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Isolation and characterization of *ERBB3*, a third member of the *ERBB*/epidermal growth factor receptor family: Evidence for overexpression in a subset of human mammary tumors

(receptor-like kinase/sequence/chromosomal mapping/expression)

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ABSTRACT **A related DNA fragment distinct from the epidermal growth factor receptor and *ERBB2* genes was detected by reduced stringency hybridization of v-*erbB* to normal genomic human DNA. Characterization of the cloned DNA fragment mapped the region of v-*erbB* homology to three exons with closest identity of 64% and 67% to a contiguous region within the tyrosine kinase domains of the epidermal growth factor receptor and ERBB2 proteins, respectively. cDNA cloning revealed a predicted 148-kDa transmembrane polypeptide with structural features identifying it as a member of the *ERBB* gene family, prompting us to designate the gene as *ERBB3*. It was mapped to human chromosome 12q13 and was shown to be expressed as a 6.2-kilobase transcript in a variety of normal tissues of epithelial origin. Markedly elevated ERBB3 mRNA levels were demonstrated in certain human mammary tumor cell lines. These findings suggest that increased *ERBB3* expression, as in the case of epidermal growth factor receptor and *ERBB2*, may play a role in some human malignancies.**

Protooncogenes encoding growth factor receptors constitute several distinct families with close overall structural homology. The highest degree of homology is observed in their catalytic domains, essential for the intrinsic tyrosine kinase activity of these proteins (1). Examples of such families include genes encoding epidermal growth factor receptor (EGF-R) and ERBB2, the colony-stimulating factor 1/platelet-derived growth factor receptors, insulin/insulin-like growth factor 1 receptors, and EPH/ELK (2–12). Growth factor receptors in several of these families play critical roles in regulation of normal growth and development. Some of these molecules have been implicated in the neoplastic process as well. In particular, both the EGF-R gene and *ERBB2* have been shown to be activated as oncogenes by mechanisms involving overexpression or mutations that constitutively activate the catalytic activity of their encoded proteins (13–16). Thus, we undertook the present studies in an effort to identify and isolate additional members of the *ERBB* protooncogene family.

MATERIALS AND METHODS

Human Cells. Mammary epithelial cells AB589 (17) and immortalized keratinocytes RHEK (18) were provided by M. Stampfer (Lawrence Berkeley Laboratory) and J. Rhim, respectively. Normal human epidermal melanocytes (NHEM) and keratinocytes (NHEK) were obtained from Clonetics (San Diego, CA). Sources for human embryo fibroblasts (19) or mammary tumor cell lines (20) have been described.

DNA and RNA Hybridization. High-stringency hybridization was conducted as described (20). Reduced-stringency hybridization of DNA was carried out in 30% (vol/vol) formamide followed by washes in 0.6× SSC, whereas intermediate stringency was achieved by hybridization in 40% (vol/vol) formamide and washing in 0.25× SSC.

Molecular Cloning. An oligo(dT)-primed human placenta cDNA library was obtained from Clontech. The oligo(dT) primed MCF-7 cDNA library was constructed in λpCEV9 (21). After plaque purification, phage DNA inserts were subcloned into pUC-based plasmid vectors for further characterization.

Nucleotide and Amino Acid Sequence Analysis. The nucleotide sequence was determined for both DNA strands by the dideoxy chain-termination method (22) using supercoiled plasmid DNA as template.‡ Amino acid sequence comparison was performed with the alignment program by Pearson and Lipman (23). Hydrophobic and hydrophilic regions in the predicted protein were identified according to Kyte and Doolittle (24).

