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The isolation and characterization of a novel cDNA demonstrating an altered mRNA level in nontumorigenic Wilms' microcell hybrid cells

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ABSTRACT

Wilms' tumor, a pediatric nephroblastoma, has been associated with genetic alterations of the 11p13 and 11p15 regions. The introduction of a der(11) chromosome into the G401 Wilms' tumor cell line has been shown previously to revert the tumorigenic phenotype. A subtractive cDNA/RNA hybridization performed between the tumorigenic parent (G401) and a nontumorigenic microcell hybrid of G401 (110.1/G401.1) containing the der(11) chromosome resulted in the identification of a single novel cDNA clone, designated QM. The cDNA is 745 nucleotides in length and encodes a predicted hydrophilic 25 kd basic protein, primarily consisting of alpha helices. The QM transcript is expressed in a wide variety of embryonic and adult tissues and demonstrates a down regulation of expression in adult kidney and heart. QM is also a member of a multigene family members of which map to chromosomes 6 and 14. The QM mRNA level is modulated between the tumorigenic and nontumorigenic cell lines and therefore may be involved in the maintenance of the nontumorigenic phenotype.

INTRODUCTION

Wilms' tumor, a pediatric nephroblastoma, originates from embryonal kidney stem cells and presents itself in either a classical form or a differentiated form[1]. The classical form appears to be composed of embryonic blastema cells, while the differentiated form may contain a variety of cell types, including: striated muscle, squamous epithelial, and cartilaginous. Wilms' tumor has also been observed to occur in either a familial or sporadic form, with the sporadic form comprising >95% of all Wilms' tumor incidence[2]. Cytogenetic analyses of Wilms' tumors from pateints with the WAGR (Wilms' tumor, aniridia, genital urinary abnormalities, and mental retardation) syndrome have identified a specific interstitial deletion of 11p13 and a loss of heterozygosity (LOH) of surrounding DNA markers. Because of the evidence of the LOH it is assumed that the region encodes a tumor suppressor gene whose inactivation is required for progression to the malignant state. A candidate 11p13 Wilms' tumor suppressor gene cDNA, termed WT-1, has been isolated[3,4] and was found to be expressed during normal kidney development[5]. However, RFLP analyses of sporadic Wilms' tumors have also indicated a LOH of DNA markers in the 11p15 region in the absence of any detectable alteration in the 11p13 region, suggesting the involvement of a second genetic locus in the etiology of Wilms' tumor[6,7], termed WT-2.

Functional confirmation for the existence of Wilms' tumor suppressive genetic material on chromosome 11 has been achieved by Weissman *et al.* (8). This group transferred a single selectable human der(11)t(X;11)(q26;q23) chromosome into a tumorigenic Wilms' tumor cell line, G401.6TG.6, via the microcell mediated chromosome transfer technique (MMCT; 9,10). The G401 cell line (11), isolated from a three month old male with Wilms' tumor, is undifferentiated and ultrastructurally has the appearance of epithelial-like cells. The cell line is pseudodiploid, with a single marker chromosome involving chromosome 12, and contains no cytogenetically detectable deletion or rearrangement of either chromosome 11 homologue. All of the resulting microcell hybrids contained the der(11) chromosome by cytogenetic analysis and were found to be nontumorigenic when assayed in nude mice. Segregation of the introduced der(11) chromosome resulted in reexpression of the tumorigenic phenotype. To eliminate the possibility that tumor suppressive information for Wilms' tumor resided on the Xq region of the der(11) chromosome and to determine whether the

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suppression of tumorigenicity was specific to chromosome 11, an X chromosome and chromosome der(13) were introduced into G401.6TG.6. The resulting microcell hybrids remained tumorigenic when assayed in nude mice. These results correlate the specific introduction of the der(11) chromosome with suppression of tumorigenicity in the G401 Wilms' tumor cell line and reexpression of the tumorigenic phenotype with its segregation. Dowdy *et al.* (12) have recently demonstrated that the genetic material suppressing the tumorigenic phenotype of the G401 Wilms' tumor cell line resides solely in the 11p terminal region, presumably due to the WT-2 gene, and specifically excludes the involvement of the WT-1 gene at 11p13. In this study, a subtractive cDNA/mRNA hybridization between the closely paired nontumorigenic Wilms' microcell hybrids (110.1/G401) and the tumorigenic Wilms' parent (G401.6TG.6) was performed in an attempt to isolate cDNAs copies of genes which demonstrated an altered mRNA level that correlated with the suppression of tumorigenicity.

