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Characterization of the *ros*1-Gene Products Expressed in Human Glioblastoma Cell Lines

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Most glioblastoma-derived cell lines express an 8.3 kb *ros*1 transcript and a 280 kD glycoprotein designated gp280^{ros1}, which can be specifically immunoprecipitated with an anti-*ROS* antibody. This 280 kD protein possesses in vitro autokinase activity and was observed in four independent glioblastoma cell lines. In a fifth glioblastoma cell line, U-118 MG, a smaller *ros*1 transcript of 4.0 kb was observed. Immunoprecipitation analysis reveals that the U-118 MG expressed a smaller, 116 kD *ros*1 gene product. cDNA cloning and sequencing of the U-118 MG *ros*1 transcript indicates it encodes the entire tyrosine kinase domain and two amino acids of the transmembrane domain of *ros*1 at its 3′ end. Sequences at its 5′ end likely arise from another gene.

KEYWORDS: *ros*1, glioblastoma, kinase, cancer

INTRODUCTION

The progression of normal cells to a neoplastic state involves alteration of the cellular genes called oncogenes. The normal cellular versions of oncogenes play a role in the control of proliferation and differentiation of normal cells (Bishop, 1985). One class of oncogenes exerts its effects by interfering with pathways regulated by growth factors (for review, see Yarden and Ullrich, 1988). Such oncogenes deregulate growth factor pathways either by coding for specific growth factors, e.g., v-*sis* (Devare et al., 1983), or by coding for altered growth factor receptor molecules, e.g., v-*erb*B (Downward et al., 1984). The same end result can perhaps be accomplished by the overexpression of a normal receptor (Slamon et al., 1987).

We have previously reported the isolation and characterization of an activated human *ros*1 gene called *mcf*3 (Fasano et al., 1984; Birchmeier et al., 1986). *mcf*3 encodes a potential transmembrane tyrosine kinase which arose from the human *ros*1 gene by a rearrangement introduced during gene transfer that deleted most of the extracellular domain of *ros*1 (Birchmeier et al., 1986). *ros*1 is the human cellular homolog of the v-*ros* oncogene, which is the transforming gene of the UR2 retrovirus (Neckameyer and Wang, 1985). *ros*1 can encode a protein similar in structure to a superfamily of known growth factor receptors (Yarden and Ullrich, 1988) and may therefore encode a growth factor receptor as well. However, the closest homolog of *ros*1 is the *Drosophila* gene *sevenless* (Hafen et al., 1987; Basler and Hafen, 1988; Bowtell et al., 1988). It has been suggested that *sevenless* does not encode a receptor for a soluble ligand. Thus, *ros*1 may encode a receptor for an as yet unidentified type of stimulus.

Recently, expression of *ros*1 has been reported in a high proportion of human glioblastoma cell lines (Birchmeier et al., 1987). In this report we describe the generation of antibodies specific for *ros*1, and the use of these antibodies for the detection of the *ros*1 protein in glioblastoma-derived cell lines. Four out of four glioblastoma cell lines that express a large *ros*1 transcript (8.3 kb) also express a 280 kD glycoprotein, which we have designated gp280^{ros1}. Another glioblastoma line, U-118 MG, contains both

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an abberrant *ros*1 transcript and gene product. U-118 MG apparently expresses a *ros*1-derived fusion protein in which the extracellular and transmembrane domains of *ros*1 have been replaced by sequences encoded by a heretofore unknown gene. The chimeric protein has a molecular weight of 116 kD.

RESULTS

Generation of Antibodies Specific for *ros1* Protein and Immunoprecipitation of *ros1* Proteins from Human Glioblastoma Lines

We previously described the isolation and characterization of an activated and rearranged *ros*1 gene, designated *mcf*3 (Birchmeier et al., 1986). To generate antibodies specific to the *ros*1 protein we adopted previously described strategies (Ruther and Mueller-Hill, 1983; Spindler et al., 1984). A cDNA fragment containing sequences that encode the C-terminal portion of *mcf*3 corresponding to the *ros*1 kinase domain was cloned into the expression vector pUR288 (see Materials and Methods). This cDNA derives entirely from an unrearranged portion of *ros*1 in *mcf*3. The resultant beta-galactosidase–*ros*1 (*lac-ros*) fusion protein was expressed in *E. coli*, purified and used to immunize rabbits (see Materials and Methods). Following labelling with [^{35}S]methionine, two protein species (indicated by arrows at the right-hand side of Fig. 1A) of relative molecular weights (M_r) 60 and 75 kD were detected in *mcf*-7-3-7, an NIH3T3 cell line transformed with the *mcf*3 oncogene (Fasano et al., 1984); these species were not detected in untransformed NIH3T3 cells.

