# Amplification of a Novel v-*erbB*–Related Gene in a Human Mammary Carcinoma

Abstract. *The cellular gene encoding the receptor for epidermal growth factor (EGF) has considerable homology to the oncogene of avian erythroblastosis virus. In a human mammary carcinoma, a DNA sequence was identified that is related to v-*erbB *but amplified in a manner that appeared to distinguish it from the gene for the EGF receptor. Molecular cloning of this DNA segment and nucleotide sequence analysis revealed the presence of two putative exons in a DNA segment whose predicted amino acid sequence was closely related to, but different from, the corresponding sequence of the erbB/EGF receptor. Moreover, this DNA segment identified a 5-kilobase transcript distinct from the transcripts of the EGF receptor gene. Thus, a new member of the tyrosine kinase proto-oncogene family has been identified on the basis of its amplification in a human mammary carcinoma.*

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The oncogenes of the acute transforming retroviruses have counterparts, designated proto-oncogenes, that are conserved within the human genome (*1*). The human *sis* proto-oncogene encodes one major polypeptide chain of platelet-derived growth factor (PDGF) (*2*), and the *erb*B proto-oncogene appears to encode the receptor for epidermal growth factor (EGF) (*3*). A number of other proto-oncogenes, like *erb*B, share nucleotide sequence homology with the tyrosine kinase–encoding *src* gene (*4*). The fact that cellular receptors for several growth factors or hormones, including the EGF receptor, possess this enzymatic activity suggests that other proto-oncogenes may encode growth factor receptors as well.

Genetic alterations affecting proto-oncogenes of the tyrosine kinase family can play a role in spontaneous tumor development. A specific translocation affecting the c-*abl* locus, for example, is associated with chronic myelogenous leukemia (*5*). Several recent studies have also documented amplification or rearrangement of the gene for the EGF receptor in certain human tumors (*6*) or tumor cell lines (*7*). We now report the detection and partial isolation of a gene that is a new member of the tyrosine kinase family and is amplified in a human mammary carcinoma. This gene is closely related to, but distinct from, the EGF receptor gene.

The identification of additional members of some proto-oncogene families has emerged from findings of related sequences amplified sufficiently in a particular tumor to allow detection (*8*). Because of the possibility that the *erb*B proto-oncogene might be related to other growth factor receptors, we used the v-*erb*B gene to probe for related genes that might be candidates for other receptor coding sequences. We selected moderate stringency hybridization conditions under which different oncogenes of the tyrosine family did not cross-hybridize. Thus, any gene detected might be expected to have a closer relationship to v-*erb*B than to other members of the tyrosine kinase family.

DNA prepared from tissue of a human mammary carcinoma, MAC117, showed a pattern of hybridization (Fig. 1A) that differed both from that observed with DNA of normal human placenta and from that observed with the A431 squamous-cell carcinoma line, which contains amplified EGF receptor genes (*7*). In A431 DNA, four Eco RI fragments were detected that had increased signal intensities compared to those of corresponding fragments in placenta DNA (Fig. 1A). In contrast, MAC117 DNA contained a single 6-kilobase pair (kbp) fragment, which appeared to be amplified compared to corresponding fragments observed in both A431 and placenta DNA's (Fig. 1A). These findings were consistent with the possibility that the MAC117 tumor contained an amplified DNA sequence related to, but distinct from, the cellular *erb*B proto-oncogene.

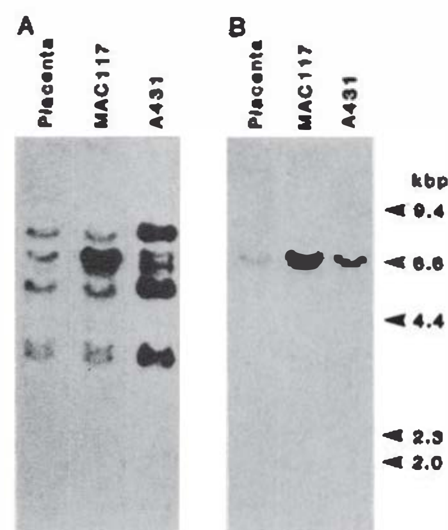


Fig. 1. Detection of v-*erb*B- and pMAC 117–specific gene fragments in normal human placenta, A431 cells, or human mammary carcinoma MAC117. DNA (15 μg) was cleaved with Eco RI, separated by electrophoresis in agarose gels, and transferred to nitrocellulose paper (*18*). Hybridization to the $^{32}$P-labeled probe (*20*) was conducted in a solution of 40 percent formamide, 0.75*M* NaCl, 0.075*M* sodium citrate, at 42°C (*19*). The v-*erb*B probe (A) was a mixture of the 0.5-kbp Bam HI–Bam HI fragment and 0.5-kbp Bam HI–Eco RI fragment of avian erythroblastosis proviral DNA. The pMAC117 probe (B) was a 1-kbp Bgl I–Bam HI fragment. After hybridization, the blots were washed first in 0.3*M* NaCl plus 0.03*M* sodium citrate at room temperature, and then in 0.015*M* NaCl, 0.0015*M* sodium citrate, and 0.1 percent sodium dodecyl sul-

