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Evidence for rearrangement, amplification, and expression of c-*myc* in a human glioblastoma

(chromosomes/oncogenes)

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ABSTRACT **Amplified cellular genes are frequently manifested in one of two cytologically recognizable forms, double minutes or homogeneously staining regions. Additionally, evidence is accumulating that aberrant expression of cellular genes (including oncogenes) may be mediated by gene amplification. We now describe the amplification and expression of the cellular oncogene c-*myc* in double-minute-containing cells from a patient with glioblastoma multiforme, and we have shown that the amplification is associated with rearrangement of the c-*myc* gene. This finding further supports the common association of the *myc* gene family in neurogenic tumors and provides evidence of *myc* gene amplification in human brain cancer.**

Amplification of sequences related to cellular oncogenes has been described in several primary human tumors (1–3) as well as established tumor cell lines (1, 4, 5). Recent studies have demonstrated that amplification of the cellular homologue of the avian erythroblastosis virus oncogene (the receptor for epidermal growth factor) occurs in up to 40% of brain tumors of glial origin (3). The postulate that cellular oncogenes contribute to neoplastic growth (for review see ref. 6) and the knowledge that expression of these cellular genes can be dramatically altered by their amplification warrant further study of oncogene amplification in nervous system tumors.

One element common to analysis of amplified DNA domains is the consistent finding of cytologically recognizable chromosome change associated with the amplification event. The finding of double minutes (DMs) (7) or homogeneously staining regions (HSRs) (4, 22) is a significant indicator of DNA sequence amplification and is increasingly being recognized as a common feature of many malignant human tumors. In detailed analysis of amplification of cellular genes in drug-resistant cell populations, it has become clear through molecular analysis of DM-enriched fractions (7) or *in situ* hybridization to HSR-bearing populations (4, 8, 23) that these aberrations reflect the chromosomal sites of DNA sequence amplification. We have sought to take advantage of this finding by "pre-selecting" cell populations for molecular biological analysis on the basis of tumor karyotype. Tumors with cytologic evidence of amplification are then screened with the technique of DNA renaturation in agarose gels to positively detect amplified sequences. Finally, tumors that show signs of amplification in the above assay are analyzed for amplification of known oncogenes. In this report, we have used the above approach to show that a rearranged c-*myc* oncogene is amplified in DM-containing cells derived from a patient with glioblastoma multiforme.

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MATERIALS AND METHODS

Tumor Cells. Culture SF-188 originated on March 31, 1980, from a biopsy sample of a right frontal brain tumor in an 8-year-old boy with no family history of neoplastic or hereditary disease. The histological features of the tumor were characteristic of a glioblastoma multiforme with nuclear and cytoplasmic atypia and pleomorphism, increased cellularity, mitoses, necrosis, and endothelial proliferation. Subsequent to the biopsy, the patient was treated with radiation and chemotherapy, but he succumbed to the tumor 18 weeks after surgery. Cells were stored in liquid nitrogen until thawing for use in this study. The SF-188 culture was characterized by indirect immunofluorescence at passages 13 and 18; all cells had HLA antigens (demonstrated by using a monoclonal antibody that recognizes a common determinant on HLA antigens of the A,B,C segregant series; Cappel Laboratories, Cochranville, PA) and were recognized by the glioma-associated monoclonal antibody GE-2 (provided by N. de Tribolet, Lausanne, Switzerland); no cells contained glial fibrillary acidic protein (GFAP) as determined by using rabbit anti-GFAP antiserum (provided by L. Eng, Palo Alto, CA). Cultures of SF-188 at passage 24 were used for the present investigations.

DNA Renaturation Analysis. The DNA renaturation method described below is modified from Roninson (9). DNA (10 μg) was cleaved with the restriction endonuclease *Hin*dIII (10 units/μg of DNA; Bethesda Research Laboratories). Next, 0.2 μg of the cleaved DNA was end labeled (90 base pairs) with 50 μCi (1 Ci = 37 GBq) of [α-^{32}P]dCTP (ICN), using the T4 DNA polymerase labeling system (Bethesda Research Laboratories). The labeled DNA (0.2 μg) was then combined with the unlabeled DNA (9.8 μg) and precipitated with ethanol. The combined DNA was separated by electrophoresis through a 1% agarose gel. The DNA was denatured in the gel by alkali treatment (0.5 M NaOH/0.6 M NaCl; twice for 30 min). Subsequently, the DNA was renatured in a 1:1 (vol/vol) solution of 10× SSPE (1.8 M NaCl/100 mM $NaPO_4$/1 mM EDTA, pH 8.0) and formamide at 45°C for 2.5 hr after four changes of buffer (20 min each). Single-stranded DNA was then degraded with S1 nuclease (Sigma) at 80 units/ml in S1 buffer (50 mM sodium acetate/0.2 M NaCl/1 mM $ZnSO_4$, pH 5.0) for 2 hr at 37°C after five changes of buffer (15 min each). This whole cycle of denaturation, renaturation, and S1 treatment was repeated a second time. The gel was dried and amplified fragments were detected by autoradiography with intensifier screens at room temperature for 60 hr.

