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Molecular cloning of the *neu* gene: Absence of gross structural alteration in oncogenic alleles

(growth factor receptor/neuroblastoma/gene amplification)

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ABSTRACT **The *neu* gene is distantly related to the *erbB* gene and encodes a cell surface protein that appears to function as a growth factor receptor. To study the mechanisms that caused the conversion of the normal *neu* gene to an oncogenic allele, we have isolated molecular clones of the *neu* oncogene as well as a clone of the corresponding protooncogene. The transforming *neu* oncogene and the proto-*neu* gene clones exhibit identical restriction enzyme patterns. Amplification of the proto-*neu* gene in NIH 3T3 cells by means of cotransfection with a dihydrofolate reductase gene resulted in methotrexate-resistant colonies that produce high levels of normal *neu*-encoded p185 protein. In contrast to cells carrying low levels of the oncogene-encoded protein, these cells appeared normal. The results suggest that the lesion that led to activation of the *neu* gene is a minor change in DNA sequence and is apparently located in the protein-encoding region of the gene.**

Rat neuro/glioblastomas induced by transplacental injection of ethylnitrosourea frequently carry an oncogene that is detectable by transfection into mouse NIH 3T3 cells (1, 2). We have designated this oncogene *neu* (3). The transfected NIH 3T3 cells display a novel 185,000-dalton tumor antigen (p185) that is not detected when these recipient cells are transformed by other oncogenes (4). The *neu* oncogene was shown to have some sequence homology with c-*erbB*, the gene encoding the structure of the epidermal growth factor receptor (EGF-r) (5). In addition, the encoded product of *neu*, the p185 antigen, is serologically related to the EGF-r (3). The *neu* gene in the normal and oncogenic forms appears to be distinct from the gene that encodes the EGF-r (6).

The genes encoding two growth factor receptors, the EGF-r and the receptor for the mononuclear phagocyte growth factor CSF-1, have been found to be activated into oncogenes (7, 8). In each case, these activations have involved major changes in the structure of these genes.

As an initial step toward studying the activating mechanism(s) of *neu*, we have used molecular cloning to isolate the oncogenic alleles of the *neu* gene from two rat neuro/glioblastomas cell lines and the normal allele from rat liver DNA. The present results indicate that oncogenic activation of the *neu* gene in these rat tumors occurred by means of quite different processes that creates minimal changes in protein structure.

MATERIALS AND METHODS

Construction and Screening of Cosmid Library. Cosmid libraries were constructed essentially according to the protocol of Ish-Horowitz and Burke (9). Genomic DNA was isolated from the cell lines, digested with *Eco*RI to completion, and loaded onto a 13-ml 1.25–5.0 M NaCl (10 mM Tris·HCl, pH 8.0/1.0 mM EDTA) exponential gradient. Centrifugation was for 4 hr at 40,000 rpm at 25°C in an SW 40.1 Beckman rotor. Twelve fractions were collected, aliquots were taken, and the size of the DNA was analyzed by gel electrophoresis. Three fractions containing DNA in the size range of 30–40 kilobases (kb) were combined.

The 30- to 40-kb *Eco*RI segments were ligated to the arms of the cosmid vector pSAE (10), as generated by partial *Eco*RI digestion. The DNA was packaged by using the extract and protocol from Promega-Biotec (Madison, WI). Transduction was carried out as described by Grosveld *et al.* (10) with *Escherichia coli* 1046 as host.

The library was screened according to published techniques (11), using as probe a gel-purified *Sac* I/*Pvu* II fragment of avian erythroblastosis virus (12). This probe is *erbB* specific and reacts with DNA containing the *neu* oncogene (3). Hybridization was carried out at low stringency (30% formamide/0.75 M NaCl/75 mM sodium citrate, pH 7.0, 42°C) for 36 hr. The filters then were washed three times at 20°C for 10 min and at 50°C for 4 hr with 0.3 M NaCl/30 mM sodium citrate/0.1% $NaDodSO_4$.

Southern Analysis. Southern blot analysis was performed essentially according to published techniques (11). 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 20°C 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% $NaDodSO_4$.

Immunoprecipitation. Immunoprecipitation of p185 was carried out by using lysates of cells that were metabolically labeled with [^{35}S]cysteine as described in detail (3).

