Identification of an Amplified, Highly Expressed Gene in a Human Glioma

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A gene, termed *gli*, was identified that is amplified more than 50-fold in a malignant glioma. The gene is expressed at high levels in the original tumor and its derived cell line and is located at chromosome 12 position (q13 to q14.3). The *gli* gene is a member of a select group of cellular genes that are genetically altered in primary human tumors.

ALTHOUGH IT HAS BEEN THOUGHT for many decades that cancer results from heritable alterations of the genome, only recently have some of the genes altered in human cancers been precisely identified (*1*). One of the challenges of current cancer research is to identify additional genes that are affected in particular forms of human cancer. In this work, we have identified a novel gene, termed *gli*, that was amplified more than 50-fold in a malignant glioma.

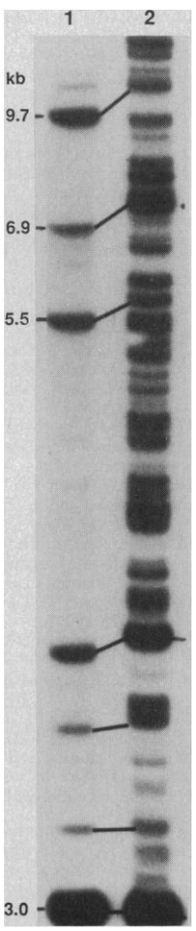

Fig. 1. Denaturation-renaturation analysis of amplified sequences. The autoradiograph shows normally repeated restriction fragments in DNA prepared from human placenta (lane 1) and amplified restriction fragments in DNA prepared from D-259 MG cells (lane 2). DNA was cleaved with Hind III, and 0.2 μg of the cleaved DNA was end-labeled (100 nucleotides at each end) with T4 DNA polymerase. The labeled DNA was then combined with 10 μg of the corresponding unlabeled DNA and precipitated with ethanol. The combined DNA was separated by electrophoresis through a 1% agarose gel. The DNA was then denatured in the gel by alkaline treatment (0.5*M* NaOH, 0.6*M* NaCl) and subsequently renatured at 45°C in a 1:1 solution of 10× SPPE (1.8*M* NaOH, 100 m*M* sodium phosphate, 10 m*M* EDTA) and formamide. Single-stranded DNA was degraded with S1 nuclease (80 unit/ml) in S1 buffer (50 m*M* sodium acetate, 0.2*M* NaCl, 1 m*M* $ZnSO_4$). The whole cycle of denaturation, renaturation, and S1 treatment was repeated (*4, 5*), and the gel was dried. Amplified fragments were visualized by autoradiography with DuPont Lightning-Plus intensifier screens and Kodak XAR-5 film at room temperature for 16 hours. The 9.7-kb and 5.5-kb bands are mitochondrial sequences; their intensities vary in different tissue because of different mitochondrial contents.

The tumor used in the current study was a malignant glioma removed prior to treatment from a karyotypically normal 46-year-old male. Chromosome analysis of the primary tumor and of the cell line established from it (D-259 MG) (*2*) revealed numerous double minute chromosomes (DMs), which suggested the presence of gene amplification (*3*). Molecular evidence for gene amplification was obtained with the denaturation-renaturation gel technique developed by Roninson (*4*). This technique allows the detection of any sequence repeated more than 20 times per haploid genome. DNA from normal cells was represented by a small number of bands corresponding to normally repeated sequences (for example, satellite and mitochondrial DNA sequences; Fig. 1, lane 1). DNA isolated from D-259 MG cells displayed numerous additional bands corresponding to amplified sequences (Fig. 1, lane 2). The sizes of these bands were summed to yield a minimum estimate of approximately 340 kb for the size of the amplification unit core. This size is within the range reported for amplification units in mammalian cells (*3, 5*).

Because of the cytogenetic and molecular evidence for gene amplification in the D-259 MG cell line, we sought to determine whether the amplification unit contained a previously described oncogene. Southern blot analysis with probes for c-*abl*, c-*erb*A, c-*erb*B1, c-*erb*B2 (*neu*), c-*fes*, c-*fms*, c-*fos*, c-*mos*, c-*myb*, c-*myc*, N-*myc*, c-*raf*, c-H-*ras*, c-K-*ras*, N-*ras*, c-*rel*, c-*ros*, c-*sis*, c-*src*, and c-*yes* revealed that the D-259 MG cell line displayed normal hybridization patterns and intensities for all sequences tested.