MATERIALS AND METHODS

Cell lines and culture conditions

The tumorigenic G401.6TG.6 and 110.1/G401.2.6TG.1 cell lines and the nontumorigenic 110.1/G401.1, -.2, -.5 microcell hybrids have been previously described (8). Briefly, the G401.6TG.6 cell line is a HPRT-derivative of the G401 Wilms' tumor cell line (ATCC) originating from a three month old male with Wilms' tumor. Three nontumorigenic microcell hybrids, 110.1/G401.1, -.2, and -.5, were derived from G401.6TG.6 by transferring a human der(11)t(X;11)(q26;q23) chromosome into the cells via the microcell-mediated chromosome transfer technique (9,10). The 110.1/G401.2.6TG.1 tumorigenic segregant was generated from 110.1/G401.2 by selection for the segregation of the der(11) chromosome via the addition of 10 μg/ml 6-thioguanine (6TG) to the regular growth medium (DMEM plus 10% FCS). All of the cell lines used during the course of this study were maintained in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS, Hazleton) plus 2 mM L-glutamine, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 0.05 μg/ml fungizone (amphotericin B). Biochemical selection of the der(11)t(X;11)(q26;q23) chromosome, which retains the hypoxanthine guanine phosphoribosyl transferase (HPRT) gene located at Xq26, was performed by the addition of 100 μM hypoxanthine, 40 μM methotrexate, and 1.6 mM thymidine (HAT) to the medium (13). All of the cell lines were routinely assayed for the presence of *Mycoplasma* (14) by cultural methods and/or by the 4,6-diamidino-2-phenylindole (DAPI) method (15), and were routinely found to be negative by both methods.

Southern and northern blot analysis

Twenty microcgrams of genomic DNA was digested with 8−12 units of restriction endonuclease (Boehringer Mannheim Biochemicals) in the manufacturer's buffer at 37°C overnight, and separated by electrophoresis through a 0.8% agarose slab gel in 1×TAE buffer (40 mM Tris-pH 7.8, 5 mM NaAcetate, and 1 mM EDTA) and the DNA was transferred to nitrocellulose and hybridized to heat-denatured ^{32}P-labeled DNA at 65°C overnight (55°C for evolutionary blot) as described (16). The filters were rinsed twice in 2×SET/0.2% SDS and washed in a large volume of 2×SET/0.2% SDS (5×SET [150 Tris-pH 8.0, 0.75 M NaCl, and 5 mM EDTA) at 65°C for 20 min, then in 0.1×SET/0.1% SDS at 65°C (55°C for evolutionary blot) for 1 hr. DNA probes were labeled by the method of Feinberg and Vogelstein (17).

Total cellular RNA was isolated from semiconfluent T-150 flasks by the guanidine hydrochloride method as described (16) or isolated from embryonic tissues as described (18). Polyadenylated RNA was isolated by passing the RNA over an oligo(dT)-cellulose (Type III, Collaborative Research) column (16). Poly A+ RNA used for subtractive cDNA/RNA hybridizations and cDNA library construction was passed through a second oligo(dT)-cellulose column after ethanol precipitation. Two micrograms of polyA+ RNA were separated by electrophoresis through a 1.4% formaldehyde-agarose gel, transferred to nitrocellulose, and hybridized to heat-denatured ^{32}P-labeled DNA probe or ^{32}P-labeled oligonucleotide probe in 1×Denhardt's, 5×SSC, 50 mM phosphate buffer-pH 7.0, and 50% deionized formamide at 42°C overnight. The filters were then rinsed twice in 2×SET/0.2% SDS and washed in 2×SET/0.2% SDS at 50°C for 20 min then in 0.1×SET/0.1% SDS at 50°C for 30 min. The tumorigenic and nontumorigenic Wilms' tumor cell lines and microcell hybrid RNA levels were normalized by rehybridizing the filters with a ^{32}P-labeled human glyceraldehyde-3-phosphate dehydrogenase (GAPDH/G3PD) cDNA probe (19). Densitometeric analysis was performed with a LKB densitometer. The RNA levels in developmental and tissue specific Northern blots were rehybridized with ^{32}P-labeled murine G3PD cDNA or murine alpha-actin cDNA (18).

Subtractive cDNA/RNA hybridization and library screening

Subtractive cDNA/mRNA hybridizations were performed essentially by the method of Davis *et al.* (20). Briefly, 2 micrograms of ^{32}P labeled 110.1/G401.1 single stranded (ss) cDNA were hybridized to 20 micrograms of poly A+ RNA from G401.6 in 0.5 M phosphate buffer-pH 7.0, 5 mM EDTA, and 0.1% SDS at 68°C for 16−20 hr. The ss cDNA was separated from the double stranded (ds) cDNA/mRNA duplexes by hydroxylapatite chromatography (HAP; BioRad), concentrated, and rereacted with a ten-fold excess of G401.6 polyA+ RNA. The HAP column flow-through (FT) was used either directly as a probe or to generate a subtracted cDNA librbary. 110.1/G401.2 cDNA used for subtracted library construction was labeled with 50 μCi of alpha dGTP, treated as described above, and then made double stranded by priming with random primers in the presence of Klenow enzyme, followed by the addition of *E. coli* DNA pol I (BMB). Eco RI linkers were ligated to the ds cDNA, digested with Eco RI, and the cDNA fragments were cloned into the Eco RI site of lambda gt10. The resulting subtracted cDNA library, 7.25scgt10, contained 7×10^5 PFU.

