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Amplified and rearranged epidermal growth factor receptor genes in human glioblastomas reveal deletions of sequences encoding portions of the N- and/or C-terminal tails

(polymerase chain reaction/gene amplification/gene mutations/cDNA)

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ABSTRACT **This study describes genomic rearrangements near the 3′ end of the epidermal growth factor receptor (EGFR) gene in eight glioblastomas displaying coamplification and expression of both normal and rearranged EGFR. In four of these cases, it was possible by PCR to amplify tumor EGFR cDNA, which allowed sequence determination of the 3′ transcript alterations associated with the rearrangements. Such analysis revealed that the four cases have in common a deletion of 255 bases that encode a portion of the receptor's cytoplasmic domain. The remaining four cases revealed genomic rearrangements in the same region of the gene as those described above and revealed aberrant EGFR transcripts lacking the same 255 bases determined to be missing in the sequenced EGFR cDNAs as well as large regions of contiguous downstream sequences. Therefore, all of the eight cases described here express transcripts that do not encode large C-terminal, intracellular portions of the receptor. In three of the eight cases, the EGFR transcripts displaying a 3′ alteration also displayed a 5′ in-frame deletion of sequences encoding a portion of the extracellular domain, and for one of the corresponding patients it was possible to determine that the two transcript alterations were acquired as separate events. We have now detected the 5′ and/or 3′ alterations in 21 of 32 cases of glioblastoma with EGFR amplification; no genetic alterations have been detected in glioblastomas without EGFR amplification. In combination with previously published reports, these data suggest the *in vivo* evolution of EGFR toward an increasingly oncogenic potential through gene amplification with subsequent and successive gene alterations.**

The involvement of the epidermal growth factor receptor (EGFR) gene with human neoplasia is perhaps most readily apparent in the case of the highly malignant central nervous system neoplasm, glioblastoma, in which the gene is amplified in ≈50% of such tumors (1–4). Predictably, EGFR gene amplification in glioblastomas has been shown to be associated with increased EGFR mRNA (1–6) and EGFR (4–6). A straightforward interpretation of the basis for this genetic alteration, therefore, is to consider it as a mechanism for activating a growth stimulatory system by increasing membrane receptor concentration.

An additional aspect of the EGFR–glioblastoma relationship, involving alteration of the EGFR gene in tumors with EGFR amplification, was initially suggested in 1985 (1). However, detailed information of such alterations was not provided until 1988, when two groups reported localization of alterations in the same region of the 5′ end of the gene in several cases of primary tumors (6) and glioblastoma xenografts (7). Recently, we reported the mRNA consequence of alterations at the 5′ end of the EGFR gene in primary tumors, and the same deletion of 801 bases coding for a portion of the receptor's extracellular domain in several glioblastomas displaying 5′ EGFR intragene deletion–rearrangement in association with EGFR amplification was determined (8). In conjunction with investigations addressing the functional and biological consequences of this alteration (7, 9), there is reason to suspect that the corresponding mutant receptor is activated and deregulated from normal cellular control mechanisms.

We now report a second "class" of EGFR mutation in glioblastomas that involves the loss of a consensus coding region near the 3′ end of the gene; previous studies suggest that this sequence encodes a portion of the receptor involved in its internalization and/or degradation and/or recycling (10–12). As was the case in identification of the 5′ EGFR alterations, the EGFR alterations characterized in this report were identified in tumors displaying amplification of both normal and aberrant EGFR genes. Since no instances of EGFR gene alteration have been detected in glioblastomas without EGFR gene amplification, these data suggest that EGFR amplification acts as a prelude to and a mechanism for inducing alteration of the gene. Furthermore, since three of the eight cases examined here revealed EGFR transcripts with the previously characterized 5′ alteration in addition to a 3′ alteration, it appears as though amplified EGFR is subject to multiple mutagenic events that can be selected for because of their functional consequences on the associated receptor.

