellular domain, transmembrane region and a highly conserved tyrosine kinase domain.

Gene alterations affecting EGFR and *erb*B-2 occur in tumor cells. A dominant cellular transforming gene activated by point mutation in chemically induced rat neuroblastomas (Shih *et al.*, 1981), *neu*, is likely to be the rat homologue of human *erb*B-2 based on comparative nucleotide sequence analysis and chromosomal localization (Schechter *et al.*, 1985; Coussens *et al.*, 1985; Yamamoto *et al.*, 1986a; Bargmann *et al.*, 1986a). In human glioblastoma, amplification and rearrangement of the EGFR gene result in extensive expression of abnormal as well as normal-sized mRNAs (Libermann *et al.*, 1985). In addition, amplification without rearrangements affecting EGFR mRNA size is frequently found in cells derived from squamous cell carcinomas (Yamamoto *et al.*, 1986b) and in two distinct mammary carcinoma cell lines (King *et al.*, 1985b; Filmus *et al.*, 1985).

Gene amplification of *erb*B-2 has been identified in a primary mammary adenocarcinoma (King *et al.*, 1985a), as well as in a salivary gland adenocarcinoma (Semba *et al.*, 1985). These findings have suggested the possibility that *erb*B-2 overexpression may contribute to neoplastic growth (King *et al.*, 1985a; Semba *et al.*, 1985).

For this study, we investigated the expression of *erb*B-2 and EGFR in 16 human mammary tumor cell lines. Our results indicate frequent overexpression of these proto-oncogenes that are related to growth factor receptors. Furthermore, analysis of the *erb*B-2 gene locus in these cell lines demonstrates that enhanced *erb*B-2 expression can occur in the presence or the absence of gene amplification, suggesting that different molecular mechanisms result in overexpression of normal size *erb*B-2 mRNA in mammary tumor cells.

Results

Isolation of erbB-2 complementary DNA

To allow a comprehensive analysis of *erb*B-2 mRNA and gene structure we isolated cDNAs with a complexity of over 4.5 kb from the mRNA (Figure 1A). An oligo (dT) primed normal human fibroblast cDNA library (Okayama and Berg, 1983) was screened with a 0.8 kbp *Acc*I DNA fragment from a genomic clone of *erb*B-2 (King *et al.*, 1985a). The largest plasmid obtained, pMAC137, carried a 2-kbp insert comprising 1.5 kbp of 3′ coding information and 3′ untranslated sequence. The remaining coding information upstream was obtained from three phage clones, λMAC30, λMAC10′ and λMAC14-1, identified in a randomly primed MCF-7 cDNA library (Walter *et al.*, 1985; Figure 1A). Nucleotide sequence analysis and restriction mapping of the entire cDNA indicated that its structure was the same as an isolate from normal human placenta (Coussens *et al.*, 1985).

Overexpression of erbB-2 or EGFR proto-oncogenes in human mammary tumor cell lines

To assess the role of *erb*B-2 in human mammary neoplasia we compared the mRNA of 16 mammary tumor cell lines to normal human fibroblasts, M413, and a human mammary epithelial cell line, HBL100. Increased expression of an apparently nor-

