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Expression and rearrangement of the *ROS1* gene in human glioblastoma cells

(tyrosine kinase/oncogene/cancer)

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ABSTRACT **The human *ROS1* gene, which possibly encodes a growth factor receptor, was found to be expressed in human tumor cell lines. In a survey of 45 different human cell lines, we found *ROS1* to be expressed in glioblastoma-derived cell lines at high levels and not to be expressed at all, or expressed at very low levels, in the remaining cell lines. The *ROS1* gene was present in normal copy numbers in all cell lines that expressed the gene. However, in one particular glioblastoma line, we detected a potentially activating mutation at the *ROS1* locus.**

We have reported the characterization of an activated *ROS1* gene which we previously called *mcf3* (1). This gene was identified using an assay for human protooncogenes based on the tumorigenicity of mouse NIH 3T3 cells cotransfected with human tumor DNA (2). *mcf3* encodes a protein-tyrosine kinase (ATP: protein-tyrosine *O*-phosphotransferase, EC 2.7.1.112) with a potential transmembrane domain. It arose from the human *ROS1* gene by a rearrangement introduced during gene transfer, which deleted the putative extracellular domain of *ROS1*, leaving potential transmembrane and intracellular protein-tyrosine kinase domains intact (1). The human *ROS1* is the closest homologue of the v-*ros* oncogene, which is the transforming gene of the avian UR2 retrovirus (3). Structurally *mcf3* and v-*ros* are similar to the v-*erbB*, v-*fms*, *neu*, and *trk* oncogenes (4–7). The cellular homologue of two genes in this group, v-*erbB* and v-*fms*, are known to encode the receptors for the epidermal growth factor and the macrophage colony-stimulating factor, respectively (8, 9). The cellular *ros1* gene may therefore encode a hormone or growth factor receptor as well.

As a first step toward characterizing the normal *ROS1* gene, we have screened for its expression in a variety of human cell lines established from normal and tumorigenic cells. *ROS1* is expressed in a high proportion of human glioblastoma cell lines and at very low levels, or not at all in the remainder of human cell lines. We did not find detectable levels of *ROS1* in either human brain or in a cell line established from a normal human glial cell. In most glioblastoma cell lines, the *ROS1*-encoded transcript was found to be 8.3 kilobases (kb) in size. Upon analysis of the *ROS1* locus by Southern blotting, an aberrant configuration due to a rearrangement in sequences encoding the putative extracellular domain, was seen in one particular glioblastoma cell line.

MATERIALS AND METHODS

***ROS1* Expression Studies.** Total RNA from various human tumor cell lines and tissues was prepared by the guanidinium/cesium chloride method (10). Human placenta was obtained from elective cesarean sections and superficially normal brain tissue (temporal lobe) from a patient undergoing surgery for epilepsy. The quantity of RNA was determined by measurements of the optical density, and the quality and quantity of the RNA preparations were verified on glyoxal gels (11). Twenty micrograms of total RNA was hybridized overnight at 52°C to ≈1 fmol of ^{32}P-labeled RNA transcript produced by Sp6 RNA polymerase (specific activity, 8×10^7 dpm/pmol) as described (12). The hybridization mixture was diluted 10-fold with buffer containing 50 mM sodium acetate (pH 4.5)/2 mM EDTA/100 mM NaCl to a final volume of 300 μl and treated with 10 units of RNase T2 for 1 hr at 30°C. The nucleic acids were precipitated with ethanol and analyzed on a 5% acrylamide gel containing Tris/borate/EDTA buffer and 8 M urea, which was autoradiographed. A complete description of the cell lines used for the expression studies, with the exception of cell lines CJ, DI, EI, SN, and HF, can be obtained from Jim Loveless, the Human Tumor Cell Line Bank, Human Tumor Cell Laboratory, Memorial Sloan–Kettering Cancer Institute. Cell lines CJ, DI, EI, and SN are human glioblastoma lines and primary glial cells, respectively, established by J. Shapiro at Memorial Sloan–Kettering Cancer Institute. HF cells are primary human foreskin fibroblasts obtained from J. Feramisco, Cold Spring Harbor Laboratory.

