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Early mutation of the *neu* (*erbB-2*) gene during ethylnitrosourea-induced oncogenesis in the rat Schwann cell lineage

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ABSTRACT **The development of malignant tumors of the peripheral nervous system (schwannomas) within a defined intracranial section of the rat trigeminal nerve ("trigeminal box") was used as a model to identify genetic alterations typically associated with the process of cell-lineage-specific oncogenesis induced by exposure to *N*-ethyl-*N*-nitrosourea on postnatal day 1. All 47 trigeminal schwannomas (and 12 extracranial neurinomas) investigated carried a T·A → A·T transversion mutation at nucleotide 2012 of the *neu* (*erbB-2*) gene sequence encoding the transmembrane domain of gp185^{neu}. This mutation was absent in all 18 tumors in the brain and spinal cord (central nervous system) isolated from the same animals. Identical observations were made in cell lines derived from *N*-ethyl-*N*-nitrosourea-induced rat schwannomas vs. brain tumors. By asymmetric PCR and mutant-specific *Mnl* I restriction fragment length analyses, cells carrying the mutant *neu* allele became detectable and could be localized within the trigeminal box as early as 7 days after the carcinogen pulse. The proliferation rate of the mutant cells strongly exceeded that of the wild-type cells up to the time of maturation of the trigeminal nerve around postnatal day 30 and thereafter to a lesser extent until the appearance of schwannomas. A specific mutation of the *neu* gene thus represents a very early, probably the first, step in the malignant conversion of immature rat Schwann cells exposed to *N*-ethyl-*N*-nitrosourea *in vivo* and is diagnostic for a subset of proliferative cells at high risk of progressing toward the expression of fully malignant phenotypes. Loss of heterozygosity for the mutant *neu* allele is a candidate event for a critical second step in the process.**

A question of particular relevance to our understanding of the process of carcinogenesis is to what extent is the involvement of specific genes that are mutationally activated, inactivated, or irregularly expressed dictated by the type of target cell lineage and its genetic program. This is not to detract from the differential capability of DNA-reactive carcinogens to induce mutations in specific gene sequences, due to the respective types of reaction products and their relative frequencies and repair in genomic DNA (1, 2). Preferential binding of carcinogens to specific bases can be further accentuated by the nature of their flanking nucleotides (3–5), and specific carcinogen adducts may selectively persist in critical gene sequences as a result of nonrandom DNA repair (6). Carcinogen-specific activating point mutations in the c-Ha-*ras* gene have been clearly demonstrated, for example, in mouse skin (7, 8) and rat mammary gland tumors (9, 10) induced by *N*-nitroso compounds and polycyclic hydrocarbons. Information remains scarce, however, regarding the relative frequencies of carcinogen-specific mutations in various types of cells exposed to the same carcinogen and on the relevance of specific mutant genes for successive steps in the process of carcinogenesis in defined cell lineages.

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Our earlier analyses of *N*-ethyl-*N*-nitrosourea (EtNU)-induced malignant rat cell lines derived from the brain and peripheral nervous system (PNS) (A. Kindler-Röhrborn, M.F.R., C. I. Bargmann, and R. A. Weinberg, unpublished data)—and observations by other groups (11, 12)—have suggested the cell lineage-specific involvement of a T·A → A·T transversion mutation at nucleotide 2012 of the transmembrane region of the *neu* (*erbB-2*) gene (13) in the malignant conversion of immature Schwann cells of the PNS. This mutation could result from misinsertion of adenine opposite an unrepaired noninstructional apurinic site (14) formed in the sense strand upon the loss (spontaneous or enzymatic) of a 3-ethyladenine residue formed in the DNA of replicating cells exposed to EtNU. Glutamic acid is thus substituted for Val-664 (15) of gp185^{neu}, a phosphoglycoprotein with tyrosine kinase activity and considerable structural homology to the epidermal growth factor receptor (16) and to the *erbB-3* gene product (17). Due to its receptor-like nature, gp185^{neu} is likely to be a transducer molecule for proliferation-controlling signals (18).

