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Genetics

In vitro loss of heterozygosity targets the *PTEN/MMAC1* gene in melanoma

GAVIN P. ROBERTSON*†, FRANK B. FURNARI*, MARY E. MIELE‡, MICHAEL J. GLENDENING§, DANNY R. WELCH¶, JANE W. FOUNTAIN§, TRACY G. LUGO‖, H.-J. SU HUANG*,**, AND WEBSTER K. CAVENEE*,**††‡‡

*Ludwig Institute for Cancer Research, **Department of Medicine, ††Center for Molecular Genetics and ‡‡Cancer Center, University of California-San Diego, La Jolla, CA 92093-0660; ‡Department of Medical Technology, University of Delaware, Newark, DE 19716; §Institute for Genetic Medicine, University of Southern California, Los Angeles, CA 90033; ¶Jake Gittlen Cancer Research Institute, Penn State University, Hershey, PA 17033-0850; and ‖Cancer Diagnosis Program, National Cancer Institute, Rockville, MD 20892

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ABSTRACT **Gross genetic lesions of chromosome 10 occur in 30–50% of sporadic human melanomas. To test the functional significance of this observation, we have developed an *in vitro* loss of heterozygosity approach in which a wild-type chromosome 10 was transferred into melanoma cells, where there was selection for its breakage and regional deletion to relieve its growth suppressive effects. The overlap of these events was at band 10q23, the site of the recently isolated *PTEN/MMAC1* tumor suppressor gene, suggesting it as a potential target. Although the gene was expressed in the parental cells, both of its chromosomal alleles contained truncating mutations. *In vitro* loss of heterozygosity resulted in loss of the chromosomally introduced wild-type *PTEN/MMAC1*, and ectopic expression of the gene caused cell growth suppression. Thus, this approach identified *PTEN/MMAC1* as a target in malignant melanoma and may provide an alternative means to localizing tumor suppressor genes.**

The mapping and identification of genes that suppress the growth of tumor cells has taken three principal approaches (1). The first approach is genetic linkage mapping and positional cloning, which has had many successes, such as the identification of the *BRCA1* and *BRCA2* genes (2), but which is unsuited for cases with genetic heterogeneity of predisposition, or for genes that primarily exert their effects in the later stages of disease progression. The second approach is a combination of cytogenetic (3) and molecular genetic (4, 5) approaches that search for deletions, translocations, isodisomy, monosomy, or mitotic recombination, which lead to chromosomal regions carrying homozygous or hemizygous mutant alleles of a growth suppressor gene. Although this approach is suitable for both sporadic and inherited tumors, the inference of growth suppressor gene inactivation is necessarily indirect. The third approach is cell fusion between normal and tumor cells coupled with cytogenetic and molecular analyses of rare tumorigenic segregants from suppressed cells (6). This approach has the handicap of severe gene dosage problems caused by the presence in the hybrid cell of two complete genome sets and the analytical complications this imposes. More focused adaptations of this approach use single chromosome transfer (7), and more elegantly, transfer of subchromosomal fragments (8, 9). Although the latter strategy does provide functional information, it is limited by the intensive labor required to generate the fragments, their individual transfer into recipient cells, and the analysis of resultant clones for growth or tumor suppression. Here we report an approach to identify genomic regions containing tumor suppressor gene(s), which uses cytogenetic and molecular analysis of tumor cells that have been selected for their escape from chromosome-mediated growth suppression. This method has the major advantage of allowing the isolation of many clones from an isogenic background and requiring only one chromosome transfer. We further show that the method, termed *in vitro* loss of heterozygosity (IVLOH), mirrors genetic alterations that occur *in vivo* and allows the localization and identification of target genes.

Approximately 90% of melanomas occur in patients without any previous familial history of melanoma tumors (10). Genetic lesions of chromosome 10 have been detected in 30–50% of these early and advanced-stage melanomas (11, 12), usually as chromosome loss leading to monosomy (11, 13). Although segmental deletions of 10q are relatively rare, they have been useful in defining a common region of loss involving the distal third of 10q (14, 15), which has been further refined by LOH to 10q22-qter (12). This region includes the recently identified *PTEN/MMAC1* tumor suppressor gene that maps to 10q23.3 and that is deleted or mutated in a proportion of a wide variety of advanced tumors (16, 17), including 30–40% of melanoma cell lines (18, 19). However, it has not been demonstrated that chromosome 10, much less the 10q23-qter segment or the *PTEN/MMAC1* gene, has functional growth-suppressing capability in melanomas.

The IVLOH system we describe clearly shows that the 10q23-qter region has growth suppressive abilities, that the *PTEN/MMAC1* gene is at least one of the genes in this region responsible for the growth suppression, and that this gene is targeted for loss during IVLOH. This approach could be of general use for localizing certain genes that suppress tumor cell growth and may be especially useful for tumors without a defined inheritance pattern, those with heterogeneity in genetic predisposition, or for genes that are involved at later stages of tumor progression.

