Identification and Characterization of Genes Differentially Expressed in Meningiomas

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Abstract
Meningiomas are common tumors derived from the thin membrane that surrounds the brain and spinal cord. Currently, the molecular mechanisms responsible for the initiation and progression of these tumors are largely unknown. Toward the elucidation of such mechanisms, we have formulated an experimental design utilizing the technique of subtractive hybridization that is aimed at identifying the changes in gene expression between intracranial meningiomas and their normal precursor cells, leptomeningeal cells. We report here the identification and initial characterization of three genes whose expression is altered in meningioma cell lines and tumors relative to cultures of normal leptomeningeal cells. Complementary DNA probes from one of these genes detect transcripts of altered size in several meningiomas relative to normal leptomeningeal cells. Another of these genes demonstrates decreased expression in meningiomas and in tumors associated with the disorder neurofibromatosis 2. A third gene isolated by this procedure is differentially expressed in both meningiomas and breast carcinomas. Therefore, the decreased expression of these genes may play roles in growth-regulatory pathways that are abrogated not only in meningiomas, but in other tumor types as well.

Introduction
Meningiomas are one of the most common tumors of the central nervous system. Derived from the leptomeningeal membrane surrounding the brain and spinal cord, these tumors account for up to 20% of all primary intracranial tumors and 25% of intraspinal neoplastic lesions (1). Clinically and histologically, meningiomas comprise a heterogeneous group of tumors, with up to nine different pathological and histological subtypes (1, 2); at present, the basis for this heterogeneity is unclear. Although meningiomas are generally solitary and sporadic, familial associations of these tumors have frequently been reported (1); these occur often, although not always, in association with the disorder NF3-2. NF-2 is an autosomal-dominantly inherited disorder predisposing affected individuals to multiple tumor types in the central nervous system, such as astrocytomas, schwannomas, meningiomas, and ependymomas; the hallmark of the disorder is considered to be the presence of bilateral vestibular schwannomas (3).

Cytogenetic studies of both sporadic and familial meningiomas (4–6), coupled with recent analyses of loss of heterozygosity of certain DNA markers in these tumors (7), have demonstrated that loss of genetic material from chromosome 22 is significantly more frequent than alterations of other chromosomes and occurs in up to 70% of meningiomas (8). These data have led to the hypothesis that the alteration of a tumor suppressor gene on chromosome 22 is involved in the genesis of both sporadic and familial meningiomas. This tumor suppressor gene, which we refer to as the meningioma susceptibility locus, or msI, has been localized cytogenetically and via deletion mapping to chromosome 22q12-qter (9–11). Because linkage analyses indicate that the NF-2 disorder is linked to loci in this region of chromosome 22 (12, 13), and deletions of loci from this chromosome have been detected in tumors from patients diagnosed with NF-2 (14), it has been hypothesized that the tumor suppressor genes involved in the initiation of meningiomas and NF-2 are allelic (15); however, some studies indicate that these tumor suppressors may represent two separate loci on chromosome 22 (16).

Cytogenetically, meningiomas are one of the best characterized solid tumors in humans. In addition to loss of genetic material from chromosome 22, these tumors display other, less frequent cytogenetic alterations. In particular, rearrangements of chromosomes 1p and 11p, as well as losses of genetic material from chromosomes 8, 14, and 17, have been documented to occur as nonrandom genetic changes in these tumors (17–19). It is generally felt that these nonrandom, secondary chromosomal aberrations and losses represent the inactivation of other tumor suppressor loci, whose functional loss may contribute to meningioma progression, aggressiveness, or chance for recurrence.

Recently, subtractive hybridization has been successfully utilized in the isolation of candidate genes for genetic disease (20, 21) and in the definition of the changes in gene expression that occur during the genesis and progression of different types of cancer, such as neuroblastoma (22) and cancers of the breast (23), liver (24), and lung (25). We reasoned that such an approach was ideal for the study of meningiomas. We describe here the use of two different sub-

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1 The abbreviations used are: NF, neurofibromatosis; LMC, leptomeningeal cells; kb, kilobase(s); bp, base pair(s); RT-PCR, reverse transcription-polymerase chain reaction; ER, estrogen receptor; IGFBP, insulin-like growth factor-binding protein; cDNA, complementary DNA; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; mac, meningioma altered expression cDNA; ATCC, American Type Culture Collection; poly(A)·, polyadenylated.
tractive hybridization protocols that served to enrich for cDNA sequences present largely exclusively in normal LMC, relative to a meningioma cell line. These protocols enabled the identification of three genes that are expressed in normal LMC but show altered or differential expression in several meningiomas. The identification and characterization of these and other such altered genes is likely to be a critical step toward uncovering the etiology and pathology of these frequent tumors and may have added implications for understanding the transformation pathways taken by other tumor types as well.

