



Imprinted *M6p/Igf2* receptor is mutated in rat liver tumors

JJ Mills^{1,3}, JG Falls¹, AT De Souza^{2,4} and RL Jirtle^{1,3}

¹Department of Radiation Oncology, Duke University Medical Center, Durham, North Carolina 27710, USA; ²Department of Safety of Medicines, Zeneca Pharmaceuticals, Alderley Park, Macclesfield, Cheshire, SK10 4TG, UK

We have previously shown that inactivation of mannose 6-phosphate/insulin-like growth factor 2 receptor (*M6P/IGF2R*) is a common early event in both human liver and breast carcinogenesis. The *M6p/Igf2r* is imprinted in mice while expression is biallelic in most humans. In this investigation the *M6p/Igf2r* gene is shown to also be imprinted in the liver of Fischer 344, Lewis and Brown Norway rats. In addition, we have identified mutations in the expressed allele of the *M6p/Igf2r* in 40% of diethylnitrosamine-initiated rat liver tumors. These results provide further evidence that the *M6P/IGF2R* functions as a liver tumor suppressor gene. They also suggest that mice and rats would be more sensitive than humans to those hepatocarcinogens in which the *M6p/Igf2r* is mechanistically involved in transformation since one rather than two alleles would need to be inactivated.

Keywords: *M6p/Igf2r*; genomic imprinting; liver cancer; tumor suppressor

Introduction

Approximately 60% of the chemicals determined by the National Toxicology Program to be carcinogenic in rats and mice give rise to liver tumors (Haseman *et al.*, 1987). We have postulated that the carcinogenic effect of at least some of these compounds results from their ability to release hepatocytes from normal hepatic growth controls (Jirtle *et al.*, 1994; Mills *et al.*, 1995). The mannose 6-phosphate/insulin-like growth factor 2 receptor (*M6P/IGF2R*) is a multifunctional cell membrane-associated glycoprotein that is involved in regulating the intracellular trafficking of lysosomal enzymes (Kornfeld, 1992; Dahms, 1996), the degradation of the mitogen, insulin-like growth factor 2 (*IGF2*; Morgan *et al.*, 1987), and the activation of the mitoinhibitor, transforming growth factor beta (*TGFβ*; Dennis and Rifkin, 1991). It has also recently been shown to be a high affinity binding receptor for retinoic acid, an agent known to have diverse biological effects in both embryogenesis and oncogenesis (Kang *et al.*, 1997). Thus, important homeostatic controls regulating cell proliferation and apoptosis would be lost with the inactivation of the *M6P/IGF2R*, suggesting that this receptor normally functions to inhibit tumor formation.

We have shown immunohistochemically that a large percentage of rodent liver tumors promoted with phenobarbital (PB; Jirtle *et al.*, 1994), Wy-14,643 (Mills *et al.*, 1995) and unleaded gasoline (Moser *et al.*, 1996) lack the *M6p/Igf2r*. Furthermore, the *M6P/IGF2R* is frequently mutated in human liver and breast tumors (De Souza *et al.*, 1995a,b; Hankins *et al.*, 1996; Yamada *et al.*, 1997), and it is a target for mismatch repair deficiency in colon, gastric and endometrial tumors (Souza *et al.*, 1996; Ouyang *et al.*, 1997). The *M6p/Igf2r* is imprinted in mice with only the maternal allele being transcribed (Barlow *et al.*, 1991). In contrast, genomic imprinting of this gene is a polymorphic trait in humans with most people having biallelic expression (Kalscheuer *et al.*, 1993; Xu *et al.*, 1993). The purpose of this study was to determine if the *M6p/Igf2r* gene is imprinted in rats, and if so, whether the expressed allele is mutated in diethylnitrosamine (DEN) initiated and DEN-initiated, PB-promoted liver tumors. The results of this investigation demonstrate that the *M6p/Igf2r* locus is imprinted in rats, and the expressed allele is frequently mutated in chemical carcinogen-induced rat hepatocellular carcinomas (HCCs).

