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Identical splicing of aberrant epidermal growth factor receptor transcripts from amplified rearranged genes in human glioblastomas

(oncogenes/central nervous system tumors/gene deletion)

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ABSTRACT **The epidermal growth factor receptor gene has been found to be amplified and rearranged in human glioblastomas *in vivo*. Here we present the sequence across a splice junction of aberrant epidermal growth factor receptor transcripts derived from corresponding and uniquely rearranged genes that are coamplified and coexpressed with non-rearranged epidermal growth factor receptor genes in six primary human glioblastomas. Each of these six tumors contains aberrant transcripts derived from identical splicing of exon 1 to exon 8 as a consequence of a deletion–rearrangement of the amplified gene, the extent of which is variable among these tumors. In spite of this intertumoral variability, each intragenic rearrangement results in loss of the same 801 coding bases (exons 2–7) and creation of a new codon at the novel splice site in their corresponding transcripts. These rearrangements do not, however, affect the mRNA sequence for the signal peptide, the first five codons, or the reading frame downstream of the rearrangement.**

The normal epidermal growth factor receptor (EGFR) gene product is a 170-kDa transmembrane glycoprotein found on many normal and malignant cells (1–7). The extracellular binding of one of its two known endogenous ligands, epidermal growth factor and transforming growth factor α, results in conformational changes of the extracellular domain (8), the activation of the receptor's intracellular tyrosine kinase activity (9, 10), and the stimulation of DNA synthesis. A constitutively activated and cell-transforming variant of this receptor, with most of the extracellular domain deleted and further carboxyl-terminal deletions and mutations, is encoded by the v-*erbB* oncogene of avian erythroblastosis virus (11, 12).

In brain tumors, EGFR gene amplification is exclusively seen in the most malignant variants of adult gliomas, especially the glioblastomas (13). Studies of DNA, mRNA, and protein from primary human glioblastomas and xenografted glioblastomas indicate that EGFR gene rearrangements are frequently associated with EGFR amplification in such tumors (1, 14–21); most results suggest rearrangements causing the loss of coding sequences for the extracellular domain (17, 18, 20).

We have studied the DNA and RNA from primary tumor tissue from six patients with brain tumors histopathologically classified as glioblastomas (22, 23) where the tumors were determined to have amplification of the EGFR gene (13). Here we demonstrate that each of these tumors has highly expressed aberrant EGFR transcripts resulting from identical coerced splicing of uniquely rearranged and amplified EGFR genes.

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MATERIAL AND METHODS

Tumor Material, DNA and RNA Isolation, and Preparation of Southern and Northern Blots. All tumors were glioblastomas of malignancy grade IV according to World Health Organization criteria (22, 23). The 6 cases were selected from the 19 cases showing amplification of the EGFR gene in a study of 35 glioblastomas. They were chosen for this detailed study because they showed obvious abnormalities of the restriction pattern of the EGFR gene. The tumor tissue was frozen for between 1 week and 2 years at −135°C. A small fragment of each tumor piece studied was examined histopathologically. For Southern blot analysis, high molecular weight DNA was isolated, digested with restriction enzymes, electrophoresed, and blotted onto nylon membranes as described (13, 24). For Northern blot analysis, total RNA was isolated from the frozen tumor tissue by Polytron (Brinkmann) homogenization in guanidinium isothiocyanate buffer followed by ultracentrifugation in a CsCl gradient. RNA (20 μg per lane) was electrophoresed in a denaturing 1% agarose gel, blotted to Hybond-N membrane (Amersham), and hybridized to radiolabeled probes. The probes used to analyze both the Northern and Southern blots included a number of synthetic oligodeoxynucleotide probes and a cDNA probe (pE7), which are detailed in Fig. 1 and its legend. A glyceraldehyde-3-phosphate dehydrogenase 50-base (bases 101–150; EMBL accession no., X01677) oligonucleotide probe was used as a "housekeeping" gene control on the Northern blots.

