Oncogenic activation of the *neu*-encoded receptor protein by point mutation and deletion

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The rat *neu* gene, which encodes a receptor-like protein homologous to the epidermal growth factor receptor, is frequently activated by a point mutation altering a valine residue to a glutamic acid residue in its predicted transmembrane domain. Additional point mutations have been constructed in a normal *neu* cDNA at and around amino acid position 664, the site of the naturally arising mutation. A mutation which causes a substitution of a glutamine residue for the normal valine at residue 664 leads to full oncogenic activation of the *neu* gene, but five other substitutions do not. Substituted glutamic acid residues at amino acid positions 663 or 665 do not activate the *neu* gene. Thus only a few specific residues at amino acid residue 664 can activate the oncogenic potential of the *neu* gene. Deletion of sequences of the transforming *neu* gene demonstrates that no more than 420 amino acids of the 1260 encoded by the gene are required for full transforming function. Mutagenesis of the transforming clone demonstrates a correlation between transforming activity and tyrosine kinase activity. These data indicate that the activating point mutation induces transformation through (or together with) the activities of the tyrosine kinase.
Key words: neu oncogene/oncogenes/receptor activation/tyrosine kinase

Introduction

The finding that the v-*erbB* oncogene encodes an altered version of the epidermal growth factor (EGF) receptor suggested that oncogenesis can occur through deregulation of the receptor molecules that ordinarily respond to mitogenic ligands (Downward *et al.*, 1984). This finding found subsequent parallels in work on the *fms*, *ros* and *neu* oncogenes, all of which also encode receptor-like proteins (Sherr *et al.*, 1985; Neckameyer *et al.*, 1986; Coussens *et al.*, 1985b; Yamamoto *et al.*, 1986; Bargmann *et al.*, 1986a). Understanding the effects of oncogenic mutations on these receptor molecules may cast light upon the mechanisms by which they are normally regulated by ligand binding.

The *neu* oncogene is frequently activated in chemically induced neuro- and glioblastomas of the rat (Schubert *et al.*, 1974; Shih *et al.*, 1981; Padhy *et al.*, 1982). The human homologue of this gene, also termed c-*erbB*-2 or HER-2 (King *et al.*, 1985; Coussens *et al.*, 1985b; Yamamoto *et al.*, 1986), is often found amplified in advanced mammary carcinomas (King *et al.*, 1985; Slamon *et al.*, 1987). The

neu-encoded protein, p185, has a predicted amino acid sequence that is co-linear and 50% identical with that of the EGF receptor (Coussens *et al.*, 1985b; Yamamoto *et al.*, 1986; Bargmann *et al.*, 1986a). Like the EGF receptor, the predicted *neu* gene product has extensive extracellular sequences, a single membrane-spanning domain and a tyrosine kinase domain followed by hydrophilic intracellular sequences. p185 has been directly demonstrated to be a transmembrane phosphoprotein with an associated tyrosine kinase activity (Stern *et al.*, 1986; Akiyama *et al.*, 1986). On the basis of its homology with the EGF receptor, it has been proposed that the normal product of the *neu* gene is the receptor for an as yet unidentified factor (Coussens *et al.*, 1985b; Yamamoto *et al.*, 1986; Bargmann *et al.*, 1986a).

In contrast with the viral v-*erbB*, v-*fms*, and v-*ros* oncogenes, which encode extensively deleted receptor-like proteins (Ullrich *et al.*, 1984; Coussens *et al.*, 1986; Neckameyer *et al.*, 1986), the transforming *neu* gene differs from its normal homologue by a single base substitution which alters a valine residue to a glutamic acid residue in the predicted transmembrane domain of p185 (Bargmann *et al.*, 1986b). Four independent ethylnitrosourea-induced tumor cell lines and 10 methylnitrosourea-induced tumors contain active *neu* genes bearing the identical substitution at this position (Bargmann *et al.*, 1986b; S.Sukumar, C.I.Bargmann, R.A. Weinberg and M. Barbacid, in preparation). The unexpected location of the activating mutations indicate a role of the transmembrane domain in regulating the activity of the *neu* oncogene product and its normal counterpart.

Biochemical characterization of the normal and transforming p185 proteins has provided some insight into the possible effect of the point mutation on the activity of p185. Under appropriate *in vitro* conditions, the normal and transforming p185 proteins differ in their associated tyrosine protein kinase activity (C.I.Bargmann and R.A.Weinberg, in preparation).

In order to understand the mechanism of activation by this point mutation, we have constructed mutations in the normal and transforming alleles of the *neu* gene. The repeated oncogene activation at residue 664 suggested that the Val → Glu alteration encoded at this site might be unique in its ability to confer transforming activity; this question was addressed by constructing point mutations in the normal *neu* gene at and around residue 664.

Differentiating between possible models of activation of p185 by the transmembrane alteration was made possible by the construction of mutants that delineate those sequences necessary for transformation by the transforming *neu* gene. This approach also critically tested the idea that the effect of the transforming mutation on the kinase activity of p185 was important for biological activity.