MATERIALS AND METHODS

Cell Culture Conditions and Chromosome Transfer. The melanoma cell lines used for these studies were derived from secondary tumors and have been described previously (20, 21). The HA(10)A cell line was generously provided by Ann M. Killary (University of Texas-M.D. Anderson Cancer Center, Houston). Normal human embryonal lung MRC-5 fibroblasts were obtained from the American Type Culture Collection. Human melanocytes were obtained from Clonetics (Walkersville, MD). Microcell-mediated chromosome transfer was as previously reported (20).

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Abbreviations: IVLOH, *in vitro* loss of heterozygosity; FISH, fluorescense *in situ* hybridization; HA, hemagglutinin A.

†To whom reprint requests should be addressed at: Ludwig Institute for Cancer Research, San Diego Branch, CMME-3080, 9500 Gilman Drive, La Jolla, CA 92093-0660. e-mail: gprobertson@ucsd.edu.

Cytogenetic and Microsatellite Analysis. G-banded metaphase spreads were prepared according to standard cytogenetic protocols (22). Either interphase nuclei or unbanded metaphase spreads were used for fluorescense *in situ* hybridization (FISH). Routine FISH protocols and strategies for labeling of probes were used (23). BAC 60C5 was obtained from Research Genetics (Huntsville, AL), and DNA to be used as a probe was extracted according to their protocols. Primers for microsatelite analysis were purchased from Research Genetics or synthesized from sequence data available in published reports or through the On-line Genome Data Base.

Vector Construction and Cloning of *PTEN*/*MMAC1* Alleles. Construction of the wild-type *PTEN*/*MMAC1* and *PTEN*/*MMAC1*-hemagglutinin A (HA) constructs has been described (24). The *PTEN*/*MMAC1* mutant allele Y76Stop-HA was created by using a similar strategy except that it was synthesized by using PCR primers (FF2A: 5′-CCCAAGCTTTGGGATCCGAATTCTACTCCCAGACATGACAGCCATC paired with 903Y76S: 5′-CTAGCCCATGGTCATGTCTTTCAGCACAAAGATT) from cDNA derived from UACC 903 cells. PCR conditions were 60°C for annealing and 72°C for extension for 35 cycles using *Taq* DNA polymerase. The PCR product was subcloned into the *Hin*dIII/*Nco*I sites of pBS-HA, subcloned into the *Eco*RI site of pBluescriptSK$^-$ to generate the Y76Stop-HA mutant construct and completely sequenced.

RNA Analysis. RNA was isolated by using the Trizol reagent (GIBCO/BRL). Northern blot hybridizations were with ^{32}P-labeled *PTEN*/*MMAC1* or 18S rRNA probes. The protocol for the RNase protection assay has been described (24).

Protein Truncation Test (PTT) and Sequencing of *PTEN*/*MMAC1* cDNAs. A TNT T7 Quick Coupled Transcription/Translation reaction kit (Promega), with the addition of 0.3 mM magnesium acetate, was used for PTT. PTT products were resolved through 15% discontinuous SDS/PAGE gels. *PTEN*/*MMAC1* cDNAs were directly sequenced by using an Applied Biosystems automated sequencer.

Transfection Assays. Transfections were performed by using TransIT-LT1 polyamine transfection reagents (PanVera, Madison, WI) for the UACC 903 cells and the calcium phosphate transfection method (25) for C8161.Cl9 cells. Transfections were performed in triplicate dishes and in a minimum of 4–5 independent experiments. Briefly, 48 h before transfection UACC 903 and C8161.Cl9 cells were seeded at 1.1×10^6 and 3×10^5 cells per dish, respectively. For transfection, 10 μg of pBP, pBP-*PTEN*/*MMAC1*, pBP-*PTEN*/*MMAC1*-HA, or pBP-*PTEN*/*MMAC1*-Y76Stop-HA were used per 10-cm dish. Transfections were terminated at 6 h, and postransfection cells were split at a 1:3 dilution the next day and maintained for 7 days in 5% DMEM containing puromycin (Calbiochem, La Jolla, CA; UACC 903 at 400 ng/ml and C8161.Cl9 at 500 ng/ml). Cells were counted on the seventh day by using a hemocytometer.

RESULTS

Functional Mapping by IVLOH. We reasoned that a chromosome carrying a wild-type allele of a trans-acting growth suppressor gene might be incompatible with growth *in vitro* and that, by analogy with LOH (4, 5), cells would use mechanisms involving chromosomal loss or rearrangement to eliminate it. If these mechanisms occurred randomly among cells, the result would be an outgrowth of a number of clones derived from a single isogenic background with overlapping alterations that would be useful for mapping purposes, and which would be analogous to the many tumors needed for LOH mapping (26) or the transfer of many chromosomal subfragments.