2×10^3 PFU of 7.25scgt10 per 150 mm plates containing LB plus 10 mM $MgSO_4$ and 0.2% maltose were screened by differential hybridization in quadduplicate (21,22). The lifts were hybridized and washed as described above, exposed to XAR5 film for 1, 3, and 7 days, and compared visually to identify potential positive plaques. The purified phage DNA from putative positives was digested with Hind III and Bgl II to release a 1.4 kb fragment encompassing the cDNA insert. The DNA sequence was determined by the chain-termination method of Sanger *et al.* (23) with the use of a modified T7 DNA polymerase (US Biochemical) and both strands of the DNA were sequenced at least three times for all clones sequenced. A full length QM cDNA was isolated by screening the G-tailed pcDB cDNA library (24) generated from the WI-38 human fibroblast cell line with

^{32}P-labeled 42gt10 oligomer derived from the unique sequence of the cDNA insert in the 1.4 kb Hind III-Bgl II fragment excluding the poly A tract. Primer extension was performed as previously described (25).

RESULTS

Subtractive cDNA hybridization

The differential hybridization of 1.2×10^5 PFU from the 7.25scgt10 subtracted cDNA library resulted in the identification of a single differentially expressed plaque. The plaque was isolated and DNA from a plate lysate was prepared. The insert size of the clone was assumed to be small, between 50 and 500 bp, due to the degradation of the cDNA during the subtractive hybridization procedure and therefore, a 1.4 kb Hind III-Bgl II fragment encompassing the cDNA insert was isolated and sequenced. The cDNA insert was determined to be 64 bp in length including a putative polyadenylation signal (AATAAA) followed by 15 nucleotides and then 22 adenine nucleotides. The DNA sequence of the insert was compared against the entire GEN:EMBL database and no entries of any significant sequence identity were determined.

A 42mer oligonucleotide, designated 42gt10, was synthesized to the negative strand of the cDNA isolate corresponding to the 42 nucleotides of unique sequence (excluding the poly A tract), ^{32}P-labeled, and hybridized to a Northern blot containing 20 micrograms RNA per lane from G401.6TG.6, 110.1/G401.1, -.2, -.5, and 110.1/G401.2.6TG.1 (Fig 1). The results demonstrated that an approximately 750 base transcript, designated QM, was specifically hybridizing to the 42gt10 probe, and that the level of the transcript was increased to a similar extent in all three of the nontumorigenic microcell hybrids (110.1/G401.1, -.2, -.5) compared to the tumorigenic parent (G401.6TG.6) and segregant (110.1/G401.2.6TG.1) cell lines. The filter was stripped and re-hybridized with ^{32}P-labeled DNA from the constitutively expressed gene GAPDH (Fig. 1B). Based on densitometeric scannings, the relative differences between the cell lines was calculated, using the tumorigenic parent G401.6TG.6 to normalize the results between each cell line (Fig. 1C). The level of QM mRNA was increased between two- to four-fold in the nontumorigenic 110.1/G401 microcell hybrids and returned to approximately 1× in the tumorigenic segregant 110.1/G401.2.6TG.1 when compared to the tumorigenic parental G401.6TG.6 cell line. These results suggest a correlation between the increased level of QM mRNA and the nontumorigenic phenotype.

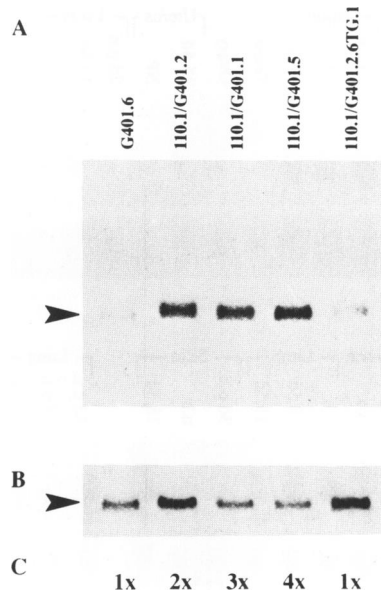

Figure 1. Expression of QM in tumorigenic parent and segregant, and nontumorigenic microcell hybrids. A: Twenty micrograms of total RNA from G401.6, 110.1/G401.1, -.2, -.5, 110.1/G401.2.6TG were separated by gel electrophoresis, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled oligonucleotide probe corresponding to position 744−703 of the negative strand of QM (refer to figure 2) which detects a 745 nucleotide transcript. B: The nitrocellulose filter was stripped and rehybridized with ^{32}P-labeled GAPDH which detects a 2.2 kb transcript. C: The relative differences between the cell lines was calculated based on densometeric scanning of panels A and B.