RESULTS

Identification of a Third Member of the *ERBB* Protooncogene Family. In an effort to detect novel *ERBB*-related genes, human genomic DNA was cleaved with a variety of restriction endonucleases and subjected to Southern blot analysis with v-*erbB* as probe. Under reduced stringency hybridization, four *Sac* I restriction fragments were detected. Two were identified as EGF-R gene fragments by their amplification in MDA-MB468 cells (Fig. 1*A*, lanes 1 and 2) known to contain EGF-R gene amplification and one as an *ERBB2*-specific gene fragment due to its increased signal intensity in *ERBB2*-amplified SK-BR-3 cells (Fig. 1*A*, lanes 1 and 3). However, a single 9-kbp *Sac* I fragment exhibited equal signal intensities in normal human thymus, A431, and SK-BR-3 DNA (Fig. 1*A*). When the hybridization stringency was raised by 7°C, this fragment did not hybridize, whereas EGF-R and *ERBB2*-specific restriction fragments were still detected with v-*erbB* as a probe (Fig. 1*B*). Taken together, these findings suggested the specific detection of another v-*erbB*-related DNA sequence within the 9-kbp *Sac* I fragment.

For further characterization we prepared a normal human genomic library from *Sac* I-cleaved thymus DNA enriched for 8- to 12-kbp fragments. Ten recombinant clones detected by v-*erbB* under reduced stringency conditions did not hybridize with human EGF-R or ERBB2 cDNA probes at high stringency. As shown in the restriction map of a repre-

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Abbreviation: EGF, epidermal growth factor.

‡The *ERBB3* nucleotide sequence has been deposited in the GenBank data base (accession no. M29366).

sentative clone with a 9-kbp insert, the region of v-*erbB* homology was localized by hybridization analysis to a 1.5-kbp segment spanning from the *Eco*RI to the downstream *Pst* I site. Nucleotide sequence analysis revealed that this region contained three open reading frames bordered by splice junction consensus sequences (Fig. 2). The predicted amino acid sequence of these three open reading frames revealed the highest identity scores of 64–67% to three regions that are continuous in the tyrosine kinase domains of v-*erbB*, as well as human EGF-R and ERBB2 proteins. Furthermore, all splice junctions of the three characterized exons in the gene were conserved with *ERBB2*. Amino acid sequence homology to other known tyrosine kinases was significantly lower, ranging from 39–46%.

A single 6.2-kb-specific mRNA was identified by Northern (RNA) blot analysis of human epithelial cells by using the 150-base pair (bp) *Spe* I–*Acc* I exon-containing fragment as probe (Fig. 2). Under the stringent hybridization conditions used, this probe detected neither the 5-kb ERBB2 mRNA nor the 6- and 10-kb EGF-R mRNAs (data not shown). All of these findings suggested that we had identified an additional functional member of the *ERBB* protooncogene family, which we tentatively designated as *ERBB3*.

Close Structural Similarity of the Predicted ERBB3 Protein with Other *ERBB* Family Members. In an effort to characterize the entire *ERBB3* coding sequence, overlapping cDNA clones were isolated from oligo(dT)-primed cDNA libraries from sources with known *ERBB3* expression, utilizing gene-specific genomic exons or cDNA fragments as probes. The clones were initially characterized by restriction analysis and hybridization to the mRNA and were subsequently subjected to nucleotide sequence analysis. The clones pE3-8, pE3-9, pE3-11, and pE3-16 contained identical 3′ ends terminating in a poly(A) stretch (Fig. 2).

The complete coding sequence of *ERBB3* was contained within a single long open reading frame of 4080 nucleotides extending from position 46 to an in-frame termination codon at position 4126. The most upstream ATG codon at position