Production of cDNA and Amplification by the Polymerase Chain Reaction (PCR). Single-stranded cDNA was produced using Moloney murine leukemia virus reverse transcriptase and random priming with hexanucleotides (26). The single-stranded cDNA was then subjected to a PCR using appropriate primers (Fig. 1). The PCR was standardized to 30 cycles, each consisting of denaturation (94°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 3 min plus 10 sec per cycle; last cycle, 10 min).

Exon Linking Strategy to Determine the Position and Size of Intron 7 in the EGFR Gene. The position and length of intron 7 were determined by using the PCR to amplify two specimens of normal DNA with the sense primer sequences in exon 7 and the antisense primer sequences in exon 8 (primers PC88 and PC89, with the assumption that exon 7 ended at base 1075). The various primers used in the PCR as well as those used to validate the results are shown in Fig. 1. After validation of the position of the intron by use of the primers PC88 and PC89, the PCR products obtained by using combinations of primers that included exon 7 or 8 sequences together with intron 7 were transferred to Southern blots to which appropriate oligonucleotides consisting of exon se-

Abbreviation: EGFR, epidermal growth factor receptor.

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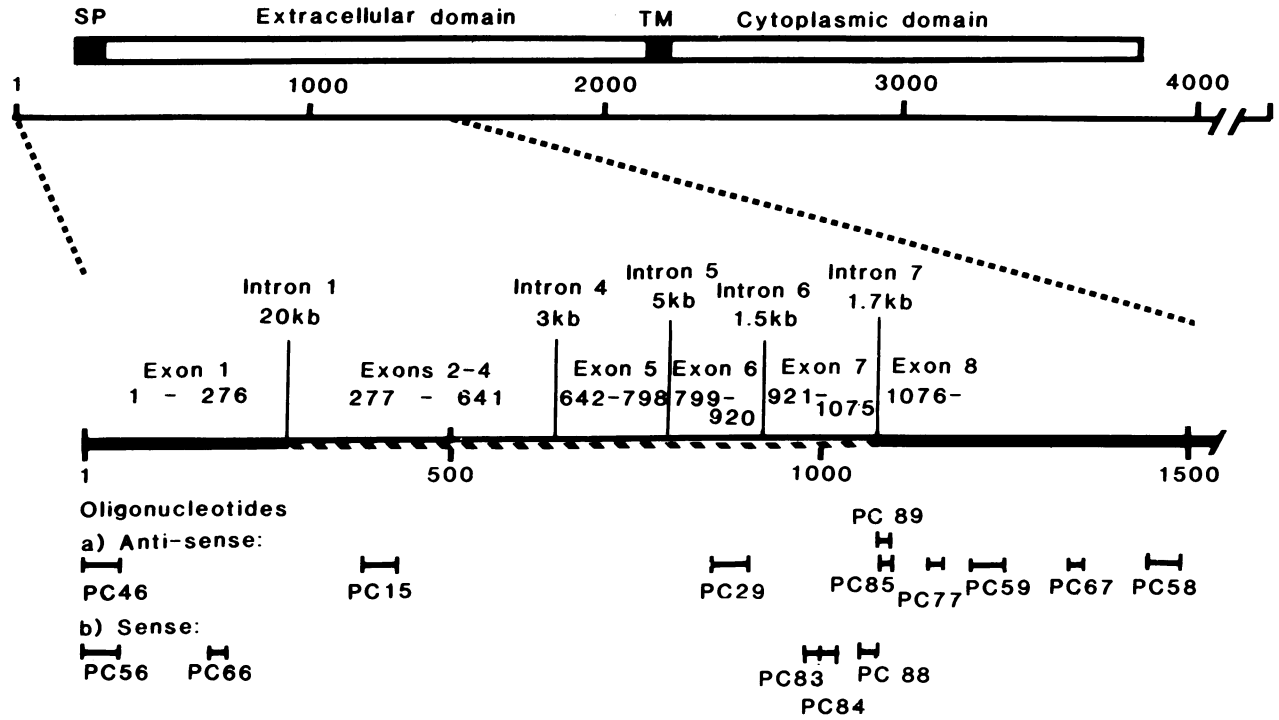

