Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 2545–2548, April 1989
Biochemistry

Amplification of the proto-*neu* oncogene facilitates oncogenic activation by a single point mutation

(gene amplification/multiple-step carcinogenesis)

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Communicated by Robert A. Weinberg, December 13, 1988

ABSTRACT **To determine whether the amplification of the proto-*neu* oncogene (also called c-erbB-2) plays a role in tumorigenicity, we previously generated an NIH 3T3 transfectant (DHFR/G-8) that carried the amplified proto-*neu* gene. The DHFR/G-8 cells exhibited normal morphology. Their growth curve was similar to that of NIH 3T3 cells but was different from that of the B104-1 cell, an NIH 3T3 transfectant that carries the activated *neu* oncogene. When injected into nude mice, B104-1 cells produced tumors within 2 weeks, whereas the DHFR/G-8 cells did not produce tumors until 3 months after injection, and the NIH 3T3 cells did not produce any tumors even after 3 months. The tumors produced by the injection of the DHFR/G-8 cells were excised and grown in culture. The cells derived from the tumors were of transformed morphology and highly tumorigenic. The DNAs from the tumor cells were transfected into NIH 3T3 cells. The transfection resulted in foci on the NIH 3T3 monolayer. Southern analysis indicated that the foci derived from the transfection contained the *neu* gene. Using oligonucleotides as probes, the *neu* gene in the foci was found to carry a single-point mutation identical to the one previously found in the rat neuroblastoma and glioblastoma induced by the ethylnitrosourea. We conclude that the DNA region encoding the transmembrane domain of *neu* is a hot spot for converting the proto-*neu* gene into an activated oncogene and that amplification of the proto-*neu* gene facilitates mutation of the hot spot.**

Rat neuro/glioblastomas induced by transplacental injection of ethylnitrosourea frequently carry an oncogene, termed *neu* (also called murine c-*erbB-2*), that is detectable by transfection into mouse NIH 3T3 cells (1, 2). The *neu* oncogene was shown to be homologous to but distinct from the gene encoding epidermal growth factor receptor (EGFR) (3–5). The mechanism that induces activation of the *neu* oncogene was found to be a single-point mutation converting adenine to thymidine. The activation mutation results in a change of valine to glutamic acid in the transmembrane domain of the *neu*-encoded protein, p185 (6).

Many human tumor DNAs contain the amplified protooncogene, suggesting its amplification may play an important role in tumorigenicity (7–12). The human homologue of the murine *neu* gene (human gene symbol *NGL* for neuro/glioblastoma-derived; has been called *ERBB2*, *HER2*, human c-*erbB-2*, or *TKR1*) has also been shown to be amplified in many human tumors (10–12). To study the possible role of amplified *neu* in tumorigenicity, we have obtained a cell line, DHFR/G-8, that is an NIH 3T3 transfectant containing approximately 100 copies of the proto-*neu* gene, the normal allele of the activated *neu* oncogene, by coamplification with the dihydrofolate reductase gene (*DHFR*) after methotrexate treatment. The DHFR/G-8 cells produce a high level of *neu*-encoded p185 protein (13, 14) but exhibit normal morphology and form monolayers when growing to confluency (15).

We studied the tumorigenicity of the DHFR/G-8 cells and found that amplification of the proto-*neu* gene in DHFR/G-8 cells facilitated a single point mutation that rendered the cells tumorigenic. The results have important implications in the pathogenesis of human tumors—namely, the amplified protooncogene in human tumors could be activated by converting one (or a small number) of the amplified protooncogenes into an activated oncogene in addition to producing a high level of protooncogene protein product.

MATERIALS AND METHODS

DNA Transfection. DNA transfection procedures were performed by the calcium phosphate precipitation technique of Graham and Van der Eb as modified by Anderson *et al.* (16). In each transfection, 75 μg of genomic DNA was applied to 8×10^5 NIH 3T3 cells (2×10 cm dishes). Cells were split in a ratio of 1:6 after 24 hr. Two weeks later, foci of morphologically transformed cells were scored and analyzed.

