

# *M6P/IGF2R* Imprinting Evolution in Mammals

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## Summary

Imprinted gene identification in animals has been limited to eutherian mammals, suggesting a significant role for intrauterine fetal development in the evolution of imprinting. We report herein that *M6P/IGF2R* is not imprinted in monotremes and does not encode for a receptor that binds IGF2. In contrast, *M6P/IGF2R* is imprinted in a didelphid marsupial, the opossum, but it strikingly lacks the differentially methylated CpG island in intron 2 postulated to be involved in imprint control. Thus, invasive placentation and gestational fetal growth are not required for imprinted genes to evolve. Unless there was convergent evolution of *M6P/IGF2R* imprinting and receptor IGF2 binding in marsupials and eutherians, our results also demonstrate that these two functions evolved in a mammalian clade exclusive of monotremes.

## Introduction

Genomic imprinting is an epigenetic form of gene regulation that results in parent of origin-dependent expression. It is stably inherited during somatic cell division but is reversed when transmitted through individuals of the opposite sex. Imprinting differs from classical Mendelian principles of inheritance because despite the equal distribution of parental autosomal genetic content to the progeny, the two alleles are differentially expressed. Thus, imprinting is a phenomenon where the expression of a genetic allele in a given generation is dependent upon whether it resided in a male or female in the previous generation.

The first two endogenous genes identified to be imprinted were *IGF2* (DeChiara et al., 1991) and *M6P/IGF2R* (Barlow et al., 1991). Homozygous *IGF2* null mice

were found to be 40% smaller than wild-type mice at birth, consistent with the growth promoting function of IGF2. Interestingly, the dwarfing phenotype was also observed in heterozygous mice, but only when the null allele was inherited from the father. This demonstrated that *IGF2* is imprinted and expressed from the paternal allele. In contrast, murine *M6P/IGF2R* is expressed exclusively from the maternal allele and is responsible for a maternally inherited lethal phenotype that maps to the *Tme* (T-associated maternal effect) locus on mouse chromosome 17 (Barlow et al., 1991; Wang et al., 1994).

*M6P/IGF2R* encodes for a receptor in viviparous mammals that binds both phosphomannosyl glycoproteins and IGF2 through independent binding sites (Kornfeld, 1992; Dahms et al., 1993a; Yandell et al., 1999b), whereas the chicken and *Xenopus* receptors lack the ability to bind IGF2 (Clairmont and Czech, 1989; Zhou et al., 1995). Evidence to date suggests that *M6P/IGF2R* does not mediate IGF2-induced cell proliferation (Korner et al., 1995); this function is performed primarily by the insulin-like growth factor I receptor (IGF1R) and the insulin receptor isoform A (Kornfeld, 1992; Frasca et al., 1999; Jirtle, 1999). The primary functions of *M6P/IGF2R* are intracellular trafficking of lysosomal enzymes, and the internalization of IGF2 and other extracellular ligands to the lysosomes for degradation (Kornfeld, 1992; Jirtle, 1999). *M6P/IGF2R* deficiency during mammalian development is associated with cardiac abnormalities, cleft palate, fetal overgrowth, and perinatal lethality (Lau et al., 1994; Wang et al., 1994; Melnick et al., 1998). Interestingly, the elimination of IGF2 expression rescues the lethal *M6P/IGF2R* null phenotype in transgenic mice (Filson et al., 1993). Thus, IGF2 bioavailability needs to be closely regulated during murine embryogenesis, and this function depends upon *M6P/IGF2R*.

A number of theories have been proposed to explain the evolutionary pressure that resulted in the formation of functionally haploid imprinted genes (Bartolomei and Tilghman, 1997; Hurst, 1997). The most actively debated model for imprint evolution arose from the finding that murine *M6P/IGF2R* and *IGF2* are paternally and maternally silenced, respectively. This reciprocal imprinting led Haig and Graham (1991) to propose that genomic imprinting resulted from a parental genetic “tug-of-war” to control maternal-dependent growth of the offspring. According to this genetic conflict model, the duration and extent of intrauterine growth and postnatal care are major driving forces for imprinting evolution.

Monotreme mammals, like the platypus, lay eggs while didelphid marsupials, such as the opossum, give birth to altricial offspring. Nevertheless, both groups of mammals have choriovitelline placentae, have comparable periods of intrauterine gestation, lack a fetal developmental stage, and provide several months of postnatal care (Griffiths, 1999; Harder and Jackson, 1999). Furthermore, the Marsupionta model of mammalian evolution that is supported by mitochondrial DNA analysis groups monotremes and marsupials into a closely related kinship that excludes eutherian mammals (Janke

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et al., 1997; Kullander et al., 1997). This inferred phylogenetic relationship coupled with comparable maternal investments in the young for both monotremes and didelphid marsupials suggests that members of these two mammalian groups have similar imprinted gene profiles.

We tested this postulate by determining whether *M6P/IGF2R* is imprinted in monotremes and didelphid marsupials, as it is in members of the eutherian group of mammals (Barlow et al., 1991; Mills et al., 1998). The species-dependent binding of IGF2 by multifunctional *M6P/IGF2R* also provides a unique opportunity to determine the relationship between receptor function and its imprinting. Our findings demonstrate that *M6P/IGF2R* is not imprinted in the platypus and echidna, whereas it is imprinted in the opossum. Since the opossum lacks a fetal stage of development, invasive placentation and intrauterine fetal growth are not required for genomic imprinting to evolve. *M6P/IGF2R* in both the monotremes and didelphid marsupials also lacks the differentially methylated CpG island in intron 2 previously postulated to be mechanistically involved in imprint control in mice (Stöger et al., 1993; Wutz et al., 1997; Birger et al., 1999). This demonstrates the existence of alternative mechanisms of *M6P/IGF2R* imprint establishment and maintenance. Our results also indicate that monotremes and marsupials are not as closely related as predicted by the Marsupionta model; instead, they support the Theria hypothesis of mammalian evolution (Gregory, 1947; Janke et al., 1997; Kullander et al., 1997; Penny and Hasegawa, 1997; Penny et al., 1999).