Results and Discussion

Genomic imprinting refers to a genetic phenomenon where certain genes are molecularly marked such that one of the parental alleles is transcriptionally silenced (for reviews see Barlow, 1995; Franklin *et al.*, 1996; De Souza *et al.*, 1997). In mice the *M6p/Igf2r* gene is expressed only from the maternal allele (Barlow *et al.*, 1991), whereas in humans, imprinting at the *M6P/IGF2R* locus is a polymorphic trait (Kalscheuer *et al.*, 1993; Xu *et al.*, 1993). Recent evidence also indicates that the *M6P/IGF2R* functions as a tumor suppressor gene in a variety of human tumors (De Souza *et al.*, 1995a; b; Hankins *et al.*, 1996; Yamada *et al.*, 1997; Souza *et al.*, 1996; Ouyang *et al.*, 1997). Thus, animals in which the *M6p/Igf2r* is imprinted would be expected to be more sensitive than humans to those carcinogens in which the *M6p/Igf2r* is mechanistically involved in the transformation process (De Souza *et al.*, 1997).

To determine whether genomic imprinting of the *M6p/Igf2r* plays a role in rat hepatocarcinogenesis, we first determined whether the *M6p/Igf2r* locus is imprinted in rat liver by utilizing a G/C DNA sequence polymorphism found in exon 48 of Lewis and F344 rats (Figure 1a and b). We have shown by reverse transcription-polymerase chain reaction (RT-PCR) and direct sequencing the presence of a single *M6p/Igf2r* RNA species in rat liver (Figure 1c), and that expression is from the maternal allele irrespective

Correspondence: RL Jirtle

³Current address: Department of Pharmacology and Toxicology, Imperial College School of Medicine at St. Mary's, Paddington, London W2 1PG

⁴Molecular Toxicology Group, Glaxo Wellcome Research & Development, Park Road, Ware, Herts SG12 0DP

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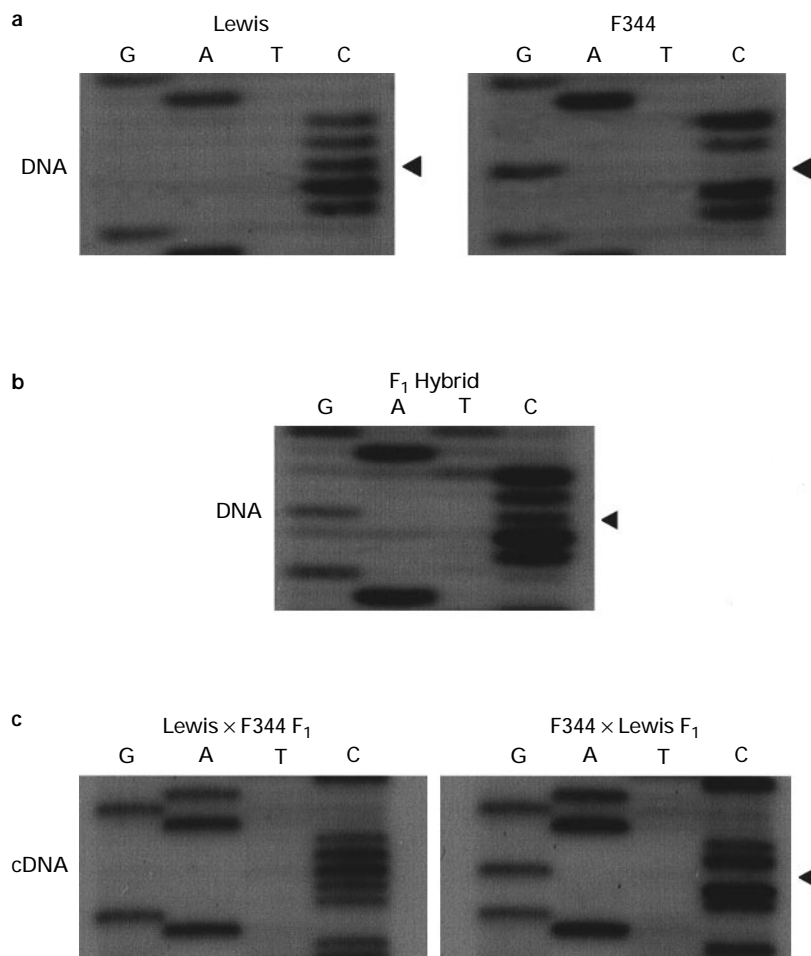