Results
Two different protocols were utilized for the construction and screening of subtraction libraries from normal leptomeningeal cells and the meningioma cell line T2898, as outlined briefly in Fig. 1. In both cases, subtractive hybridization was performed such that these libraries would be enriched for sequences found largely or exclusively in normal LMC, relative to the meningioma cell line T2898. The construction of the plasmid T2898 subtraction library incorporated one round of hybridization and subtraction, as shown in Fig. 1; this library was screened with similarly made “subtracted probes” made from mRNA from normal LMC and another meningioma cell line, designated T2966. In an effort to increase the efficiency of subtraction, the phage T2898 subtraction library was generated; the construction of this library incorporated two rounds of hybridization and subtraction, each followed by amplification of LMC-enriched sequences via the polymerase chain reaction. The phage T2898 subtraction library was differentially screened, as described in “Materials and Methods.”

From subtracted probe and differential screenings of these two subtraction libraries, 30 plasmid and phage clones were recovered for secondary Northern analysis. Of these, four unique cDNA clones detected a pattern or level of hybridization in normal leptomeningeal cell total RNA that was different in the T2898 and/or T2966 meningiomas. Two of these clones, although of distinct sequence, hybridized to the same pattern of RNA in Northern analyses and likely represent the same gene. The three cDNA clones presented in this study, containing inserts of 1.1, 0.42, and 0.3 kb, were designated mac25, mac30, and macP1, respectively.

The mac25 Gene Is Differentially Expressed in Meningiomas, but not in Other Tumors of Neuroectodermal Origin, Including Those Associated with NF-2. The cDNA clone designated mac25 hybridizes to a single abundant transcript of approximately 1.1 kb in RNA isolated from cultures of normal leptomeningeal cells (Fig. 2A, Lane 1) and from the LTAg2B immortalized line of these cells (Lane 10), as well as in RNA isolated directly from normal leptomeningeal tissue (Lane 14). However, as depicted in Fig. 2, this cDNA detects decreased levels of the 1.1-kb transcript in RNA isolated from several meningioma cell lines (Fig. 2A, Lanes 2, 3, and 11–13). Additionally, the mac25 cDNA probe detects the added presence of a higher molecular weight transcript, of approximately 4.0 kb, in the spinal meningioma T2966 (Fig. 2A, Lane 3). Furthermore, transcripts hybridizing to the mac25 probe are virtually undetectable in RNA isolated from several meningioma solid tumors (Fig. 2A, Lanes 4–9). In contrast, these same samples exhibit relatively low levels of transcripts for β2-microglobulin (Fig. 2A, B3M, Lanes 1–14), as well as ribosomal RNA (data not shown).

Transcript levels of the mac25 gene were not found to be significantly decreased in RNA prepared from other neuroectodermally derived tumors or tumor cell lines, relative to levels in normal LMC. As shown in Fig. 2B, significant levels of mac25 transcript are present in RNA isolated from a neuroblastoma (Lane 3), a neurofibroma (Lane 5), and a glioma cell line (Lane 6). Additionally, in an effort to extend these studies to other tumors found in the disorder neurofibromatosis 2, we analyzed the level of expression of mac25 in RNA isolated from a spinal schwannoma from a NF-2 patient, as well as in a sporadic spinal schwannoma (Fig. 2B, Lanes 4 and 6, respectively); in both samples, levels of mac25 RNA were indistinguishable from that in cultured LMC (Lane 1). Therefore, the decreased expression of the mac25 gene appears limited to certain meningioma cell lines and solid tumors and is not common to all neuroectodermally derived tumors, nor to all tumors associated with the disorder NF-2.

Northern blot analysis of RNA from normal mouse tissues indicates that the mac25 gene is expressed in a broad spectrum of tissues, such as brain, lung, heart and skeletal muscle, testes, ovary, and pregnant uterus; the highest levels of mac25 transcripts were found in the latter two tissues (data not shown).