To investigate the cell lineage-specific role of the *neu* gene mutation in the process of EtNU-induced neurooncogenesis, we placed the carcinogen pulse into a developmental "window" (24 h postnatally) ensuring a high yield of trigeminal schwannomas relative to tumors derived from other neuroectodermal cell lineages and sufficient synchrony in tumorigenesis (19, 20). Due to the feasibility of reproducible microsurgical dissection, we restricted our analyses to the Schwann cell population of an intracranial segment of the trigeminal nerve (termed the "trigeminal box"). The trigeminal box encompasses 1×10^6 cells on postnatal day 1. It extends from the brain–PNS junction to the distal end of the Gasserian ganglion and can be subsectioned into vertical "section levels." Morphological analyses could thus be performed and mutant *neu* cells could be identified and localized within the trigeminal box from the period of carcinogen exposure until the appearance of schwannomas.

MATERIALS AND METHODS

Animals and Carcinogen Administration. Rats of the inbred strain BDIX (21) were used. A single dose of EtNU [80 μg/g (body weight)] was administered s.c. 24 h postnatally (19).

Preparation of Tissues for Histological and DNA Analyses. Tissue samples dissected under a stereo operation microscope (Zeiss) were either stored in liquid nitrogen or fixed in

Abbreviations: ASO, allele-specific oligonucleotide; EtNU, *N*-ethyl-*N*-nitrosourea; PNS, peripheral nervous system.

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phosphate-buffered 10% (vol/vol) formalin and embedded in paraffin/celloidin. Sections were stained with hematoxylin and eosin, and additional staining procedures (Foot's argentaffin stain, van Gieson's, periodic acid–Schiff, Alcian blue, or Heidenhain's hematoxylin) were applied when required.

Cell Lines. The EtNU-induced malignant neural rat cell lines GV1C, TV1C (and subclones TV1Ca and TV1Cb), NV1C (and subclones NV1Cb and NV1Cc), BT3C, BT4C, and BT7C (22); RT4-AC, RT4-D6, and RT4-E5 (23); and B104 (24); and cell lines SCTL-1 (derived from an EtNU-induced BDIV-rat ovarian Sertoli cell tumor; ref. 25) and B104.1.1 (secondary NIH 3T3 mouse fibroblast transfectant from line B104; ref. 13) were maintained as monolayer cultures.

Isolation of DNA for PCR Analyses. DNA from tissues and cells was isolated as described (26). For analyses of DNA from histologically selected areas of tissue, ten 8-μm-thick frozen sections (followed by two sections for morphological examination) were placed into Eppendorf tubes and digested according to ref. 27.

PCR. The conventional PCR was as described (28). Two reaction mixtures for restriction fragment length analyses were used. (*i*) The conventional mixture included the 5′-end ^{32}P-labeled primer (0.2 μM), and amplification with recombinant *Taq* polymerase (AmpliTaq; Perkin–Elmer/Cetus) was performed as described (28). (*ii*) The reaction mixture for the asymmetric PCR (29) contained the primers 410 and 402 at 0.08 μM and 0.4 μM, respectively, a 1:5 ratio. Amplification was performed in 35 cycles. Thereafter, fresh reaction mixture (20% of original volume) was added that contained 0.2 μM 5′-end ^{32}P-labeled inner primer 408 (complementary to the DNA strand amplified in excess). One to 5 additional PCR cycles were performed (94°C for 1 min, 55°C for 1.5 min, and 72°C for 2 min; final extension at 72°C for 10 min).

Restriction Fragment Length Analysis. Amplified ^{32}P-labeled DNA (4 μl) was combined with 1 unit of *Mnl* I [recognition sequence, 5′-CCTC(N)$_7$; New England Biolabs]. After a 1-h digestion at 37°C, samples were electrophoresed for 4.5 h at room temperature on nondenaturing 12% polyacrylamide gels [acrylamide/*N*,*N*′-methylenebisacrylamide, 19:1 (wt/vol)] at 27 V/cm in TBE (0.089 M Tris borate, pH 8.3/0.025 M EDTA). Gels were exposed to Kodak X-Omat-AR or X-Omat-R films with two intensifying screens.