Finally, deletions were constructed in the normal *neu* gene in order to determine whether the gene could be activated
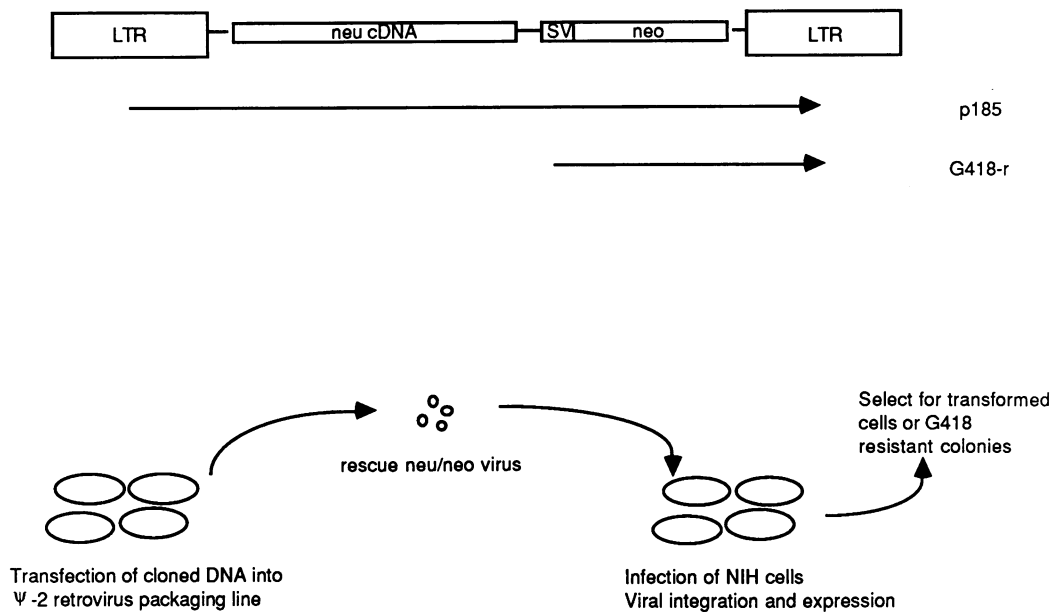
Fig. 1. Method for measuring biological activity of mutated *neu* clones. (**Top**) Diagram of the expression vector pDOL/neu. A full-length normal rat *neu* cDNA (Bargmann *et al.*, 1986b) was inserted into the *Sal*I site of pDOL (Korman *et al.*, 1987); following cloning, only the *Sal*I site 3' to the inserted cDNA was restored. Below the construct are shown the two mRNAs which should result from transcription initiated at each of the two promoters contained within pDOL. The longer transcript, which encodes p185, can be packaged into virions. The shorter transcript confers G418 resistance. (**Bottom**) the Ψ-2 retrovirus packaging line (Mann *et al.*, 1983) was transfected with the pDOL/neu clone and helper-free virus supernatants rescued and used to infect NIH-3T3 cells. The NIH-3T3 cells were split following infection and either selected in G418 or grown without selection to assess focus formation.

by the mechanisms responsible for activating the v-*erbB* and v-*ros* genes in naturally occurring retroviruses.

Results

Evaluation of mutant *neu* alleles

The chemically induced mutation in the activated *neu* oncogene, which causes the replacement of a valine residue at amino acid 664 with a glutamic acid residue, leads to > 100-fold increase in the transforming potential of the cDNA clones (Bargmann *et al.*, 1986b). We expected that other mutations introduced into *neu* might lead to an intermediate level of biological activity. For this reason, a vector system that permitted quantitation of the relative efficiencies of different *neu* clones was developed.

The strategy of mutagenesis and measurement of resulting biological activity is outlined in Figure 1. The normal rat *neu* cDNA (Bargmann *et al.*, 1986b) was inserted into the *Sal*I cloning site of a modified version of the pDOL retrovirus vector (Korman *et al.*, 1987). Upon introduction into mammmalian cells, the single DNA clone pDOL/neu or the virus derived from it can direct expression of both the selectable G418-resistance marker and the unselected *neu* cDNA.

In order to test each mutated *neu* clone, plasmid DNA was transfected into the packaging cell line Ψ-2 (Mann *et al.*, 1983) and viral supernatants containing RNA copies of the pDOL/neu clone were rescued from these cells. These viruses were subsequently used to infect NIH-3T3 cells. Some of the infected cells were plated into a non-selective medium and scored for the appearance of foci of transformed cells; others were introduced into a selective G418-containing medium and scored for the total number of G418-resistant colonies. The ratio of transformed foci to G418-resistant

Table I. Biological activity of *neu* clones bearing point mutations at amino acids 663−665

Virus	Foci per 1000 *neo* colonies
Experiment 1	
Nml	10
Asp-664 (2)	10, 10
Lys-664 (2)	<5, <5
Gly-664	1
Gln-664 (2)	1000, 1000
Glu-664	1000
Experiment 2	
Nml*	1
Asp-664*	20
Gly-664*	5
Lys-664*	<5
Gln-664*	600
Glu 664*	2500
Experiment 3	
Nml*	3
Asp-664* (3)	9, 9, 8
Gly-664*	3
Lys-664*	0.1
Gln-664*	700
Glu-664*	1300
His-664	2
Tyr-664	10
Glu-663	5
Glu-665 (2)	4, 4
Asp-665	8

The number of independently derived virus producer cell lines is given in parentheses following the virus. Asterisks denote clonal producer cell lines.

colonies should give a measurement of the specific transforming activity of each *neu* allele.

This strategy relies on the assumption that a single integrated retrovirus will express both the drug resistance marker and the non-selected marker. This assumption was verified using the normal (Val-664) and transforming (Glu-664) cDNAs. Twelve G418-resistant cell lines isolated following infection with the Val-664 virus were screened for expression of p185 by immunoprecipitation; each of these cell lines contained at least 20 000 molecules of p185 per cell (not shown). In addition, cell lines containing the transforming p185 clone have been expanded from transformed foci generated under nonselective conditions, and all of these cell lines were resistant to G418. Since clonal cell lines should contain a single integrated copy of the pDOL/neu provirus, it appears that the vector is indeed able to direct expression of both the *neu* and the *neo* genes.

The level of p185 expression induced by this vector is not greatly influenced by the presence of the various point mutations affecting the structure of p185. At least three G418-resistant cell lines bearing each point-mutated *neu* allele (see below) were tested for p185 expression and found to express p185 in the same range. The levels of immunoprecipitable p185 from metabolically labelled cells were compared to levels in B104-1-1 cells and DHFR-G8 cells, which have been shown to contain 2×10^5 and 10^6 molecules of p185 per cell, respectively (Stern *et al.*, 1986). Of a total of 48 cell lines expressing various pDOL/neu alleles that have been examined, 45 appear to contain between 50 000 and 200 000 molecules of p185 per cell.

Biological activity of point-mutated *neu* genes

Oligonucleotide-directed mutagenesis was used to introduce nine different point mutations into the portion of the normal *neu* cDNA that specifies the transmembrane domain. Following mutagenesis, the region of each mutation was sequenced to verify the alteration. Either one (3/9) or two (6/9) independent isolates of each mutation were isolated and tested in the biological assay as described above. The results of these experiments are given in Table I.

The Val-664 virus which carries the normal *neu* allele was tested first. It was found to yield 1 − 10 transformed foci per 1000 G418-resistant colonies. In contrast, the Glu-664 virus, which carries the *neu* allele activated by chemical carcinogenesis *in vivo*, yields 1000 foci per 1000 G418-resistant colonies.

Viruses encoding six other substitutions at amino acid residue 664 were also tested for biological activity. Of these, only one, the virus bearing Gln-664, had a transforming activity comparable to that of the Glu-664 virus. This mutant yielded at least 600 foci per 1000 G418-resistant colonies. In contrast, the viruses specifying Gly-664, Lys-664, His-664 and Tyr-664 substitutions yielded < 10 foci per 1000 G418-resistant colonies. These clones are essentially without transforming activity; in each case, the numbers were not significantly above the level of variability of the mock-infected controls.