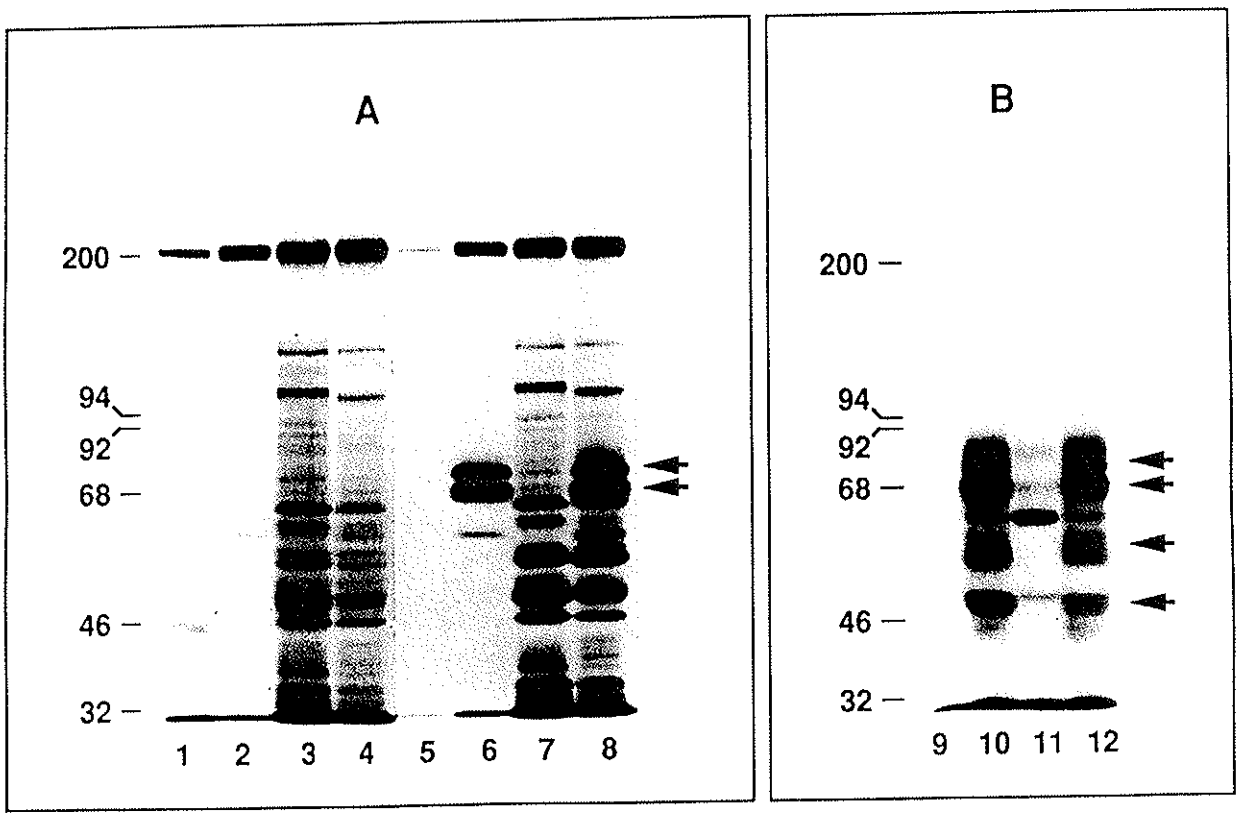


FIGURE 1. Immunoprecipitation of transforming *ros*1 protein. (A) Fluorogram of an SDS–polyacrylamide gel from immunoprecipitates of [^{35}S]methionine-labelled normal NIH3T3 cells (lanes 1–4) and *mcf*-7-3-7 cells, an NIH3T3 cell line transformed by the truncated *ros*1 gene called *mcf*3 (lanes 5–8) (exposure time, 24 hr). (B) Autoradiogram of an SDS–acrylamide gel of in vitro autokinase assays on immunoprecipitates from *mcf*-7-3-7 cells (exposure time, 2 hr). Immunoprecipitation of each cell line is represented by four lanes. Lanes 1, 5 and 9: immunoprecipitation with preimmune serum; lanes 2, 6 and 10: immunoprecipitation with anti-*lac-ros* antiserum; lanes 3, 7 and 11: immunoprecipitation with anti-*lac-ros* antiserum in the presence of competing *lac-ros* fusion protein; lanes 4, 8 and 12: immunoprecipitation with anti-*lac-ros* antiserum in the presence of *lac* protein. The positions of *ros*-specific bands are indicated by arrows at the right-hand side. At the left-hand side, the relative mobility of molecular weight (M_r) markers is indicated.

Immunoprecipitation of these proteins is competed by the addition of an excess of soluble *lac-ros* fusion protein, but not by beta-galactosidase (*lac*) itself. We presume, therefore, that these are the protein products of the rearranged *ros*1 gene in *mcf*-7-3-7.

The human *ros*1 gene is homologous to other genes encoding transmembrane tyrosine kinases (Birchmeier et al., 1986), and the v-*ros* product has been shown to have tyrosine kinase activity (Feldman et al., 1982). Many transmembrane tyrosine kinases are capable of autoprosphorylation when incubated with ATP in vitro. We therefore attempted to visualize *ros*1 products using in vitro immune complex kinase assays as described previously (Konopka and Witte, 1985). Figure 1B shows the results of in vitro autokinase assays performed on immunoprecipitates from *mcf*-7-3-7. Polypeptides of M_r 48 and 55 kD were observed in addition to the 60 and 75 kD species that were previously observed by [^{35}S]methionine labelling (Fig. 1A). It is possible that these additional species are *ros*1-specific peptides that are more readily detected by the in vitro kinase assay. Heterogeneity of *ros*1 products from *mcf*-7-3-7 was expected due to the heterogeneity of *ros*1 transcripts seen in this cell line (Birchmeier et al., 1986). However, we cannot exclude the additional possibility that some of the observed polypeptides are proteolytic degradation products.

*ros*1 mRNA is expressed in greater than 60% of human glioblastoma cell lines analyzed (Birchmeier et al., 1987). The vast majority of these express an 8.3 kb mRNA. [^{35}S]methionine labelling and immunoprecipitation analysis revealed a single specific polypeptide of M_r 280 kD (Fig. 2A, C) in cell lines expressing the 8.3 kb RNA. The cell lines SW1088 and U-105 MG were tested in this way. Similar-sized proteins were visualized by ^{32}P-labelling (by autokinase assays) from these cells (Fig. 2B, D), and [^{3}H]mannose labelling (Fig. 3B). A 280 kD polypeptide was also detected in the two other glioblastoma cell lines, SW1783 and U-343 MG (Fig. 3A). These lines also expressed the 8.3 kb *ros*1 transcript. A similarly migrating phosphorylated protein species

