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Methylation and Downregulated Expression of mac25/Insulin-like Growth Factor Binding Protein-7 Is Associated with Liver Tumorigenesis in SV40T/t Antigen Transgenic Mice, Screened by Restriction Landmark Genomic Scanning for Methylation (RLGS-M)

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Restriction landmark genomic scanning for methylation (RLGS-M) was used to detect alterations in DNA methylation associated with murine SV40 T/t antigen-induced hepatocarcinogenesis. An altered locus/spot (*S130*) was cloned and found to correspond to sequences in the 5′ flanking region and 5′ portion of the cDNA for the murine mac25/insulin-like growth factor binding protein-7 (*Igfbp-7*) gene. IGFBPs are believed to be capable of binding insulin, Igf1, and Igf2 and modulating mitogenic effects. Previous studies have shown that Igf2 has an important role in promoting liver tumorigenesis. Quantitative PCR was used to access the methylation status of the *Not*I site just 5′ to the coding region and the expression level of the *mac25/igfbp-7* gene. The results indicated that the degree of methylation was inversely related to the expression level and is consistent with a role for DNA methylation in silencing *mac25/Igfbp-7* gene expression and function for *mac25/Igfbp-7* as a tumor suppressor gene. © 2000 Academic Press

Regional DNA hypermethylation of CpG islands is believed to contribute to tumor progression by silencing expression of tumor suppressor genes including *p16/Ink4a, VHL, E-cadherin,* and the estrogen receptor (1–5). In this respect, methylation of the CpG islands and promoter regions appears to be an alternative to mutation or deletion for inactivating gene function.

Restriction landmark genomic scanning (RLGS-M) (6–9) can effectively be used for genome-wide screening for alterations in DNA methylation (10, 11). RLGS-M was previously used to screen for alterations in DNA methylation associated with hepatocarcinogenesis in transgenic mice containing a MUP (mouse major urinary protein) enhancer/promoter driving expression of the SV40 T/t early region (12–14). In the above study, 14 RLGS loci/spots were cloned that appeared to have alterations of DNA methylation status.

One of these loci was identified as a gene previously reported to be involved in cancer, *p16/Ink4a.* The region of the *murine p16/Ink4a* gene that was found to be methylated corresponds to the same region which is methylated in human tumors and is associated with transcriptional silencing (15–17).

Further analysis of the DNA sequences of the spot clones obtained in that study indicated that spot *S130* had strong homology to the *human mac25* gene, also known as *Igfbp7* and *Igfbp-rp1* (18, 19). Insulin-like growth factor binding proteins are thought to be capable of modulating growth by binding to IGFs, modifying the interaction with the cognate receptor, and thereby inhibiting the mitogenic activity. In addition, the down-regulation of the human *mac25/Igfbp-7* gene

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has been reported to be associated with disease progression in breast carcinomas (20).

Reactivation of Igf2 expression is a common event during murine hepatocarcinogenesis in transgenic mice suggesting a role in malignant growth (21). More recently, the role of Igf2 in hepatocarcinogenesis was investigated by crossing a MUP/SV40 T/t Antigen transgenic line with mice containing an Igf2 null allele. The development of large tumors was reduced approximately 15-fold in the absence of Igf2 expression, indicating an important role for Igf2 in tumor growth and progression (22). The mitogenic effects of Igf2 are thought to be mediated primarily through binding to Igf1 receptor. In addition, the transformation of mouse embryonic fibroblasts by SV40 T Antigen requires a functional Igf1 receptor (23). These results indicate that Igf2 expression and binding to the Igf1 receptor may be an important progressive event in hepatocarcinogenesis in MUP/SV40 T/t Antigen transgenic mice. The potential ability of mac25/Igfbp-7 to modulate and block the mitogenic response to the interaction of Igf2 with the Igf1 receptor suggests that mac25/Igfbp-7 could function as a tumor suppressor gene in this context. Therefore, the possible role of DNA methylation in silencing mac25/Igfbp-7 was investigated further. Our results indicate that there is an inverse relationship between the degree of methylation and expression of mac25/Igfbp-7. This is consistent with a role for DNA methylation in silencing *mac25/Igfbp-7* gene expression and a function for mac25/Igfbp-7 as a tumor suppressor gene.

MATERIALS AND METHODS

Mice and SV40 T/t Antigen Transgenic Line

The MT-D2 transgenic line, which contains the SV40 early region under control of MUP enhancer/promoter has been described previously (12, 13). The MT-D2 transgene was made congenic in a C57BL/6J (B6) genetic background (MT-D2/B6) and mated with *M. spretus* to produce F1 progeny. Multiple tumors develop which are histopathologically characterized as hepatocellular carcinomas and adenomas. Normal liver was from B6 and M. spretus (44–48 weeks of age) for control.

