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A skeletal muscle-specific mouse *Igf2* repressor lies 40 kb downstream of the gene

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SUMMARY

***Igf2* and *H19* are closely linked and reciprocally expressed genes on distal chromosome 7 in the mouse. We have previously shown that a 130 kb YAC transgene contains multiple tissue-specific enhancers for expression of both genes during embryogenesis. The YAC also contains all the crucial elements responsible for initiating and maintaining appropriate parent-of-origin-specific expression of these genes at ectopic sites, with expression of *Igf2* after paternal inheritance and of *H19* after maternal inheritance. Located centrally between *Igf2* and *H19* are two prominent DNaseI hypersensitive sites, and two stretches of sequence that are conserved between mouse and human. In this study, we have deleted, from the transgene, a one kb part of the intergenic region that contains the hypersensitive sites and one of the homologous stretches. We demonstrate that this deletion results in loss of maternal *Igf2* repression in skeletal muscle cells, most strikingly in the tongue, late in embryogenesis. We propose that the intergenic region functions as a tissue-specific repressor element, forming an integral part of the complex regulatory mechanism that controls monoallelic gene expression in this domain.**

Key words: *Igf2*, *H19*, Imprinting, Silencer, YAC transgene, Tongue, Skeletal muscle

INTRODUCTION

Genomic imprinting in mammals results in the differential expression of a limited number of autosomal genes. This process is dependent on parental origin, whereby the imprint is reset during gametogenesis in every generation. To date, nearly 40 imprinted genes have been identified in the mouse, and many of these are clustered within large domains (summarised at www.mgu.har.mrc.ac.uk). This suggests that the imprinting process may be controlled by higher order regulation of chromatin structure, and may use common regulatory elements within a domain (Ainscough and Surani, 1996; Reik and Walter, 1998; Surani, 1998).

We have been investigating a group of imprinted genes on distal chromosome 7 in the mouse (Ainscough et al., 1998), which includes the reciprocally expressed insulin-like growth factor 2 (*Igf2*) and *H19* genes (Bartolomei et al., 1991; DeChiara et al., 1991). These genes lie approximately 70 kb apart (Koide et al., 1994; Zemel et al., 1992). Expression of *Igf2* is predominantly from the paternal allele, while that of *H19* is from the maternal allele. However, their patterns of expression are similar, both spatially and temporally, indicating that they may be controlled by the same regulatory elements (Bartolomei et al., 1993).

Within the *Igf2*/*H19* domain are a number of prominent epigenetic features that are characteristic of important regions, such as expressed sequences or regulatory elements. These features include areas of CpG-rich sequence, which are differentially methylated on the two parental chromosomes, and regions that are hypersensitive to nucleases (Bartolomei et al., 1993; Feil et al., 1994; Ferguson-Smith et al., 1993; Khosla et al., 1999; Sasaki et al., 1992). Some regulatory elements from the *Igf2*/*H19* domain have been identified and partially characterised, and are invariably associated with one or more of these epigenetic markers (Khosla et al., 1999; Koide et al., 1994; Yoo-Warren et al., 1988).

The available evidence suggests that *Igf2* and *H19* share some regulatory elements, most notably the enhancers just downstream of *H19*, which direct expression of both genes in liver and gut endoderm (Leighton et al., 1995a). We have recently shown that at least some of the mesoderm specific enhancers, including those for skeletal muscle, are located on the 130 kb YAC transgene, YZ (Ainscough et al., 1997, 2000a). This YAC also contains sequences that control reciprocal imprinting of these genes with high fidelity (Ainscough et al., 1997). These sequences include silencers (Brenton et al., 1999; Drewell et al., 2000) and boundary elements (Bell and Felsenfeld, 2000; Hark et al., 2000; Szabo et al., 2000; Thorvaldsen et al., 1998). The mechanism regulating imprinted expression of *Igf2* and *H19* is therefore highly complex and uses regulatory elements that are distributed throughout the region.

Here, we report on the function of a previously identified genomic region located centrally between *Igf2* and *H19* (Koide et al., 1994). This region was characterised as unmethylated and hypersensitive to nuclease on both alleles, and showed a

high degree of conservation between mammalian species. One possibility is that the region contains an additional gene. Alternatively, it may contain additional control elements required for regulating expression of *Igf2*, *H19*, or both. As a first step towards deciphering the role of this region, we deleted the central 1 kb sequence from the YAC transgene. This 1 kb region contains the hypersensitivity sites, a stretch of homology between mouse and human, and part of the unmethylated GC-rich sequence. Our investigation demonstrates that this region regulates repression of the maternal *Igf2* allele in a highly tissue specific manner.

MATERIALS AND METHODS

Mouse strains and polymorphism analysis

(C57BL/6 × CBA) F_1 and BALB/c mice were used throughout. The YAC clone was from C3H mice. $F_1 \times F_1$ crosses were used for analysis of *lacZ* expression. BALB/c mice were crossed with F_1 mice for polymorphic analysis of *H19* expression as described (Ainscough et al., 1997, 2000a).