Molecular Analysis. DNA was cleaved with restriction endonuclease *Eco*RI or *Hin*dIII and was analyzed by agarose

Abbreviations: DM, double minute; HSR, homogeneously staining region; kb, kilobase(s).

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gel electrophoresis and Southern blotting as described previously (14, 23). Digested DNA from SF-188 cells was analyzed with probes representing 18 known oncogenes (c-Ha-*ras*, c-Ki-*ras*, N-*ras*, c-*myc*, N-*myc*, *mos*, *erb*-B, *erb*-A, *rel*, *yes*, *fes*, *fos*, *abl*, *src*, *raf*, *fms*, *myb*, and *sis*).

RNA was purified by using guanidium isothiocyanate followed by centrifugation through cesium chloride (10, 11). For blot hybridization, RNA was separated by electrophoresis through a formaldehyde agarose gel (12, 13) and transferred to nitrocellulose by the method of Southern (14). Probes used for the blotting experiments presented in Fig. 2 were a 5.5-kilobase (kb) *Bam*HI fragment of the mouse c-*myc* gene, provided by I. R. Kirsch (15), and the actin cDNA insert from plasmid pA1, provided by D. Cleveland (16).

Cytogenetic Analysis. Chromosomal banding was analyzed by utilizing Q- and G-banding techniques as previously described (17).

RESULTS

Cytologic Evidence of Gene Amplification in SF-188 Cells. Cytogenetic examination revealed a number of clonal chromosomal alterations as well as multiple copies of DMs (found in ≈70% of all cells examined) (Fig. 1). The number of copies of DMs per cell varied between 10 and $>10^2$, with significant heterogeneity in the size of DMs observed (Fig. 1*C*). In addition to multiple copies of DMs, this tumor also displayed a near-triploid modal number, with ≈20 unidentified "marker" chromosomes per cell. Among the identifiable clonal structural chromosome alterations were the deletion of the long arm of chromosome 6 [del(6)(q15)]; and isochromosome formation of the long arm of chromosome 1 [iso(1q)] (Fig. 1*A Inset*). Of interest, on the average, 3 copies of an apparently normal chromosome 8 were present in all cells analyzed, with no evidence for structural alteration of 8q24 (the single-copy chromosomal locus of the c-*myc* oncogene) observed. Finally, no HSR was found in any cell examined.

DNA Renaturation Evidence for Sequence Amplification in SF-188 Cells. The karyotypic evidence described above suggested that gene amplification had occurred in SF-188 cells. To obtain molecular evidence for gene amplification, we employed the technique recently developed by Roninson (9). In this technique, genomic DNA is cleaved with a restriction endonuclease, end-labeled and subjected to electrophoresis through an agarose gel. DNA in the gel is then subjected to alkaline (denaturing) conditions followed by renaturation conditions. Only sequences that are repeated more than about 20 times per haploid genome will renature under the conditions employed, and unrenatured sequences are subsequently degraded by S1 nuclease. Results of such an experiment are shown in Fig. 2. DNAs from normal human lymphocytes (lane 1) and normal brain (lane 2) display several bands, corresponding to repeated sequences normally present in the human genome. DNA from SF-188 cells, in addition to the normal bands, displays several new fragments (the most prominent of which are indicated by arrows adjacent to lane 3), consistent with the presence of an amplified domain of DNA.

Evidence of c-*myc* Amplification and Rearrangement in SF-188 Cells. Because many of the DNA amplifications that occur in cancer cells have been found to encompass oncogenes, we sought to determine whether the amplified domain in SF-188 included a known oncogene. *Eco*RI- or *Hin*dIII-digested DNA from SF-188 cells was screened by Southern blots with probes representing 18 known oncogenes (c-Ha-*ras*, c-Ki-*ras*, N-*ras*, c-*myc*, N-*myc*, *mos*, *erb*-B, *erb*-A, *rel*, *yes*, *fes*, *fos*, *abl*, *src*, *raf*, *fms*, *myb*, and *sis*). Of all the genes screened, only the c-*myc* gene was found to be amplified in SF-188 DNA.