MATERIALS AND METHODS

Tumor Material. All tumors were classified as glioblastomas, malignancy grade IV, according to the criteria established by the World Health Organization (13) and others (14). Specimens were collected fresh from surgery and subdivided into macroscopically pure subsections. Each subsection was sampled histopathologically to assess tumor cell content and then frozen, along with a specimen of normal tissue from each patient (peripheral blood leukocytes), and stored at −135°C for between 1 month and 2 years.

DNA and RNA Isolation and Analysis. High molecular weight DNA was isolated from tumor specimens and peripheral blood leukocytes, digested with restriction enzymes, electrophoresed, blotted onto nylon membranes, and hybridized with radiolabeled probes as were Northern filters (Hybond N membrane) to which 20 μg of total tumor RNA had

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Abbreviations: EGFR, epidermal growth factor receptor; ss, single stranded.

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been blot-transferred after electrophoresis in a denaturing 1% agarose gel (8). The probes used to analyze both the Northern and Southern blots included the oligonucleotide, cDNA, and genomic probes shown in Fig. 1. The numerical sequence identities of the oligonucleotides (5′ to 3′), based on Ullrich *et al.* (15), are as follows: 46, 50–1; 66, 172–193; 15, 428–379; 29, 914–865; 59, 1250–1201; 67, 1356–1335; 58, 1490–1441; 54, 1850–1801; 55, 1979–1930; 17, 2100–2051; 64, 2500–2549; 120, 2801–2822; 63, 2899–2850; 135, 3001–3020; 75, 3100–3051; 47, 3299–3250; 136, 3832–3813; 34, 3699–3650; 45, 4050–4001; 82, 5501–5462. The genomic probes Int7, IntY, and IntZ were produced on a normal DNA template by PCR amplification with oligonucleotides 1054–1075 and 1095–1076, 3110–3131 and 3155–3134, and 3434–3455 and 3479–3458, respectively. The X1 probe (double stranded, 2801–3832) was produced by amplifying EGFR cDNA from A431 cells by PCR using the 120 and 136 oligonucleotides as primers. A 50-base oligonucleotide probe complementary to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (bases 101–150; EMBL accession no. X01677) was used as a control on Northern filters to assess RNA loading variation.

Production of cDNA, PCR Amplification, and Sequencing. Single-stranded (ss) cDNA was produced by using Moloney murine leukemia virus reverse transcriptase and random priming with hexanucleotides (16). The ss cDNA was then subjected to a PCR using primer 120 or 135 together with 136 (see Fig. 1). The PCRs were standardized to 30 cycles: 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min plus 10 sec per cycle; last cycle, 10 min. The cDNA was then electrophoresed and isolated from the 1% agarose gel by using GeneClean, and 1.5 ng of the double-stranded cDNA was then asymmetrically amplified in an unbalanced PCR (same cycle parameters as described above) using 50 pM primer 120 and 1.5 pM primer 136 to produce a sense ss cDNA template, which was isolated from a 1% agarose gel by freeze–thawing (17). Approximately 100 ng of this ss cDNA was then primed with oligonucleotide 136 (0.6 pM) and sequenced by the chain-termination procedure with a Sequenase kit version 2.0 (United States Biochemical) according to the manufacturer's recommendations.

RESULTS

Seventy glioblastomas were assayed for EGFR gene amplification by Southern blot analysis using the genomic, cDNA, and "oligoprobes" indicated in Fig. 1 as well as with other probes previously described (4, 8). Thirty-two of these tumors revealed EGFR gene dosage values >10 times (>20 EGFR gene copies per cell average) that of normal cellular DNA (N.S., A.J.E., and V.P.C., unpublished data). Hybridization of the mRNA complementary oligoprobes (Fig. 1) to patient normal–tumor DNA pairs revealed two regions of the gene for which amplified, tumor-specific restriction fragments were apparent in several of the patients with EGFR gene amplification. Sixteen of the 32 patients with EGFR amplification displayed aberrant *Hin*dIII and/or *Sac* I restriction fragments from hybridization of probes 59 and/or Int7; probe–enzyme combinations previously determined to be diagnostic for the 801-base-pair (bp) coding sequence deletion associated with 5′ alterations (ref. 8; see Fig. 2*a*). Eight of these 32 patients revealed aberrant *Hin*dIII fragments from hybridization of probes 34, 75, and X1 (see Fig. 2*a*); three of the tumor DNAs from these patients also displayed the 5′ alteration so that 21 of the 32 patients revealed one or both of the alterations.