Southern and RNA Blot Analysis. Southern blot analysis was done as described (13). RNA for blotting was isolated by the guanidinium/cesium chloride method and was subsequently purified on oligo(dT) columns (11). RNA blotting was done essentially as described (14). For hybridization, the same *ROS1* probe synthesized by Sp6 RNA polymerase and described was used. The RNA blot was hybridized for 36 hr at 62°C in 50% formamide/5× standard saline citrate (SSC) (1× SSC is 0.15 M sodium chloride/0.015 M sodium citrate)/10 mM EDTA/10 μg of carrier DNA per ml/5× Denhardt's solution (1× Denhardt's solution is 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin), and washed in 0.1× SSC/0.1% $NaDodSO_4$ once for 2 min at room temperature and four times for 30 min at 64°C.

RESULTS

We previously reported the isolation of a cDNA corresponding to the transcript of an activated *ROS1* gene, *mcf3* (1). A portion of this cDNA encoding amino acids 140–295 was subcloned into pSp65, a vector containing a Sp6 RNA polymerase promoter (12). A ^{32}P-labeled Sp6 RNA polymerase transcript of the overall length of 730 nucleotides (nt), containing 460 nt of *mcf3* sequence complementary to *ROS1*-encoded mRNA and 270 nt of sequence derived from the vector was synthesized and gel purified. This transcript was hybridized in solution to total RNA prepared from a variety of human tumor cell lines and was then digested with RNase (12). The RNA hybrids protected from nuclease digestion were subsequently analyzed on denaturing acrylamide gels. Fig. 1 shows, as an example, the analysis of RNA from six

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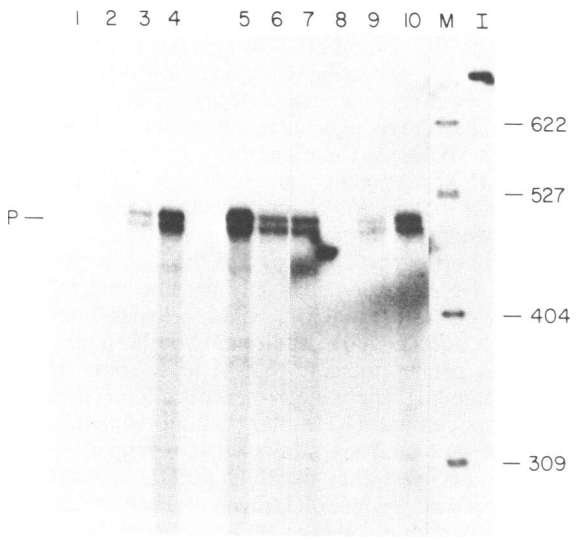

FIG. 1. Expression of *ROS1* in glioblastoma cells. Total RNA from human glioblastoma and mouse MCF-7-3-7 cells was hybridized to the ^{32}P-labeled RNA transcript synthesized by Sp6 RNA polymerase as described. After digestion of the single-stranded RNA with RNase T2, the nucleic acids were analyzed on an acrylamide gel under denaturing conditions. The figure was photographed from an autoradiogram exposed for 36 hr. Numbers at right, size of DNA marker fragments in nt. Bar at left (P), fragment protected from RNase T2 digestion by *mcf3* or *ROS1*-encoded RNA. Lanes: 1, 0.033 μg of RNA from MCF-7-3-7 and 20 μg of yeast tRNA; 2, 0.1 μg of RNA from MCF-7-3-7 and 20 μg of yeast tRNA; 3, 0.33 μg of RNA from MCF-7-3-7 and 20 μg of yeast tRNA; 4, 1 μg of RNA from MCF-7-3-7 and 20 μg of yeast tRNA; 5, 10 μg of RNA from human U-343 MG cells and 10 μg of yeast tRNA; 6, 20 μg of RNA from human SW-1088 cells; 7, 20 μg of RNA from human U-118 MG cells; 8, 20 μg of RNA from human U-251 MG cells; 9, 20 μg of RNA from human U-105 MG cells; 10, 20 μg of RNA from human U-138 MG cells; M, pBR322 DNA cut with *Msp* I; and I, labeled-RNA transcript before RNase T2 treatment.