Figure 1 Genomic imprinting of the *M6p/Igf2r* in rat liver. (a) Direct PCR sequencing showing a G/C sequence polymorphism (arrow heads) in exon 48 (base number 7176) between Lewis and F344 rats. (b) Liver genomic DNA from a F344 \times Lewis F₁ hybrid cross shows the presence of both parental alleles (arrow head) in the F₁ genome. (c) Liver cDNA from both Lewis \times F344 and F344 \times Lewis F₁ hybrid crosses demonstrates the presence of a single mRNA species expressed from the maternal allele (arrow head)

of whether the Lewis or Fischer 344 (F344) rat is the mother. The presence of a single RNA species that is expressed only from the maternal allele demonstrates that the *M6p/Igf2r* gene is imprinted in the liver of rats as in mice. We have also observed genomic imprinting of the *M6p/Igf2r* gene in the liver of Brown Norway rats (data not shown).

The precise molecular mechanism for genomic imprinting of the *M6p/Igf2r* is presently unknown. Methylation of a CpG rich region in intron 2 of the expressed maternal *M6p/Igf2r* allele has been shown to carry the imprint signal for this gene (Stöger *et al.*, 1993; Wutz *et al.*, 1997). Furthermore, this region appears to normally function as the promoter of an antisense transcript that is expressed only from the repressed paternal allele indicating that a form of expression competition regulates imprinting of the *M6p/Igf2r* gene (Wutz *et al.*, 1997). Whether intron 2 of the *M6p/Igf2r* gene in rats also contains a maternal specific CpG methylation as observed in mice is presently being investigated.

Mutations in the *M6p/Igf2r* allele were seen in 2/6 (33%) liver tumors that arose in F344 rats treated with DEN alone, while 5/11 (45%) liver tumors were mutated in F344 rats initiated with DEN and then promoted with PB; this difference in mutational frequency is not statistically significant ($P > 0.1$). Of

the 17 liver tumors analysed, genomic deletions were observed in 6/17 tumors (Figures 2 and 3), and a point mutation was observed in 1/17 tumors (Figure 4). Thus, the overall mutation frequency of the *M6p/Igf2r* gene in these liver tumors is 7/17 or 41%. Additionally, examination of seven DEN-induced liver tumors from Lewis \times F344 F₁ hybrid rats revealed monoallelic expression in the *M6p/Igf2r* in all of the tumors (i.e. imprinting was maintained). Three of these tumors (43%) had deletions in the maternally expressed allele of the *M6p/Igf2r* gene (data not shown). This mutation frequency is not significantly different ($P > 0.1$) from that observed in the F344 rats. This is the first report of mutations in the imprinted *M6p/Igf2r* tumor suppressor gene in rodent liver tumors induced with a chemical carcinogen.

Deletions T1 to T4 flank the region of the *M6p/Igf2r* that contains the IGF2 binding domain (Figures 2 and 3). These deletions remove 477, 802, 861 and 852 bases at the RNA level, respectively. Deletions in T1 to T3 are in frame leading to predicted *M6p/Igf2r* protein lengths of 2322, 2197 and 2192 amino acids rather than the full length 2480 amino acid receptor protein. The deletion in T4 is out of frame leading to a stop codon after 1420 amino acids. This truncated receptor would be expected to be secreted into the extracellular space since it lacks a transmembrane domain. These four