Allele-Specific Oligonucleotide (ASO) Hybridization. Amplified DNA was spotted onto nylon filters (Schleicher & Schüll) and hybridized with a ^{32}P-labeled oligomer probe as described (28). The temperature of the stringent wash was 63.5°C. For quantitative analyses, DNA was denatured in 0.4 M NaOH/25 mM EDTA, transferred with a slot-blot device (Schleicher & Schüll) in 200 μl per slot, and hybridized (28). Blotted DNA was quantitated on films by densitometry,

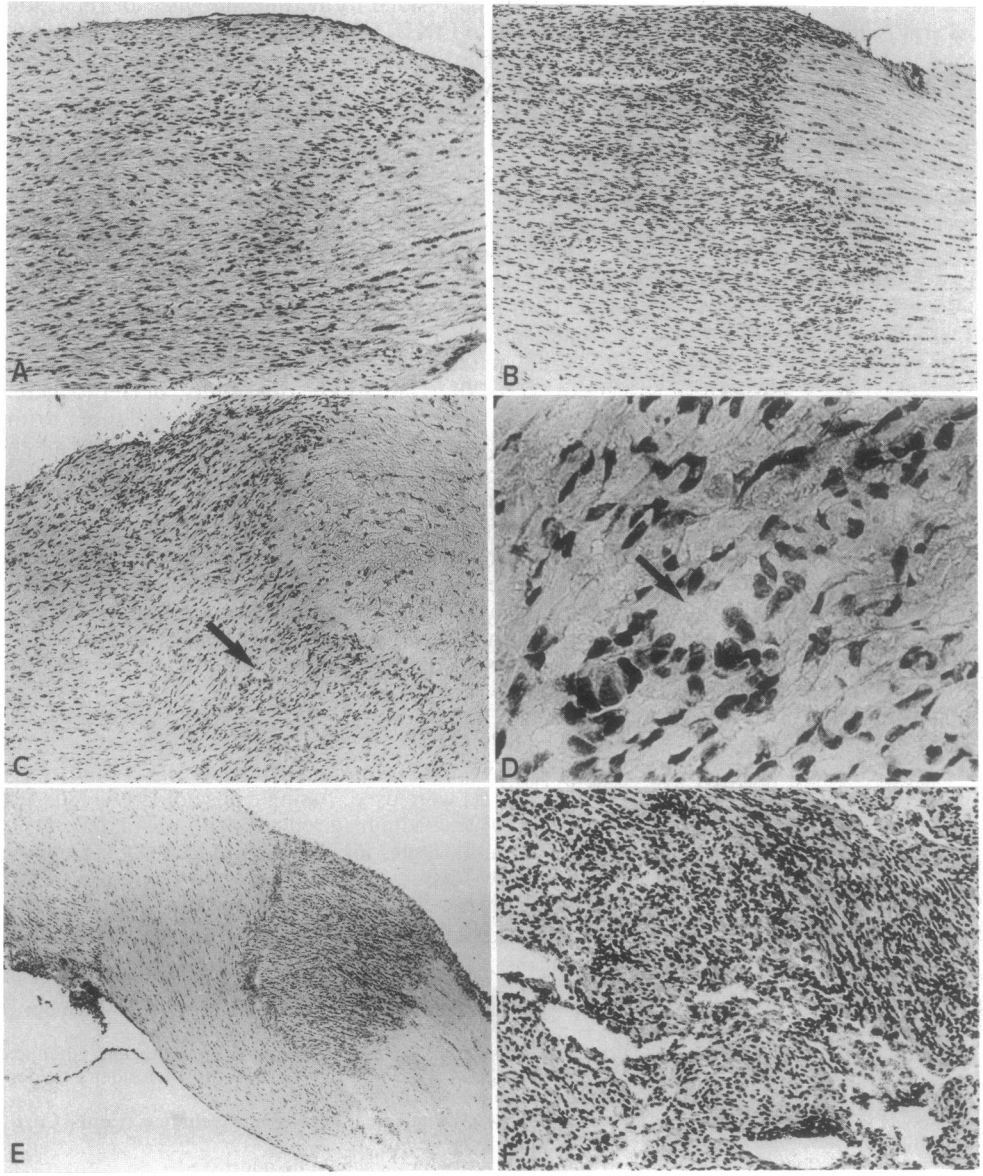

FIG. 1. Morphological changes in the BDIX rat intracranial trigeminal nerve exposed to EtNU *in vivo* on postnatal day 1. (*A*) Brain–PNS junction in a control rat (postnatal day 22). (*B*) Brain–PNS junction in an EtNU-treated rat (21 days after EtNU exposure); note increased cellularity when *B* and *A* are compared. (*C*) Cellular polymorphism (arrow) in the trigeminal nerve adjacent to the brain–PNS junction (day 28). (*D*) Higher magnification of the area shown in *C*. Note the atypical cells and excess nonfilamentous extracellular matrix (arrow). (*E*) Early microtumor in the trigeminal nerve adjacent to the brain–PNS junction (day 49). (*F*) Poorly differentiated schwannoma with occasional "palisade" and "stream-like" structures (day 203). (Hematoxylin and eosin. *A–C* and *F*, ×75; *D*, ×380; *E*, ×30.)

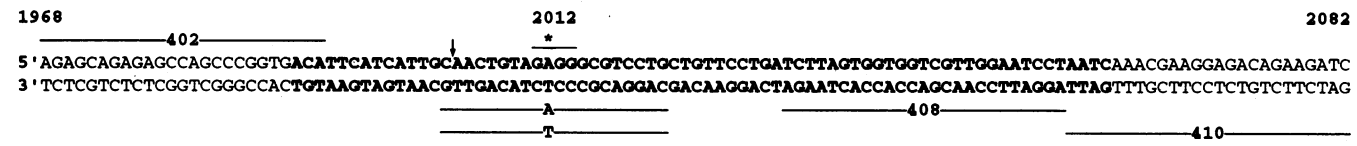

FIG. 2. Part of the rat *neu* gene cDNA (boldface type, nucleotides 1990–2061 encoding the transmembrane domain of gp185^{neu}) with the T·A → A·T transversion mutation at nucleotide 2012 (*). Also shown are the PCR primers (402 and 410), the inner primer used for extension after asymmetric PCR (408), and the hybridization oligonucleotides for the wild-type (A) and mutant *neu* (T) alleles. The mutant *neu*-specific *Mnl* I recognition sequence (GAGG) is overlined and the restriction site is marked ↓.