The mac25 Gene Is Also Differentially Expressed in Breast Carcinomas and Has Homology to Genes Encoding Insulin-like Growth Factor-binding Proteins. There are numerous reports in the literature that indicate a statistically significant clinical association between the occurrence of meningiomas and breast carcinoma (26–28). Therefore, in addition to examining the expression of isolated mac genes in tumors associated with the disorder NF-2, we were also interested in examining the expression of these genes in breast carcinoma samples. As depicted in Fig. 3, we found
that the mac25 gene is expressed at high levels in some breast carcinoma cell lines, yet it is undetectably expressed in others (Lanes 2–8). In total, we found mac25 expressed at high levels in four breast carcinoma cell lines, but absent in expression in three. It is unlikely that the differences in levels of this transcript represent tissue-specific changes, as all seven of these cell lines represented advanced stage carcinomas from mammary ductal epithelium.

We noted that two of the mac25 nonexpressing breast carcinoma cell lines had been previously reported to be positive for estrogen receptor protein (29). Therefore, we sought to determine whether the expression of these two genes might be inversely correlated in this cell type. As shown in Fig. 3, we found that the presence of ER mRNA, as measured in a RT-PCR assay, negatively correlates with mac25 gene expression in a cell line from normal breast tissue (Hs 578Bst; Fig. 3, Lane 1), and in seven of eight breast carcinoma cell lines (Lanes 2–8). One cell line (MDA-MB-453) expressed neither RNA, however (data not shown), nor were we able to detect the presence of ER mRNA in any of our meningiomas (Fig. 3, Lanes 9–11). The inverse correlation between ER and mac25 gene expression in breast car-

Fig. 2. A, Northern analysis of mac25 gene expression in meningioma cell lines and tumors. Ten μg of total RNA are present in each lane; equal loadings and RNA integrity are controlled by levels of ribosomal RNA, as well as RNA for β2-microglobulin (B2M). RNA samples in each lane are: Lane 1, normal leptomeningeal cells; Lane 2, T2898 meningioma cell line; Lane 3, T2966 meningioma cell line; Lanes 4–9, meningioma tumors ND1, ND2, A287B, A244, A809, and A188, respectively; Lane 10, LTAg2B immortalized LMC; Lanes 11–13, meningioma cell lines T2896, T2921, and T2891; Lane 14, normal leptomeningeal tissue. B, Northern analysis of mac25 expression in: Lane 1, LTAg2B leptomeningeal cells; Lane 2, a sporadic spinal schwannoma; Lane 3, a neuroblastoma; Lane 4, a spinal schwannoma from a patient diagnosed with NF-2; Lane 5, a neurofibroma; Lane 6, U-373Mg glioma cell line. Ten μg of total RNA are loaded in each lane.

Fig. 3. Northern analysis of mac25 gene expression in breast carcinomas and meningiomas, compared to the positive (+) or negative (−) presence of mRNA for the estrogen receptor in these cell lines. The presence of ER mRNA was measured by a RT-PCR assay. Lane 1, RNA from normal breast line Hs 578Bst; Lanes 2–8, breast carcinoma cell lines Hs 578T, ZR-75-30, ZR-75-1, MDA-MB-157, MDA-MB-231, BT-483, and T-47D, respectively; Lane 9, ND1 meningioma; Lane 10, T2888 meningioma cell line; Lane 11, LTAg2B leptomeningeal cells.
cinoma cell lines may indicate that the mac25 gene is estrogen regulated in these cells; however, the absence of detectable ER mRNA in our meningiomas suggests that other variables must contribute to the decreased expression of this gene in these tumors.

A search of the GenBank database with the full-length nucleotide sequence of the mac25 cDNA indicates that the sequence of this gene has not been reported previously. A protein homology search revealed that the 281-amino acid protein representing the largest open reading frame of this gene possesses a significant degree of homology at the amino terminus to the amino termini of several members of the IGFBP family. Importantly, this homology, detailed for IGFBP-1 in Fig. 4, includes 11 of the 12 cysteine residues known to be critical for the function of insulin-like growth factor-binding proteins (30). As might be expected with a putative member of the IGFBP family, the mac25 gene demonstrates a very high species conservation; several genomic fragments hybridize to the mac25 cDNA probe in multiple species, such as rat, mouse, dog, and monkey, at washing stringencies as high as 0.3x SSC at 55°C (data not shown).