To test this possibility, we used microcell-mediated chromosome transfer to introduce a normal chromosome 10 into two independently isolated melanoma cell lines, UACC 903 and C8161.Cl9. Before transfer, each cell line was extensively characterized to determine its endogenous chromosome 10 content by G-band analysis, FISH using chromosome 10 painting, and a centromere-specific probe (D10Z1) as well as genotypic analysis using microsatellite markers spanning the length of chromosome 10. UACC 903 cells contained two cytogenetically intact copies of chromosome 10 that were homozygous for all 48 markers (10 from 10p and 38 from 10q) examined, suggesting that they had lost one parental copy of chromosome 10 and duplicated the other, resulting in uniparental disomy. In contrast, C8161.Cl9 cells contained one intact copy and a second that had a deletion from 10q25-qter. Five markers in the 10q25-qter region also supported the presence of only a single copy of this region because in each case only a single allele was present. In contrast, seven of 11 loci examined in the 10q21–24 region of chromosome 10 were heterozygous in the C8161.Cl9 cell line, suggesting the presence of two distinct copies proximal to the 10q25 region.

Hybrids containing an intact copy or a portion of the transferred chromosome 10 were established for both cell lines. An intact chromosome 10 had been introduced into each of the four UACC 903 microcell hybrids; partial G-band karyotypes showed the presence of three cytogenetically intact copies of chromosome 10 in a proportion of cells from each of the four microcell hybrids, indicating that these clonally derived cell lines initially contained three intact copies of chromosome 10 (Fig. 1*A*). Chromosome painting, G-band analysis, and limited molecular genotyping showed only fragments of the transferred chromosome 10 in three C8161.Cl9 microcell hybrids. An example of this latter analysis for the marker *D10S1739* is shown for hybrid Cl9(10n)2 (Fig. 1*B*). In addition to *D10S1739*, donor alleles were present for markers *D10S1687*, *D10S215*, *D10S1765*, *D10S677*, *D10S1735*,

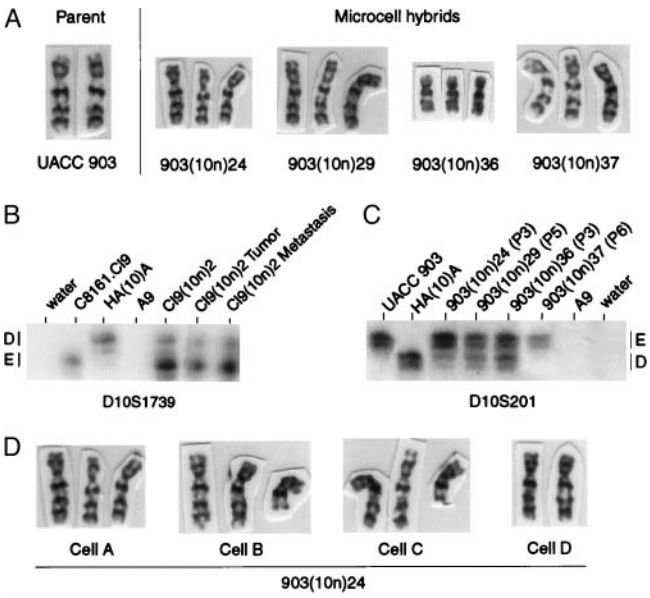

FIG. 1. Genetic analysis of microcell hybrids. (*A*) Partial karyotypes, derived from the four UACC 903 microcell hybrid cell lines, show the presence of three intact copies of chromosome 10 after chromosome transfer. (*B*) *D10S1739* marker analysis for microcell hybrid Cl9(10n)2. The donor allele for *D10S1739* was present in the original hybrid line and in a tumor and metastasis that derived from this hybrid. *D* and *E* indicate the locations of the donor and endogenous alleles, respectively. (*C*) *D10S201* polymorphism analysis of UACC 903 microcell hybrids. A donor-derived *D10S201* allele was present in hybrids 903(10n)24, 29, and 36 but the band intensity was reduced for hybrid 903(10n)24. (*D*) Fragmentation of the donor chromosome in microcell hybrid 903(10n)24. Examples of four independent partial metaphase spreads showed either an intact (Cell A), partially deleted (Cells B and C), or absent donor chromosome 10 (Cell D). Cells B and C are examples where breakage of the donor chromosome occurred at band 10q23.

D10S185, and *D10S173*, suggesting that microcell hybrid C19(10n)2 retained portions of the transferred chromosome, including the 10q23–24 region and at least a portion of the p-arm (10p15) containing the selectable neo marker. The transferred 10q23–24 segment, containing the *PTEN/MMAC1* gene, in this hybrid appeared stable in culture as well as during growth *in vivo*, as donor alleles were present in material derived from a tumor and a metastasis after growth in nude mice, and so the fragment appeared to either not contain a tumor suppressor or to contain one(s) that did not suppress the *in vivo* growth of these cells (Fig. 1*B*).