YAC manipulations and transgenic mice

The yeast artificial chromosome (YAC) clone YZ (Ainscough et al., 1997) was modified by homologous recombination using alkali-cation yeast transformation kit (Bio 101), and vectors constructed in this laboratory. High quality intact YZ DNA was purified for microinjection as described (Ainscough et al., 2000b). Dialysed YAC DNA was injected at a concentration of <1 ng/μl into fertilised eggs from $F_1 \times F_1$ matings following standard procedures (Allen et al., 1987). Surviving eggs were cultured overnight in T6 medium supplemented with 4 mg/ml BSA, and transferred at the two-cell stage to oviducts of day 1 pseudopregnant F_1 females.

Transgene copy number

DNA from pup tail samples was digested with *Pvu*II, separated through 1.3% agarose, blotted onto hybond N+ membrane and hybridised with a [^{32}P] dCTP-labelled 1 kb *Bam*HI-*Xho*I (BX) probe located adjacent to the *lacZ* insert in the *Igf2* gene. YAC copy number was determined by densitometry of the hybridising bands using Image Quant phosphorimager software, normalised for the endogenous signal as two copies.

Cre-induced loxP recombination

Fertilised eggs from superovulated F_1 females, mated with homozygous transgenic males, were injected with a Cre-recombinase-expressing plasmid (courtesy of S. Aparicio) at 0.1 ng/μl, and transferred to the uterus of pseudo-pregnant foster mothers at the two cell stage. DNA was prepared from tail biopsies of live born pups and typed for the deleted allele by polymerase chain reaction (PCR) using primers external to the loxP sites. Confirmation of deletion was by Southern-blot analysis (see Fig. 2C). Animals containing the recombined allele were used as founders for investigating the effect of the deletion after germline transmission. To study the deletion effects without germline transmission, homozygous transgenic females were mated with heterozygous CMV-Cre males (courtesy of S. Aparicio) and embryos were removed at day 17 of gestation for typing and *lacZ* expression analysis.

lacZ expression

Embryos and tissues for *lacZ* analysis, using Blue-Gal (Melford Laboratories Ltd), were washed with cold PBS and fixed for 1 hour at 4°C in 5% formaldehyde, 0.8% glutaraldehyde, 0.02% NP40, 1 mM $MgCl_2$, 0.1 mg/ml Sodium deoxycholate in PBS. Samples were then washed in PBS and incubated for 48 hours at 30°C in Blue-Gal staining solution (1 mg/ml Blue-Gal, 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM magnesium chloride in PBS). After further washing with PBS, samples were fixed overnight in 4% formaldehyde, and stored in 70% ethanol.

In situ hybridisation analysis

In situ hybridisation analysis was performed as described (John et al., 1999), using in vitro transcribed, DIG labelled probes from a 2 kb *H19* cDNA clone. *H19* null mice, used for in situ analysis, were as described (Ripoche et al., 1997).

RESULTS

The A6A4 intergenic region

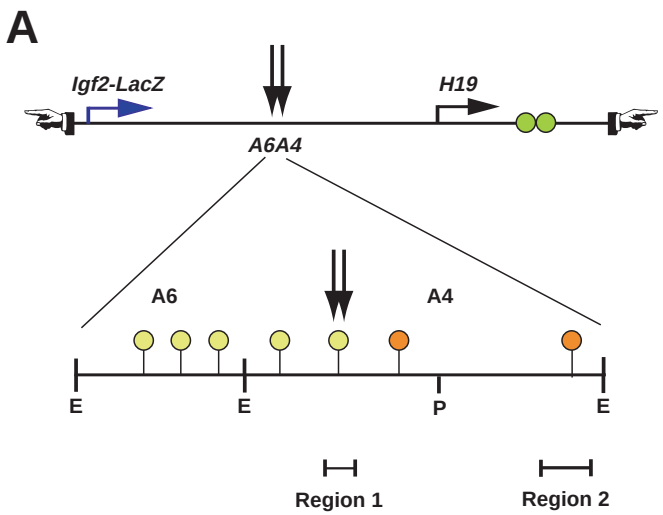
We have previously identified a region (A6A4) located centrally between *Igf2* and *H19* that is GC rich, unmethylated, hypersensitive to DNase I enzyme and shows cross-species conservation (Fig. 1) (Koide et al., 1994). The GC-rich DNA containing the unmethylated CpG sites spans approximately 1 kb, from the A6 fragment up to and including the hypersensitive sites. Outside of this region the DNA is progressively less GC rich and more methylated (Koide et al., 1994).