Isolation of the QM cDNA

^{32}P-end labeled 42gt10 oligomer was used to screen 2.4×10^5 colonies from the pcDB cDNA library. Seven independent positives were identified and isolated. Four isolates remained positive after subsequent secondary screening with ^{32}P-end labeled 42gt10. Bam HI restriction endonuclease digestion of

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   1                                                                  59
   CTCTTTCCCTT CGGTGTGCCA CTGAAGATCC TGGTGTCGCC ATG GGC CGC CGC CCC GCC
                                                MET GLY ARG ARG PRO ALA

 60                                                                  113
 CGT TGT TAC CGG TAT TGT AAG AAC AAG CCG TAC CCA AAG TCT CGC TTC TGC CGA
 ARG CYS TYR ARG TYR CYS LYS ASN LYS PRO TYR PRO LYS SER ARG PHE CYS ARG

 114                                                                 167
 GGT GTC CCT GAT GCC AAG ATT CGC ATT TTT GAC CTG GGG CGG AAA AAG GCA AAA
 GLY VAL PRO ASP ALA LYS ILE ARG ILE PHE ASP LEU GLY ARG LYS LYS ALA LYS

 168
 GTG GAT GAG TTT CCG CTT TGT GGC CAC ATG GTG TCA GAT GAA TAT GAG CAG CTG
 VAL ASP GLU PHE PRO LEU CYS GLY HIS MET VAL SER ASP GLU TYR GLU GLN LEU

 222                                                                 275
 TCC TCT GAA GCC CTG GAG GCT GCC CGA ATT TGT GCC AAT AAG TAC ATG GTA AAA
 SER SER GLU ALA LEU GLU ALA ALA ARG ILE CYS ALA ASN LYS TYR MET VAL LYS

 276                                                                 329
 AGT TGT GGC AAA GAT GGC TTC CAT ATC CGG GTG CGG CTC CAC CCC TTC CAC GTC
 SER CYS GLY LYS ASP GLY PHE HIS ILE ARG VAL ARG LEU HIS PRO PHE HIS VAL

 330                                                                 383
 ATC CGC ATC AAC AAG ATG TTG TCC TGT GCT GGG GCT GAC AGG CTC CAA ACA GGC
 ILE ARG ILE ASN LYS MET LEU SER CYS ALA GLY ALA ASP ARG LEU GLN THR GLY

                                                                     437
 ATG CGA GGT GCC TTT GGA AAG CCC CAG GGC ACT GTG GCC AGG GTT CAC ATT GGC
 MET ARG GLY ALA PHE GLY LYS PRO GLN GLY THR VAL ALA ARG VAL HIS ILE GLY

 438                                                                 491
 CAA GTT ATC ATG TCC ATC CGC ACC AAG CTG CAG AAC AAG GAG CAT GTG ATT GAG
 GLN VAL ILE MET SER ILE ARG THR LYS LEU GLN ASN LYS GLU HIS VAL ILE GLU

 492                                                                 545
 GCC CTG CGC AGG GCC AAG TTC AAG TTT CCT GGC CGC CAG AAG ATC CAC ATC TCA
 ALA LEU ARG ARG ALA LYS PHE LYS PHE PRO GLY ARG GLN LYS ILE HIS ILE SER

 546                                                                 599
 AAG AAG TGG GGC TTC ACC AAG TTC AAT GCT GAT GAA TTT GAA GAC ATG GTG GCT
 LYS LYS TRP GLY PHE THR LYS PHE ASN ALA ASP GLU PHE GLU ASP MET VAL ALA

 600                                                                 653
 GAA AAG CGG CTC ATC CCA GAT GGC TGT GGG GTC AAG TAC ATC CCC AGT CGT GGC
 GLU LYS ARG LEU ILE PRO ASP GLY CYS GLY VAL LYS TYR ILE PRO SER ARG GLY

 654                                                                 706
 CCT CTG GAC AAG TGG CGG GCC CTG CAC TCA TGA GGGCTTCCAA TGTGCTGCCC
 PRO LEU ASP LYS TRP ARG ALA LEU HIS SER ***

 707                                 744
 CCCTCTTAAT ACTCACCAAT AAATTCTACT TCCTGTCC (A)n
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Figure 2. DNA sequence and predicted peptide sequence of the QM cDNA insert in p201.3. The complete cDNA sequence was determined by dideoxy sequencing and the deduced coding strand is represented. The predicted peptide sequence is listed below the cDNA sequence with the stop codon denoted by ***. The predicted polyadenylation signal sequence is denoted by underlining and the polyadenylated tailed denoted by $(A)_n$.