A plasmid library was then constructed with DNA enriched for amplified sequences by the gel denaturation-renaturation technique (*6*). Ten of 31 clones tested from the library were specifically amplified in D-259 MG DNA. Three of these plasmids (pKK324, pKK354, and pKK380) had unique inserts that were free of repeated sequences and could detect single-copy sequences in normal DNA by Southern blot analysis. These cloned fragments were then used to estimate the level of amplification in the D-259 MG cell line and to determine whether the gene amplification event had occurred in the original tumor. Plasmids pKK380 and pKK354 were used as probes in Southern blot analysis to demonstrate that the amplification unit was repeated approximately 75 times per haploid genome in the D-259 MG cell line. Southern blot analysis of DNA prepared from a biopsy of the original tumor indicated that the fragment in pKK380 was amplified in the original tumor to an extent similar to that in the D-259 MG cell line (Fig. 2A). Specifically, 100 ng of DNA from the original tumor (Fig. 2A, lane 2) resulted in a signal comparable to that from 5 μg of DNA (Fig. 2A, lane 1 or 3) isolated from normal lymphocytes; the slight difference in migration between the restriction fragment detected in normal cell DNA and in the tumor DNA is a result of the difference in the amount of DNA loaded onto the gel.

We next determined the normal chromosomal location of the amplified sequences. The three cloned fragments were used as probes for Southern blot analysis of a panel of mouse-human and hamster-human somatic hybrids (*7*). For all three fragments, the hybridization signal unique to human DNA segregated without discordancies in hybrids positive for isoenzymes assigned to chromosome 12; the other 23 chromosomes each showed at least one discordancy. We then used the fragment of pKK380 as a probe to analyze a series of human-hamster somatic hybrids that contained various portions of chromosome 12 (*8*). The hybridization pattern obtained indicated that the sequences in pKK380 are normally located on 12(cen–q14.3) (Fig. 2B).

Independent confirmation and refined assignment of this chromosome location was obtained by in situ hybridization to normal metaphase chromosomes. Forty-nine metaphase spreads were examined by in situ hybridization with the ^{3}H-labeled insert from pKK380 as probe. Twelve percent of

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the silver grains were localized to chromosome 12, and 35% of the cells had one or more grains on chromosome 12. Within chromosome 12, 60% of the grains (12 of 20) were clustered over the proximal long arm (band region q13 to q15; Fig. 2C). These results, showing that the amplified sequences in D-259 MG cells were normally located at chromosome 12 position (q13 to q14.3), prompted us to test whether the putative oncogene *int*-1 or the insulin-like growth factor–1 (IGF-1) genes were amplified in the D-259 MG cell line, since both genes are located on chromosome 12 (*9, 10*). Southern blot analysis with probes for *int*-1 and IGF-1 indicated that neither gene was amplified in D-259 MG. Furthermore, since L-*myc*, *ski*, B-*lym*, *met*, *bcl*-1, *bcl*-2, *tcl*-1, *tcl*-2, and p53 have been assigned to other chromosomes (*11*) they could not represent the amplified gene. Thus, the D-259 MG cell line contained amplified sequences that had not been previously implicated in human tumorigenesis.

We then cloned a large part of the amplification unit and determined which regions were expressed. A genomic library was constructed from D-259 MG DNA by means of a cosmid vector (*12*) and screened for cosmids containing amplified sequences that used DNA isolated from DMs as a hybridization probe (*13*). Forty-eight cosmids were isolated that were positive through two sequential screenings. In order to confirm the validity of this screening method, 13 of these cosmids were used as probes for Southern blot analysis; 12 of the 13 cosmids were found to contain inserts that were specifically amplified in D-259 MG cells.

Two lines of evidence indicated that the 48 cosmids selected contained a large proportion of the sequences present in the amplification unit of D-259 MG cells. First, the 48 cosmids contained approximately 1900 kb of amplified sequences (48 cosmids × 40 kb per cosmid), whereas the size of the amplification unit core was estimated to be 340 kb. The core represents those sequences present in the majority of amplification units (*5*). Hence, these cosmids should redundantly cover the core of the amplification unit. Second, restriction fragment patterns of the cosmids indicated that several related cosmids had been isolated two to five times. This was substantiated by hybridization of four plasmids containing amplified sequences to the 48 cosmid clones; at least two cosmid clones contained sequences found in each of these plasmids. These data suggested that the cosmid clones represented the amplification unit with a two- to fivefold redundancy.