In contrast, genetic analysis of UACC 903 microcell hybrid cells showed that the introduced chromosome 10 underwent breakage and deletion over time in cell culture. The identity of the chromosome that had undergone loss was determined by microsatellite marker analysis (example in Fig. 1*C*); a cytogenetic depiction is shown in Fig. 1*D*. The donor-derived allele for marker *D10S201*, which maps to 10q22.3, was present in three of four hybrids but was absent from hybrid 903(10n)37 at passage 6. Band intensity differences suggested that the *D10S201* donor allele was present at slightly reduced levels in hybrid 903(10n)24 and analysis of markers *D10S1744*, *D10S215*, *D10S1735,* and *D10S1765*, in the 10q23 region, showed barely detectable donor alleles, indicating its presence in only a low percentage of cells. This finding provided a molecular localization for the breakpoint between *D10S201* and *D10S215* in the 10q22–23 region of the donor chromosome.

To determine the extent of loss undergone by the introduced chromosome 10, a minimum of 20 metaphase spreads were examined from each microcell hybrid after various times in culture. Several metaphase spreads contained intact copies of the introduced chromosome whereas others had undergone breakage at different locations along the length of the chromosome. An example of the product of this evolution is shown in Fig. 1*D* for hybrid 903(10n)24 and the extent of fragmentation increased with time in culture (Table 1). For hybrid 903(10n)29 and 903(10n)37, the percentage of cells with an intact transferred chromosome 10 dropped from 85% to 10% and 100% to 0% over 14 and six passages, respectively. Increasing numbers of karyotypes showed progressive loss of 10q. However, three chromosome 10 centromeres were observed in approximately 80% of late-passage hybrid cells, after D10Z1 FISH analysis, indicating retention of at least the centromere and p-arm.

Stable cell lines that had lost most of the introduced chromosome 10q were established by subcloning 903(10n)37 cells. In all cases the heterozygosity observed immediately after chromosomal transfer was lost (i.e., a partial reversion to homozygosity or partial IVLOH). Twenty-one stable subclones were analyzed for 34 informative loci along the length of chromosome 10 and were homozygous for all markers distal to *D10S581*, which maps to 10q21, indicating nearly complete loss of 10q. Certain of the subclones only retained the p-arm and the centromeric region of chromosome 10.

Characterization of the chromosome breakpoints and areas of deletion along the introduced chromosome were used to determine where the initial region of breakage and deletion occurred, and then to identify the minimal region of loss, which extended from 10q23 to 10qter (Table 1) and included the *PTEN/MMAC1* locus. No breakpoints were identified distal to the 10q23 region but were observed in each chromosomal band proximal to this region. Thus, this IVLOH system allowed the generation of several chromosomal breakpoints for elimination of regions containing genes incompatible with continued cell growth. These converged on a region observed to be deleted for *in vivo* melanoma tumors with an initial region of breakage occurring at band 10q23.

Candidacy of *PTEN/MMAC1* in IVLOH. Because the IVLOH chromosome breakage and loss data implicated the region of chromosome 10 encoding *PTEN/MMAC1,* subsequent studies focused on the candidacy of this gene as a functional melanoma growth suppressor and target of IVLOH. FISH analysis, using the BAC 60C5 that encompasses the *PTEN/MMAC1* gene, showed that UACC 903 and C8161.Cl9 cells contained signals for *PTEN/MMAC1* on each chromosome 10 at 10q23.3, as well as on each chromosome 9 (27) (Fig. 2*A*). Both cell lines also exhibited RNA expression patterns similar to those of normal human melanocytes and MRC-5 fibroblasts (Fig. 2*B*) and similar to those published previously (24). To examine this more carefully, cDNA from each cell line was sequenced and used as a template for coupled *in vitro* transcription and translation to search for point mutations, especially those leading to truncated protein products. C8161.Cl9 cells contained full-length wild-type sequence whereas UACC 903 had two identical mutant copies of *PTEN/MMAC1.* Both copies contained point mutations that convert a tyrosine codon to a stop (Y76Stop), severely truncating the protein at amino acid 76. Partial sequence showing the point mutation is illustrated in Fig. 2*C*. *In vitro* transcription and translation (Fig. 2*D*) yielded wild-type-sized protein (i.e., approximately >47 kDa) from the C8161.Cl9 cells, but not from the mutant allele-containing UACC 903 cells where a protein of approximately 31 kDa was seen to increase in intensity. This species likely represents one that initiates at methionine residue 134 (which also contains an appropriate Kozak sequence) downstream from the stop codon caused by the mutation.