An intermediate activity was observed with the virus specifying an Asp-664 substitution. The number of foci obtained, 10 − 20 per 1000, was consistently above the background level or the level obtained with Val-664, but still far lower than the number of foci obtained with Glu-664 or Gln-664.
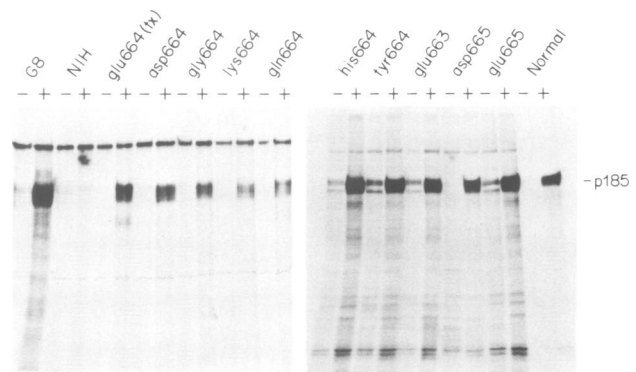

Fig. 2. Expression of p185 in cell lines bearing pDOL/neu. ^{35}S-labelled lysates of cells were incubated either with (+) or without (−) monoclonal anti-p185 antibody 7.16.4. (Drebin *et al.*, 1984) and collected on Protein A Sepharose. G8 (DHFR G8 cells) NIH cells transfected with a normal genomic *neu* gene (Hung *et al.*, 1986). NIH, uninfected NIH-3T3 cells. Glu-664 (tx), Asp-664, Gly-664, Lys-664, Gln-664, His-664, Tyr-664, Glu-663, Asp-665, Glu-665 and normal, clonal cell lines expanded from G418-resistant colonies resulting from infection with pDOL/neu viruses bearing each of the point-mutated *neu* genes.

The low activity of the Asp-664 virus was examined further by generating clonal Ψ-2 cell lines (Mann *et al.*, 1983) that produce this virus. Such lines should produce only one or a few different viruses. If the activity of Asp-664 in the earlier experiments were due to the generation of occasional highly transforming viruses through additional mutations, some of these cloned producer lines should produce a highly transforming virus and others would yield no transforming viruses. If however, all Asp-664 viruses have a low but significant probability of inducing foci, a low but constant frequency of transforming viruses should be present in each virus population. As shown in Table I, each Asp-664 virus yielded a similar ratio of transformed foci to G418-resistant colonies. We conclude that the Asp-664 allele has a low but detectable level of transforming activity.

In order to explore further the specific requirements for activation of the *neu* gene, mutations were made which affected amino acid positions adjacent to residue 664. The valine residue at amino acid residue 663 was converted to a glutamic acid residue to generate the mutant Glu-663, and the glycine at position 665 was changed either to aspartic acid (Asp-665) or glutamic acid (Glu-665). The presence of a glutamic acid residue at either position 663 or 665 was insufficient to activate the *neu* gene (Table I). A small number of foci have been observed in response to infection with the Asp-665 virus.

The morphologies of cell lines expanded from G418-resistant colonies resulting from infection with each virus correlate with the observed focus-forming ability. Thus, cell lines resulting from infection with Val-664, Gly-664, His-664, Tyr-664, Lys-664, Glu-663 or Glu-665 were essentially all normal in morphology, while all cell lines resulting from infection with viruses Glu-664 or Gln-664 consisted of refractile, morphologically transformed cells. Most of the cell lines containing Asp-664 or Asp-665 were morphologically normal, but a small fraction (2 − 5%) of the G418-resistant colonies were observed to be refractile in morphology. These presumably represent the subfraction of infected cells which can give rise to transformed foci in a monolayer.

Table II. Cell surface expression of point-mutated *neu* clones

Virus	Activity/well
Normal	1000
DHFR-G8	1871
NIH-3T3	0
Asp-664	288
Gly-664	626
Lys-664	88
Gln-664	220
His-664	520
Tyr-664	209
Glu-664	570
Glu-663	89
Glu-665	623
Asp-665	608

Cells were incubated with antibody 7.16.4 followed by [^{125}I]Protein A, washed extensively, solubilized and counted. Non-specific binding was measured by omitting the antibody; non-specific binding was substracted from specific binding in all cases. A description of the activity units is given in the Materials and methods section.

Characterization of p185 in cell lines bearing mutated neu genes

The described biological experiments suggested that only two of the 11 mutant *neu* alleles have transforming activity. However, the remaining *neu* alleles might encode a functional, normal p185 or completely aberrant molecules whose lack of transforming activity was trivially caused by defects in the synthesis or maturation of p185. In order to address this question, the proteins encoded by the different *neu* alleles were compared with the normal p185 protein and each other.

Clonal cell lines were expanded from G418-resistant colonies carrying mutant *neu* alleles and screened for production of p185. As shown by the immunoprecipitation in Figure 2, the p185 protein was present in similar amounts in all of these cell lines. The proteins comigrated with each other and with the p185 present in the control transfected cell line DHFR G8 (lane G8). Thus, no gross rearrangement of the coding sequences of the *neu* gene is likely to have occurred during the generation of these cell lines.

The normal and transforming p185 molecules are both predominantly expressed at the cell surface (Drebin *et al.*, 1984, 1985; Stern *et al.*, 1986). If any of the mutated *neu* clones encoded a p185 molecule that failed to be expressed at the cell surface, such a clone might be non-transforming for this reason alone. This was a particular concern since some of the substitutions caused the replacement of hydrophobic residues in the transmembrane domain with potentially charged residues.

To monitor cell-surface localization of p185, whole cells from each of these cell lines were incubated with monoclonal anti-p185 antibody 7.16.4 (Drebin *et al.*, 1984) followed with ^{125}I-labelled protein A (Table II). The specific binding of the labelled protein A in the presence of anti-p185 antibody indicated that at least some of the p185 present in each cell line was expressed at the surface of living cells, although the fraction of total protein so expressed may vary due to changes in processing or turnover.

