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Novel Activating Mutations in the *neu* Proto-oncogene Involved in Induction of Mammary Tumors

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Amplification of the Neu/c-erbB-2 receptor tyrosine kinase has been implicated as an important event in the genesis of human breast cancer. Indeed, transgenic mice bearing either an activated form of *neu* or the wild-type proto-oncogene under the transcriptional control of the mouse mammary tumor virus promoter-enhancer frequently develop mammary carcinomas (L. Bouchard, L. Lamarre, P. J. Tremblay, and P. Jolicoeur, Cell 57:931–936, 1989; C. T. Guy, M. A. Webster, M. Schaller, T. J. Parson, R. D. Cardiff, and W. J. Muller, Proc. Natl. Acad. Sci. USA 89:10578–10582, 1992; W. J. Muller, E. Sinn, R. Wallace, P. K. Pattengale, and P. Leder, Cell 54:105–115, 1988). Induction of mammary tumors in transgenic mice expressing the wild-type Neu receptor is associated with activation of the receptor's intrinsic tyrosine kinase activity (Guy et al., Proc. Natl. Acad. Sci. USA 89:10578–10582, 1992). Here, we demonstrate that activation of Neu in these transgenic mice occurs through somatic mutations located within the transgene itself. Sequence analyses of these mutations revealed that they contain in-frame deletions of 7 to 12 amino acids in the extracellular region proximal to the transmembrane domain. Introduction of these mutations into a wild-type *neu* cDNA results in an increased transforming ability of the altered Neu tyrosine kinase. These observations suggest that oncogenic activation of Neu in mammary tumorigenesis frequently occurs by somatic mutation.

The *neu* (c-*erbB-2* or *HER2*) proto-oncogene encodes a receptor tyrosine kinase belonging to the epidermal growth factor receptor family (2, 9). Oncogenic activation of Neu can occur through multiple molecular mechanisms, including a point mutation in its transmembrane domain (3) and deletion of the extracellular domain (4). Amplification and consequent overexpression of *neu*/c-*erbB-2* have also been observed in a significant proportion of human breast and ovarian cancers (14, 24, 27). In fact, several studies have shown that the degree of amplification is inversely correlated to a poor clinical outcome for breast cancer patients in whom the cancer has not spread to the lymph nodes (11, 22). Although elevated levels of *neu*/c-*erbB-2* are observed in these human cancers, no comparable mutations have been detected in these tumor samples (15).

Consistent with these clinical observations, expression of activated or wild-type *neu* targeted to the mammary epithelia of transgenic mice results in the efficient induction of mammary tumors (5, 12, 19). In mice expressing elevated levels of the wild-type Neu protein, the appearance of mammary tumors is associated with a high incidence of metastatic disease (12). Mammary gland-specific expression of Neu results in the appearance of focal mammary tumors after a variable latency period (12). Both the focal nature and long latency period suggest that additional genetic events are needed to transform the mammary epithelium. Mammary tumorigenesis in these transgenic strains is correlated with elevated intrinsic Neu tyrosine kinase activity and the de novo tyrosine phosphorylation of several cellular proteins (12). The elevated Neu tyrosine kinase activity cannot simply be accounted for by overexpression of Neu in tumors by comparison with expression in the adjacent epithelia because many of the tumors and adjacent epithelia express comparable levels of transgene RNA and protein (12).

While it is clear that activation of Neu plays an important role in the induction of mammary tumors in these transgenic-mouse models, the precise mechanism by which this occurs is unclear. Because catalytic activation of Neu can occur through a single point mutation in the transmembrane domain (3), we examined whether this alteration was responsible for the elevated levels of Neu tyrosine kinase activity observed in tumors induced by the *neu* proto-oncogene. To this end, total RNA derived from both mammary tumor and adjacent mammary epithelium tissues was subjected to reverse transcription (RT) followed by PCR with oligonucleotides flanking the transmembrane domain of Neu. Hybridizations were then carried out with oligonucleotides specific for both the wild-type *neu* sequence and the activating point mutation. The results showed that while there was no evidence of the point mutation, many of the PCR products derived from the Neu-induced tumors possessed deletions. Sequence analyses of the altered products revealed that these deletions are in frame and situated in the extracellular domain of Neu proximal to the transmembrane domain. To test whether these alterations were responsible for activating Neu, the deletions were inserted into a wild-type *neu* expression cassette and tested for the capacity to transform Rat-1 fibroblasts. Expression of the altered *neu* cDNAs resulted in the induction of transformed foci that expressed elevated levels of tyrosine-phosphorylated Neu. These observations suggest that activation of Neu in these mammary tumors occurs through mutations located in the extracellular domain.

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MATERIALS AND METHODS

DNA constructs. PCR products were initially cloned as *Bst*1107I-*Eag*I fragments into pSL301 (Invitrogen) to facilitate sequence analyses. Those PCR products which contained