plasmid DNA from each clone determined that two clones, 2A-2 and 2B-3, contained the largest inserts. Clone 2A-2 was arbitrarily chosen for further analysis. ^{32}P-labeled 2A-2 DNA specifically hybridized to an approximately 750 base transcript and demonstrated the identical increase in mRNA level in the nontumorigenic microcell hybrids as the 42gt10 oligomer (data not shown). Partial DNA sequence analysis identified the presence of the original 42 bases from the subtracted cDNA clone. Primer extension, using an oligonucleotide primer (QM5P2, 28mer, position 97 to 69) derived from the partial DNA sequence of the 5′ end of 2A-2, resulted in a cDNA of 97 nucleotides in length (data not shown) which corresponded to the first nucleotide of the 2A-2 cDNA insert (Fig 2). To facilitate the DNA sequencing of the QM cDNA insert in 2A-2, the cDNA insert was subcloned into the Bam HI site of pUC18 including approximately 140 bp of 5′ and approximately 70 bp of 3′ flanking plasmid DNA due to the tailing. Both strands of the QM cDNA were sequenced a minimum of three times. The complete full length nucleotide sequence of the QM cDNA insert in p201.3 is presented in Figure 2.

The coding strand was determined based on the ability of the QM5P2 and 42gt10 oligonucleotide probes to hybridize to the QM transcript in Northern blot analysis. The cDNA is 745 nucleotides in length, and contains an ATG at position 42 within a sequence closely related to the Kosak (26) translational initiating consensus sequence. The putative AUG is followed by a translational open reading frame of 214 codons encoding a putative peptide of 24.7 kD and terminates with an opal termination codon at position 683. The polyadenylation signal, AATAAA, occurs at position 724 followed by 15 nucleotides prior to the polyadenylation track 44 nucleotides in length. The ogriginal 42 nucleotides of the 42gt10 oligomer correspond to position 745 to 703. The QM cDNA sequence or sequences related to it were not present in the GEN:EMBL database (release 66.0; 12-15-90).

The putative peptide contains 22 arginine and 23 lysine basic residues, and 10 aspartic acid and 10 glutamic acid residues, giving a calculated pI near 12. The basic and acidic residues are spread throughout the length of the peptide. The peptide contains 8 dispersed cysteine residues that may be capable of forming intra- and/or inter-molecular disulfide bonds to stabilize the protein structure. The peptide was also analyzed for putative active site motifs which resulted in the identification of no known active sites. A SV40 type of nuclear localization signal occurs from residues 37 to 42. Hydropathy (27) values were calculated and plotted for the predicted QM peptide (Fig. 3A). The plot shows a hydrophilic amino terminal sequence consisting of 23 residues, 11 of which are basic. The peptide does not contain an obvious membrane spanning region or membrane associated region, nor does the putative protein contain any glycosylation sites. The secondary structure of the putative peptide has been calculated using both the Chou-Fasman (28) and Garnier-Osguthorpe-Robson (GOR; 29) algorithms (Fig. 3B). Both algorithms predict a peptide composed primarily of three alpha helical regions, interrupted with regions containing strong turn values. The predicted peptide sequence from the QM cDNA sequence was not present in the Protein Identification Resource database (release 26.0; 10-90).

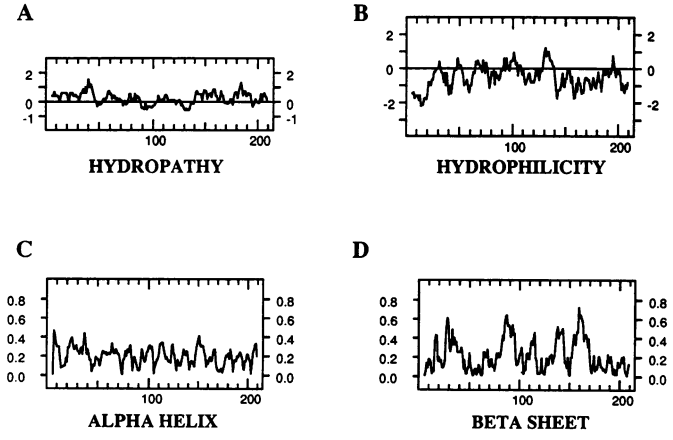

Figure 3. Hydropathy and secondary structure predictions of the putative QM peptide. A: Kyte and Dolittle (KD) hydropathy values were determined from the predicted QM peptide and plotted. B: Antigenic index and flexibilty values were determined using the Hopp and Woods algorithm. C: Alpha helical and D: beta sheets were calculated from the Chou-Fasman (CF) and Garnier-Osguthorpe-Robson (GOR) algoritms and are indicated as increases in the base line for each plot. The numbering of the X-axis is from the initiating methionine residue (1) to the carboxy terminal serine residue (214).