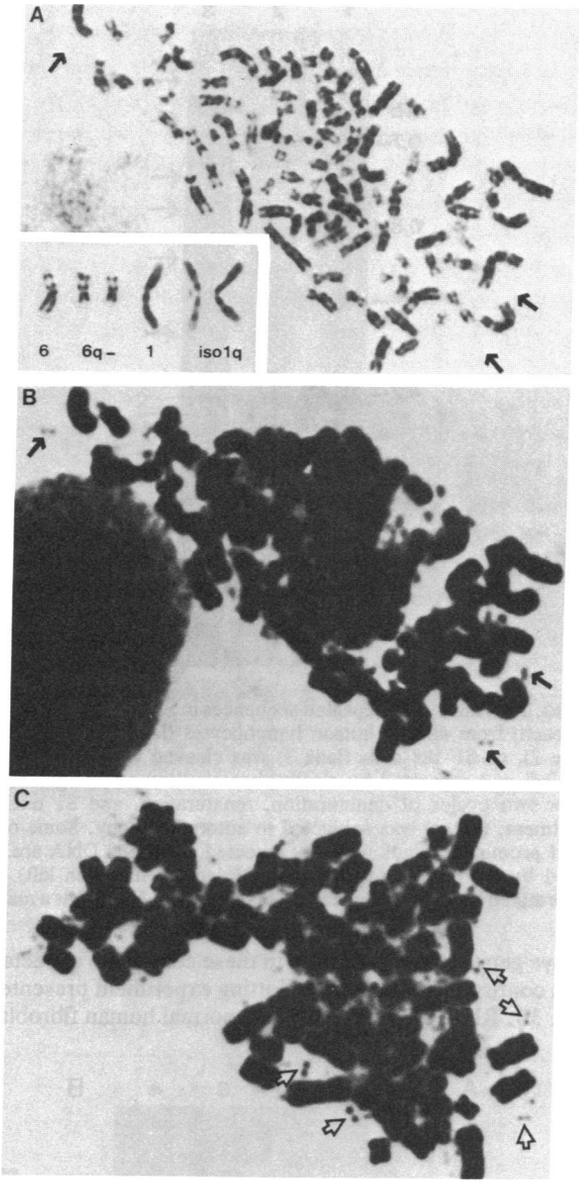

FIG. 1. Cytologic evidence for gene amplification in SF-188 cells. (*A*) G-banded metaphase demonstrating near-triploid chromosome number with multiple structural rearrangements. (*Inset*) Two of the clonal structural alterations, del(6)(q15) and iso(1q) (see text). (*B*) Same cell presented in *A*, photographically overexposed to demonstrate DMs (arrows). Note, arrows in *A* illustrate the difficulty in recognizing DMs in G-banded preparations. (*C*) Giemsa-stained cell with multiple copies of DMs (open arrows). Note their marked heterogeneity in size.

In addition to the amplification of the c-*myc* gene, a rearrangement was observed. Fig. 3*A* demonstrates two fragments, 10.0 and 7.1 kb, present in SF-188 DNA digested with *Hin*dIII, instead of a single normal 10-kb fragment. Digestion with *Eco*RI also revealed two fragments, 12.5 and 14.0 kb, instead of a single normal 12.5-kb fragment (data not shown). In both the *Eco*RI and *Hin*dIII digests of SF-188 DNA, each of the two fragments hybridizing to the c-*myc* probe was amplified approximately 25-fold per haploid genome. Although our results clearly indicate a genetic rearrangement has accompanied the amplification, it is not clear from this experiment whether the rearrangement preceded or followed the initial amplification event.

Evidence for c-*myc* Expression in SF-188 Cells. If c-*myc* were amplified in SF-188 cells, then one would expect the

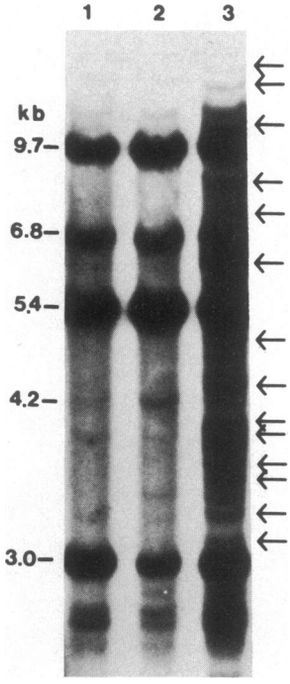

FIG. 2. Analysis of repeated sequences in SF-188 DNA. DNA (10 μg each) from normal human lymphocytes (lane 1), normal brain (lane 2), or SF-188 cells (lane 3) was cleaved with *Hin*dIII, end-labeled, and separated by electrophoresis through an agarose gel. After two cycles of denaturation, renaturation, and S1 nuclease treatment, the gel was subjected to autoradiography. Some of the most prominent bands uniquely detected in SF-188 DNA are indicated by the arrows on the right. Molecular sizes (on left) were determined by using λ phage DNA digested with *Hin*dIII as a marker.