To characterise the degree of conservation between mouse and human, a human cosmid clone was isolated using the mouse A4 fragment as a probe (Koide et al., 1994). A4-positive restriction fragments were subcloned and sequenced. Comparison of the mouse (accession number: AF263830) and human (AF263831) sequences revealed two regions of homology (Regions 1 and 2) within the A4 fragment, separated by a region of highly divergent sequence (Fig. 1A). Region 1 spans approx. 100 bp and lies in the area which shows strong hypersensitivity, while Region 2 (approx. 200 bp) lies towards the *H19* side of A4 (Fig. 1A,B). In humans this sequence lies approx. 37 kb upstream of the *H19* gene (see AC004556). We could not detect any convincing evidence for expression of either the mouse or human sequence, suggesting that this region is probably not a new gene. This was supported by extensive reverse transcriptase (RT)-PCR analysis using RNA from a range of tissues and developmental stages, which detected no products (not shown). It is therefore possible that these small conserved regions have independent regulatory functions for *Igf2* and/or *H19*.

To begin to understand the role of this region we have investigated the function of the hypersensitive sites contained within Region 1, by flanking them with loxP sites on the YAC transgene, YZ (Ainscough et al., 1997), allowing their deletion under controlled conditions.

YZΔΔ transgenic lines

We previously demonstrated that *Igf2-lacZ* imprinting from the YZ transgene was copy number dependent, whereas *H19* imprinting was not, as *Igf2-lacZ* only imprinted when integrated as one, or at most two copies (Ainscough et al., 1997). In this study, we used the same YAC clone, but with the addition of flanking the 1 kb Region 1 containing sequence with loxP (Δ) sites (designated YZΔΔ, Fig. 2A), using established homologous recombination techniques in yeast (Rothstein, 1991). Eight transgenic lines were established, ranging in copy number from one to six (Fig. 2B). These were assessed by analysing a polymorphic *Pvu*II site introduced by the *Igf2-lacZ* gene (Ainscough et al., 1997). Southern-blot



B

Region 1 (86%)

M AAATTTTATTGTCTCTTGCGCCCCCGTGAGGTCAGCGGTTAGCATAACAACCAT
H AAATTTTgTTGTCTCcTGCGCCCCCGTGAGGTCAG tGGgcAGCATgACAcCCAT

M TTGGCGACTGAGGTGGGGTTCCTGGGGGGGATGCAGGTGGAAAATGCCCC
H TTGGC cACTGAGGTGGGGcTCCT aGGGGGGgTGCAGGTGGgAAgcGaCCC

Region 2 (87%)

M AGCCCAGCTGGGACAGCAAACAAACCTCT -GGGTCTCTCCTTTCCCTTGCCT
H AGCCCAGCTGGGACAGCAAACAggCCTCTgGGGTCTCTCCTTTCCCTTGCCT

M GCCCACCACAGGGGTCTGGCTGGGGCCAGAGCTGGGGGAGACTTCCTCGAT
H GCCCAC tgCAGGGGgCTGGC cGGGG -CAGgGCcGGGGGAGACTTCCTCGAT

M TTATCTCTGCAGCCGGTGGCCAGCCCTAAGCCTGGAATCCCGGGTCAAGCTA
H TTATCTCTGCAGCC aGcGG aCAGCC tacAGCCTGGAATC tg GGGT tAgGCTg

M AGGTTTGCCTGCCCTGCTGTGAGCAGCTTGGAGCTGGTCAGAAGG
H AGGTTTGCgTG tCCTGCTGTGAGCAGCTTG cAGCaGGaCAGAAGG

Fig. 1. Structure of the A6A4 intergenic region. (A) The YAC transgene spans 130 kb and encompasses the *Igf2-lacZ* gene, the A6A4 region, the *H19* gene and the enhancers downstream of *H19* (green circles). Vertical arrows indicate DNase I hypersensitive sites. Horizontal arrows show direction of transcription. YAC arms are shown at each end of the transgene. The A6A4 region (magnified) spans 3 kb and contains seven methylation sensitive restriction sites (circles). Unmethylated sites are yellow, and partially methylated sites are red. Location of two homologous stretches between mouse and human are indicated (Regions 1 and 2). (B) Sequence comparison between mouse (blue) and human (red) DNA in regions 1 and 2. Degree of identity is shown. Mis-matches are shown in lower case on the human sequence. E, *Eco*RI; H, human sequence; M, mouse sequence; P, *Pst*I.

analysis using cosmid clones cAH, cCH and cEIG (which span the entire YAC; Koide et al., 1994); plasmid clones as probes; and YAC arm specific PCR, revealed no evidence of rearrangement of the YAC in any of the lines (not shown).

Igf2-lacZ activity is not affected by introduction of the loxP sites

Preliminary assessment of *Igf2-lacZ* activity was performed on embryos at day 12 of gestation after paternal transmission. The staining pattern was highly consistent with that reported in our previous study, showing strong activity in the limb buds and liver, somites, developing ribs, and cranio-facial muscle precursors (Ainscough et al., 1997) (Fig. 3). Although we also detected strong staining in the yolk sac after transmission through the paternal germline, no staining was seen in the placenta (Fig. 4). This suggests that the enhancers for *Igf2* expression in yolk sac are present on the YAC, while those for placental expression are not. No activity was seen in the developing nervous system. Therefore, introduction of the loxP sites onto the YZ transgene did not disrupt any major elements regulating expression of *Igf2*.

