MOLECULAR AND CELLULAR BIOLOGY, June 1990, p. 3247–3252
0270-7306/90/063247-06$02.00/0

Vol. 10, No. 6

Transformation of NIH 3T3 Cells by Overexpression of the Normal Coding Sequence of the Rat *neu* Gene

EDDI DI MARCO, JACALYN H. PIERCE, CHARLES L. KNICLEY, AND PIER PAOLO DI FIORE*

Laboratory of Cellular and Molecular Biology, National Cancer Institute, Building 37, Room 1E24, Bethesda, Maryland 20892

Received 27 November 1989/Accepted 13 February 1990

While the normal human *erbB-2* gene is potently transforming when overexpressed in NIH 3T3 cells, its rat homolog, the *neu* gene, seems to acquire transforming properties only upon alteration of its coding sequence. In this study, we compared the effects of different levels of expression of normal *erbB-2* and *neu* in NIH 3T3 cells. Our results revealed that the normal rat *neu* gene acts as a potent oncogene when sufficiently overexpressed in NIH 3T3 cells.

The *erbB-2/neu* gene has been implicated in the genesis of a number of experimentally induced (14, 18) or naturally occurring (7, 11, 12, 17, 20, 21, 26) malignant diseases. The rat *neu* gene was first identified as a dominant transforming gene in rat neuroblastomas induced by ethylnitrosourea (14, 18). Subsequently, Bargmann et al. (2) reported that oncogenic activation was due to a single point mutation in the region coding for the transmembrane domain of the *neu* proto-oncogene (henceforth referred to as *neuN*). The human homolog of the *neu* gene is known as *erbB-2*. It was originally identified owing to its amplification in a mammary adenocarcinoma (11) and has been subsequently found amplified or overexpressed or both at a high frequency in human carcinomas (7, 17, 20, 21, 26). *erbB-2* amplification in mammary and ovary adenocarcinomas may correlate with a more aggressive disease course (20, 21). The *erbB-2/neu* gene encodes for a growth factor receptor-like protein, gp185, with intrinsic tyrosine kinase activity (1, 3, 4, 16, 22, 23) which shares extensive structural and sequence homology with the receptor for epidermal growth factor (5). Recently, evidence for the existence of an *erbB-2/neu* ligand has been provided (25).

Studies have been performed to investigate the effect of overexpression of the normal *erbB-2* and *neu* coding sequences in model systems in vitro. We (6) and others (9) demonstrated that the human normal *erbB-2* gene acts as a potent oncogene when overexpressed in NIH 3T3 cells. Furthermore, we showed that a critical level of overexpression is necessary to achieve transformation of NIH 3T3 cells (6). Overexpression of the normal rat *neu* gene has not been reported to cause cell transformation (2, 3, 10). The basis for the apparent discrepancy in transforming potential of human *erbB-2* and normal rat *neu* genes has not been resolved. However, in the normal rat *neu* gene, no attempt has been made to estimate the level of overexpression of its product, $gp185^{neuN}$ (2, 3, 10), compared with the levels of $gp185^{erbB-2}$ required to induce cell transformation.

In this study, we endeavored to study the effects of different levels of overexpression of $gp185^{neuN}$ on the phenotype of NIH 3T3 fibroblasts. Our findings demonstrated that normal rat *neu* acts as a potent oncogene when sufficiently overexpressed in NIH 3T3 cells.

Engineering of LTR-based expression vector for normal rat *neu* cDNA. In previous studies, we have demonstrated that different promoters can control the transcription of the human normal *erbB-2* cDNA in NIH 3T3 cells with different efficiency (6). By use of simian virus 40 (SV40) early region and Moloney murine leukemia virus long terminal repeat (LTR) promoters, we were able to obtain around 10- and 100-fold overexpression, respectively, of $gp185^{erbB-2}$ (6). Therefore, we engineered a eucaryotic expression vector, LTR-2/*neuN*, in which the transcription of the normal rat *neu* gene was controlled by Moloney murine leukemia virus LTRs. The strategy followed to engineer this construct is illustrated in Fig. 1. The open reading frame (ORF) of the normal rat *neu* cDNA (*neuN* [2]) was cloned into the previously described LTR-2 vector (6) (Fig. 1). This vector was created from the proviral form of Moloney murine leukemia virus by engineering an internal deletion spanning from the *Pst*I site at position 568 of the original sequence (19) to the *Bam*HI site at position 6538 and replacing this sequence with the one coding for a unique *Xho*I cloning site. This deletion removes the *gag* and *pol* genes and a large fraction of the *env* gene, including its putative translation start site (19). In addition, the translation initiation codon of the *gag* gene is removed and no other ATGs followed by an ORF are available upstream from the unique *Xho*I cloning site. Therefore, translation has to start at the natural ATG present in the cDNA to be expressed.