Each of the cell lines was also examined for p185-associated tyrosine kinase activity. Both normal and transforming proteins possess such an activity in an immune complex autophosphorylation assay (Stern *et al.*, 1986). A similar analysis of each of the point-mutated *neu* genes is
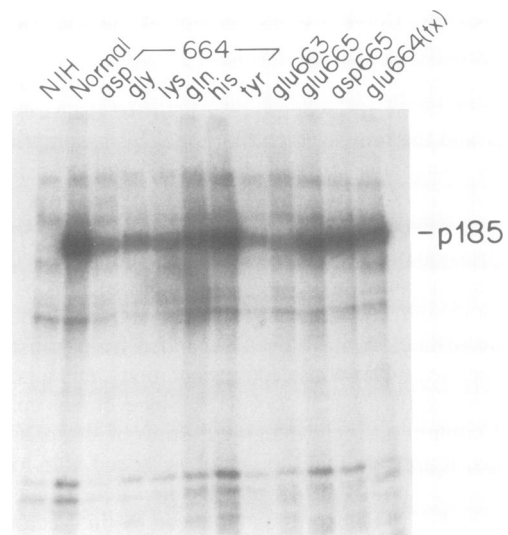

Fig. 3. Tyrosine kinase activity of point-mutated *neu* genes. p185 was immunoprecipitated using 7.16.4 and incubated with [γ-^{32}P]ATP as described (Stern *et al.*, 1986). NIH, uninfected NIH-3T3 cells. Other lines are the same cell lines shown in Figure 2, clonal cell lines which express each of the point-mutated *neu* genes.

shown in Figure 3. Incubation of antibody-purified p185 from each cell line with [γ-^{32}P]ATP led to the phosphorylation of a band that comigrated with the p185 molecule found in DHFR G8 cells (not shown). No activity was present in uninfected fibroblasts (lane NIH). These various analyses indicate that each of the point-mutated *neu* clones encodes a p185 protein that is comparable to the normal protein in expression, cell surface localization, and tyrosine kinase activity.

Domains of neu which are necessary for transformation

Under different conditions than were used above, there is an elevated tyrosine kinase activity associated with transforming alleles at p185 (C.I.Bargmann and R.A.Weinberg, in preparation). Transformation by other oncogenes has also been demonstrated to correlate with tyrosine kinase activity (Hunter and Cooper, 1985). We sought to verify that the tyrosine kinase domain was indeed essential for the oncogenic effects of the point mutation and to determine whether other collaborating domains of the protein might also be necessary for these effects. To do this, we further modified the transforming (Glu-664) *neu* oncogene allele in order to define domains of p185 essential for transformation. Figure 4 diagrams the deletion and linker insertion mutations that were constructed as further modifications of the Glu-664 *neu* allele. Each of the mutated *neu* genes was tested for its biological activity in either transfection assays, infection assays, or both (Table III).

Deletion of virtually the entire extracellular domain of p185 (amino acid residues 30−628, mutant 5'Tx) did not result in a substantial diminution in biological activity of the transforming *neu* allele. A second large deletion encompassing amino acid residues 1007−1248 (mutant 3'Tx), including most of the proposed sites of tyrosine autophosphorylation in p185, also did not affect the transforming activity of the *neu* gene. 5'3'Tx, a *neu* clone bearing both of these deletions, was also a fully transforming clone. When inserted
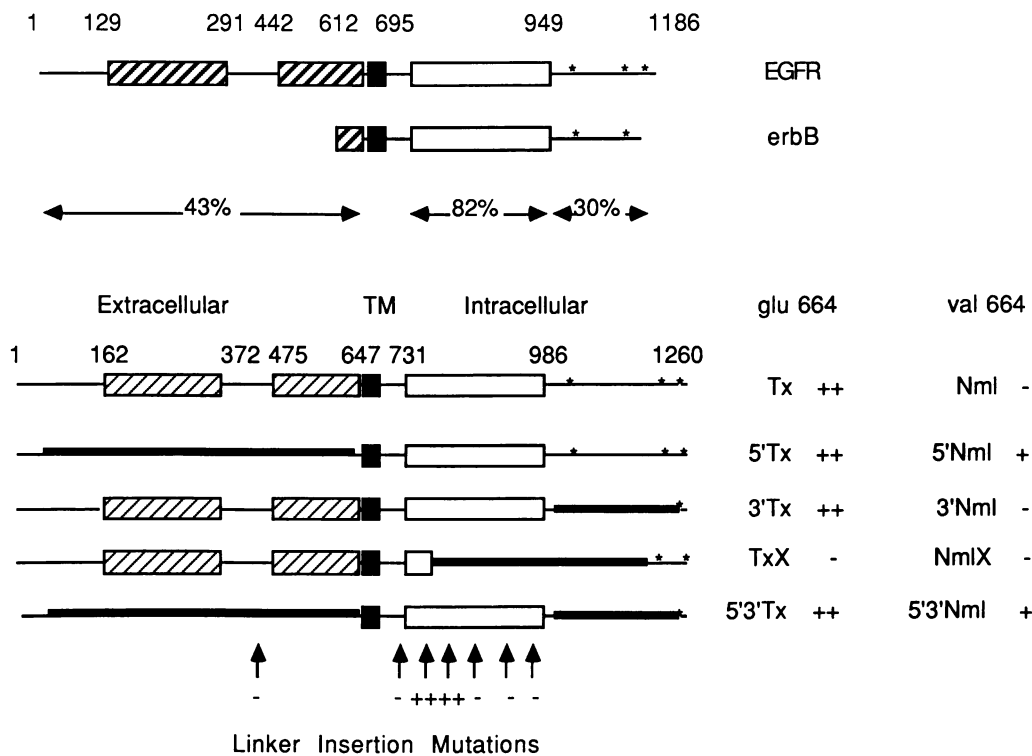
1   129   291 442 612 695   949   1186

EGFR

erbB

◄——————43%——————► ◄—82%—► ◄30%►

Extracellular   TM   Intracellular   glu 664   val 664

1   162   372 475 647 731   986   1260

Tx ++   Nml -

5'Tx ++   5'Nml +

3'Tx ++   3'Nml -

TxX -   NmlX -

5'3'Tx ++   5'3'Nml +

↑   ↑↑↑↑ ↑↑

-        - ++++ -   - -

Linker Insertion Mutations

Fig. 4. Deletion and insertion mutations constructed in the *neu* gene. At the top are the EGF receptor and v-*erbB*, with the percentage amino acid identity between the EGF receptor domains and those of *neu* given underneath. Numbers above the EGF receptor and *neu* are amino acid residue numbers. Beneath are shown schematic figures of the mutations made in *neu*. The hatched boxes represent cysteine-rich extracellular domains, the open boxes represent tyrosine kinase domains, and the black boxes represent transmembrane domains. Thick black lines represent sequences deleted in a particular clone. Each of the deletion mutations was constructed in a clone bearing the glutamic acid resiude at 664 and a clone bearing valine at position 664. At the right are names and the biological activities of each clone, where − means non-transforming, + means weakly transforming, and + + means strongly transforming (see text and Table III). Linker insertion mutations were made in a clone bearing a glutamic acid residue at position 664. The biological activity of each clone is summarized at − or + +. From left to right, the mutations are referred to as B455, B719, B745, X774, P800, X898 or S933 in the text.