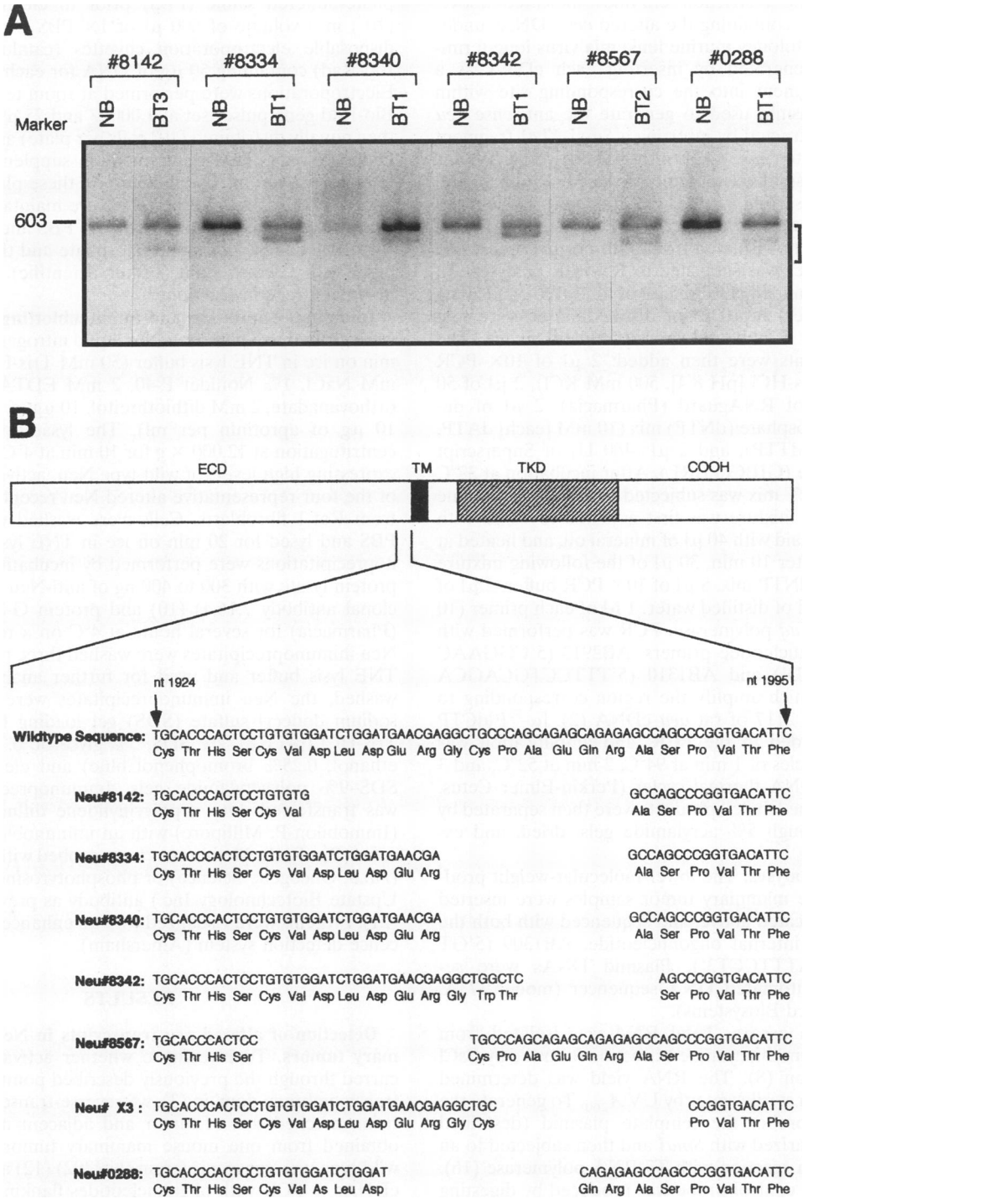

FIG. 1. Detection of in-frame deletions within the *neu* proto-oncogene. (A) Total RNA was isolated from transgenic mice expressing an MMTV wild-type *neu* fusion gene (N#202 line) and subjected to RT-PCR analysis. RT was used to prepare first-strand cDNAs from matched sets of RNA from both mammary tumor tissue (BT) and adjacent epithelium tissue (NB). PCR was performed with primers AB2913 and AB1310, which amplify a region of *neu* between nucleotides 1492 and 2117 (see Materials and Methods). [α-^{32}P]dCTP was included in the PCR, and the labeled products were separated on 5% acrylamide gels. The gels were then dried and visualized by autoradiography. The upper band present in each lane corresponds to the predicted wild-type PCR product, while the bracket indicates the altered mobility products. (B) Nucleotide and amino acid sequence encoded by the altered PCR products. The lower-molecular-weight products from each of the tumor samples were rescued into plasmid vectors and subjected to automated DNA sequence analysis. Partial sequences from each of the isolated deletions are shown and compared with the wild-type sequence of the *neu* cDNA. ECD, extracellular domain; TM, transmembrane domain; TKD, tyrosine kinase domain; COOH, carboxyl terminus.

alterations were then rescued into a wild-type version of the *neu* cDNA with the restriction enzymes indicated above. Expression plasmids containing the altered *neu* cDNAs under the control of the Moloney murine leukemia virus long terminal repeat were generated by inserting each cDNA as a *Hin*dIII-*Eco*RI fragment into the corresponding site within pJ4Ω (17). The plasmid used to generate the antisense *neu* riboprobe was constructed by inserting a *Sma*I-*Xba*I fragment (nucleotides 1684 to 2332) (2) into pSL301. The PGK-1 internal control plasmid was obtained from M. Rudnicki and was generated by inserting an *Acc*I-*Pst*I fragment (nucleotides 939 to 1633) (18) into the *Pst*I site of pSP64 (Promega).

RT-PCR. Total RNA isolated from both normal breast and breast tumor samples was subjected to RT-PCR analyses. To synthesize single-stranded cDNA, 5 μg of total RNA and 20 ng of oligo(dT) (18-mer) in 10 μl of distilled water were first heated to 90°C for 5 min and quickly chilled on ice. The following components were then added: 2 μl of 10× PCR buffer (200 mM Tris-HCl [pH 8.4], 500 mM KCl), 2 μl of 50 mM $MgCl_2$, 1 μl of RNAguard (Pharmacia), 2 μl of deoxynucleoside triphosphate (dNTP) mix (10 mM [each] dATP, dCTP, dGTP, and dTTP), and 2 μl (400 U) of Superscript reverse transcriptase (GIBCO BRL). After incubation at 37°C for 2 h, the entire RT mix was subjected to PCR. The volume of the RT reaction mixture was first adjusted to 70 μl with distilled water, overlaid with 40 μl of mineral oil, and heated at 95°C for 10 min. After 10 min, 30 μl of the following mixture was added: 2 μl of dNTP mix, 8 μl of 10× PCR buffer, 2 μl of 50 mM $MgCl_2$, 15 μl of distilled water, 1 μl of each primer (10 mM), and 1 μl of *Taq* polymerase. PCR was performed with the following oligonucleotide primers: AB2913 (5′CGGAAC CCACATCAGGCC3′) and AB1310 (5′TTTCCTGCAGCA GCCTACGC3′), which amplify the region corresponding to nucleotides 1492 to 2117 of rat *neu* cDNA (2). [α-^{32}P]dCTP (10 μCi) was included in the PCR, and amplification was performed for 30 cycles of 1 min at 94°C, 2 min at 52°C, and 3 min at 72°C in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). The labeled products were then separated by electrophoresis through 5% acrylamide gels, dried, and exposed to X-ray film.