The efficiency of transcription in this vector is strongly affected by the amount of 5′ untranslated information present in the cDNA to be expressed (P. P. Di Fiore, unpublished observations). Therefore, we removed all the 5′ untranslated sequence preceding the translation initiation codon of the *neuN* cDNA before insertion in the LTR-2 vector. To do this, the *Bal*I site present at position 3887 after the stop codon of the *neuN* cDNA was modified to *Sal*I and a *Bcl*I (position 19)-*Sal*I (position 3887) fragment of the *neuN* cDNA was cloned in the *Xho*I site of the LTR-2 vector (Fig. 1). The *Bcl*I site at position 19 overlaps the translation initiation codon of the normal *neu* cDNA. Therefore, an adapter oligonucleotide was used to join this *Bcl*I site to the 5′ *Xho*I cloning site, which also restored the proper *neu* ORF with an intact initiation codon (Fig. 1).

Effect of *neu* expression vectors on phenotype of NIH 3T3 cells. To assess the transforming potential of the normal rat *neu* cDNA, we transfected the LTR-2/*neuN* and SV40/*neuN* (2) expression vectors into NIH 3T3 cells. For comparison, we also transfected the analogous plasmids engineered with the human *erbB-2* gene, LTR-2/*erbB-2* (6) and SV40/*erbB-2*

* Corresponding author.

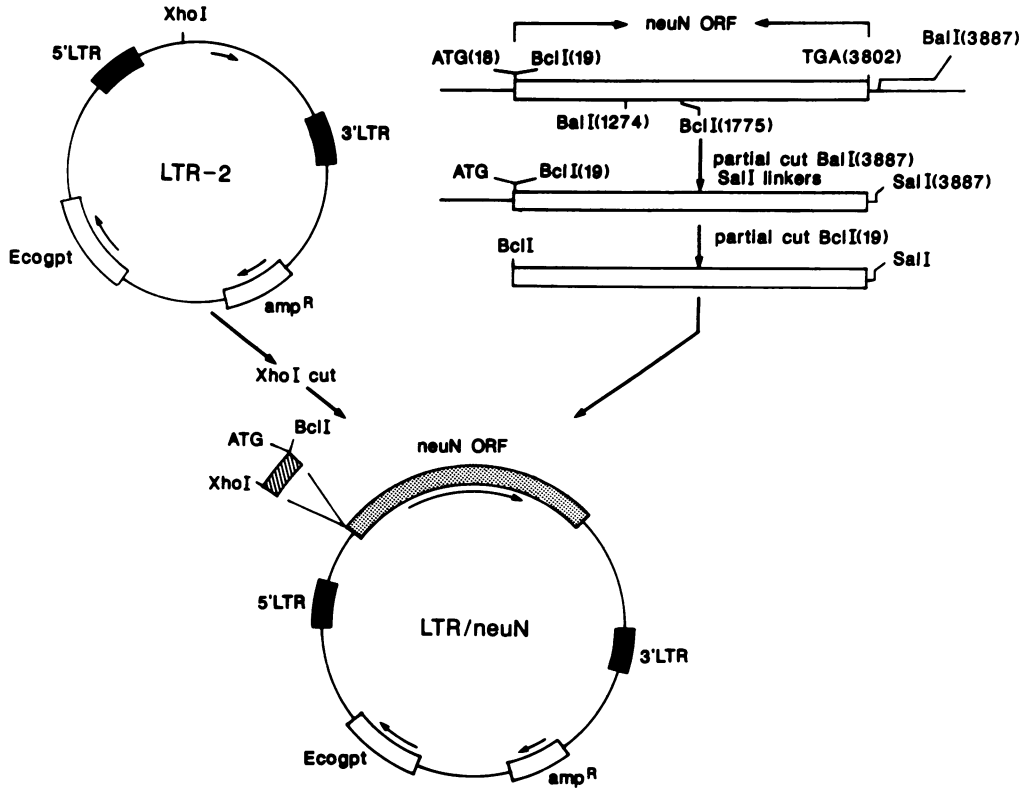

FIG. 1. Engineering of the LTR-2/*neuN* eucaryotic expression vector. The modalities of engineering are detailed in the text. Arrows indicate the transcriptional orientation of the individual promoters. ▨, Oligonucleotide used to restore the ORF of the *neu* cDNA. The adapter oligonucleotide had the following structure: 5'-AATTCGCAGCGTCGACGCCGCAAT-3'. The LTR-2/*neuN* construct was sequenced in the regions which underwent genetic manipulations to verify that the predicted structure was obtained. The region coding for the transmembrane domain of the *neu* protein was also sequenced to ensure that the normal coding sequence was present.