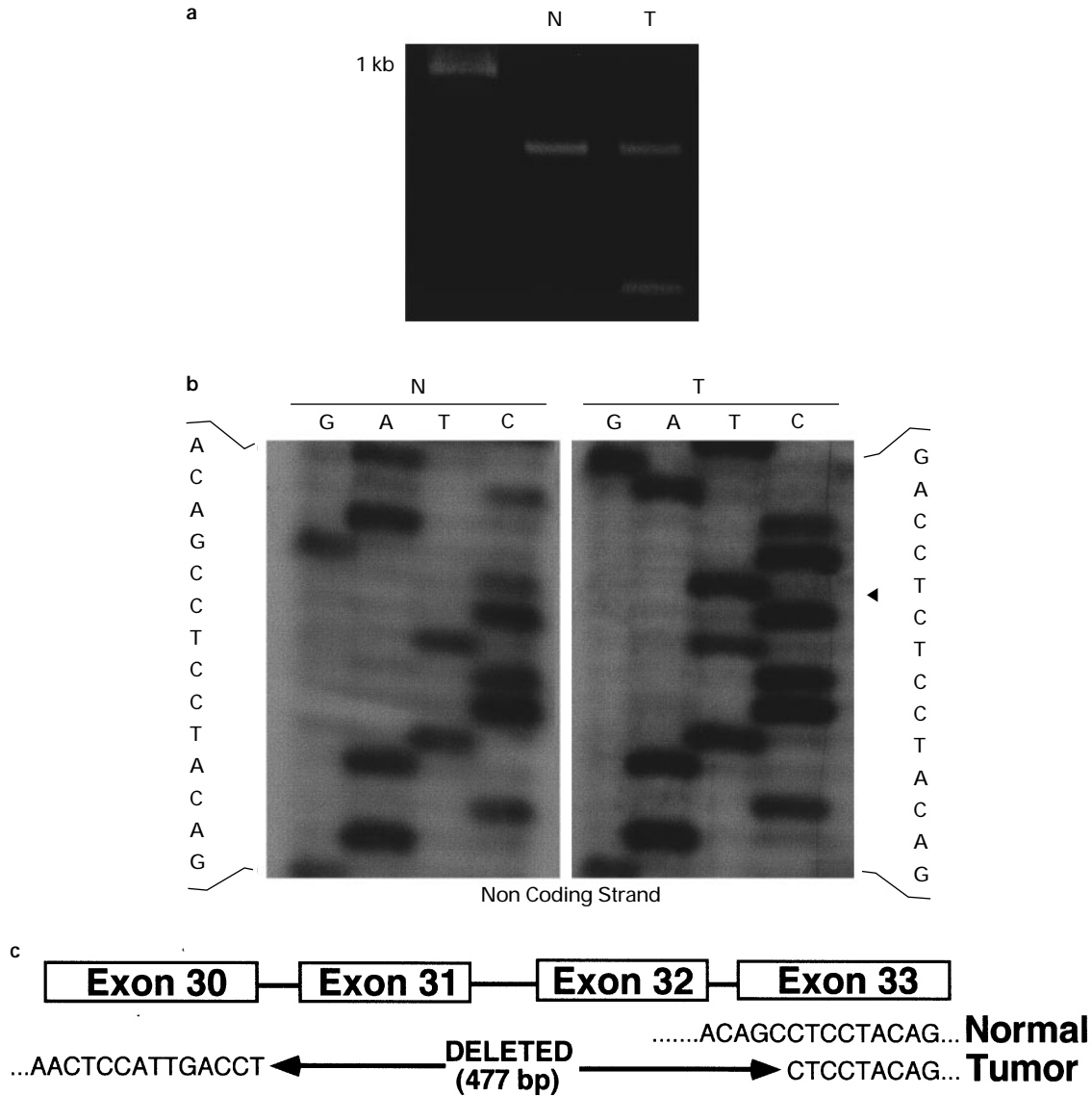


Figure 2 RT-PCR amplification of the M6P and IGF2 binding domains (Kornfeld, 1992; Dahms, 1996) of the *M6p/Igf2r* gene from a DEN-initiated rat liver tumor. (a) The tumor (T) has a lower band which is absent in surrounding normal tissue (N). (b) Gel extraction and PCR sequencing of the lower band identifies an in frame deletion of 477 bp from nucleotide 4192 to 4669 (arrow head). (c) The deletion begins in exon 30 and ends in exon 33, removing the IGF2 binding site, but leaving both M6P binding sites intact

deletions result in the loss of the IGF2 binding site. Mutations in this region have also been frequently observed in both human HCCs and breast tumors (De Souza *et al.*, 1995b; Hankins *et al.*, 1996; Yamada *et al.*, 1997). The combined frequency for mutations in this region in rat and human tumors is 11/33 (33%) suggesting that degradation of IGF2 by the M6P/IGF2R is important in maintaining a normal cell phenotype. This supports recent findings of Ludwig *et al.* (1996) who demonstrated that in mutant mice, *Igf2* turnover during embryogenesis is regulated by the M6p/Igf2r, and targeted disruption of the *M6p/Igf2r* results in *Igf1r* overstimulation by *Igf2*.

Deletions in tumors T5 and T6 involve a loss of genetic material in the 5' end of the *M6p/Igf2r* gene (Figure 3). DNA is lost from exon 3 to exon 27 in tumor 5. This would result in a truncated RNA that would code for a putative protein that is shifted out of reading frame after 134 amino acids and is terminated with a stop codon after 163 amino acids. Interestingly, tumor 6 is deleted from 15 base pairs into the open reading frame to

exon 26 leading to a putative protein of seven amino acids. This deletion also includes intron 2 which is postulated to contain the maternal specific imprint mark in mice (Stöger *et al.*, 1993; Wutz *et al.*, 1997).