These 48 cosmids were subsequently tested to determine which of them contained sequences transcribed in D-259 MG cells. Complementary DNA (cDNA) prepared from polyadenylated RNA isolated from D-259 MG cells was labeled by nick translation, and repeated sequences were removed from the probe by preannealing to human DNA (*13*). Four cosmids contained fragments that gave strong hybridization signals when the labeled cDNA was used as a hybridization probe (Fig. 3B, cosmid 36) indicating homology to polyadenylated RNA. Some other cosmids produced faint hybridization signals (Fig. 3B, cosmid 38);

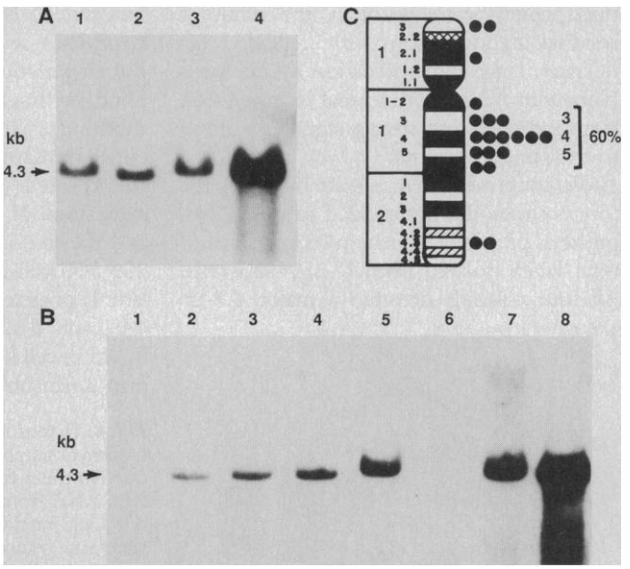

Fig. 2. Amplification and chromosome localization of sequences from clone pKK380. (**A**) Southern blot analysis of the D-259 MG tumor and the cell line derived from it. Lanes 1 and 3, 5 μg of DNA from normal lymphocytes of two different individuals; lane 2, 0.1 μg of DNA from a surgical biopsy of the original D-259 MG tumor; lane 4, 1.0 μg of DNA prepared from the D-259 MG cell line. (**B**) Southern blot analysis of DNA from hamster-human hybrids. Lane 1, 7.5 μg of DNA from K1 (a Chinese hamster ovary [CHO] cell line); lanes 2 to 6 contain DNA prepared from somatic cell hybrids between human cells and the K1 CHO cell lines (*8*); lane 2, 7.5 μg of DNA from cell line E4E (contains only human chromosome 12 in addition to hamster chromosomes); lane 3, 7.5 μg of DNA from cell line 16-43 [contains chromosome 12 with a deletion in the short arm and with a deletion of 12(q14.3 to qter)]; lane 4, 7.5 μg of DNA from 37A9 [contains chromosome 12 del (pter to p12.05)]; lane 5, 7.5 μg of DNA from F11-1 (contains long arm of chromosome 12 only); lane 6, 7.5 μg of DNA from F11-13 (contains short arm of chromosome 12 only); lane 7, 7.5 μg of DNA from normal lymphocytes; lane 8, 1 μg of DNA from the D-259 MG cell line. (**C**) Summary of in situ chromosome hybridization data. For (A) and (B), DNA was cleaved with Hind III, separated by electrophoresis through a 1% agarose gel, and transferred to a nitrocellulose membrane. Hybridization was performed as described (*20*) with the 4.3-kb insert from pKK380 as probe. For (C), the cloned fragment from pKK380 was labeled with [^{3}H]deoxynucleotide triphosphates by the oligo-labeling method. In situ chromosome hybridization and autoradiography was performed as described (*21*), except that hybridization was performed for 16.5 hours with a final DNA concentration of 0.6 μg/ml.