Microcell hybrids were examined after transfer to determine whether *PTEN/MMAC1* was transiently expressed and selected against during passaging. At different cell passages after chromosome transfer, cDNA was isolated, sequenced, and evaluated by *in vitro* transcription and translation assays. Fig. 2*C* shows that both wild-type and mutant *PTEN/MMAC1* sequence were found in three of the four UACC 903 microcell hybrids. Sequence profiles of these three hybrids are shown at

Table 1. Cytogenetic losses occurring on the donor chromosome 10 in UACC 903 microcell hybrids

		Region of donor chromosome retained					
Microcell hybrid line	Passage number	Intact 10	10pter-q23	10pter-q22	10pter-q21	10pter-q11	None
903(10n)24	3	2/24	5/24	3/24	7/24	4/24	3/24
903(10n)29	4	17/20	1/20	0/20	1/20	1/20	0/20
	14†	2/20	2/20	0/20	15/20	1/20	0/20
903(10n)36	6	1/20	0/20	3/20	5/20	6/20	5/20
903(10n)37	3	20/20	0/20	0/20	0/20	0/20	0/20
	6	0/20	0/20	0/20	8/20	12/20	0/20
	17†	0/22	0/22	0/22	3/22*	3/22	2/22

*Fourteen of 20 metaphase spreads contained a copy of chromosome 10 translocated at 10q21.

†Chromosome 10 painting was used to confirm the cytogenetic analysis; all 903(10n)29 (P14) cells contained two intact copies of chromosome 10 and a chromosome 10 fragment that was completely painted. 903(10n)37 (P17) cells contained two intact copies of chromosome 10 and a translocated chromosome 10 fragment in 72% of the cells examined.