FIG. 1. (*Upper*) EGFR cDNA [3816-base-pair (bp) coding sequence] described with respect to the regions coding for structural/functional domains of the corresponding protein according to Ullrich *et al.* (25). SP, signal peptide; TM, transmembrane. (*Lower*) Enlargement of the first 1500 bases, showing approximate positions and sizes of the exons and of introns 1–7. Intron sizes are given in kilobases (kb). Striping indicates coding sequences eliminated from aberrant transcripts in the six glioblastomas examined. Locations of oligonucleotides used in the study are indicated. Numerical sequence identities of oligonucleotides (5′ to 3′) based upon the data of Ullrich *et al.* (25) are as follows: PC46, 50–1; PC56, 1–50; PC66, 172–193; PC15, 428–379; PC29, 914–865; PC83, 979–1000; PC84, 1000–1021; PC88, 1054–1075; PC89, 1097–1076; PC85, 1099–1078; PC77, 1167–1146; PC59, 1250–1201; PC67, 1356–1335; PC58, 1490–1441. In addition, a cDNA probe, pE7 [including approximately bases 650–3000 (14)], and probe Int7, produced by PCR of normal DNA using the primers PC85 and PC88, were used to screen *Sac* I-digested DNA from the tumors for aberrant restriction fragments. Oligonucleotides used to examine amplified genes 3′ of the deleted region (not shown; all antisense) included the following: PC54, 1850–1801; PC17, 2100–2051; PC64, 2549–2500; PC63, 2899–2850; PC47, 3299–3250; PC34, 3699–3650.

quences could be hybridized. The origin and identification of the ≈1.8-kb product could thus be confirmed. In addition, the PCR with primers PC85 and PC88 was used to produce an intron 7 probe (Int7) for hybridization to Southern blots of normal and tumor DNA (see Fig. 1 legend).

Production of Single-Stranded DNA by PCR and Sequencing. Double-stranded cDNA was produced and amplified as described above. The DNA was then electrophoresed and isolated from the 1% agarose gel by use of Gene-Clean (Bio 101). cDNA (1.5 ng) was then used in an unbalanced PCR reaction (30 cycles as described above) with primers PC66 (50 pM) and PC67 (1.5 pM) to produce a single-stranded sense cDNA template, which was isolated from a 1% agarose gel by freeze–thawing (27). Approximately 100 ng of this sense-strand cDNA was then primed with PC77 (0.6 pM) and sequenced by the dideoxy chain-termination method, using a Sequenase kit (United States Biochemical) according to the manufacturer's recommendations.

RESULTS

EGFR Gene. Hybridization of the cDNA probe pE7 (Fig. 1) to Southern blots of corresponding, restriction enzyme-digested normal (peripheral white blood cell) and tumor (glioblastoma) DNA pairs consistently revealed restriction fragments with decreased relative signal response in the glioblastoma DNAs. Since this cDNA probe produces a complex restriction fragment pattern as a result of its spanning 18 exons over ≈57 kb of genomic DNA, we used a series of 50-base oligonucleotide probes (Fig. 1) on the same Southern blots to localize the corresponding regions of the gene. It was possible to determine the regions that displayed a reduced level of amplification (sequences complementary to PC15 and PC29), such as the 10-kb restriction fragment (recognized by PC15) in the tumor DNA of patient P_1 (Fig. 2). According to Haley *et al.* (28), this region lies between intron 1 and intron 7. Oligonucleotide probe PC59, whose sequence corresponds to the 3′ end of exon 8 (Fig. 1), revealed an amplified, tumor DNA-specific, rearranged *Sac* I restriction fragment in some of the cases (Fig. 2, P_1 and P_2). An amplified normal 1.75-kb restriction fragment recognized by PC59 (*Sac* I recognition sites in introns 7 and 8) was present in addition to the aberrant restriction fragments, but its amount varied between tumors. The ratio of the amplified rearranged restriction fragment to the normal restriction fragment was unique to each tumor. Some patients, including patient P_3 (Fig. 2), showed no rearrangement of the 1.75-kb *Sac* I restriction fragment. However, for patient P_3, a rearrangement of the contiguous 5′ normal 3-kb restriction fragment was identified by the Int7 and PC29 probes (Fig. 2).