## Results and Discussion

There are three phylogenetic groups of living mammals: Prototheria (monotremes), Metatheria (marsupials), and Eutheria (placental mammals). Genomic imprinting has been described in eutherians, but it is unknown whether it exists in monotremes and marsupials. Progeny from interspecies hybrid laboratory crosses are known to harbor global disruptions in imprinted gene expression and epigenetic markings (O'Neill et al., 1998; Vrana et al., 1998). Therefore, we chose to study imprinting evolution by determining whether *M6P/IGF2R* is imprinted in native populations of platypus (*Ornithorhynchus anatinus*), echidna (*Tachyglossus aculeatus*), and the American opossum (*Didelphis virginiana*).

The platypus and echidna are nonplacental mammals that resemble surreal hybrids between eutherian mammals and reptiles and birds (Hughes and Hall, 1998). Their characteristic fur, nursing of young with milk, and thermoregulation place these monotremes within the mammalian kingdom. Nevertheless, the females lay eggs, embryonic cleavage is meroblastic, and male spermatogenesis is bird-like. Unlike other egg-laying vertebrates, the monotreme embryo initially develops in utero (Griffiths, 1999). The egg stretches and grows during a 3- to 4-week period as it accumulates nutrients from maternal endometrial secretions. Thus, in contrast to other oviparous animals, the egg yolk in monotremes contributes little to overall development (Hill, 1933, 1941). Monotreme young are then reared by their mothers for several months after hatching.



Figure 1. Biallelic *M6P/IGF2R* Expression in the Platypus and Echidna

(A) PCR-amplified genomic DNA (panel 1) and cDNA of spleen, liver, and kidney (panels 2–4) demonstrate equal transcript intensities (arrowheads) for the 8557A/G transition polymorphism in the platypus (arrow) (sequenced in reverse direction).

(B) PCR-amplified genomic DNA (panel 1) and cDNA of kidney, liver, skin, intestine, and muscle (panels 2–6) demonstrate equal transcript intensities (arrowheads) for the 702A/G transition polymorphism in the echidna (arrow) (sequenced in reverse direction).

The American opossum is a marsupial characterized by a 13-day in utero gestation. This is the shortest gestation period for any marsupial and is even shorter than the 23- and 28-day gestations of the echidna and platypus, respectively (Manger et al., 1998; Griffiths, 1999; Harder and Jackson, 1999). Unlike true placental mammals, the placenta of the opossum is choriovitelline and does not invade the secretory uterine epithelium (Krause and Cutts, 1985; Roberts and Breed, 1994). Thus, both monotreme and didelphid marsupial development is characterized by in utero nourishment derived from maternal uterine secretions followed by extensive postnatal care in the absence of a father. The overall postzygotic maternal investment in the progeny of the platypus, echidna, and opossum is therefore comparable even though monotremes lay eggs. This suggests that *M6P/IGF2R* imprinting and IGF2 binding would be identical in these nonplacental mammals.

### *M6P/IGF2R* Imprinting Status in Monotremes and Marsupials

We analyzed *M6P/IGF2R* single-nucleotide polymorphisms (SNPs) in 18 different animals including 16 platypuses and 2 echidnas from either mainland Australia or Tasmania to determine whether this gene is imprinted in monotremes. cDNAs corresponding to the entire 9.1 kb platypus and 4.5 kb echidna *M6P/IGF2R* transcripts were sequenced, and 4 platypus and 3 echidna SNPs were identified. The genomic DNA for 8 of the 16 platypus skin biopsy samples was heterozygous (i.e., informative) at one or more of these SNPs. Liver, kidney, and spleen tissues were also available from 2 of the 8 informative platypuses (Figure 1A). The two echidnas were also informative; spleen tissue was available for

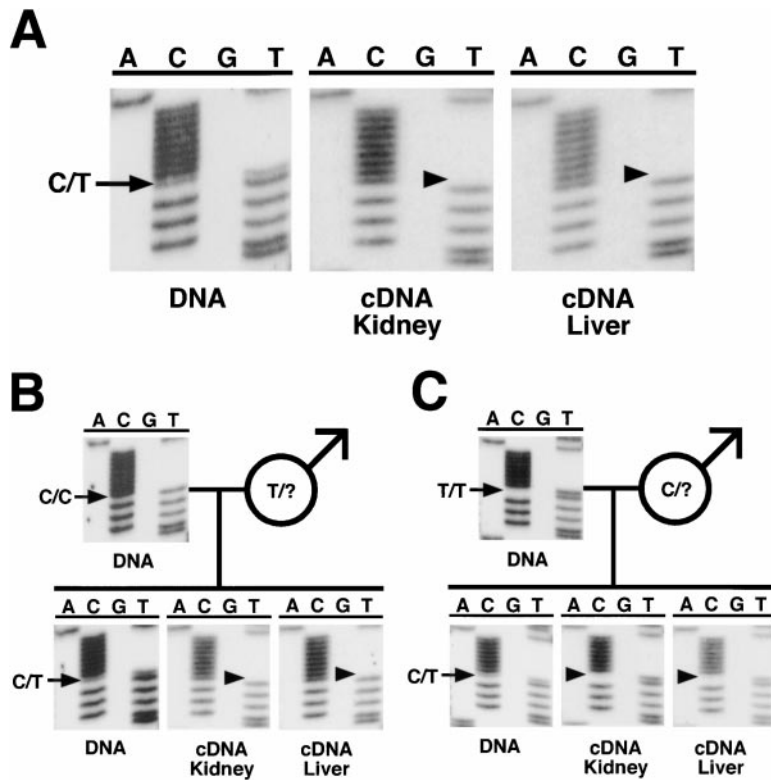


Figure 2. Maternal-Specific *M6P/IGF2R* Expression in the Opossum

(A) A C/T transition polymorphism (panel 1) in genomic DNA at nucleotide position 839 in putative exon 48 (arrow) demonstrates that the *M6P/IGF2R* allele containing the C but not the T (arrowhead) nucleotide is expressed in the kidney (panel 2) and liver (panel 3) of the adult opossum.