into the pDOL/neu virus described above and tested in focus assays, each of these deletion mutants gave over 800 transformed foci per 1000 G418-resistant colonies (Table III). Protein products of these deleted genes of 110 (5'Tx), 140 (3'Tx), and 55 kd (5'3'Tx) have been identified in cell lines expressing these *neu* viruses (not shown). Thus, the effects of the Glu-664 point mutation are still manifested even in the absence of co-existing amino- and carboxy-terminal regions of p185. (As is described later, such deletions on their own do not duplicate the observed activity of the point-mutated oncogene).

In contrast, a deletion of sequences including the predicted tyrosine kinase domain of p185 (TxX) completely destroyed the biological activity of the *neu* gene product (Figure 4). These results are consistent with the idea that the kinase activity is necessary for transformation.

To refine the latter observation, linker insertion mutations within the predicted tyrosine kinase domain were generated and tested for their biological activity (Figure 4). Each of the linker insertion mutations led to the in-frame insertion of four amino acids within the predicted tyrosine kinase domain. Four out of six mutated clones were non-transforming (clones B719, P800, X898 and S933, <5% of wild-type biological activity) and two out of six mutated clones were transforming (clones B745, X774, >30% of wild-type biological activity). Since these clones were only assayed in the transfection assay, more quantitative assessments of their biological activity were not obtained.

Table III. Biological properties of 5' and 3'-deleted normal and transforming *neu* cDNA clones

Clone	Foci per 1000 *neo* colonies	
	Infection	Transfection
Nml	2	10
5'Nml	45	20
3'Nml	15	20
5'3'Nml	55	170
Tx	1000	650
5'Tx	850	210
3'Tx	1000	1300
5'3'Tx	1000	600

Each of the linker-insertion mutations was co-transfected into NIH-3T3 cells with pSV2neo (Southern and Berg, 1982) and cell lines containing each mutated DNA were established and tested for expression of p185. Cell lines containing clones B719, B745, X774 or P800 contained substantial amounts of immunoprecipitable p185. However, only small amounts of p185 could be detected in cell lines derived from transfection of X898 or S933. The reason for this low expression was not explored further. It appears that these two linker insertions interfere with expression of p185, perhaps by destabilizing the protein.

Representative cell lines expressing each of the other four linker insertion mutations were tested for the presence of
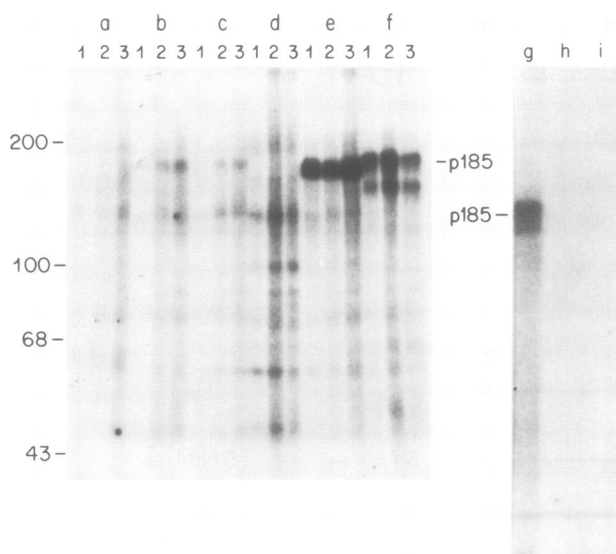
Fig. 5. Tyrosine kinase activity of *neu* genes bearing linker insertions. Cell lines expressing comparable levels of the mutant p185 protein (as assessed by [^{35}S]cysteine labelled protein) were used in each case. Lysates were incubated with antibody 7.16.4, immune complexes collected on Protein A-Sepharose, washed and incubated with ^{32}P-labelled ATP as described (Stern *et al.*, 1986). Lane a, cells containing clone B719. Lane b, cells containing clone B745. Lane c, cells containing clone X774. Lanes d and h, NIH cells (no transfected *neu* gene). Lane e, cells containing clone B455. Lanes f and g, B104-1-1 cells expressing a (Glu-664) transforming *neu* clone. Lane i, cells containing clone P800. In lanes a–f, the incubation was carried out either at 30°C (lanes marked 1), 37°C (lanes marked 2) or 42°C (lanes marked 3).

Fig. 6. *neu*-encoded proteins derived from clone 5'NML. [^{35}S]cysteine-labelled cells were lysed and incubated with antibody α-21N, directed against the carboxy-terminus of the predicted amino acid sequence of p185. An excess of cold peptide 21N was added to those lanes marked +. Lanes a and h, cells transfected with neo-r marked alone. Lanes b–f, i and j, cells transfected with the 5'NML clone. Lanes g and k, cells transfected with the 5'TX clone. Lane l, DHFR G8 cells, which express the normal *neu* gene. The cell lines corresponding to lanes b, d, g and k are morphologically transformed.

a kinase activity associated with p185. Each cell line contained about the same amount of p185 as the control transformed cell line B104-1-1 (not shown). The p185 proteins produced by B745 and X774 were active in a kinase assay (Figure 5, lanes b and c), although both were less active than the wild-type p185 protein (Figure 5, lanes f and g). Less than 1% of wild-type kinase activity was associated with the p185 protein produced by mutants B719 and P800 (Figure 5, lanes a and i). Thus, only those genes that had an associated kinase activity were transforming.

While kinase activity may be necessary for transformation, it is apparently not sufficient. The mutant B455, which contains a linker insertion outside of the tyrosine kinase domain, totally lacks transforming activity despite its high tyrosine kinase activity (Figure 5, lane e). Cells containing this protein do not appear to express p185 at their surface when analyzed either by ^{125}I-protein A binding or by fluorescence-activated cell sorting (not shown). We suggest that proper subcellular localization as well as kinase activity is necessary for transformation mediated by p185.