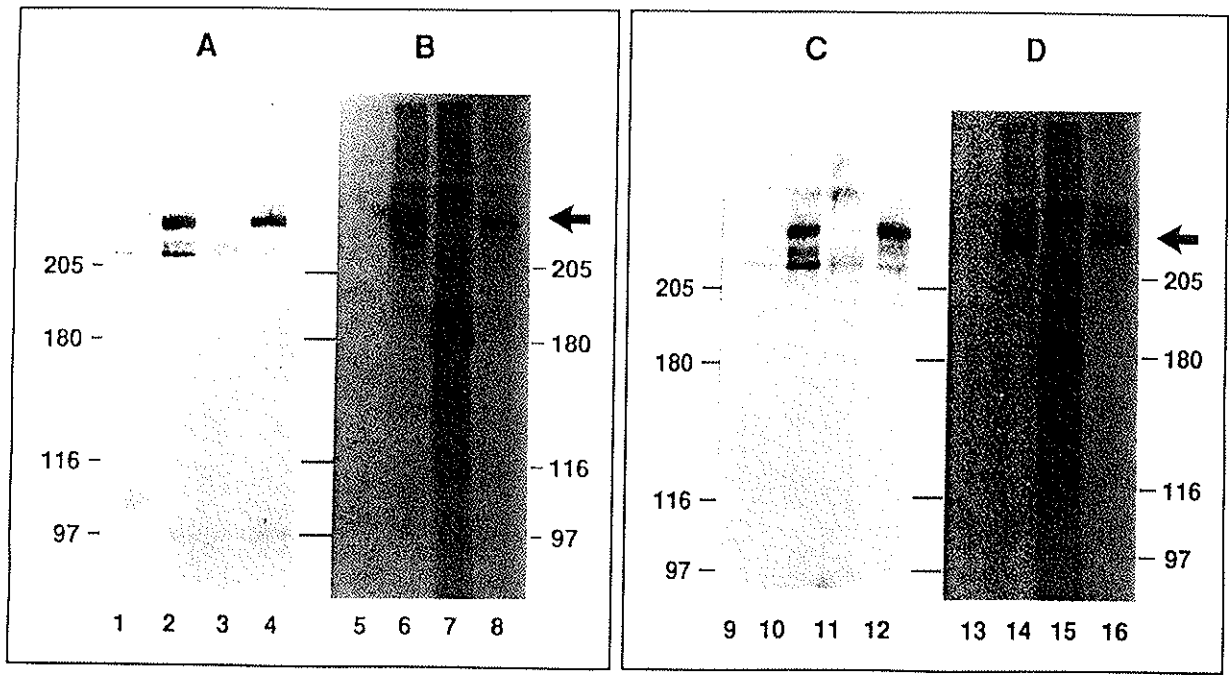


FIGURE 2. Immunoprecipitation of *ros*1 protein from cell lines expressing the 8.3 kb transcript. (A) and (C) Fluorograms of SDS-polyacrylamide gels from immunoprecipitates of [^{35}S]methionine-labelled cells SW1088 and U-105 MG, respectively (exposure time, 3 weeks). (B) and (D) SDS-polyacrylamide gel analysis of autokinase assays of immunoprecipitates from cells SW1088 and U-105 MG, respectively (exposure time, 12 hr). (A) and (B) represent two halves of one gel, as do (C) and (D). Lanes 1, 5, 9 and 13: immunoprecipitation with control pre-immune serum; lanes 2, 6, 10 and 14: immunoprecipitation with anti-*lac-ros* antiserum; lanes 3, 7, 11 and 15: immunoprecipitation with anti-*lac-ros* antiserum in the presence of competing *lac-ros* fusion protein; lanes 4, 8, 12 and 16: immunoprecipitation with anti-*lac-ros* antiserum in the presence of *lac* protein. The position of the *ros*1-specific band is indicated by an arrow. At the left-hand sides of (A) and (C) and the right-hand sides of (B) and (D), the relative mobility of the molecular weight (M_r) markers is indicated.

of 280 kD was observed when the immunoprecitation and in vitro kinase assay on U-343 MG cell extracts was performed with a second antiserum raised against a different *lac-ros* fusion protein containing sequences from the extracellular domain of the *ros*1 product (data not shown). In summary, a 280 kD protein is consistently observed when the 8.3 kb transcript is expressed.

One glioblastoma line, U-118 MG, contains a rearranged *ros*1 gene and expresses a 4.0 kb *ros*1 transcript (Birchmeier et al., 1987). To obtain further information about the protein products in these cell lines, immunoprecipitation analyses were performed (Fig. 4). [^{35}S]methionine labelling followed by immunoprecipitation from the U-118 MG cell lines revealed a single *ros*1-specific polypeptide of M_r 116 kD (Fig. 4A). Figure 4B shows the results of in vitro kinase assays performed on immunoprecipitates from U-118 MG cells. A single specific polypeptide of an approximate M_r 116 kD was observed.

Further Characterization of the *ros1* Product of the Glioblastomas U-118 MG Line

The sequence of cDNAs to the *mcf*3 gene found in the transformed NIH3T3 cell line *mcf*-7-3-7 has already been published. cDNA cloning and sequencing of the large 8.3 kb mRNA from the human glioblastoma cell line SW1088 is in progress, and will be presented when it is complete. Preliminary results indicate that the 8.3 kb mRNA can indeed encode a large protein, with a maximum molecular weight of 300 kD. The encoded protein would contain a putative transmembrane domain, and have a large extracellular domain homologous to the *Drosophila sevenless* gene product.

We have previously provided preliminary evidence that the *ros*1 locus in the glioblastoma cell line U-118 MG is rearranged (Birchmeier et al., 1987). Thus the 116 kD *ros*1 product of this line might arise entirely from the *ros*1 locus or be the result of a gene fusion. To resolve this, we cloned cDNA copies of the U-118 MG *ros*1 transcript and compared them

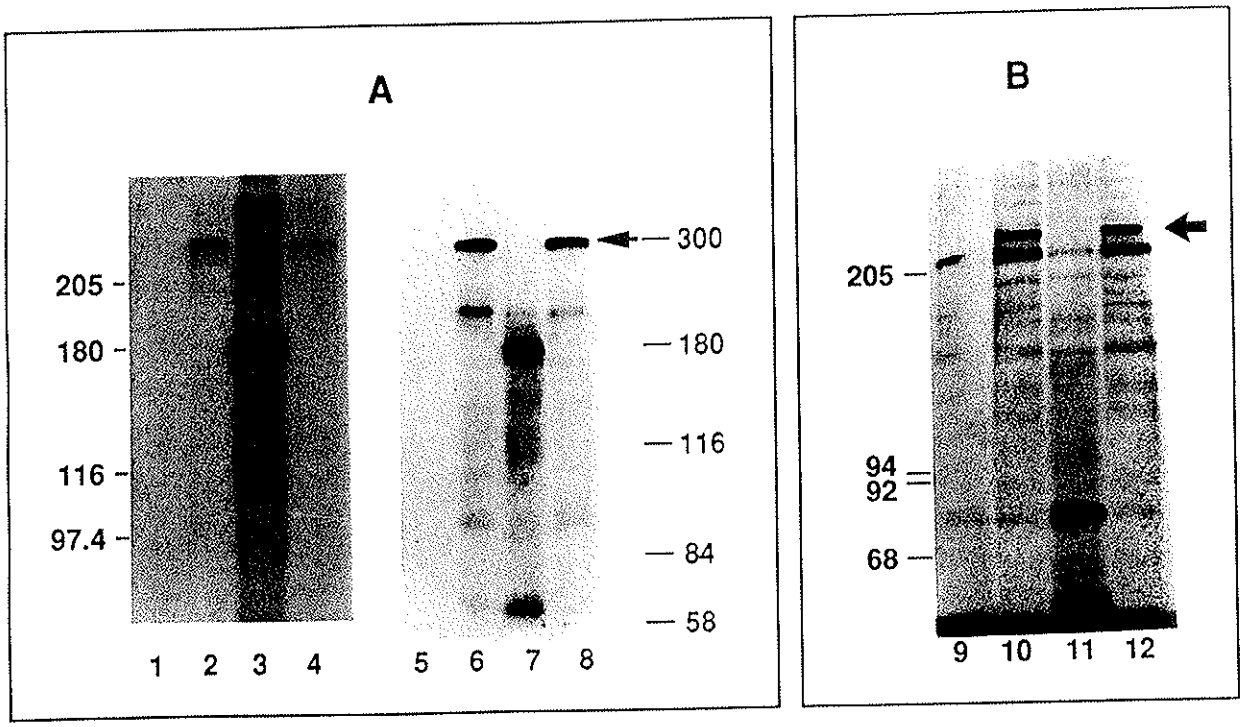


FIGURE 3. Analysis of *ros*1 proteins from cell lines expressing the 8.3 kb transcript by in vitro kinase assays (A) and [^{3}H]mannose labelling (B). Lanes 1–4 represent immunoprecipitates from the glioblastoma cell line SW 1783 and lanes 5–12 are immunoprecipitates from the cell line U-343 MG. Lanes 1, 5 and 9: immunoprecipitation with control pre-immune serum; lanes 2, 6 and 10: immunoprecipitation with anti-*lac-ros* antiserum; lanes 3, 7 and 11: immunoprecipitation with anti-*lac-ros* antiserum in the presence of competing *lac-ros* fusion protein; lanes 4, 8 and 12: immunoprecipitation with anti-*lac-ros* antiserum in the presence of *lac* protein. The position of the *ros*1-specific band is indicated by an arrow. Positions of the molecular weight standards are indicated on the left-hand side of (A) and (B) and also on the right-hand side of (A).

