91°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min. The probe was purified from unincorporated nucleotides using a NAP™-5 Column (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The specific activity of the probe ranged from 0.7–1 × 10^7 counts per minute (cpm)/ng.

Total RNA was purified from the human melanoma cell lines A375 and C8161 by cellular lysis in 4 M guanidinium isothiocyanate (GITC) followed by a CsCl gradient (2) and then separated on a 1% agarose gel containing 1.1% formaldehyde. The RNA was transferred to Hybond™-N paper (Amersham Pharmacia Biotech), UV fixed and pre-hybridized in QuikHyb Hybridization Solution (Stratagene, La Jolla, CA, USA). Equal loading of the RNA was assessed by ethidium bromide staining of the agarose gel (data not shown). Ten nanograms or approximately 300 μL of the purified probe were added, hybridized and washed as per the manufacturer’s instructions. The probe generated a single band by Northern blot analysis for both the A375 and C8161 cell lines of approximately 8 kb in size, which is in agreement with the published MCSP cDNA sequence (Figure 1). The relative intensity of the bands for the different cell lines can be explained by the fact that A375 cells express much more of the MCSP protein than the C8161 cells (Figure 2).

The timesavings associated with this technique are significant in that all that is needed to generate a DNA probe for Northern blot analysis is the DNA sequence of interest. It is not necessary to generate a purified dsDNA template by reverse transcription (RT)-PCR or by cloning to make a probe. This should save days if not weeks of probe preparation time. It has proven to be an effective method in our laboratory for a number of other target mRNA species for Northern analysis. In addition, we believe this labeling technique should have wide spread utility for probes for Southern blots and in situ hybridization.

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Method to Distinguish Between the MMAC1/PTEN Gene and Its Pseudogene in RT-PCR Analysis of Point Mutations

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The candidate tumor-suppressor gene MMAC1/PTEN, a dual-phosphatase gene with homology to the cytoskeletal protein tensin and to auxilin, is frequently inactivated by homozygous deletions and mutations in a variety of advanced cancers including brain, prostate, breast, thyroid and endometrium cancers and malignant melanoma (1–3,5,6,12–16). Germ-line mutations in the MMAC1/PTEN gene give rise to Cowden disease, which is typified by the formation of multiple benign tumors and increased susceptibility to malignant cancers (7,9) and were also detected in patients with Bannayan-Zonana syndrome (8) and juvenile polyposis coli (10).

In most of these studies, mutational analysis of MMAC1/PTEN was performed by polymerase chain reaction (PCR) amplification of exon sequences (using intronic primers), followed by detection of mutated exons with aberrant mobility by single-stranded conformation polymorphism (SSCP) or denaturing gradient gel electrophoresis (DGGE) analysis. Suspected mutant exons were subjected to sequence analysis (1–3,5–10,12,14–16). This approach is cumbersome because one must amplify all nine exons of the MMAC1/PTEN gene and analyze each of them in a multistep process. An alternative and more efficient approach to screen for mutations in the coding region is based on the analysis of MMAC1/PTEN mRNA. The procedure includes reverse transcription (RT) of the mRNA into first-strand cDNA, followed by PCR amplification of the MMAC1/PTEN coding region using unique primers and direct sequencing of the PCR product.

However, this strategy is prone to mistakes due to the presence of an intron-less MMAC1/PTEN pseudogene,
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on chromosome 9p21-q13 [located on bacterial artificial chromosomes (BACs) 145c22 and 188122] (3,4). In addition, minute amounts of contaminating genomic DNA in the RT-PCR can result in amplification of the intronless MMAC1/PTEN pseudogene. The authors of the only previous studies using RT-PCR to screen for MMAC1/PTEN mutations were unaware of the existence of a MMAC1/PTEN pseudogene, and some of the mutations they reported reflect sequence differences between MMAC1/PTEN and the pseudogene (13,16).