Igf2-lacZ is appropriately imprinted

To see if the modifications affected *Igf2-lacZ* imprinting we examined expression following maternal transmission (Figs 3 and 4). Consistent with our previous investigation, we found that inserts containing one or two copies of the transgene exhibited imprinting, where *Igf2-lacZ* was repressed following maternal transmission, and reactivated after paternal transmission. Both of the single copy, and two of the two-copy lines showed robust imprinting (Fig. 3A). Indeed, after maternal transmission *Igf2-lacZ* activity was only seen in the meningial layers of the brain at later stages in development (see Fig. 5C). This is entirely consistent with the bi-allelic *Igf2* expression seen in this tissue at the endogenous locus. For the remaining transgenic lines, which contained two or more copies of the transgene, β-galactosidase (β-gal) staining was seen in all appropriate tissues after both paternal and maternal transmission (Fig. 3B). These results support our previous observations demonstrating a copy number dependency for *Igf2-lacZ* imprinting in the embryo (Ainscough et al., 1997).

Imprinting in yolk sac is robust

In addition to expression in the embryo, we also detected expression of *Igf2-lacZ* in the yolk sac. In this tissue, however, the *Igf2-lacZ* gene was expressed only after paternal inheritance (Fig. 4). It is particularly striking that the parent-of-origin-dependent expression occurred irrespective of transgene copy number, or site of integration, which is clearly different from imprinting of the *Igf2-lacZ* gene in the embryonic tissues. The reasons for this highly robust imprinting in the yolk sac, but not in the embryo, are not clear at present.

Importantly, the results of this initial analysis demonstrate that the loxP sites flanking the intergenic hypersensitive locus had no notable deleterious effect on expression or imprinting of *Igf2-lacZ*. We were therefore able to commence our investigation into the function of the hypersensitive sites located in the intergenic region, by deletion of this region in situ.

Deleting the intergenic hypersensitive sites

Having established a number of transgenic lines, with appropriate expression and imprinting of the *Igf2-lacZ* gene, we next deleted the intergenic hypersensitive sites in situ, by Cre-enzyme mediated recombination (see Materials and Methods). Two imprinting transgenic lines were chosen, one containing a single copy of the YZΔΔ transgene (ΔΔ19) and the other containing two copies (ΔΔ17). Following Cre treatment live-born progeny were checked for deletion of the intergenic 1 kb region from the YAC, by PCR (not shown). The