Northern analysis (Figs. 2*b* and 3) of tumor mRNAs revealed that in all but two of the tumors with 3′ EGFR DNA

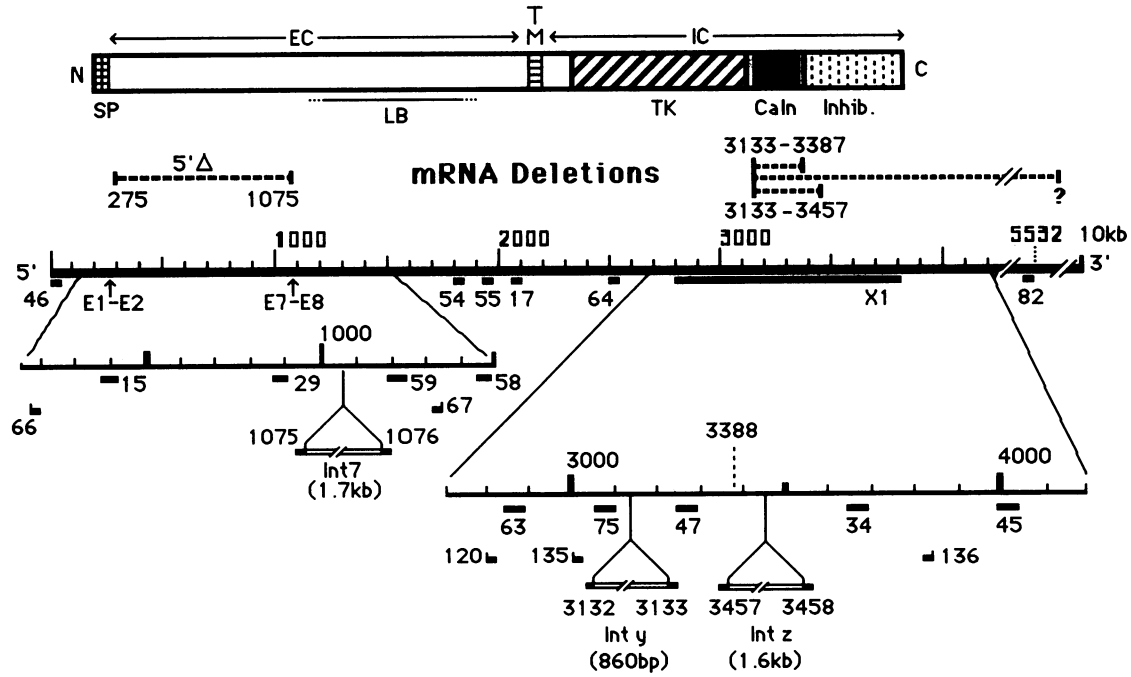

FIG. 1. EGFR cDNA (3630-bp coding sequence) described with respect to the regions coding for structural and functional domains of the corresponding protein (15, 18). The first 1500 bases for the extracellular domain and bases 2800–4200 for the intracellular domain and part of the 3′ untranslated portion of the message (beginning at base 3817) have been enlarged. N, N terminus; C, C terminus; EC, extracellular domain; TM, transmembrane domain; IC, intracellular/cytoplasmic domain; SP, signal peptide; LB, ligand binding region; TK, tyrosine kinase domain; CaIn, Ca^{2+} regulatory/internalization domain; Inhib., inhibitory domain. The sequences determined to be deleted from the aberrant transcripts are shown as dotted lines with the first and last deleted base indicated. The positions of the exon 1–exon 2 (E1–E2) border and the exon 7–exon 8 (E7–E8) border are indicated on the cDNA sequence. The following probes were used in the genomic and mRNA examination of EGFR: 46, 66, 15, 29, 59, 67, 58, 54, 55, 17, 64, 120, 63, 75, 135, 47, 136, 34, 45, and 82 (oligonucleotides listed in 5′ to 3′ order); Int7, IntY, and IntZ (intron probes); X1 (cDNA probe).