Activation of the neu gene by deletion

The events which led to the activation of the *erbB*/EGF receptor, *fms*/CSF-1 receptor and *ros* oncogenes included a truncation of either amino- or carboxy-terminal sequences of the encoded protein (Ullrich *et al.*, 1984; Coussens *et al.*, 1986; Neckameyer *et al.*, 1986). These deletions are thought to deregulate the activities of the associated tyrosine kinases, thereby causing oncogene activation. We constructed deletions in the *neu* gene to see whether the amino- and
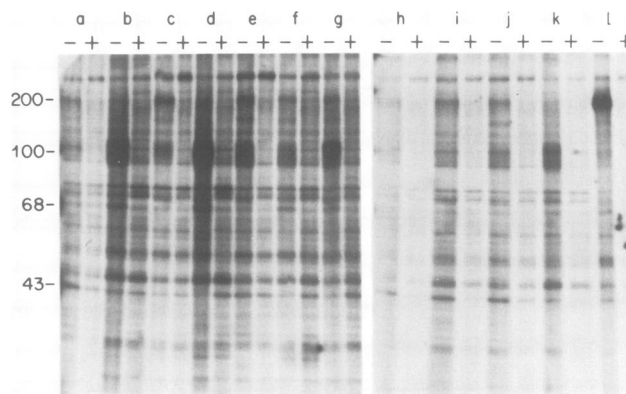
carboxy-terminal sequences of p185 might also deregulate the activity of this protein and lead to the creation of an active oncogene.

The normal (Val-664) *neu* cDNA was modified using the same deletion strategy as was described above for the transforming *neu* cDNA (Figure 4). Since the deletion of carboxy-terminal or amino-terminal sequences of the transforming *neu* cDNA did not affect its transforming activity (see above), it seemed likely that the corresponding normal (Val-664) mutants would specify a stable and properly localized protein product. Indeed, cell lines expressing all of the deleted *neu* alleles have been examined for the presence of *neu*-encoded proteins, and proteins of the expected mol. wt have been identified in all cases. DOL/neu viruses bearing each of these deleted *neu* alleles were generated and assayed for their biological activity (Table III). Deletion of the carboxy-terminus of the normal *neu* gene had little effect on its transforming activity (15 foci generated per 1000 G418-resistant colonies), but deletion of the amino-terminus (in the 5'NML clone) led to an appreciable increase in the number of foci in an NIH-3T3 monlayer (45 foci per 1000 G418-resistant colonies). These foci were smaller than those formed in response to the transforming Glu-664 clone, and took several days longer to be detected in an NIH-3T3 monolayer. G418-resistant colonies generated following introduction of the 5'NML clone were variable in morphology; in a typical experiment, two out of 10 cell lines were morphologically transformed and eight out of 10 were normal.

The proteins present in cell lines bearing the 5'NML (amino terminally deleted) clone were examined to explore the difference between the morphologically normal and transformed cell lines. Figure 6 shows an immuno-precipitation of protein from two transformed and five non-transformed cell lines transfected with this clone. Lysates from these cells were incubated with a polyclonal anti-peptide antibody (a gift from W.Gullick) directed against the carboxy-terminus of p185. A broad protein band present in the 5'NML-containing cells is recognized by this serum

(Figure 6, lanes b−f, i and j). This protein comigrates with the protein present in cells transfected with 5'TX, the corresponding deleted clone containing the Glu-664 mutation (lanes g and k), is not present in cells transfected with the neo-resistance marker alone (lanes a and h) and is not precipitated if the antiserum is preincubated with the immunogenic peptide before the immunoprecipitation (+ lanes). These are the properties expected of the protein encoded by 5'NML. Lanes b and d are lysates from the two cell lines that are morphologically transformed. These two cell lines appear to contain the same protein as the other lines, but at a higher level of expression. These data suggest that 5'NML must be expressed at a high level estimated at 500 000 molecules/cell) to transform cells. In contrast, 5'TX is fully transforming even when expressed at ∼10−20% of that level (lane k).

The number of foci formed by the doubly deleted clone (5'3'NML) was slightly higher than that formed by the 5'NML clone deleted only at its amino-terminus (Table III); the foci formed by the doubly deleted clone are larger and more morphologically transformed. A larger difference between the 5'NML and 5'3'NML clones is observed following transfection rather than infection. The 5'3'NML clone yields many more foci (170/1000 G418-resistant colonies) than the 5'NML clone in this assay (Table III). It appears that removal of the amino-terminal sequence of *neu* can weakly activate the transforming potential of the gene, and that this activation can be marginally increased by concomitant carboxy-terminal deletion.

Discussion

Previous work has demonstrated that the *neu* oncogene is frequently activated by a point mutation altering the predicted transmembrane domain of p185, and that this alteration correlates with an increase in the tyrosine kinase activity associated with p185 (Bargmann *et al.*, 1986b, C.I.Bargmann and R.A. Weinberg, in preparation). The experiments described here were designed to distinguish between models describing the effect of the activating mutation on the activity of the *neu* gene product, and to establish more firmly the correlation between activation of the tyrosine kinase activity and increased transforming potential.

Point mutations

Nine distinct point mutations were generated in the normal *neu* gene affecting residues 663−665 (residue 664 is the site of the chemically induced activating mutations of the *neu* gene). To date, the best-characterized example of activation of a proto-oncogene by point mutation has been that of the *ras* oncogene (reviewed in Varmus, 1984). The c-H-*ras* proto-oncogene has been mutagenized so that each of the 20 amino acids was present at amino acid 12 (Seeburg *et al.*, 1984). A total of 18 out of 20 possible clones were transforming; only proline and the natural glycine at position 12 did not convert the c-H-*ras* gene into an oncogene. Indeed, deletion of amino acid 12 also converts the c-H-*ras* proto-oncogene to an oncogene (Chipperfield *et al.*, 1985). These and other results (McGrath *et al.*, 1984; Manne *et al.*, 1985; Sweet *et al.*, 1984) suggest that these *ras* mutations act by destroying an activity of the *ras* gene product (perhaps a

negative regulatory activity), and that virtually any change in this region disrupts this activity and thereby achieves oncogene activation.

The c-*src* gene can be activated by a point mutation altering a tyrosine at amino acid position 527 of c-*src* to a phenylalanine (Cartwright *et al.*, 1987; Piwnica-Worms *et al.*, 1987; Kmiecik and Shalloway, 1987). Since phosphorylation of the *src* gene product at the tyrosine residue at position 527 decreases its tyrosine kinase activity (Cooper *et al.*, 1986; Cooper and King, 1986), it seems that a site of negative regulation of the proto-oncogene is altered in its activation.