Preparation of Genomic Clones and Screening of the Full-Length cDNA Library

B6 genomic liver DNA was partially digested with *Mbo*I and cloned into lambda DASH II (Stratagene, U.S.A.). The full-length cDNA library from normal B6 liver, kidney, and lung was constructed as reported previously (24). The high-density filter was prepared by dotting the full-length liver cDNA library to Biodyne B (PALL, U.S.A.) by Q-Bot (GENETIX, UK). Dot blot analysis was performed by screening the high-density filter probed with the phage clone, M33, which contains the mac25/Igfbp-7genomic clone.

RLGS Analysis and Sequence Analysis

RLGS Analysis was described previously (6–9). Sequence analysis was performed using dye-primer reaction chemistry with LIC-4200L(S) system sequencer (Aloka, Japan).

Preparation of Total RNA

Total RNA was prepared from 0.1–0.4 g of tumor sample. The tissues were homogenized in 4 ml Solution D (25) followed by the addition of 0.4 ml 2 M sodium acetate, pH 4.0, and one volume of phenol/chloroform (5:1). After extraction, RNA was precipitated from the aqueous phase by adding one volume of isopropanol, and centrifuged at 5500 rpm for 15 min at 4°C. The pellet was washed with 70% ethanol and resuspended in water. Selective cetyltrimethylammonium bromide (26) precipitation was performed (24) to remove polysaccharides. RQ-1 (Promega, U.S.A.) was added with an RNase inhibitor (Wako, Japan) and incubated 1 h at 37°C to remove contaminating DNA. The mixture was then extracted again with one volume of phenol/chloroform (5:1), followed by ethanol precipitation. The pellet was resuspended in water.

Northern Blot Analysis

Total RNA (10 μg) was electrophoresed in 0.8% agarose-formaldehyde denaturing gel and transferred to Biodyne B nylon membranes. The probes for hybridization were labeled with [α-^{32}P]dCTP by PCR. The solution for hybridization contained 10% PEG 8000, 1.5× SSPE, and 7% SDS. The membrane was washed in 1× SSPE, 0.1% SDS, followed by 0.5× SSPE, 0.1% SDS and then autoradiographed with BAS film and analyzed using the BAS2000 system (FUJI, Japan).

Quantitative PCR for RNA

The primers and TaqMan probe were designed by Primer Express (Perkin–Elmer, U.S.A.) software. The forward primer was 5′-TAAGGA GGACGCTGGAGAGTATG-3′, the reverse primer was 5′-CGCGGAAGCCTGCC-3′ and the TaqMan probe was 5′-TGCCACGCATCCAACTCCCA-3′. It was labeled at the 5′ end with the reporter dye molecular FAM (6-carboxy-fluorescein) and at the 3′ end with the quencher dye molecule TAMRA (6-carboxy-tetramethyl-rhodamine). For an internal control, we used the primers and TaqMan probe for the mouse GAPDH gene. The forward primer was 5′-CAAAGTGGAGATTGTTGCC-3′, the reverse primer was 5′-CTGGAACATGTAGACCATGTAGT-3′, and the TaqMan probe was 5′-TCAACGACCCCTTCATTGACCTC-3′. It was labeled at the 5′ end with FAM and at the 3′ end with TAMRA. Amplification reactions (50 μl) contained 50 ng total RNA, TaqMan EZ Buffer, 3 mM manganese acetate, 300 nM dNTP, 200 nM forward and reverse primers, 100 nM TaqMan probe, 5 U rTth DNA polymerase, 0.5 U AmpErase UNG. Thermal cycler parameters included 2 min at 50°C, 30 min at 60°C, 5 min 95°C, and 40 cycles of 20 s at 94°C and 1 min. at 60°C. The real-time data was collected with the ABI PRISM 7700 SDS analytical thermal cycler (PE Applied Biosystems).

Quantitative PCR for Methylation

Preparation of template DNA. Genomic DNA from tumors and normal B6 and *M. spretus* liver (10 μg each) were digested with *Pst*I (Nippon Gene, Toyama, Japan) in a volume of 100 μl for 3 h. Following phenol/chloroform extraction and ethanol precipitation, the DNA was resuspended in buffer containing 0.01% BSA and 0.01% triton and an aliquot (50 μl) digested with *Not*I (Nippon Gene, Toyama, Japan) in a final volume of 100 μl for 3 h. The other aliquot (50 μl) was treated in the same manner except it was not digested with *Not*I. The templates were then extracted again with phenol/chloroform and the DNA precipitated with ethanol. For each sample, complete digestion with *Not*I was confirmed by the electrophoresis pattern of the plasmid *pBS II* (0.1 μg) which was mixed with the normal or tumor DNA *Not*I digestion solution and incubated for 3 h.