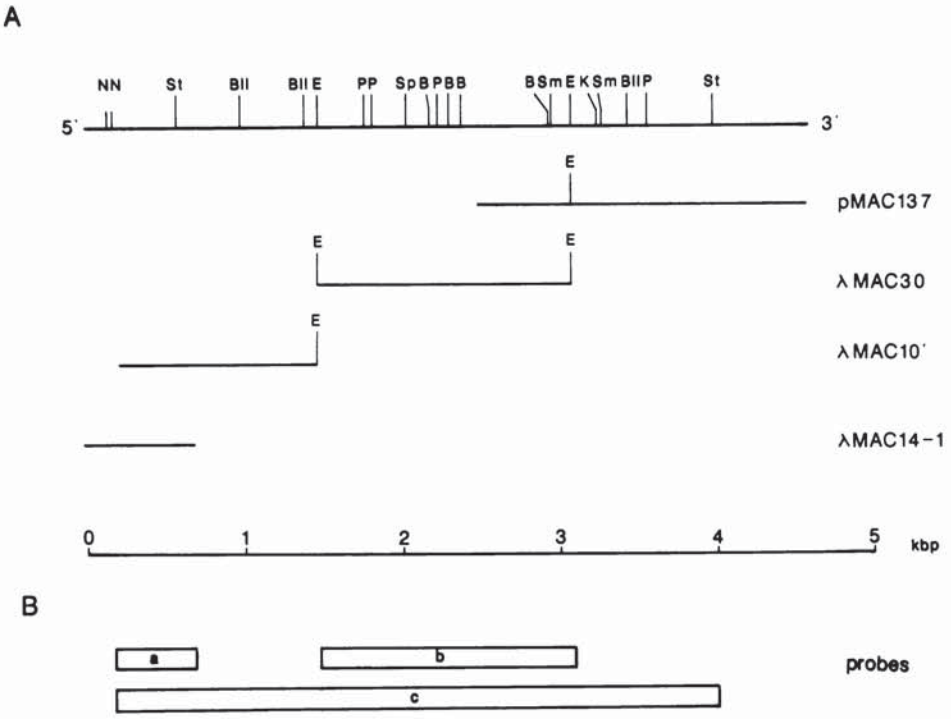

Fig. 1. (A) Isolation and restriction mapping of *erb*B-2 cDNA. Clone pMAC137 was isolated from an oligo (dT) primed normal human fibroblast cDNA library (Okayama and Berg, 1983). Clones λMAC30, λMAC 10′ and λMAC14-1 were subsequently obtained from a randomly primed MCF-7 cDNA library (Walter *et al.*, 1985). Restriction sites: B = *Bam*HI, BII = *Bst*EII, E = *Eco*RI, N = *Nco*I, P = *Pst*I, Sm = *Sma*I, Sp = *Sph*I and St = *Stu*I. **(B)** cDNA probes used in hybridization analysis.

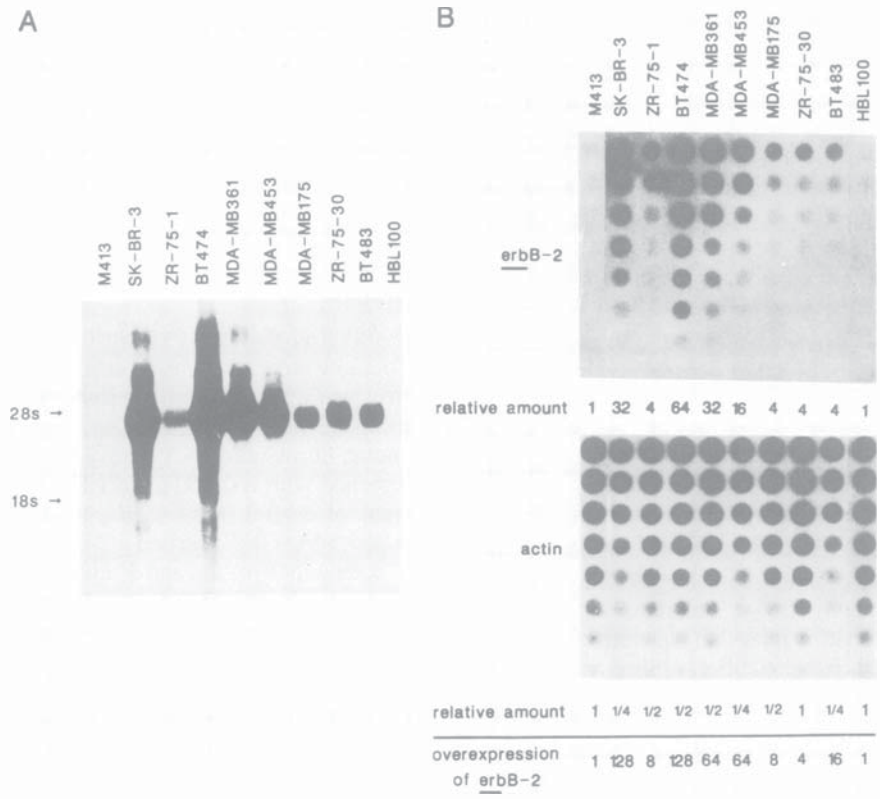

Fig. 2. Overexpression of *erb*B-2 in human mammary tumor cell lines. **(A)** Northern blot analysis. Total cellular RNA (10 μg) of mammary tumor cell lines, normal human fibroblasts M413 and HBL100 was hybridized with a cDNA probe derived from the 5′ end of the *erb*B-2 coding region (Figure 1B, probe a). M413 and HBL100 cells contain *erb*B-2 specific mRNA detectable after longer autoradiographic exposures. **(B)** Quantitation of *erb*B-2 mRNA levels. Serial 2-fold dilutions of total RNA were applied to nitrocellulose. Replicate filters were hybridized with either a *erb*B-2 cDNA probe (Figure 1B, probe b) or human β-actin which served as control for RNA amounts present on the nitrocellulose filter. Relative amounts detected with each probe are indicated in