(6). SV40/*neuN* and SV40/*erbB-2* did not induce detectable foci of morphologically transformed cells (Fig. 2). In striking contrast, the LTR-2/*neuN* construct induced transformation with an efficiency of around 10^3 foci per pmol of added DNA (Fig. 2; Table 1). This effect was comparable to that obtained with the LTR-2/*erbB-2* expression vector (Fig. 2; Table 1). As previously reported (2), the SV40/*neuT* plasmid, which contains a transmembrane mutation that activates the *neu* product (2), was also capable of inducing cell transformation (Table 1). The LTR-2 vector was not transforming when transfected alone or when cotransfected at different molar ratios with the SV40/*neuN* construct (Fig. 2; Table 1).

As an independent approach to assess the transformed phenotype of LTR-2/*neuN* transfectants, we investigated their ability to display anchorage-independent growth, a property known to correlate well with malignancy. LTR-2/*neuN* transfectants were able to form colonies in soft agar at high efficiency (Table 1), comparable to that observed for LTR-2/*erbB-2* and v-H-*ras* transfectants. NIH 3T3 cells transfected with SV40/*neuN* or SV40/*erbB-2* constructs did not display any anchorage-independent growth under the same conditions (Table 1). Transforming activity in vitro paralleled the results obtained in a tumorigenicity assay in nude mice. LTR-2/*neuN* and LTR-2/*erbB-2* transfectants displayed comparable levels of malignancy in vivo and were as tumorigenic as cells transformed by the highly potent v-H-*ras* oncogene (Table 1). Of note, tumors appeared with a short latent period and grew rapidly, excluding the possibility that secondary alterations selected for in vivo were responsible for the tumorigenic phenotype of these cells. SV40/*neuN* and SV40/*erbB-2* transfectants did not form tumors even when 10^6 cells were inoculated (Table 1).

Expression of gp185^{neu} in NIH 3T3 transfectants. To analyze the levels of gp185^{neuN} and gp185^{erbB-2} in the various NIH 3T3 transfectants, we obtained mass cell populations after selection of the transfectants with mycophenolic acid (13). An antiserum (M1 antibody) has been generated against a peptide spanning residues 866 to 880 of the predicted human *erbB-2* product (5), a region identical to that in the predicted *neu* protein. This antibody recognized both proteins with similar efficiency in Western blot (immunoblot) analysis. As shown in the immunoblot in Fig. 3A, a 185-kilodalton protein was identified in SV40/*neuN*, LTR/*neuN*, and LTR-2/*erbB-2* transfectants. This recognition was specifically blocked by preabsorption of the antibody with the specific peptide (data not shown). Densitometric scans of the autoradiogram in Fig. 3A were performed on several exposures taken in the linear range of signal intensity. They revealed that the levels of expression of the *erbB-2*/*neu* product were comparable in LTR-2/*erbB-2* and LTR-2/*neuN* transfectants and about 20-fold higher than those detected in SV40/*neuN* transfectants (ratio in arbitrary units, 1:19.6:20.7 for SV40/*neuN*, LTR/*neuN*, and LTR/*erbB-2*, respectively; see also legend to Fig. 3A).

In another series of experiments, we isolated one SV40/*neuN*-transfected mass population which displayed high levels of expression of gp185^{neuN} by cotransfection with a high ratio of SV40/*neuN* and pSV2/*gpt* (13). When this cell