Recently an 8 G polynucleotide repeat in the *M6P/IGF2R* gene was reported to be a target for microsatellite instability in human gastric, colon and endometrial tumors (Souza *et al.*, 1996; Ouyang *et al.*, 1997). No mutations were seen in the rat microsatellite homologue of this human sequence in these rat liver tumors. In addition, no point mutations were detected in the IGF2 and M6P binding domains or in the transmembrane and intracellular trafficking regions (Kornfeld, 1992; Dahms, 1996). However, a T:A→A:T transversion was found in one liver tumor that substituted an arginine for a stop codon in exon 48 resulting in the addition of 57 amino acids to the full length protein (Figure 4). Such a change would be expected to alter the intracellular trafficking of the receptor since the trafficking signals are at the C-terminal portion of the receptor (Kornfeld, 1992).

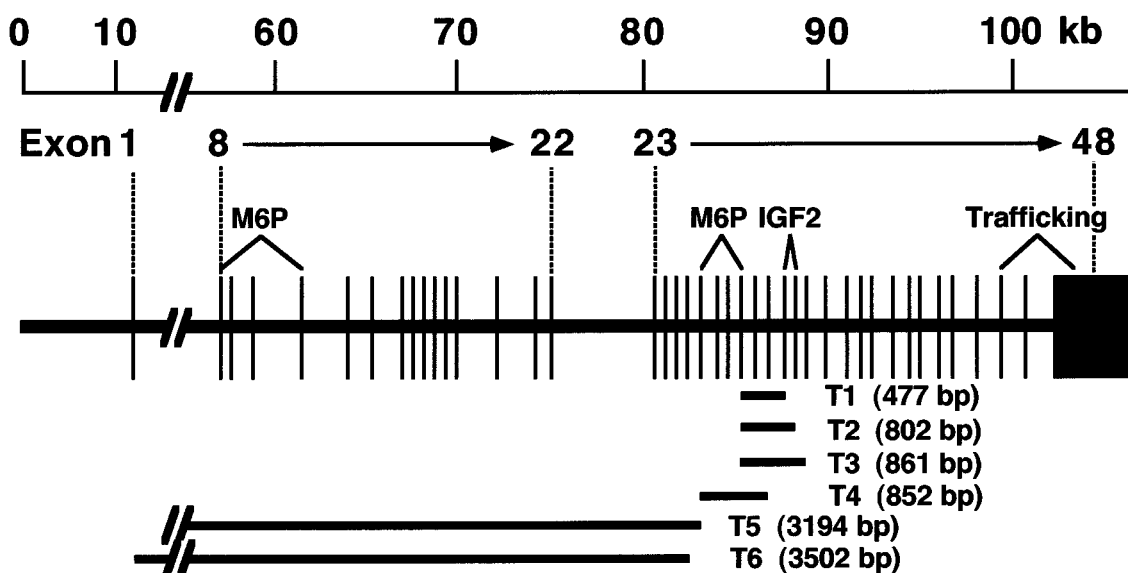
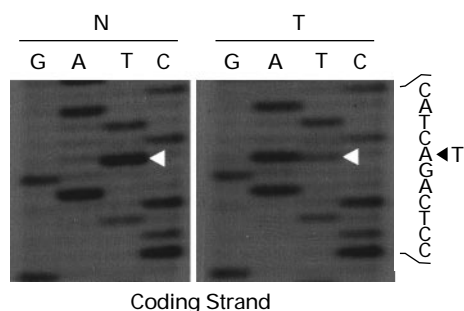


Figure 3 Genomic map of the mouse *M6p/Igf2r* gene (Szebenyi and Rotwein, 1994) showing the extent of deletions (horizontal lines) observed in DEN-initiated rat liver tumor cDNAs T1 to T6. Exons and introns are depicted by the vertical bars and intervening thick horizontal lines, respectively. The number and location of bases deleted from each tumor cDNA is shown below the genomic map. Functionally important regions of the *M6p/Igf2r* gene (i.e. M6P and IGF2 binding sites and receptor trafficking region) are as indicated



NormalGACCTCCTACACATCTGAActccgtg.....
AAD L L H I *

TumorGACCTCCTACACATCAGACTCCGT.....
AAD L L H I R G A....