(B) Pedigree demonstrating maternal-specific *M6P/IGF2R* expression in opossum kidney and liver. The maternal genotype is C/C at polymorphism 839 (arrow), and a pouch young from that mother is a C/T heterozygote (arrow). The T allele of this offspring is derived from the father, and it is not expressed in the kidney and liver (arrowheads).

(C) Pedigree demonstrating maternal-specific *M6P/IGF2R* expression in opossum kidney and liver. The maternal genotype is T/T at polymorphism 839 (arrow), and a pouch young from that mother is a C/T heterozygote (arrow). The C allele of this offspring is derived from the father, and is not expressed in the kidney and liver (arrowheads).

one animal, and kidney, liver, skin, intestine, and muscle tissues were available for the other animal (Figure 1B). Thus, a total of 20 informative monotreme tissue samples from 6 different organs and 10 individual animals were used to determine the imprint status of *M6P/IGF2R* in monotremes. Using RT-PCR followed by nucleotide sequence analysis to examine each polymorphism, we found that *M6P/IGF2R* expression was biallelic in all platypus (Figure 1A) and echidna (Figure 1B) samples.

To determine whether *M6P/IGF2R* is imprinted in marsupials, an 8.8 kb cDNA fragment of the *M6P/IGF2R* transcript from the American opossum was isolated, sequenced, and analyzed for polymorphisms. Two distinct populations were studied, including animals from Hyde and Wilson Counties, North Carolina. Using an SNP identified at position 839 of putative exon 48, monoallelic *M6P/IGF2R* expression was found in the kidney and liver of 3/3 informative adult female opossums (Figure 2A). DNA and RNA extracted from females and their pouch young were also used to determine the parental origin of the expressed allele. A homozygous DNA genotype was present in 4 of 8 mothers in which one or more pouch young were heterozygotes (Figures 2B and 2C). Quantitative RT-PCR expression analysis showed that expression was derived exclusively from the maternal allele in the kidney and liver of the offspring of these four informative kinships. Thus, opossum *M6P/IGF2R* is maternally expressed, as in eutherian mammals (Barlow et al., 1991; Mills et al., 1998). The reciprocal imprinting of *M6P/IGF2R* and *IGF2* (O'Neill et al., 2000) in marsupials demonstrates that invasive placentation and intrauterine fetal development are not required for genomic imprinting to evolve since these reproductive characteristics are absent in the opossum despite the presence of imprinting.

#### Putative *M6P/IGF2R* Intron 2 Imprint Control Element Is Absent in Monotremes and Marsupials

Murine *M6P/IGF2R* contains a 1.5 kb CpG island and imprinting box in intron 2 that debatably are critical for the imprinting of this gene (Figure 3A) (Stöger et al., 1993; Hu et al., 1999). This CpG island is methylated in mouse oocytes but not in sperm, and its deletion from yeast transgenes alters the expression of YAC-derived transcripts in a parent of origin-dependent manner (Stöger et al., 1993; Wutz et al., 1997; Birger et al., 1999). We sequenced the complete intron 2 of the platypus to determine whether it also contains these regulatory elements. Initial PCR amplification of the platypus *M6P/IGF2R* intron 2 demonstrated that it is approximately 8.3 kb, a length comparable to that of the mouse (Szebenyi and Rotwein, 1994). Despite this similar length, our sequence data demonstrate that intron 2 in the platypus does not contain a vertebrate CpG island, nor does it include the putative imprinting box (Figure 3B) (Gardiner-Garden and Frommer, 1987; Bernardi, 1989). G/C boxes, a hallmark of CpG islands, are also entirely missing from the *M6P/IGF2R* intron 2 sequence of the platypus. Intron 2 of platypus *M6P/IGF2R* is instead comprised of a large number of MIR (mammalian interspersed repeat) and LINE (long interspersed nuclear elements) retroposon repeat motifs. In contrast, retroposons are largely absent in the mouse intron 2 sequence.

We then determined whether the *M6P/IGF2R* differentially methylated putative imprinting control region in intron 2 of mice is present in the opossum (Figure 3C). The complete opossum *M6P/IGF2R* intron 2 was amplified, cloned, and sequenced, which revealed a 4.3 kb intron completely lacking a CpG island. Indeed, there