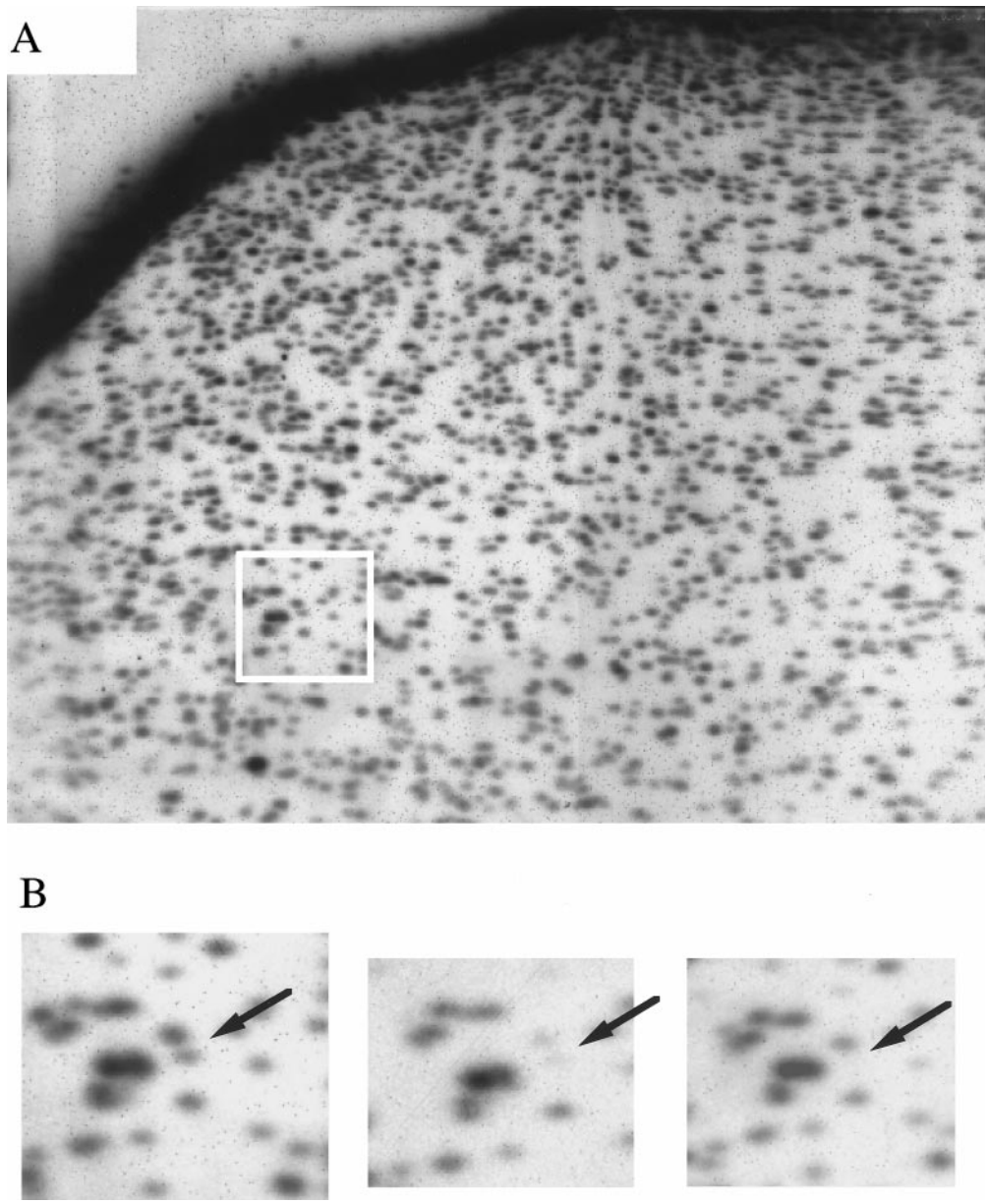

FIG. 1. RLGS-M profile of B6 X *M. spretus* F1 normal liver DNA using the restriction enzyme combination *Not*I, *Pvu*II, and *Pst*I. The region containing the *S130* spot is indicated by the rectangle in A. B shows a magnification of this region in RLGS profiles of normal liver DNA (left) and tumor DNA in which the intensity of the *S130* spot was reduced (middle) or absent (right). The *S130* spot was reduced or completely absent in 23 or 29 tumor samples.