The series of oligonucleotides PC54, PC17, PC64, PC63, PC47, and PC34 (see Fig. 1 legend for details), which recognize exon sequences 3′ of exon 8, did not detect any abnormalities in these cases.

EGFR Transcripts. Northern blot analysis showed variable high expression of the normal 10-kb transcript in all cases. In addition, overexpressed aberrant transcripts were detected in at least five of the cases, two examples of which are shown in Fig. 3*a*. The aberrant transcripts hybridized to all antisense oligonucleotides (Fig. 1) except PC29 (Fig. 3*a*) and PC15 (data not shown). As will be shown below, the aberrant transcripts lacked an internal stretch of 801 bases. An oligonucleotide of 51 bases spanning the aberrant splice site recognized the aberrant transcripts only (data not shown).

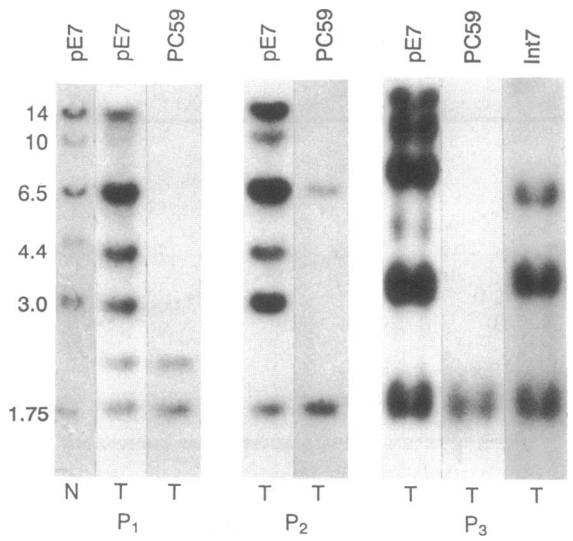

FIG. 2. Genomic analysis of EGFR rearrangements. *Sac* I-digested normal DNA (N; from peripheral blood leukocytes; shown only for patient P_1) and tumor DNA (T) from three patients, P_1–P_3, were hybridized with pE7, PC59, or the Int7 probe (see Fig. 1 and legend) as indicated above each lane. The same blot with tumor DNA was used for all hybridizations. Hybridization of pE7 to each of the tumor DNAs revealed a relative depletion of the normal 10-kb *Sac* I restriction fragment in all cases. The 1.75-kb restriction fragment identified by pE7 in normal DNA was also identified by PC59. For tumor DNAs from P_1 and P_2, hybridization with PC59 revealed additional, tumor-specific restriction fragments of 2.4 kb (P_1) and 6.5 kb (P_2). These aberrant bands were also detected by pE7, although the aberrant 6.5-kb restriction fragment of P_2 was somewhat obscured by a normal restriction fragment of similar size. These aberrant restriction fragments resulted from the deletion–rearrangement eliminating the *Sac* I site defining the 5′ end of the 1.75-kb fragment, located in intron 7. Patient P_3 showed no rearrangement of the 1.75-kb fragment. However, Int7, a PCR-synthesized probe containing the last 22 bases of exon 7, all of intron 7 and the first 24 bases of exon 8, revealed a 5-kb aberrant fragment in P_3 tumor DNA, indicating that the 3′ end of the rearrangement in this tumor occurred 5′ of the *Sac* I site in intron 7.