DNA sequence analyses. The lower-molecular-weight products observed in the mammary tumor samples were inserted into pSL301, as indicated above, and sequenced with both the T7 primer and an internal oligonucleotide, AB1309 (5′GT CAACTGCAGTCATTTCCT3′). Plasmid DNAs were sequenced with an automated DNA sequencer (model 373A, version 1.2.0; Applied Biosystems).

RNase protection assays. Total RNA was isolated from tissues by guanidinium thiocyanate extraction followed by CsCl gradient fractionation (8). The RNA yield was determined after resuspension in sterile water by UV A_{260}. To generate the antisense *neu* riboprobe, the template plasmid (described above) was first linearized with *Sma*I and then subjected to an in vitro transcription reaction with T7 RNA polymerase (16). The PGK-1 internal control probe was produced by digesting the template plasmid (described above) with *Eco*NI and transcribing the product with SP6 RNA polymerase. The RNase protection assays were performed by hybridizing the above-mentioned probes to 20 μg of total RNA as previously described (16). The protected fragments were separated on a 6% denaturing gel and subjected to autoradiography. The DNA markers correspond to *Hae*III-digested ϕX174 molecular weight standards (GIBCO BRL) which were end labeled with [γ-^{32}P]dATP.

Focus assays. Rat-1 fibroblasts were harvested for electroporation when the cells had reached approximately 60 to 70% confluence. The cells were washed three times in 1× phosphate-buffered saline (PBS) prior to electroporation. Cells (10^7) in a volume of 600 μl of 1× PBS were aliquoted into disposable electroporation cuvettes (catalog no. 165-2088; Bio-Rad) containing 50 μg of DNA for each construct tested. Electroporations were performed at room temperature with a Bio-Rad gene pulser set at 1,000 V and 25 μF. The cells were then equally distributed (10^6 cells per plate) among 10 plates in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. The medium on these plates was changed once every 3 days, and the cells were maintained for 14 days. The cells were washed once with 1× PBS and fixed for 30 min with 10% buffered formalin phosphate and then stained overnight with Giemsa stain (Fisher Scientific) by following the manufacturer's instructions.

Immunoprecipitation and immunoblotting. Tissue samples were ground to a powder under liquid nitrogen and lysed for 20 min on ice in TNE lysis buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM sodium orthovanadate, 2 mM dithiothreitol, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml). The lysates were cleared by centrifugation at 12,000 × *g* for 10 min at 4°C. Stable cell lines expressing high levels of wild-type Neu, activated Neu, or one of the four representative altered Neu receptors were derived from Rat-1 fibroblasts. Cells were washed with ice-cold 1× PBS and lysed for 20 min on ice in TNE lysis buffer. Immunoprecipitations were performed by incubating 500 μg of the protein lysate with 300 to 400 ng of anti-Neu antibody (monoclonal antibody 7.16.4) (10) and protein G-Sepharose beads (Pharmacia) for several hours at 4°C on a rotating platform. Neu immunoprecipitates were washed three to four times with TNE lysis buffer and used for further analysis. After being washed, the Neu immunoprecipitates were resuspended in sodium dodecyl sulfate (SDS) gel loading buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 5% glycerol, 0.7 M 2-mercaptoethanol, 0.25% bromophenol blue) and electrophoresed on SDS–9% polyacrylamide gels. Immunoprecipitated protein was transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore) with an immunoblot transfer apparatus (Bio-Rad). Membranes were probed with either anti-Neu (Ab.3; Oncogene Science) or phosphotyrosine-specific (4G10; Upstate Biotechnology Inc.) antibody as previously described (21). Proteins were visualized with an enhanced chemiluminescence detection system (Amersham).

RESULTS

Detection of altered *neu* transcripts in Neu-induced mammary tumors. To investigate whether activation of Neu occurred through the previously described point mutation in the transmembrane domain (3), we reverse-transcribed total RNA from matched sets of tumor and adjacent mammary tissues obtained from one mouse mammary tumor virus (MMTV) wild-type *neu* transgenic strain (N#202) (12) and subjected the cDNAs to PCR with oligonucleotides flanking the transmembrane domain. The resulting PCR products were hybridized to radiolabeled oligonucleotides specific for either the wild-type *neu* sequence or the transmembrane mutation in *neu* (3). Although these analyses failed to detect the transmembrane mutation in *neu* (data not shown), several of the PCR products which hybridized to the wild-type *neu* oligonucleotide had electrophoretic mobilities different from that of the wild-type product (Fig. 1A). Although the expected wild-type PCR product could be detected in samples of both adjacent mammary epithelia and mammary tumors, it is interesting that the altered products were observed only in tumor RNA samples