to *ros*1-specific cDNAs we have cloned from *mcf*-7-3-7 and the SW1088 cell lines. A cDNA library constructed from U-118 MG mRNA was screened with *ros*1-specific cDNA probes. We identified several cDNA clones hybridizing to *ros*1 sequences. A composite restriction map of the U-118 MG *ros*1 cDNA is shown in Fig. 6. The 0.45 kbp EcoRI fragment in the U-118 MG *ros*1 cDNA corresponds to an 0.45 kb fragment found in cDNAs from SW1088 and *mcf*-7-3-7. cDNAs obtained from U-118 MG, SW1088 or *mcf*-7-3-7 have the same composite EcoRI restriction endonuclease map 3′ to this 0.45 kbp fragment (data not shown). However, U-118 MG *ros*1 cDNA contains a 1.8 kbp EcoRI fragment 5′ to the 0.45 kbp fragment, whereas the *ros*1 cDNA from SW1088 has a 1.4 kbp EcoRI fragment at this equivalent position (C. Birchmeier, unpublished data). This suggests that the 1.8 kbp EcoRI fragment on U-118 MG *ros*1 cDNA diverges from cDNAs of the larger transcripts.

sequence of *mcf*3 cDNA and cDNAs from the 8.3 kb transcript. First of all, we find a 42 nucleotide insert in U-118 MG *ros*1 cDNA which we do not see in *ros*1 cDNAs from either *mcf*-7-3-7 or from SW1088. These nucleotides occur precisely between splice donor and acceptor sites within *ros*1 (Matsushime et al., 1986). Southern blot analysis with oligonucleotide probes indicates that the 42 extra nucleotides do derive from the *ros*1 locus, and may represent a cryptic exon. Secondly, and more importantly, while *ros*1 cDNAs from *mcf*-7-3-7 and SW1088 can encode a transmembrane domain, all but two amino acids of this domain are missing in the U-118 MG *ros*1 product. Rather, new sequences are found precisely 5′ to a splice junction within the transmembrane coding region.

In order to characterize the unexpected sequences found at the 5′ end of the U-118 MG *ros*1 cDNA, the two EcoRI fragments (0.45 and 1.8 kbp) were used as probes to analyze Northern blots of RNAs

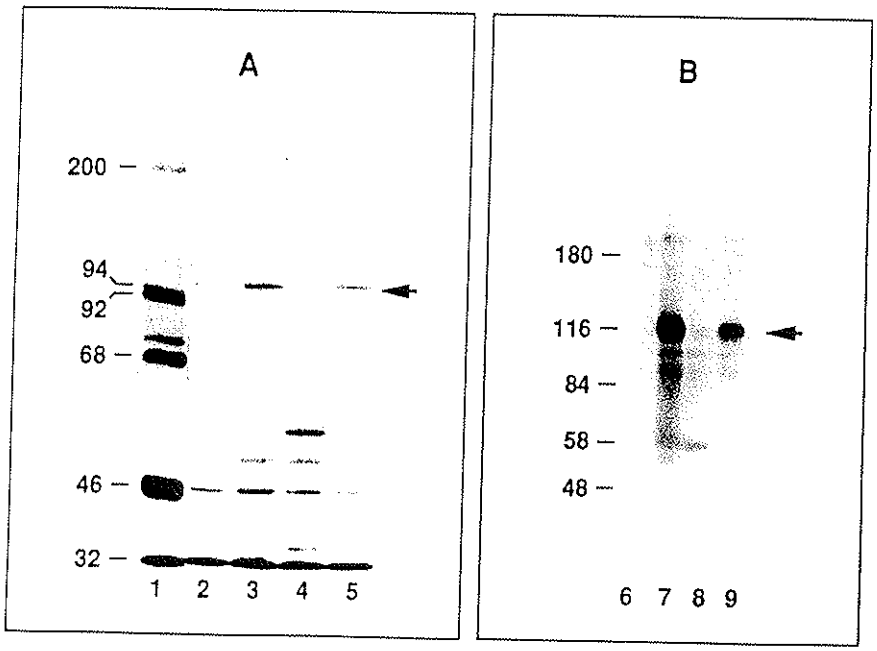
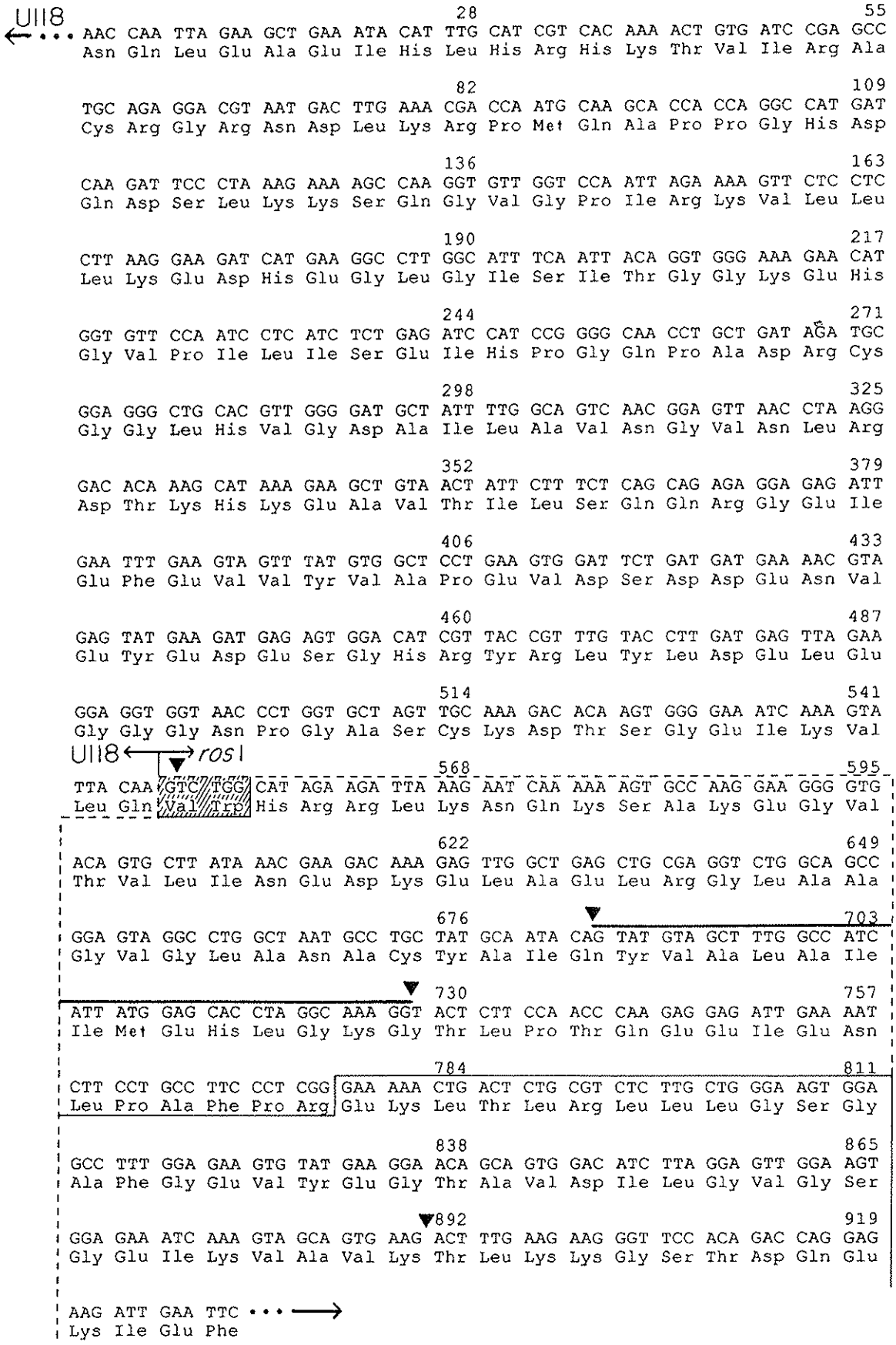