The results described here with the *neu* gene suggest a different mechanism of activation. Only two out of eight residues tested at amino acid position 664 lead to full activation of the oncogenic potential of the gene, and a third gives weak activation; five out of eight substituents tested do not substantially activate the gene. Some of these substituted residues have side chains quite distinct from that of the valine, so it seems very unlikely that the *neu* gene is activated by destroying some specific property of the normally present valine residue. Rather, a novel or augmented activity must be ascribed to the presence of the activating glutamic acid and glutamine residues at this position.

Since the glutamic acid residue found in the transforming version of p185 has the potential to be negatively charged, it might be imagined that the role of the activating mutation is to insert a charged residue into the otherwise fully hydrophobic transmembrane domain, thereby distorting its conformation. However, since lysine and histidine are not at all transforming and aspartic acid only weakly so, we conclude that the presence of a potentially charged residue *per se* cannot activate the *neu* protein. Furthermore, the presence of a negative charge cannot be the important determinant of activation, since the uncharged glutamine at 664 is much more active than the charged aspartic acid. The high degree of specificity required for the activating mutation is further underscored by the fact that glutamic acid residues substituted at the neighbouring positions 663 or 665 fail to release the transforming potential of the *neu* protein.

The critical determinant of activation must be a property which is shared by glutamic acid and glutamine, which is deficient in aspartic acid and altogether absent in the other tested amino acids. We believe that the biological activities of the mutated *neu* genes favor a model in which glutamic acid/glutamine enhances or decreases a specific intermolecular interaction that is important in receptor activation. This interaction might be sterically more favored by glutamic acid or glutamine than the slightly smaller aspartic acid residue. One possibility is that the activated p185 is clustered. There is some evidence that self−self clustering is involved in the activation of the EGF receptor kinase by its ligand (Yarden and Schlessinger, 1987), and the high degree of homology between the gene encoding the EGF receptor and *neu* supports the notion that the two proteins are regulated in similar ways. Nonetheless, it is not obvious that a small change in the transmembrane domain would be able to induce clustering of such a large protein.

Alternatively, the transmembrane domain of p185 might normally interact with a second distinct protein or other molecule that has a domain within the membrane, such as

a regulatory subunit of the kinase. Such heterotypic interactions might be altered in the activated protein.

The role of the kinase domain in transformation

Mutagenesis of the transforming allele of *neu* supports the idea that the alteration of the transmembrane domain acts through its ability to activate the tyrosine kinase activity of p185. The basal kinase activity of p185 seen in the immune complex kinase assay (Figure 3) is similar between all of the mutants; however, in membrane kinase assays, Glu-664 and Gln-664 p185 forms display an increased tyrosine-specific protein kinase activity relative to the non-transforming mutants (CI.Bargmann and R.A.Weinberg, in preparation, and data not shown). In addition, those linker insertion mutations that destroy the tyrosine kinase activity of p185 also destroy the biological activity of the transforming *neu* clone.

The activated v-*src* protein is associated with an increased tyrosine kinase activity (Iba *et al.*, 1985; Coussens *et al.*, 1985a) and the strong correlation between tyrosine kinase activity and transformation observed upon study of a number of different viral oncogenes supports the importance of this activity in transformation by this class of proteins (Hunter and Cooper, 1985).

Virtually all of the extracellular sequences of p185 can be deleted without inhibiting the strongly transforming activity of the point-mutated *neu* clone. Therefore, the transmembrane domain is probably not activating p185 by altering its ligand specificity or affinity. Nor is the extensive carboxyterminal domain of the transforming *neu* protein necessary for transformation. Deletion of both amino- and carboxyterminal domains leaves only 420 amino acids of the original 1260, yet leaves the protein fully competent for transformation. It seems that all activities important for transformation must be sought within this residual, highly truncated protein.

Activation of neu by deletion

A measurable degree of activation of *neu* could be detected in response to amino-terminal truncation of the normal gene. The EGF receptor, when truncated at its amino terminus, can transform erythroblasts in an avian system, although additional alteration is required for fibroblast transformation (Gamett *et al.*, 1986). Similarly, truncation of the amino-terminal sequences of c-*ros* in viruses or by transfection appear to activate the gene (Birchmeier *et al.*, 1986; Neckameyer *et al.*,1986). These results suggest that the extracellular domains of each of these proteins exert a negative influence on the intracellular sequences that can be bypassed by deletion, and is, perhaps, normally relaxed in response to ligand binding.

Mutations constructed in the normal human *neu*/c-*erbB*-2 gene have recently been described (Di Fiore *et al.*, 1987). In that work, the human *neu*/c-*erbB*-2 gene was shown to transform cells when expressed at high levels. We observe substantially less transformation by the normal rat *neu* gene. This difference may be due to the higher level of expression of the human cDNA in those experiments, or to differences in the biological activities of the human and rat clones in mouse and cell lines. Di Fiore *et al.* (1987) also observed an increase in the transforming activity of the human *neu*/c-*erbB* gene following amino-terminal truncation. Although the incidence of transformation was higher than is seen with

the deleted rat *neu*/c-*erbB* clone, the overall result of activation of the normal gene by this deletion is similar in their experiments and these. In addition, we found that deletion of carboxy-terminal sequences from a normal *neu* clone which was additionally deleted at its amino terminus increased its biological activity.

A most striking result revealed by deletion analysis of the normal *neu* cDNA is that the difference in biological activity between the normal (Val-664) and transforming (Glu-664) cDNAs was substantial even following deletion of two thirds of the coding sequences of the *neu* gene. Even the most active of the deleted normal clones, the doubly deleted 5'3'NML, directed the formation of < 10% of the number of foci seen with the Glu-664 clones, although comparable levels of protein expression were observed in all cases (not shown). These experiments indicate that the effect of the point mutation is not merely to uncouple the kinase domain from one of these potential negative regulatory domains, and emphasize the difference between the Glu-664 mutation which activates *neu* and those deletion mutations which have been characterized in other systems, each of which involves sequences similar to those that have been deleted in the 5'3'NML clone.