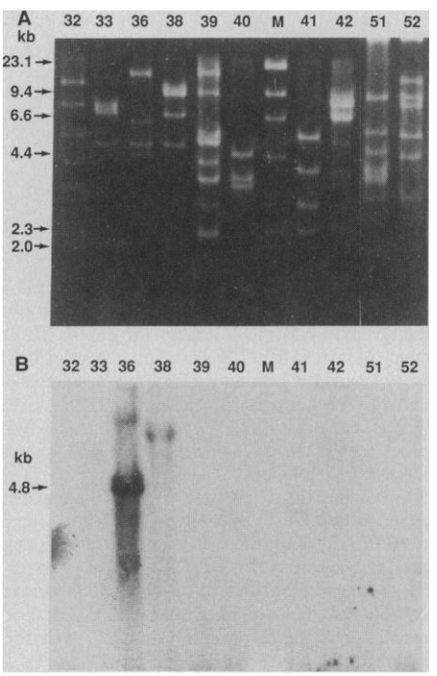

Fig. 3. Assay for expressed sequences in cosmids containing portions of the D-259 MG amplification unit. (**A**) Ethidium bromide-staining patterns of Hind III–cleaved DNA from ten cosmids (numbered above lanes) containing sequences amplified in D-259 MG cells. Lane M contains λ DNA (0.5 μg) cleaved with Hind III. (**B**) The gel shown in (A) was transferred to nitrocellulose and hybridized with a ^{32}P-labeled cDNA probe prepared from D-259 MG cell RNA. The cosmid clone cKK36 reveals a 4.8-kb Hind III fragment that hybridized to the cDNA probe. Approximately 1 μg of DNA from each cosmid was cleaved with the restriction endonuclease Hind III, and the fragments were separated by electrophoresis through a 1% agarose gel and subsequently transferred to a nitrocellulose membrane. Polyadenylated RNA was isolated from D-259 MG cells, and double-stranded cDNA was synthesized with an oligo(dt) primer and the ribonuclease H/pol I method (*22*). The cDNA was labeled with [^{32}P]deoxycytidine 5′-triphosphate by nick translation. Repeated sequences were removed as described (*13*). Hybridization, washing, and autoradiography were performed as described (*20*).

these signals were probably the result of residual hybridization with repeated sequences, since rehybridization of the same blots with ^{32}P-labeled normal human DNA as probe produced strong signals localized to the same fragments.

We confirmed these results by using the four cosmids that hybridized to the cDNA probe as probes in Northern blot analysis of total RNA isolated from D-259 MG cells. All four cosmids detected a major 4.8-kb messenger RNA (mRNA) and a minor 1.9-kb mRNA in D-259 MG cells. A 1.55-kb Pst I restriction fragment from the 4.8-kb Hind III fragment of cosmid cKK36 was subcloned. This subclone (pKK36P1) contained transcribed sequences and was devoid of repeated sequences. It detected the expression of the major 4.8-kb transcript and the minor 1.9-kb transcript in the D-259 MG cell line grown in vitro (Fig. 4A, lane 4) or as xenograft in a nude mouse (Fig. 4A, lane 5). These transcripts were not found in cell lines from two malignant gliomas, a neuroblastoma, a pancreatic carcinoma, and two small cell lung carcinomas (Fig. 4 and data not shown). Integrity of RNA in these preparations was demonstrated by hybridization of duplicate Northern blots with a probe for β-actin (Fig. 4B). The pKK36P1 subclone was also used to analyze the same panel of human-hamster somatic cell hybrids containing portions of chromosome 12 as was used in Fig. 2B. The hybridization signal from pKK36P1 segregated with the same hybrids as did the signal from pKK380 (Fig. 2B), indicating that pKK36P1 also originated from chromosome 12(cen–q14.3).