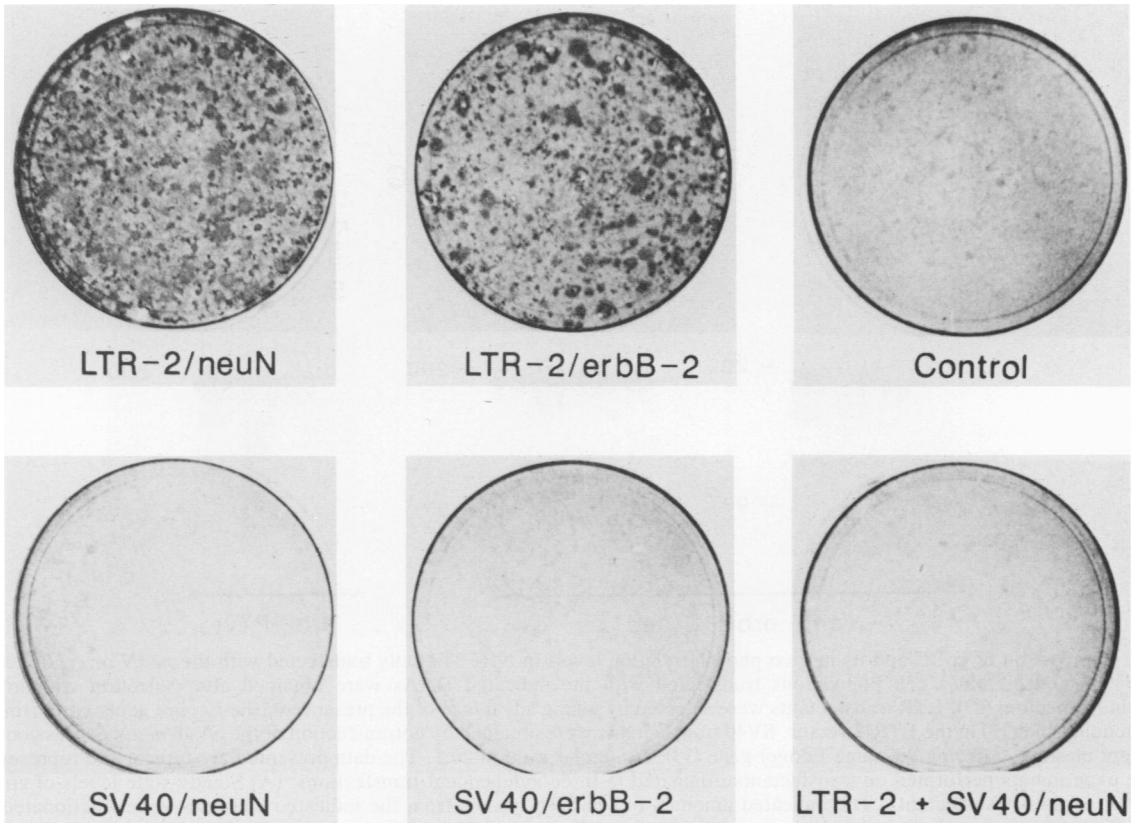

FIG. 2. Comparison of transforming activities of *erbB-2* and *neuN* under LTR or SV40 control. NIH 3T3 cells were transfected with 1 μg of DNA from each construct by the calcium phosphate precipitation technique (8, 24). Plates were stained with hematoxylin at 21 days after transfection. Control was calf thymus DNA. LTR-2 + SV40/*neuN*; Plates were transfected with 5 μg of both constructs. The data presented are typical and representative of four independent experiments performed in duplicate.

line, designated NIH SV40/*neuNM*, was assayed in immunoblot, it was found to express gp185^{neuN} at levels almost as high as those detectable in LTR/*neu* transfectants and more than 10-fold higher than those in SV40/*neuN* transfectants (see the legend to Fig. 3B for densitometric measurements). The generation of an SV40/*neuN* transfectant expressing high levels of gp185^{neuN} was a rare event. In fact, only one such a transfected line was isolated during our experiments. Interestingly, NIH SV40/*neuNM* cells were tumorigenic in vivo, forming tumors in four of four mice inoculated with 10^5 cells, and displayed high cloning efficiency of around 35% in semisolid medium. Thus, the higher levels of *erbB-2*/*neu* expression, achieved under LTR or sporadically under SV40 influence, correlated with the appearance of transformed phenotype.

It has been previously demonstrated that tyrosine phosphorylation of gp185^{erbB-2} and gp185^{neu} correlates well with their specific kinase activity and transforming potency (3, 4, 13, 16). Thus, we also analyzed the levels of in vivo tyrosine phosphorylation of gp185^{erbB-2} and gp185^{neuN} in various transfectants. The specific level of tyrosine phosphorylation of gp185^{neu} and gp185^{erbB-2} was similar, in that the reactivity with antiphosphotyrosine correlated with receptor number (Fig. 3C). Thus, we conclude that analogous to *erbB-2* (6, 16), a certain level of kinase activity dependent on the level of protein expression is required for gp185^{neuN} to exert its transforming effect.