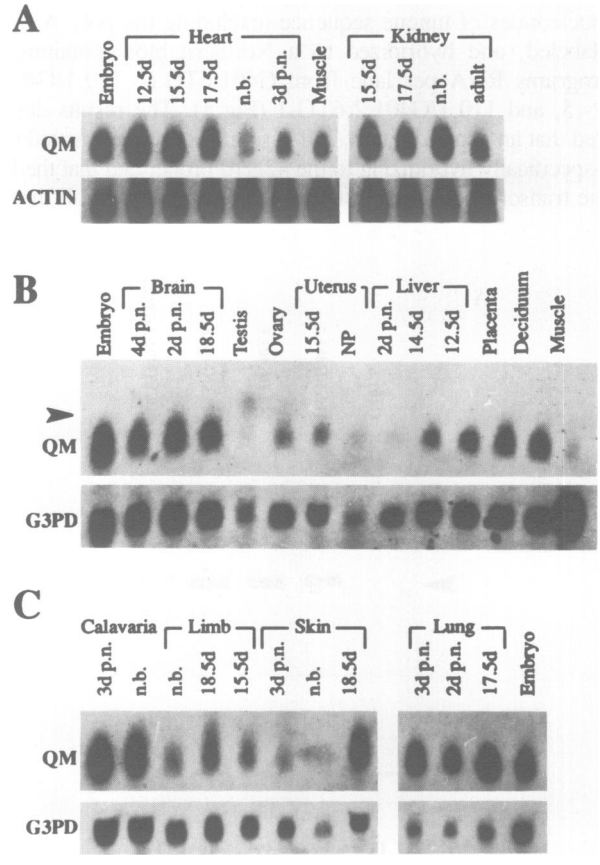

Figure 4. Developmental and Tissue Specific Expression of QM in Mouse Tissues. A, B, C: Twenty micrograms of total RNA isolated from murine heart, kidney, muscle, brain, testis, ovary, uterus, liver, placenta, deciduum, calavaria, limb, skin, lung, and total embryo was hybridized with ^{32}P-labeled QM cDNA. The membranes were then stripped and rehybridized with either alpha-actin or G3PD as designated. Ages are given as days post coitum (d), days postnatal (p.n.), and newborn (n.b.), lanes marked embryo are 16.5d post coitum. The muscle RNA in panel A. is newborn and adult in panel B. NP denotes nonpregnant uterus and the arrow denotes increased QM transcript length in testis.

Developmental tissue specific expression of QM

The developmental and tissue specific expression of the QM gene was determined by Northern blot analysis of RNA isolated from embryonic, newborn, neonatal, and adult murine tissues were hybridized with ^{32}P-labeled QM cDNA. The results from these experiments showed that the QM gene was expressed at different levels in all of the tissues analyzed as early as 8.5 days post coitum, including cardiac and striated muscle, brain, testis, ovary, pregnant and nonpregnant uterus, liver, placenta, deciduum, calavaria, whole limb, skin, lung, kidney and total embryo (Fig. 4). The apparent highest expression of QM mRNA was detected in kidney and cardiac muscle and both of these tissues demonstrated a developmental decrease in the mRNA level of the QM transcript. QM expression was high in embryonic and newborn kidney and was significantly reduced in adult kidney, which is consistent with the origin of the G401.6TG.6 cell line being infant human kidney. QM expression was also high in embryonic cardiac muscle and significantly reduced in newborn and adult cardiac muscle. The 750 base transcript was absent in testis; however, a novel transcript of approximately 1.1 kb in length was present. These results suggest that the level of the QM mRNA is altered in a developmental fashion. In addition, the presence of a larger QM transcript in testis leaves open the possibility of a complex mechanism for gene expression.

Southern blot analysis of QM

^{32}P-labeled QM cDNA was hybridized to a Southern blot containing human and mouse genomic DNA digested with Eco RI or Hind III (Fig. 5). The Southern blot results revealed 14 and 22 different fragments present in human DNA digested with Hind III and Eco RI, respectively (the QM cDNA does not contain sites for either of these enzymes). The Hind III bands ranged in size from 1.2 to >18 kb. These data suggest that the QM gene is either a member of a multigene family, potentially composed of actively transcribed gene(s) and pseudogenes, or that the QM gene contain a large number of small exons. QM also strongly cross hybridized to mouse DNA, suggesting that the QM gene is conserved evolutionarily between mouse and man. The chromosomal map location of QM was attempted using a human-hamster somatic cell hybrid panel digested with Hind III. Due to the multiple genomic copies and high conservation between rodent and man, the chromosomal location of all of the Hind III bands could not be determined at this time. However, two Hind III fragments were mapped, one to chromosome 6 (1.2 kb) and a second (3.5 kb) to chromosome 14 (data not shown).

Evolutionary conservation of QM

To determine the evolutionary conservation of the QM gene, a Southern blot containing genomic DNA digested with Eco RI, representing a range of species from *Drosophila* to man was hybridized with ^{32}P-labeled QM cDNA. The filter contained genomic DNA from *Drosophila*, chicken, mouse, rat, hamster, capuchin, baboon, chimpanzee, and man. The results show the presence of multiple QM sequences in all of the species except chicken and *Drosophila* (Fig. 6). The intensity of the signals is reduced in the rat and mouse lanes compared to the other lanes, potentially suggesting further evolutionary distance of the QM gene between these species and hamster. In addition, all of the lanes containing primate DNA show some conservation of restriction fragment length, especially between man and chimpanzee. These results demonstrate that the QM gene is strongly conserved in all of the mammalian species analyzed as a multigene family.