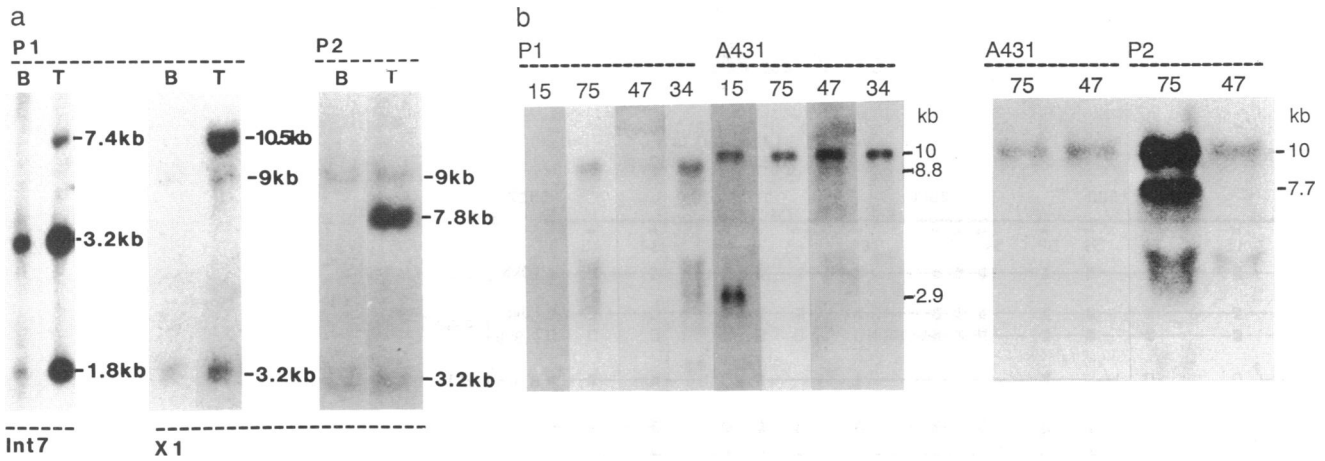

FIG. 2. (*a*) Autoradiograms revealing 5′ (extracellular) and 3′ (cytoplasmic) alterations in tumor DNA of patient P1 and 3′ alterations in tumor DNA of patient P2. *Sac* I-digested normal and tumor DNA was electrophoresed through 0.8% agarose, transferred to nylon membranes, and hybridized with the probes indicated below the autoradiogram. Rearrangement of the 5′ end of the gene is indicated by the presence of the tumor-specific aberrant Ab1 band of the Int7 autoradiogram (8), while rearrangements at the 3′ ends of the gene in both patients are indicated by the presence of the tumor-specific aberrant bands (Ab1) of the X1 (P1 and P2) autoradiograms. P1 tumor DNA revealed normal restriction fragment patterns when hybridized with probes from all regions of the gene other than those displayed here. B, constitutional (normal) DNA pattern; T, tumor DNA pattern. (*b*) Autoradiogram composites revealing absence of 5′ and/or 3′ coding sequences in aberrant EGFR transcripts of glioblastomas from P1 and P2. Tumor RNAs were electrophoresed, blot-transferred to nylon filters, and subjected to repeated rounds of hybridization with oligonucleotides 15, 75, 47, and 34. Aberrant tumor signal responses for each probe can be compared relative to that resulting from probe hybridization to the normal EGFR transcript (10 kb) from A431 cells. Autoradiograms demonstrate the lack of hybridization of oligoprobes 15 and 47 to the aberrant 8.8-kb transcript of the tumor of P1 and the lack of hybridization of oligoprobe 47 to the aberrant 10- and 7.7-kb transcripts of the tumor of P2.