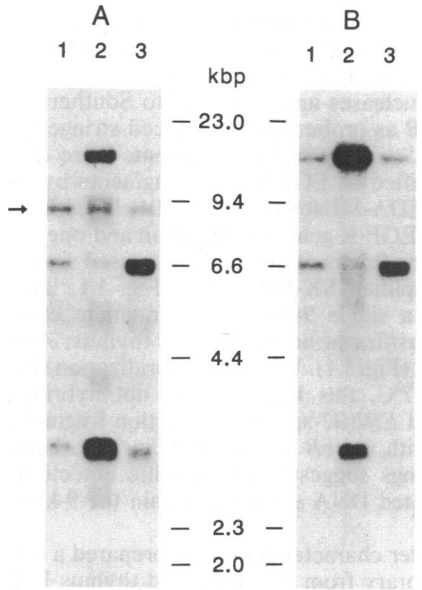

FIG. 1. Detection of v-*erbB*-related gene fragments in normal human thymus (lane 1), MDA-MB468 (lane 2), SK-BR-3 (lane 3). DNAs were restricted with *Sac* I and hybridized with a v-*erbB*-specific probe spanning from the upstream *Bam*HI to the *Eco*RI site in avian erythroblastosis proviral DNA (25). Hybridization was conducted at reduced (*A*) or intermediate (*B*) stringency conditions. The arrow denotes a 9-kbp *ERBB*-related restriction fragment distinct from those of EGF-R and *ERBB2*.

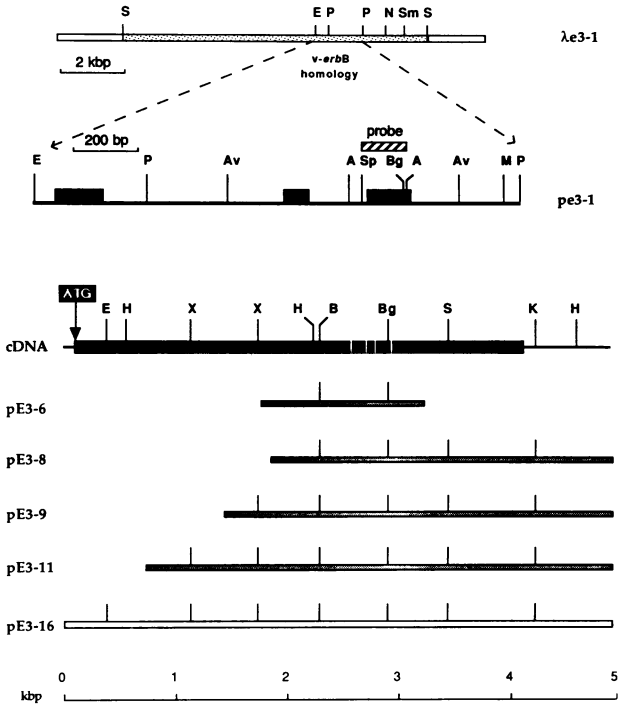

FIG. 2. Genomic and cDNA cloning of *ERBB3*. The region of v-*erbB* homology within the genomic 9-kbp *Sac* I insert of λe3-1 was subcloned into pUC (pe3-1) and subjected to nucleotide sequence analysis. The three predicted exons are depicted as solid boxes. ERBB3 cDNA clones were isolated from normal human placenta (shaded bars) and MCF-7 (open bar) oligo(dT)-primed libraries. The entire nucleotide sequence was determined for both strands on ERBB3 cDNA from normal human placenta and upstream of the 5′ *Xho* I site on pE3-16. The coding sequence is shown as a solid bar, and splice junctions of the three characterized genomic exons are indicated by vertical white lines. Solid lines in the cDNA map represent untranslated sequences. Restriction sites: A, *Acc* I; Av, *Ava* I; B, *Bam*HI; Bg, *Bgl* II; E, *Eco*RI; *H*, *Hin*dIII; K, *Kpn* I; M, *Mst* II; P, *Pst* I; S, *Sac* I; Sm, *Sma* I; and Sp, *Spe* I.

100 was the likely initiation codon, as it was preceded by an in-frame stop codon at nucleotide position 43 and fulfilled Kozak's criteria (26) for an authentic initiation codon. The open reading frame comprised 1342 codons, predicting a 148-kDa polypeptide. As shown in Fig. 3, the deduced amino acid sequence of the ERBB3 polypeptide predicted a transmembrane receptor tyrosine kinase most closely related to EGF-R and ERBB2. A hydrophobic signal sequence of ERBB3 was predicted to comprise the 19 amino-terminal amino acid residues. Cleavage of this signal sequence between Gly-19 and Ser-20 would generate a processed polypeptide of 1323 amino acids with an estimated molecular mass of 145 kDa. A single hydrophobic membrane-spanning domain encompassing 21 amino acids was identified (Fig. 3).