using a model CS930 dual-wavelength Chromato-Scanner (Shimadzu, Kyoto, Japan). Nonlinearity of the autoradiographic response was corrected by serial dilutions of DNA and by exposing the films for various times. The normalization of oligomer labeling and hybridization efficiency values was based on measurements of known amounts of target DNA. Mean values (±SEM) were calculated for samples containing the mutant *neu* allele.

RESULTS AND DISCUSSION

Yield of Trigeminal Schwannomas and Other Neural Tumors. All 29 rats (15 males and 14 females) exposed to EtNU developed at least one neural tumor during the time of observation (84–322 days after the EtNU pulse; total number of neural tumors, 87). Ninety percent (26/29) of the animals developed trigeminal schwannomas after a mean time of 179 ± 25 days, with both trigeminal nerves being affected in >50% of the animals (total number of trigeminal schwannomas, 47). Because many tumors were poorly differentiated, those accepted as schwannomas either originated from nerve trunks or presented histological features characteristic of schwannomas, such as fibrillar and reticular patterns of Antony types A and B, palisade-like structures, and cyst formation (Fig. 1*F*). Twenty of the trigeminal schwannomas were "microtumors" diagnosed microscopically. Of the 12 nontrigeminal schwannomas, 9 were localized in the cauda equina and in the roots of the spinal cord, 2 were in the plexus brachialis, and 1 was located retroperitoneally. Brain tumors (13) and spinal cord tumors (5) were classified as oligodendrogliomas (8), astrocytomas (2), ependymomas (5), or mixed gliomas (3). Of 10 tumors classified as being of an uncertain type, 7 were found near the spinal cord and 3 were in the mediastinum.