different glioblastoma cell lines by this technique. The original transcript of 760-nt length used for hybridization is shown in lane I. RNA from MCF-7-3-7, a mouse NIH 3T3 cell line transformed by *mcf3* (1), protects a fragment of 460 nt from digestion by RNase because only 460 nt of the labeled probe are complementary to *mcf3*-encoded RNA (Fig. 1, lane 1–4). RNA from glioblastoma cell lines protected a fragment identical in length (Fig. 1, lanes 5–10). Thus *ROS1* transcripts are presented in the tested glioblastoma lines. As can be seen in Fig. 1, the protected probe resolved into a doublet on denaturing acrylamide gels. This was reproducibly observed with RNAs from all cell lines that protected this probe. The appearance of this doublet might either reflect a heterogeneity in the *mcf3*- and *ROS1*-encoded mRNA or might be an artifact due to strong secondary structures within the protected fragment.

The intensity of the band on the autoradiogram corresponding to the protected fragment can be used to quantify the amount of *ROS1* transcripts in the tested RNA. For standardization, we used RNA from cell line MCF-7-3-7 and determined the counts present in the protected fragment after analysis by scintillation counting. Five micrograms of total RNA from MCF-7-3-7 cells protected 5000 dpm of the labeled probe (specific activity, 8.10^7 dpm/pmol) or 0.06 fmol; 0.06 fmol corresponds to 50 pg of the 2.5-kb *mcf3*-encoded mRNA. Assuming that hybridization went essentially to completion, 50 pg of *mcf3* mRNA was present in 5 μg of the total RNA. Assuming that 2% of total RNA is mRNA, this amount of *mcf3* mRNA represents 0.05% of the mRNA; and assuming 400,000 mRNA molecules per cell, this amount would correspond to ≈200 molecules of *mcf3*-encoded mRNA per cell. *mcf3* transcripts were still detected on 0.025 μg of the total RNA from MCF-7-3-7 cells after autoradiograms were exposed for 1 week, which indicated that the detection limit of this assay was 0.25 pg of *mcf3* mRNA. In all experiments, RNA from MCF-7-3-7 was used in parallel to estimate the levels of *ROS1* transcripts in tested RNAs. Table 1 summarizes the result of these experiments and includes name and tissue of origin of tested cell lines together with the approximate number of *ROS1* transcripts found in these cells.

Table 1. *ROS1* transcripts in tested human cell lines and tissues

Human cell line or tissue	Origin	*ROS1* transcripts per cell,* ≈no.
U-343 MG	Glioblastoma multiforme	60
U-118 MG	Glioblastoma multiforme	10
E1	Glioblastoma multiforme	10
U-105 MG	Astocytoma, grade III	3
U-178 MG	Astocytoma, grade II	<1
U-251 MG	Astocytoma, grade I	<1
U-138 MG	Glioblastoma, not graded	10
SW-1088	Glioblastoma, not graded	10
DI	Glioblastoma multiforme	ND
CJ	Glioblastoma, astocytoma, medium grade	ND
A-172	Glioblastoma, not graded	ND
A-382	Glioblastoma, not graded	ND
SN	Normal glial cells	ND
Wiltu-1	Kidney, Wilm tumor	ND
SK-NEP-1	Kidney, Wilm tumor	ND
Caki-1	Kidney, carcinoma	ND
RD-2	Rhabdomyosarcoma	ND
575A	Bladder, carcinoma	ND
TCCSUP	Bladder, carcinoma	<1
VM-CUB-2	Bladder, carcinoma	<1
486P	Bladder, carcinoma	ND
MCF7	Breast, carcinoma	ND
734B	Breast, adenocarcinoma	<1
SK-Br-3	Breast, carcinoma	ND
SK-N-MC	Neuroblastoma	ND
SK-N-SH	Neuroblastoma	ND
SK-HEP-1	Liver, adenocarcinoma	ND
LS-174T	Colon, adenocarcinoma	ND
HT-29	Colon, adenocarcinoma	ND
SW-480	Colon, adenocarcinoma	ND
8387	Bone, sarcoma	<1
SK-MEL-3	Melanoma	ND
RPMI-4445	Melanoma	ND
C-4II	Cervix, carcinoma	ND
RPMI-2650	Nasal, squamous cell carcinoma	ND
Fadu	Pharynx, squamous cell carcinoma	ND
RPMI-8402	Leukemia, lymphoplastic	ND
SK-L3	Leukemia, myelomonocytic	ND
U-698 M	Lymphoma	ND
NB-58B	Normal lymphocyte	ND
Calu-3	Lung, adenocarcinoma	ND
SK-MES-1	Lung, squamous cell carcinoma	ND
SW-982	Fibrosarcoma	ND
HF	Normal fibroblasts	ND
A-431	Epidermoid carcinoma	ND
Brain, temporal lobe		ND
Placenta		ND