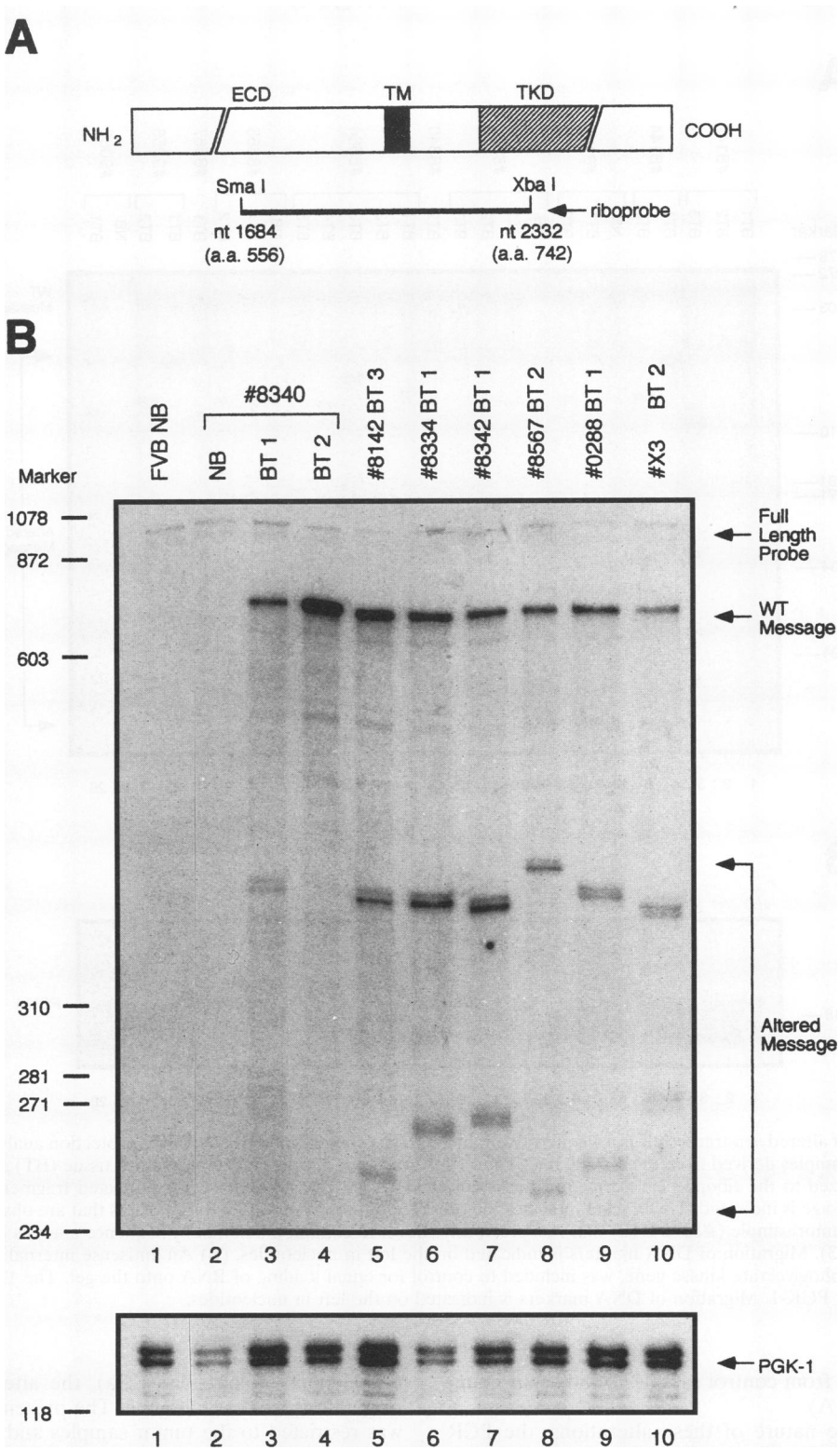

FIG. 2. RNase protection analyses demonstrating the presence of altered *neu* transcripts in Neu-induced tumors. (A) Schematic diagram indicating the region of the *neu* cDNA used to generate the antisense riboprobe. The riboprobe includes the region of the rat *neu* cDNA (2) between nucleotides 1684 and 2332. ECD, extracellular domain; TM, transmembrane domain; TKD, tyrosine kinase domain; COOH, carboxyl terminus. (B) RNase protection analyses of RNA derived from tumor tissue (BT) and adjacent mammary epithelium tissue (NB). The full-length, undigested probe (890 nucleotides) and the protected wild-type (WT) *neu* transcript (640 nucleotides) are indicated by the arrows. Protected fragments corresponding to the altered *neu* transcripts are indicated by the bracket. Normal mammary RNA from both a nontransgenic animal (lane 1) and an MMTV wild-type *neu* transgenic mouse (lane 2) were included as negative controls. In addition, RNA from a breast tumor sample which did not display alterations by the RT-PCR approach was also used as a negative control (lane 4). An antisense riboprobe, directed against the mouse phosphoglycerate kinase gene, was used to control for equal loading of RNA onto the gel. The PGK-1 probe protects a 124-nucleotide fragment, as indicated by the arrow in the lower panel. Migration of DNA markers is indicated on the left in nucleotides. FVB, Friend leukemia virus strain B.