FIGURE 4. Immunoprecipitation of truncated *ros*1 protein from glioblastoma cell line U-118 MG. Fluorogram of SDS-polyacrylamide gel from immunoprecipitates of [^{35}S]methionine-labelled U-118 MG cells (A) and in vitro autokinase assays on immunoprecipitates from U-118 MG cells (B). Lane 1: [^{14}C]-labelled molecular weight markers; lanes 2 and 6: immunoprecipitation with control preimmune serum; lanes 3 and 7: immunoprecipitation with anti-*lac-ros* antiserum; lanes 4 and 8: immunoprecipitation with anti-*lac-ros* antiserum in the presence of competing *lac-ros* fusion protein; lane 5 and 9: immunoprecipitation with anti-*lac-ros* antiserum in the presence of *lac* protein. The position of the *ros*1-specific band is indicated by an arrow. At the left-hand side of (A) and (B), the relative mobility of molecular weight (M_r) markers is indicated.

In order to better understand the events that created the 4.0 kb *ros*1-derived transcript in the glioblastoma line U-118 MG, the 1.8 kbp cDNA fragment was subcloned into a pUC118 vector and sequenced. The sequence of part of the cDNA and its encoded amino acid sequence are shown in Fig. 5. This DNA can encode one long open reading frame. Two major differences are apparent when the U-118 MG *ros*1 cDNA is compared to the known

derived from tumor cell lines of glial origin (Fig. 6). Blots probed with the 0.45 kbp cDNA fragment (Fig. 6) reveal the *ros*1-specific transcript of 8.3 kb in all the glioblastoma cell lines tested (lanes 1, 3, and 4), with the exception of U-118 MG itself (Fig. 6A, lane 2), for which the 4.0 kb transcript is evident. When reprobed with the 1.8 kbp EcoRI fragment, the blot showed a different pattern of hybridization (Fig. 6B). Weak hybridization to the 8.3 kb *ros*1 transcript is


U118
28
55
AAC CAA TTA GAA GCT GAA ATA CAT TTG CAT CGT CAC AAA ACT GTG ATC CGA GCC
Asn Gln Leu Glu Ala Glu Ile His Leu His Arg His Lys Thr Val Ile Arg Ala
82
109
TGC AGA GGA CGT AAT GAC TTG AAA CGA CCA ATG CAA GCA CCA CCA GGC CAT GAT
Cys Arg Gly Arg Asn Asp Leu Lys Arg Pro Met Gln Ala Pro Pro Gly His Asp
136
163
CAA GAT TCC CTA AAG AAA AGC CAA GGT GTT GGT CCA ATT AGA AAA GTT CTC CTC
Gln Asp Ser Leu Lys Lys Ser Gln Gly Val Gly Pro Ile Arg Lys Val Leu Leu
190
217
CTT AAG GAA GAT CAT GAA GGC CTT GGC ATT TCA ATT ACA GGT GGG AAA GAA CAT
Leu Lys Glu Asp His Glu Gly Leu Gly Ile Ser Ile Thr Gly Gly Lys Glu His
244
271
GGT GTT CCA ATC CTC ATC TCT GAG ATC CAT CCG GGG CAA CCT GCT GAT AGA TGC
Gly Val Pro Ile Leu Ile Ser Glu Ile His Pro Gly Gln Pro Ala Asp Arg Cys
298
325
GGA GGG CTG CAC GTT GGG GAT GCT ATT TTG GCA GTC AAC GGA GTT AAC CTA AGG
Gly Gly Leu His Val Gly Asp Ala Ile Leu Ala Val Asn Gly Val Asn Leu Arg
352
379
GAC ACA AAG CAT AAA GAA GCT GTA ACT ATT CTT TCT CAG CAG AGA GGA GAG ATT
Asp Thr Lys His Lys Glu Ala Val Thr Ile Leu Ser Gln Gln Arg Gly Glu Ile
406
433
GAA TTT GAA GTA GTT TAT GTG GCT CCT GAA GTG GAT TCT GAT GAT GAA AAC GTA
Glu Phe Glu Val Val Tyr Val Ala Pro Glu Val Asp Ser Asp Asp Glu Asn Val
460
487
GAG TAT GAA GAT GAG AGT GGA CAT CGT TAC CGT TTG TAC CTT GAT GAG TTA GAA
Glu Tyr Glu Asp Glu Ser Gly His Arg Tyr Arg Leu Tyr Leu Asp Glu Leu Glu
514
541
GGA GGT GGT AAC CCT GGT GCT AGT TGC AAA GAC ACA AGT GGG GAA ATC AAA GTA
Gly Gly Gly Asn Pro Gly Ala Ser Cys Lys Asp Thr Ser Gly Glu Ile Lys Val
U118
ros1
568
595
TTA CAA GTC TGG CAT AGA AGA TTA AAG AAT CAA AAA AGT GCC AAG GAA GGG GTG
Leu Gln Val Trp His Arg Arg Leu Lys Asn Gln Lys Ser Ala Lys Glu Gly Val
622
649
ACA GTG CTT ATA AAC GAA GAC AAA GAG TTG GCT GAG CTG CGA GGT CTG GCA GCC
Thr Val Leu Ile Asn Glu Asp Lys Glu Leu Ala Glu Leu Arg Gly Leu Ala Ala
676
703
GGA GTA GGC CTG GCT AAT GCC TGC TAT GCA ATA CAG TAT GTA GCT TTG GCC ATC
Gly Val Gly Leu Ala Asn Ala Cys Tyr Ala Ile Gln Tyr Val Ala Leu Ala Ile
730
757
ATT ATG GAG CAC CTA GGC AAA GGT ACT CTT CCA ACC CAA GAG GAG ATT GAA AAT
Ile Met Glu His Leu Gly Lys Gly Thr Leu Pro Thr Gln Glu Glu Ile Glu Asn
784
811
CTT CCT GCC TTC CCT CGG GAA AAA CTG ACT CTG CGT CTC TTG CTG GGA AGT GGA
Leu Pro Ala Phe Pro Arg Glu Lys Leu Thr Leu Arg Leu Leu Leu Gly Ser Gly
838
865
GCC TTT GGA GAA GTG TAT GAA GGA ACA GCA GTG GAC ATC TTA GGA GTT GGA AGT
Ala Phe Gly Glu Val Tyr Glu Gly Thr Ala Val Asp Ile Leu Gly Val Gly Ser
892
919
GGA GAA ATC AAA GTA GCA GTG AAG ACT TTG AAG AAG GGT TCC ACA GAC CAG GAG
Gly Glu Ile Lys Val Ala Val Lys Thr Leu Lys Lys Gly Ser Thr Asp Gln Glu
AAG ATT GAA TTC
Lys Ile Glu Phe