ND, not detected.
*For calculations, it was assumed that there were 400,000 mRNA molecules per cell and that 2% of the total RNA represents mRNA.

In addition to glioblastoma cell lines, four other cell lines (TCCSUP, VM-CUB-2, 734B, and 8387) expressed detectable levels of *ROS1* transcripts, albeit at low levels, estimated to correspond to <1 transcript per cell. We did not find detectable levels of *ROS1* expression in the remaining 28 cell lines, including SN, a cell line established from normal human glial cells; nor did we detect *ROS1* expression in the two analyzed tissues, placenta and brain.

We have previously shown that the human *ROS1* gene can be activated by either deletion of the putative extracellular domain and/or by amplification (1). It was therefore of interest to determine whether the *ROS1* genes in the human tumor cell lines that express *ROS1* are present in normal copy number and in normal configuration. In all Southern blots the fragments detected by the *ROS1* probe were of similar intensities in placental and tumor DNAs, indicating *ROS1* genes in the tumor DNAs to be present in normal copy number. To study potential rearrangements, we confined our analysis of the genomic *ROS1* DNA to the part encoding the junction of the putative extracellular and transmembrane domains. For Southern blotting, we used a genomic *Eco*RI fragment 5.2 kb in length containing three exons that encode 70 amino acids of the putative extracellular domain, the potential transmembrane domain, and 42 amino acids of the putative intracellular domain (1, 15) (see diagram at bottom of Fig. 2). In placental DNA, this probe hybridized mainly to a 9.5-kb *Bam*HI fragment (Fig. 2, lane 1). The position of this fragment within the *ROS1* locus is depicted in the diagram at the bottom of Fig. 2. A fragment identical in size was seen in the DNAs from all tested cell lines with the exception of cell line U-118 MG, where a 20-kb fragment was detected (Fig. 2); U-118 MG is a cell line derived from a glioblastoma and contains ≈10 *ROS1* transcripts per cell. To exclude that altered mobility of this fragment was due to a restriction endonuclease-fragment length polymorphism, the same probe was used for Southern blot analysis of DNA digested with a different restriction enzyme, *Hin*dIII. In placental DNA and DNA from six other cell lines, two fragments, 1.5 kb and 5.5 kb, were recognized by this probe (Fig. 3). In DNA from U-118 MG cells, the common 1.5-kb fragment and an unusual 2.25-kb fragment were seen (Fig. 3, lane 6). This demonstrates that the *ROS1* gene in cell line U-118 MG DNA has an unusual conformation and is probably rearranged. Further support of this conclusion is the fact that in cell line U-118 MG DNA we detected only the aberrant DNA fragments, demonstrating that U-118 MG cells are homozygous for the altered *ROS1* gene. Additional Southern blotting data with cDNA probes indicate that at least some of the sequences encoding the putative extracellular domain are missing from cell line U-118 MG (data not shown).

Southern blot analysis allowed us to map the point of divergence between the placental and U-118 MG *ROS1* alleles within the 5.2-kb *Eco*RI fragment. Prolonged exposure of the Southern blots shown in Fig. 2 revealed a second *Bam*HI fragment that was 10 kb in length and common to all cell lines except cell line U-118 MG (data not shown). The position of this *Bam*HI fragment within the *ROS1* locus is shown in the diagram at the bottom of Fig. 2. In U-118 MG DNA, *Bam*HI fragments of 9.5 kb and 10 kb are both altered; the 1.5-kb *Hin*dIII fragment is intact, whereas the 5.5-kb *Hin*dIII fragment is altered. The altered *Hin*dIII fragment spans the *Bam*HI site within the 5.2-kb *Eco*RI fragment. Therefore, this *Bam*HI site is apparently lost. The point of divergence is thus located within the same fragment we have previously seen rearranged in *mcf3*.