Role of *erbB-2*/*neu* overexpression in in vitro transformation and in human tumors. The present studies demonstrated that the normal rat *neu* gene acts as a potent oncogene when overexpressed at sufficient levels in NIH 3T3 cells. Several lines of evidence demonstrated that the transforming action of the LTR-2/*neuN* construct was due to the high levels of gp185^{neuN} expressed and not to any other alteration induced during the gene transfer manipulations. First, the method of engineering of the LTR-2/*neuN* vector makes it extremely unlikely that any ORF other than that for the *neuN* was translated into this vector. Accordingly, the LTR-2 vector alone did not induce morphological alterations of NIH 3T3 cells or render them tumorigenic, even if cotransfected with the SV40/*neuN* construct. In addition, we were able to obtain a rare NIH SV40/*neuN* transfectant (NIH SV40/*neuNM*) which was tumorigenic upon injection into nude mice. This tumorigenicity correlated well with high levels of gp185^{neuN}, comparable to those normally obtained in LTR-2/*neuN* transfectants. Thus, irrespective of the method of achieving overexpression, gp185^{neuN} levels above a certain threshold invariably correlated with the acquisition of the transformed phenotype.

Our results are at variance with previous reports which did not detect transformation by the normal gp185^{neuN} (2, 3, 10). It is possible that quantitative variations might in part explain the observed discrepancies. This may be the case for the experiments performed with the normal rat *neu* cDNA under SV40 promoter influence (2). Our present results indicate that the level of overexpression normally achieved