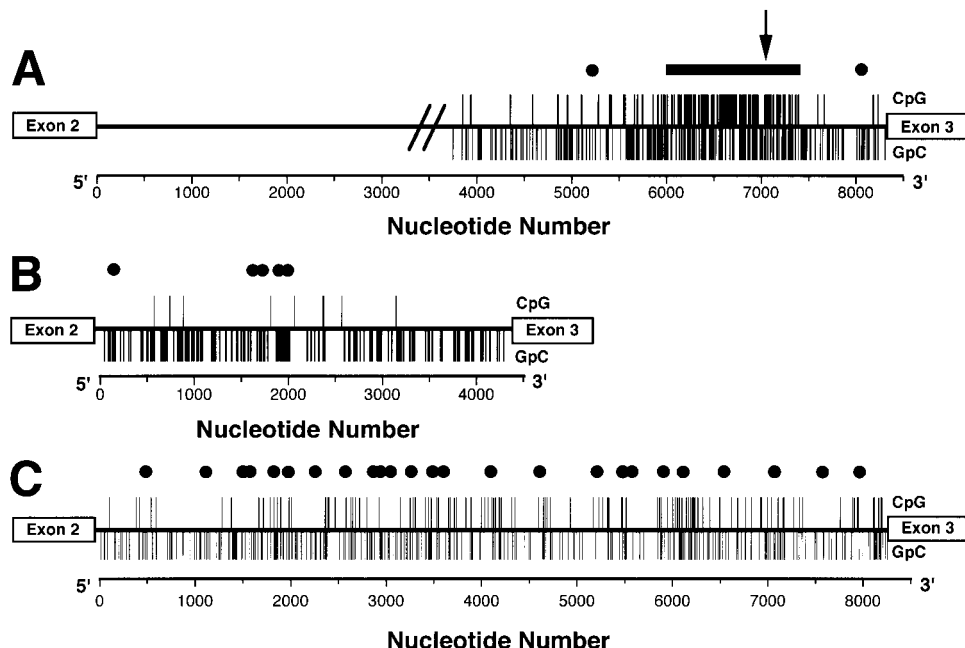


Figure 3. Mouse, Opossum, and Platypus *M6P/IGF2R* Intron 2 Sequence Showing the Distribution of CpG and GpC Dinucleotides (A) The positioning of CpG and GpC sites in the mouse *M6P/IGF2R* intron 2 reveals a 1.5 kb CpG island (Wutz et al., 1997) indicated by a solid horizontal bar. We identified flanking retroposon motifs (circle) (Tomilin, 1999) by analyzing murine intron 2 (GenBank accession number AF151173; Stoger et al., 1993). The location of the putative 113 bp imprinting box (Birger et al., 1999) is indicated by an arrow. (B) The opossum *M6P/IGF2R* intron 2 lacks CpG islands, imprinting boxes, or differentially methylated elements. (C) The platypus *M6P/IGF2R* intron 2 is notable for the absence of CpG islands or homology to the murine imprinting box. In contrast, the platypus intron is largely composed of retroposon motifs.

are only nine CpG dinucleotides within the entire intron. To investigate the possibility that these nine CpGs might be differentially methylated, opossum genomic DNA from four different pouch young was bisulfite treated, and the intronic CpGs were amplified and sequenced. There was no parent of origin-dependent methylation of these CpGs (data not shown). All nine CpGs were substantially methylated, as is normally observed for CpGs outside of CpG islands. The stark absence of a CpG island and differentially methylated CpG elements in the opossum *M6P/IGF2R* intron 2 demonstrates that either the opossum controls *M6P/IGF2R* imprinting differently than does the mouse, or they share an imprinting mechanism whose fundamental features are not yet known.

#### M6P/IGF2R Binding of IGF2 in Monotremes and Marsupials

Sequence duplications of a gene ancestral to both the cation-dependent mannose 6-phosphate receptor (CDM6PR) and *M6P/IGF2R* appear to have resulted in the formation of the 15 repeat regions in *M6P/IGF2R* (Dahms et al., 1987; Szebenyi and Rotwein, 1994). The extracellular region of CDM6PR and all 15 repeat domains in *M6P/IGF2R* are homologous, but only repeat domains 3 and 9 have conserved an M6P-binding site (Dahms et al., 1987, 1993b; Lobel et al., 1988). Furthermore, repeat 11 of *M6P/IGF2R* in eutherians and marsupials gained the ability to bind the growth factor IGF2 (Dahms et al., 1994; Schmidt et al., 1995; Garmroudi et al., 1996). It has been proposed that IGF2 binding by this

receptor evolved in response to high levels of paternally produced IGF2 (Haig and Graham, 1991). According to this model, once *M6P/IGF2R* acquired its ability to bind IGF2 for degradation, natural selection would have favored inactivating the gene when it was paternally derived. This predicts that monotreme *M6P/IGF2R* does not bind IGF2 since the platypus and echidna are not imprinted at this locus. It also follows that the amino acid sequence in the binding domain of monotreme repeat 11 would be divergent from viviparous mammals and would more closely resemble that of the chicken, whose *M6P/IGF2R* does not bind IGF2 (Zhou et al., 1995).

Multiple species amino acid sequence alignment was performed to investigate the basis for M6P (Figure 4) and IGF2 (Figure 5) binding by *M6P/IGF2R*. Repeat domains 3 and 9 were aligned for human, cow, rat, mouse, opossum, platypus, and chicken as shown in Figures 4A and 4B, respectively. Despite some variability in putative  $\beta$  strand 1 and the loop region between strands 1 and 2 in repeat 3 (Figure 4A) and the loop region between strands 2 and 3 in repeat 9 (Figure 4B) (Roberts et al., 1998), there is an overall high homology between all species in these two repeat domains. Furthermore, the four amino acids necessary for M6P binding in CDM6PR and the cysteine residues required for disulfide bonds are completely conserved across all species (Roberts et al., 1998). These findings are consistent with the idea that *M6P/IGF2R* originated as a phosphomannosyl glycoprotein-binding receptor (Dahms et al., 1987; Szebenyi and Rotwein, 1994).