Histological Observations During Trigeminal Tumorigenesis. While searching for morphological alterations within the trigeminal box after the EtNU pulse, we observed an increase in cellularity and mitotic rate from 21 days onward (Fig. 1 *A* and *B*). In agreement with other reports (30, 31), clear deviations from the normal appearance of the developing trigeminal nerve were first seen on day 28 (Fig. 1*C*). These alterations were mostly localized in areas from which microtumors originated at later stages (Fig. 1*E*) (i.e., adjacent to the brain–PNS junction and in the Gasserian ganglion). Typically, multiple groups of irregularly distributed cells with an increased nuclear/cytoplasmic ratio were seen (Fig. 1*D*). In some cases, microtumors were found as early as 50 days after the EtNU pulse. Cells from microtumors formed tumors within a few weeks after s.c. reimplantation into syngeneic hosts.

Association of the Mutant *neu* Gene with the Type of Neural Tumors. DNA fragments [115 base pairs (bp)] containing the T·A → A·T transversion site at N2012 of the *neu* gene were amplified by the PCR and analyzed by ASO hybridization (Fig. 2). While the mutant *neu* gene was present in all 59 PNS tumors, it was not detectable in any of the 18 brain and spinal cord tumors (see Fig. 3). Two of the 10 tumors classified as being of an uncertain type lacked the mutant *neu* gene, signaling the origin of these tumors from cells not committed for the Schwann cell lineage.

Concordant with these observations, the mutant *neu* gene was not detected in cell line GV1C (derived from an EtNU-induced mixed glioma in the brain of a BDIX rat; ref. 22) or in cell lines BT3C, BT4C, and BT7C (derived from fetal BDIX rat brain cells in culture after exposure to EtNU *in vivo*; ref. 22) (Fig. 3). Moreover, the mutant *neu* gene was not detected in brain and liver DNA of both EtNU-exposed and untreated control rats, isolated on postnatal days 1, 3, 7, 14, 21, 28, 49, 77, 105, 133, 161, 189, 217, and 245, or in the DNA of lung, kidney, stomach, colon ascendens, spleen, thigh muscles, mediastinal adipose tissue with lymph nodes, pituitary and adrenal glands, testis, or uterus isolated on postnatal days 49 and 133.

As expected, the mutant *neu* gene was also present in cell lines TV1C and NV1C (22) and RT4-AC (23), which originate from EtNU-induced PNS tumors of BDIX rats, and in subclones of lines NV1C (NV1Cb and NV1Cc) and RT4-AC (RT4-D6 and RT4-E5) (Fig. 3). Interestingly, all of these lines were found to be homozygous (or hemizygous) for the mutant *neu* allele, a feature previously noted (15) in four other lines derived from EtNU-induced neural tumors in BDIX rats (24), including line B104 (Fig. 3). In contrast to 700 other mutant *neu* subclones grown in semisolid agar medium from cells of the trigeminal schwannoma-derived line TV1C, one subclone (TV1Ca) contained only the wild-type *neu* gene. However, as all of the cell lines and subclones analyzed, TV1Ca cells were tumorigenic upon s.c. reimplantation of 1×10^5 cells into newborn syngeneic animals. This might indicate gene con-