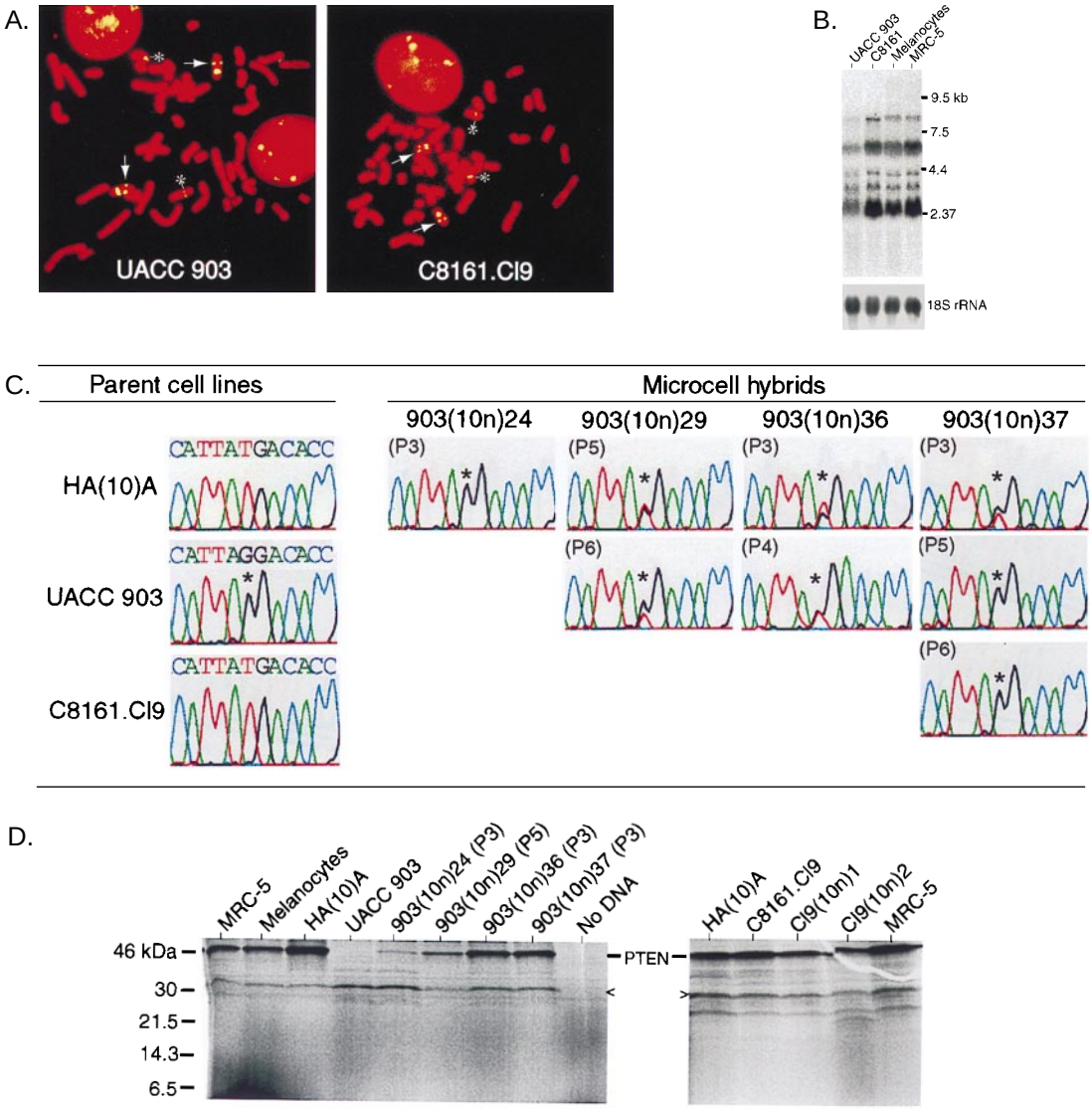

FIG. 2. Status of *PTEN/MMAC1* in cell lines and microcell hybrids. (*A*) FISH analysis shows the presence of the chromosome 10 centromere and the *PTEN/MMAC1* gene (arrow) in both copies of chromosome 10 derived from parental UACC 903 and C8161.Cl9 cell lines. * indicates the signals on chromosome 9. (B) Northern blot showing *PTEN/MMAC1* expression patterns in UACC 903, C8161.Cl9, human melanocytes, and MRC-5 fibroblast cells. (*C*) *PTEN/MMAC1* sequence profiles (from nucleotides 223 to 234) derived from each cell line or microcell hybrid line. The wild-type profiles for HA(10)A and C8161.Cl9 cells are shown for comparison. * shows the location of the T to G point mutation occurring in UACC 903 cells. Similar profiles are shown for each of the four UACC 903 microcell hybrids and for hybrids 903(10n)29, 36, and 37 at later passages (P number refers to cell passages). (*D*) Generation of an *in vitro* transcription and translation *PTEN/MMAC1* product. A full-length ^{35}S-labeled protein product was produced from cDNA derived from MRC-5 fibroblasts, melanocytes, HA(10)A donor cells, and all four UACC 903 microcell hybrids. The arrow shows the location of a 31-kDa species predicted to originate from methionine 134, which is associated with the next Kozak consensus sequence downstream of the truncating mutation. An 8-kDa truncated *PTEN/MMAC1* product that might have arisen from the truncating mutation was not observed in UACC 903 cells. Also shown are the full-length *PTEN/MMAC1* proteins produced from C8161.Cl9 cells and two microcell hybrids.

different passages. Consistent with the karyotype and microsatellite marker data, later passages showed reduced amounts of wild-type sequence. Microcell hybrid 903(10n)37 quickly lost all expression of *PTEN/MMAC1* during growth in culture. A full-length protein product was detected by *in vitro* transcription and translation for early passages of all four hybrids, although only a small amount was observed for hybrid 903(10n)24 (Fig. 2*D*). In contrast, full-length *PTEN/MMAC1* was present and expressed in the C8161.Cl9 parent cell line and in both hybrids (Fig. 2*D*), consistent with the genetic analysis described above.

Direct Functional Evidence Implicates *PTEN/MMAC1* in Melanoma. The foregoing experiments showed a consistent correlation between endogenous *PTEN/MMAC1* mutations, continued growth, IVLOH, and loss of wild-type *PTEN/MMAC1*. We next directly tested whether the *PTEN/MMAC1* gene could actually function as a growth suppressor in melanoma cells and whether the mutation present in UACC 903 cells eliminated such activity. Vectors containing full-length *PTEN/MMAC1* or mutant *PTEN/MMAC1* truncated at codon 76, each tagged with HA, were transfected into both C8161.Cl9 and UACC 903 cells. After puromycin selection, surviving cells were counted 7 days posttransfection. HA-tagged or native wild-type *PTEN/MMAC1* caused dramatic reductions (80–90%) in the number of UACC 903 cells (Fig. 3*A*), whereas vector alone or the truncated and HA-tagged mutant allele had no effect on their growth. In sharp contrast, little effect was seen on the number of surviving cells after transfer of *PTEN/MMAC1* into C8161.Cl9 cells, which were endogenously wild type (Fig. 3*B*). RNase protection assays showed comparable expression of the transfected *PTEN/MMAC1* or truncated mutant construct in cells 2 days posttransfection