Figure 4 Translational stop codon mutation in a DEN-initiated, PB-promoted F344 rat liver tumor. The normal stop codon (TGA) in exon 48 is mutated to arginine (AGA) (arrow head), which results in the addition of 57 amino acids to the full length protein (not shown)

It is possible that other mutations present in the *M6p/Igf2r* gene in these rat liver tumors have gone undetected. Because of the large size of the *M6p/Igf2r* locus (≈ 100 Kbp) and corresponding mRNA (9.1 Kb) (Szebenyi and Rotwein, 1994), only those regions of the mRNA known to code for sites involved in receptor function (Kornfeld, 1992; Dahms, 1996) were directly sequenced for mutations. Genomic deletions that started or finished outside the coding region or deletions in the 3' untranslated region of the receptor, which may affect RNA stability or intracellular trafficking, would not be identified by the screening strategy employed in this study. Also, we did not screen for mutations or methylation changes in that region of intron 2 reported to control genomic

imprinting in mice (Stöger *et al.*, 1993; Wutz *et al.*, 1997) or in the promoter region that controls gene expression. Thus, the frequency of mutations described in this report is the minimum present in DEN-initiated rat liver tumors.

In conclusion, the data reported here provides further evidence that the *M6P/IGF2R* functions as a liver tumor suppressor gene. Furthermore, the imprinted *M6p/Igf2r* represents a susceptibility locus for chemical carcinogens in mice and rats since only one allele needs to be inactivated. In contrast, humans would be expected to be less susceptible to the loss of *M6P/IGF2R* function since most people have biallelic expression. These findings raise potentially important regulatory issues concerning the extrapolation of carcinogenesis results from rodents to humans.

Materials and methods

Animals

Male F344 and Lewis \times F344 F₁ hybrid rats weighing 80–100 g (Charles River Laboratories, Raleigh, NC) were fed Purina rodent chow No. 5010 (PMI Feeds, St. Louis, MO) and given water *ad libitum*. They were maintained in a temperature- and humidity-controlled room under a 12 h light/dark cycle. Following 1 week of acclimatization, the rats were treated with DEN (Sigma, St. Louis, MO) at 50 p.p.m. in the drinking water for 1 month. The animals were then randomized; one half of the animals were given regular water while the remaining animals received 0.1% PB (Mallinckrodt, Paris, KY) in their drinking water for 4 months. The exposure regimen resulted in the formation of liver tumors 1–2 cm in diameter in both treatment groups, but there were more in the PB-promoted animals than in the non-promoted animals. After 4 months of PB treatment, all the rats were killed. Tumors and normal liver tissue surrounding the tumors were excised and snap frozen in liquid nitrogen for RNA extraction. All animal use was in full compliance with NIH guidelines for humane

care, and was approved by the Duke University Medical Center Animal Use Committee.

Determination of genomic imprinting

The imprinted status of the *M6p/Igf2r* in rat liver was determined by utilizing a single base sequence polymorphism between F344 and Lewis rats in the 5' end of exon 48 (base number 7176) (Figure 1). DNA and total RNA were simultaneously extracted from F344 × Lewis and Lewis × F344 F₁ hybrid rat livers (Harlan Sprague Dawley, Indianapolis, IN) by the RNA/DNA-Stat-60 method (Tel-Test B, Friendswood, TX), a modification of the single-step acid guanidinium thiocyanate-phenol-chloroform method. Briefly, 50 mg of liver tissue was homogenized using a rotor/stator homogenizer in 1 ml of RNA STAT-60 reagent until disaggregated. After 5 min at room temperature, 200 µl of chloroform was added, the homogenate was vortexed for at least 15 s, stored at room temperature for 2–3 min, and then centrifuged at 12 000 g for 15 min at 4°C. After centrifugation the upper, aqueous phase was transferred to a 1.5 ml microcentrifuge tube, 0.8 ml of isopropanol added and the mixture incubated at room temperature for 5–10 min. Following a further centrifugation at 12 000 g for 15 min at 4°C, the supernatant was removed, the RNA pellet washed with 75% ethanol, vortexed and centrifuged at 7500 g for 5 min at 4°C. The supernatant was discarded and the RNA pellet air-dried before dissolution in 50–100 µl of water.