Here, we present a fast and reliable strategy for distinguishing between MMAC1/PTEN and its pseudogene in RT-PCR-based analysis of mutations. Our strategy is based on sequence differences between the MMAC1/PTEN gene and its pseudogene (16) and is illustrated in Figure 1. The sequence differences allowed us to selectively digest the pseudogene cDNA, without digesting the MMAC1/PTEN cDNA, and to distinguish between the genes by size differences using gel electrophoresis.

In our procedure, the coding regions of MMAC1/PTEN and the pseudogene were reverse-transcribed into two overlapping cDNA fragments: fragment 1 (602 bp) and fragment 2 (752 bp) (Figure 1A). For PCR amplification and sequence analysis of MMAC1/PTEN cDNA fragments, we used the following primers: forward primer F-1, 5′-AGCTTCTGCCATCTCTCTCC-3′ (starting at nucleotide 985) (GenBank® Accession No. U92436) (14), and reverse primer R-1, 5′-GATTCTTAACAGGTAGCTA-3′ (starting at nucleotide 1587). For PCR amplification and sequence analysis of the fragment 2 MMAC1/PTEN cDNA, we used the following primers: forward primer F-2, 5′-AGGGAGTAACTATTCCCAGTC-3′ (starting at nucleotide 1525) and reverse primer R-2, 5′-TGGTGTTTATCTCCTCTTGA-3′ (starting at nucleotide 2277). In each case, as a control for a successful RT of the RNA, we amplified a 626-bp β-actin cDNA fragment, using previously published primers that do not amplify the β-actin pseudogene (data not shown) (11). Restriction enzyme digestion of fragment 1 with NsiI and fragment 2 with PfI MI cleaves only the pseudogene (Figure 1A).

Next, we tested the ability of our procedure to distinguish between MMAC1/PTEN and its pseudogene in 3 bladder carcinoma cell lines, UC-13 (Figure 1B, lanes 1, 4, 7 and 10), UC-11 (Figure 1B, lanes 2, 5, 8 and 11) and UC-3 (Figure 1B, lanes 3, 6, 9 and 12). Total RNA was extracted from each of the cell lines using TRIzol® Reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer’s protocol. For RT-PCR, 1–2 µg of total RNA was first treated with 10 U of DNase I, RNase-free (Boehringer Mannheim, Indianapolis, IN, USA) at 37°C for 30 min in 1× PCR buffer (20 mM Tris-HCl, pH 8.4 and 50 mM KCl), 2.5 mM MgCl2 and 20 U of RNasin® Ribonuclease Inhibitor (Promega, Madison, WI, USA) in a total vol of 10 µL. This treatment eliminated the residual genomic DNA. One microliter of 25 mM EDTA was added, and the mixture was incubated at 68°C for 15 min to inactivate the DNase I. The RNA was then reverse-transcribed in the presence of 1 mM dithiothreitol (DTT), 50 ng of random hexamer, 0.5 mM each of the four deoxytriphosphate nucleotides and 200 U of SuperScript™ II RNase H- Reverse Transcriptase in total vol of 20 µL according to the manufacturer’s protocol (Life Technologies). To remove residual RNA, which can interfere with the PCR amplification, 1 µL of Escherichia coli RNase H