*M6P/IGF2R* imprinting may have evolved to thwart the

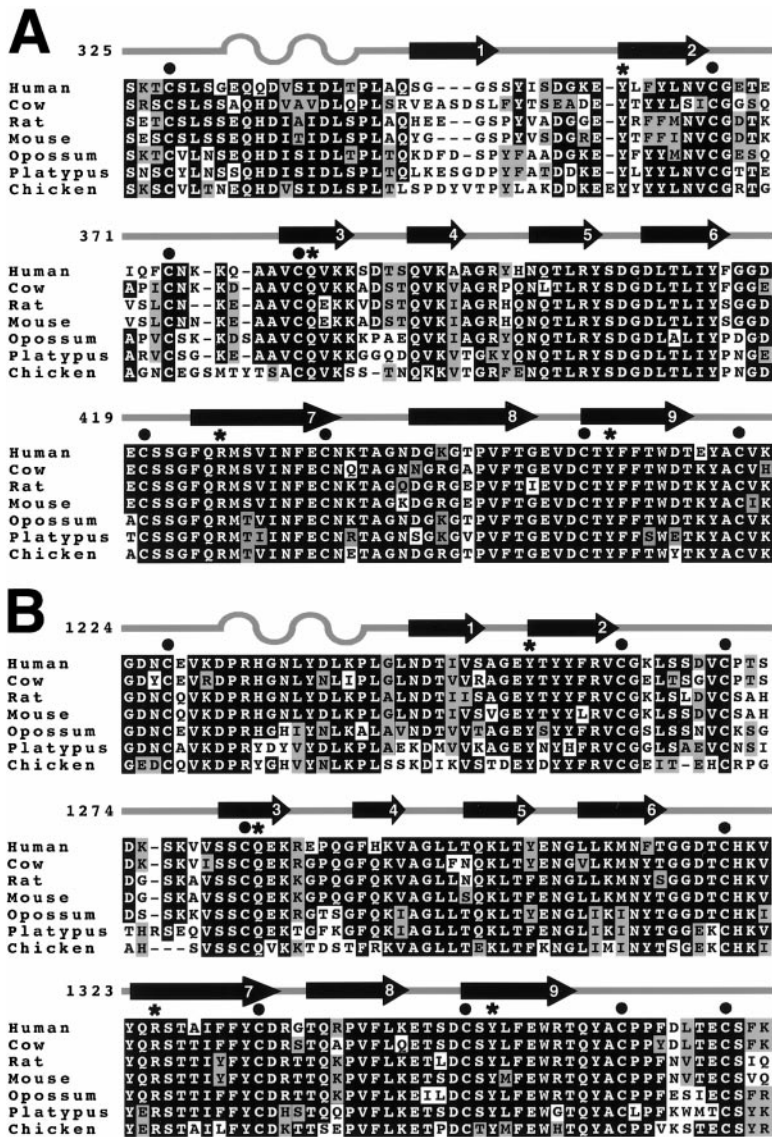


Figure 4. Conservation of the Receptor M6P-Binding Domains

Multiple species alignment of the M6P-binding regions of repeat domains 3 (exons 8–10) (A) and 9 (exons 27–29) (B) of *M6P/IGF2R* (Lobel et al., 1987; Morgan et al., 1987). Translational alignment of the human, cow, rat, mouse, opossum, platypus, and chicken sequences in repeat domains 3 and 9 of M6P/IGF2R demonstrates amino acid sequence identity (black), homology (gray), and divergence (white) between the different species. The secondary receptor structure shown above the sequence alignment is that predicted by the three-dimensional structure of CDM6PR (Roberts et al., 1998). An arrow represents a  $\beta$  strand, and a curved line signifies an  $\alpha$  helix. Cysteine residues (circle) and putative M6P-binding residues (star) are shown. Amino acid numbering is based upon the human sequence (Morgan et al., 1987).

interaction of growth-regulatory ligands, like the latent complex of TGF $\beta$ 1, that utilize the M6P rather than the IGF2 binding ability of this multifunctional receptor (Kovacina et al., 1989). Since TGF $\beta$ 1 activation is M6P/IGF2R dependent (Dennis and Rifkin, 1991), the paternal silencing of *M6P/IGF2R* would reduce the bioavailability of this growth inhibitor, a result also consistent with the genetic conflict model of imprint evolution (Haig and Graham, 1991). Phosphomannosyl glycoprotein binding of M6P/IGF2R (Kornfeld, 1992; Jirtle, 1999), and the amino acids required for M6P binding (Figures 4A and 4B) are conserved in species phylogenetically ranging from chickens to humans. In contrast, *M6P/IGF2R* is imprinted in eutherians (Barlow et al., 1991; Mills et al., 1998) and marsupials (Figures 2A and 2B), but not in monotremes (Figures 1A and 1B). Thus, although M6P binding is an important function of this receptor, it cannot be the driving force for the evolution of *M6P/IGF2R* imprinting.

It has been demonstrated previously that human, cow,

rat, kangaroo, and opossum M6P/IGF2Rs bind IGF2 (Kornfeld, 1992; Dahms et al., 1993a; Yandell et al., 1999b) while the chicken and *Xenopus* receptors lack this function (Clairmont and Czech, 1989; Zhou et al., 1995). Although the insulin receptor, IGF1R, and IGF2-binding proteins also bind IGF2 with high affinity, none of these proteins are structurally related to M6P/IGF2R (Kornfeld, 1992; Krywicki and Yee, 1992; Frasca et al., 1999; Jirtle, 1999). Therefore, they do not assist in defining its IGF2-binding site. The IGF2-binding domain of M6P/IGF2R has been narrowed to amino acids residues 1508–1650 in the N-terminal portion of repeat 11 (Dahms et al., 1994; Schmidt et al., 1995), and Ile-1572 has been shown to be necessary for IGF2 binding (Figure 5) (Garmoudi et al., 1996; Byrd et al., 1999; Devi et al., 1999). By performing a phylogenetic amino acid comparison, we have identified additional residues within repeat 11 that appear to be necessary for M6P/IGF2R low- and high-affinity IGF2 binding in marsupials and eutherians, respectively (Figure 5).