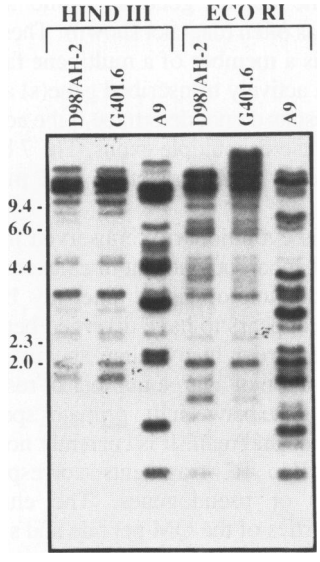

Figure 5. Southern blot analysis of human and mouse DNA demonstrating the presence of multiple DNA fragments containing QM sequences. Twenty micrograms of human and mouse genomic DNA was digested with Hind III or Eco RI, as denoted, and hybridized with ^{32}P-labeled QM cDNA. The markers denote DNA fragment size in kilobases.

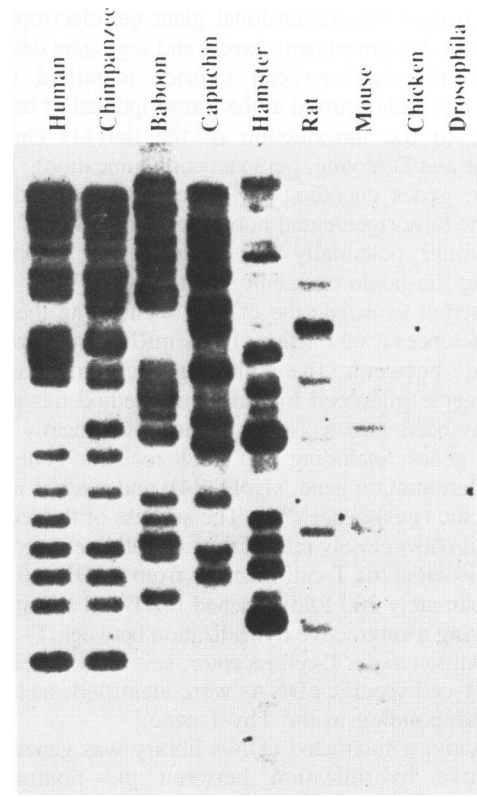

Figure 6. Southern blot analysis demonstrating the evolutionary conservation of QM. Genomic DNA isolated from *Drosophila*, chicken, mouse, rat, hamster, capuchin, baboon, chimpanzee, and man was digested with Eco RI, transferred to nitrocellulose, and hybridized with ^{32}P-labeled QM cDNA.

DISCUSSION

Weissman *et al.* (8) contributed to an understanding of the function of the Wilms' tumor suppressor gene by transferring a der(11) chromosome into the G401 Wilms' tumor cell line via MMCT. The resulting microcell hybrids were nontumorigenic when assayed for tumorigenic potential *in vivo* in nude mice. The specific segregation of the der(11) chromosome from the nontumorigenic microcell hybrids resulted in reexpression of the tumorigenic phenotype. The introduction of an ×chromosome or a chromosome der(13) into G401.6 did not alter its tumorigenic potential. These results directly correlated the introduction of a single copy of normal genetic material specifically present on chromosome 11, with the suppression of tumorigenicity in the G401 Wilms' tumor cell line in an *in vivo* functional assay. Dowdy *et al.* (12) demonstrated that the genetic material suppressing the tumorigenic phenotype of the G401 Wilms' tumor cell line resides solely in the 11p terminal region, presumably due to the WT-2 gene, and specifically excludes the involvement of the WT-1 gene at 11p13.

Although Weissman *et al.* (8) suppressed the tumorigenic potential of the G401 Wilms' tumor cell line by the introduction a human der(11) chromosome, this group was unable to identify an *in vitro* correlate associated with the *in vivo* nontumorigenic phenotype, including differences between the nontumorigenic and tumorigenic cell types in soft agar colony formation, morphology, production of plasminogen activator, fibronectin expression, and proto-oncogene expression. Thus, it appeared that the introduction of a chromosome 11 into Wilms' tumor cells has had little effect on *in vitro* properties previously associated with malignancy (30−32). In fact two-dimensional giant gel electrophoresis of proteins from the tumorigenic parent and segregant cell lines and the nontumorigenic microcell hybrids identified only four alterations that had occurred at the transcriptional or translational levels due to the introduction of the der(11) chromosome (T.Wheeler and D.Young, personal communication). Therefore, any of the genes encoding the observed protein differences between the tumorigenic and nontumorigenic Wilms' microcell hybrids would potentially be of significant importance in maintaining the nontumorigenic phenotype.