Transfection of Cloned DNA. DNA transfection into mouse NIH 3T3 cells was carried out by the calcium phosphate precipitation technique of Graham and van der Eb (13) as modified by Anderson *et al.* (14). After 2 weeks, foci of morphologically transformed cells were scored and analyzed. Cotransfection with pSV2-DHFR* plasmid (kindly provided by Heidi Stuhlmann) was performed at a molar ratio of 1:10 with the cNeu-p clone carrying the normal allele of *neu* according to published procedures (15). The pSV2-DHFR* plasmid contains an altered mouse dihydrofolate reductase (DHFR) cDNA (15) that displays abnormally low affinity for methotrexate (MTX). NIH 3T3 cells (8×10^5) in a 10-cm plate were exposed to 10 μg of the cNeu-p clone and 0.1 μg of the pSV2-DHFR* clone. After 24 hr the cells were split into 10 10-cm plates and grown in the presence of 0.6 μM MTX in Dulbecco's modified Eagle's medium containing 10% dialyzed calf serum. The medium was changed every 3–4 days.

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Abbreviations: DHFR, dihydrofolate reductase; MTX, methotrexate; kb, kilobase(s); EGF-r, epidermal growth factor receptor.

The MTX-resistant colonies were subcloned with glass cylinder cloning rings after 12–14 days.

RESULTS

We wished to obtain a molecular clone of the *neu* oncogene that demonstrated transforming activity. Our previous work showed that the biological activity of the *neu* oncogene is not inactivated by endonuclease *Eco*RI (1). This suggested that the entirety of the gene lay within a single *Eco*RI segment whose size we estimated was >23 kb (3). Consequently, we attempted to isolate by molecular cloning this *Eco*RI segment with the hope that it would contain a biologically intact *neu* oncogene.

In devising a cloning strategy, an accurate size determination of this *Eco*RI segment was needed. To obtain this, we studied DNAs from B103-1 and B104-1 cell lines that had been transfected with B103 and B104 tumor DNAs, respectively. These DNAs were digested with *Eco*RI and size-fractionated by electrophoresis through low-composition (0.4%) agarose gels. Analysis by Southern blotting using an *erbB* probe (12) revealed that the large *neu*-containing *Eco*RI segments were about 33 kb in size (data not shown). Because λ phage vectors are unable to carry DNA segments of this size, we therefore turned to a cosmid vector capable of packaging DNA within the 30- to 40-kb size range. In particular, we chose to employ the cosmid vector pSAE (10) since arms generated by partial *Eco*RI cleavage of its DNA should be able to carry insert DNA in the range of 30–40 kb.

Libraries were constructed from the genomes of the two NIH 3T3 transfectants deriving from transfer of the B103 and the B104 neuroblastoma DNAs since these transfections resulted in the insertion of multiple copies of the *neu* oncogene in the recipient cell genomes (3). We further attempted to package only *Eco*RI-digested DNA of 30–40 kb in size that had been enriched by gradient centrifugation. The resulting cosmid libraries were screened with an *erbB* probe that is crossreactive with *neu* (3). Each of the positive clones, designated cNeu-103 and cNeu-104, contains a 33-kb *Eco*RI insert.

We wished to determine whether these clones were capable of oncogenic transformation of NIH 3T3 cells. To that end, we transfected 1 μg of the cloned DNA by the calcium phosphate precipitation technique (13) and after 2 weeks scored foci of transformed cells. Approximately 300 foci appeared in the monolayer cultures of NIH 3T3 cells that had been transfected with the cNeu-103 or the cNeu-104 clone. These foci largely contained cells that were extremely refractile. Fig. 1 shows representative cell lines derived from such foci.

Further work confirmed that these foci of transformed cells contain the intact transfected *neu* DNA and the encoded tumor antigen p185. Fig. 2 shows a Southern blot analysis of the DNAs of these cell lines and immunoprecipitation of metabolically labeled cell lysates. As can be seen, the putative transfectants all carry the 33-kb *Eco*RI DNA segments associated with the *neu* oncogene and express the oncogene-encoded p185 protein.