Preliminary chromosomal mapping studies using a rodent/human somatic cell hybrid panel indicate that the mac25 gene maps to human chromosome 4 (data not shown).

The mac30 Gene Demonstrates Decreased Expression in Meningiomas and Schwann Cell Tumors. The mac30 cDNA, like mac25, was isolated as a positive clone in the screening of the plasmid T2989 subtraction library with subtracted probes made from the T2966 meningioma cell line. This 421-bp cDNA hybridizes to two transcripts of approximately 2.3 and 2.8 kb in RNA isolated from normal leptomeningeal cells (Fig. 5A, Lane 1) and from immortalized cultures of these cells (Lanes 6 and 11). When hybridized to a Northern panel containing total RNA isolated from five meningioma cell lines (Fig. 5A, Lanes 2, 5, and 7–10), as well as RNA prepared directly from six meningioma tumors (Lanes 3, 4, and 12–14), this cDNA probe detects significantly decreased expression of this gene. In contrast, these samples demonstrate comparable levels of β2-microglobulin (Fig. 5A, Lanes 1–14) and ribosomal RNA (data not shown). Thus far, transcripts hybridizing to mac30 have been detected at levels comparable to that in normal LMC in over 30 cell lines from normal and transformed human tissue (see, for example, Fig. 5B, Lanes 1–11), including all eight breast carcinoma cell lines (see, for example, Lane 9), as well as RNA isolated directly from leptomeningeal tissue (Lane 11). These data indicate that the mac30 gene is likely to be normally ubiquitously expressed, and furthermore, that its down-regulation is not common to all transformed cells.

In efforts to again extend these studies to the disorder neurofibromatosis 2, we analyzed several NF-2 tumors for expression of mac30. Using a RT-PCR assay to more accurately detect mac30 transcripts in tumors, we found expression of mac30 to be dramatically decreased in RNA preparations from two vestibular schwannomas and one spinal schwannoma, all from patients diagnosed with neurofibromatosis 2 (Fig. 6A, Lanes 2–4), in a sporadic vestibular schwannoma (Lane 5), and in a sporadic spinal schwannoma (Lane 6). Somewhat surprisingly, we also found the mac30 gene to be similarly decreased in expression in several neurofibromas, which are Schwann cell tumors of the peripheral nervous system characteristic of the disorder NF-1 (Ref. 31; Fig. 6A, Lanes 8–10). No decrease in levels of mac30 RNA were found in other tumors of neural crest origin [for example, in a neuroblastoma (Fig. 6A, Lane 7)]; furthermore, a transcript hybridizing to this cDNA is clearly present in RNA isolated from cultures of immortalized rat Schwann cells (Fig. 6B, Lane 1). Therefore, decreased expression of this gene appears to be limited to meningioma tumors and cell lines, as well as to Schwann cell tumors, including those associated with the disorders NF-1 and NF-2.

Sequence analysis of a 2.0-kb mac30 cDNA and a search of the GenBank database indicate that the sequence of this gene has not been previously reported and, furthermore, that mac30 sequences do not contain any significant homologies to previously reported genes. Chromosomal mapping using a rodent/human somatic cell hybrid panel indicates that this gene resides on human chromosome 17 (data not shown). Further mapping with the somatic cell hybrid panel indicates that this gene resides on chromosome 17q21–qter, indicating that this gene resides on chromosome 17q21–qter (data not shown).

The macP1 cDNA Detects Transcripts of Altered Size in Many Meningiomas. In order to increase the sensitivity of our subtractive hybridization protocol, we generated a
Fig. 6. A. RT-PCR analysis of RNA levels of mac30 and β-actin in tumors associated with the disorders NF-2 and NF-1. Lane 1, normal LMC; Lanes 2 and 3, NF-2 vestibular schwannomas; Lane 4, NF-2 spinal schwannoma; Lane 5, sporadic vestibular schwannoma; Lane 6, sporadic spinal schwannoma; Lane 7, neuroblastoma; Lanes 8–10, NF-1 neurofibromas; Lane 11, minus-reverse transcriptase control; Lane 12, water control. B. Northern analysis of mac30 expression in RNA from an immortalized rat Schwann cell line (C4+Zn; Lane 1) and LTag28 LMC (Lane 2). RNA blot was washed in 0.3× SSC at 55°C and exposed to film for 48 h. Arrow: size of the mac30 transcript in rat cells; similar levels of a transcript of this size are evident in RNA from adult mouse brain as well as in leptomeningeal cultures (not shown).