To clone the 6-kbp fragment, we digested DNA from MAC117 with Eco RI, ligated it into bacteriophage λgtWES, packaged it in vitro, and transferred it to *Escherichia coli* strain BNN45 by infection. A library of 4 × $10^5$ bacteriophages was screened by plaque hybridization with radioactive *v-erb*B DNA. Ten of 14 hybridizing phages contained a 6-kbp Eco RI fragment. Figure 2 shows the physical map of one of these phages, λMAC117, and pMAC117, a pUC12 subclone containing a 2-kbp Bam HI fragment of λMAC117 that hybridized with v-*erb*B probes. The region of pMAC117 to which v-*erb*B hybridized most intensely was flanked by Acc I and Nco I sites. Human repetitive sequences were also localized (Fig. 2, region demarcated by arrows).

By digestion of pMAC117 with Bgl I and Bam HI, it was possible to generate a single-copy probe homologous to v-*erb*B. This probe detected a 6-kb Eco RI fragment that was amplified in MAC117 DNA and possibly increased in A431 cellular DNA relative to normal DNA (Fig. 1B). The sizes of the fragments corresponded to the amplified 6-kb Eco RI fragment detected in MAC117 DNA by means of v-*erb*B (Fig. 1A). Hybridization to Southern blots containing serial dilutions of MAC117 genomic DNA indicated an approximate amplification of 5- to 10-fold when compared to human placenta DNA.

The nucleotide sequence of the portion of pMAC117 located between the Nco I and Acc I sites contained two regions of nucleotide sequence homologous to v-*erb*B separated by 122 nucleotides (Fig. 3). These regions shared 69 percent nucleotide sequence identity with both the v-*erb*B and the human

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acid sequence of these regions was 85 percent homologous to two regions that are contiguous in the EGF receptor sequence (*7*). Furthermore, these two putative coding regions of the MAC117 sequence were each flanked by the AG and GT dinucleotides that border the exons of eukaryotic genes (*9*). These findings suggest that the sequence shown in Fig. 2 represents two exons, separated by an intron, of a gene related to the *erb*B/EGF receptor gene.

The predicted amino acid sequence of the λMAC117 putative exons is homologous to the corresponding sequences of several members of the tyrosine kinase family. The most striking homology was observed with the human EGF receptor or *erb*B (Fig. 3). In addition, we observed 42 percent to 52 percent homology with the predicted amino acid sequences of other tyrosine kinase–encoding genes. At 25 percent of the positions there was identity among all the sequences analyzed (Fig. 3). A tyrosine residue in the λMAC117 putative coding sequence, conserved among the tyrosine kinases analyzed, is the site of autophosphorylation of the *src* protein (*10*).

The availability of cloned probes of the MAC117 gene made it possible to investigate its expression in a variety of cell types. The MAC117 probe detected a single 5-kb transcript in A431 cells (Fig. 4). Under the stringent conditions of hybridization utilized, this probe did not detect any of the three RNA species recognized by EGF receptor complementary DNA. Thus, MAC117 represents a new functional gene within the tyrosine kinase family, closely related to, but distinct from, the gene encoding the EGF receptor.

There is precedent for the identification of genes related to known oncogenes on the basis of their amplification in human tumors. For example, the high degree of amplification of N-*myc* in certain malignancies made it detectable by means of the *myc* gene as a molecular probe (*8*). In the present study, a five- to tenfold amplification of a v-*erb*B–related gene in the MAC117 mammary carcinoma made it possible to identify this sequence against a complex pattern of EGF receptor gene fragments. Analysis of DNA from ten additional mammary carcinomas has not revealed amplification of the MAC117 gene. However, extensive studies will be required to determine the frequency of MAC117 gene amplification in different human malignancies.