Cell Culture. NIH 3T3 cells and transfectant clones derived from NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. The methotrexate-resistant or -sensitive properties were analyzed by plating 1×10^5 cells in a 10-cm plate dish with 10 ml of DMEM containing 10% calf serum and 0.6 μM methotrexate as described (15). After 2 weeks, the sensitive cells were completely killed, and the resistant cells were allowed to grow to confluency.

Southern Analysis. Southern blot analysis was performed essentially by published techniques (17) as described (18). The nitrocellulose filters were hybridized at high stringency (50% formamide/0.75 M NaCl/75 mM sodium citrate, 42°C) for 36 hr and then were washed twice at room temperature for 5 min in 0.3 M NaCl/30 mM sodium citrate and at 65°C for 1 hr in 15 mM NaCl/1.5 mM sodium citrate/0.1% $NaDodSO_4$.

Oligonucleotide Hybridization. The oligonucleotides initially were provided by Cornelia Bargmann at MIT and later synthesized on the Applied Biosystems 380A DNA synthesizer at M. D. Anderson Cancer Center. Full-length oligonucleotides were purified by polyacrylamide gel electrophoresis, and 100 ng of each oligonucleotide was phosphorylated by polynucleotide kinase by using the published technique (17). Unincorporated nucleotide was removed by chromatography over a Sephadex G-25 column. The oligonucleotide hybridization was carried out essentially by the procedures described by Bargmann *et al.* (6).

RESULTS

We had previously generated an NIH 3T3 transfectant, the DHFR/G-8 cell line, containing approximately 100 copies of

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Abbreviations: DHFR, dihydrofolate reductase; EGFR, epidermal growth factor receptor.

the rat genomic proto-*neu* gene—a normal and unmutated allele of the rat *neu* oncogene—by cotransfection with pSV2-DHFR plasmid and selection with methotrexate (15). The DHFR/G-8 cell line expresses high levels of the *neu*-encoded p185 protein but exhibits normal morphology and forms monolayers in culture. To further investigate if the overexpression of proto-*neu* gene could play a role in tumorigenicity, the DHFR/G-8 cells were injected into nude mice. The DHFR/G-8 cells produced tumors only after a long lag period of 3 months; the control cell line, B104-1, an NIH 3T3 transfectant containing the mutation-activated *neu* oncogene, produced tumors rapidly within 2 weeks after injection, and the NIH 3T3 cells did not produce any tumor even 3 months after injection (Table 1).

When two independent tumors induced by injection of the DHFR/G-8 cells were excised and grown in culture, they (PNT-1 and PNT-2) exhibited typical transformed morphology similar to that of B104-1 cells. The PNT-1 and PNT-2 cells produced tumors within 2 weeks after injection (Table 1). Since the morphology and tumorigenicity of PNT-1 and PNT-2 cells were different from those of parental DHFR/G-8 cells, it is conceivable that a second hit involving genetic alteration in the DHFR/G-8 cell occurred during the 3-month lag period.

We reasoned that the genetic alteration by the second hit might activate some protooncogene and then convert the DHFR/G-8 cells into the transformed PNT-1 and PNT-2 cells. To test this possibility, we carried out the NIH 3T3 focus-forming assay. The DNAs prepared from PNT-1, PNT-2, and DHFR/G-8 cells were transfected into NIH 3T3 cells. Both PNT-1 and PNT-2 DNA produced foci and DHFR/G-8 DNA did not. Four independent foci (PNT-1.1, PNT-1.2, PNT-2.1, and PNT-2.2) were picked, and they all exhibited transformed morphology (data not shown). All four foci were further shown to be tumorigenic two weeks after injection. The results suggest that PNT-1 and PNT-2 indeed contained activated oncogene.

Since the DHFR/G-8 cell line is an NIH 3T3 transfectant containing 100 copies of the rat proto-*neu* gene and since only injection of DHFR/G-8 cells but not NIH 3T3 cells produced tumors in the nude mice, we suspected that the target of the second hit might reside in the amplified rat proto-*neu* gene. If so, we expected the foci generated by PNT-1 and PNT-2 would contain the rat *neu* gene. The DNAs from the four independent foci were analyzed by Southern blot with a rat *neu*-specific probe (Fig. 1). All four foci exhibited the rat *neu*-specific restriction pattern, indicating that the target of the second hit was the amplified rat proto-*neu* gene in the DHFR/G-8 cells.