phage subtraction library that incorporated two rounds of subtraction, each followed by amplification of LMC-enriched sequences by the polymerase chain reaction. Differential screening of this cDNA library yielded the isolation of the mac30 cDNA, which hybridizes to transcripts of approximately 5.0, 3.5, and 3.0 kb in normal and immortalized leptomeningeal cell RNA (Fig. 7, Lanes 1 and 2). In RNA from the meningioma T2898, the mac30 probe detects decreased levels of a transcript that is consistently reduced in size relative to the normal 3.0-kb message (Fig. 7, Lane 3). In RNA samples run longer for greater separation, this size difference appears on the order of 100–200 bp (data not shown). Therefore, in addition to being decreased in expression in the T2898 meningioma, the 3.0-kb mac30 transcript may also contain sequences present in normal LMC that are deleted in the T2898 tumor. In the T2966 meningioma cell line, the major hybridizing mac30 transcript has a molecular size greater than 3.0 kb, whereas the normal 5.0-kb message is undetectable (Fig. 7, Lane 4). Additionally, RNA isolated from another meningioma contains an abundant transcript of approximately 6 kb that hybridizes to mac30 probe (Fig. 7, Lane 5); yet, other meningiomas have demonstrated a pattern of hybridization indistinguishable from normal LMC (see, for example, Fig. 7, Lane 6).

Analysis of the hybridization pattern of the mac30 gene in several normal and transformed human cell lines, as well as RNA isolated directly from nonmeningeal tumors, indicates that the expression of the 5.0-kb transcript may be ubiquitous, whereas the expression of the 3.0- and 3.5-kb transcripts may be restricted to normal LMC and some meningiomas (see, for example, Fig. 7, Lanes 7–9). At present, it has not been determined whether the three transcripts that hybridize to mac30 in normal LMC RNA are derived from the same or homologous genes. Sequence analysis of the 300-bp mac30 cDNA clone, as well as a 2.5-kb cDNA from this gene, has revealed no significant sequence homology to sequences in the GenBank database.

Discussion

In this report, we describe the use of subtractive hybridization to identify mRNA differences between a population of normal leptomeningeal cells and a meningioma cell line. It was expected that cDNA clones isolated in this study could be classified into at least one of the following categories: (a) clones representing genes in which the alteration in expression is accompanied by genomic mutation; (b) genes whose expression is lost due to the initial loss of function of the ms1 gene product; and (c) genes whose expression is lost in meningiomas due to a change in differentiation state in these tumors relative to normal LMC. The first class of genes includes candidate tumor suppressor genes; the understanding of the function and characteristics of the latter two classes of genes will be critical toward the understanding of the function of the normal ms1 gene and may further elucidate some aspects of the biology of meningiomas.

For example, the existence of a hormonal influence on the clinical course of meningiomas has long been acknowledged. These tumors, which are more frequent in women, have often been found to become clinically symptomatic following pregnancy or menstruation (1). Furthermore, the occurrence of meningiomas has been found to be statistically associated with the incidence of breast carcinoma in women (26–28). The basis for these associations is incompletely understood, however; numerous studies have failed to find conclusive evidence in meningiomas for the widespread presence of functional receptors for either estrogen or progesterone, although the presence of androgen receptors has been reported (33, 34). We have found that the mac25 gene has the potential to encode a protein with homology to a family of growth factor-binding proteins and is altered in expression in both meningiomas and breast carcinomas. Furthermore, the expression of mac25 is inversely correlated in breast carcinomas with the presence of mRNA for the estrogen receptor. Additional studies on the normal function of the mac25 gene may help elucidate both the nature of the
hormonal component to meningioma growth as well as the clinical association between these tumor types.