c-*myc* gene to be transcribed in these cells. This expectation was confirmed by the RNA blotting experiment presented in Fig. 3*B*. RNA from SF-188 cells, normal human fibroblasts, or human placenta was separated by electrophoresis through a denaturing agarose gel and, after transfer to nitrocellulose, hybridized with the c-*myc* probe. A 2.3-kb RNA transcript was observed in the RNA from SF-188. This transcript is the same size as reported for the c-*myc* RNA in normal human cells (18). The content of c-*myc* RNA in SF-188 was 2.5-fold and at least 20-fold higher than that contained in RNA from fibroblasts and placenta, respectively. In contrast, placenta and fibroblasts contained an equal or greater concentration of a control RNA (actin) than SF-188 cells. Hence, SF-188 contained significant amounts of a normal size c-*myc* RNA. Since there is no normal cell available corresponding to the exact cell type from which the SF-188 glioblastoma tumor arose, we could not compare the c-*myc* RNA content of SF-188 to that of its appropriate progenitor cell.

DISCUSSION

Strategy for Combining Cytogenetic and Molecular Biologic Techniques. In this report we have combined three "levels" of cytogenetic and molecular biologic analysis in a coordinated effort to recognize DNA sequences likely to be of significant interest. First, cytogenetic analysis provides a "first-line screen' to identify tumors likely to harbor amplification events. However, the presence of putative HSRs or DMs, though suggestive of gene amplification, is not conclusive evidence (especially in tumors displaying significant karyotypic alterations). Accordingly, the ability to corroborate or refute the significance of cytogenetic observations at the molecular level is very important. Therefore, DNA renaturation analysis is a "second-line screen" that is capable of confirming the cytologic evidence of amplification, without requiring any knowledge of the nature of the amplified sequences. Further, it is possible to clone representative fragments directly from the DNA renaturation gels for further analysis. Finally, Southern and RNA blotting experiments using available probes for DNAs likely to participate in amplification events (e.g., cellular oncogenes) provide a