To study the activating mechanism of *neu*, we isolated a molecular clone of the normal allele of *neu* by screening a cosmid library constructed from normal BDIX rat liver DNA. The resulting clone, designated cNeu-p, also carries a 33-kb *Eco*RI insert that exhibits the same restriction pattern as the cNeu-103 (Fig. 3). In fact, analysis of 50 restriction enzyme sites revealed no differences between the normal and oncogenic clones of the gene (not shown).

The oncogenic transforming ability of the normal clone of *neu* was tested by transfection into NIH 3T3 cells. No foci could be detected. Cotransfection of this normal clone with the neomycin-resistance gene resulted in colonies that produce a p185 antigen that migrates similarly to the antigen found in oncogene-transformed cells (see below). These cells were not morphologically altered. Thus, NIH 3T3 cells that have obtained the normal rat allele by means of transfection express a normal version of the rat p185 that apparently lacks the ability to induce morphological transformation.

These data suggested that a subtle change in the structure of the *neu* gene resulted in its conversion to an active oncogene. Other growth factor receptor genes have been converted to active oncogenes following gene amplification or mutations that cause substantial rearrangement in protein

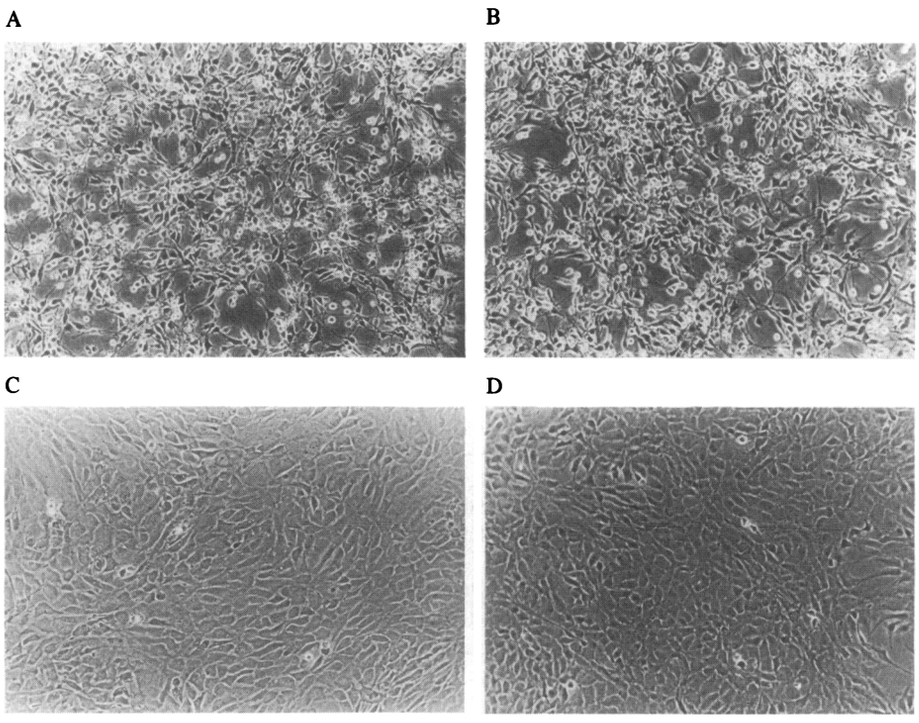

FIG. 1. Morphology of *neu* NIH 3T3. (*A*) cNeu-103-1, a transfectant containing the cNeu-103 clone. (*B*) cNeu-104-1, a transfectant containing the cNeu-104 clone. (*C*) DHFR/G-8, a transfectant containing the cNeu-p clone and pSV2-DHFR* plasmid. (*D*) Untransfected recipient NIH 3T3 cells. (×50.)