still observed in the glioblastoma lines (lanes 5, 7, and 8). However, in addition to the 8.3 kb band, strong hybridization to a transcript of 4.4 kb is evident in most glioblastoma lines (lanes 5, 7, and 8). This 4.4 kb transcript was absent in the U-118 MG cell (lane 6). Instead, strong hybridization to the 4.0 kb transcript was detected in this line (lane 6). These results strongly suggest that the 1.8 kbp EcoRI fragment contains sequences from two normally separate transcription units, one of which is *ros*1 and one of which normally produces a 4.4 kb RNA species frequently expressed in glioblastomas. In U-118 MG these transcription units are fused to form a single 4.0 kb transcript.

DISCUSSION

We have previously reported that *ros*1-specific mRNA is expressed in approximately 60% of the glioblastomas that we have analyzed, but not in a normal glial cell line, in normal brain tissue, nor in the vast majority of tumor cell lines of diverse origins which we have studied (Birchmeier et al., 1987). We have demonstrated in this report that a *ros*1-specific protein is also expressed in glioblastoma cell lines. Most glioblastoma cell lines express an 8.3 kb transcript. Using polyclonal antisera raised against a *ros*1 fusion protein, we find that these cell lines express a 280 kD glycoprotein (gp280^{ros1}) which can be labelled in vivo with [^{35}S]methionine, [^{3}H]mannose, and in vitro with [^{32}P]ATP. If, as its

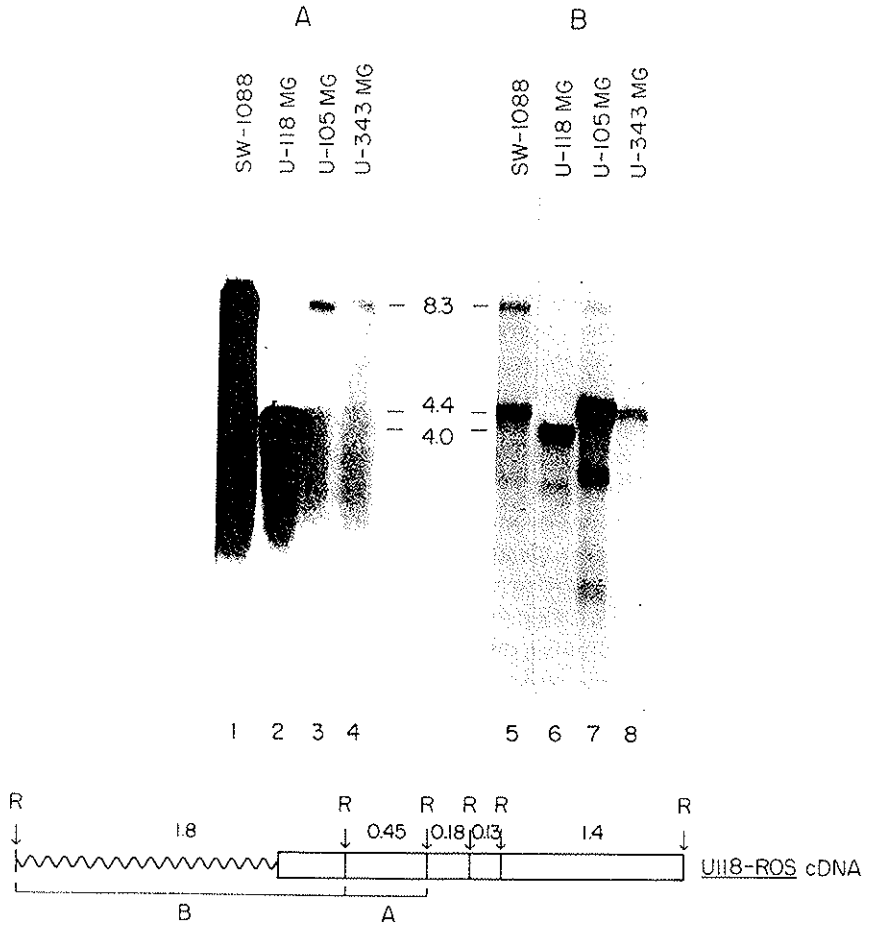


FIGURE 6. Northern blot analysis of *ros*1 transcript in human glioblastoma cell lines. Autoradiograms of the same Northern blot hybridized twice with different probes. (A) Autoradiogram of Northern blot hybridized with a *ros*1-specific probe, indicated as fragment A below. (B) Autoradiogram of Northern blot hybridized with fragment B from U-118 MG cDNA (see below) containing both *ros*1 and fused sequences. The name of the glioblastoma cell line used to prepare the RNA is indicated at the top of each lane. The size of the different hybridizing transcripts is indicated in kilobases. At the bottom of the figure, the EcoRI restriction map of the U-118 cDNA is depicted. The numbers indicate the sizes of the EcoRI fragments in kbp. The wavy line indicates the fused sequences of the cDNA, which are not *ros*1-specific.

FIGURE 5. Partial sequence of *ros*1 cDNA from U-118 MG cells. The sequence of part of the cDNA, together with its deduced amino acid sequence, is shown. The point of fusion at position 548 between the sequences of unknown origin and the *ros*1-specific sequences is indicated. The *ros*1-specific sequences are boxed with dotted lines, and the sequences encoding the tyrosine-specific protein kinase domain are boxed with solid lines. The two amino acids remaining of the putative transmembrane domain of *ros*1 are indicated by a cross-hatched box. The positions of exon-intron junctions are indicated by triangles, and the pseudoexon between nucleotides 685 and 726 is indicated by a solid line above the sequence.

structure suggests, *ros*1 encodes a transmembrane receptor, this receptor would be one of the largest known. Because of its characteristic expression pattern in glioblastomas, the *ros*1 receptor might provide a useful target antigen for diagnostic purposes and, possibly, therapeutic intervention. It is likely that the *ros*1 product presents an external membrane antigen, since we have shown that $p280^{ros1}$ can be labelled with [^{3}H]mannose, indicating that it is glycosylated.