Thus far, we have found no evidence for gross genomic alteration of either the mac25 or mac30 genes in any of our meningioma tumors or cell lines, indicating that the decreased expression of these genes may be caused by a potential loss of differentiation state in these tumors or that the low expression status of these genes may be a marker for a particular cell type in the leptomeninges that is predisposed to tumor formation. Alternatively, the decreased expression of these genes may result from the inactivation of an “upstream” tumor suppressor gene. Our finding that the mac30 gene demonstrates markedly reduced expression in both meningiomas and other tumors associated with the disorder NF-2 raises the possibility that the upstream gene influencing mac30 expression may be the meningioma susceptibility locus, assuming that these two disorders are caused by inactivation of the same tumor suppressor gene; this hypothesis obviously awaits the cloning of the msl gene. We were quite surprised to find that the mac30 gene similarly decreased in expression in Schwann cell tumors associated with NF-1; the decreased expression of this gene in these tumors alone, but not in cultures of rat Schwann cells, suggests that meningiomas and Schwann cell tumors may share a common pathway of transformation that involves or necessitates the decreased expression of mac30.

To date, we have not yet determined the nature of the size alterations in the macP1 gene in our meningiomas. Although it is possible that this gene has suffered mutations that affect transcript size in these tumors, it is also possible that this gene is subject to extensive alternative splicing in these cells. In the latter event, it might be expected that different splice forms of this gene could be correlated with phenotypic variables, such as meningioma subtype, making this gene useful as a phenotypic marker in clinical studies. Additionally, our finding that the major transcript for the macP1 gene is likely to be limited in expression to leptomeningeal cells facilitates its use as a marker specific for meningiomas.

Our underlying hypothesis for this study was that genes found to be consistently down-regulated or otherwise altered in expression in meningiomas relative to their precursor cell type would be candidates for genes intimately involved with mechanisms of growth control and differentiation. This hypothesis is validated by our finding that two of the genes identified in this study have been found to be altered in expression in other tumor types as well. Further characterization of these three genes, as well as identification and characterization of other such aberrantly expressed genes, will undoubtedly lead to a better understanding of the transformation pathway utilized by these common tumors.

### Materials and Methods

#### Cell Lines and Cell Culture

Normal human leptomeningeal cells were established, characterized, and maintained as described (35). The LTag2B line of immortalized leptomeningeal cells was established from primary cultures by transfection with an SV40 large T antigen expression construct, as detailed (35). Meningioma cell lines T2898, T2966, T2896, T2921, T2891, and T2888 were established and characterized by one of us (K. D. Z.); these cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal bovine serum and 100 units/ml of penicillin and streptomycin. The cell line C4+Zn is an immortalized rat peripheral Schwann cell line kindly provided by George DeVries (Medical College of Virginia) and was cultured as described (36). The cell line Tu-87, from a rhabdoid tumor of the brain, was kindly provided by Kuang Lin Ying (Children’s Hospital of Los Angeles) and was cultured as described (37). Breast carcinoma studies utilized ATCC cell lines HS 5788bt (normal human breast), HS 578t, and BT-483, maintained in Dulbecco’s modified Eagle’s medium; ZR-75-1, ZR-75-30, and T-47D, maintained in RPMI 1060; and MDA-MB-157, MDA-MB-231, and MDA-MB-453, maintained in Liebovitz’s L-15 medium; all media were supplemented with 10% fetal bovine serum and antibiotics. Other cell lines examined for expression of certain genes included ATCC cell lines RD-ES, HeLa, Colo32DM, K-562, Sk-mel-2, and U-373Mg; these lines were cultured according to ATCC guidelines.