The putative ERBB3 ligand-binding domain was 43% and 45% identical in amino acid residues with the predicted ERBB2 and EGF-R protein, respectively. Within the extracellular domain, all 50 cysteine residues of the processed ERBB3 polypeptide were conserved and similarly spaced when compared with the EGF-R and ERBB2. Forty-seven cysteine residues were organized in two clusters containing 22 and 25 cysteines, respectively, a structural hallmark of this tyrosine kinase receptor subfamily (2–4). Ten potential N-linked glycosylation sites were localized within the ERBB3 extracellular domain. In comparison with the EGF-R and ERBB2 proteins, five and two of these glycosylation sites were conserved, respectively. Among these, the site proximal to the transmembrane domain was conserved among all three proteins (Fig. 3).

Within the cytoplasmic domain, a core of 277 amino acids from position 702–978 revealed the most extensive homology with the tyrosine kinase domains of EGF-R and ERBB2. In this region 60 or 62% of amino acid residues were identical and 90 or 89% were conserved, respectively. This stretch of amino acid homology coincides with the minimal catalytic domain of tyrosine kinases (1). There was significantly lower homology with other tyrosine kinases (Fig. 3). The consensus sequence for an ATP-binding site Gly-Xaa-Gly-Xaa-Xaa-Gly (1) at amino acid position 716–721 as well as a lysine residue located 21 amino acid residues farther carboxyl-terminal were conserved between the three ERBB-related receptors. Taken together, these findings defined the region between amino acid position 702 and 978 as the putative catalytic domain of the ERBB3 protein (Fig. 3).

The most divergent region of ERBB3 compared with either EGF-R or ERBB2 was its carboxyl terminus, comprising 364 amino acids. Tyrosine residues at positions 1197, 1199, and 1262 matched closest with the consensus sequence for putative phosphorylation sites (28). The peptide one-letter sequence YEYMN, encompassing Tyr-1197 and Tyr-1199, was repeated at positions 1260–1264 and was at both locations surrounded by charged residues. These observations render Tyr-1197, Tyr-1199, and Tyr-1262 likely candidates for autophosphorylation sites of the ERBB3 protein.

Chromosomal Mapping of Human *ERBB3*. We determined the chromosomal location of the *ERBB3* gene by *in situ* hybridization (29) with an ^{3}H-labeled plasmid containing the *ERBB3* amino-terminal coding sequence. A total of 110 human chromosome spreads were examined prior and subsequent to G banding for identification of individual chromosomes. One hundred forty-two grains were localized on a 400-band ideogram. We observed specific labeling of chromosome 12, where 38 out of 51 grains were localized to band q13 (data not shown). Thus, the genomic locus of *ERBB3* was assigned to 12q13. In this region of chromosome 12, several genes have previously been mapped including the melanoma-associated antigen ME491 (30), histone genes (31) and the gene for lactalbumin (32). In addition, two protooncogenes, *INT1* (33) and *GLI* (34), are located in close proximity to *ERBB3*.

***ERBB3* Expression in Normal and Malignant Human Cells.** To investigate its pattern of expression, we surveyed a number of human tissues for the ERBB3 transcript. The 6.2-kb ERBB3-specific mRNA was observed in term placenta, postnatal skin, stomach, lung, kidney, and brain, but it was not detectable in skin fibroblasts, skeletal muscle, or lymphoid cells (data not shown). Among the fetal tissues analyzed, the ERBB3 transcript was expressed in liver, kidney, and brain but not in fetal heart or embryonic lung fibroblasts. These