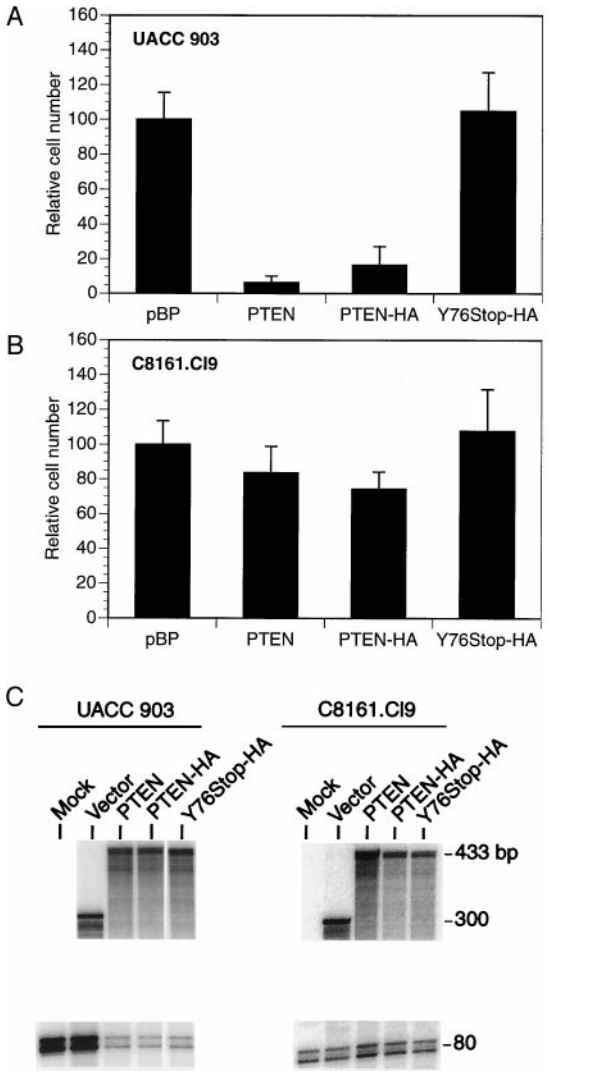

FIG. 3. Growth suppression after transfection of *PTEN/MMAC1* into human melanoma cell lines UACC 903 and C8161.Cl9. Empty vector (pBP), or vector containing wild-type *PTEN/MMAC1*, *PTEN/MMAC1-HA*, or mutant allele Y76stop-HA were transfected into (*A*) UACC 903 (mutant *PTEN/MMAC1* background) or (*B*) C8161.Cl9 (wild-type *PTEN/MMAC1* background), selected with puromycin for 7 days, and then counted. The number of cells remaining in the vector control was set at 100%, and all other cell numbers were normalized to this value. The results shown are combined from four separate experiments, each performed in triplicate; bars, SD. Similar results were obtained by using different plasmid preparations. (*C*) Ribonuclease protection analysis of RNA levels of exogenous *PTEN/MMAC1* in UACC 903 and C8161.Cl9 transfected cells. The protected band at 433 bp represents an exogenous *PTEN* fragment that at 300 bp is a vector fragment and that at 80 bp is an 18S RNA fragment.

(Fig. 3*C*). Western blotting of extracts from parallel transfections probed with anti-HA antibodies showed expression of the exogenous HA-tagged full-length *PTEN/MMAC1* protein products in the transfectants. The HA-tagged mutant protein product was not detected in the transfectants, despite being well transcribed (Fig. 3*C*), perhaps because of instability of the truncated product (Fig. 2*D*). UACC 903 transfectants could not be used for *in vivo* tumorigenicity assays because wild-type *PTEN/MMAC1* expression was rapidly lost from stably selected mass populations. Thus, wild-type *PTEN/MMAC1* acts as a potent growth suppressor in melanoma cells harboring endogenous mutant *PTEN/MMAC1* alleles (i.e., UACC 903 cells), but not in those with endogenous wild-type alleles (i.e., C8161.Cl9 cells).

DISCUSSION

We have exploited chromosome transfer technology to develop a system that mirrors *in vitro* the pattern of chromosome 10 breakage and deletion that occurs in 30–50% of sporadic malignant melanomas (11, 12). The UACC 903 cell line used to establish this model had lost a copy of chromosome 10, with duplication of the remaining one. Therefore, any mutations or deletions would be present in both copies of chromosome 10, supporting the two-hit model for tumor suppressor genes (5, 28). Under these conditions, introduction of a wild-type chromosome would restore a copy of the mutated or deleted melanoma growth suppressor gene. Because this condition might not favor *in vitro* growth, we postulated that the microcell hybrids would lose portions of the introduced chromosome, thereby defining a region(s) containing the candidate tumor suppressor gene(s). In fact, the introduced wild-type chromosome 10 underwent evolution in culture, as postulated, and chromosome breakage and loss centered on the 10q23-qter region. Because cytogenetic and LOH studies of melanoma tumors have documented deletions involving the distal third of 10q (11, 12), the IVLOH results were reflective of the *in vivo* situation. However, monosomy is the predominant alteration of chromosome 10 in melanomas (13, 29). Because our UACC 903 microcell hybrids eventually lost most of 10q it is possible that the whole chromosome might have been lost if retention of the selectable neomycin marker, which was inserted at 10p15, had not been required for hybrid cell survival. In fact, fragments of the transferred chromosome were undetectable in some karyotypes. These cells likely retain a small neo-containing fragment translocated onto another chromosome that was not cytogenetically detectable. Therefore, IVLOH appears to provide a functional and experimentally malleable method to localize and identify certain tumor suppressor genes, or loci that play a role in tumor progression rather than predisposition.