#### RNA Isolation and Northern and Southern Blots

Total cellular RNA was isolated from exponentially growing tissue culture cells with guanidine hydrochloride, as described (38), and poly(A)+ RNA was prepared using Poly(A)Quick columns, according to the manufacturer’s instructions (Stratagene). Snap-frozen meningiomas, normal leptomeningeal tissue, vestibular schwannomas (acoustic neuromas), peripheral neurofibromas, and a spinal schwannoma were provided by the Cooperative Human Tissue Network, part of the National Disease Research Interchange. Additional NF-2 tumors were kindly provided by Vincent Riccardi (NF Institute); neuroblastoma tumors were kindly provided by Roger Kennett (University of Pennsylvania). RNA from tumor samples was isolated with RNAzol, according to recommendations made by the supplier (Cinna-Biotecx), except that frozen tumor tissue was thinly sliced with a sterile scalpel just prior to homogenization. In some cases, these RNA samples were treated with RNase-free DNase I (Pharmacia) to eliminate contaminating genomic DNA, using protocols derived from the supplier. Northern and Southern blots were prepared according to previously published protocols (39, 40), modified slightly to include hybridization of nitrocellulose filters in the presence of heparin (41). cDNA fragments used as probes on Northern and Southern blots were labeled using random oligonucleotides (42). Probes used in these studies consisted of either the full-length 1.1-kb mac25 cDNA, or bases 146–567 of the 2.0-kb mac30 cDNA, or the full-length 300-bp macP1 cDNA. RNA filters were washed in 0.3X SSC (0.045 M NaCl-0.0045 M sodium citrate)-0.1% SDS at 55°C. Chromosomal mapping studies utilized DNA from the National Institute of General Medical Sciences human/rodent somatic cell hybrid mapping panel no. 1 (Coriell Institute for Medical Research), along with DNA from the chromosome hybrid VII-2 HAT, which contains a single human translocation chromosome containing sequences from 17q21-qter (32). DNA blots were washed in 0.1X SSC-0.1% SDS at 65°C.

#### cDNA Library Construction and Subtraction Library Construction

cDNA libraries were generated in the plasmid vector pcDNAII (Invitrogen) from poly(A)+ RNA from primary cultures of leptomeningeal cells and the meningioma cell line T2898, according to standard protocols (38). These libraries were randomly and oligo dT primed and were size selected above 100 bp. Single-stranded cDNA was isolated from these libraries using the M13 rescue technique (43) with the phage R408 using protocols from the pcDNAII manufacturer, with the following modifications: 200 µl of each unamplified cDNA library were used to inoculate 20 µl of LB broth-50 mg/ml ampicillin-10 µM magnesium sulfate and incubated with shaking to an absorbance of A600 = 0.3. This was inoculated with 1 x 10¹³ plaque-forming units
of R408 helper phage and incubated with shaking at 37°C for 30 min. Then 80 ml of LB-ampicillin-10 mg/ml magnesium sulfate were added, and this was incubated with shaking for 6-9 h. All manipulations of single-stranded DNA were carried out using siliconized plasticware (Sigma).

Double-stranded DNA was removed from single-stranded preparations using a magnesium-phenol extraction and restriction endonuclease digestion protocol, as described (44). Biotinylation of single-stranded DNA from the tumor library using photobiotin acetate (Sigma), and removal of excess biotin, was performed as detailed (44). To make the subtraction libraries, 2 µg of LMC single-stranded cDNA were added to 60 µg of biotinylated T2898 tumor cDNA, along with 250 ng of a biotinylated 350-bp piece of DNA that is present as an insert only in nonrecombinant forms of the vector pcDNAII (Invitrogen). These DNAs were ethanol precipitated together, resuspended in 5 µl of dH2O, and added to 5 µl of 2× hybridization solution [1 nM NaCl-100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.6-4 mM EDTA-80% deionized formamide (44)]; this solution was heated to 100°C for 3 min, snap cooled, and incubated at 52°C for 2 h. Subtraction of nonrecombinant and excess biotinylated sequences was performed exactly as described (45), except that 100 µg of yeast tRNA (GIBCO/BRL) were used as a carrier to aid in precipitation of ethanol. Precipitated cDNA, enriched for LMC-specific sequences, was either incubated with T7 promoter primer and Klenow polymerase (Pharmacia) to generate double-stranded DNA and used to transform competent DH5αF' cells (GIBCO/BRL) to generate a plasmid subtraction library (38), or was amplified by PCR for use in generating a phage subtraction library.

The phage subtraction library was generated by PCR amplification of nonsubtracted sequences with primers specific for the Sp6 and T7 promoters of the pcDNAII vector. PCR was carried out in a 100-µl reaction that was 25 mM Tris-HCl, pH 9.5, 50 mM potassium chloride, 10 mM magnesium chloride, 0.2 mM each dCTP, dATP, dGTP, and dTTP, 5 ng/µl each primer, and 3 units of Hot Tub polymerase (Amersham), using the following program: 94°C for 60 s, 40°C for 60 s, and 72°C for 120 s for 35 cycles, followed by 5 min at 72°C. Amplified sequences were extracted with phenol-chloroform (1:1), digested with XmnI to remove vector sequences, as recommended (New England Biolabs), and blunt ended with Klenow polymerase (38). EcoRI adapters (Stratagene) were ligated to recovered cDNA (38); unligated adapters were eliminated after separation on a 1% agarose gel (Seakem FMC). This cDNA was isolated from agarose using a gel extraction system (Qiagen) and resubtracted with 60 µg of biotinylated tumor cDNA exactly as outlined above.