Table I. Overexpression of *erb*B-2 and EGFR proto-oncogenes in human mammary neoplasia

	*erb*B-2		EGFR	
	Overexpression of mRNA[a]	Gene amplification	Overexpression of mRNA[a]	Gene amplification
M413	1	1	1	1
HBL100	1	1	1	1
MCF-7	1	1	<1	1
SK-BR-3	128	4–8	1	1
BT474	128	4–8	1	1
MDA-MB361	64	2–4	1	1
MDA-MB453	64	2	<1	1
ZR-75-1	8	1	1	1
ZR-75-30	4	<1	<1	1
MDA-MB175	8	1	<1	1
BT483	8	<1	<1	1
BT20	1	1	16	4–8
MDA-MB468	1	–	32	32

[a]Overexpression above normal fibroblast and HBL100.

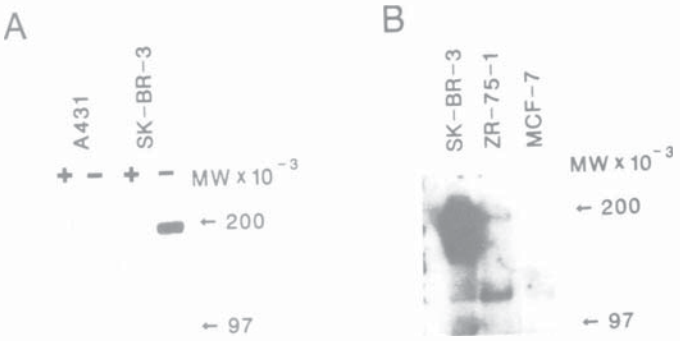

Fig. 3. Elevated *erb*B-2 protein levels in mammary tumor cell lines. 40 μg total cellular protein was separated by electrophoresis and transferred to nitrocellulose filters. The *erb*B-2 protein was detected with an antipeptide antibody coupled to ^{125}I protein A. The specificity of antibody detection was determined by pre-incubation of the antibody with excess amounts of peptide prior to immunodetection. (+) preincubation with peptide, (–) no peptide. In panel **B**, nonspecific bands at 110 kd are observed in longer exposures of peptide-blocked immunoblots (panel **A**).

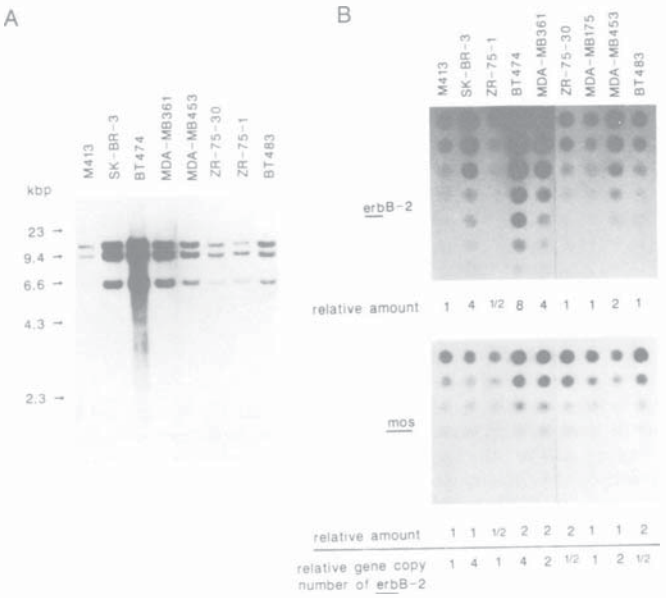

Fig. 4. Gene amplification of *erb*B-2 in mammary tumor cell lines. **(A)** Southern blot analysis. For each lane 10 μg genomic DNA were restricted with *Xba*I and hybridized with a probe comprising the entire coding region of *erb*B-2. *Hin*dIII restriction fragments of lambda DNA served as mol. wt standards. **(B)** DNA dot-blot analysis. Genomic DNA (10 μg) digested with *Eco*RI was applied in serial 2-fold dilutions to nitrocellulose filters. Filters were hybridized either with *erb*B-2 (Figure 1B, probe b) or *mos*, which served as a control for DNA amounts applied to replicate nitrocellulose filters. Gene copy numbers of *erb*B-2 relative to M413 indicate the minimal extent of gene amplification detected in DNA from mammary tumor cell lines.