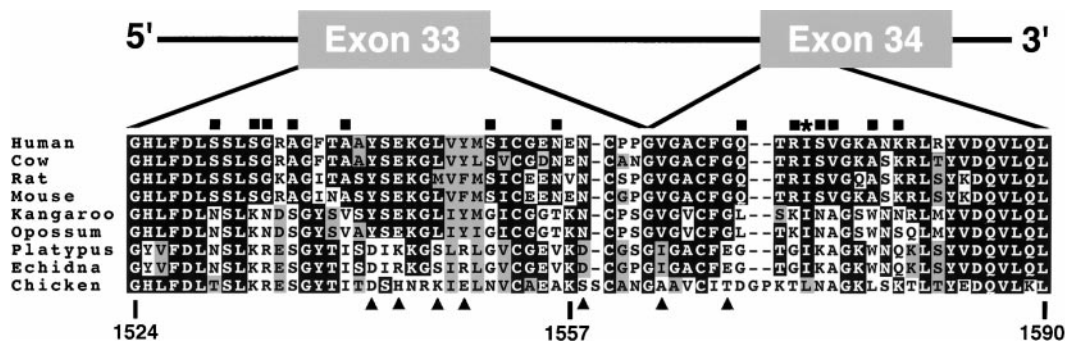


Figure 5. Divergence of the Receptor IGF2-Binding Domain

Multiple species alignment of the IGF2-binding region in repeat domain 11 (exons 33–35) of *M6P/IGF2R* (Lobel et al., 1987; Morgan et al., 1987). Translational alignment of the human, cow, rat, mouse, kangaroo, opossum, platypus, echidna, and chicken sequences 1524–1590 in repeat domain 11 of *M6P/IGF2R* demonstrates amino acid sequence identity (black), homology (gray), and divergence (white) between the different species. The amino acids predicted to have participated in the sequential evolution of low-affinity (triangle) and high-affinity (square) IGF2 binding are shown, as is Ile-1572 (star), previously shown to be required for IGF2 binding (Garmroudi et al., 1996; Byrd et al., 1999; Devi et al., 1999). The genomic structure of extracellular repeat domain 11 is identical for human (Killian and Jirtle, 1999), mouse (Szebenyi and Rotwein, 1994), and chicken (GenBank accession numbers AF225878-93). The numbering is based upon the human sequence (Morgan et al., 1987).

The opossum and kangaroo receptors bind IGF2, but their binding affinity is significantly lower than that of placental mammals (Dahms et al., 1993a; Yandell et al., 1999b). This suggests that acquisition of a high-affinity IGF2-binding site is specific to the eutherian lineage. The sequence comparison in Figure 5 further narrows the high-affinity IGF2-binding site in higher mammals to within amino acid positions 1530–1579 since these residues are highly divergent while both flanking regions are conserved. Acquisition of this high-affinity IGF2-binding site is unlikely to have resulted from exon shuffling or recombination with other IGF2-binding proteins since the exon/intron boundaries for repeat domain 11 are identical for IGF2 binding (mouse and human) and nonbinding (chicken) receptor homologs (Figure 5). Multiple species *M6P/IGF2R* alignment suggests that seven amino acids within this refined region were involved in the formation of a low-affinity IGF2-binding site in marsupials (Figure 5, triangles). These amino acids are divergent in the chicken, platypus, and echidna species that do not bind IGF2, but are highly conserved in marsupials and eutherians. Therefore, a small number of critical amino acid changes in this domain are predicted to have formed the low-affinity IGF2-binding site. Additional conserved amino acid changes also occurred in this same region within the eutherian lineage (Figure 5, squares). These alterations are predicted to enable *M6P/IGF2R* to bind IGF2 with high affinity.

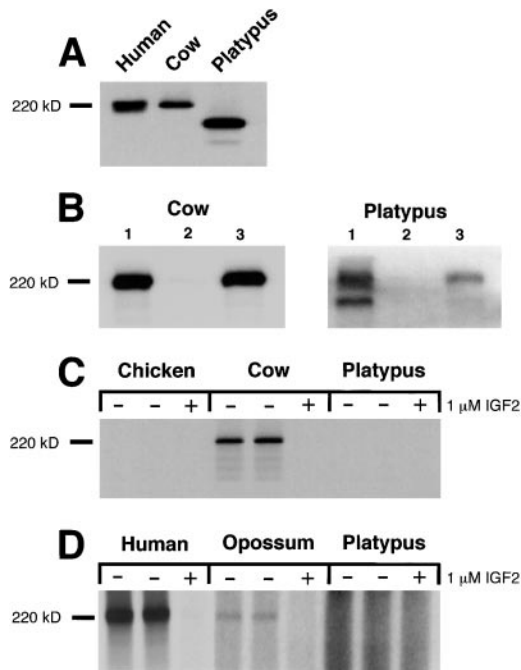
It can be seen in Figure 5 that the monotreme amino acid sequence for repeat 11 is more similar to the chicken than to both the marsupials and eutherians, suggesting that monotreme *M6P/IGF2R* does not bind IGF2. To directly test this hypothesis, we determined the ability of platypus *M6P/IGF2R* to bind IGF2. Human IGF2 was used in these assays rather than platypus IGF2 since Yandell et al. (1999a, 1999b) showed that mammalian and nonmammalian IGF2s are “functionally very similar” regarding their abilities to bind to *M6P/IGF2R*. Furthermore, we have cloned and sequenced platypus *IGF2* and found that it is 94% homologous and 86% identical to human *IGF2* (data not shown).

Western blots using plasma membranes prepared from platypus liver demonstrated a protein that interacted with an *M6P/IGF2R*-specific antibody (Figure 6A). Platypus *M6P/IGF2R* bound to pentamannose phosphate (PMP) attached to Sepharose resin in an *M6P*-dependent manner, confirming the conservation of the receptor’s *M6P* binding function (Figure 6B). In contrast, when we attempted to directly cross-link radiolabeled human IGF2 to chicken, cow, human, opossum, and platypus *M6P/IGF2R*, only the cow, human, and opossum homologs specifically bound IGF2 (Figures 6C and 6D). Further analysis also revealed that platypus *M6P/IGF2R* was incapable of binding IGF2 covalently attached to Sepharose resin (data not shown). Because monotreme *M6P/IGF2R* does not bind IGF2, it follows that it cannot be involved in regulating IGF2-driven embryonic or postnatal growth. Therefore, there would be no evolutionary pressure to imprint this locus in accordance with the genetic conflict model. The lack of *M6P/IGF2R* imprinting in the platypus and echidna, however, does not exclude the possibility that other genes involved in growth are imprinted in monotremes.

## Conclusions

We have shown that *M6P/IGF2R* has undergone significant changes within the mammalian lineage in both its imprint status and ability to bind IGF2. It is striking that *M6P/IGF2R* is imprinted in marsupial opossums but not in monotremes since they share a number of important reproductive characteristics. We have found, however, that monotreme *M6P/IGF2R* does not bind IGF2. Therefore, it is not involved in regulating IGF2-dependent intrauterine growth in this species, and a selective advantage to the paternal germline would not be provided by extinguishing the expression of its *M6P/IGF2R*.