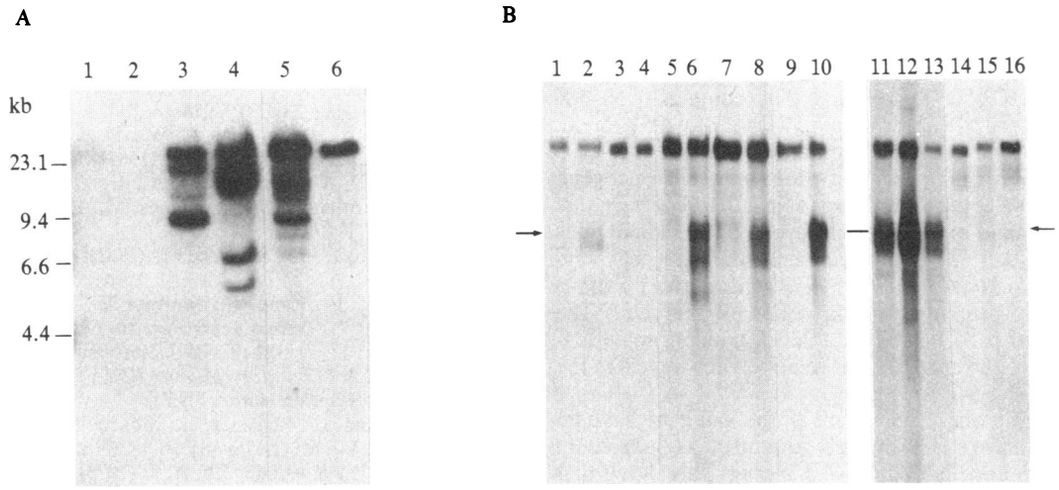

FIG. 2. (*A*) Southern analysis of *neu* NIH 3T3 transfectants. DNA was isolated from indicated sources, digested with *Eco*RI, and analyzed by Southern blotting probing with a 4.0-kb *Bam*HI segment of cNeu-103 that has been shown to be *erbB* homologous (3). Ten micrograms of DNA was loaded on each lane. Lane 1, BDIX rat liver; lane 2, NIH 3T3 cell; lane 3, cNeu-103-1, a transfectant containing the cNeu-103 clone; lane 4, cNeu-104-1, a transfectant containing the cNeu-104 clone; lane 5, DHFR/G-8, a cell line derived from cotransfection of cNeu-p and pSV2-DHFR*; lane 6, B104-1. (*B*) Immunoprecipitation of p185. Lysates of [^{35}S]cysteine-labeled cells were incubated with either preimmune (lanes 1, 3, 5, 7, 9, and 14–16) or anti-p185 monoclonal antibody 7.16.4 (16) (lanes 2, 4, 6, 8, and 10–13) and subsequently precipitated and analyzed by NaDodSO$_4$ gel electrophoresis. Lanes 1 and 2, Rat-1 cells; lanes 3 and 4, NIH 3T3 cells; lanes 5 and 6, cNeu-103-1; lanes 7 and 8, cNeu-104-1; lanes 9, 10, 13, and 16, B104-1; lanes 11 and 14, DHFR/G-6; lanes 12 and 15, DHFR/G-8; DHFR/G-6 and DHFR/G-8 are two independent cell lines containing cNeu-p and pSV2-DHFR*. The arrows show the position of p185.

structure (7, 8, 17, 18). We wished to see whether a deregulation of *neu* expression, such as that which might arise following gene amplification, might explain the transforming powers of this gene. We therefore selected cells containing a high copy number of the normal *neu* clone by cotransfection of this clone with the pSV2-DHFR*, a clone of the DHFR gene.

