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Genetics

Expression of the *neu* protooncogene in the mammary epithelium of transgenic mice induces metastatic disease

(mammary tumorigenesis/protein-tyrosine kinase/cancer metastasis)

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ABSTRACT **Overexpression and amplification of the *neu* (c-*erbB2*, *ERBB2*) protooncogene have been implicated in the development of aggressive human breast cancer. To directly assess the effect of mammary gland-specific expression of the *neu* protooncogene, transgenic mice carrying unactivated *neu* under the transcriptional control of the mouse mammary tumor virus promoter/enhancer were established. By contrast to the rapid tumor progression observed in several transgenic strains carrying the activated *neu* transgene, expression of unactivated *neu* in the mammary epithelium resulted in the development of focal mammary tumors after long latency. The majority of the mammary tumors analyzed expressed elevated levels of *neu*-encoded mRNA and protein. Overexpression of *neu* in the mammary tumors was also associated with elevated neu intrinsic tyrosine kinase activity and the *de novo* tyrosine phosphorylation of several cellular proteins. Interestingly, many of the tumor-bearing transgenic mice developed secondary metastatic tumors in the lung. These observations suggest that overexpression of the unactivated neu protein can induce metastatic disease after long latency.**

The *neu* protooncogene encodes a 185-kDa transmembrane protein that is a member of the epidermal growth factor receptor family (1–4). Oncogenic activation of *neu* can occur through a point mutation in the transmembrane domain (5), deletion of the extracellular domain (6), or overexpression (7–9). Amplification and overexpression of the human homologue of *neu* (c-*erbB2*; human gene symbol, *ERBB2*) have been observed in a large percentage of primary breast cancers (1, 10–13) and appear to be inversely correlated with the survival of the patient (11, 13).

Given the close correlation between *neu* overexpression and mammary carcinogenesis, a number of laboratories have been interested in directly testing the tumorigenic potential of the *neu* oncogene in the mammary epithelium of transgenic mice. Initially, this was accomplished by generating several lines of transgenic mice carrying the activated rat *neu* oncogene under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter/enhancer (14–16). In several strains of MMTV/activated *neu* mice, early onset of transgene expression in the mammary epithelium of female mice was associated with the synchronous development of tumors involving the entire mammary epithelium. These results suggested that expression of activated *neu* requires few, if any, additional genetic events to transform the mammary epithelial cell (14).

These studies suggested that activated *neu* can act as a potent oncogene in the mammary epithelium. However, overexpression of the unactivated neu protein may be the primary mechanism contributing to human breast cancer, since examination of primary breast cancer biopsy samples has thus far failed to reveal comparable activating mutations (13, 17). To directly test the oncogenic potential of the unactivated neu protein in the mammary epithelium, six lines of transgenic mice carrying a MMTV/unactivated *neu* fusion gene were derived. Overexpression of the unactivated *neu* product in the female mammary epithelium of five of these lines resulted in appearance of focal mammary tumors that metastasized with high frequency. These observations support the hypothesis that elevated expression of *neu*-associated tyrosine kinase activity in the mammary epithelium induces metastatic disease.

MATERIALS AND METHODS

DNA Constructions and Generation of Transgenic Mice. The recombinant plasmid pMMTVneuN was established by inserting the *Hin*dIII–*Eco*RI fragment encoding the unactivated *neu* cDNA and simian virus 40 (SV40) polyadenylylation and splicing signals from pSV2neuN (5) into the corresponding *Hin*dIII–*Eco*RI sites of the plasmid pMMTVneuNT (14). The plasmid pASV, which contains SV40 early splice and polyadenylylation signals, was constructed as described (14). The β-casein riboprotection probe was obtained from J. Rosen (Baylor College of Medicine) and was cloned as a 205-base-pair *Pst* I fragment in the plasmid pSP64 (Promega). The internal control plasmid rpL3227.3.7 was obtained from M. Shen (Harvard Medical School) and encodes the *Xho* II–*Dra* I fragment of the mouse ribosomal protein gene *rpL32* inserted into the corresponding sites of the plasmid pBluescript KS (Stratagene). All plasmid DNAs were isolated and purified as described by Sinn *et al.* (18). To derive transgenic mice carrying the MMTV/unactivated *neu* transcription unit, the pMMTVneuN *Sph* I–*Eco*RI fragment was microinjected into the pronuclei of FVB/N fertilized one-cell zygotes as described (18). Transgenic progeny were identified by probing genomic DNA after Southern blot transfer (19) with a *neu* cDNA-specific probe that was radiolabeled with [α-^{32}P]dCTP by random priming (20).