To determine the consequences of these rearrangements on the EGFR mRNA, a pair of oligonucleotide primers (PC66 and PC67) containing sense sequences 5′ (exon 1) and antisense sequences 3′ (exon 8) of the deleted region were used for PCR amplification of EGFR cDNA from each tumor. This procedure should normally result in the amplification of an 1185-bp cDNA fragment (bases 172–1356 of mRNA). However, in all cases an abnormal fragment of ≈380 bp was observed in addition to the normal 1185-bp fragment (Fig. 3*c*). The yield of the two bands relative to one another varied between the tumors. The normal 1185-bp fragment was always obtained, although it was only possible to show its presence in some of the cases by blotting the PCR product and hybridizing this Southern blot with oligonucleotide probes within the amplified sequence. As anticipated, probes PC15 and PC29 hybridized to the normal fragment but not to the shorter, aberrant fragment (Fig. 3*d*), whereas PC59 detected both fragments (Fig. 3*c*). We concluded that this 380-bp fragment included the flanking coding sequences on either side of the deletion and that all the tumors had lost approximately the same number of bases in their aberrant EGFR transcripts. Sequencing of this 380-bp fragment (Fig. 4) revealed that the last nucleotide of the first exon (base 274; refs. 28 and 29) had been spliced to base 1076 in each of the six tumors.

Determination of the Position and Size of Intron 7 in the EGFR Gene. Base 1076 lies 52 bases into exon 8 according to the published data (28). To clarify this further, the position