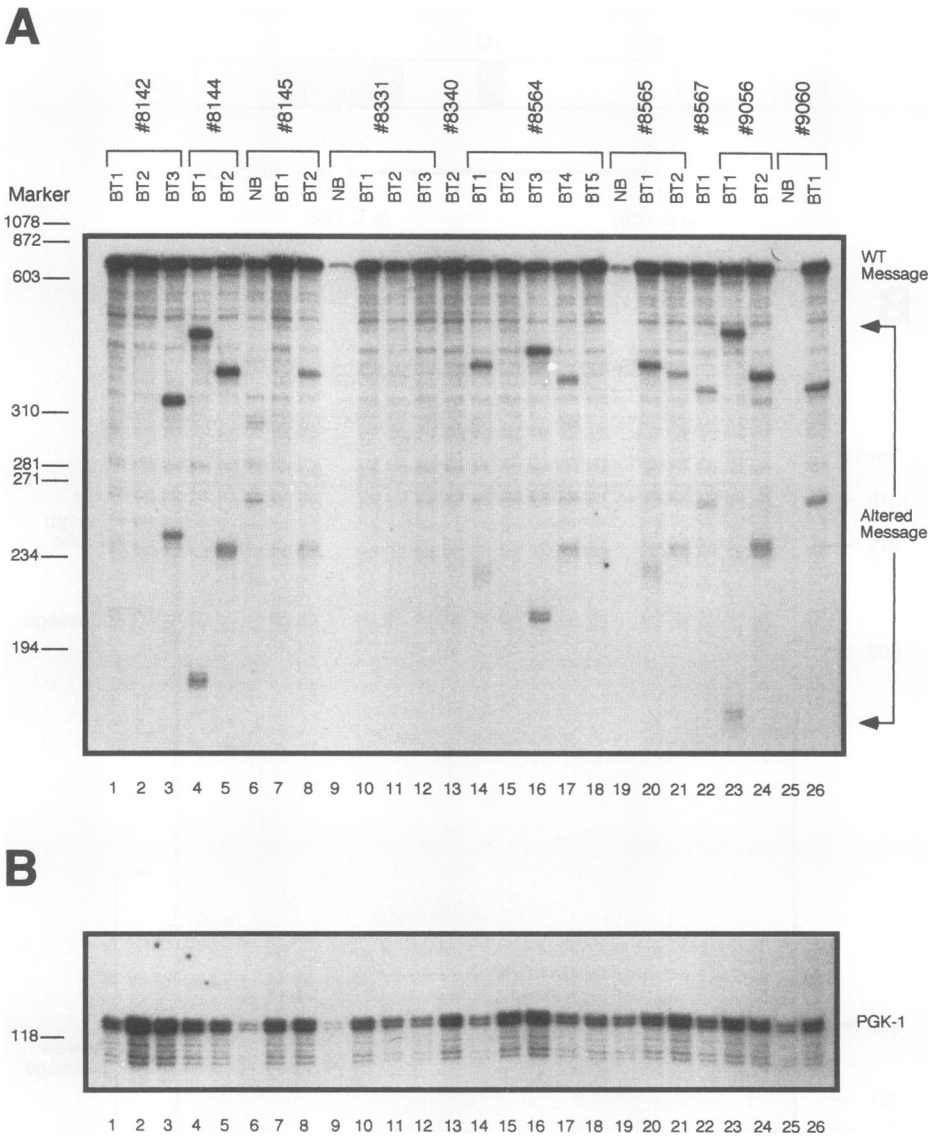

FIG. 3. Expression of altered *neu* transcripts is a frequent event in mammary tumorigenesis. (A) RNase protection analyses were used to screen an additional 25 RNA samples derived from the N#202 line. Total RNA was isolated from mammary tumor tissue (BT) and adjacent epithelium tissue (NB) and hybridized to the riboprobe described in the legend to Fig. 2A. The 640-nucleotide protected fragment corresponding to the wild-type (WT) *neu* message is indicated. The bracket indicates the presence of the various altered transcripts that are observed only in the tumor samples. RNA from a tumor sample (#8142 BT3) that was previously shown to contain a deletion by sequence analysis (Fig. 1) was included as a positive control (lane 3). Migration of DNA markers is indicated on the left in nucleotides. (B) An antisense internal control probe, directed against the mouse phosphoglycerate kinase gene, was included to control for equal loading of RNA onto the gel. The 124-nucleotide protected fragment is indicated by PGK-1. Migration of DNA markers is indicated on the left in nucleotides.

and did not originate from control samples of adjacent mammary epithelia (Fig. 1A).

To characterize the nature of these alterations, the PCR products from samples of both tumors and matched samples of adjacent mammary epithelia were inserted into plasmid vectors and subjected to DNA sequence analyses. For each matched pair of samples, several independent subclones were analyzed. These PCR products contained deletions ranging from 21 to 36 bp (7 to 12 amino acids) that were clustered in a region of the extracellular domain proximal to the transmembrane domain (Fig. 1B). Significantly, the deletions maintained the reading frame of the protein. Because the transgene contains the rat *neu* cDNA which differs in sequence from the endogenous mouse gene (23a), the altered transcripts must originate from the transgene. The presence of these deletions was restricted to the tumor samples and was not observed in the samples of adjacent normal mammary epithelia.

Because the RT-PCR analyses indicated that the deleted products were coexpressed with wild-type *neu* transcripts, we assessed the relative amounts of mutant versus wild-type RNAs in the tumors by employing a sensitive RNase protection assay. Total RNA derived from the mammary tumors was subjected to RNase protection analyses with a radiolabeled probe spanning the transmembrane region of rat *neu* (Fig. 2A). The results showed that protected bands corresponding to both the wild-type and mutant transcripts could readily be detected