Although interparental genetic conflicts can operate at any stage of postzygotic maternal care, fetal development in utero was thought to be particularly important in the evolution of imprinting (Haig, 1993). Our finding



**Figure 6.** Ligand Binding Characteristics of Platypus M6P/IGF2R (A) An ~200 kDa platypus M6P/IGF2R was detected on Western blot with an antibody specific for M6P/IGF2R. The migration differences between the platypus and the human and cow receptors may represent variation in disulfide bonding or receptor conformation. (B) Immunoblots showing that cow (panel 1) and platypus (panel 2) M6P/IGF2R bind M6P residues specifically. Lane 1, acid-washed plasma membranes; lane 2, unbound fraction following incubation of plasma membranes with PMP-Sepharose resin; lane 3, fraction following M6P elution from the PMP-Sepharose resin. (C) IGF2 affinity labeling of plasma membrane preparations from chicken, cow, and platypus. Cross-linking of liver plasma membranes to <sup>125</sup>I-labeled human IGF2 in the absence (-) or presence (+) of 1 μM unlabeled human IGF2. (D) IGF2 affinity labeling of plasma membrane preparations from human, opossum, and platypus. Cross-linking of plasma membranes to <sup>125</sup>I-labeled human IGF2 in the absence (-) or presence (+) of 1 μM unlabeled human IGF2.

that opossum *M6P/IGF2R* is maternally expressed demonstrates that imprinting is not unique to mammals with an intrauterine fetal growth stage or those with invasive placentation. Thus, if genetic conflicts drove *M6P/IGF2R* imprinting in the opossum, they operated at the embryonic and/or the postnatal stages of development. The opossum also lacks the intronic structures thought to be necessary for *M6P/IGF2R* imprinting in mice (Stöger et al., 1993; Wutz et al., 1997; Birger et al., 1999). Marsupials therefore either developed an alternative imprinting system or they share an imprinting mechanism with eutherians whose fundamental features have not yet been defined. Comparative sequence and chromatin structure analysis of the *M6P/IGF2R* locus in eutherians and marsupials will facilitate the resolution of this important regulatory issue.

The Theria hypothesis of mammalian evolution argues for the early divergence of monotremes from a natural clade that includes marsupials and eutherians while the Marsupionta model purports an early divergence of placental mammals from a lineage that ultimately gave rise

to monotremes and marsupials (Gregory, 1947; Janke et al., 1997; Kullander et al., 1997; Penny and Hasegawa, 1997; Penny et al., 1999). Unless there was convergent evolution of both *M6P/IGF2R* imprinting and IGF2 binding in marsupials and eutherians, our findings support the Theria hypothesis of mammalian evolution.

#### Experimental Procedures

##### Tissue Samples

Tasmanian and mainland Australian platypus (*Ornithorhynchus anatinus*) visceral organs (i.e., spleen, liver, and kidney) and skin biopsies were obtained from wild animals that were a victim of dog attack and were under surveillance, respectively. Echidna (*Tachyglossus aculeatus*) tissues were harvested from animals that were accidentally killed by automobiles. Kidney and liver tissues were taken from eight healthy adult female American opossums (*Didelphis virginiana*) and their pouch young following euthanasia by the North Carolina Wildlife Commission officials as part of a predator removal/disease epidemiology study in Hyde and Wilson Counties, NC (Stoskopf et al., 1999). Samples were transported on dry ice from either the University of Tasmania or Hyde and Wilson Counties, NC, to Duke University where they were maintained at -80°C until protein, DNA, and RNA extraction. Fresh chicken and cow livers were obtained from Albertson's supermarket (Omaha, NE) prior to membrane preparation at the University of Nebraska.

##### Isolation and Sequencing of Platypus, Echidna, and Opossum *M6P/IGF2R*

Total RNA was isolated from platypus, echidna, and opossum tissues by homogenization in RNA-Stat 60 (Tel-Test, Friendswood, TX). First-strand cDNA was synthesized from 1–5 μg of total RNA using SuperScript II (Life Technologies, Baltimore, MD). Forty non-degenerate cross-species RT-PCR primers were designed based upon human, cow, rat, mouse, and chicken *M6P/IGF2R* sequence alignment (<http://www.geneimprint.com>), and two cross-species primer sets were identified. With cDNA as a template, the forward primer 5'-TGTGTGCATTACTTTGAATGGAGGAC-3' and reverse primer 5'-GGCATACTCAGTGATCCACTC-3' were used to amplify exons 3–8 of the echidna, opossum, and platypus *M6P/IGF2R* using 1.5 U Platinum Taq DNA polymerase (Life Technologies, Baltimore, MD), 15 pmol primers, 1.5 mM MgCl<sub>2</sub>, and 100 μM dNTPs in a 30 μL PCR reaction volume (94°C × 15 s, 55°C × 5 s, and 72°C × 45 s for 30–35 cycles). Alternatively, the Titan 1 Tube RT-PCR (Roche Boehringer Mannheim, Indianapolis, IN) system was used according to the manufacturer's directions with these primers to amplify exons 3–8 directly from total RNA in a single 35 cycle round of RT-PCR. The forward primer 5'-GCAGACATGCACTCTCTTCTTCTCITGGC-3' and reverse primer 5'-AAGCCTCATAACCACAGTGGC-3' were used to amplify *M6P/IGF2R* exon 34, intron 34, and exon 35 from 100 ng echidna, opossum, and platypus genomic DNA using the PCR conditions described above. RT-PCR and PCR products were analyzed by electrophoresis on a 1.5% agarose gel, and the appropriately sized fragments were excised and gel extracted (GenElute, Sigma Chemical Co., St. Louis, MO). Following direct sequencing of these RT-PCR and PCR products according to the manufacturer's protocol (Thermo Sequenase, USB Corporation, Cleveland, OH), gene-specific primers were designed to connect exon 8 with 34 by cDNA amplification using Expand Long Template PCR (Roche Boehringer Mannheim, Indianapolis, IN) with buffer #3 (94°C × 15 s, 68°C × 2 min for 32 cycles). Other gene-specific primers were designed for use in 3'RACE and 5'RACE as recommended by the manufacturer (Life Technologies, Baltimore, MD) to complete the *M6P/IGF2R* cDNA sequence. Intron 2 of opossum and platypus *M6P/IGF2R* (Figure 3) were amplified by long-template PCR (Roche Boehringer Mannheim, Indianapolis, IN) with the use of primers to putative exons 2 and 3. The distribution analysis of CpG and GpC sites in intron 2 of mouse, opossum, and platypus *M6P/IGF2R* was performed using Gene Jockey II (Biosoft, Cambridge, UK). The absence of differential methylation in opossum intron 2 was determined by DNA sequencing following bisulfite treatment as described in the manufacturer's protocol (CpGenome DNA Modification Kit,