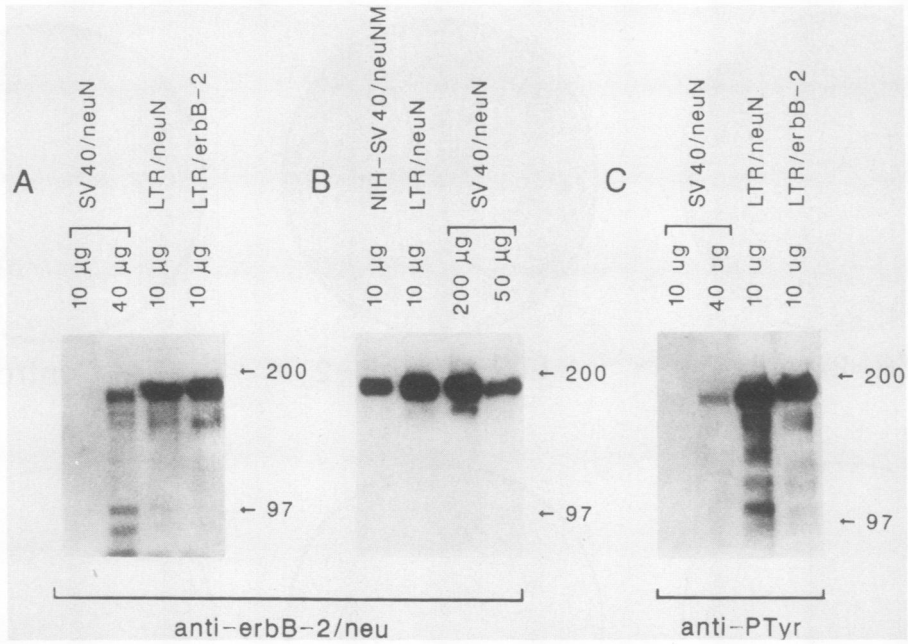

FIG. 3. Comparison of gp185 and its in vivo phosphorylation levels in NIH 3T3 cells transfected with the *neuN* or *erbB-2* cDNA under LTR or SV40 control. Mass cell populations transfected with the indicated DNAs were obtained after selection with mycophenolic acid-containing medium (13). LTR transfectants were selected by taking advantage of the presence of the Eco*gpt* gene, conferring resistance to mycophenolic acid (13) in the LTR-2 vector. SV40 transfectants were obtained by cotransfection of the SV40/*neuN* expression vector with the pSV2/*gpt* plasmid, carrying the same Eco*gpt* gene (13), at a molar ratio of 20:1. The data presented are typical and representative of at least three experiments performed on transfectants originated in three independent transfections. (A) Steady-state levels of $gp185^{neuN}$ and $gp185^{erbB-2}$ in various transfectants. The indicated amounts of total cell lysates from the indicated cell lines were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by immunoblot with the M1 antibody as previously described (6, 12). Molecular size markers are shown in kilodaltons. (B) Steady-state levels of $gp185^{neuN}$ in NIH SV40/*neuNM* cells in comparison with LTR/*neuN* and SV40/*neuN* transfectants. The indicated amounts of total cell lysates were analyzed as described for panel A. Molecular size markers are indicated in kilodaltons. (C) In vivo tyrosine phosphorylation. The indicated amounts of total cell lysates were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by immunoblot with a specific antiphosphotyrosine (anti-PTyr) antibody prepared as described by Pang et al. (15). The specificity of the reaction was determined by immunostaining identical blots with the antiphosphotyrosine antibody preabsorbed with phosphotyrosine, phosphoserine, or phosphothreonine. Molecular size markers are shown in kilodaltons. Densitometric scans of the autoradiograms in panels A to C were performed on several exposures taken in the linear range of signal intensity. Relative protein levels were calculated on the basis of the areas of the individual peaks, calculated in optical density × millimeters, and converted to arbitrary units after normalization for the amount of protein loaded. In the autoradiogram in panel A, for example, areas of 1.18, 5.78, and 6.11 optical density × mm were obtained for the lanes SV40/*neu* (40 µg) LTR/*neu* (10 µg), and LTR/*erbB-2* (10 µg). After correction for the amount of protein loaded, we obtained a ratio, in arbitrary units, of 1:19.6:20.7 for SV40/*neuN*, LTR/*neuN*, and LTR/*erbB-2* transfectants, respectively. Similarly, scans of the autoradiogram in panel B yielded values of 1:12.8:24.6 for SV40/*neuN*, NIH SV40/*neuNM*, and LTR/*neuN*. Scans of the autoradiogram in panel C yielded values of 1:26.8:25.5 for SV40/*neuN*, LTR/*neuN*, and LTR/*erbB-2* transfectants, respectively.

under these conditions would not be sufficient to induce the transformed phenotype. Direct attempts to further increase the level of $gp185^{neuN}$ expression have also been made (3, 10), which resulted in higher levels of $gp185^{neuN}$ expression but no detectable transformation. In the absence of a direct comparison between the levels of overexpression achieved under our conditions and those obtained in the previously published studies (3, 10), this difference is not readily explainable and warrants further investigation.

Overexpression of *erbB-2*, with or without gene amplification, is the most frequently detected alteration affecting this gene in human tumors (7, 11, 12, 17, 20, 21, 26). There are several reasons to believe that the $gp185^{erbB-2}$ overexpressed in some human tumors is normal. First, $gp185^{erbB-2}$ expressed in human tumor cell lines possesses normal intrinsic kinase activity compared with the enhanced activity displayed by activated transmembrane mutants (16). In addition, the *erbB-2* gene cloned from two human tumors possesses the normal transmembrane sequence (21). Therefore, our present and previously published results (6, 16) lend support to the hypothesis that overexpression of the normal product of the *erbB-2/neu* gene contributes to the development of some human tumors. However, the question remains as how the oncogenic potential of *erbB-2/neu* is activated under conditions of overexpression. In this regard, it is interesting that, unlike other growth factor receptors, the normal *erbB-2/neu* product displays constitutive levels of kinase activity in the absence of exogenously added ligand (3, 4, 16, 23). This could reflect production of its putative ligand (25) by an array of cell types or a constitutive unstimulated enzymatic activity. This latter possibility is supported by our recent finding that the chronic activation of $gp185^{erbB-2}$ is, at least in part, due to a strong upregulation of kinase activity exerted by its carboxy-terminal domain (6a). Although the participation of a ligand cannot be excluded at the present, these results indicate that control of the levels of $gp185^{erbB-2}$ expression is critical to regulate the signalling ability of its constitutively active kinase. An imbalance in the

TABLE 1. Transforming activity of *neu* and *erbB-2* expression vectors

DNA	Transforming efficiency (focus-forming units/pmol)[a]	Growth in agar (%)[b]	Tumorigenicity[c]		
			No. of cells injected	Tumor incidence	Latency period (wks)
SV40/*neuN*	$<10^0$	<0.01	10^6	0/8	
SV40/*neuT*	3.0×10^3	37	10^5	8/8	1
SV40/*erbB-2*	$<10^0$	<0.01	10^6	0/8	
LTR/*neuN*	1.4×10^3	28	10^5	8/8	2
LTR/*erbB-2*	2.6×10^3	32	10^5	8/8	2
LTR-2	$<10^0$	<0.01	10^6	0/8	
LTR-2 + SV40/*neuN*[d]	$<10^0$	<0.01		NT[e]	
v-H-*ras*	4.2×10^3	45	10^5	8/8	1