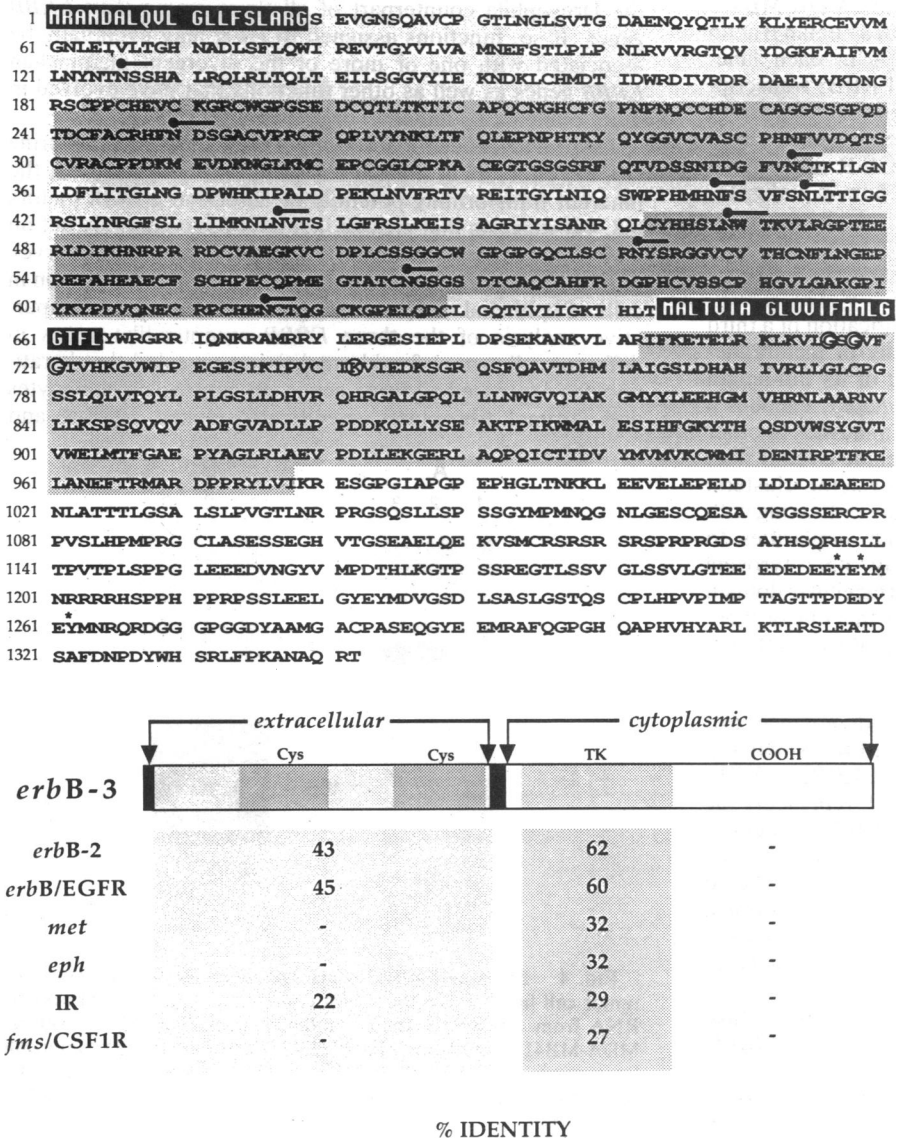

FIG. 3. (*Upper*) Predicted amino acid sequence of the ERBB3 polypeptide (as deduced from the GenBank sequence M29366) and comparison with other receptor-like tyrosine kinases. The amino acid sequence is shown in single-letter code and is numbered at left. The putative extracellular domain (light shading) extends between the predicted signal sequence (solid box) at the amino terminus and a single hydrophobic transmembrane region (solid box) within the polypeptide. The putative ATP-binding site at the amino terminus of the TK domain is circled. Potential autophosphorylation sites within the carboxyl-terminal domain (COOH) are indicated by asterisks. Potential N-linked glycosylation sites (●—) are marked above the amino acid sequence. (*Lower*) The two cysteine clusters (Cys) in the extracellular domain and the predicted tyrosine kinase domain (TK) within the cytoplasmic portion of the polypeptide are outlined by dark shading. The percentage of amino acid homology of ERBB3 in individual domains with ERBB2 (4), EGF-R (2), MET (27), EPH (11), insulin receptor [IR (9)], and FMS (5) is listed below. Less than 16% identity is denoted by -.

observations indicated the preferential expression of the ERBB3 transcript in epithelial tissues and brain.