To determine the size of the *ROS1* transcripts in the glioblastoma cell lines, RNA blotting of different lines was done. RNA from cell line SK-MES-1 served as a negative

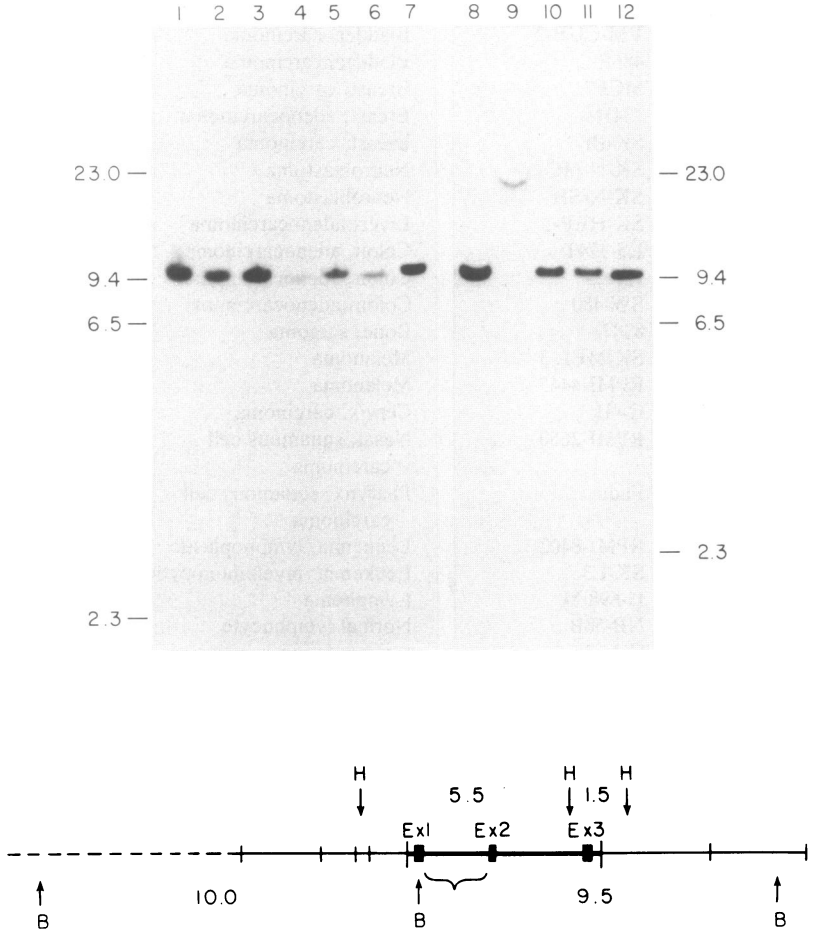

FIG. 2. Southern blot analysis of the *ROS1* locus in different cell lines expressing *ROS1*. Genomic DNA (≈10 μg) and DNA (0.1 ng) from a *ROS1* cosmid clone (3P7) derived from human placental DNA were digested with *Bam*HI and analyzed by Southern hybridization. As a probe, the 5.2-kb *Eco*RI fragment, indicated by a bold line in the lower diagram was used; positions of the three exons within this fragment are indicated. In addition, the restriction map of the *ROS1* locus around this fragment is shown. The cloned piece of the *ROS1* locus is depicted by a solid line, and the mapped part is depicted by a broken line. The *Eco*RI sites in the cloned region are shown by vertical lines. *Bam*HI sites and relevant *Hin*dIII sites are indicated by arrows and the letters B and H, respectively. Numbers between arrows indicate the size of the *Hin*dIII and *Bam*HI fragments in kb pairs. The bracket between exon 1 and exon 2 shows the location of the rearrangement in *mcf3*. Lanes: 1, placental DNA; 2, DNA from MCF7 cells; 3, DNA from VM-CUB-2 cells; 4, DNA from TCCSUP cells; 5, DNA from 734B cells; 6, DNA from SW-1088 cells; 7, DNA from 8387 cells; 8, DNA from U-105 MG cells; 9, DNA from U-118 MG cells; 10, DNA from U-138 MG cells; 11, DNA from U-343 MG cells; and 12, 3P7 DNA. Intensity difference of the hybridizing fragments between the lanes reflects differing amounts of DNA applied, as judged by ethidium bromide staining of the gel before transfer. Numbers at left and right of the Southern blot indicate DNA marker size. The second hybridizing fragment in lane 12, which is ≈19 kb long, corresponds to a fragment containing cosmid sequences that hybridize to the plasmid sequences of the probe.