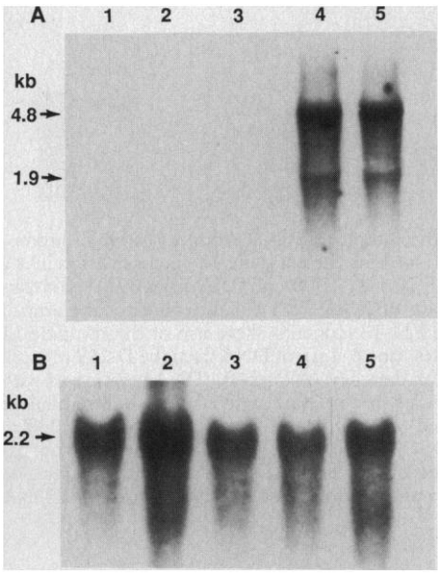

Fig. 4. Detection of RNA transcripts encoded by sequences amplified in D-259 MG cells. Duplicate Northern blots were prepared with 10 μg of total RNA from the following: lane 1, the small cell lung carcinoma line HUT82; lane 2, the pancreatic cancer cell line T_3M_4; lane 3, the malignant glioma cell line D319; lane 4, the D-259 MG cell line; lane 5, a nude mouse xenograft of D-259 MG. Hybridization was performed with the insert from the plasmid pKK36P1 (**A**) and the β-actin cDNA insert from plasmid pA1 (**B**) (*23*). The mRNA sizes were determined by comparison with ribosomal RNA markers. Total cellular RNA was isolated by the guanidium isothiocyanate method (*24*), separated on a denaturing 1% 2-(*N*-morpholino)ethane sulfonic acid and formaldehyde agarose gel, and transferred to nitrocellulose. Labeling of the probes, hybridization, washing, and autoradiography were performed as described (*20*). The pKK36P1 contains a 1.55-kb Pst I insert from the 4.8-kb Hind III fragment of the cosmid cKK36.

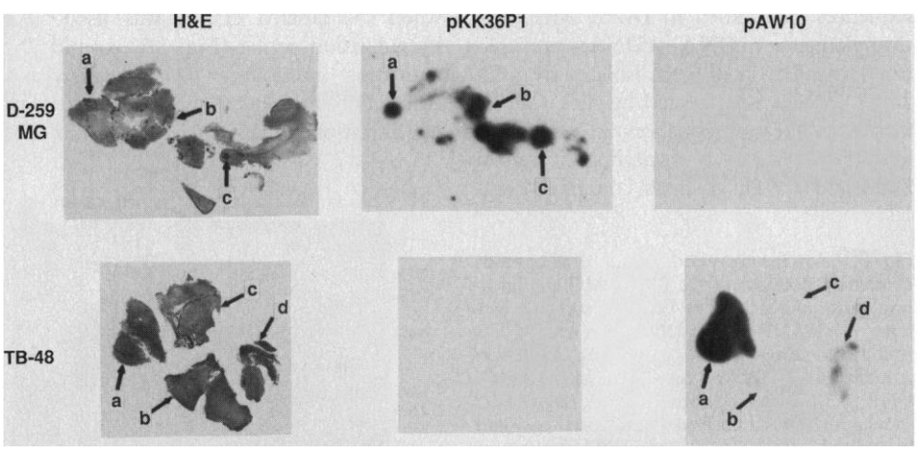

Fig. 5. Detection of RNA transcripts in the D-259 MG tumor by in situ hybridization to fixed tissue. Sections were prepared from the surgical biopsy of the malignant glioma from which D-259 MG was established or from the malignant glioma TB-48, a tumor that contained an amplified EGF-receptor gene (*14*). In D-259 MG sections, arrows a, b, and c point to clusters of tumor cells. In TB-48 sections, arrows a and d point to tumor tissue whereas arrows b and c point to normal brain. Sections were stained with hematoxylin and eosin (H&E) or hybridized with a probe for the *gli* gene (pKK36P1) or with a probe for the EGF-receptor gene (pAW10). Sections (6 μm) of paraffin blocks were baked onto slides coated with gelatin, deparaffinized, rehydrated, treated with proteinase K, and acetylated (*25*). RNA transcripts labeled with [^{32}P]cytidine 5′-triphosphate to be used as probes were prepared with SP6 polymerase. The plasmid pAW10 contained a 1.6-kb Hind III/Eco RI insert from pE7 [a cDNA clone of the EGF-receptor gene (*26*)] cloned in the vector pGEM3. Sections were hybridized overnight at 50°C in a solution containing probe, 10% dextran sulfate, 50% formamide, 2× SSC (standard saline citrate), salmon sperm DNA (1 mg/ml), carrier RNA (0.5 mg/ml), and bovine serum albumin (2 mg/ml). Slides were washed in 2× SSC, 50% formamide for 1 hour at 54°C, washed three times in 2× SSC at room temperature, incubated with 8 μg/ml of ribonuclease A in 2× SSC at 37°C for 1 hour, washed with 2× SSC, 50% formamide at 54°C twice for 1 hour each, dehydrated, and exposed to Kodak XAR-5 film for 16 hours at −70°C.