The MAC117 coding sequence, as determined by nucleotide and predicted

related to the *erb*B/EGF receptor among known members of the tyrosine kinase family. The two genes are distinct, on the basis of sequence diversity and transcript size. Detailed structural analysis of the complete coding sequence should give insights into the possible functions of this v-*erb*B–related gene. Nevertheless, because of its close relationship to the sequence of the EGF receptor, it is possible to speculate that the MAC117 coding sequence may also be derived from a gene encoding a growth factor receptor. An oncogene in a chemically

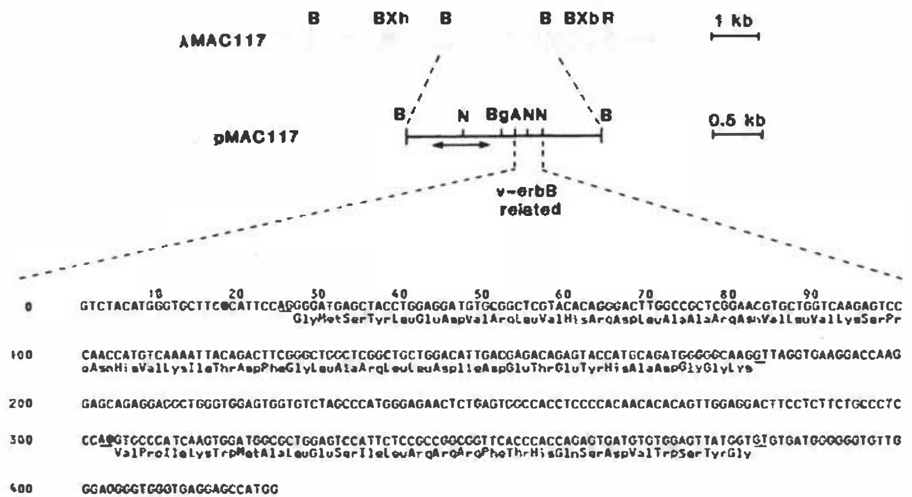


Fig. 2. Restriction-site map of λMAC117 and plasmid pMAC117. A, Acc I; B, Bam HI; Bg, Bgl I; N, Nco I; R, Eco RI; X, Xba I; Xh, Xho I. The sites were located by electrophoretic analysis of the products of single and double digestion. Regions homologous to v-*erb*B or human repetitive sequences (region flanked by arrows) were located by Southern blot hybridization (*18*) with the v-*erb*B probe or total human DNA made radioactive by nick translation (*20*). Hybridization conditions were as described in Fig. 1A. The nucleotide sequence of pMAC117 between the Acc I site and the Nco I sites and regions of encoded amino acid sequence homologous to the EGF receptor are shown. The AG or GT dinucleotides flanking the putative coding regions are underlined. To determine the sequence, Nco I, Hinf I, and Sau 96 I fragments were labeled at the 3′ termini by means of the large fragment of *E. coli* DNA polymerase, separated into single strands by gel electrophoresis, and chemically degraded (*21*).

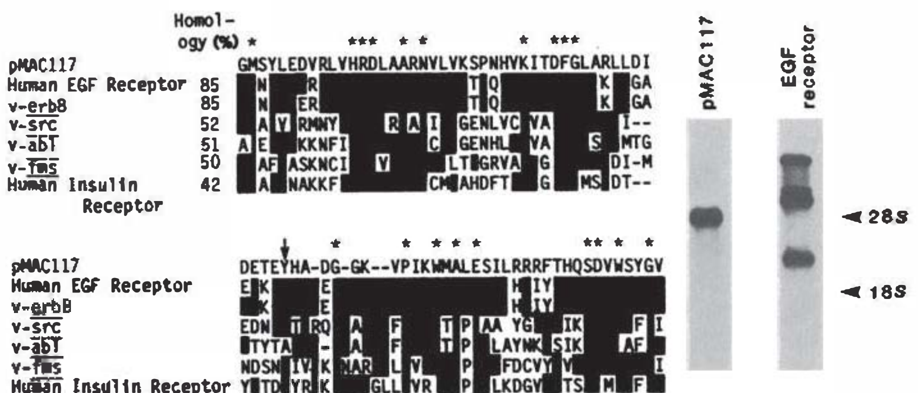


Fig. 3 (left). Comparison of the putative encoded amino acid sequence in pMAC117 with known tyrosine kinase sequences. Black regions represent homologous amino acids. Differing amino acid residues are shown in one-letter code (*22*). Amino acid positions conserved in all sequences are denoted by ⋆. The tyrosine homologous to that autophosphorylated by the v-*src* protein (*10*) is shown by an arrow. The v-*abl* sequence contains a tyrosine residue in this region displaced by two positions. The amino acid sequences of human EGF receptor (*7*), v-*erb*B (*4*), v-*src* (*23*), v-*abl* (*24*), v-*fms* (*4*), and human insulin receptor (*25*) were aligned by the computer program described (*26*). The homology observed with the predicted amino acid sequences of v-*yes* and v-*fes* was 51 percent and 48 percent, respectively. Fig. 4 (right). Detection of distinct messenger RNA species derived from the λMAC117 gene and the human EGF receptor gene. Polyadenylated messenger RNA of A431 cells was separated by denaturing gel electrophoresis in formaldehyde (*23*), transferred to nitrocellulose (*18*), and hybridized under stringent conditions (50 percent formamide, 0.075*M* NaCl, 0.75*M* sodium citrate, at 42°C) with $^{32}$P-labeled probe from pMAC117 (Bgl I–Bam HI fragment) or human EGF receptor complementary DNA (PE7: 2-kb Cla I inserted fragment). Filters were washed under conditions of high stringency (0.015*M* NaCl plus 0.0015*M* sodium citrate at 55°C). Hybridization was detected by

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induced rat neuroblastoma has been detected by DNA transfection analysis (*11*). This oncogene, designated *neu*, appears to encode a protein immunologically related to the EGF receptor (*12*). Whether the MAC117 coding sequence and *neu* represent the same or different cellular genes awaits further characterization.