The role of the *ros*1 product in the etiology of glioblastomas is not yet clear. However, both the v-*ros* and *mcf*3 genes are oncogenic (Balduzzi et al., 1981; Fasano et al., 1984). Judging by the events which accompany the acquisition of the transforming potential of *ros*1, *met*, *trk*, and other proto-oncogenes encoding transmembrane tyrosine kinases (Birchmeier et al., 1986; Martin-Zanca et al., 1986; Park et al., 1987; Yarden and Ullrich, 1988), deletion of the extracellular coding domain of *ros*1 can sometimes activate its transforming potential. Deletion of the extracellular coding domain of *ros*1 appears to have occurred in the cell line U-118 MG. This cell line has rearranged the *ros*1 locus and produces a shortened 4.0 kb transcript. It also expresses a smaller 116 kD *ros*1-specific protein. We cloned and sequenced the U-118 MG *ros*1 cDNA and compared it to the *mcf*3 cDNAs and *ros*1 cDNA sequences found in SW1088. *ros*1 transcripts from U-118 MG do not contain the sequences encoding the extracellular and most of the transmembrane domains of *ros*1. The deleted sequences are replaced by coding sequences of unknown origin. No homology to these new sequences is found in current data bases (Genebank Data Base or Protein Identification Resource). These new sequences occur in the U-118 MG *ros*1 transcript precisely at splice junctions (Matsushime et al., 1986). The U-118 MG *ros*1 cDNA recognized two separate transcripts in most glioblastomas: the 8.3 kb *ros*1 transcript and a new 4.4 kb transcript. Only a single transcript of 4.0 kb is recognized in U-118 MG. These results suggest that the U-118 MG *ros*1-specific transcript results from the fusion of two separate transcription units. The overall features of this event closely resemble the event which gave rise to the *met* oncogene (Dean et al., 1985; Park et al., 1987), and support the hypothesis that expression of *ros*1 contributes to the development of glioblastomas. Further support for this hypothesis comes from work in progress that indicates additional abnormalities in the *ros*1 product expressed in glioblastomas.

MATERIALS AND METHODS

Cells

mcf-7-3-7 cells were obtained by transforming NIH3T3 cells with the activated *ros*1 gene *mcf*3 (Birchmeier et al., 1986). Cell lines SW1088, SW-1783, U-105 MG, U-118 MG, and U-343 MG were from the Human Tumor Cell Line Bank, Human Tumor Cell Laboratory, Memorial Sloan-Kettering Cancer Institute, and were kindly provided to us by Jim Loveless.

RNA and Northern Blot Analysis

RNA was prepared and analyzed as described (Thomas, 1980). DNA fragments used as probes, were nick-translated using standard procedures provided by the supplier (Bethesda Research Labs).

Construction of *ros1* Expression Plasmid and Generation of Antisera

A gene fusion between β-galactosidase and *ros*1 (*lac-ros*) was constructed by cloning a cDNA fragment (EcoRI/SalI) encoding amino acids 140 to 496 and including the 3′ untranslated region of *mcf*3 (Birchmeier et al., 1986) into the expression vector pUR288 (Ruther and Mueller-Hill, 1983). The resultant *lac-ros*-containing vector was transformed into the JM101 strain of *E. coli*. Transformants expressing the *lac-ros* fusion protein were identified essentially as described (Ruther and Mueller-Hill, 1983). The *lac-ros* fusion protein was purified by differential extraction as described (Birkel et al., 1983), followed by preparative SDS–polyacrylamide gel electrophoresis. The fusion protein was excised, eluted and used to immunize rabbits essentially as described previously (Spindler et al., 1984).

Immunoprecipitation and *in vitro* Kinase Assays

Typically about 5×10^7 cultured animal cells were grown as sub-confluent monolayers, washed twice in phosphate-buffered saline (PBS), and metabolically labelled with [^{35}S]methionine (100 μCi/ml) in 3 ml of methionine minus Dulbecco's Modified Eagles Medium (DMEM) for 4 hr. Labelling was terminated by washing the cells once in ice-cold PBS. Cell extracts were prepared by adding 1 ml of lysis buffer (1% Trition X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl and 0.05 M Tris,

pH 8.0). Cell lysates were clarified by spinning in a microfuge (16,000 ×*g*) at 4°C for 15 min. The cleared lysates were divided equally into 4 tubes and to the first tube was added 0.3% (v/v) of pre-immune serum. The second tube received 0.3% (v/v) of rabbit polyclonal serum directed against the *lac-ros* product (designated anti-*lac-ros*). To the third tube was added anti-*lac-ros* antibody (0.3% v/v) that was preincubated with about 30 μl of cell lysates prepared from *E. coli* expressing the *lac-ros* fusion. The fourth tube received anti-*lac-ros* antibody (0.3% v/v) that was preincubated with about 30 μl of cell lysates from *E. coli* that overexpressed the β-galactosidase gene. Immunoprecipitation reactions were allowed to proceed at 4°C for 8–10 hr. The antigen antibody complexes were collected on 0.3% protein A Sepharose CL-4B (Pharmacia) and pelleted by centrifugation. The pellets were washed sequentially with wash buffer I (650 mM NaCl, 50 mM Tris, pH 7.4, 5 mM EDTA), wash buffer II (150 mM NaCl, 50 mM Tris, pH7.4, 5 mM EDTA, 1% NP40, 0.1% SDS) and wash buffer III (10 mM Tris, pH 7.4, 0.1% NP40). The proteins were solubilized in sample buffer (10% glycerol, 10 mM 2-mercaptoethanol, 6% SDS, 10 mM Tris, pH 6.8), boiled for 5 min and separated by electrophoresis on an SDS–polyacrylamide gel (5% for the large *ros*1 and 7.5% for the shorter *ros*1 products). The gels were subjected to fluorography (for [^{35}S]methionine) and autoradiography.

For the in vitro kinase assay, cell extraction, immunoprecipitation, in vitro phosphorylation and gel electrophoresis were performed as described previously (Konopka and Witte, 1985). Briefly, about 5×10^7 cells were washed in ice-cold PBS and pelleted by low-speed centrifugation (100 ×*g*). The cell pellet was resuspended in 50 μl of ice-cold PBS and lysed by the addition of 4 ml of ice-cold kinase–lysis buffer containing 1% Triton X-100, 0.05% SDS, 10 mM phosphate buffer, 15 mM NaCl, 5 mM EDTA and 5 mM PMSF. Homogenates were subjected to high-speed centrifugation (100,000 ×*g*), the supernatant was divided into 4 aliquots and immunoprecipitated as described above. Immunoprecipitated complexes, collected on protein A Sepharose, were washed twice in ice-cold kinase–lysis buffer lacking SDS and once in 50 mM Tris (pH 7.0). Immunoprecipitated complexes were then resuspended in kinase buffer containing 20 mM $MnCl_2$ and 20 mM PIPES (piperazine-*N*,*N*′-bis[2-ethane sulfonic acid]) (pH 7.0). Kinase reactions were initiated by the addition of 50 μCi of [γ-^{32}P]ATP (Amersham; specific activity, 3000 Ci/mmol) and incubated at 35°C for 60 m. Kinase reactions were terminated by the addition of EDTA to a final concentration of 50 mM. The pellets were washed in RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 0.15 M NaCl and 0.05 M Tris, pH 8.0), and then sequentially in wash buffers I, II and III as described above. The proteins were solubilized in Laemmli's sample buffer and subjected to electrophoresis on 5% polyacrylamide–SDS gels at 40 mA for 4 h. At the end of the run the gels were dried down and subjected to autoradiography from 8 to 24 h.