It was previously shown that a single point mutation at the DNA region encoding the transmembrane domain is sufficient to convert the proto-*neu* gene into an activated *neu* oncogene in rat neuro/glioblastomas (6). Since the PNT-1 and PNT-2 cells carry transfected *neu* genes, it was of interest to determine whether the second hit in the DHFR/G-8 cells contained the same mutation. To answer this question, we used synthetic oligonucleotides as probes to search for the

Table 1. Tumorigenicity assay

Cell line	No. of tumors/ no. of injections*	Time to develop tumors†
B104-1	6/6	2 weeks
DHFR/G-8	6/6	3 months
NIH 3T3	0/6	3 months
PNT-1	6/6	2 weeks
PNT-2	6/6	2 weeks

*Each mouse was injected s.c. with 10^6 cells in 0.2 ml of phosphate-buffered saline.
†The tumors were at least 1 cm in diameter.

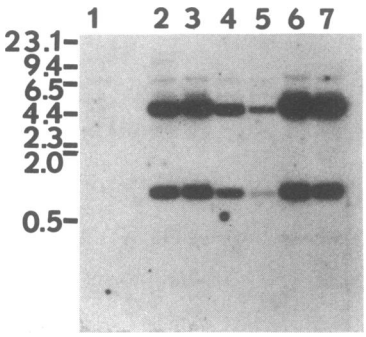

FIG. 1. Southern blot analysis of DNA isolated from the indicated sources. Ten micrograms of DNA was digested with *Bam*HI and hybridized with a 0.4-kilobase (kb) *Bam*HI fragment of rat neu cDNA (5). Lanes: 1, NIH 3T3 cells; 2–5, PNT-2.1, PNT-2.2, PNT-1.1, and PNT-1.2 cells, respectively (four independent transfectant cell lines derived from PNT-2 and PNT-1 by NIH 3T3 focus-forming assay); 6 and 7, PNT-1 and PNT-2 cells (lines derived from tumors that were excised from the tumor-bearing mice 3 months after injection of DHFR/G-8 cells). Phage λ *Hin*dIII size markers in kb are indicated to the left.

potential mutation in the same region that contains the mutation in rat neuro/glioblastomas.

Oligonucleotides corresponding in sequence to the wild type or the adenosine-to-thymidine mutant version of the *neu* gene were as described (6). These two 20-mers were hybridized under stringent conditions to dried agarose gels containing DNAs that had been digested with appropriate restriction endonucleases. The hybridization condition was shown to be able to distinguish the wild-type and the mutant version of *neu* gene from each other (6). The 20-mer corresponding to the wild-type sequence hybridized with DNA from DHFR/G-8 but not with DNA from B104-1 or any of the four independent foci (Fig. 2). When the gel was stripped of probe and rehybridized with the 20-mer corresponding to the mutant sequences, positive signals were observed for DNAs from B104-1 and the four independent foci but not for DNA from DHFR/G-8. Since it is known that transfectants usually contain multiple copies of the transfected gene, the different intensities shown by the four independent foci (Fig. 2*b*, lanes 3–6) are due to different copy numbers of the transfected *neu* gene in the foci. From these results we conclude that the second hit of DHFR/G-8 cells is a single point mutation that is identical to the one previously found in the rat neuro/glioblastoma (6). We also tried to determine how many copies of the proto-*neu* gene were mutated to become activated *neu* oncogene in the PNT-1 and PNT-2 cells. The majority of the *neu* genes in PNT-1 and PNT-2 cells were normal versions of the *neu* gene, since the hybridization signals with the wild-type 20-mer were similar to that of DHFR/G-8. When the mutant 20-mer was hybridized with DNA from PNT-1 and PNT-2, we detected only a very weak signal after a long exposure of x-ray film. However, a signal with similar intensity was also produced by cross-hybridization of the mutant 20-mer to the 100 copies of proto-*neu* gene in DHFR/G-8 cells (19/20-nucleotide match) under the same conditions (data not shown). Since it is difficult to distinguish the hybridization signal of the mutant 20-mer to a single copy of mutated *neu* oncogene (20/20-nucleotide match) from the cross-hybridization signal to 100 copies of the proto-*neu* gene (19/20-nucleotide match) in the PNT-1 and PNT-2 cells (see below), we could not accurately estimate exactly how many copies of the proto-*neu* gene were mutated. However, the results suggest that at most only a very few copies of proto-*neu* in PNT-1 and PNT-2 cells were mutated to become activated *neu* oncogene.