[a] Transfection was performed with 40 μg of calf thymus DNA as the carrier and 10-fold serial dilutions of specific circular plasmid DNA (10 to 0.001 μg) by the calcium phosphate precipitation technique (8, 24). Focus formation on NIH 3T3 cells was scored at 14 to 21 days on duplicate plates. Transforming efficiency was calculated in focus-forming units per picomole of added DNA based on the relative molecular weights of the respective plasmids and after normalization for the efficiency of colony formation in parallel dishes treated with mycophenolic acid containing medium for LTR-based constructs. Typically, we obtained around 5×10^3 mycophenolic acid-resistant colonies per pmol of DNA when circular LTR-based constructs were used. Data represent average of four independent experiments performed in duplicate. In two experiments, three independent clonal isolates of the LTR/*neuN* expression vector were used. The three plasmid clones yielded identical results.

[b] All transfectants were isolated from plates that received 1 μg of cloned DNA and were selected for their ability to grow in the presence of mycophenolic acid (13). For the anchorage-independent growth assay in soft agar, cells were plated at 10-fold serial dilution in 0.45% soft agar medium containing 10% calf serum. Visible colonies comprising >100 cells were scored at 14 days. Data represent average of three independent experiments performed in duplicate.

[c] NFR nude mice were injected subcutaneously with the indicated mass cell populations from two independent transfections selected as indicated in footnote *b*. Tumor formation was monitored at least twice weekly for up to 30 days.

[d] Cotransfection of the LTR-2 vector with the SV40/*neuN* expression vector was performed at 1:1 and 10:1 molar ratio (LTR-2:SV40/*neuN*), using 1 and 5 μg of SV40/*neuN*.

[e] NT, Not tested.

equilibrium caused by overexpression above a critical threshold might, therefore, contribute to the progression of the cell along the malignant pathway.

We are deeply in debt to S. A. Aaronson for continuous support and encouragement during this work and for critically reviewing this manuscript. We thank C. I. Bargmann and R. A. Weinberg for providing the SV40/*neuN* and SV40/*neuT* eucaryotic expression vectors. We also thank Paul Arnstein for performing the tumorigenicity studies.

LITERATURE CITED

1. **Akiyama, T., C. Sudo, H. Ogawara, K. Toyoshima, and T. Yamamoto.** 1986. The product of the human c-*erb*B-2 gene: a 185-kilodalton glycoprotein with tyrosine kinase activity. Science **232**:1644–1646.
2. **Bargmann, C. I., M. C. Hung, and R. A. Weinberg.** 1986. Multiple independent activations of the *neu* oncogene by a point mutation altering the transmembrane domain of p185. Cell **45**:649–657.
3. **Bargmann, C. I., and R. A. Weinberg.** 1988. Oncogenic activation of the *neu*-encoded receptor protein by point mutation and deletion. EMBO J. **7**:2043–2052.
4. **Bargmann, C. I., and R. A. Weinberg.** 1988. Increased tyrosine kinase activity associated with the protein encoded by the activated *neu* oncogene. Proc. Natl. Acad. Sci. USA **85**:5394–5398.
5. **Coussens, L., T. L. Yang-Feng, Y. C. Liao, E. Chen, A. Gray, J. McGrath, P. H. Seeburg, T. A. Libermann, J. Schlessinger, U. Francke, A. Levinson, and A. Ullrich.** 1985. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. Science **230**:1132–1139.
6. **Di Fiore, P. P., J. H. Pierce, M. H. Kraus, O. Segatto, C. R. King, and S. A. Aaronson.** 1987. *erb*B-2 is a potent oncogene with overexpressed in NIH/3T3 cells. Science **237**:178–182.

6a. **Di Fiore, P. P., O. Segatto, F. Lonardo, F. Fazioli, J. H. Pierce, and S. A. Aaronson.** 1990. The carboxy-terminal domains of *erbB-2* and epiderminal growth factor receptor exert different regulatory effects on intrinsic receptor kinase function and transforming activity. Mol. Cell. Biol. **10**:2749–2756.