RNA from the original tumor was not sufficiently intact for Northern blot analysis. However, we demonstrated expression in the original tumor by using the pKK36P1 fragment as a probe for in situ hybridization to RNA in formaldehyde-fixed specimens of the tumor. A high level of expression of the gene was detected by pKK36P1 in the original tumor, but not in surrounding normal tissue or in another malignant glioma (TB-48; *14*) that had amplified the gene for epidermal growth factor (EGF) (Fig. 5). All clusters of D-259 MG tumor cells were strongly positive, even those that were composed of only 100 to 200 cells. This was consistent with the fact that nearly all of the metaphase cells examined from the original tumor contained DM chromosomes (*2*). No signal above background was detected when the sense strand of pKK36P1 was used as a control probe. In contrast, when a probe for the EGF receptor gene was used, TB-48 hybridized strongly, whereas no signal was detected for D-259 MG (Fig. 5, pAW10 probe).

We will refer to the gene detected by the pKK36P1 clone as *gli* (for glioma). The approach taken here demonstrates the feasibility of isolating genes that are genetically altered in human cancers, even when these genes are not active in standard transfection assays (*15*) and are not related to known oncogenes. Two arguments suggest the importance of the sequences amplified in D-259 MG in the development of this tumor. First, gene amplification is rapidly lost in the absence of selective pressure (*3*). The presence of high levels of gene amplification in the D-259 MG tumor both in vivo (human and mouse hosts) and in vitro suggest that these sequences must have provided a significant growth advantage. Second, the amplification of the *gli* gene was not unique to one malignant glioma; *gli* was amplified over tenfold in one other malignant glioma in addition to D-259 MG (*16*). It is possible that *gli* did not represent the target gene of the amplification unit but was a coamplified gene. However, the facts that only four cosmids containing expressed sequences

were identified, and that each of these cosmids detected transcripts of the same size in Northern blot experiments, suggest that only one highly expressed gene (that is, *gli*) was present within the amplification unit.

The assignment of the sequences amplified in D-259 MG cells to chromosome 12 (q13 to q14.3) is provocative. A fragile site of the folic acid type has been described at 12q13 (*17*); such sites have been hypothesized to colocalize with proto-oncogenes and play a role in tumor development in affected individuals (*18*). In addition, translocations in the 12(q13 to q14) region have been described in human myxoid liposarcomas and salivary gland tumors (*19*). There is a precedent for genes that are amplified in some tumors to be translocated in other tumors and thus to show similar increases in expression (*1*). This hypothesis can now be tested in liposarcomas and salivary gland tumors with probes for *gli*.