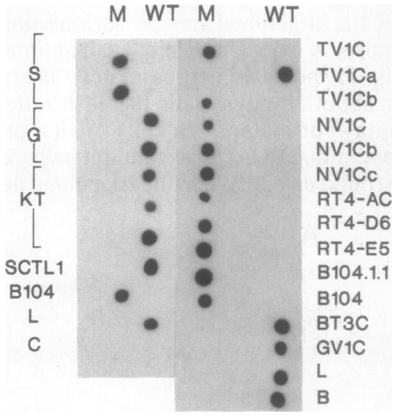

FIG. 3. Representative examples of PCR–ASO hybridization demonstrating the mutant (lanes M) and wild-type (lanes WT) *neu* genes in EtNU-induced BDIX rat schwannomas (S) and glial brain tumors (G); EtNU-induced rat kidney tumors (KT); rat cell lines derived from EtNU-induced BDIX rat tumors of the PNS (TV1C, TV1Ca, TV1Cb, NV1C, NV1Cb, NV1Cc, RT4-AC, RT4-D6, and RT4-E5); the EtNU-induced malignant BDIX rat neural line B104; cell line B104.1.1 derived from an NIH 3T3 secondary transfectant with the mutant *neu* gene and cell lines derived from BDIX rat brain cells malignantly transformed by EtNU (GV1C and BT3C); line SCTL-1 derived from an EtNU-induced BDIV rat ovarian Sertoli cell tumor; and in liver (L) and brain (B) of untreated control rats. C, mock control (PCR without DNA).

version at low frequency, and the possibility that the mutant *neu* gene may not be obligatory for the tumorigenic phenotype of schwannoma cells; however, more trivial explanations such as the presence of a minor contaminant subpopulation of non-Schwann-cell-derived tumorigenic cells cannot be ruled out.

First Detection, Localization, and Proliferative Behavior of Mutant *neu* Cells in the Trigeminal Box. In DNA isolated from the total trigeminal box and analyzed by ASO hybridization after PCR, the mutant *neu* gene was first detected 14 days after the EtNU pulse. The distribution of mutant *neu* cells was investigated by PCR on DNA isolated from frozen sections of defined regions of the trigeminal box and subsequently by mutant-specific *Mnl* I restriction fragment length analysis (Fig. 4). Asymmetric PCR followed by annealing of the inner primer provided additional proof for the specificity of the restriction pattern. By these more sensitive procedures, mutant *neu* cells were detected as early as 7 days after the EtNU pulse and appeared to be clustered near the brain–PNS junction and in the region of the Gasserian ganglion. No mutant *neu* cells were detectable in the brain area proximal to the brain–PNS junction. At the time of first observation, mutant *neu* cells thus show a "microclone-like" nonrandom localization in those areas that later exhibit distinct morphological alterations.

Mutational activation of $gp185^{neu}$ is associated with increased tyrosine kinase activity (32, 33) and with the induction of the *fos–jun* transcription factor complex, the glucose transporter, and ornithine decarboxylase activity (34). These observations and the results of other studies (ref. 18 and references therein) suggest a role of $gp185^{neu}$ in signal transduction. Mutational activation might either "lock" the putative neu receptor into a mode mimicking the binding of a proliferation stimulatory ligand or prevent the binding of an inhibitory ligand (35). In either case the abrogation of control factor dependence could lead to excessive proliferation of mutant *neu* cells [i.e., to a high-risk condition for undergoing further alterations such as malignant conversion (22, 36)].

To characterize the proliferative behavior of the mutant *neu* cell subpopulation(s) and to obtain an estimate of the number of mutant *neu* cells initially formed, the ratio of mutant to wild-type *neu* alleles was recorded in bulk DNA isolated from the trigeminal box at various times after the carcinogen pulse. A logarithmic plot against time after EtNU exposure (Fig. 5) shows an approximately linear increase of this ratio for about 30 days. This increase reflects a proliferative advantage of mutant *neu* cells (with a possible additional component due to G_0 phase mutant cells recruited into cell cycle). Around day 30, when the trigeminal nerve reaches its mature state (37, 38), the doubling time of the ratio slows down ≈4-fold from its initial value (≈3.5 days) but continues to increase with a quasilinear slope until day 140. At least part of the mutant *neu* cell subpopulation, therefore, assumes a reduced proliferation rate or ceases to proliferate during this period of the process. This might be due to a partially retained responsiveness to growth factors (39, 40) no longer present in the mature trigeminal nerve, to a partially lost sensitivity to proliferation inhibiting factors (41, 42), or to both.