Primers for the *mac25/Igfbp-7* gene were mac003 (5′-TCGATCCAGCCACCTTATGATG-3′) and mac010 (5′-GGCATCCGAAGAGGAGGAAG-3′), which generated a 110-bp DNA fragment containing the *Not*I sequence but not the *Pst*I sequence. Primers for the *mouse β-actin* gene which served as an internal control were β-actin 338-F(5′-GGCATTGTGATGGACTCCGG-3′) and β-actin 338-R(5′-AAAGAGCCTCAGGGCATCGG-3′), which generated a 338 bp DNA fragment containing no *Not*I or *Pst*I recognition sequences. PCR conditions: Amplifications were performed in 20-μl reaction mixtures containing 50 ng genomic DNA, 5% DMSO, 160 nM dATP, dGTP, dTTP, 80 nM dCTP, [α-^{32}P]dCTP, 500 nM primers for mac25/Igfbp-7 sequences, 50 nM primers for β-actin sequences, ExTaq buffer and TaKaRa ExTaq polymerase (TAKARA, Japan) using the PTC200 DNA Engine (M. J. Research, U.S.A.) under the following conditions: 3 min at 95°C, 30 cycles of 15 s at 96°C 30 s at 61°C, 30 s at 72°C, and 1 min at 72°C.

PCR products were analyzed in 7.5% polyacrylamide gels. The gels were exposed to BAS IP film and the radioactivity of each lane was quantified by BAS 2000 system.

Statistical Analysis

To determine the correlation between methylation status at the mac/Igfbp-7 *Not*I site and the expression levels in tumor, a linear regression analysis was performed. Data were analyzed with Statview J-4.5 software (Abacus Concepts, Inc, CA).