mal size 5-kb transcript was detected in 8 of 16 tumor cell lines, when total cellular RNA was subjected to Northern blot analysis. Figure 2A shows the results using a cDNA probe comprising the coding sequences of the amino-terminal extracellular domain of *erb*B-2 (Figure 1B, probe a). These results of overexpression of normal-sized mRNA were confirmed by hybridization of poly(A)$^+$ selected RNA using several *erb*B-2-specific probes comprising coding information for the transmembrane and tyrosine kinase domains. An aberrantly sized *erb*B-2 mRNA was not detected in any of the cell lines analyzed.

To quantitate more precisely the amount of *erb*B-2 transcript in eight mammary tumor cell lines which overexpress *erb*B-2, serial 2-fold dilutions of total cellular RNA were subjected to dot blot analysis using human β actin as a control for the amount of RNA applied to the nitrocellulose filters. As shown in Figure 2B, the highest levels of *erb*B-2 mRNA, which ranged from 64- to 128-fold over that of our controls, were observed in the cell lines MDA-MB453, SK-BR-3, MDA-MB361 and BT474. Moreover, *erb*B-2 mRNA levels were increased 4- to 8-fold in four cell lines including BT483, MDA-MB175, ZR-75-30 and ZR-75-1 (Table I).

To determine if the overexpression of *erb*B-2 mRNA resulted in a steady state increase of its encoded gene product, we developed a specific immunoblot assay. Antisera were raised against a synthetic peptide whose sequence corresponded to a portion of the putative *erb*B-2 tyrosine kinase domain. As this region is partially conserved between the encoded proteins of the EGFR and *erb*B-2 genes, we tested its specificity using A431 and SK-BR-3 cell lines which overexpress EGFR or *erb*B-2 mRNA, respectively. As shown in Figure 3A, a specific band of ~185 kd was detected in extracts of SK-BR-3 but not in A431 cells. This band was not detected when the antibody was pre-incubated with the synthetic peptide corresponding to its antigen. The human *erb*B-2 and rat *neu* products have been reported to be glycoproteins of 185 kd (Akiyama *et al.*, 1986; Stern *et al.*, 1986).

To estimate the relative amounts of *erb*B-2 protein in different mammary tumor cell lines, immunoblot analysis was conducted using equivalent amounts of total cellular protein. As shown in Figure 3B, an intense band of protein was detected in extracts of SK-BR-3 and a less intense but readily detectable band in extracts of MCF-7, a mammary tumor cell line, that did not display overexpression of *erb*B-2 mRNA. We interpret these results to indicate that substantially more *erb*B-2 protein is found in both SK-BR-3 and ZR-75-1 than in MCF-7 cells where the amount of protein escapes the sensitivity of the assay. Dilution experiments suggest that SK-BR-3 contains between 5- and 10-fold more *erb*B-2 protein than does ZR-75-1 (data not shown).

We also analyzed total cellular RNAs of the same mammary tumor cell lines for evidence of EGFR receptor mRNA overex-