Expression Analyses. RNA was isolated from tissues by the procedure of Chirgwin *et al.* (21), using a CsCl sedimentation gradient modification. RNA probes were made with either pBluescript (Stratagene, San Diego, CA) or pGEM (Promega) vectors, and RNase protection was done according to Melton *et al.* (22), with 10 μg of total cellular RNA per assay. Western analyses with rabbit anti-phosphotyrosine antibodies were performed as described (23). To detect the presence of the neu protein in these tumors, 300 μg of total cellular protein was

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Abbreviations: MMTV, mouse mammary tumor virus; SV40, simian virus 40.
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electrophoresed in an SDS/8% polyacrylamide gel, transferred to nitrocellulose, and probed with the anti-neu monoclonal antibody 7.16.4 (24). The proteins were visualized with the enhanced chemiluminescence (ECL) detection system (Amersham). *In vitro* kinase assays were conducted as described (16).

Histological Evaluation. Complete autopsies were performed and both gross and microscopic examinations were done. Tissues were fixed in 4% paraformaldehyde, blocked in paraffin, sectioned at 4 μm, routinely stained with hematoxylin and eosin, and examined as indicated in Fig. 1.

RESULTS

Expression of the Transgene Correlates with Tumor Development. To test the oncogenic potential of the unactivated *neu* product in the mammary epithelium, a hybrid transcription unit comprising the MMTV promoter/enhancer and *neu* cDNA was microinjected into mouse zygotes. The MMTV/unactivated *neu* transgene is isogenic to the MMTV/activated *neu* described previously (14), except for the absence of the activating mutation in the transmembrane domain and the presence of a *Sal* I restriction endonuclease site between the *neu* cDNA and the SV40 polyadenylylation/splicing signals. A total of eight transgenic founders were generated carrying the MMTV/unactivated *neu* transgene. Of the eight strains, six passed the transgene to their progeny in a Mendelian fashion.

To assess the tissue specificity of transgene expression, 10 μg of total RNA derived from 10 different tissues isolated from both male and female carriers of the various founder strains was subjected to RNase protection with a transgene-specific probe comprising the SV40 polyadenylylation/splicing signals (pASV) (14). Representative results from these RNase protection experiments for the MMTV/*neu* strains are summarized in Table 1. Consistent with the observations made with other transgenic strains bearing MMTV/oncogenes, transgene expression was noted in mammary glands of female transgenic mice in five of six lines. Lower amounts of transgene transcript were detected in other tissues such as the salivary gland, spleen, thymus, and lung after longer exposure of the autoradiograms (Table 1).

Although low-level expression of *neu* in the mammary epithelium did not initially affect mammary gland function or development, focal mammary tumors began to appear in these strains at 4 months of age. Histological examination of the tumors revealed focal mammary adenocarcinomas surrounded by hyperplastic mammary epithelium (Fig. 1*B*). These tumors were composed of solid nests of pale intermediate cells that were morphologically identical to tumors associated with activated *neu* (25). Transplantation of the tumor cells into the mammary fat pads of syngeneic recipients resulted in the appearance of tumors, confirming their neoplastic potential. In our best characterized line, N#202, 50% of female carriers developed mammary tumors by 205 days (Fig. 1*A*). The appearance of tumors in this particular line was not strictly dependent on pregnancy, because virgin female transgenic mice also developed mammary tumors (data not shown). Furthermore, female transgenic mice derived from the other transgenic strains (N#169, N#510, N#721, and N#732) also developed tumors, albeit with later onset (Table 1). However, male MMTV/*neu* carriers did not exhibit any phenotypic abnormalities.

Because overexpression of *neu* (*ERBB2*) in human breast cancer has been implicated as an important step in tumor progression, we compared the level of transgene expression in the tumors and in the adjacent mammary epithelium. To this end, 10 μg of total RNA derived from mammary tumors and adjacent mammary epithelium isolated from female N#202 transgenic mice was hybridized with a radiolabeled transgene-specific probe (pASV) and subjected to RNase digestion (Fig. 2). To ensure that equal quantities of RNA were loaded, an *rpL32* antisense probe was also included in the hybridization reactions. Representative results for several sets of matched tumor and adjacent mammary epithelium for the N#202 line are shown in Fig. 2. Densitometric measurement of these autoradiograms revealed that many of the tumors ($n = 16$) expressed higher (10- to 50-fold) levels of transgene RNA than the adjacent mammary epithelium. However, several other matched sets of normal and tumor tissues ($n = 7$) expressed equivalent amounts of *neu* RNA (e.g., N#5741; Fig. 2). While there was some variation in transgene expression in the normal mammary epithelium, all mammary tumors examined expressed elevated levels of the *neu* transgene (Table 1).