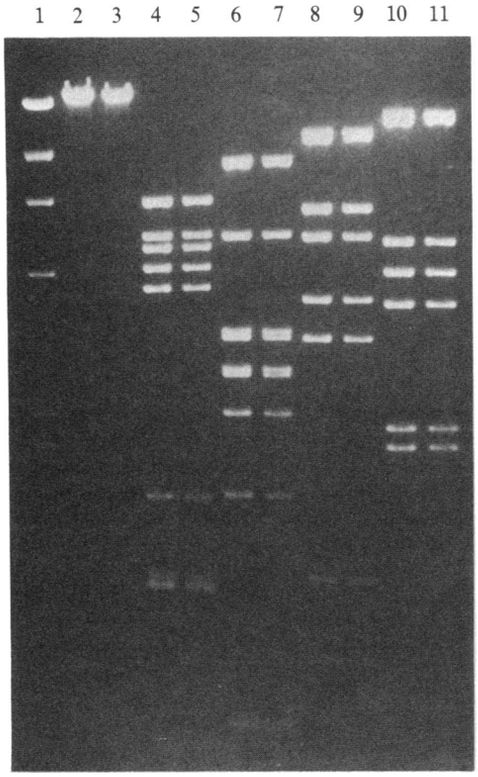

FIG. 3. Restriction pattern of cNeu-103 and cNeu-p clones. The cNeu-103 and cNeu-p clones were digested with *Eco*RI, and the 33-kb *Eco*RI inserts were gel-purified. The 33-kb *Eco*RI inserts were run on the agarose gel (lanes 2 and 3) or digested with *Bam*HI (lanes 4 and 5), *Bgl* I (lanes 6 and 7), *Hin*dIII (lanes 8 and 9), and *Xho* I (lanes 10 and 11) and then fractionated by gel electrophoresis. Lanes 2, 4, 6, 8, and 10, cNeu-103; lanes 3, 5, 7, 9, and 11, cNeu-p. λ *Hin*dIII markers are shown in lane 1.

Transfection of the DHFR clone followed by application of MTX allows selection of cells that have acquired a high copy number of the DHFR gene and of the cotransfected DNA (19). Six of 12 MTX-resistant colonies that were picked contained amplified *neu* sequences and high levels of p185. Fig. 2 shows the analysis of protein lysates from two cell lines derived from these colonies. These two cell lines exhibit the highest levels of gene amplification and antigen expression.

One of these two cell lines contains a 50- to 100-fold higher gene copy number than that of normal rat liver DNA and about 10-fold higher levels of p185 antigen than do colonies transformed by the cNeu-103 or cNeu-104 clone. Importantly, this colony was morphologically indistinguishable from untransfected NIH 3T3 cells (Fig. 1). The fact that very high level expression of the normal p185 antigen cannot alter the morphological phenotype of NIH 3T3 cells suggests that mutations leading to overexpression of the *neu* gene will not lead to oncogenic activation. We conclude that the alterations that activated the *neu* oncogene likely affect the structure of p185 rather than its regulation. They should eventually be found in the protein-encoding region of this gene.

DISCUSSION

We present here the molecular cloning of biologically active transforming and normal alleles of the *neu* gene. The fact that the entire *neu* gene resides in a single piece of 33-kb *Eco*RI fragment made it easy to construct a library that contains highly enriched *neu* gene. Using the 30- to 40-kb *Eco*RI fragments isolated from the primary mouse transfectant to construct the cosmid library, we found that ≈1 of 1000 colonies in the library contained the *neu* oncogene.

A high level of gene amplification of certain genes has been achieved by cotransfection with the DHFR gene followed by long-term growth in the presence of continually increasing concentrations of MTX (19). Such selection can be laborious and time-consuming. We describe here the success of gene amplification with a high copy number in a single step

selection. By cotransfecting the cNeu-p and pSV2-DHFR* clones and directly selecting the MTX-resistant colonies in relatively high concentrations (0.6 μM) of MTX, we have isolated the DHFR/G-8 cell line that contains ≈50–100 copies of cNeu-p and expresses a high level of p185 protein.

The DHFR/G-8 cells that contained amplified cNeu-p produced 10-fold higher levels of p185 than cells oncogenically transformed by cNeu-103 or cNeu-104. The fact that this very high level expression of normal p185 antigen does not morphologically alter the recipient NIH 3T3 cells suggests that the activation of the *neu* gene derives from a mutation in the protein-encoding region of the gene. The resulting altered p185 protein is able to induce transformation, even when it is expressed at relatively low levels in the cell.

The normal and oncogenic forms of the *neu* gene have no detectable differences in overall structure, as judged by extensive restriction endonuclease mapping. This shows that activation of the gene in the rat neuro/glioblastomas involved a very subtle change in DNA sequence. This idea is further supported by the data showing that the molecular weights of the p185 protein encoded by the normal and transforming alleles are very similar in size, as judged by $NaDodSO_4$ gel electrophoresis (Fig. 2).

One further clue concerning the nature of the activating lesion is provided by the fact that the carcinogen used to induce the rat neuro/glioblastomas was ethylnitrosourea, a known point mutagen (20). Taken together with the other data presented here, this suggests that subtle changes in p185 protein structure, like those found in the *ras*-encoded p21 proteins (21–28), may contribute to the outgrowth of certain types of tumors.

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