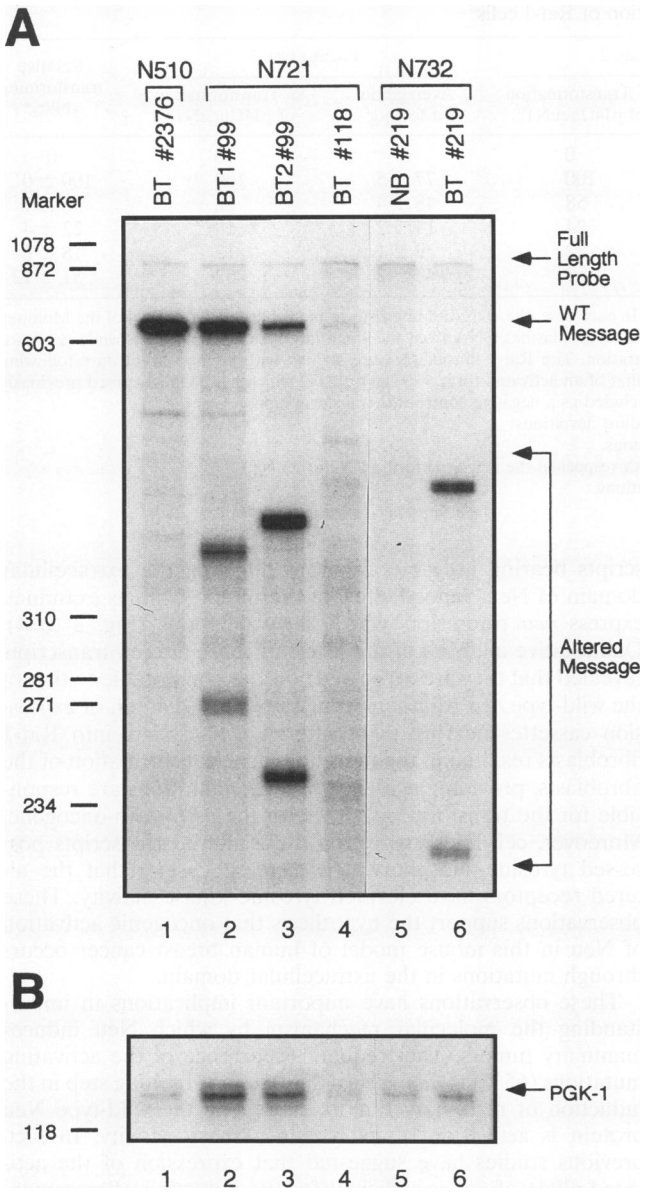

FIG. 4. Altered *neu* transcripts are detected in multiple transgenic lines. (A) Total RNA from mammary tumor tissue (BT) and adjacent epithelium tissue (NB) from three independently derived transgenic lines (N#510, N#721, and N#732) were subjected to RNase protection analysis with the riboprobe described in the legend to Fig. 2A. The 890-nucleotide protected fragment corresponding to the undigested (full-length) probe and the 640-nucleotide protected fragment representing the wild-type (WT) *neu* message are indicated by arrows. The bracket indicates the protected fragments corresponding to the altered *neu* transcripts. Migration of DNA markers is indicated on the left in nucleotides. (B) An antisense internal control probe, directed against the mouse phosphoglycerate kinase gene, was included to control for equal loading of RNA onto the gel. The 124-nucleotide protected fragment is indicated by PGK-1. Migration of DNA markers is indicated on the left in nucleotides.

in these tumors (Fig. 2B, lanes 3 and 5 to 10). The altered transcripts were expressed at levels ranging from 60 to 170% of that of the wild-type transcript as determined by PhosphorImager analyses (data not shown). Consistent with the DNA sequence data, the RNase protection analyses failed to detect the presence of the altered transcripts in normal mammary epithelia derived from wild-type or transgenic animals (Fig. 2B, lanes 1 and 2). In each case, the length of the altered protected fragments observed in the tumor-derived RNA corresponded with the predicted size of the identified deletions. These observations provide evidence that the altered transcripts are expressed in the tumors at levels comparable to that of the wild-type transgene product.

Expression of altered *neu* transcripts is a frequent event in mammary tumorigenesis and occurs in multiple independent transgenic strains. To determine whether the occurrence of these altered transcripts is a frequent event in Neu-induced tumorigenesis, identical RNase protection analyses of an additional 25 RNA samples from the N#202 strain were conducted. As shown in Fig. 3, these analyses showed that 12 of the 25 N#202 samples possessed deletions within this region of the *neu* transcript. Again, quantitative analyses revealed that the levels of altered transcripts ranged from 25 to 170% of that of the wild-type *neu* transcript (23a). No altered products were detected in the other 10 tumor samples or in the 3 matched samples of adjacent mammary epithelia (Fig. 3, lanes 9, 19, and 25).

To exclude the possibility that the occurrence of these mutations was restricted to one MMTV wild-type *neu* transgenic line (N#202), five tumor samples from three other lines (N#510, N#721, and N#732) (12) were also examined by RNase protection analysis. The results revealed that three of the five tumor samples possessed deletions within this region of the *neu* transcript (Fig. 4). In total, 22 of 34 tumor samples obtained from MMTV wild-type *neu* transgenic mice displayed evidence of these deletions. However, no instance of the transmembrane point mutation (3) was observed either by direct sequence analysis or by oligonucleotide hybridization. Taken together, these observations argue that deletion mutations in the *neu* transgene in mammary tumorigenesis occur frequently and in multiple independent transgenic strains.

Established cell lines expressing the altered Neu proteins are morphologically transformed and express elevated levels of tyrosine-phosphorylated Neu. The observation that the deletions preserved the Neu open reading frame suggested that the transcripts may encode functional Neu proteins. In addition, the alterations were observed only in tumor tissue and not the adjacent mammary epithelium, raising the possibility that these mutations might activate Neu. To directly test whether these alterations in *neu* were responsible for its oncogenic activation, several of the deleted regions were inserted into a wild-type *neu* expression cassette and tested for the capacity to transform Rat-1 fibroblasts. To accomplish this, expression plasmids bearing the wild-type *neu* cDNA (pJ4ΩneuN), the transforming point mutation (pJ4ΩneuNT), and four representative deletion mutants were electroporated into Rat-1 cells and assessed for the capacity to induce transformed foci. These experiments were repeated three times with two independent plasmid DNA preparations (Table 1). Whereas expression of wild-type *neu* was unable to transform Rat-1 cells, expression of the altered *neu* cDNAs resulted in the induction of transformed foci (Fig. 5 and Table 1). Comparison of the altered *neu* cDNAs with the transforming allele of *neu* harboring the point mutation in the transmembrane domain (pJ4ΩneuNT) revealed that these altered *neu* cDNAs transformed Rat-1 cells at 18 to 56% of the frequency observed with the activated *neu* allele (Fig. 5 and Table 1).

To test whether the primary tumors expressing these altered transcripts possessed constitutively activated Neu, protein extracts derived from tumor or adjacent mammary epithelium tissue were immunoprecipitated with Neu-specific antibodies

TABLE 1. Transformation of Rat-1 cells[a]

Expression plasmid	Focus assay 1		Focus assay 2		Focus assay 3		Relative transforming ability[b]
	Average no. of foci/μg[c]	% Transformation of pJ4ΩneuNT[d]	Average no. of foci/μg[e]	% Transformation of pJ4ΩneuNT[d]	Average no. of foci/μg[c]	% Transformation of pJ4ΩneuNT[d]	
pJ4ΩneuN	0	0	0	0	0	0	0
pJ4ΩneuNT	136 ± 4	100	74 ± 5	100	77 ± 5	100	100 ± 0
pJ4Ωneu8142	65 ± 3	48	43 ± 2	58	48 ± 4	62	56 ± 7
pJ4Ωneu8340	34 ± 3	25	18 ± 2	24	14 ± 2	18	22 ± 4
pJ4Ωneu8342	29 ± 4	21	16 ± 1	22	9 ± 1	12	18 ± 6
pJ4Ωneu8567	53 ± 3	39	39 ± 3	53	48 ± 2	62	51 ± 12

[a] Three independent focus assays were performed with established Rat-1 fibroblasts. In each case, the cDNA of interest was placed under the control of the Moloney murine leukemia virus long terminal repeat. The first two experiments were conducted with plasmid DNA from the same large-scale preparation, while the third experiment was performed with DNA obtained from an independent plasmid preparation. The Rat-1 fibroblasts were stained with Giemsa at 14 days following electroporation. The transforming ability of the various mutants were compared with that of an activated form of Neu (NeuNT) which has been described previously (3). Expression of the wild-type Neu receptor (NeuN) in Rat-1 fibroblasts was also included as a negative control for transformation.