alterations, both normal and aberrant transcripts were clearly expressed and at ratios reflecting the relative levels of normal and aberrant gene amplification. Only aberrant EGFR transcripts could be clearly distinguished in RNA from tumors P1 and P3, and these tumors also displayed the highest ratio of aberrant over normal gene amplification. Repeated rounds of hybridization using 15 oligoprobes complementary to various regions of normal EGFR mRNA (Fig. 1) permitted a rough mapping of the deleted regions from the aberrant transcripts (Fig. 3). Transcripts with interstitial deletions of coding sequence for the extracellular domain were found in three cases: P1 (Fig. 2*b*), P2, and P7b, with each displaying loss of sequences complementary to oligoprobes 15 and 29. Aberrant transcripts from tumors P1–P4 revealed interstitial deletions of a region encoding the cytoplasmic part of the receptor (Fig. 2*b*). Tumors P5–P8 displayed transcripts with losses of large regions downstream of a cytoplasmic domain sequence recognized by oligoprobe 75 (the extent of the loss of sequences in these cases has only been approximated by the Northern filter analysis of transcript size); P2 also revealed overexpression of such transcripts in addition to transcripts with an interstitial 3′ deletion.

As demonstrated previously with aberrantly spliced EGFR transcripts resulting from 5′ genomic rearrangements, primers flanking deleted mRNA sequences can be used to amplify cDNA for direct sequencing (8); for the tumors at issue here, primers flanking both the 5′ (oligoprimers 66 and 67) and the 3′ (oligoprimers 120 and 136) interstitial deletions were used to amplify EGFR cDNA for direct sequencing by PCR. Amplification of cDNA from tumors P1, P2, and P7b with the 5′ primer pair revealed a normal-length and a shortened PCR product in each case (Fig. 4*a*). Sequencing of the aberrant PCR products revealed the in-frame deletion of bases 275–1075 (data not shown; see ref. 8). Amplification of cDNA from tumors P1–P4 with the 3′ primer pair revealed fragments reduced by ≈250 (P1) or ≈300 (P2–P4) bp relative to the normal 1032-bp band (Fig. 4*a*). Sequencing of these PCR products revealed the in-frame deletion of 255 coding bases (bases 3133–3387) from the P1 transcript. Sequencing of cDNAs from tumors P2, P3, and P4 revealed identical frameshift deletions of bases 3133–3457 and the introduction of a stop codon at bases 3464–3466.

For each of the four cases whose 3′ cDNA alterations could be sequenced, the combined DNA and RNA analysis suggested that the altered transcripts resulted from aberrant splicing secondary to gene rearrangement. In fact, the results suggested the same mechanism as that associated with the 5′ alteration, an intragene deletion–rearrangement resulting in a genomic elimination and reorganization of coding sequences (data not shown). Since the last coding base before each transcript deletion is 3132 and since base 3458 marked the point at which the normal transcript sequence resumed in three of the cases, we determined to see whether these positions marked the last and first bases, respectively, of exons that had been juxtaposed as a result of the gene rearrangements. To address this point, the PCR exon–exon linkage strategy (8, 19) was applied to amplify the proposed introns between coding bases 3132 and 3133 and between bases 3457 and 3458 (primer pairs 3113–3132/3152–3133 and 3438–3457/3477–3458) from normal DNA templates. This approach revealed an 860-bp intron (IntY) between bases 3132 and 3133 and a 1.6-kilobase (kb) intron (IntZ) between bases 3457 and 3458. Comparison of the exon–intron boundaries and intron sizes with existing physical maps of the EGFR locus (18, 20) provided no clear indication of their precise location within the gene. Because of the results of P1, the possibility of an intervening sequence between bases 3387 and 3388 was also investigated. Sequencing of PCR-amplified normal human DNA has established, however, that there is no genomic separation of these bases and that a cryptic splice site 56 bases into an exon is used in forming the P1 aberrant transcript (data not shown).