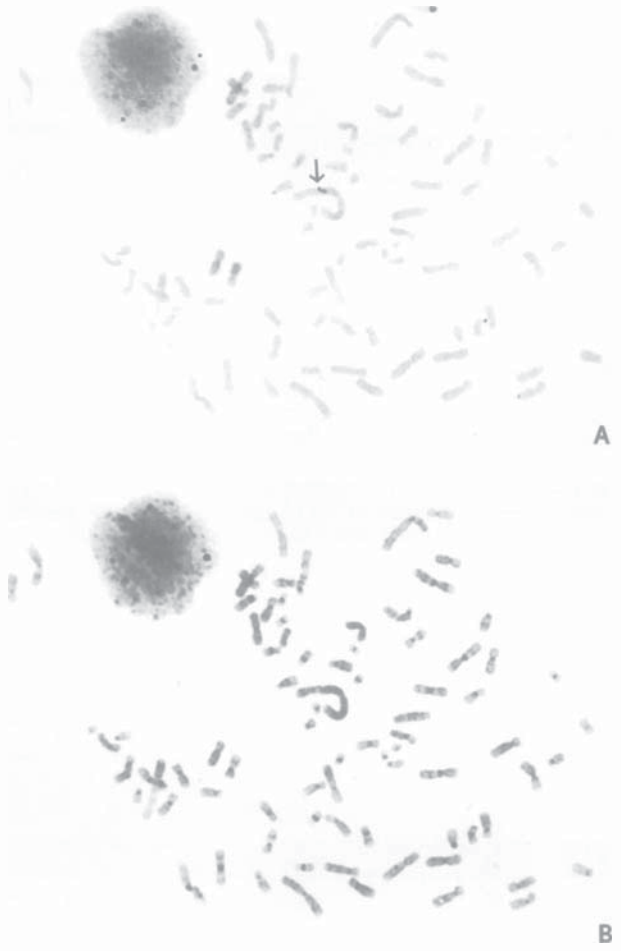

Fig. 5. Representative metaphase from the SK-BR-3 carcinoma cell line after *in situ* hybridization with an *erb*B-2 cDNA probe, autoradiography and G-banding. **(A)** A chromosome spread exhibiting silver grains on a distinctive, highly rearranged chromosome (arrow). **(B)** The same spread after trypsin/EDTA treatment to produce G-bands. Detailed G-banding analysis did not indicate that the site of hybridization contains a homogeneously stained region (HSR).

mRNA were observed in BT20 and MDA-MB468. These two cell lines have previously been shown to contain amplified EGFR genes. EGFR transcripts were elevated 16-fold in BT20 and 32-fold in MDA-MB468 above the level seen in normal human fibroblasts as determined by RNA dot-blot analysis (Table I).

Genetic abnormalities associated with elevated erbB-2 expression

To investigate alterations of the *erb*B-2 gene associated with mRNA overexpression, we examined *Xba*I-restricted high mol. wt DNA by Southern blot analysis using a probe comprising the entire coding sequence of *erb*B-2 (Figure 1B, probe c). The normal restriction pattern of *Xba*I fragments was detected in all DNA samples analyzed indicating that gene rearrangements in proximity of the *erb*B-2 coding region did not occur in these cell lines. When compared with normal human fibroblast DNA (Figure 4A, lane 1) the *erb*B-2-specific *Xba*I restriction fragments appeared clearly amplified in the cell lines SK-BR-3, BT474 and MDA-MB361 (Figure 4A, lanes 2–4). Similar results were obtained with restriction enzymes *Eco*RI and *Sac*I (data not shown).

Quantitation of *erb*B-2 gene copy number was accomplished using DNA dot-blot analysis. These studies revealed a 4- to 8-fold *erb*B-2 gene amplification in SK-BR-3 and BT474 relative to normal human DNA and a 2- to 4-fold *erb*B-2 gene amplification in MDA-MB361. In addition, a 2-fold *erb*B-2 gene amplification was identified for the cell line MDA-MB453 by DNA dot-blot analysis. Thus, gene amplification was associated with overexpression in the four tumor cell lines with the highest levels of *erb*B-2 mRNA (Table I). In contrast, gene amplification could not be detected by Southern blot analysis or DNA dot-blot analysis in the four tumor cell lines with *erb*B-2 transcript increased to intermediate levels.

To examine the nature of any chromosomal abnormalities associated with overexpression we used *in situ* hybridization to localize the *erb*B-2 gene in two cell lines which either do or do not contain amplified gene copies. The *erb*B-2 gene has been mapped in normal human cells on chromosome 17q11.2-22 (Schechter *et al.*, 1985; Coussens *et al.*, 1985; Fukushige *et al.*, 1986). Mammary tumor cell line SK-BR-3 contains 4- to 8-fold gene amplification and 128-fold overexpression of *erb*B-2 mRNA. In SK-BR-3, G-banding showed no copies of normal chromsome 17. *In situ* hybridization of the *erb*B-2 gene to these cells revealed accumulations of grains on two large abnormal marker chromosomes derived from complex rearrangements involving at least three chromosomes. An average of three grains was observed at each labelled site (Figure 5). These results indicate that the amplification of the *erb*B-2 gene occurs in an abnormal chromosomal location and is not associated with either a homogeneously stained region or double minute chromosomes, abnormalities diagnostic for gene amplification (Biedler and Spengler, 1976; Levan *et al.*, 1977). Mammary tumor cell line ZR-75-1 showed no evidence of gene amplification and an 8-fold overexpression of mRNA (Table I). Chromosome 17 was present in one or two copies per cell. Analysis of 50 cells after *in situ* hybridization with a *erb*B-2 cDNA probe revealed the largest accumulation of grains on chromosome 17 with 85% of these clustered on chromosome bands 17q11.2-21, the normal location of the *erb*B-2 gene. This indicates that overexpression of the *erb*B-2 gene can occur in the absence of detectable structural abnormalities of chromosome 17.