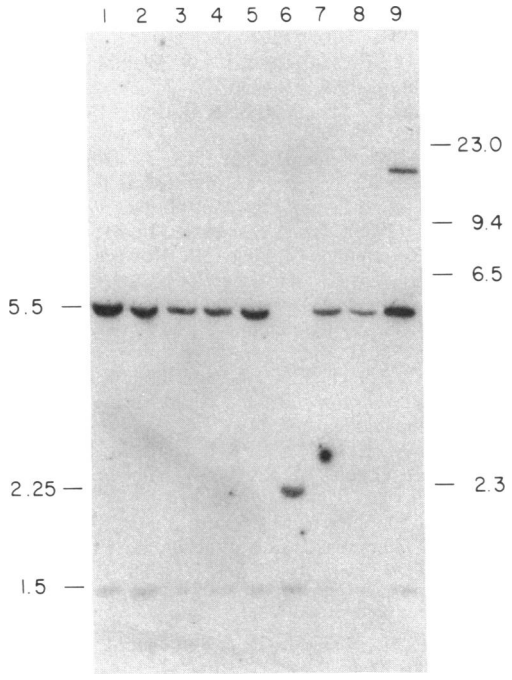

FIG. 3. Aberrant configuration of the *ROS1* locus in U-118 MG DNA. Genomic DNA (≈10 μg) and DNA (0.1 ng) of a cosmid clone (3P7) were cut with *Hin*dIII and analyzed by Southern hybridization. As a probe, a plasmid containing the 5.2-kb *Eco*RI fragment, indicated by a bold line in the diagram of Fig. 2 was used. Numbers at left, sizes of hybridizing fragments; numbers at right, sizes of DNA marker fragments. Lanes: 1, DNA from placenta; 2, DNA from MCF-7; 3, DNA from SW-1088; 4, DNA from SW-1783; 5, DNA from U-105 MG; 6, DNA from U-118 MG; 7, DNA from U-138 MG; 8, DNA from U-343 MG; 9, DNA from 3P7. The second hybridizing fragment in lane 9, ≈17 kb, corresponds to a fragment containing cosmid sequences that hybridize to the plasmid sequences of the probe.

control (Fig. 4, lane 1). In three glioblastoma lines, SW-1088, U-343 MG, and U-105 MG, the structure of the *ROS1* gene appeared normal by Southern blot analysis. In RNA of these three cell lines, we detected comigrating *ROS1*-encoded

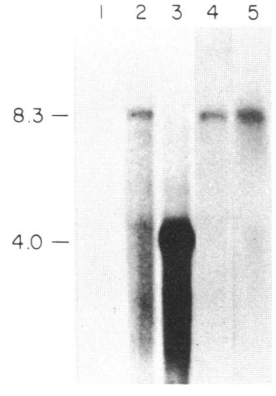

FIG. 4. RNA analysis from glioblastoma cell lines. Different RNA preparations were fractionated by gel electrophoresis, transferred to nitrocellulose, and hybridized to a *ROS1*-derived cDNA probe. Lanes: 1, 10 μg of poly(A)$^+$ RNA from SK-MES-1; 2, 10 μg of poly(A)$^+$ from U-105 MG;3, 10 μg of poly(A)$^+$ RNA from U-118 MG; 4, 10 μg of poly(A)$^+$ RNA from SW-1088; and 5, 20 μg of total RNA from U-343 MG. Lanes 1–3 and lanes 4 and 5 originate from two different RNA blots; lanes 4 and 5 were photographed from autoradiograms exposed for different times. Transcript sizes were determined by comparison with the mobility of ^{35}S-labeled RNA transcripts produced by Sp6 RNA polymerase of 7.5 kb, 5.3 kb, 4.6 kb, 4.2 kb, and 3.2 kb.

transcripts of 8.3-kb (Fig. 4, lanes 2, 4, and 5). In the fourth glioblastoma cell line, U-118 MG, which contains an aberrant configuration of the *ROS1* gene, a smaller 4-kb *ROS1* transcript, is seen (Fig. 4, lane 3).