To establish whether the elevated expression of transgene transcripts observed in the tumor tissues resulted in a corresponding increase in neu protein levels, Western analyses with neu-specific antibodies were conducted on protein extracts of normal and neoplastic tissues. The level of neu

Table 1. Transgene expression and onset of tumors in MMTV/unactivated *neu* mice

Line	Sex	Expression							Onset of tumor formation, days	% metastatic* tumors	Tumor type†
		M.gl.T	M.gl.N	Sal	L	SV	T	Ep			
N#169	F	+++	+	+	−				337 ($n = 4$)	50 ($n = 2$)	M.gl.Ad.
	M	−	+	+	−	−	+	+	NA	NA	No tumor
N#202	F	+++	+	+	+				205 ($n = 57$)	72 ($n = 41$)	M.gl.Ad.
	M	−	+	+	+	−	+	+	NA	NA	No tumor
N#204	F	−	−	−	−				NA	NA	No tumor
	M	−	−	−	−	−	−	−	NA	NA	No tumor
N#510	F	+++	+	+	−				367 ($n = 2$)	50 ($n = 1$)	M.gl.Ad.
	M	−	−	−	−	−	−	−	NA	NA	No tumor
N#721	F	+++	+	+	−				261 ($n = 21$)	11 ($n = 2$)	M.gl.Ad.
	M	−	+	+	−	+	−	+	NA	NA	No tumor
N#732	F	+++	+	+	−				268 ($n = 7$)	43 ($n = 3$)	M.gl.Ad.
	M	−	+	+	−	+	−	+	NA	NA	No tumor

RNase protection analysis was performed on 10 μg of total RNA isolated from a variety of organs from both male (M) and female (F) carriers derived from the MMTV/*neu* transgenic lines. The probe used in this analysis is directed against the SV40 component of the transgene and yields a 784-nucleotide protected fragment (see Fig. 2). Relative levels of transgene expression are indicated by − (not detected), + (low), or +++ (high). M.gl.T. refers to mammary gland tumor, whereas M.gl.N. represents normal adjacent epithelium. Other tissues examined for expression of the transgene included salivary glands (Sal), lung (L), seminal vesicles (SV), testes (T), and epididymis (Ep). NA, not applicable; *n*, number of animals analyzed.

*Percentage of tumor-bearing mice over 8 months of age possessing lung metastases.

†M.gl.Ad., mammary gland adenocarcinoma.