In an attempt to isolate the cDNA(s) encoding these limited protein differences a subtractive cDNA/mRNA hybridization was performed between the tumorigenic parental and a nontumorigenic microcell hybrid. This method has previously successfully been used to isolate several biologically important regulatory genes, including the T-cell receptor gene (33); the muscle differentiation gene, *myo*D (34); and a series of growth-arrest-specific (*gas*) genes (34). The success of the technique is dependent on how closely related the two cell lines used. Hedrick *et al.* (33) isolated the T-cell receptor from a cDNA library that was approximately 200-fold enriched for T-cell specific species by performing a subtractive hybridization between T- and B-cell lines. In addition to the T-cell receptor, several other biologically important T-cell specific cDNAs were identified, including one cDNA corresponding to the Thy-1 gene.

In this study, a subtracted cDNA library was generated from a subtractive hybridization between the nontumorigenic 110.1/G401.2 microcell hybrid and the tumorigenic G401.6TG.6 parent which resulted in an approximate 100-fold enrichment for nontumorigenic species specific cDNAs. The screening resulted in the identification and isolation of a single differentially expressed cDNA, designated QM. The QM cDNA contains an open reading frame of 214 residues encoding a putative basic 24.7 kD hydrophilic peptide with no known active site motifs but a potential nuclear localization signal. Secondary structure predictions have identified three alpha helical regions spread throughout the peptide, interrupted by regions containing potential turns.

Developmental Northern blot analysis identified a variety of expression levels of the QM mRNA in different tissues with the highest levels in kidney and cardiac muscle. The most dramatic alteration was observed with the decreased level of the QM transcript in adult kidney and cardiac muscle tissue compared to fetal kidney and cardiac muscle. The alteration of the QM mRNA level in kidney is not an unexpected result given the origin of the G401 parent cell line being infant kidney. The novel 1.1 kb QM transcript detected in testis suggests that a complex mechanism of transcription potentially exists for this gene. The testis transcript could have arisen by a variety of mechanisms such as the splicing of a testis specific exon, the read-through of the polyadenylation signal, or the transcriptional activation of a related member of the QM gene family that has diverged to include additional coding sequences. Lyons *et al.* (18) have noted that the *Vgr*-1 gene of the TGF-Beta gene superfamily encodes a transcript of 1.8 kb in mouse testis; however, the *Vgr*-1 gene also transcribes the normal 3.5 kb transcript in this tissue. Furthermore, the formal possibility exists that the transcripts detected in tissues other than kidney may be due to a related member of the QM multigene family and not the specific QM cDNA isolated during the course of this study.

Southern blot analysis was performed as a first approach to characterize the genomic organization of the QM gene. The results indicated that QM is either composed of a large number of small exons or, more likely, that multiple identical or related copies of QM exist in the genome. Two of the QM Hind III bands map to specific chromosomes, namely 6 and 14, while the location of the remaining 12 Hind III bands is unknown, primarily due to the comigration of hamster QM DNA fragments at the same molecular weight. Southern blot analysis with subfragments of the QM cDNA indicated that 5′ sequences of QM are present in 7 copies in the human genome, while 3′ sequences are represented twice as often (data not shown). These results strongly suggest that QM is a member of a multigene family, potentially composed of both actively transcribed gene(s) and pseudogenes. However, these results do not determine if the actively transcribed gene(s) contain single or multiple exons. The 7 bands hybridizing exclusively to the 3′ subfragment of QM may be processed pseudogenes (36).

The QM gene is evolutionarily conserved in mammals from mouse to man and apparently absent in chicken and Drosophilia. Based on the reduction of signal intensity by Southern blot analysis, the gene appears to have diverged between mouse and rat, and the higher order mammalian species. It is also worth noting the conservation of a large number of restriction fragment lengths of the QM gene between the primates species, particularly between chimpanzee and man. It is currently not known whether these conserved Eco RI fragments correspond to acitvely transcribed genes or pseudogenes. The elucidation of the biochemical properties of the QM peptide and structural features of the QM gene should aid in determining its function and/or involvement in maintaining the nontumorigenic phenotype in the Wilms' tumor microcell hybrids.

In summary, the transfer of a single selectable chromosome into a malignant cell via the microcell mediated chromosome transfer technique has provided an excellent approach to analyze

the biological and molecular nature of tumor suppression in Wilms' nephroblastoma. The use of the subtracted hybridization technique has identified a gene that demonstrates an altered level of mRNA in the nontumorigenic microcell hybrids. The direct or indirect involvement of the QM gene product in maintaining the nontumorigenic phenotype has not yet been determined; however, given the limited number of observed protein differences between the tumorigenic and nontumorigenic phenotypes any gene demonstrating a difference in expression level would presumably be an important gene.

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