DISCUSSION

We have found expression of the *ROS1* gene in human tumor cell lines. In cell lines established from glioblastomas we frequently detected *ROS1* transcripts ranging from below one transcript to 60 transcripts per cell. By contrast, we found only low levels of *ROS1* transcripts (below one transcript per cell) in 4 out of 32 other cell lines. The weakly positive cell lines originated from two bladder carcinomas, one breast carcinoma, and one osteosarcoma. In the remaining cell lines, levels of *ROS1* transcripts were below our detection limit. In a similar survey of human tumor cell lines, other workers failed to detect expression of *ROS1* in any lines (16), although that survey may not have included glioblastoma cells. Moreover, we see no expression of a *ROS1* gene in normal brain tissue. Thus the high level of *ROS1* expression in glioblastomas seems specific.

A transcript in low abundance that hybridizes to a v-*ros* probe has been previously reported in RNA from kidneys of 7- to 14-day-old chicken. Due to RNA degradation, the size of this transcript could not be determined (17). Discovery of cells that express a *ROS1* gene in abundance has allowed us to determine the size of the *ROS1* transcript. As determined by RNA blotting, the size of the *ROS1*-encoded mRNA in three glioblastoma lines was found to be 8.3 kb and the mRNA has, thus, the potential to encode a large protein. Preliminary immunoprecipitation studies indicate this is the case (S.S., unpublished work). The *ROS1* transcript in one other glioblastoma cell line, U-118 MG, was found to be 4 kb. As determined by Southern blotting, the *ROS1* locus of U-118 MG does have an aberrant configuration and therefore was expected to produce an altered *ROS1* transcript.

Southern blot analysis indicated that *ROS1* was present in normal copy number in all tumor cell lines expressing the gene. However, an aberrant *ROS1* locus was found in one of the glioblastoma cell lines, U-118 MG. The point of divergence between the normal and U-118 MG *ROS1* genes was located to that portion of *ROS1* that encodes the junction between the putative extracellular and transmembrane domains. Our analysis indicated that sequences downstream from the point of divergence were intact. This region encodes the putative intracellular domain of *ROS1*. However, at least part of *ROS1* encoding putative extracellular sequences are missing in U-118 MG DNA, and preliminary data indicate that U-118 MG cells do produce high levels of an altered *ROS1*-encoded protein (S.S., unpublished work). Interestingly, the point of rearrangement in U-118 MG DNA is very close to the point of rearrangement that created the *mcf3* gene. In *mcf3*, this leads to deletion of all but eight amino acids of the putative extracellular domain of *ROS1* (1). Loss of the putative extracellular domain is also seen in v-*ros* (3). Thus deletions of this domain may be an important event in the activation of the oncogenic potential of the *ROS1* gene.

Presently, we cannot be sure of the contribution of *ROS1* expression to the pathogenesis of glioblastomas, although several observations suggest a possible role. First, expression of *ROS1*, a potential oncogene, appears specific to glioblastomas. We do not see expression of *ROS1* in normal brain RNA. Second, one glioblastoma cell contains in all likelihood an "activating" deletion within the *ROS1* gene and is homozygous for this mutation. For these reasons we propose that *ROS1* expression contributes to the development of glial tumors. *ROS1* is not the only potential oncogene expressed in this cell type. The epidermal growth factor receptor (EGFR) gene is frequently amplified and expressed

at high levels in primary human glioblastomas, and an aberrant configuration of the EGFR gene has been seen in one particular tumor of glial origin (18).

Sequence data strongly suggest that *ROS1* is a membrane-spanning protein and therefore specifies an extracellular antigen. Thus, antibodies recognizing the putative extracellular domain might be clinically useful in diagnosis or imaging. Moreover, because *ROS1* does not seem to be ubiquitously expressed, such antibodies might also find application in the specific treatment of tumors of glial origin.

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