Since the mutations of the *neu* gene in these four independent foci were identical to that in B104-1, it might be possible that the DHFR/G-8 cells used for injection were contam-

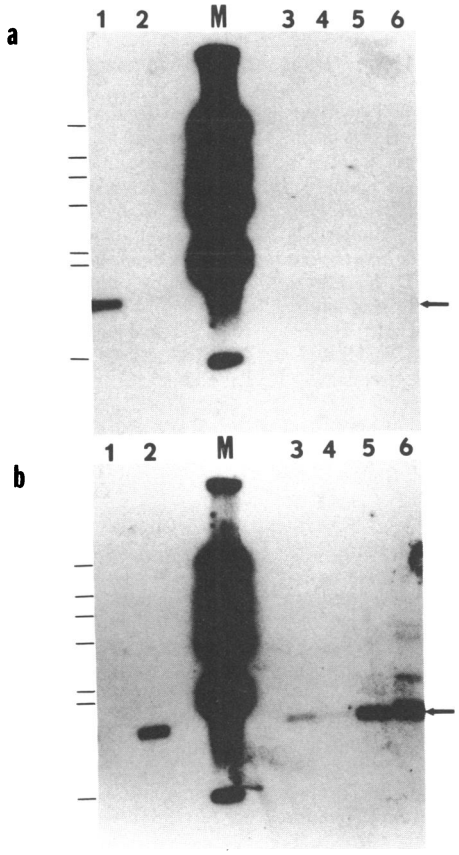

FIG. 2. Oligonucleotide hybridization with tumor cell lines and *neu* transfectants. DNA was digested with *Bgl* II and hybridized either with wild-type oligonucleotide (*a*) or mutant oligonucleotide (6) (*b*). The arrows to the right indicate the hybridization signals. Lanes: 1, DHFR/G-8; 2, B104-1; 3–6, PNT-1.1, PNT-1.2, PNT-2.1, and PNT-2.2, respectively; M, overexposed phage λ *Hin*dIII size markers that are indicated to the left.

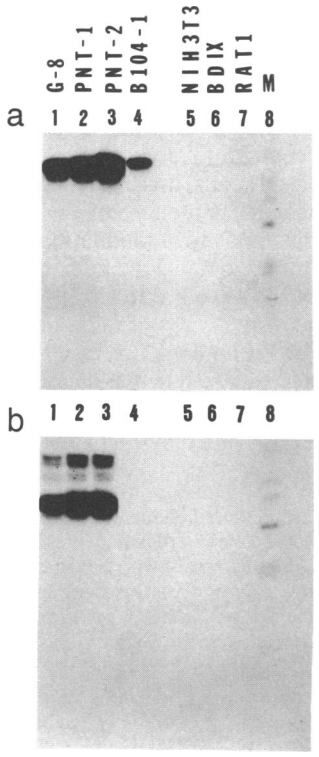

FIG. 3. Southern blot analysis of DNA isolated from the indicated sources. Ten micrograms of DNA was digested with *Eco*RI and hybridized with either the 0.4-kb *Bam*HI fragment of rat neu cDNA (*a*) or a DHFR DNA probe derived from pSV2-DHFR (*b*). Lanes: 1, DHFR/G-8 cells; 2, PNT-1 cells; 3, PNT-2 cells; 4, B104-1 cells; 5, NIH 3T3 cells; 6, BDIX rat liver cells; 7, Rat-1 cells; 8, phage λ *Hin*dIII size markers in kb.

inated with a small fraction of B104-1 cells, and the PNT-1 and PNT-2 cells were derived from the contaminating B104-1 cells. To rule out this possibility, the PNT-1 and PNT-2 cells were shown to be of DHFR/G-8 origin. Like DHFR/G-8, both PNT-1 and PNT-2 were methotrexate-resistant and contained highly amplified pSV2-DHFR DNA and the *neu* gene (Fig. 3); B104-1 did not contain pSV2-DHFR and was methotrexate-sensitive. When compared to the BDIX rat liver and Rat1 cells that presumably contained two copies of the *neu* gene (Fig. 3), the copy number of the *neu* gene in B104-1 was approximately 20, which was much lower than those seen in PNT-1, PNT-2, and DHFR/G-8, which contained approximately 100 copies.