Doublely subtracted cDNA was amplified by PCR as above with the EcoRI adaptor-specific oligo 5'-CGCTAGGATTCGGCACGAG-3' using the following program: 94°C for 60 s, 32°C for 60 s, and 72°C for 120 s for 15 cycles, followed by 94°C for 60 s, 60°C for 60 s, 72°C for 120 s for 25 cycles, and 5 min at 72°C. This PCR product was digested with EcoRI as recommended (New England Biolabs) and used to generate a phage subtraction library in Lambda Zap II (Stratagene) following protocols furnished by the supplier.

Subtraction cDNA Library Screening. Duplicate lifts of the phage subtraction library were differentially screened using radiolabeled cDNA from LTAg2B or T2898 cells that was generated as described (46), except that 7 µl of random hexamers (Pharmacia; 90 a260 units/ml) and 3 µg of oligo-dT (Pharmacia) were used to prime reverse transcription, and 500 µCi of [32P]dCTP were added (Amersham; >3000 Ci/mmol). Duplicate filters were hybridized at 5 × 105 cpm/ml of hybridization solution, washed at 55°C in 0.3× SSC-0.1% SDS, and exposed to Kodak XAR-5 film for 12-36 h.

Subtracted probes, used to screen the plasmid T2898 subtraction library, were generated by hybridizing radiolabeled cDNA from LTAg2B cells to a 5-fold excess of poly(A)+ RNA from the T2966 cell line and subtracting as outlined above. After subtraction, precipitated cDNA was treated at 45°C for 20 min with 0.5 nM NaOH, reprecipitated with ethanol, and used directly as a probe. Filters were hybridized at >1 × 105 cpm/ml, washed at 55°C in 0.3× SSC-0.1% SDS, and treated with a Proteinase K solution to reduce background hybridization (47); these filters were exposed on Kodak XAR-5 film for 21 days.

Reverse Transcription-Polymerase Chain Reaction. Five µg of total RNA were reverse transcribed with murine Moloney leukemia virus reverse transcriptase using conditions recommended by the supplier (GIBCO/BRL) in a 30-µl reaction, using 4 µl of 90 a260/ml random hexamers (Pharmacia) as primers. One µl of this reaction was used in a 100-µl PCR reaction. PCR was performed as described above using the following program: 94°C for 60 s, 60°C for 60 s, and 72°C for 60 s for 20 cycles, followed by 72°C for 10 min. Samples were extracted with phenol-chloroform (1:1) and ethanol precipitated before electrophoresis on 1% agarose; for mac30, these gels were blotted overnight and hybridized as described (40). Primers for mac30 were 5'-GCTGGTGAACTGGTGGCCACGAA-3' (antisense) and 5'-TAGGGGTCTCCGCAATGAAA-3' (sense). Primers for actin were 5'-CTCAATAGGTGCGTGGCCG-3' (sense) and 5'-CAGGGCTCCAGAGGATGCG-3' (antisense). Primers for the human estrogen receptor, kindly provided by C. Richard Lytle (University of Pennsylvania), were 5'-GGACATGAGAGCCGACAC-3' (sense) and 5'-CCAGCACAGTGGAGATC-3' (antisense). All reactions were monitored for contamination with genomic DNA by inclusion of a control incubated without reverse transcriptase.

cDNA Library Screening and Sequence Analysis of cDNA. cDNA libraries screened for larger cDNA clones included a skin fibroblast cDNA library (Stratagene) and a HeLa cDNA library (courtesy of Thomas Kadesch, University of Pennsylvania). DNA sequencing was performed using the dideoxy chain termination method of Sanger et al. (48), with Sequenase enzyme (U.S. Biochemical). Sequence analyses and database searches were performed using the Wisconsin GCG program (49).

Nucleotide Sequence Accession Numbers. Nucleotide sequence data for the 1.1-kb mac25 cDNA and the 2.0-kb mac30 cDNA were submitted to the GenBank database and assigned the accession numbers L19182 and L19183, respectively.

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References