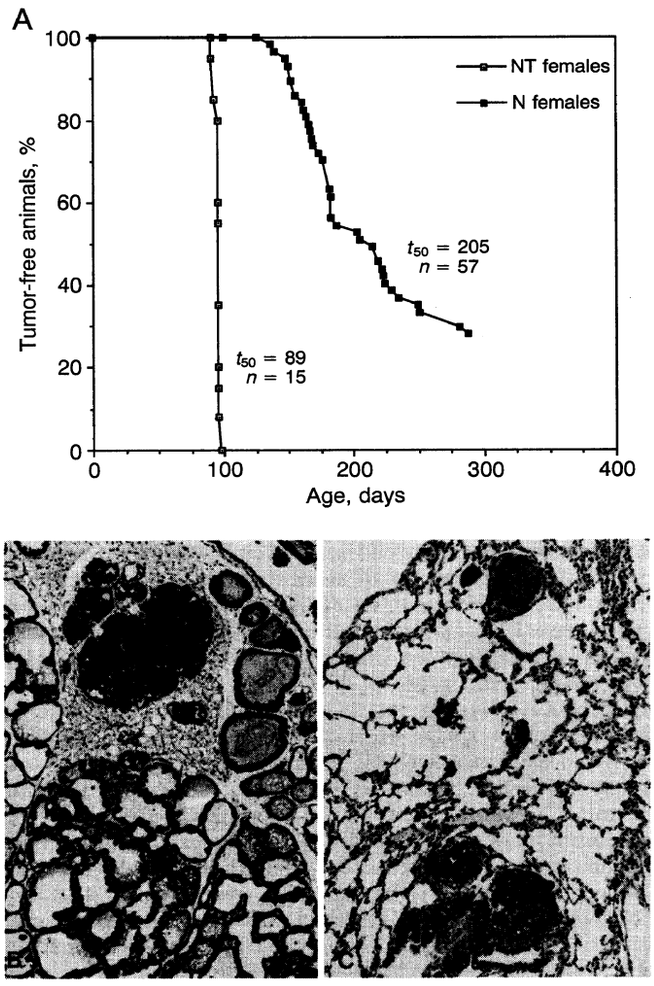

FIG. 1. Kinetics of tumor occurrence and histopathology of the MMTV/unactivated *neu* transgenic mice. (*A*) Comparison of the kinetics of tumor formation between female transgenic carriers bearing the MMTV/activated *neu* (NF line) (14) and females carrying the MMTV/unactivated *neu* construct N (N#202 line). The age at which 50% of mice were found to have tumors (t_{50}) and the number of mice examined (n) are indicated. (*B*) Photomicrograph of a hematoxylin/eosin-stained slide illustrating the expansile focus of solid tumor arising in the midst of a hyperplastic, dysplastic mammary gland (N#128, 210 days of age). Note that the dysplastic mammary cells lining dilated luminal spaces do not show lipid production. (×48.) (*C*) Photomicrograph of a hematoxylin/eosin-stained slide demonstrating a typical nest of mammary adenocarcinoma cells metastatic in the lung (N#202, 221 days of age). Note that the metastasis is in solid nests which resemble those found in the primary tumors such as those illustrated in *B*. (×48.)

protein in the tumors was higher than in the adjacent epithelium (Fig. 3*A*). Taken together, these observations suggest that elevated expression of *neu* may be an important step for tumorigenesis.

Mammary Gland-Specific Expression of *neu* Is Associated with the Induction of Metastatic Disease. Surprisingly, a high percentage of tumor-bearing MMTV/*neu* animals developed metastases to the lung (Fig. 1*C*). Histological examination of lung tissue in these affected animals revealed the presence of multiple foci of metastatic mammary adenocarcinoma lodged in pulmonary vessels (Fig. 1*C*). Like the primary mammary tumors, these metastatic lung tumors also expressed elevated levels of the *neu* transgene RNA (Fig. 2). The extent of metastatic involvement in these lines was particularly remarkable with respect to its penetrance. For example, in the N#202 line, 72% of the tumor-bearing mice that lived to an age of 8 months or older developed metastatic disease. Similar proportions of older tumor-bearing mice from the N#169, N#510, N#721, and N#732 lineages also developed metastatic disease (Table 1). Consistent with these observations, metastatic foci could also be detected in lung tissue after transplantation of the primary tumors into the fat pads of normal syngeneic recipients.

To confirm that the tumors detected in the lung were of mammary origin, RNase protection analyses with a probe specific to the mammary differentiation marker, β-casein, were performed on RNA derived from both primary and metastatic tumors (Fig. 2) (26). Both the primary mammary tumors and lung metastases derived from the N#202 line expressed moderate levels of β-casein RNA. However, normal lung tissue isolated from a nontransgenic female sibling did not express detectable amounts of β-casein RNA. Together with the histological observations, these results demonstrate that expression of unactivated *neu* in the mammary epithelium leads to the development of metastatic disease.

Induction of Mammary Tumors by *neu* Results in Elevated neu Tyrosine Kinase Activity. Because the transforming potential of the neu protein is closely correlated with its intrinsic tyrosine kinase activity, we were interested in measuring neu kinase activity in tumor and adjacent mammary epithelium derived from female animals of the N#202 line. To accomplish this, protein extracts derived from normal and tumor tissues were subjected to *in vitro* kinase assays using a monoclonal antibody directed against the rat neu protein. A prominent 185-kDa phosphorylated band was observed when extracts of mammary tumors and their derived metastases were assayed (Fig. 3*B*). By contrast, no comparable autophosphorylated species could be detected when extracts of nontransgenic control tissues or the adjacent mammary epithelium were assayed for kinase activity (Fig. 3*B*). Because neu protein could readily be detected in the mammary epithelium adjacent to the tumor tissue (Fig. 3*A*), these observations indicate that the *neu*-induced tumors possess higher neu-associated tyrosine kinase activity than the adjacent mammary epithelium.