Figure 1. Discrimination between MMAC1/PTEN cDNA and its pseudogene. (A) The overall strategy of selective digestion of the MMAC1/PTEN pseudogene cDNA. The procedure is initiated by RT of the mRNA followed by PCR amplification of two overlapping cDNA fragments. NsiI and PfI MI can cleave only MMAC1/PTEN pseudogene cDNA fragments. (B) MMAC1/PTEN and its pseudogene expression in three bladder carcinoma cell lines; UC-3 (lanes 3, 6, 9 and 12), UC-11 (lanes 2, 5, 8 and 11) and UC-13 (lanes 1, 4, 7 and 10). M, molecular weight markers, φX174 DNA-HaeIII digest.
DNA denaturation at 94°C). The amplification consisted of forward and reverse primers and 2.5 U of each dNTP, 50 pmol of each of the forward and reverse primers and 2.5 U of Taq DNA Polymerase (Life Technologies, Gaithersburg, MD, USA). Each 50-μL PCR amplification mixture contained 2 μL of the reverse-transcribed, first-strand cDNA in 1× PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂); 200 μM each dNTP, 50 pmol of each of the forward and reverse primers and 2.5 U of Taq DNA Polymerase (Life Technologies, Gaithersburg, MD, USA). In this sequencing procedure, an average of 800 bp of DNA is sequenced using the PE BigDye® Terminator Cycle Sequencing Kit (BIO 101, Vista, CA, USA) and analyzed with a Model 373 DNA Analysis System (both from PE Applied Biosystems, Foster City, CA, USA). In this sequencing procedure, an average of 800 bp of DNA is sequenced in a single step.

Overall, when both the MMAC1/PTEN gene and its pseudogene are expressed, this procedure allows fast discrimination between MMAC1/PTEN and its pseudogene and efficient (100%) detection of point mutations in the MMAC1/PTEN coding region by sequence analysis of two overlapping cDNA fragments.

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Use of a Promoterless Renilla Luciferase Vector as an Internal Control Plasmid for Transient Co-Transfection Assays of Ras-Mediated Transcription Activation

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One problem in studies of gene regulation is selecting an appropriate internal control for transfection efficiency and extract preparation whose expression remains constant during treatments that cause changes in the expression of the gene being studied. This is an especially difficult problem with studies involving activated ras, because most internal control reporters are driven by viral promoters and almost all of these are transactivated by oncogenic ras or other nonnuclear

Figure 1. Ras-mediated transactivation of the human MCSF receptor promoter using either pRL-0 or pRL-CMV as an internal control plasmid. (A) The MCSF receptor promotor in pRL-0 and the CMV promotor in pRL-0 are both activated by oncogenic ras, whereas the promoterless vector pRL-0 is not. For transactivation assays, 2 x 10^5 CV1 cells (CCL-70; ATCC, Rockville, MD, USA) were plated in 4 mL of Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in 60-mm tissue culture plates 18 h before transfection. Cells were transfected by the calcium phosphate method as previously described with: (i) the reporter plasmid: 10 µg of the promoterless firefly luciferase vector pXP2; 10 µg of the 159-bp human MCSF receptor promoter (pMCSFR) (-88 to +71 with respect to the major monocytic transcription start site) in pXP2; 0.002 µg of the CMV promotor in the firefly luciferase vector pXP2; 0.002 µg of an internal control plasmid using a CMV promotor subcloned in pRL-0 (Renilla luciferase gene driven by the CMV promotor) (11) or 0.2 or 0.8 µg of the pRL-0 vector for Renilla luciferase, which lacks eukaryotic promototer and enhancer elements upstream of Renilla luciferase; (ii) 1 µg of the expression vector: oncogenic pMT3-H-Ras(L61) or the empty expression vector; and (iii) sheared salmon sperm DNA to a total of 15 µg DNA per plate. The medium was changed after 16 h, and firefly luciferase activities for pXP2 alone and pCMV in pXP2 and Renilla luciferase activities for pCMV in pRL-0 and pRL-0 alone were determined 40 h after the initiation of the transfection protocol with the Dual-Luciferase™ Reporter Assay System (Promega). Results were normalized for extract protein content and are given as mean plus standard error of the mean (SEM) of at least 6 independent experiments. (B) Using pRL-0 as an internal control plasmid results in an accurate calibration of the 4-fold transactivation of the MCSF receptor promotor by activated ras, whereas pRL-CMV cannot be used as an internal control. Firefly luciferase activities of pMCSFR in pXP2 were normalized to the Renilla luciferase values of either pCMV in pRL-0, 0.2 µg pRL-0 or 0.8 µg pRL-0 (data from Panel A).