Mammary tumor cell lines overexpressing erbB-2 do not contain readily detectable transforming genes

In chemically induced rat neuroblastomas, a point mutation within the transmembranous domain activates the rat homologue of *erb*B-2, *neu*, to transforming activity readily detectable in the NIH/3T3 transfection assay (Bargmann *et al.*, 1986b). Previous transfection analysis of 21 mammary tumors and tumor cell lines did not reveal activation of *erb*B-2 as a transforming gene in human mammary neoplasia (Kraus *et al.*, 1984). To investigate whether an activating lesion similar to the rat *neu* gene was associated with the overexpression of *erb*B-2 in human mammary tumor cell lines, we transfected genomic DNA of these cell lines into mouse NIH/3T3 cells. Under conditions where high mol. wt DNA from the cell line T24 which is known to contain an activated H-*ras* oncogene induced 4–8 foci/plate, genomic DNA from eight mammary tumor cell lines which overexpress *erb*B-2 did not induce detectable morphological transformation (Table II).

Discussion

Analysis of 16 human mammary tumor cell lines for the EGF receptor and the related *erb*B-2 gene revealed frequently increased transcript levels of either member of this family of growth fac-

Table II. DNA transfection of human mammary tumor cell lines

Source of genomic DNA	FFU/plate[a]
T24 prep 1	32/4
T24 prep 2	19/4
T24 prep 3	16/4
SK-BR-3	0/12
BT474	0/12
MDA-MB361	0/2
MDA-MB453	0/4
ZR-75-1	0/8
ZR-75-30	0/4
BT483	0/4
MDA-MB175	0/8

[a]FFU/plate = focus-forming units/number of transfected plates. 30 μg high mol. wt DNA were coprecipitated with calcium phosphate and transfected onto NIH/3T3 fibroblasts as previously described (Wigler *et al.*, 1977). Genomic T24 DNA served as positive control for each assay.

of normal size EGF receptor gene transcripts is associated with gene amplification (King *et al.*, 1985b; Filmus *et al.*, 1985). Increased transcript levels of *erb*B-2 were detected in the presence and absence of gene amplification, indicating that *erb*B-2 overexpression in human mammary tumor cell lines can be caused by different molecular mechanisms. The absence of aberrantly sized *erb*B-2 mRNA in Northern blot analysis suggests that a normal size mRNA is overexpressed in mammary tumor cell lines rather than a rearranged form. Moreover, the fact that genomic DNA from these cell lines lacked the ability to transform NIH/3T3 cells by transfection indicates that point mutations similar to those that can activate the rat *neu* gene did not occur. These observations provide evidence that a structurally normal coding sequence of *erb*B-2 is overexpressed as mRNA in human mammary tumor cell lines. Protein analysis of representative samples with *erb*B-2 overexpression suggests that elevated *erb*B-2 transcript levels are translated into *erb*B-2 proteins.

Several lines of evidence link the overexpression of proto-oncogenes to the neoplastic process. The increased transcription of normal coding sequences of either the human c-*sis*/PDGF gene or the H-*ras* gene using a viral long terminal repeat promoter induces transformation of NIH/3T3 cells in culture (Chang *et al.*, 1982; Gazit *et al.*, 1984). Moreover, in human tumors *myc* or N-*myc* amplification correlates with increased malignancy (Kohl *et al.*, 1983; Schwab *et al.*, 1983; Nau *et al.*, 1984). Our results link *erb*B-2 overexpression to the neoplastic growth of mammary tumor cells.