Intergen Company, Purchase, NY). Translational alignments of repeat domains 3, 9, and 11 of M6P/IGF2R were performed with ClustalW followed by Boxshade-assisted shading (BCM server). Exon numbering is based upon the genomic structure of the human homolog (Killian and Jirtle, 1999). All gene-specific PCR primers and conditions are available upon request.

#### Determination of M6P/IGF2R Imprinting in the Platypus, Echidna, and Opossum

The imprint status of M6P/IGF2R in the echidna, opossum, and platypus was determined using SNPs. M6P/IGF2R polymorphisms were identified in these animals by direct sequence analysis of RT-PCR products (Thermo Sequenase, USB Corporation, Cleveland, OH). Four M6P/IGF2R SNPs were detected in the platypus (cDNA positions 4534C/T, 7812A/G, 8409A/G, and 8557A/G), three in the echidna (cDNA positions 230A/G, 702A/G, and 1051C/T), and one in the opossum (C/T at nucleotide position 839 in putative exon 48). Genomic DNA was then extracted from different animals (DNA-Stat 60, Tel-Test, Friendswood, TX), and those that were polymorphic (i.e., informative) were selected for quantitative RT-PCR imprinting analysis (Kelsey and Reik, 1998). Total RNA was extracted from tissues from the informative animals and reverse transcribed as described above. PCR oligonucleotides designed to cross spliced exon junctions (data not shown) were used to amplify fragments from the cDNA that contained a polymorphism; PCR amplification was a single round of 30–35 cycles. The absence of contaminating genomic DNA was confirmed by agarose gel electrophoresis combined with direct sequencing across exon–exon junctions. Mixing studies further demonstrated unbiased amplification of the two polymorphic alleles (data not shown). The imprint status of M6P/IGF2R was determined by comparing the genomic DNA and cDNA sequence at each polymorphic nucleotide position.

#### M6P/IGF2R Ligand Binding

Chicken, cow, opossum and platypus plasma membranes were prepared from 2 g of liver tissue (Oppenheimer and Czech, 1983). Plasma membranes expressing recombinant human M6P/IGF2R were prepared from transfected 293T cells (Devi et al., 1998). Equal amounts of liver plasma membranes from cow and platypus (200 µg) or 100 µg of transfected 293T cell membranes were electrophoresed on 6% nonreducing gels, transferred to BA-85 nitrocellulose, probed with a polyclonal antibody to cow M6P/IGF2R (gift of P. Lobel, Piscataway, NJ), and detected with <sup>125</sup>I-Protein A (Dupont NEN, Boston, MA). Endogenous ligands were removed by washing plasma membranes twice for 15 min with 0.5 M NaCl, 0.2 M acetic acid followed by two washes with HEPES-buffered saline (HBS) (pH 7.4). Equal volumes (200 µL) of a 5 mg/ml cow or platypus membrane protein extract were exposed to 100 µl of packed PMP-Sepharose resin for 3 hr at 3°C. The PMP-Sepharose resin was washed twice with HBS containing 0.05% Triton X-100 (HBST) followed by receptor elution with 200 µl HBST + 10 mM M6P. Prebound, washed, and postelution aliquots were electrophoresed and immunoblotted, and the receptor was detected with an antibody to cow M6P/IGF2R. IGF2 affinity labeling was conducted as described previously (Devi et al., 1998). In brief, 200 µg of chicken, cow, opossum, and platypus plasma membranes or 100 µg of recombinant human M6P/IGF2R plasma membranes from transfected 293T cells were incubated with 2 nM <sup>125</sup>I-labeled human IGF2 + 100 nM IGF1 for 16 hr at 3°C, followed by the addition of 0.25 mM disuccinimidyl suberate for 30 min at 3°C. Cross-linking was terminated by adding 0.1 M Tris–HCl (pH 7.4). Cross-linked IGF2-receptor complexes were resolved on 6% SDS-PAGE. Recombinant human IGFs were provided by M. H. Niedenthal (Lilly Research Laboratories, Indianapolis, IN). Native *Hansenula holstii* Y-2448 O-phosphomannan was a gift from M. E. Slodki (Midwest Area Northern Regional Research Center, Peoria, IL). Carrier-free Na<sup>125</sup>I (Amersham, Arlington Heights, IL) was used to radioiodinate IGF2 with precoated iodogen tubes (Pierce, Rockford, IL).

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#### GenBank Accession Numbers

GenBank accession numbers are as follows: AF151171 and AF225877, respectively, for the entire intron 2 of platypus and opossum *M6P/IGF2R*; AF151173 for a portion of the mouse *M6P/IGF2R* intron 2; AF151172 for the full-length platypus *M6P/IGF2R* cDNA homolog; AF225894 and AF225895, respectively, for the 4.5 kb of the echidna and 8.8 kb of the opossum *M6P/IGF2R* cDNA homologs; AF225878–93 for chicken *M6P/IGF2R* exons 29–39; and AF225876 for platypus *IGF2*.