DISCUSSION

Our observations provide direct evidence that expression of the protooncogenic form of *neu* results in a heritable development of metastatic mammary tumors. In five independent strains of MMTV/unactivated *neu* mice (N#202, N#169, N#510, N#721, and N#732), expression of the transgene resulted in the appearance of focal mammary adenocarcinomas which eventually metastasized to the lung. Tumorigenesis in these lines was correlated with elevated expression of the *neu* transgene and an increase in neu-associated tyrosine kinase activity. These observations support the hypothesis that elevated expression of *neu* can induce the production of metastatic mammary adenocarcinomas.

Overexpression of *neu* has been frequently observed in human primary breast cancers (10–13) and derived cell lines (1, 12, 27). In a large percentage of these human samples, overexpression of *neu* was associated with gene amplification. Consistent with these observations, RNase protection and Western analyses of tumor tissue (Figs. 2 and 3*A*) also revealed evidence of elevated expression of *neu* in mammary tumors. However, unlike human breast cancer samples, these tumors exhibited no evidence of amplification of the transgene or endogenous *neu* gene (data not shown). Conceivably, an increased transcription rate or an increase in mRNA stability of the transgene product could account for elevated *neu* expression. Indeed, several human mammary tumor cells express elevated levels of *neu* RNA in the absence of detectable gene amplification (27).

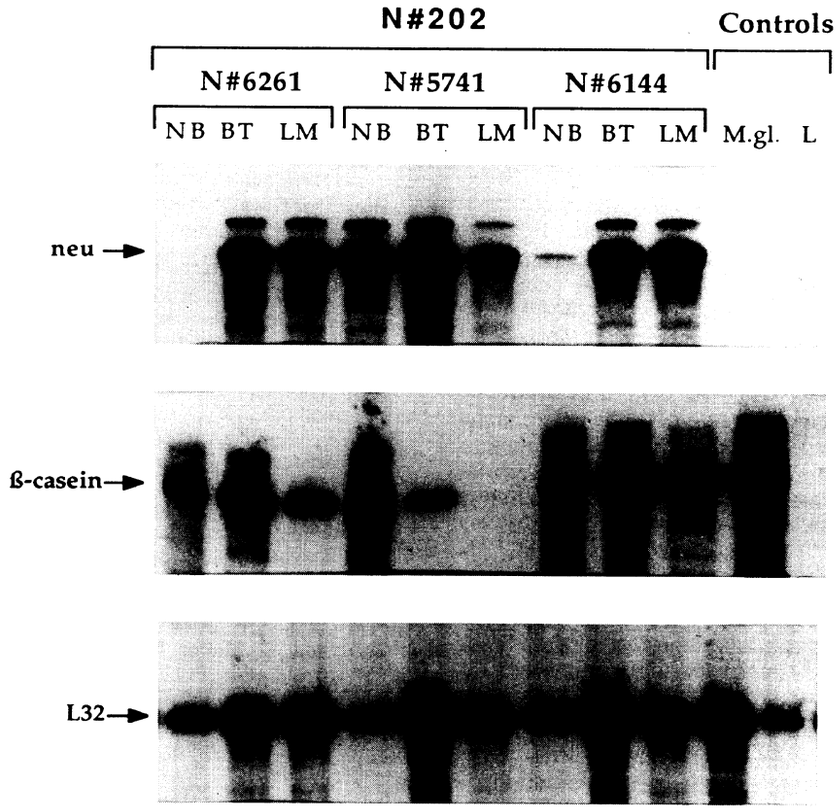

FIG. 2. RNase protection analysis of expression of the transgene RNA in tumor and adjacent mammary epithelium. RNA from control and transgenic tissues hybridized with probes directed to the transgene (neu), β-casein, and the *rpL32* ribosomal protein gene. The control tissues were isolated from lactating mammary glands (M.gl.) and lung (L) of normal female FVB mice. Transgenic tissues derived from multiparous female N#202 carriers (N#6261, 301 days of age; N#5741, 342 days of age; and, N#6144, 295 days of age) include primary mammary tumor (BT), adjacent mammary epithelium (NB), and lung metastases (LM). The 784-nucleotide protected fragment corresponds to the SV40 component of the transgene (neu), the 205-nucleotide protected fragment for β-casein, and the 278-nucleotide protected fragment for the *rpL32* control are indicated by arrows.