For DNA isolation, 800 µl of DNA STAT-60 reagent was added to the lower, nonaqueous phase obtained after the initial centrifugation of the tissue homogenate. This sample was vortexed for at least 15 s, stored at room temperature for 2–3 min and centrifuged at 12 000 g for 15 min at 4°C. The DNA isolation then followed the same protocol as that for RNA. Forward (GTCAGCAAGGAGGAGGAGACAG) and reverse (GGGGCTGTGAGCTTTCCACCTC) primers, and genomic DNA as template, were used to amplify the portion of exon 48 containing the polymorphism by the PCR conditions described below.

For RT-PCR, first strand cDNA synthesis was performed using: 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.5 mM 4dNTP mix, 1 µM reverse primer (GCTGAAGTCTTATTTGGTTGGAG), 2–5 µg total RNA, and 200 units M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD) in a total volume of 20 µl. RNA was mixed and heated to 65°C for 10 min, and then chilled on ice prior to the addition of the other components. The final mixture was incubated at 37°C for 60 min and then at 65°C for 10 min. Protein and excess primer were removed from the RT reaction using QIAquick PCR Purification columns (Qiagen, Chatsworth, CA) with the cDNA resuspended in 50 µl H₂O.

To ensure that PCR amplification and subsequent purification of cDNA was not contaminated with genomic DNA, RT-PCR primers were designed to span intron sequences. Thus, amplification of genomic DNA would generate a template containing introns whereas these introns would be absent in the smaller templates generated from cDNA. The reaction conditions for polymerase chain reaction (PCR) were as follows: 10 mM Tris-HCl, pH 8.3

(at 25°C), 50 mM KCl, 1.5 mM MgCl₂, 200 µM 4dNTP mix, 200 nM forward (TGTGGACTTTGATGATGAGAATGC) and reverse (GGGGCTGTGAGCTTTCCACCTC) primers, 5 µl RT template, and 2.5 units Taq DNA polymerase (Perkin Elmer, Norwalk, CT) in a total volume of 100 µl. Hot-start PCR was carried out under the following conditions: 1 min denaturing at 94°C, 1 min annealing at 60°C, and 1 min extension at 72°C for 35 cycles with an additional 5 min extension for cycle 35 on a DNA Thermal Cycler (Perkin Elmer, Norwalk, CT). The cDNA generated by PCR was characterized by agarose gel electrophoresis, and the expected bands gel extracted using a QIAquick Gel Extraction kit (Qiagen, Chatsworth, CA). 10 µl of the gel extracted material was used as a template for direct PCR sequencing using the AmpliCycle kit (Perkin Elmer, Norwalk, CT).

Identification of M6p/Igf2r mutations

RT-PCR MisMatch detection (Ambion, Austin TX) and direct PCR sequencing were used to determine the mutational frequency in the 9.1 Kb mRNA derived from rat liver tumors. The protein coding region of the cDNA was initially screened for mutations by MisMatch detection as previously described (De Souza *et al.*, 1995b). In addition, direct PCR sequencing was used to screen the translational start site, the known IGF2 and M6P protein binding regions, the transmembrane domain and the intracellular trafficking regions for genetic alterations (Kornfeld, 1992; Dahms, 1996). For RT-PCR and direct PCR sequencing, first strand cDNA synthesis and PCR were performed by the methods described above. Briefly, first round primers were used to amplify overlapping 2 Kb fragments, which formed the templates for overlapping nested 500 bp second round amplifications. For nested PCR amplifications, excess primers and other contaminants were removed from the first round amplification using QIAquick PCR Purification columns (Qiagen, Chatsworth, CA). The DNA generated by PCR was characterized by agarose gel electrophoresis and the expected bands gel extracted using a QIAquick Gel Extraction kit (Qiagen, Chatsworth, CA). Ten µl of the gel extracted material was used as a template for direct PCR sequencing using the AmpliCycle kit (Perkin Elmer, Norwalk, CT). Because the fidelity of Taq DNA polymerase is such that errors can be introduced during the PCR process, a number of precautions were taken to address this potential problem. Mutant templates were amplified in two or more independent PCR reactions. In all cases the corresponding normal template was amplified in parallel with the tumor template, and the tumor mutations were confirmed by direct sequencing in both directions.

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