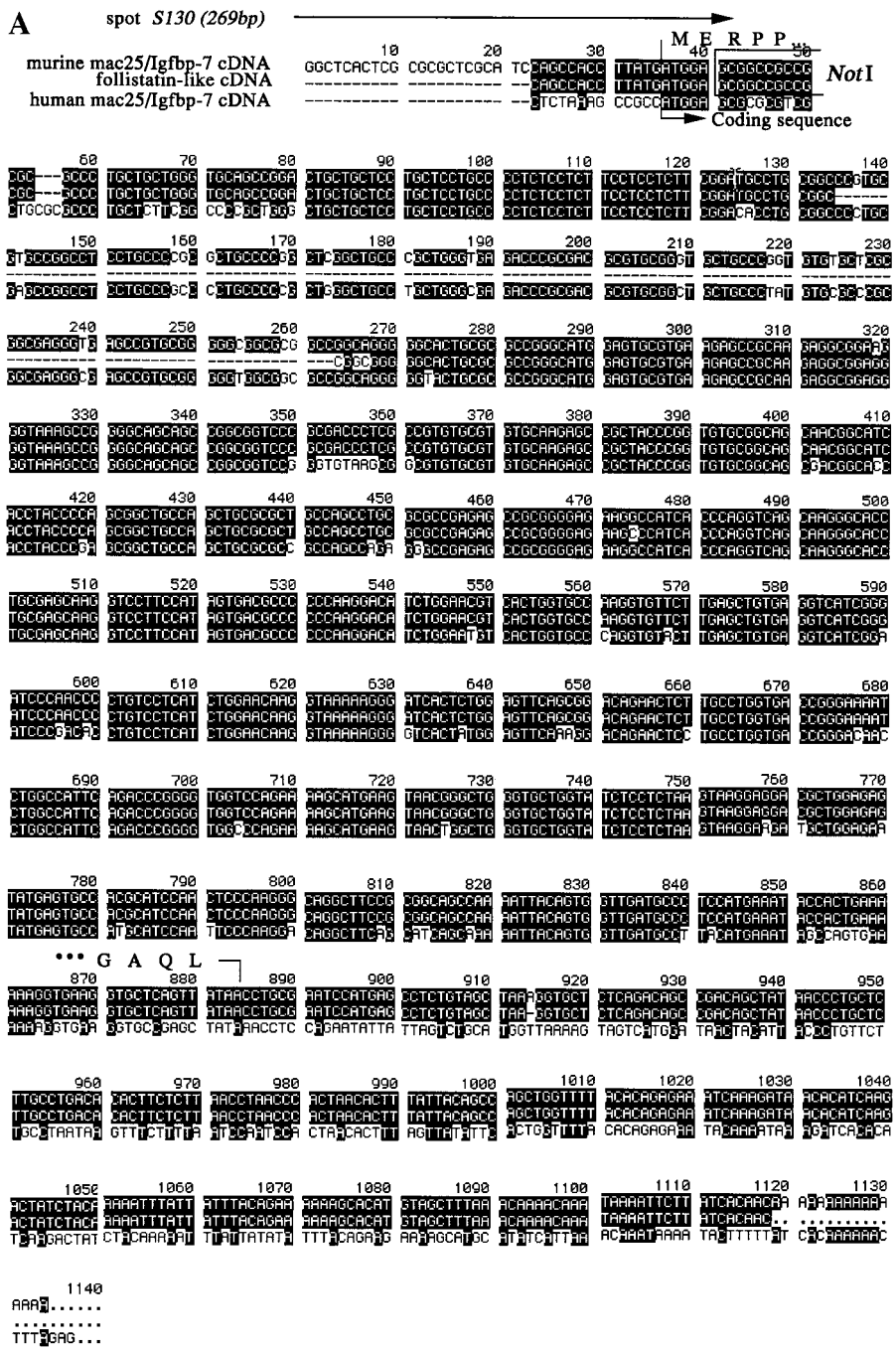

FIG. 2. (A) Alignment of the full length *mac25/Igfbp-7* cDNA (our sequence) with *follistatin-like* cDNA (Kato *et al.*, 1996), and *human mac25* cDNA (Murphy *et al.*, 1993; GenBank Accession No. L19182). The position of the *Not*I recognition sequence and the region corresponding to the *S130* spot clone are indicated. The genomic *S130* spot clone contains the identical 47 bp sequence from the 5′ end of the *mac25/Igfbp-7* cDNA. The beginning and the end of amino acid coding sequence of *murine mac25* cDNA are also indicated. (B) Alignment of the murine MAC25 with the human MAC25. The differences in amino acid sequence were restricted to a few AA residues in the C-terminus and the N-terminus.

RESULTS

Correspondence of S130 to the mac25/Igfbp-7 Gene

The *spretus*-specific spot, *S130*, which had been cloned from the RLGS gel (Fig. 1A) was reduced or absent (Fig. 1B) in 23 of 29 tumor samples (14) indicating that this RLGS locus/spot was methylated and/or deleted in the tumor samples. This locus/spot had been previously mapped to a region on chromosome 5 (*D5Rik124*) (8). The spot clone (*S130*) was used

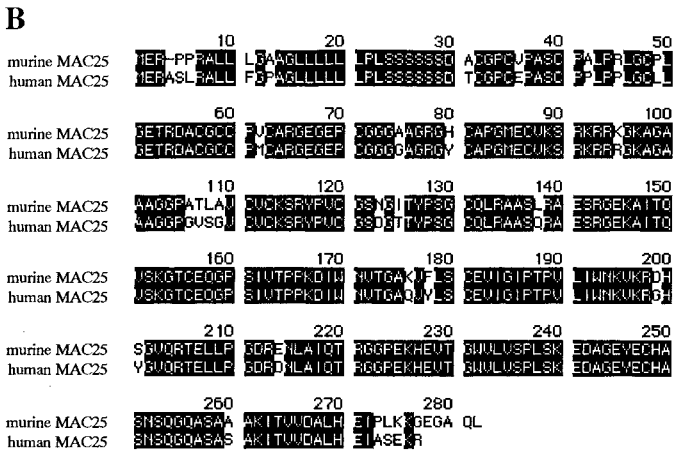

FIG. 2—*Continued*

as a probe for colony hybridization to identify a lambda *DASH II* phage genomic clone (*M33*) containing the *S130* sequence and 18 kb of adjacent genomic DNA. The *M33* genomic clone was hybridized to a high-density filter containing a liver full-length cDNA library (24) which identified a cDNA clone of 1131 bp. The 5′ end of this cDNA clone contained a 47 bp sequence, which was identical to the region of *Not*I end of the *S130* spot clone. The *Not*I sequence in the genomic clone was 6 bp from the start of the coding sequence. The murine cDNA clone (GenBank Accession No. AB012886) had high homology to the human *mac25/Igfbp-7*. It also had fairly high homology to the *murine follistatin* gene as previously noted but differed somewhat from the murine mac25 sequence previously reported (27).

Homology to Human mac25/Igfbp-7 Gene

The cDNA clone had high homology to human *Mac25* gene located at 4q12 (20, 28) (Fig. 2A). Furthermore, the RLGS locus (*D5Rik124*) maps to a region of mouse chromosome 5, which is syntenic with human chromosome 4q12. In the sequence of the human *Mac25* gene, the *Not*I recognition sequence present in the mouse just upstream of coding region was absent. The major differences in amino acid sequence between murine *mac25/Igfbp-7* gene and human homologue were a few amino acid residues in both the N-terminus and C-terminus (Fig. 2B). Full-length cDNA clones from both kidney and lung cDNA libraries were isolated, sequenced, and found to be identical to that of the liver cDNA. The differences between the *mac25/Igfbp-7* sequence reported here and that reported by Kato *et al.* could be a strain specific difference. Alternatively, it is possible that the clone reported by Kato *et al.* has a deletion or is a splicing variant. The later appears to be ruled out since there was no apparent consensus sequence for splicing in this region and Northern blot of RNAs from normal B6 mouse tissues showed a single band (Fig. 3).

Expression of Murine mac25/Igfbp7 Gene

A Northern blot of RNAs from various normal B6 mouse tissues is shown in Fig. 3. Each tissue showed a single band. The *murine mac25/Igfbp-7* gene was highly expressed in lung, kidney, small intestine, testis and uterus. The gene was moderately expressed in the liver.