Since both PNT-1 and PNT-2 cells were derived from DHFR/G-8 cells and since all four foci derived from PNT-1 and PNT-2 cells contain the same mutation-activated *neu* oncogene, we conclude that amplification of the proto-*neu* gene facilitated oncogenic activation by a single point mutation and that this point mutation, which is identical to the one found in the rat neuro/glioblastomas, is a "hot spot" for activating the proto-*neu* gene.

DISCUSSION

We have shown that amplification of the proto-*neu* gene can facilitate oncogenic activation by a single point mutation in some of the amplified proto-*neu* genes. Our results indicate that one way to convert a protooncogene into an activated oncogene is through a two-step mechanism—namely, amplification and then mutation.

In the present study, the mutation responsible for activation is a single point mutation. However, it is conceivable that amplification could facilitate rearrangement or deletion, too. The recent results reported by E. Helseth (personal communication) and colleagues support the concept. They found *EGFR* (formerly called *ERBB1*; also called human c-*erbB-1*) was amplified and overexpressed in a human carcinoma cell line, T-CAR-1, derived from the adrenal cortex. In addition to the high level of normal EGFR (170 kDa), they detected a 50-kDa protein that interacted with antibody against the C-terminal domain of EGFR. Their results suggest that the 50-kDa protein may be a mutated form of EGFR. From our model system in the present study, we would suggest that *EGFR* was amplified in the carcinoma, and later one of the amplified *EGFR* genes was mutated to produce the 50-kDa protein. Since the virus-associated oncogene v-*erbB* is known to encode a truncated form of EGFR, the 50-kDa protein may be activated in a similar way to the v-erbB protein.

Several protooncogenes have been shown to be amplified in many human tumors (7–12, 19, 20). Our study raises an interesting question: "could some of the amplified protooncogenes bear activating mutations?" If the mutated alleles can be frequently found in those human tumor DNAs, containing amplified *neu* (*ERBB2*; *NGL*) or *EGFR* (*ERBB1*), our present model may be appropriate for human tumors—i.e., amplification of the protooncogene may facilitate additional oncogenic mutation, and amplification and mutation may form a two-step mechanism in multistepped carcinogenesis.

In vitro mutagenesis studies have shown that mutations that convert valine to glutamine or aspartic acid can also activate the proto-*neu* gene into an activated *neu* oncogene (21). However, the point mutations induced by ethylni-

trosourea and spontaneous mutation in the present study are all thymidine-to-adenosine transversions that convert valine to glutamic acid. The codon for the specific valine is GTG (6), which thus requires only one thymidine-to-adenosine mutation to mutate valine to glutamic acid. However, double mutations are required to convert the specific valine to glutamine or aspartic acid. This difference probably explains why all of the oncogenic mutations of *neu* detected *in vivo* so far are valine-to-glutamic acid mutations.

Recently, overexpression of the human homologue of the *neu* gene has been shown to transform NIH 3T3 cells (22, 23). It is not yet clear why overexpression of the rat proto-*neu* gene in DHFR/G-8 cells does not result in a transforming phenotype. It could be possible that the rat proto-*neu* gene is less potent than the human counterpart or that DHFR/G-8 cells do not produce enough normal P185 to transform NIH 3T3 cells.

These studies were initially conducted in the Whitehead Institute laboratory of Dr. Robert Weinberg, whom we thank for enthusiastic advice and support. We also thank Ms. Carolyn Davis and Ms. Jean Eakins for typing this manuscript. This work was supported in part by Grant CA-45265 of the National Institutes of Health, Grant CD-360 of The American Cancer Society, and Award 5-621 of the Basil O'Connor Starter Scholar Research Award from the March of Dimes Birth Defects Foundation (to M.C.H.).

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