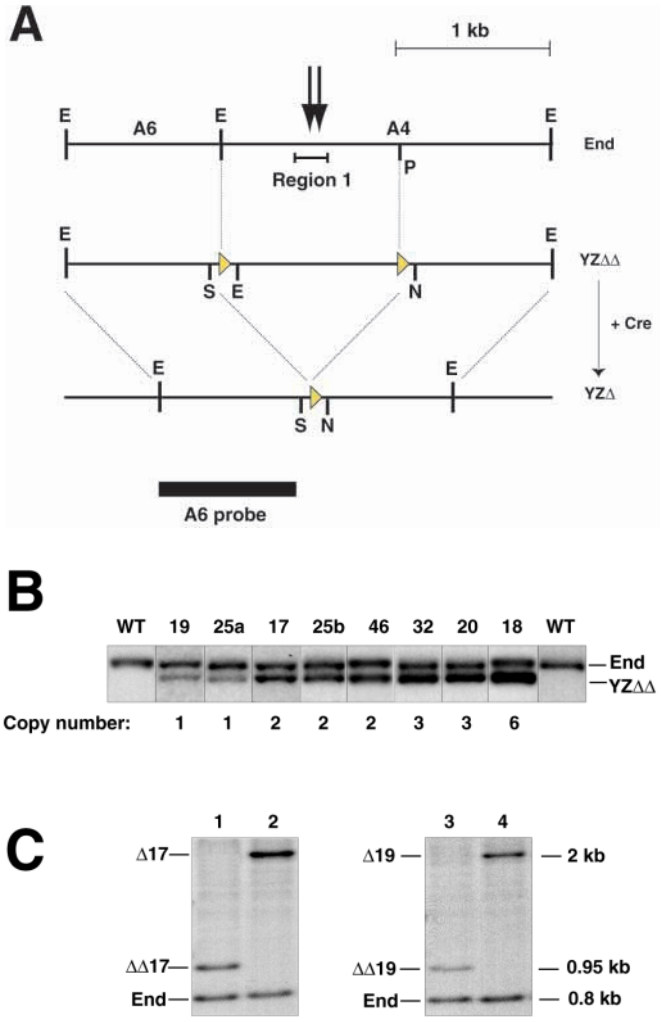


Fig. 2. Position of loxP sites (yellow triangles) on the transgene, and characterisation of transgenic animals. (A) loxP sites were introduced to flank Region 1 and the hypersensitive sites, at the *Eco*RI (E) and *Pst*I (P) sites, resulting in transgene YZΔΔ. After exposure to Cre recombinase, the intergenic region is deleted as shown (designated YZΔ). (B) Transgene copy number in each line was assessed by *Pvu*II digestion and hybridisation with probe BX (Ainscough et al., 1997), to show the endogenous 2.0 kb fragment (2 copy) and the transgenic 1.8 kb fragment. Numbers above the lanes indicate transgenic line. (C) Successful deletion following Cre treatment was confirmed by Southern blot analysis on *Eco*RI digested DNA, hybridised with the A6 probe shown in (A), in one single copy line (19) and one two copy line (17). S, *Spe*I; N, *Not*I; WT, nontransgenic DNA.

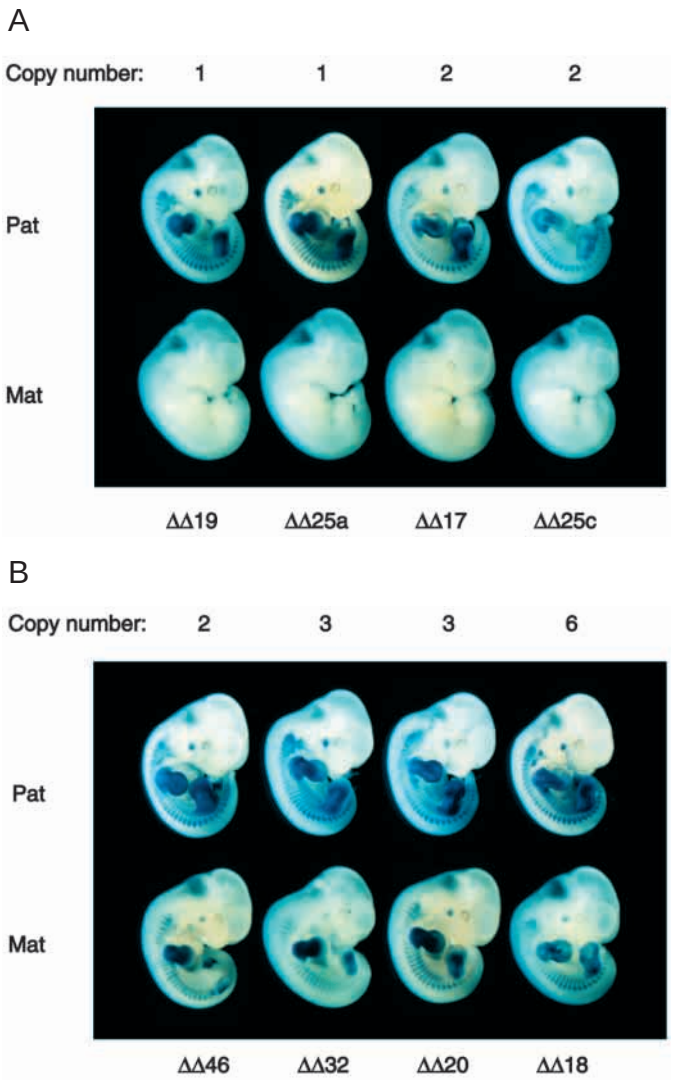


Fig. 3. *Igf2-lacZ* imprinting in YZΔΔ transgenic embryos. (A) Four low copy number lines show excellent imprinting of *Igf2-lacZ* at day 12 of embryogenesis. After paternal transmission (Pat), *Igf2-lacZ* is activated, resulting in β-galactosidase (β-gal) activity in the expected tissues. Maternal transmission (Mat) resulted in appropriate repression of β-gal activity in these lines, at all stages in development. (B) Four lines, containing two or more copies of the transgene, showed the correct pattern of β-gal activity in the embryo, but maternal transmission failed to repress the transgene.

results were confirmed by Southern-blot hybridisation (Fig. 2C). Founder animals with the deleted intergenic region were designated Δ19 and Δ17, respectively. Interestingly, the transgene in line Δ17 remained two copy, verified using the *Pvu*II polymorphism in the *Igf2-lacZ* gene. Thus, Cre treatment in this line resulted in deletion of the intergenic region independently from each copy, with no recombination between the two remaining loxP sites (Fig. 2C - lane 2).