The Relation between the Methylation Status and the Expression Level of the mac25/Igfbp-7 Gene

The *murine mac25/Igfbp-7* gene has a *Not*I site 6 bp from the transcription start site. A set of PCR primers was designed so that the PCR product included the *Not*I site. Although *S130* is a *spretus*-specific allele, sequence analysis of PCR products indicated that both B6 and *spretus* alleles contain the *Not*I site. Thus, the PCR assay described below access the methylation status of both the B6 and *spretus* alleles in tumors from the MT-D2B6 X M. *spretus* F1 animals. Aliquots of Genomic DNA, either digested with *Not*I or undigested, were amplified and the level of products determined by quantitative PCR (Fig. 4A). The ratio of the products produced from each template, expressed as a percentage, is shown in Fig. 4B. Amplification of β-actin (338 bp) was used as an internal control to correct for slight variations in amplification in different samples. The average extent of methylation of the *Not*I site in normal B6 liver DNA was 31.1 ± 5.1% (mean ± SD, $n = 4$) and the average in *M. spretus* liver DNA was 20.2% ($n = 2$). The average methylation of the *Not*I site in the eleven tumor samples was 67.4 ± 25.4% (mean ± SD). The expression level of the *mac25/Igfbp-7* gene in tumor and control samples was examined by real-time PCR using TaqMan technology

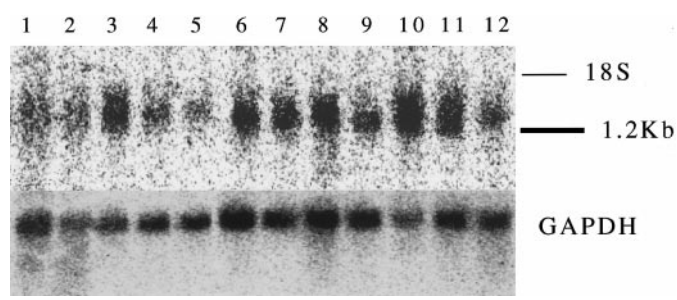

FIG. 3. Northern blot analysis of *mac25/Igfbp-7* mRNA in normal B6 tissues. Lanes 1–12 are: 1, brain; 2, thymus; 3, lung; 4, liver; 5, spleen; 6, kidney; 7, stomach; 8, small intestine; 9, large intestine; 10, testis; 11, uterus; 12, skin. A probe to *mouse GAPDH* mRNA was hybridized to the blot to confirm approximately equal loading.