Interestingly, the ratio of mutant to wild-type *neu* alleles reaches a value of 10^{-1} around day 50 and even surpasses 10^{0} around day 110. This indicates the acquisition of homozygosity for the mutant *neu* gene by initially heterozygous cells of the mutant *neu* subpopulation during a postinitiation stage of the process. The time of occurrence and the frequency of this loss of heterozygosity remain to be determined. However, the homozygosity for the mutant *neu* gene found in all cell lines derived from schwannomas (see above) and similar observations on cells from EtNU-induced primary trigeminal tumors (A.Y.N. and M.F.R., unpublished results) suggests that this loss of heterozygosity may represent a critical "second event" preceding the emergence of fully malignant phenotypes (43).

By assuming the proliferation rate of heterozygous mutant *neu* cells to be constant prior to day 14 and by discounting amplification of the mutant *neu* allele, the curve shown in Fig. 5 may be extrapolated to day 2 after the carcinogen pulse [i.e., to the time when mitotic cells reappeared in the trigeminal box after the temporary inhibition of cell division caused by exposure to EtNU (this study and refs. 44 and 45)]. This extrapolation indicates the presence of up to ≈100 mutant cells among the 1×10^6 cells of the trigeminal box 2 days after *in vivo* exposure to EtNU at 80 μg/g (body weight), in agreement with the plurifocal alterations of tissue architecture observed by histological analysis.

Conclusions. The present data show that a well-characterized DNA-reactive compound, EtNU (2, 4, 5, 22, 44), belonging to a major class of endogenously formed and environmental carcinogens (1), induces a specific mutation in a gene (*neu*) likely to be involved in the control of proliferation and differentiation (46). This mutation is found selectively and without exception in malignant cells deriving from EtNU-exposed cells of a particular lineage (Schwann cells) of the developing nervous system. It may thus represent a cell lineage- and carcinogen-specific initiating event in the process of carcinogenesis. The first mutant *neu* cells are detected within a few days after carcinogen exposure in a small model compartment (trigeminal box) that contains 1×10^6 cells on postnatal day 1 and can be reproducibly isolated for analysis