We also investigated *ERBB3* expression in individual cell populations in comparison to EGF-R and ERBB2 transcripts. As shown in Table 1, mRNA levels of each were relatively high in keratinocytes and low but similar in cells derived from glandular epithelium. These findings are consistent with growth regulatory roles of all three receptor-like molecules in squamous and glandular epithelium. Whereas ERBB2 and EGF-R transcripts were also readily seen in normal fibroblasts, the same cells lacked detectable ERBB3 mRNA. In contrast, normal human melanocytes, which expressed both ERBB3 and ERBB2 at levels comparable with human keratinocytes, lacked detectable EGF-R transcripts. Thus, the expression patterns of these receptor-like molecules were different in specialized cell populations derived from epidermal tissues.

The ERBB3 transcript was detected in 36 of 38 carcinomas and 2 of 12 sarcomas, whereas 7 tumor cell lines of hematopoetic origin lacked measurable ERBB3 mRNA. Markedly elevated levels of a normal-sized transcript were observed in 6 of 17 tumor cell lines derived from human mammary carcinomas. By Southern blot analysis, neither gross gene rearrangement nor amplification was detected in the cell lines (data not shown). Fig. 4*A* shows the results of Northern blot analysis with control AB589 nonmalignant human mammary epithelial cells (lane 1) and two representative human mammary tumor lines, MDA-MB415 (lane 2) and MDA-MB453 (lane 3). Hybridization of the same filter with a human β actin probe (Fig. 4*B*) verified levels of mRNA in each lane. Densitometric scanning indicated that the ERBB3 transcript in each tumor cell line was elevated more than 100-fold above that of the control cell line. Thus, overexpression of this third member of the *ERBB* family, as for the EGF-R and *ERBB2* genes, may play an important role in some human malignancies.

DISCUSSION

In the present report, we describe the identification of a third member of the *ERBB*/EGF receptor family of membrane-spanning tyrosine kinases and the cloning of its full-length coding sequence. This gene, designated *ERBB3*, encodes a predicted protein with striking structural similarities to other members of this family. These features include overall size, extracellular domain with two signature cysteine clusters, and its uninterrupted tyrosine kinase domain, exhibiting with 81% and 83% significantly greater overall similarities to EGF-R and ERBB2 products than to any other tyrosine kinase. The structural relatedness of its extracellular domain with that of the EGF-R raises the possibility that one or more of an increasing number of EGF-like ligands (36) may interact with the ERBB3 product.

Distinct regions within the predicted *ERBB3* coding sequence revealed relatively higher degrees of divergence. For example, its carboxyl-terminal domain failed to exhibit significant colinear identity scores with either ERBB2 or EGF-R. Within the tyrosine kinase domain, which represents the most conserved region of the predicted ERBB3 protein, a short stretch of 29 amino acids carboxyl-terminal to the ATP-binding site differed from regions of the predicted ERBB2 and EGF-R coding sequence in 28 and 25 positions, respectively. Such regions of higher divergence in their cytoplasmic domains may confer functional specificity to these closely related receptor-like molecules.

Chromosomal mapping localized *ERBB3* to human chromosome 12q11-13, whereas the related EGF-R and *ERBB2* genes are located on chromosomes 7p12-13 (37) and 17p12-21.3 (3, 29), respectively. Thus, each appears to localize to regions containing different respective homeobox (38, 39) and collagen gene (40) loci. Keratin type I and type II genes also map to regions of 12 and 17 (41, 42), consistent with localization of *ERBB3* and *ERBB2*, respectively.