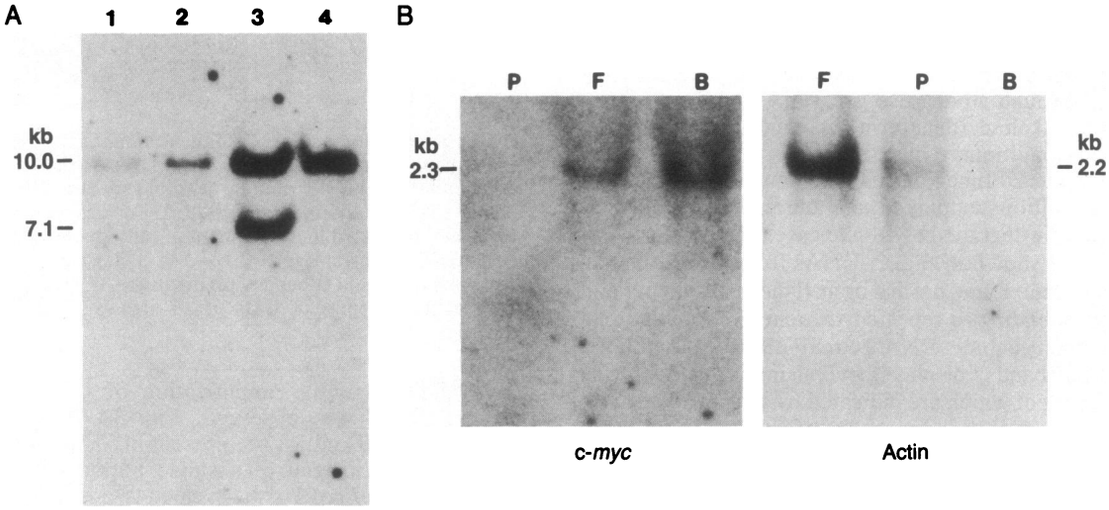

FIG. 3. Evidence for amplification, rearrangement, and expression of c-*myc* oncogene in SF-188. (*A*) Southern blot analysis of DNA from SF-188 cells digested with *Hin*dIII, using c-*myc* gene probe. DNA samples (5 μg) were from the following sources: lane 1, normal human lymphocytes; lane 2, Hut 23, a human lung cancer cell line with the c-*myc* gene amplified 5-fold; lane 3, SF-188; lane 4, NCI-417, a human small cell carcinoma cell line with the c-*myc* gene amplified ≈20-fold (from D. Carney, National Cancer Institute). Molecular sizes were determined by using λ phage DNA digested with *Hin*dIII as a marker. (*B*) Blot hybridization analysis of RNA from SF-188 cells. Total cellular RNA was purified and separated by electrophoresis through a denaturing agarose gel. After transfer to nitrocellulose, RNAs were hybridized with a ^{32}P-labeled c-*myc* probe (left three lanes) or an actin probe (right three lanes). Ten micrograms of RNA was used for hybridization with the c-*myc* probe and 0.5 μg of RNA was used for hybridization with the actin probe. RNA was derived from normal human fibroblasts (lanes F), placenta (lanes P), or SF-188 cells (lanes B). Fragment sizes were determined by using synthetic RNA markers made with the Riboprobe Gene Analysis System (Promega Biotec, Madison, WI).

logical "third-line screen" readily employed for initial characterization of recognized amplification events.

It is not expected that every amplified domain identified by this strategy will correspond to a known cellular oncogene. We anticipate that this combination of methods will facilitate recognition of new members of known cellular oncogene families, distant homologous sequences of retroviral oncogenes, as well as perhaps identifying novel genes that may have transforming or growth-promoting properties.

Nature of the Amplified Domain Containing c-*myc*. As described earlier, results of DNA gel renaturation studies provided evidence for several amplified restriction fragments in DNA from SF-188 not repeated in the normal human genome (Fig. 2). Using various conditions of gel electrophoresis, we could identify a total of more than 25 of these new amplified restriction fragments in SF-188 DNA. These restriction fragments were repeated 25–50 times per haploid genome, as estimated from the densities of these bands, compared with the densities obtained in reconstruction experiments using various amounts of phage λ DNA mixed with normal human DNA. Accordingly, the degree of amplification estimated by DNA gel renaturation techniques was consistent with the estimate obtained by using Southern blots of genomic DNA probed with c-*myc*. It is known that amplified domains containing drug resistance genes ordinarily include various amounts of flanking sequences amplified together with the "selected" gene (19, 20, 24, 25). As judged on the basis of knowledge of the gene structure of the c-*myc* gene (21), our results are consistent with significant co-amplification of c-*myc* flanking sequences occurring in this tumor.

The presence of both a nonrearranged and a rearranged amplified *myc* gene in SF-188 suggested that a gene duplication preceded the initial amplification event. The possibility that the amplified nonrearranged and rearranged c-*myc* gene are contained in two independent amplification units cannot be ruled out, but this seems unlikely since both copies were amplified to the same extent and there was no evidence for transcriptional activity of the rearranged *myc* gene (see below). Restriction mapping of a cloned rearranged 7.1-kb *Hin*dIII fragment containing *myc* sequences has indicated that the rearrangement occurred within 300 base pairs of the 3′ boundary of the second exon (K.K. and B.V., unpublished data). Preliminary studies have also shown that the sequences that recombined with the *myc* gene are normally located on chromosome 8, as is the *myc* gene. These results are thus consistent with an unequal sister chromatid exchange or an intrachromosomal rearrangement. The fact that an abnormally sized c-*myc* transcript was not seen on blot hybridization analysis indicated that the amplified rearranged *myc* gene may not be expressed.

Frequency of Amplification Events in Glioblastoma. It is important to recognize that although evidence for amplification of known cellular oncogenes is becoming increasingly recognized in a large number of human cancers, the frequency of amplification within a given tumor type may vary substantially. For example, gene amplification is extremely common in neuroblastoma, where the overwhelming majority of cell cultures (1), as well as many late stage tumor specimens (1, 2), can be shown to display amplification of the N-*myc* oncogene. In contrast, most other reports of oncogene amplification have been described in isolated cases of many different tumor types, with no other tumor type to date displaying the apparent specificity or frequency of DNA amplification in neuroblastoma.

Our current report provides additional support to the notion that gene amplification is of biologic and ultimately clinical importance in nervous system tumors, and particularly human brain tumors. Our finding of the amplification of c-*myc*, coupled with the recent findings of Liberman *et al.* (3) of c-*erb*-B amplification, leads us to suggest that selective pressures important during growth or differentiation of tumors of glial origin may facilitate DNA sequence amplification. Although the exact role of gene amplification in general, and c-*myc* amplification in particular, is currently indeterminate for human brain tumors, this area of investigation appears especially encouraging for future study.

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