Reactivation of *Igf2-lacZ* from the maternal allele

To assess the effect of the 1 kb deletion on expression of *Igf2-lacZ* we first tested β-gal activity at day 12 of embryogenesis. Surprisingly, we detected no obvious effect at this stage, after transmission through the paternal or maternal germline. After paternal transmission, both lines showed the established pattern of staining, while repression of *Igf2-lacZ* remained unchanged after maternal transmission. Further investigation prior to day 17 of embryogenesis revealed similar results. However, after day 17 the normally repressed *Igf2-lacZ* allele was strikingly activated after maternal transmission. Activity was strongest in the tongue, but also evident in other skeletal muscles throughout the body (Fig. 5A). In contrast, no reactivation was seen in liver or other cell types, demonstrating that the reactivation was specific for skeletal

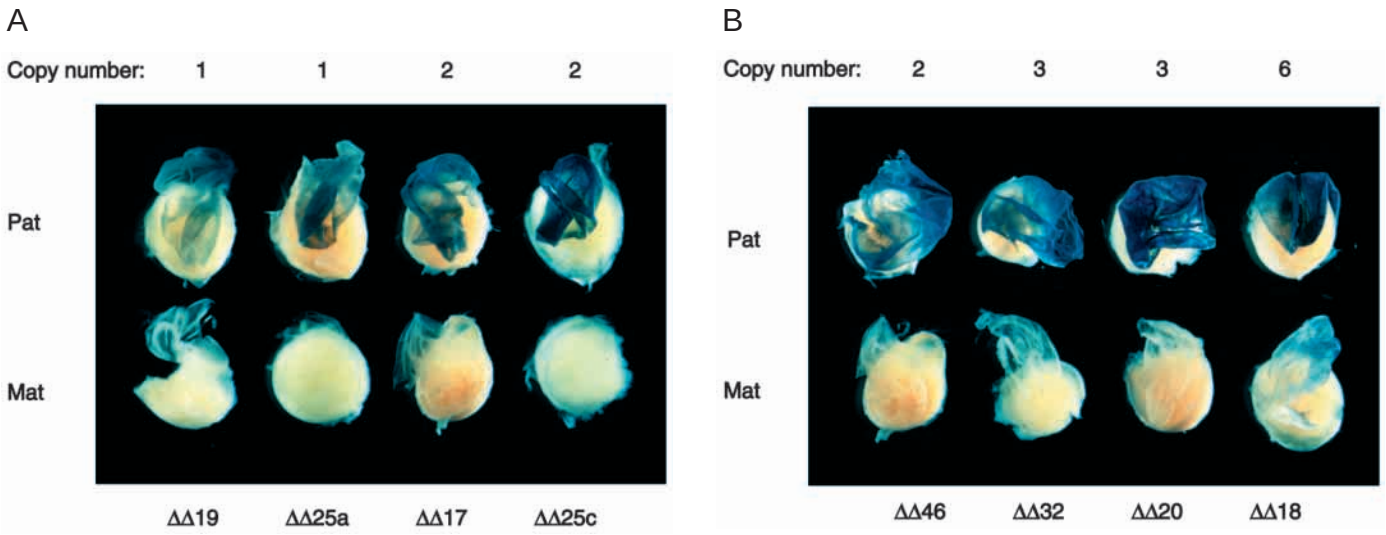


Fig. 4. *Igf2-lacZ* imprinting in YZΔΔ transgenic extra-embryonic tissues. (A) Extra-embryonic tissues from embryos shown in Fig. 3A. (B) Extra-embryonic tissues from embryos shown in Fig. 3B. In all lines, strong β-gal activity was seen in yolk-sac following paternal transmission. Activity was very weak, or absent in the yolk-sac after maternal transmission. No expression was seen in placenta of any line.

muscle cells. Between embryonic day 17 and day 5 of neonatal development, maternal *Igf2-lacZ* activity increased in skeletal muscles throughout the body, but never reached levels comparable with those after paternal transmission (Fig. 5B,D). Indeed, with the exception of the tongue, little activity was seen in the cranio-facial muscles prior to neonatal day 5 (see later), indicating a lag in reactivation of maternal *Igf2-lacZ* in these muscles (Fig. 5B). At all stages of development, the maternal *Igf2-LacZ* allele was repressed from the nondeleted YAC, except in the meningial layers of the brain (Fig. 5C). As the reactivation pattern was identical in both lines, the maternal *Igf2-lacZ* activity seen in skeletal muscle cells was caused by deletion of the 1 kb intergenic hypersensitive region.

To investigate whether transmission through the maternal germline is pre-requisite for establishing repression, we next studied the effect of deleting the region after essential germline specific modifications (parental imprints) are imposed. If the