Materials and methods

Oligonucleotide-directed mutagenesis
Oligonucleotides were synthesized on an Applied Biosystems oligonucleotide synthesizer by Wayne Brown of the Whitehead Institute. An *RsaI* fragment of pSV2neuN corresponding to nt 1881–2419 of the sequence was cloned into the *SmaI* site of pUC13 so that the *HindIII* site of the polylinker fell at the 5' end of the inserted sequences and mutagenized as described (Chipperfield *et al.*, 1985). The identity of all point mutations was confirmed by DNA sequencing (Maxam and Gilbert, 1980). The plasmid bearing each novel mutation was cleaved with *NdeI* and *BglI*, which liberate a fragment between nt 1899 and 2387 (the mutations are at position 2008–2016). This fragment was isolated and ligated to two other DNA fragments to regenerate a pDOL/neu clone. Most DNA techniques were as described in Maniatis *et al.* (1982).

Linker insertion mutagenesis
Plasmid pSV2neuT, which contained the entirety of the coding region of the transforming *neu* gene in the pSV2 expression vector, was partially digested with restriction enzymes which cleaved within the tyrosine kinase domain, blunted where appropriate, and ligated to DNA linkers which regenerated the correct reading frame of p185.

Mutant P800. A *Bam*HI 12mer was inserted into a *Pvu*II site. This mutation led to the replacement of a Gln residue with Arg−Gly−Ser−Ala residues.

Mutant X898. A *Bam*HI 12mer was inserted into an *Xmn*I site. This mutation led to the replacement of a Ser residue with Ala−Asp−Pro−Arg residues.

Mutants B455, B719 and B745. *Sal*I 8mers were inserted into *Bam*HI sites that had been blunted with Klenow fragment. Each of these mutations led to the replacement of an Ile residue with Gly−Arg−Pro−Ile residues.

Mutant X774. *Bam*HI 8mers were inserted into an *Xba*I site that had been blunted with Klenow fragment. This mutation led to the replacement of a Leu residue with Ala−Asp−Pro−Leu residues.

Mutant S933. *Bam*HI 12mers were inserted into a *Sma*I site. This mutation led to the replacement of the amino acids Ala−Arg with Pro−Arg−Ile−Arg−Gly.

Construction of deletion mutations
Deletion of 5' sequences of pSV2neuN and pSVneuT (mutants 5'Tx and 5'Nml). 10mer oligonucleotide *HindIII* linkers (Biolabs) were inserted between an *RsaI* site at the 3' end of the sequences encoding the signal sequence of p185 and a blunted *NdeI* fragment containing the carboxy-terminal sequences of either the normal or transforming *neu* gene.

Deletion of the kinase domain. pSV2neuT or pSV2neuN was digested to completion with *Xba*I and religated.

Deletion of 3' sequences

Mutant 3'Tx. A *Pst*I 8mer linker was ligated to the blunted *Nco*I site at positon 3035 of the *neu* gene. The 5' end of the gene up to the *Pst*I site was ligated to a *Pst*I fragment containing the sequences from the *Pst*I site at 3760 to the end of the gene.

Construction of other 3' deleted clones. The *Bgl*I fragment of pDOL/3'Tx bearing the 3' end of the gene was ligated into *Bgl*I-cut, phosphated DNAs containing the 5' ends and vector sequences of pDOL/5'Tx, pDOL/5'Nml, and pDOLneu(NML) to generate the 3' deleted clones pDOL/Tx5'3', pDOLNml5'3' and pDOL3'Nml.

Transfection and cell culture

NIH-3T3 cells and transfectants clones deriving from NIH-3T3 cells were grown in Dulbecco's modified minimal essential medium (DME) supplemented with 10% calf serum. Transfections were as described previously (Shih *et al.*, 1981; Bargmann *et al.*, 1986b).

Virus harvesting and infection. Supernatants were harvested from subconfluent 10 cm dishes of Ψ-2 retrovirus producer lines. NIH cells were infected at a density of 5×10^4 to 2×10^5 per 10 cm dish, 12−48 h after splitting, with 3 ml of 10% calf serum/DME/4−8 μg/ml polybreen containing up to 1/5 volume of Ψ-2 supernatant. 24 h later cells were fed with either 5% calf serum/DME or 10% calf serum/DME/0.6−0.8 ng/ml G418. In some experiments, an additional infected dish was split after 24 h into 5% calf serum/DME, G418-containing medium, or 0.325% agarose/calf serum/DME.

Immunoprecipitation

Cells growing in 10 cm dishes were labelled with [^{35}S]cysteine (New England Nuclear) for 5−6 h at 37°C and immunoprecipitated as described (Stern *et al.*, 1986) either with 1 μl of a 60 $\times$ concentrated hybridoma supernatant containing the mouse anti-p185 monoclonal antibody 16.4 (Drebin *et al.*, 1984) or 5 μl of a polyclonal rabbit antiserum directed against the carboxy-terminal amino acids of the *neu* gene product, with or without preincubation with the peptide (this serum, α-21N, was a generous gift of Dr William Gullick of the Institute for Cancer Research, Chester Beatty Laboratories, London, UK).

Immune complex kinase assays

These were as described (Stern *et al.*, 1986). In some experiments, the temperature of the assay was 37 or 42°C.

Antibody[^{125}I]protein A binding

Cells (10^5) were plated into a 16 mm microtiter well and permitted to attach. Cells were washed once in ice-cold binding buffer (DME, 0.5% BSA, 50 mM Hepes, pH 7.5) and incubated at 0°C in 200 μl of binding buffer with or without a 1:1000 dilution of monoclonal antibody 7.16.4 for 2−4 h. Antibody was aspirated and the cells incubated with 200 μl of ice-cold binding buffer with a 1:100 dilution of [^{125}I]Protein A (15 μCi/μg, New England Nuclear) for 0.5−2 h at 0°C. Cells were washed extensively with binding buffer, solubilized in 0.5N NaOH, and counted in a gamma counter. Specific counts were calculated by subtracting the number of counts bound in the absence of primary antibody from the total number of counts bound. All experiments were completed in duplicate. Since the specific activity of the protein A varied in different experiments, all numbers were normalized to the value of the cell line expressing the normal (Val-664) *neu* gene, which was included in each experiment; its value was arbitrarily set at 1000. Thus in one experiment, there were 63 745 and 88 066 c.p.m. specifically bound and 3261 and 3430 c.p.m. non-specifically bound to the cell line expressing the normal (Val-664) p185, for an average of 72 560 c.p.m. specifically bound per well; the cell line expressing Asp-664 bound 13 713 and 22 905 c.p.m. specifically and 1913 and 1925 c.p.m. non-specifically, for an average of 16 390 c.p.m. specifically bound per well. If the Val-664 value is set at 1000, the Asp-664 cell line value is 16 390/72 560 $\times$ 1000 = 225.

Acknowledgements

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