Table 1. Normal expression pattern of human *ERBB* gene family members

Source	Relative transcript levels		
	ERBB3	ERBB2	EGF-R
Embryonic fibroblast (M426)	−	+	+
Skin fibroblast (501T)	−	+	+
Immortal keratinocyte (RHEK)	++	++	++
Primary keratinocyte (NHEK)	+	+	++
Glandular epithelium (AB589)	(+)	(+)	(+)
Melanocyte (NHEM)	++	++	−

Replicate Northern blots were hybridized with equal probe counts of similar specific activity for ERBB3, ERBB2, and EGF receptor, respectively. Relative signal intensities were semiquantitatively estimated: −, not detectable; (+), weakly positive; +, positive; ++, strongly positive.

Recent studies in *Drosophila* have emphasized how critical and multifunctional are developmental processes mediated by ligand–receptor interactions. An increasing number of *Drosophila* mutants with often varying phenotypes have now been identified as being due to lesions in genes encoding such proteins (43, 44) including the *Drosophila* EGF-R homologue, designated DER. It is not yet known whether DER is the Drosophila counterpart of all three mammalian *ERBB* genes. If so, functions assigned to DER may eventually be associated with one or more of the divergent mammalian *ERBB* genes as well as other functions that have evolved in more complex mammalian organisms.

There is evidence for autocrine (45, 46) as well as paracrine (19, 47) effectors of normal cell proliferation. However, the inherent transforming potential of autocrine growth factors (48, 49) suggests that growth factors most commonly act on their target cell populations by a paracrine route. Our survey of *ERBB3* gene expression indicated its normal expression in cells of epithelial and neuroectodermal derivation. Comparative analysis of the three *ERBB* receptor-like genes in different cell types of epidermal tissue revealed that keratinocytes expressed all three genes. In contrast, melanocytes and stromal fibroblasts specifically lacked EGF-R and

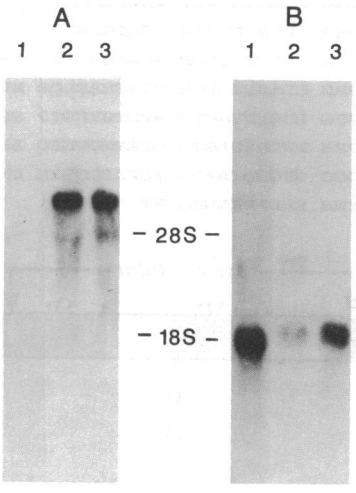

FIG. 4. Elevated ERBB3 transcript levels in human mammary tumor cell lines. A Northern blot containing 10 μg of total cellular RNA from AB589 mammary epithelial cells (lane 1), as well as MDA-MB415 (lane 2) and MDA-MB453 (lane 3) mammary tumor cell lines was hybridized with an ERBB3 cDNA probe (*A*). After signal decay the same blot was rehybridized with a human β actin cDNA probe (35).

ERBB3 transcripts, respectively. Thus, melanocytes and stromal fibroblasts may be sources of paracrine growth factors for EGF-R and ERBB3 products, respectively, that are expressed by the other cell types residing in close proximity in epidermal tissues.

To date, both *ERBB*/EGFR and *ERBB2* have been causally implicated in human malignancy. EGF-R gene amplification and/or overexpression in tumors has been demonstrated in squamous cell carcinomas and glioblastomas (50). *ERBB2* amplification and/or overexpression has been observed in human breast and ovarian carcinomas (51, 52), and *ERBB2* overexpression has been reported to be an important prognostic indicator of particularly aggressive tumors (52). Thus, our present findings that the ERBB3 transcript is overexpressed in a significant fraction of human mammary tumor cell lines raises the possibility that this new member of the ERBB/EGF receptor family may also play an important role in some human malignancies.

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