7. **Fukushige, S., K. Matsubara, M. Yoshida, M. Sasaki, T. Suzuki, K. Semba, K. Toyoshima, and T. Yamamoto.** 1986. Localization of a novel v-*erb*B related gene, c-*erb*B-2, on human chromosome 17 and its amplification in a gastric cancer cell line. Mol. Cell. Biol. **6**:955–958.
8. **Graham, F. L., and A. J. Van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
9. **Hudziak, R. M., J. Schlessinger, and A. Ullrich.** 1987. Increased expression of the putative growth factor receptor p185HER2 causes transformation and tumorigenesis of NIH 3T3 cells. Proc. Natl. Acad. Sci. USA **84**:7159–7163.
10. **Hung, M. C., A. L. Schechter, P. Y. Chevray, D. F. Stern, and R. A. Weinberg.** 1986. Molecular cloning of the *neu* gene: absence of gross structural alteration in oncogenic alleles. Proc. Natl. Acad. Sci. USA **83**:261–264.
11. **King, C. R., M. H. Kraus, and S. A. Aaronson.** 1985. Amplification of a novel v-*erb*B-related gene in a human mammary carcinoma. Science **229**:974–976.
12. **Kraus, M. H., N. C. Popescu, S. C. Amsbaugh, and C. R. King.** 1987. Overexpression of the EGF receptor-related proto-oncogene *erb*B-2 in human mammary tumor cell lines by different molecular mechanisms. EMBO J. **6**:605–610.
13. **Mulligan, R. C., and P. Berg.** 1981. Selection for animal cells that express the Escherichia coli gene coding for xanthine-guanine phosphoribosyltransferase. Proc. Natl. Acad. Sci. USA **78**:2072–2076.
14. **Padhy, L. C., C. Shih, D. Cowing, R. Finkelstein, and R. A. Weinberg.** 1982. Identification of a phosphoprotein specifically induced by the transforming DNA of rat neuroblastomas. Cell **28**:865–871.
15. **Pang, D. T., B. R. Sharma, and J. A. Shafer.** 1985. Purification of the catalytically active phosphorylated form of insulin receptor kinase by affinity chromatography with O-phosphotyrosyl-binding antibodies. Arch. Biochem. Biophys. **242**:176–186.
16. **Segatto, O., C. R. King, J. H. Pierce, P. P. Di Fiore, and S. A. Aaronson.** 1988. Different structural alterations upregulate in vitro tyrosine kinase activity and transforming potency of the *erbB-2* gene. Mol. Cell. Biol. **8**:5570–5574.
17. **Semba, K., N. Kamata, K. Toyoshima, and T. Yamamoto.** 1985. A *verb*B-related proto-oncogene, c-*erb*B-2, is distinct from the c-*erb*B-1/epidermal growth factor-receptor gene and is amplified in a human salivary gland adenocarcinoma. Proc. Natl. Acad. Sci. USA **82**:6497–6501.
18. **Shih, C., L. C. Padhy, M. Murray, and R. A. Weinberg.** 1981. Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. Nature (London) **290**:261–264.
19. **Shinnick, T. M., R. A. Lerner, and J. G. Sutcliffe.** 1981. Nucleotide sequence of Moloney murine leukaemia virus. Nature (London) **293**:543–548.
20. **Slamon, D. J., G. M. Clark, S. G. Wong, W. J. Levin, A. Ullrich, and W. L. McGuire.** 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. Science **235**:177–182.
21. **Slamon, D. J., W. Godolphin, L. A. Jones, J. A. Holt, S. G. Wong, D. E. Keith, W. J. Levin, S. G. Stuart, J. Udove, A. Ullrich, and M. F. Press.** 1988. Studies of the HER-2/*neu* proto-oncogene in human breast and ovarian cancer. Science **244**:707–712.

22. **Stern, D. F., P. A. Heffernan, and R. A. Weinberg.** 1986. p185, a product of the *neu* proto-oncogene, is a receptorlike protein associated with tyrosine kinase activity. Mol. Cell. Biol. **6:** 1729–1740.
23. **Stern, D. F., M. P. Kamps, and H. Cao.** 1988. Oncogenic activation of p185*neu* stimulates tyrosine phosphorylation in vivo. Mol. Cell. Biol. **8:**3969–3973.
24. **Wigler, M., S. Silverstein, L. S. Lee, A. Pellicer, Y.-C. Cheng, and R. Axel.** 1977. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell **11:**223–232.
25. **Yarden, Y., and R. A. Weinberg.** 1989. Experimental approaches to hypothetical hormones: detection of a candidate ligand of the *neu* protooncogene. Proc. Natl. Acad. Sci. USA **86:**3179–3183.
26. **Yokota, J., T. Yamamoto, K. Toyoshima, M. Terada, T. Sugimura, H. Battifora, and M. J. Cline.** 1986. Amplification of c-*erb*B-2 oncogene in human adenocarcinomas in vivo. Lancet **i:**765–767.