Cloning and Sequencing *ros1* cDNAs

cDNAs were synthesized from poly(A)$^+$ mRNA isolated from U-118 MG cells (Watson and Jackson, 1984). The cDNA library was constructed in a λgt10 vector and phages containing *ros*1 cDNAs were isolated by plaque hybridization (Huynh et al., 1984). The dideoxy sequence method (Sanger et al., 1977), with [α-^{35}S]dATP as a substrate (Biggin et al., 1983) was carried out in combination with the unidirectional progressive deletion method (Henikoff, 1984) on the phagemid vector pUC118 (Vieira and Messing, 1987).

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REFERENCES

Basler K., and Hafen E. (1988). Control of photoreceptor cell fate by the *sevenless* protein requires functional tyrosine kinase domain. Cell **54**: 299–311.

Balduzzi P.C., Notter M.F.D., Mirgan H.R., and Shibuya M. (1981). Same biological properties of two new avian sarcoma viruses. J. Virol. **40**: 268–275.

Biggin M., Gibson T., and Hong G. (1983). Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. Proc. Natl. Acad. Sci. USA **80**: 3963–3965.

Birchmeier C., Birmbaum D., Waitches G., Fasano O., and Wigler M. (1986). Characterization of an activated human *ROS* gene. Mol. Cell. Biol. **6**: 3109–3116.

Birchmeier C., Sharma S., and Wigler M. (1987). Expression and rearrangement of the *ros*1 gene in glioblastoma cells. Proc. Natl. Acad. Sci. USA **84**: 9270–9274.

Birkel I., Roberts T.M., Bladon M.T., Green R., Amann E., and Livingston D.M. (1983). Purification of biologically active simian virus 40 small tumor antigen. Proc. Natl. Acad. Sci. USA **80**: 906–910.

Bishop J.M. (1985). Viral oncogenes. Cell **42**: 23–38.

Bowtell D., Simon M., and Rubin G. (1988). Nucleotide sequence and structure of the *sevenless* gene of *Drosophila melanogaster*. Genes Dev. **2**: 620–634.

Dean M., Park M., Lebeau M.M., Robins T., Diaz M.O., Rowley J.D., Blair D.G., and Vande Woude G.F. (1985). The human *MET* oncogene is related to the tyrosine kinase oncogenes. Nature **318**: 385–388.

Devare S.G., Reddy E.P., Law D.J., Robbins K.C., and Aaronson S.A. (1983). Nucleotide sequence of the simian sarcoma virus genome: demonstration that its acquired cellular sequences encode the transforming gene product $p28^{sis}$. Proc. Natl. Acad. Sci. USA **80**: 731–735.

Downward J., Yarden V., Mayes E., Scrace G., Totty N., Stockwell P., Ullrich A., Schlessinger J., and Waterfield M.D. (1984). Close similarity of the epidermal growth factor receptor and v-*erb*B oncogene protein sequence. Nature **307**: 521–527.

Fasano O., Birnbaum D., Edlund L., Fogh J., and Wigler M. (1984). New human transforming genes detected by a tumorigenicity assay. Mol. Cell. Biol. **4**: 1695–1705.

Feldman O., Wang L.-H., Hanafusa H., and Balduzzi P.C. (1982). Avian sarcoma virus UR2 encodes a transforming protein which is associated with a unique protein kinase activity. J. Virol. **42**: 228–236.

Hafen E., Basler K., Edstroem J.-E., and Rubin G.M. (1987). *Sevenless*, a cell-specific homeotic gene of *Drosophila*, encodes a putative transmembrane receptor with a tyrosine kinase domain. Science **236**: 55–63.

Henikoff S. (1984). Undirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28**: 351–359.

Huynh T.V., Young R.A., and Davis R.W. (1984). Construction and screening cDNA libraries in λgt10 and λgt11. *In:* DNA cloning: a practical approach, Glover D., Ed. (Oxford: IRL Press), pp. 49–78.

Konopka J.B., and Witte O.N. (1985). Detection of c-*abl* tyrosine kinase activity in vitro permits direct comparison of normal and altered *abl* gene products. Mol. Cell. Biol. **5**: 3116–3123.

Martin-Zanca D., Hughes S., and Barbacid M. (1986). A human oncogene formed by the fusion of a truncated tropomyosin and protein kinase sequences. Nature **319**: 743–748.

Matsushime H., Wang L.-H., and Shibuya M. (1986). Human c-*ros*1 gene homologous to the *v-ros* sequence of UR2 sarcoma virus encodes for a transmembrane receptor-like molecule. Mol. Cell. Biol. **6**: 3000–3004.

Neckameyer W.S., and Wang L.-H. (1985). Nucleotide sequence of avian sarcoma virus UR2 and comparison of its transforming gene with other members of the tyrosin protein kinase oncogene family. J. Virol. **53**: 879–884.

Park M., Dean M., Kaul K., Braun M.J., Gonda M.A., and Vande Woude G. (1987). Sequence of *MET* protooncogene cDNA has features characteristic of the tyrosine kinase family of growth factor receptors. Proc. Natl. Acad. Sci. USA **84**: 6379–6383.

Ruther U., and Mueller-Hill B. (1983). Easy identification of cDNA clones. EMBO J. **2**: 1791–1794.

Sanger F., Nicklen S., and Coulson A. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**: 5463–5467.

Slamon D.H., Clar G.M., Wong S.G., Levin W.J., Ullrich A., and McGuire W.L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. Science **235**: 177–182.

Splindler K.R., Rosser D.S.E., and Berk A.J. (1984). Analysis of adenovirus transformation proteins from early regions 1A and 1B with antisera to inducible fusion antigens produced in *Escherichia coli*. J. Virol. **49**: 132–141.

Thomas P.S. (1980). Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77**: 5201–5206.

Vieira J., and Messing J. (1987). Production of single-stranded plasmid DNA. Methods Enzymol. **153**: 3–11.

Watson C.J., and Jackson J.F. (1984). An alternative procedure for the synthesis of double-stranded cDNA for cloning in phage and plasmid vectors. *In:* DNA cloning, a practical approach, Glover D., Ed. (Oxford: IRL Press).

Yarden Y., and Ullrich A. (1988). Growth factor receptor tyrosine kinases. Annu. Rev. Biochem. **57**: 443–478.