Attempts to amplify aberrant cDNA from patients P5, P7, and P8 (there was no remaining RNA from patient P6 for PCR amplification) by using downstream primers complementary to the sections of the published sequence (15) for the untranslated portion of the EGFR message (beginning at base 3817) were unsuccessful, even though the use of the antisense primers on Northern filters revealed their presence in normal, amplified 10-kb EGFR transcripts (data not shown). The

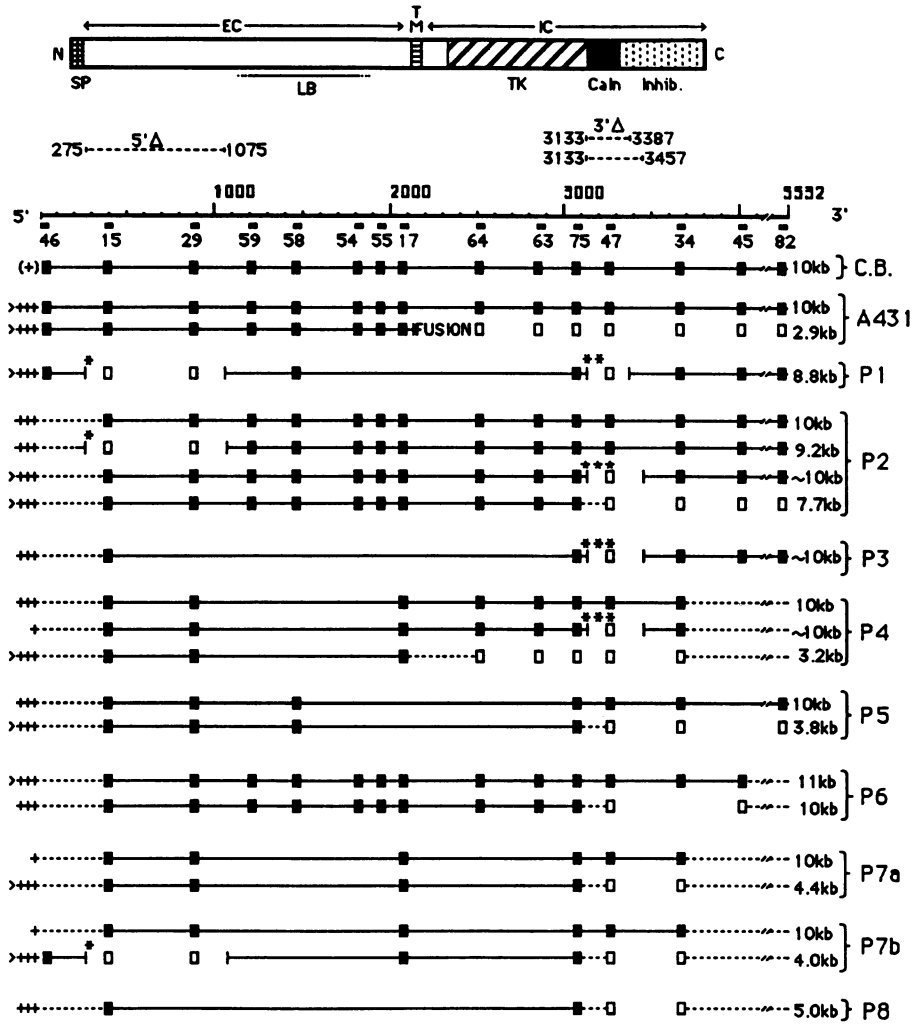

FIG. 3. Summary of the EGFR transcript data showing size (kb) and relative amounts and types of EGFR transcripts in the tumors studied. Expression scale: +, control brain; >+++, highly overexpressed (4). Open boxes denote lack of sequences in transcripts; solid boxes denote sequences that are present, as inferred by oligonucleotide hybridization patterns to aberrant transcripts (see Fig. 2). Asterisks denote cases involving 5' 801-bp (*), 3' 255-bp (**), and 3' 325-bp (***) interstitial deletions of EGFR coding sequences. Dashed lines denote tumors in which the exact point of transcript reinitiation on the 3' side of the deleted sequences have yet to be determined. Transcript sizes are noted.