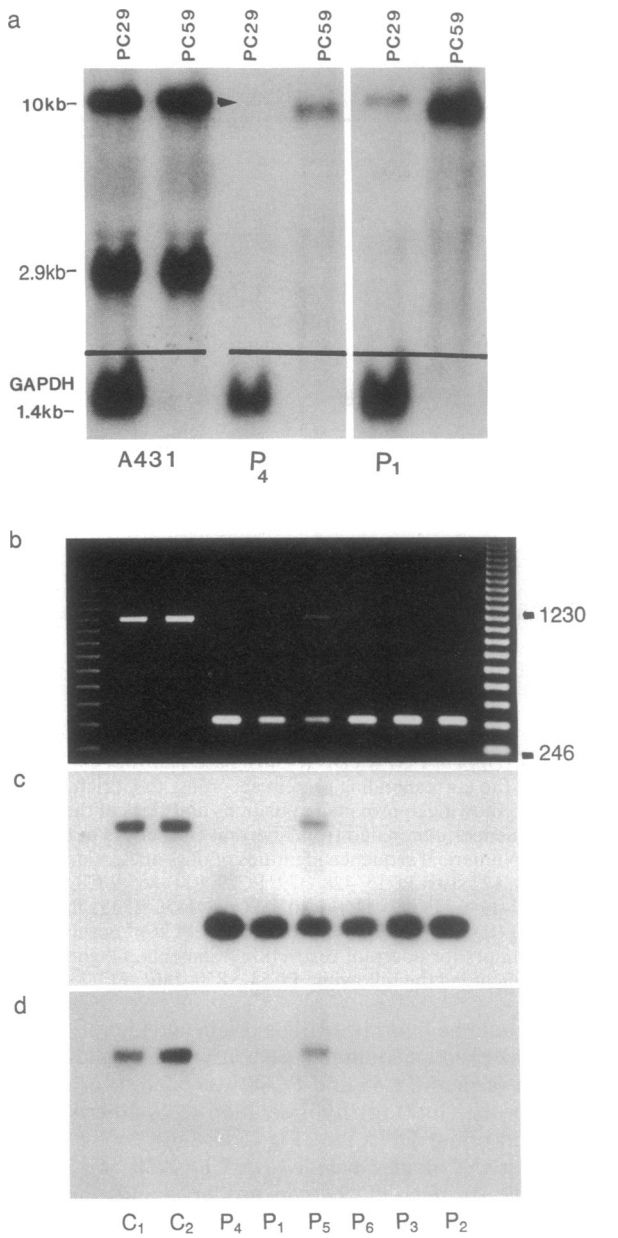

FIG. 3. (*a*) An autoradiogram composite indicating aberrant EGFR transcripts in glioblastomas from patients P_1 and P_4. The upper part was constructed by superimposing horizontally displaced autoradiograms (open lane between samples on gel) resulting from hybridization of PC29 and rehybridization of PC59 to the same filter. A third autoradiogram, resulting from rehybridization of a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) oligonucleotide probe (50 bp) to the same filter, has been vertically aligned with the PC29 autoradiogram to verify sample quantity. A431 human epidermoid carcinoma cells, which have an amplified EGFR gene and show overexpression of a 10-kb and a 2.9-kb transcript (11), were included as a control. P_4 tumor expressed a normal 10-kb transcript recognized by probe PC29 (visible only at long exposures) as well as an aberrant, overexpressed, shortened transcript detected with PC59. P_1 showed an overexpressed 10-kb transcript as well as the same phenomenon as P_4. (*b*) Ethidium bromide-stained gel of the products from PCR amplification of EGFR cDNA with primer pair PC66 and PC67 (Fig. 1). Outermost lanes, 123-bp "ladder" (size markers); lanes C_1 and C_2, products from two specimens of non-neoplastic brain, removed in the surgical treatment of epilepsy; lanes P_4, P_1, P_5, P_6, P_3, and P_2, products from the six tumors. (*c*) Southern blot of *b* probed with PC59. In autoradiograms exposed longer, the normal 1185-bp band was detected in all samples. (*d*) Southern blot of *b* probed with PC29. Hybridization with PC15 (data not shown) gave similar results (in autoradiograms exposed longer, the 1185-bp band was seen in all samples).

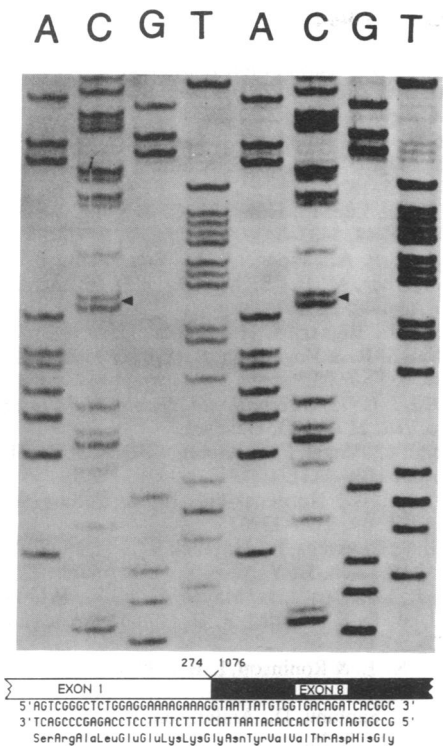

FIG. 4. (*Upper*) Autoradiogram of a sequence gel (reading antisense) from P_6 (*Left*) and P_1 (*Right*). (*Lower*) A comparison of this sequence with that reported (28, 29) for the first exon of EGFR shows that the last 70 nucleotides (only 26 shown) before the 5′ splice site in the sense sequence are identical to the last 70 nucleotides in exon 1. The aberrant splicing (arrowhead) occurs after the first base in EGFR codon 6 (last base in exon 1) and continues with the last two bases from EGFR codon 273 (first two bases of exon 8; see text); thus the rearranged mRNA remains in frame with the loss of 267 codons and the production of a glycine codon (GGT). The aberrant mRNA codes for the normal signal peptide and a shortened EGFR consisting of the first five N-terminal amino acids, a glycine (instead of valine) at position 6, and the amino acids read in frame from codon 274 onward.

and size of intron 7 were determined by using an exon–exon connection strategy (30). A contiguous primer pair, one (PC88) ending at sense base 1075 (hypothesized to be the last nucleotide in exon 7) and the second (PC89) ending at antisense base 1076 (hypothesized to be the first nucleotide in exon 8, with the assumption that normal splice sequences are used), was used in a PCR to amplify noncoding DNA that might interrupt these sequences. The PCR product obtained by using these primers on two different normal DNA templates (white blood cells) was ≈1780 bp (data not shown). This result is consistent with our hypothesis that intron 7 lies between bases 1075 and 1076 and that authentic splice sequences are utilized. To show that this 1780-bp PCR product represents an authentic part of the EGFR gene, 5′ sense and 3′ antisense primers lying outside the region defined by PC88 and PC89 were substituted for one or the other of the original primers to amplify a slightly larger genomic fragment containing known coding sequences (≈1850 bp). As expected, the probes internal to the primers used in each case hybridized to the amplified product after Southern blotting.