Because the *PTEN/MMAC1* gene was located at the chromosomal band where breakage occurred and within the deleted IVLOH region of chromosome 10, we hypothesized that it might be one of the tumor growth suppressors functioning in this subset of sporadic melanomas. By using Northern blotting, cDNA sequencing, and *in vitro* transcription and translation, we found that human melanocytes expressed *PTEN/MMAC1,* and both UACC 903 and C8161.Cl9 cell lines expressed comparable levels of RNA. However, UACC 903 cells contained two copies of *PTEN/MMAC1* with an identical truncating point mutation within exon 4 at codon 73 of the tensin/auxilin/phosphatase homology domain, which essentially eliminates the entire catalytic region of the dual-specificity phosphatase. Mutations within the *PTEN/MMAC1* phosphatase domain from patients with Cowden's disease (30, 31), Bannayan-Zonana syndrome (32), and several tumor types (16, 17), including melanomas (18, 19), are predicted to disrupt phosphatase function. A recent report has confirmed these predictions by showing significant decreases of *in vitro* phosphatase activity by certain of these mutants (33). Therefore we postulate that endogenous mutant alleles that disrupt the dual-specificity phosphatase domain might eliminate functional *PTEN/MMAC1* in melanomas.

In contrast, the C8161.Cl9 cell line, representative of the 60–70% of sporadic melanomas that express wild-type *PTEN/MMAC1*, stably maintained a transferred 10q23–24 region of chromosome 10 containing an additional copy of the *PTEN/MMAC1* gene. This finding might indicate the possibility that additional melanoma genes reside on this chromosome, that genes on chromosome 10 were not involved during the evolution of the C8161.Cl9 tumor, or that unidentified members of the *PTEN/MMAC1* pathway are nonfunctional in this cell line. Consistent with the first possibility, the region distal to 10q25 is missing from one copy of chromosome 10 in

C8161.Cl9 cells and might encode a gene(s) relevant in this particular tumor type. In addition, LOH studies of gliomas (34–36) and prostate cancers (37) have detected several regions of loss on chromosome 10, supporting the possibility of multiple suppressors on this chromosome. Furthermore, a role for loci on 10p is implicated in growth suppression because hybrid UACC 903 cells retaining only the short arm exhibit a somewhat decreased growth rate (unpublished data). Growth suppression also has been reported after the transfer of 10p or portions of 10p into both prostate and glioma cell lines (38, 39).

The validity of the assertion that the *PTEN/MMAC1* gene is a specific suppressor of melanoma cell growth was shown through transfection of ectopic *PTEN/MMAC1*. None of the constructs affected the growth of C8161.Cl9 cells, consistent with the microcell hybrid results, further eliminating a role for *PTEN/MMAC1* in the evolution of this particular melanoma tumor cell type. This finding is also consistent with published results that document no observable effect on cell growth, after transfection of *PTEN/MMAC1* into glioma cells that express endogenous wild-type *PTEN/MMAC1* (24). In contrast, transfection of *PTEN/MMAC1* into UACC 903 cells dramatically inhibited growth, causing an 80–90% reduction in cell number. Consistent with the observations from the UACC 903 microcell hybrids, the only surviving transfectant cells did not express wild-type *PTEN/MMAC1*.

This report establishes a functional role for genes on human chromosome 10 in the development of human malignant melanomas. The transfer of a single copy of human chromosome 10, followed by the progressive *in vitro* loss of regions of this chromosome, particularly distal to 10q23, provided insight to the genes involved. One of the major candidate genes implicated by the IVLOH system was the dual-specificity phosphatase, *PTEN/MMAC1.* Because UACC 903 cells contained two mutant copies of *PTEN/MMAC1* and wild-type *PTEN/MMAC1* expression was lost from UACC 903 microcell hybrids and transfectants during culture, its involvement in a subset of sporadic human melanomas lacking endogenous wild-type activity is strongly suggested. In contrast, not all melanomas contain *PTEN/MMAC1* mutations and the C8161.Cl9 cell line represents an example from this category in which additional exogenous *PTEN/MMAC1* expression does not appear to have an dramatic biological effect.

Thus this study establishes an IVLOH model system for localizing candidate tumor suppressor genes, implicates *PTEN/MMAC1* as a putative gene in this model system, and provides functional evidence suggesting the involvement of *PTEN/MMAC1* in the development of as many as 30–40% of sporadic human malignant melanomas.

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