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6. Genomic DNA (200 μg) was cleaved with Hind III, and the fragments were separated by electrophoresis through a 1% agarose gel. While in the gel, the DNA was denatured and renatured as described for the analytical assay for amplified sequences (see Fig. 1). However, instead of treating the gel with S1 nuclease, a region of the gel previously shown to have a large number of amplified restriction fragments (2.6 to 5.3 kb) was excised, and the DNA was electroeluted from the gel [I. B. Roninson, H. T. Abelson, D. E. Housman, N. Howell, A. Varshavsky, *Nature (London)* **309**, 626 (1984)]. The eluted DNA was ligated into the Hind III site of the vector pKB358 [T. M. Roberts, S. L. Swanberg, A Poteete, G. Riedel, K. Backman, *Gene* **12**, 123 (1980)]. Only DNA that was double-stranded after the denaturation-renaturation step could recombine with pKB358 to form a viable plasmid, and the library prepared from this DNA was enriched for amplified sequences.
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12. DNA from D-259 MG was partially cleaved with Mbo I and size-fractionated by centrifugation through a sucrose gradient. Size-fractionated DNA (30 to 45 kb) was cloned in the cosmid vector pTL5 [M. Steinmetz, A. Winoto, K. Minard, L. Hood, *Cell* **28**, 489 (1982)].
13. DMs were isolated from D-259 MG cells by differential centrifugation of metaphase chromosomes as described by C. C. Lin *et al.* [*Chromosoma* **92**, 11 (1985)]. DNA prepared from these DMs contained the equivalent of more than a 1000-fold amplification (that is, 15-fold enrichment by differential centrifugation × 75-fold original amplification). Therefore, an amplified sequence should have had a signal 1000 times as strong as that of any single-copy sequence when labeled DM DNA was used as a hybridization probe. However, hybridization signals from repeated DNA sequences in the DM DNA probe would mask signals from amplified sequences because such repeated sequences occur over 10,000 times in the human genome. This problem with repeated sequences was eliminated by preannealing the hybridization probe to a large excess of human placental DNA as described by P. G. Sealey, P. A. Wittaker, and E. M. Southern [*Nucleic Acids Res.* **13**, 1905 (1985)].
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15. No transforming activity was detected in DNA from the D-259 MG cell line in NIH 3T3 transfection experiments performed in collaboration with S. Needleman.
16. DNA was purified from frozen or paraffin-embedded malignant gliomas [as described in A. J. Wong *et al.*, *Science* **233**, 461 (1986)], slot blotted onto nitrocellulose filters, and screened by hybridization with the pKK36P1 probe. Two glioblastoma multiforme tumors (D-259 MG and one other tumor) out of the 45 tested contained more than a tenfold amplification of the sequences detected by pKK36P1. This was confirmed by Southern blotting of Pst I–cleaved DNA, which showed that a 1.55-kb Pst I fragment homologous to pKK36P1 was amplified in both tumors.
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27. We thank C. Jones for providing DNA for hybrid lines F11-1 and F11-13, S. Needleman for performing the NIH/3T3 transformation assay, and P. White and S. Smith for preparing the manuscript. Supported by a National Cancer Institute training grant CA-09243 (K.W.K. and A.J.W.), and NIH grants CA-43722 (S.H.B.), NS-20023 (S.H.B. and D.D.B.), CA-11898 (D.D.B.), CA-29476 (J.M.T.), CA-41183 (J.M.T.), GM-33903 (M.L.L.), and CA-43460 (B.V.).

23 October 1986; accepted 15 January 1987

Superconductivity in Alkaline Earth–Substituted La_2CuO_{4-y}

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La_2CuO_{4-y} ceramics containing a few percent of Ca^{2+}, Sr^{2+}, and Ba^{2+} ions have been prepared. Resistivity and susceptibility measurements exhibit superconductive onsets (as in earlier Ba^{2+}-containing samples). The onset temperature La_2CuO_{4-y} with Sr^{2+} is higher and its superconductivity-induced diamagnetism larger than that found with Ba^{2+} and Ca^{2+}. This is proof that the electronic change resulting from alkaline earth–doping, rather than the size effect, is responsible for superconductivity. The ionic radius of Sr^{2+} is close to that of La^{3+} for which it presumably substitutes.

RECENTLY, BEDNORZ AND MÜLLER (*1*) reported the possible onset of superconductivity in the metallic BaLaCuO system with transition temperatures T_c in the 30 K range. Subsequent dc susceptibility measurements in our laboratory (*2*) supported this, and resistivity and low-field susceptibility data from Tanaka's group (*3*) and Chu's (*4*) group substantiated this finding. The early BaLaCuO samples consisted of up to three phases (*2*); by combining x-ray and diamagnetic susceptibility measurements (*5, 6*), it could be shown that one of them, the La_2CuO_{4-y}:Ba phase, becomes partly superconducting. Identification of flux trapping and the existence of a superconductive glass state in the ceramics was demonstrated by nonergodic magnetic responses (*7*).

Leading to the discovery of superconductivity in the La_2CuO_{4-y} were, first, the known existence of high electron-phonon coupling in oxides such as $Li_xTi_{2-x}O_4$ (*8*) spinel and $BaPb_{1-x}Bi_xO_3$ (*9*) perovskite, and, second, the expected high electron-phonon coupling occurring in polaronic systems as emphasized by Chakraverty (*10*). The existence of Jahn-Teller polarons in a linear chain model was proposed by Höck *et al.* (*11*): If the Jahn-Teller stabilization energy is of the same magnitude as the bandwidth in a metal, the effective mass of the itinerant electrons or holes will become large, that is, a large electron-phonon coupling exists. As a result, such a polaronic system can be favorable for the occurrence of superconductivity. The Cu^{2+} ions in the itinerant compound La_2CuO_{4-y}

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