[b] Values are the mean transforming abilities from all three experiments ± the standard deviations.

[c] Values are the mean numbers of foci counted on six plates ± the standard deviations.

[d] Values are the ratios of the mean numbers of foci obtained for each construct with respect to the activated form of Neu (neuNT).

[e] Values are the mean numbers of foci counted on five plates ± the standard deviations.

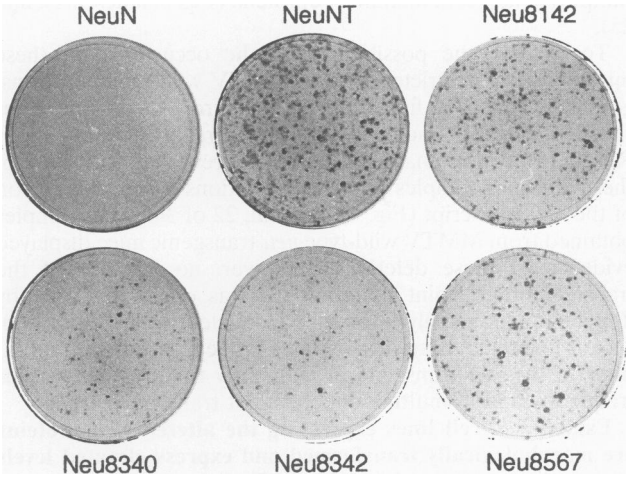

FIG. 5. Expression of altered Neu receptors results in morphological transformation. Shown are representative plates from focus assay 2 (see Table 1) illustrating the transforming ability of the indicated altered Neu receptors compared with those of both wild-type (NeuN) and activated (NeuNT) Neu.

and then subjected to immunoblot analyses with antiphosphotyrosine antibodies. As shown in Fig. 6A, tyrosine-phosphorylated Neu was observed only in the tumor samples (lanes 2, 4, 6, 8, and 9 to 11). To further determine whether the deletions resulted in the activation of the Neu tyrosine kinase, we measured the state of tyrosine phosphorylation of Neu in several independent cell lines expressing the mutant constructs. As shown in Fig. 6C, tyrosine-phosphorylated Neu could be detected in transformed cell lines expressing the altered *neu* transcripts but was not detected in cell lines expressing elevated levels of wild-type Neu. Because immunoblot analyses revealed that the various cell lines expressed comparable levels of Neu protein (Fig. 6B), the increase in tyrosine-phosphorylated Neu likely reflects the catalytic activation of the altered receptors which results from the deletions occurring within the extracellular domain.

DISCUSSION

We have shown that mammary tumors arising in transgenic mice carrying the *neu* proto-oncogene possess altered transcripts bearing in-frame deletions affecting the extracellular domain of Neu. Indeed, 65% of the tumor samples examined express *neu* transcripts which carry deletions (Fig. 2 to 4). Quantitative analyses of the levels of these altered transcripts revealed that they are expressed at levels comparable to that of the wild-type *neu* transgene transcript. Introduction of expression cassettes bearing these altered *neu* cDNAs into Rat-1 fibroblasts resulted in the morphological transformation of the fibroblasts, providing evidence that the mutations are responsible for the transforming activity of the *neu* proto-oncogene. Moreover, cell lines expressing these altered transcripts possessed tyrosine-phosphorylated Neu, suggesting that the altered receptors have elevated tyrosine kinase activity. These observations support the hypothesis that oncogenic activation of Neu in this mouse model of human breast cancer occurs through mutations in the extracellular domain.

These observations have important implications in understanding the molecular mechanism by which Neu induces mammary tumors. The frequent occurrence of the activating mutations (65%) in *neu* argues that the rate-limiting step in the induction of mammary tumors expressing the wild-type Neu protein is activation of its tyrosine kinase activity. In fact, previous studies have suggested that expression of the activated allele of *neu* is sufficient for the induction of mammary tumors in mice (19, 28). Although these mutations occur at a high frequency in this transgenic-mouse model, 35% of the tumors still lack detectable alterations in this region of *neu*. It is conceivable that activation of Neu in the remaining tumors occurs through other types of mutations. For example, deletion of the carboxy-terminal regulatory domain of Neu can result in its oncogenic activation (1). Alternatively, it is possible that activation of Neu could occur through a mechanism involving overexpression of its cognate ligand(s).

There is considerable evidence to suggest that activation of receptor tyrosine kinases such as Neu through somatic mutations is a frequent occurrence in both animal and human cancers. In chemically induced rat neuroblastomas, Neu is constitutively activated by a single point mutation in the transmembrane domain (3). Whereas the mechanism by which this point mutation influences the Neu tyrosine kinase is not clearly understood, it is believed that the mutant receptors demonstrate an increased capacity to homodimerize in a ligand-independent fashion (6, 7, 29). Interestingly, germ line mutations in the transition between the ligand binding and transmembrane domains of the RET receptor tyrosine kinase