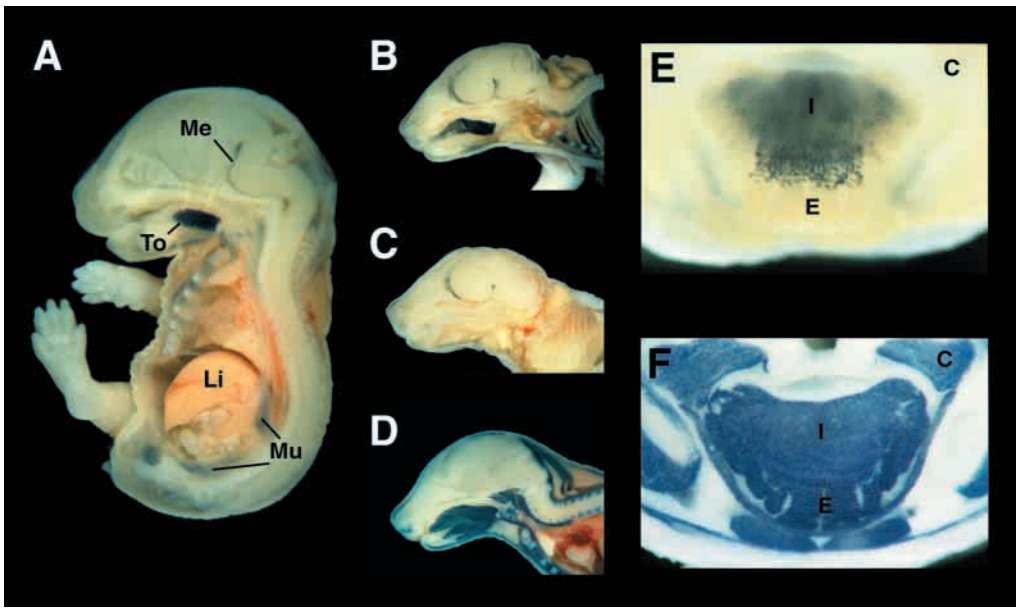


Fig. 5. Intergenic element deletion results in maternal *Igf2-lacZ* re-activation. (A) Bisected embryo at day 17 of embryogenesis from line Δ17, after maternal transmission, showing strong reactivation of *Igf2-lacZ* in the tongue (To), and weak re-activation in other skeletal muscles (Mu) throughout the body. Expression was also seen in the meninges (Me). No re-activation was seen in the liver (Li). (B) As in A, at neonatal day two. Reactivation is strong in the tongue, and has increased in other skeletal muscles of the body, but not in cranio-facial muscles. (C) In contrast, the only activity that can be seen at neonatal day two in ΔΔ17 mice (intact transgene) after maternal transmission is in the meninges. (D) After paternal transmission of Δ17 strong activity is seen in all skeletal muscles at neonatal day two. Strong activity is seen in the liver (not shown). No difference in expression could be detected between deleted and nondeleted transgenes after paternal transmission. (E) Coronal section through the tongue in Δ17 transgenic mice, at neonatal day two, after maternal transmission. Activity is restricted to intrinsic muscle cells (I) at this stage. No activity is seen in extrinsic muscle cells (labeled E). No activity is seen in other cranio-facial muscles (labeled C). (F) Comparative section to that in (E), after paternal transmission. Activity can be seen throughout the cranio-facial muscles, including both intrinsic and extrinsic muscles of the tongue.

region contains an imprinting element then deletion postfertilisation may have no effect on repression of *Igf2* after maternal transmission. If, however, the region contains a repressive element (maintenance function), then deletion postfertilisation should result in loss of repression, indistinguishable to that seen after maternal germline transmission (described above). To test these possibilities, embryos exposed to Cre at fertilisation (see Materials and Methods) were removed at day 17 of gestation and stained for β-gal activity. Embryos in which the region was deleted showed strong staining in the tongue (not shown). This demonstrates that continued presence of the intergenic region is required, at least through early stages of embryogenesis, to maintain full repression of the maternal *Igf2* allele.

Igf2-lacZ activity after maternal inheritance is cell type-specific

Re-activated maternal *Igf2-lacZ* activity was weaker than activity following paternal inheritance. However, individual muscles are composed of different cell types, including fast and slow twitch fibres. These are at different stages of differentiation during late embryonic/early neonatal development. Muscles also contain other cell types, including connective tissues, in varying proportions. It is therefore possible that the low-level activation of *Igf2-lacZ* seen in the muscles is associated with a specific state of differentiation, or cell type determination. Restricted reactivation was most clearly evident in the tongue (Fig. 5A,B), which contains distinct muscle bundles lying in different planes to one another. Detailed examination of the reactivation pattern showed that activity was restricted to intrinsic muscle cells at neonatal day 2 (Fig. 5B,E). Cells of the extrinsic muscles such as the genioglossus and the hyoglossus showed no *Igf2-lacZ* activity at this stage.

Delayed reactivation in cranio-facial muscles

Most muscles showed reactivation of *Igf2-lacZ* prior to birth. The notable exception were the cranio-facial muscles (excluding the intrinsic muscles of the tongue) (Figs 5B and 6E). We therefore extended our analysis for some time after birth to investigate temporal changes in expression. Increased activity was seen in cranio-facial muscles from around neonatal day 5 (Fig. 6F). This was transient and completely lost by neonatal day 11. Indeed, all activity was lost from the maternally inherited deleted transgene by neonatal day 14. By contrast, at no stage of development was skeletal muscle specific activity detected from the intact transgene after maternal inheritance (Figs 5C and 6C,D). Thus, the temporally regulated *Igf2-lacZ* activation seen throughout the body was deletion specific.