failure of these primers to amplify this region of the aberrant cDNAs from cases P5, P7, and P8 is consistent with the estimated decreases in size for the aberrant transcripts in these cases (Fig. 3) and strongly suggests that the sequences for these transcripts resumes at some point further downstream than the 3' terminus of the 5.6-kb transcript. It is important to note, however, that the aberrant transcripts from these tumors, as well as P6, displayed the same type of oligoprobe hybridization pattern observed near the 5' boundary of the interstitial coding sequence deletions determined for aberrant EGFR transcripts in tumors P1–P4 (Fig. 3): the presence of sequences recognized by probe 75 (bases 3051–3100) and the absence of sequences recognized by probe 47 (bases 3250–3299). It is therefore quite likely that the consensus region of deletion (bases 3133–3387), determined for the transcripts with interstitial coding sequence deletions from tumors P1–P4, has also been eliminated from the aberrant transcripts noted in tumors P5–P8.

DISCUSSION

Observation of the frequent amplification of the EGFR gene in glioblastoma was initially reported in 1985 by Libermann *et al.* (1), and this association has been confirmed on several subsequent occasions (2–4). As the amplified gene in such tumors has been studied in greater detail (6–9), however, it has become increasingly apparent that alterations of the gene secondary to amplification may be important toward the oncogenic effect of EGFR (21, 22).

We have now analyzed in total 70 glioblastomas for EGFR gene amplification and alteration. The frequency of amplification in this series, 46% (32 of 70), is consistent with that reported by other groups (1–3). Some of the novel information in this report stems from the estimated frequency of its alteration in these tumors: 21 of the 32 tumors (66%) with gene amplification display the 5' and/or 3' alterations. Of these two alterations, the 5' is the more common, having been detected in 50% of the tumors with amplification; the 3' change was detected in 25% of these tumors. Use of the probes depicted in Fig. 1 indicates that other coding portions of the EGFR gene are infrequent targets of mutations. The two cases in which other "types" of EGFR genomic alterations have been detected in our series have yet to be characterized with respect to their associated transcripts.

Due to its well-documented involvement with cell proliferation, the biological and functional aspects of EGFR have been extensively studied. An approach frequently used in this regard has involved the functional analysis of EGFR mutants, including *erbB*, the retroviral homologue of this oncogene. Sequence alterations of normal EGFR have been shown to change the conditions necessary for EGFR cell transformation and/or the inherent kinase activity of the receptor. For instance, ligand-independent transformation and receptor kinase activation have been demonstrated with EGFR constructs lacking sequences encoding portions of the protein's extracellular domain (23). For the cytoplasmic domain, ligand-sensitized transformation has been conferred by constructs lacking sequences that encode a portion of the receptor important for receptor internalization and degradation (between residues 991 and 1022) and thus down regulation of the receptor (10, 11); all of the mutants characterized here lack this region. As these alterations are found to be amplified