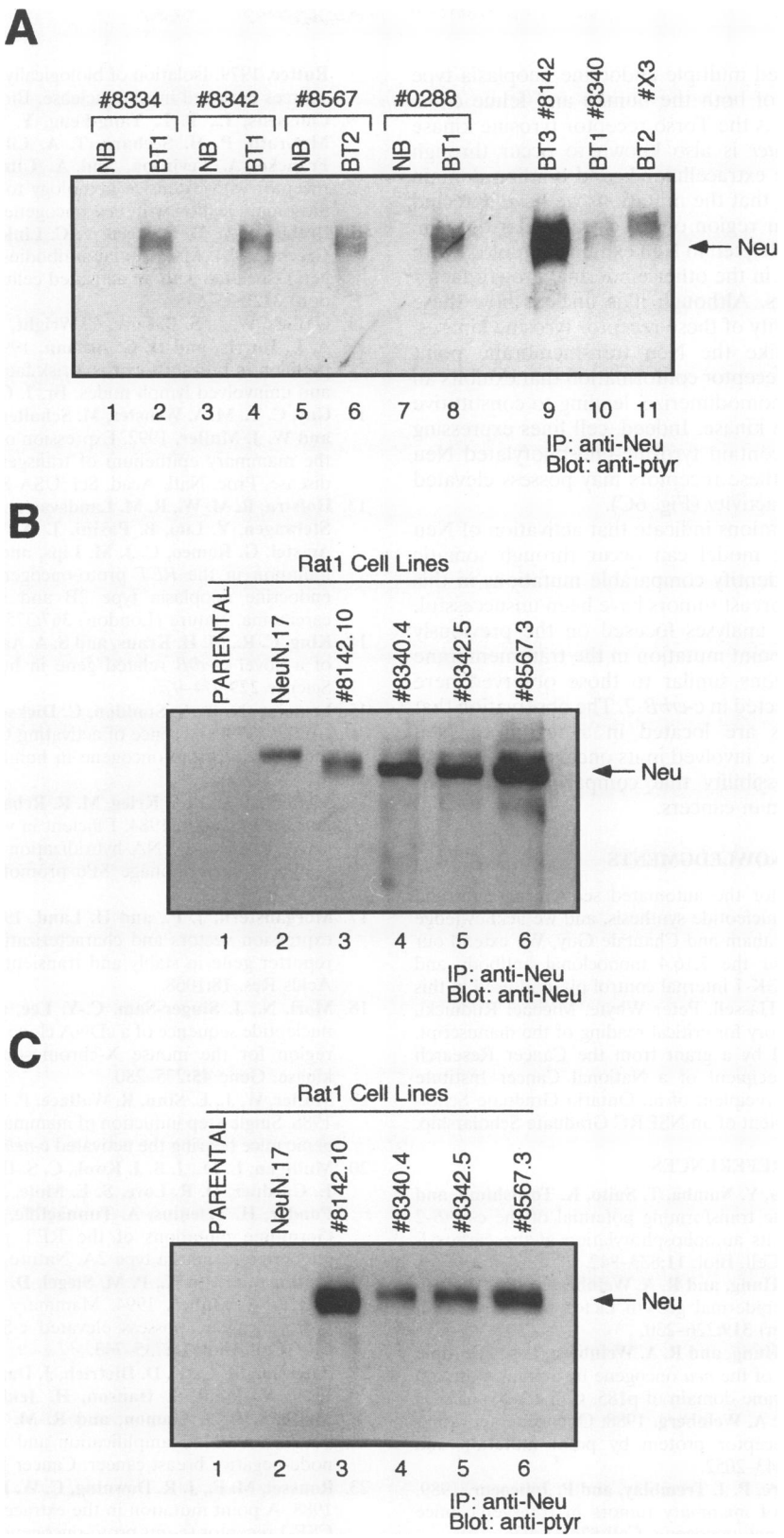

FIG. 6. Altered Neu proteins are constitutively tyrosine phosphorylated in vivo. (A) Protein lysates from tumor tissue and adjacent mammary epithelium tissue were immunoprecipitated (IP) for Neu with a monoclonal antibody (7.16.4) (anti-Neu) and then subjected to an immunoblot analysis (Blot) with an antiphosphotyrosine antibody (4G10) (anti-ptyr). The position of the tyrosine-phosphorylated Neu protein is indicated by the arrow. (B) Stable cell lines overexpressing wild-type Neu protein NeuN) and cell lines each overexpressing one of the four altered receptors were derived from Rat-1 fibroblasts. Parental refers to the original Rat-1 cell line, which does not contain any of the Moloney murine leukemia virus expression plasmids. Lysates from these cell lines were immunoprecipitated for Neu (monoclonal antibody 7.16.4), and 50% of the immunoprecipitate was subjected to immunoblot analysis with a Neu-specific antibody (Ab3) (anti-Neu). The position of the Neu protein is indicated by the arrow. (C) The remaining 50% of the Neu immunoprecipitate was subjected to immunoblot analysis with phosphotyrosine-specific antibody (4G10). The position of the tyrosine-phosphorylated Neu protein is indicated by the arrow.

are implicated in inherited multiple endocrine neoplasia type 2A (13, 20). Activation of both the human and feline c-*fms* proto-oncogenes as well as the Torso receptor tyrosine kinase in *Drosophila melanogaster* is also known to occur through mutations located in the extracellular ligand binding domain (23, 26, 30). It is striking that the mutations we have detected in Neu reside in a similar region of the extracellular domain. Interestingly, the region deleted in Neu exhibits homology with the comparable domains in the other epidermal growth factor receptor family members. Although it is unclear how these mutations affect the activity of these receptor tyrosine kinases, it is conceivable that like the Neu transmembrane point mutation, they induce a receptor conformation that exhibits an increased propensity to homodimerize, leading to constitutive activation of the tyrosine kinase. Indeed, cell lines expressing mutant Neu molecules contain tyrosine-phosphorylated Neu protein, suggesting that these receptors may possess elevated levels of tyrosine kinase activity (Fig. 6C).

Although these observations indicate that activation of Neu in this transgenic-mouse model can occur through somatic mutations, attempts to identify comparable mutations in this region of Neu in human breast tumors have been unsuccessful. However, because these analyses focused on the previously characterized activating point mutation in the transmembrane domain (15, 25), mutations similar to those observed here would not have been detected in c-*erbB-2*. The observation that the identified mutations are located in a region of Neu previously not known to be involved in its oncogenic activation raises the intriguing possibility that comparable mutations might be detected in human cancers.

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