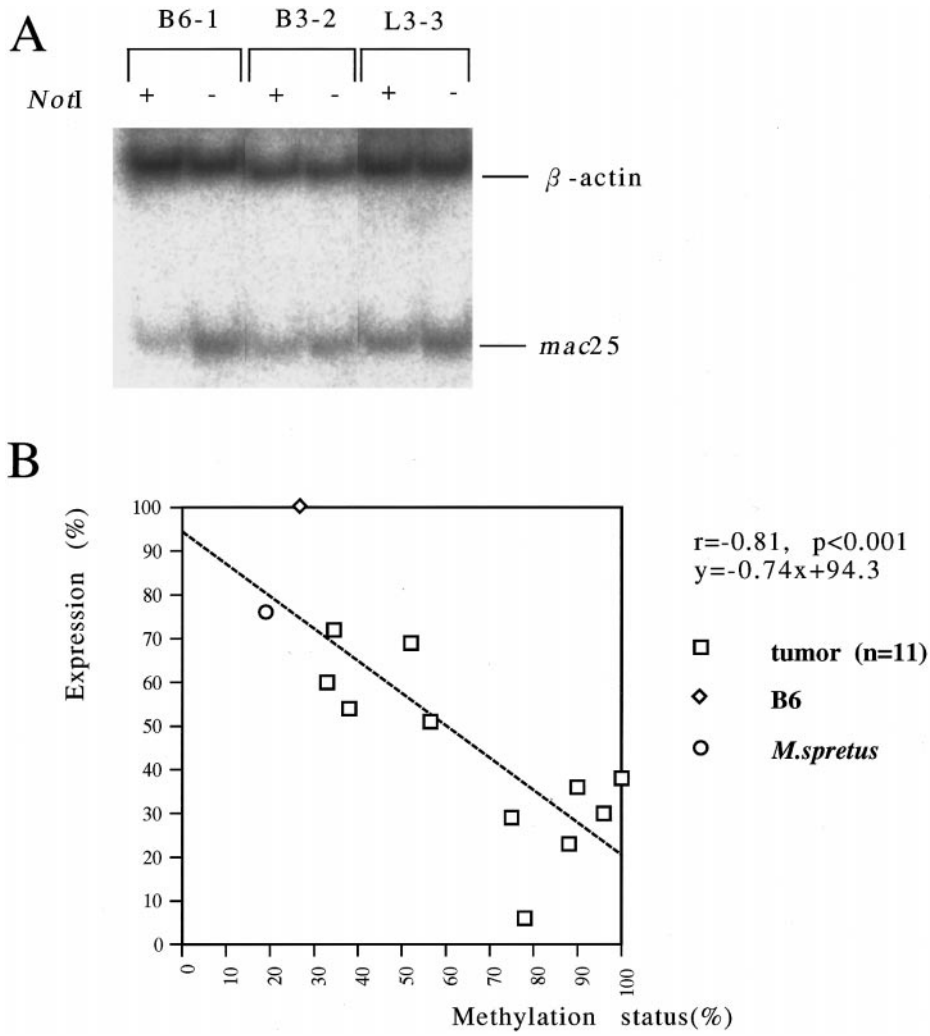

FIG. 4. Quantitative PCR for methylation using sets of primers for mac25 (110 bp) and for β-actin (338 bp) as an internal control. Nontransgenic B6 liver DNA (B6-1) and DNA from two liver tumors (B3-2 and L3-3) from MTD2B6 X *M. spretus* F1 progeny are shown. The template DNAs were either digested (+) or not digested (−) with *Not*I prior to amplification. The PCR products were analyzed on 7.5% polyacrylamide gels and exposed to BAS IP film. Correlation between Methylation status of *Not*I recognition sites assayed by quantitative PCR and expression of *murine mac 25/Igfbp-7* gene assayed by real-time RT-PCR. Twelve tumors from MTD2B6 X *M. spretus* F1 progeny, nontransgenic B6, and *M. spretus* were assayed. There was an inverse relation between methylation status and expression level ($r = -0.81$, $p < 0.001$, $y = -0.74x + 94.3$). *X* axis: Methylation status; the percent methylation is determined by the ratio of PCR products produced from template DNA digested with *Not*I to that produced from undigested template DNA. *Y* axis: The relative expression levels of *mac25/Igfbp-7* in each tumor sample were compared with that in normal B6 liver (100%) and are expressed as a percent.

(29, 30). *GAPDH* gene expression levels were used as an internal control to correct for differences in RNA preparations. The *mac25/Igfbp-7* gene was underexpressed in all tumor RNA ($n = 11$; Fig. 4B). The methylation status and expression levels of *mac25/Igfbp-7* in normal, non transgenic B6 liver were 27.2 and 100% respectively, and those in *M. spretus* liver were 18.9% and 76% respectively. The average expression level in tumor RNA relative to RNA from normal B6 liver was 42.5 ± 20.5% (mean ± SD). The results indicate that the expression levels in tumor and normal liver are inversely related to the methylation status at the *mac25/Igfbp-7 Not*I site ($r = -0.81$, $p < 0.001$, $y = -0.74x + 94.3$; Fig. 4B).

DISCUSSION

When using *Not*I as the restriction landmark, which has two CpGs in its recognition sequence, RLGS targets gene rich CpG islands. Since *Not*I is methylation sensitive, RLGS-M can be used to detect alterations in methylation associated with tumori-

genesis. RLGS-M was previously used to identify regions of the genome that are frequently methylated in liver tumors from MUP/SV40 T/t Antigen transgenic mice (14). In that study, 14 loci were cloned, sequenced and utilized for a DNA data base search (BLAST). One of the loci was identified as *p16/Ink4a* gene reported to be involved in tumorigenesis. The region methylated in the murine *p16* tumor suppressor gene included exon 1, which is hypermethylated in human tumors and results in transcriptional silencing (15).

In this study, we found one of the other spot clones (*S130*) corresponds to *mac25/Igfbp-7*. This conclusion is supported by the fact that full-length murine Mac25/Igfbp-7 cDNA contains sequences identical to *S130* and *S130* (D5Rik124) maps to a region of chromosome 5 syntenic to human chromosome 4q12, the locus for the human *Mac25* gene (28).

The *Not*I site that becomes methylated in the tumors is just 6 bp downstream of the putative translation initiation site. Using quantitative PCR, the expression of the *mac25/Igfbp-7* gene was found to be inversely related to the methylation status of the *Not*I site indicating that the *mac25/Igfbp-7* gene is down-regulated during hepatocarcinogenesis and may function as a tumor suppressor gene. The partial methylation of the *Not*I site in normal B6 liver DNA (31.1%) and in normal *M. spretus* liver DNA (20.2%) may be due to the methylation of the *mac25/Igfbp-7* gene in some cell types contained in the liver tissue. The lack of complete methylation and silencing within the tumors may be due to tumor heterogeneity and/or contaminating normal tissue. It may be possible to distinguish these possibilities by using laser capture microdissection technology (31).