DISCUSSION

These data demonstrate that there is a frequent rearrangement of the EGFR gene when it is amplified in glioblastomas. At least 6 of the 19 (32%) glioblastomas with amplification of the EGFR gene in the tumor series we were studying have rearrangements affecting the area including exons 2–7 with a resultant identical transcript. Preliminary data from a screening study indicate that the frequency may be higher (N.S., A.J.E., and V.P.C., unpublished data). We have also seen rearrangements that affect other parts of the gene, but these occur less frequently. The findings also demonstrate the variability in the genomic rearrangements; the location of both the 3′ and the 5′ end of the intragenic deletion–rearrangement may differ in the individual cases. The variation in the sizes of the aberrant fragments detected by PC59 in tumors from patients P_1 and P_2 (the difference between the two was 4.1 kb) cannot be accounted for by differences in the position of the rearrangement site within the 1.75-kb normal fragment. It seems reasonable, from the present results, that the 5′ ends of the rearrangements occur within the large (20-kb) first intron.

On the basis of published findings, our results indicate that these aberrant transcripts contain the entire exon 1 (28, 29) followed by a sequence starting at base 52 of the 150-base-long exon 8 (28). However, our exon-linking data indicate that intron 7 lies between cDNA bases 1075 and 1076. We therefore conclude that the sequence observed in the aberrant mRNA transcripts results from the coerced splicing of exon 1 to exon 8. This splice results in the creation of a glycine codon (GGT) at codon 6 and the in-frame apposition of what would normally be codon 274 so that it becomes codon 7 of the aberrant transcript. That this transcript arises from an aberrant splicing explains why the sequence is the same despite the variable sequence losses in the amplified genes.

The aberrant EGFR gene/transcript has been found only in tumors also having a nonrearranged amplified gene and therefore presumably arose during the amplification process. In 66 gliomas we have studied, no gross genomic or transcript rearrangement has been observed in the absence of amplification (A.J.E., V.P.C., C.D.J., R. F. Pettersson, B. Seliger, and W. K. Cavenee, unpublished data). Tumors with an amplified, rearranged gene coexpress normal and aberrant transcripts. It remains to be seen whether similar rearrangements occur in the other tumor types that show amplification of the EGFR gene (3, 5).

The finding of identical abnormal splicing of exon 1 to exon 8 in the primary glioblastomas of six different patients suggests that the resulting loss of part of the EGFR polypeptide has biological significance. Our results indicate that the abnormal splicing results in the loss of most of the amino-terminal, cysteine-rich domain, without involvement of the major ligand-binding domain, which has been determined to lie between residues 294 and 543 (31). More recent studies have shown residues 321–367, and particularly 328–337, to be primarily involved in ligand binding (32). However, it is impossible to predict the impact of the loss of 267 residues, ≈50 amino acids away from the major ligand-binding region, on the function of the receptor or its ability to bind its ligands.

The presence of the signal peptide with five N-terminal amino acids and the in-frame coding sequence downstream of the rearrangement should ensure proper sorting and membrane insertion, respectively, of the putative aberrant protein. The aberrant transcript with its loss of 801 bases reported here would code for a protein of ≈140 kDa. It is interesting that this was also the molecular mass of an aberrant EGFR reported in two xenografted human glioblastomas (18). Cell membrane preparations from these two xenografts containing the aberrant 140-kDa EGFR protein showed a significant increase in tyrosine kinase activity in the absence of ligand (18), suggesting that the receptor was constitutively activated. Expression of an EGFR with an isolated extracellular deletion including the ligand-binding domain, and without any cytoplasmic-domain abnormalities,

has been shown to induce transformation of immortalized rodent fibroblasts (33). The effects of the expression of such a protein at relatively high levels (the aberrant genes are amplified) *together* with the overexpression of the normal protein (also amplified) in a cell with the genomic abnormalities of a glioma cell (13, 34) are impossible to anticipate and require further investigation.

Note Added in Proof. An identical aberrant EGFR transcript has been recently reported in xenografted human glioblastomas (35, 36).

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