Previous studies have demonstrated that transgenic mice expressing the activated *neu* oncogene in the mammary epithelium also develop mammary tumors which are cytologically indistinguishable from those associated with the *neu* protooncogene (14, 15). However, malignant progression in strains carrying the MMTV/activated *neu* gene differs from the process observed here in the MMTV/unactivated *neu* transgenic mice. Mammary gland-specific expression of the activated *neu* oncogene in these strains was associated with the rapid and synchronous development of multifocal mammary tumors in both sexes (14). These studies suggested that expression of activated *neu* was sufficient for mammary tumorigenesis. By contrast, expression of unactivated *neu* is not sufficient, since mammary epithelium adjacent to tumors expresses appreciable levels of neu protein (Fig. 3*A*). It is conceivable that differences in the activity of the neu tyrosine kinase in transgenic mice expressing either the activated or the unactivated *neu* transgene account for this phenotypic variation. Consistent with this hypothesis is the observation that the *neu*-induced tumors possess higher tyrosine kinase activity than the adjacent mammary epithelium (Fig. 3*B*). Whereas the product of the activated *neu* transgene is constitutively active, the wild-type neu kinase may require additional events. For example, increased expression of neu receptor or its ligand could constitutively activate the neu kinase. Alternatively, neu kinase activity might be influenced by mutation or by the action of other cellular proteins. Consistent with the hypothesis that increased neu tyrosine kinase activity is required for tumor formation, a number of phosphotyrosine-containing proteins, including proteins of 185 kDa and 56 kDa, were specifically detected in tumor tissue by Western blot analyses with anti-phosphotyrosine

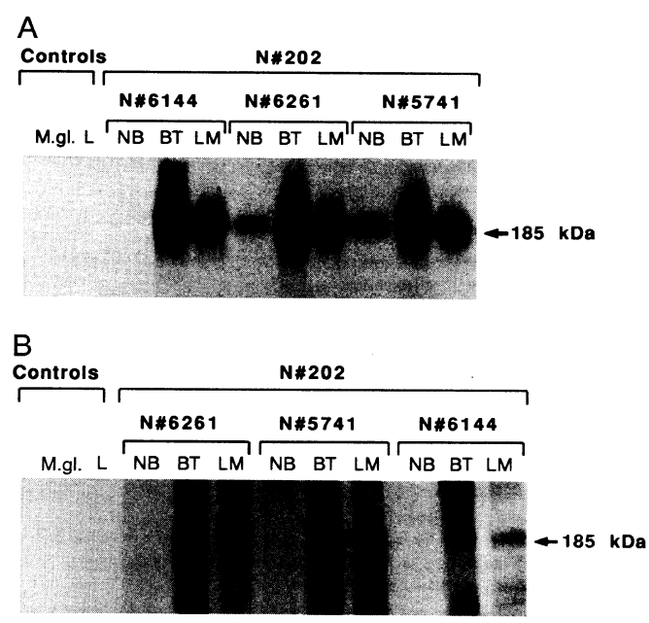

FIG. 3. Expression of neu protein and associated kinase activity in tumor and adjacent mammary epithelium. (*A*) Western analyses of control and transgenic tissues with a neu-specific monoclonal antibody. The control and transgenic tissues were isolated from the sources described in Fig. 2. The 185-kDa neu protein is indicated. (*B*) *In vitro* kinase activities of the same samples. Protein extracts were incubated with neu-specific monoclonal antibody, and the immunocomplexes were incubated with [γ-^{32}P]ATP. The 185-kDa phosphorylated neu species is indicated.

antibodies (data not shown). Because *in vitro* kinase analyses had demonstrated elevated neu kinase activity in tumor tissue (Fig. 3*B*) the 185-kDa protein very likely represents the autophosphorylated form of neu.

The unexpected finding that many of the older tumor-bearing *neu* transgenic animals developed pulmonary metastases may have important clinical implications. The observation that overexpression of *neu* in human breast cancer is associated with poor clinical outcome in node-negative women (28, 29) and the results of these transgenic experiments suggest that overexpression of *neu* can confer an enhanced metastatic potential upon the mammary tumor cell. Pulmonary metastases were also observed in transgenic strains carrying the MMTV/activated *neu* transgene (14). However, metastasis in these mice was relatively infrequent. Because these activated *neu* tumors involve the entire mammary epithelium and thus considerably shorten the animals' survival, it is conceivable that the further steps necessary for metastatic progression do not have sufficient time to occur in these MMTV/activated *neu* mice. Consistent with this notion, metastasis in the MMTV/unactivated *neu* lines is observed only in mice that have carried mammary tumors for several months (Table 1). Determination of the mechanism by which the neu kinase is involved in the induction of metastatic disease awaits further analysis.

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