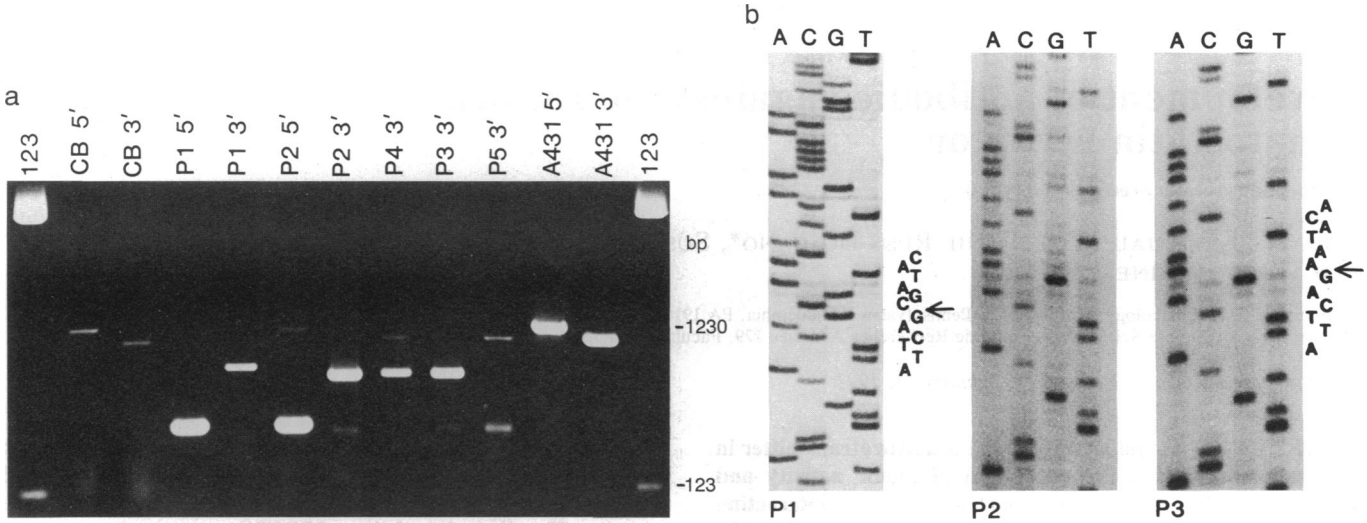

FIG. 4. (*a*) Ethidium bromide-stained gel of the products from PCR amplification of EGFR cDNA revealing deletion of coding sequences from aberrant glioblastoma transcripts. Tumor mRNAs were reverse transcribed into ss cDNA. ss cDNA was subjected to PCR by using primer pairs designed to detect 5′ (oligonucleotides 66 and 67) and 3′ (oligonucleotides 120 and 136) deletions of coding sequences from EGFR transcripts. The products of such reactions were electrophoresed through 0.8% agarose and the mobility of double-stranded cDNA was compared against the normal-length PCR products derived from control brain tissue (removed at operation for epilepsy and not required for diagnosis) and from A431 EGFR transcripts (123, 123 ladder; CB, control brain; P1–P5, patient nos.; A431, A431 cell line. All samples are suffixed by 3′ or 5′ depending on which region was being PCR amplified.) All methods are as described (8). (*b*) Autoradiograms of sequence gels (reading sense) from the aberrant transcripts of P1, P2, and P3. The 5′ breakpoint in the sequence (numbered according to ref. 15) occurs after base 3132 in each case (arrow), while the 3′ side resumes at base 3388 in the case of P1, and at base 3458 in the cases of P2 and P3. In P1, the transcript will remain in-frame and encode a protein with an interstitial deletion of 85 amino acids. P2 and P3 transcripts lose 325 bases and go out of frame.

in glioblastomas, it seems plausible to suspect that such changes are of biological/functional significance. In fact, two studies directed at addressing the consequences of the 5′ (801-bp deletion) alteration seem to support this hypothesis (7, 9). Further examination of EGFR alterations occurring in glioblastomas is therefore likely to provide important information concerning the manner in which EGFR affects cell proliferation. With regard to the transcript alterations described here, studies designed to address their associated protein functional consequences have been initiated.

A final point of significance associated with the results presented here is the inference that these tumor cell-specific genes are producing tumor cell-specific proteins. In the case of the 5′ alteration this appears to be the case, as it has recently been demonstrated that polyclonal antibodies can be raised against a mutant EGFR produced in glioblastoma xenografts lacking the same 267 amino acids predicted as missing in proteins produced by transcripts lacking bases 275–1075 of the EGFR gene (24). The development of monoclonal antibodies against this and other common EGFR variants may prove clinically useful in glioblastoma diagnosis and/or therapy.

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