The *mac25/Igfbp-7* gene is a member of a family of insulin-like growth factor binding proteins which are capable of specifically binding insulin, Igf1, or Igf2 and modulating their interaction with their cognate receptor (32). Depending on Igfbp and cell type, the interaction of Igfs and Igfbp can either enhance or suppress the mitogenic response. The binding affinities of different Igfs for different Igfbps vary. Human IGFBP 1-6 bind IGFs with high affinity whereas mac25/Igfbp-7 binds IGFs at somewhat lower affinity. The human IGFBP-7 protein was shown to bind insulin with high affinity and block insulin action (33). However, even with somewhat lower affinity for binding IGFs, mac25/Igfbp-7 should still be capable of modulating interaction with their receptor (34).

Kato *et al.* suggests that the murine mac25/Igfbp-7 may function as an activated *follistatin-like* gene and act as a growth suppressor by modulating signaling by the transforming growth factor-β (TGFβ) family. The N-terminal region of mac25/Igfbp-7 has considerable homology to other members of the Igfbp family and contains the GCGCCxxC IGFBP motif. However, the C-terminus of mac25/Igfbp-7 lacks a conserved domain present in Igfbp 1-6. This may account for its somewhat lower affinity for Igfs than Igfbp 1-6. Kato *et al.* also suggests that the lack of this C terminal region may impart an activated follistatin activity. However, evidence for binding of TGFβ is currently lacking whereas there is clear evidence that mac25/Igfbp-7 specifically binds Igfs (32). Thus, current evidence favors an Igf binding function for mac25/Igfbp-7.

Although mac25/Igfbp-7 binds Igfs with somewhat lower affinity than insulin, the binding is specific. In addition, mac25/Igfbp-7 has a wide distribution in normal tissues and expression is reduced in a variety of tumor cell lines, suggesting that *mac25/Igfbp-7* may function as a tumor suppressor gene (34). The *mac25/Igfbp-7* gene was found to be expressed in normal breast tissue, but down-regulated in hyperplastic and DCIS (ductal carcinoma in situ), and there was no detectable expression in invasive carcinoma (20). In addition, mac25/Igfbp-7 was found to be greatly up-regulated in senescent normal mammary epithelial cells, further supporting a role in growth suppression (28).

Igf1 and Igf2 have important growth stimulating roles in normal development and have potent mitogenic activity in a variety of tumor cell types. The growth stimulatory effects of Igf2 are through binding to the Igf1 and insulin receptors (35, 36). Moreover, the targeted disruption of the Igf1 receptor prevents transformation of mouse embryonic fibroblasts by SV40 T Antigen. The ability to transform these cells can be restored by transfection with a plasmid expressing the human IGF1 receptor (23).

There is abundant evidence that Igf2 has an important role in tumorigenesis. Igf2 is paternally expressed imprinted gene, which plays a major role in tissue overgrowth and tumorigenesis associated with Beckwith Wiedemann syndrome (37). The loss of Igf2 imprinting (LOI) resulting in biallelic expression of Igf2 has been observed in a number of tumor types (38).

The overexpression and reactivation of Igf2 expression is frequently observed in hepatocellular carcinomas from humans (39) and transgenic mice (21, 40, 41) suggesting a role in promoting hepatocarcinogenesis. This possibility was tested directly by creating Igf2 (+/−) MUP SV40 T/t Antigen transgenic mice (22). These mice were essentially null for Igf2 expression since the paternal Igf2 allele is null and imprinting normally precludes expression from the maternal allele. Hepatocarcinogenesis in these mice was strongly suppressed. Male mice exhibit a 15-fold reduction in the frequency of large tumors indicating that re-expression of Igf2 is a selective event in hepatocarcinogenesis. Similar results indi-

cated that the absence of Igf2 expression strongly suppressed development of insulinomas in Rat Insulin Promoter/SV40 T Antigen (RIP-Tag) transgenic mice (42). The reactivation of the normally silent maternal Igf2 allele in Tgfα induced hepatocellular carcinomas in mice is also consistent with a major role for Igf2 in hepatocarcinogenesis (43).

Recently published results provide additional evidence that Igfbps can modulate liver tumorigenesis in SV40 T antigen transgenic mice (44). These results indicate that over-expression of TIMP-1 (Tissue Inhibitor of Metalloproteinase 1) inhibits SV40 T antigen induced hepatocarcinogenesis by blocking degradation of Igfbp-3. Thus, in this case, the post transcriptional up-regulation of an Igfbp suppresses Igf2 promoted tumorigenesis.

The potential ability of mac25/Igfbp-7 to bind Igf2 and inhibit its interaction with the Igf1 receptor indicates that the mac25/Igfbp-7 could act as a tumor suppressor gene in this context. The results presented here, indicating that the expression of *mac25/Igfbp-7* is down-regulated as a consequence of DNA methylation, is consistent with a role as a tumor suppressor gene.

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