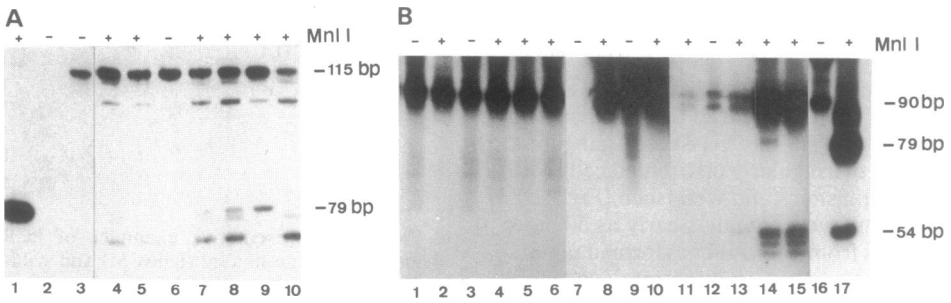

FIG. 4. (*A*) Conventional PCR–restriction fragment length analysis with a ^{32}P-labeled primer (410; see Fig. 2) of DNA isolated from various regions of the trigeminal box 7 days after EtNU exposure before (−) and after (+) *Mnl* I digestion. Lanes: 1, line TV1C (homozygous for the mutant *neu* allele; see Fig. 3); 2, mock control (PCR without DNA); 3–5, brain; 6–9, region adjacent to brain–PNS junction; 10, Gasserian ganglion region. The 79-bp fragment is specific for the mutant *neu* allele. (*B*) Detection by asymmetric PCR–restriction fragment length analysis (mutant-specific *Mnl* I) of the 54-bp diagnostic fragment among the 90-bp fragments of DNA isolated from the trigeminal box region adjacent to the brain–PNS junction 5 days (lanes 1–6) and 7 days (lanes 8–15) after EtNU exposure. Lanes: 1, 2, and 8–10, untreated control rats; 3–6 and 11–15, EtNU-exposed rats; 7, mock control; 16 and 17, line B104 (homozygous for the mutant *neu* allele). The 79-bp band results from digestion of the 115-bp PCR product (flanking primers 402 and 410; Fig. 2). Lanes 11–13 are one cycle of extension after annealing of the inner primer. In all other cases, extension was repeated five times.

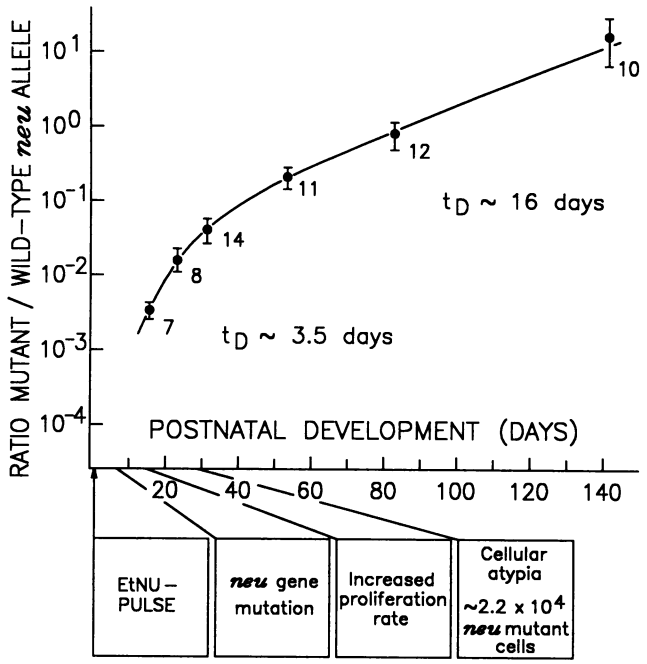

FIG. 5. Mutant to wild-type *neu* allele ratio, as determined by PCR–ASO hybridization in the cells of the trigeminal box as a function of time after exposure to EtNU *in vivo* on postnatal day 1. The ratio was calculated from densitometric measurements on autoradiograms of mutant *neu*- and wild-type-specific ^{32}P-labeled oligonucleotides hybridized to filters carrying the amplified DNA after correction for hybridization efficiency of oligomers and nonlinearity of the autoradiographic response. t_D, Doubling time. Error bars, SEM (with numbers of individual determinations shown).

in vitro. The *neu* gene mutation thus becomes diagnostic for a subset of cells with a proliferative advantage and, therefore, at high risk of undergoing further changes associated with malignant transformation. The aberrant proliferative behavior of the mutant *neu* cell subpopulation can be monitored (and hopefully be exploited for the isolation of intact mutant *neu* cells for selective analysis) up to the stage of the emergence of malignant phenotypes. Loss of the normal *neu* allele in cells initially heterozygous for the mutant *neu* allele is a candidate event for a critical postinitiation step. It is not yet clear whether—after *in vivo* exposure to EtNU—the *initial frequency* of mutant *neu* cells in the Schwann lineage is the same as or different from the frequencies in cell lineages giving rise to malignant tumors in the central nervous system (which lack the *neu* gene mutation) or in nonneural cell lineages. These initial mutation frequencies need to be determined precisely to appreciate the lineage-specific involvement of the mutant *neu* gene in EtNU-induced neurooncogenesis. Of equal importance for this evaluation will be a detailed characterization of the functional role of the *neu* gene in PNS vs. central nervous system development and differentiation.

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