Overexpression of proto-oncogenes can cause cell transformation in culture and may function in the development of human tumors. Amplification of a normal *ras* gene or its increased expression under the control of a retroviral long terminal repeat (LTR) induces transformation of NIH 3T3 cells (*13*). Expression of the normal human *sis*/PDGF-2 coding sequence in NIH 3T3 cells, which do not normally express their endogenous *sis* proto-oncogene, also leads to transformation (*14*). In Burkitt lymphoma, a chromosomal translocation involving *myc* places its normal coding sequence under the control of an immunoglobulin gene regulatory sequence (*15*). The resulting alteration in *myc* expression is likely to be causally related to tumor development (*16*). The observation of amplification of *myc* or N-*myc* in more malignant phenotypes of certain tumors has supported the idea that overexpression of these genes can contribute to the progression of such tumors (*8, 17*). The *erb*B/EGF receptor gene is amplified or overexpressed in certain tumors or tumor cell lines (*6*). The five- to tenfold amplification of our v-*erb*B-related gene in a mammary carcinoma suggests that increased expression of this gene may have provided a selective advantage to this tumor. The isolation of a new member of the tyrosine kinase gene family amplified in a human mammary carcinoma provides an opportunity to investigate the potential role of this gene in human malignancy.

*Note added in proof*: Recently, Semba *et al.* (*28*) independently detected a v-*erb*B-related gene that was amplified in a human salivary gland adenocarcinoma. Nucleotide sequence analysis of this gene indicates its identity to the MAC117 gene in the regions compared.

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22. The following abbreviations were used for amino acids: A, alanine; C, cysteine, D, aspartic acid; E, glutamic acid; F. phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.
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29. We thank I. Pastan and G. Merlino for providing the human EGF complementary DNA clone PE7 and P. di Fiore for the polyadenylated A431 RNA.

22 April 1985; accepted 3 June 1985


## The *neu* Gene: An *erb*B-Homologous Gene Distinct from and Unlinked to the Gene Encoding the EGF Receptor


Abstract. *The* neu *oncogene, identified in ethylnitrosourea-induced rat neuroglioblastomas, had strong homology with the* erbB *gene that encodes the epidermal growth factor receptor. This homology was limited to the region of* erbB *encoding the tyrosine kinase domain. It was concluded that the* neu *gene is a distinct novel gene, as it is not coamplified with sequences encoding the EGF receptor in the genome of the A431 tumor line and it maps to human chromosome 17.*



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Rat neuroglioblastomas induced by exposure in utero to ethylnitrosourea frequently carry an oncogene detectable upon transfection into NIH 3T3 mouse cells (*1*). This oncogene (which we have termed *neu*) was found to be related to c-*erb*B (*2*), a gene that encodes the receptor for epidermal growth factor (EGF-r) (*3*). The *neu* oncogene induces the synthesis of a tumor antigen, p185, which is serologically related to the EGF-r (*2*).

Southern blot analysis of rat DNA

which contained the *neu* oncogene in a biologically active form (*2*). It remained unclear whether the same or other DNA segments encode the EGF-r. Other analysis uncovered differences between the products of the two genes. While polyclonal sera to the EGF-r recognized p185, monoclonal antibodies to p185 did not react with the EGF-r. Moreover, there was an apparent molecular weight difference of 15,000 daltons between the two proteins (*2*).

These data raised several possibilities regarding the relationship between the *neu* and c-*erb*B genes. The *neu* oncogene might be a mutated allele of the normal c-*erb*B gene, or it might be derived from a normal gene, the sequences of which overlap with those of c-*erb*B. Alternatively, the *neu* oncogene might have arisen from a gene that is totally separate and distinct from *erb*B.

We used three subclones of human c-*erb*B complementary DNA (cDNA) (*4*) and a 0.7-kilobase (kb) subclone of the v-*erb*B oncogene that had been transduced by the genome of avian erythroblastosis virus (*5*) for these studies. All of the *neu* oncogene lies within a 34-kb Eco RI segment that is present in the genomes of normal and tumor rat cells as well as in


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