Gene amplification of multidrug resistance genes is observed in cells selected for the ability to grow in media containing certian metabolic inhibitors (Alt *et al.*, 1978). There is evidence that overexpression of these genes can precede gene amplification in the development of multidrug resistance (Shen *et al.*, 1986). Our observation of elevated *erb*B-2 transcript levels in the presence and absence of gene amplification may reflect a similar pathway. Overexpression of *erb*B-2 may confer an initial selective growth advantage to the tumor cell and subsequent gene amplification cause a further step where the selective growth advantage is enhanced and stabilized. *erb*B-2 overexpression is consistently higher in those samples with gene amplification when compared with cell lines lacking gene amplification. Interestingly, the amount of overexpression per gene copy is approximately constant in the cell lines that overexpress *erb*B-2 (Table I). This suggests that deregulated *erb*B-2 expression due to different molecular mechanisms in mammary tumor cell lines may be part of a multistep process which confers selective growth advantage to a mammary tumor cell.

In all cell lines examined we detected no abnormalities of *erb*B-2 gene structure by Southern blot hybridization. Moreover, in ZR-75-1, in which overexpression occurs without gene amplification, the *erb*B-2 gene was located at its normal site on chromosome 17. These results indicate that mechanisms of deregulation are unlikely to involve the type of rearrangements responsible for activation of the *myc* gene in Burkitt's lymphoma (Taub *et al.*, 1982; Dalla-Favera *et al.*, 1983). Deregulation of *erb*B-2 gene expression may therefore involve subtle changes in *cis*-acting control sequences, changes involving transacting regulatory elements, or changes which enhance mRNA stability.

Abnormalities of *erb*B-2 and EGFR genes are not restricted to mammary tumor cells in culture, as we and others have identified *erb*B-2 or EGFR gene amplification in several samples of mammary tumor tissue (King *et al.*, 1985a; Yokota *et al.*, 1986, and unpublished observation). The precise action of growth factor receptor gene overexpression in the neoplastic process of mammary tumor cells has yet to be established. However, since tumorigenicity of mammary tumor cells can be enhanced by steroid hormones or polypeptide growth factors (Kasid *et al.*, 1985; Dickson *et al.*, 1986), it is tempting to hypothesize that the overexpression of polypeptide growth factor receptors increases tumorigenicity by stimulation of the same or similar growth signalling pathways. Forty percent of mammary tumor cell lines analyzed did not exhibit overexpression of *erb*B-2 or EGFR. Heterogeneity of steroid receptor expression is of considerable therapeutic and prognostic value in human mammary neoplasia (McClelland *et al.*, 1986; DeSombre *et al.*, 1986). Our results strongly suggest an additional level of heterogeneity defined by overexpression of the *erb*B-2 or EGFR gene.

In the cell line SK-BR-3, where we identified an amplified and overexpressed *erb*B-2 gene, a *myc* gene amplification with overexpression has been previously reported (Kozbor and Croce, 1984). The *myc* gene has been shown to cooperate with a *ras* gene in the transformation of primary fibroblasts (Land *et al.*, 1983). It will be of interest to determine whether overexpression of *erb*B-2 and *myc* can complement each other in promoting the growth of mammary tumor cells. In addition, systematic analysis of mammary tumors will determine whether overexpression of *erb*B-2 or the EGFR gene can be associated with a particular clinical manifestation of mammary carcinoma.

Materials and methods

Tumor cell lines

Human mammary tumor cell lines were obtained from ATCC (American Type Culture Collection) and cultured according to the supplier's recommendation.

RNA blotting

Total cellular RNAs were isolated by CsCl-gradient centrifugation (Chirgwin *et al.*, 1979). For Northern blot analysis 10 μg total RNA were denatured and electrophoresed in 1% formaldehyde gels (Lehrach *et al.*, 1977). Following mild alkali degradation (50 mM NaOH at 24°C for 30 min) RNA was transferred to nitrocellulose filters using 1 M ammonium acetate as a convectant. RNA dot-blot analysis was conducted by applying serial 2-fold dilutions of total RNA in 1 M ammonium acetate to replicate nitrocellulose filters in a dot-blot manifold.

DNA blotting

For Southern blot analysis DNA samples (10 μg) were restricted with *Xba*I and subjected to electrophoresis in 0.8% agarose gels. DNAs were transferred to nitrocellulose filters by the method of Southern (Southern, 1975). For dot-blot analysis DNAs were cleaved with *Eco*RI, denatured with 0.1 M NaOH and neutralized with 1 M ammonium acetate. Serial 2-fold dilutions of each sample were applied to replicate nitrocellulose filters using a dot-blot manifold.

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