When the deleted transgene was transmitted through the male germline no changes in expression were seen. *Igf2-lacZ* retained full activity in all active cell types, including skeletal muscle, liver and the epithelial cell layers (Figs 5D and 6A,B). Shortly after birth, the endogenous paternal *Igf2* allele is repressed in most tissues, except skeletal muscle, by an unknown mechanism. We found that temporal regulation of *Igf2-lacZ* expression was highly consistent with this endogenous profile, suggesting that the mechanism involved in controlling *Igf2* repression from the paternal allele after birth is intact on the YAC transgene.

The paternal *H19* allele is not reactivated by the intergenic deletion

Activity of the *H19* gene is closely associated with that of *Igf2*, and there is much evidence to suggest that their reciprocal expression is mechanistically linked (Ainscough et al., 1997; Leighton et al., 1995a,b). We therefore investigated whether the intergenic element had any effect on *H19*.

To examine allele specific *H19* expression an *Msp*1 polymorphism between transgenic and BALB/c DNA was used, as previously described (Ainscough et al., 1997, 2000a). RNA from neonatal tongues, containing paternally inherited ΔΔ17 and Δ17 transgenes, was subjected to semi-quantitative RT-PCR analysis. High level expression was detected only from the endogenous maternal allele, suggesting that the paternal *H19* allele was not reactivated in the tongue. Additional studies, where we crossed the ΔΔ17 and Δ17 transgenes onto an *H19* null background (Ripoche et al., 1997), supported this observation. *H19* expression analysis, by in situ hybridisation on embryos at day 17 of gestation, showed no evidence of *H19* activation from the paternal allele (not shown). This indicates that activity of the intergenic element may be restricted to the *Igf2* gene.

DISCUSSION

We have previously demonstrated that the YAC contains enhancers for *Igf2* and *H19* expression in many cell types, in

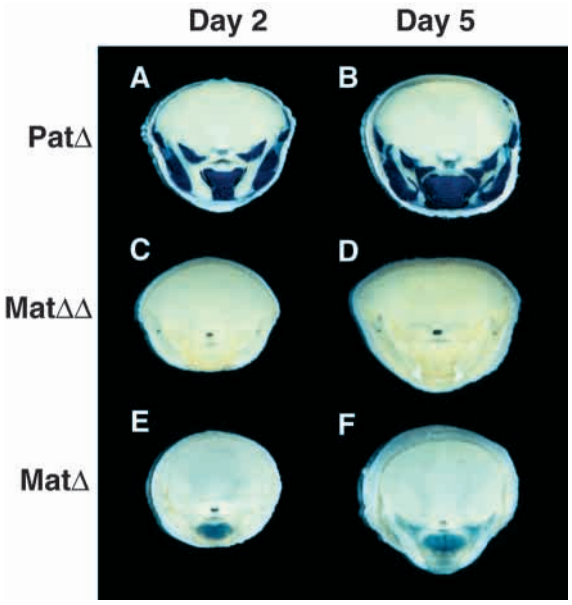


Fig. 6. Temporal control of *Igf2-lacZ* reactivation in cranio-facial muscles after birth. Coronal sections through the head at neonatal days two (A,C,E) and five (B,D,F). (A,B) After paternal transmission *Igf2-lacZ* activity is strong throughout the cranio-facial muscles, from both the deleted (Δ17 – A,B) and the non-deleted (ΔΔ17 – not shown) transgene. (C,D) After maternal transmission of ΔΔ17, *Igf2-lacZ* activity is completely repressed in the cranio-facial muscles. (E,F) After maternal transmission of Δ17 *Igf2-lacZ* is active only in the intrinsic muscle cells of the tongue at neonatal day two (E), but by day five weak activity can also be seen in the other cranio-facial muscles (F).

addition to elements for appropriate imprinting of both genes (Ainscough et al., 1997, 2000a). Here, we confirm a copy number dependency for *Igf2-lacZ* imprinting from the YAC. Cumulative data from all studies shows that seven lines with 1 or 2 transgene copies appropriately imprint *Igf2-lacZ*, while five lines containing three or more copies do not. However, one of four two-copy lines (ΔΔ46) also failed to fully imprint *Igf2-lacZ*. We are currently investigating this loss of imprinting (LOI), which seems to be related to the relative orientation of transgene(s) when more than one copy has integrated. This affects the location of regulatory elements, relative to the gene (J. F-X. A. and M. A. S., unpublished data).

In this study, we also analysed the extra-embryonic tissues. Strong expression was seen in yolk sac, but not in the placenta. This suggests that placental enhancers for *Igf2* expression are not on the YAC. What is particularly noteworthy is that *Igf2-lacZ* imprinting in yolk sac occurred irrespective of copy number or integration site, unlike that in embryonic tissues. This indicates that regulatory elements for *Igf2* activity in yolk sac are physically separate from those regulating expression in the embryo. The location of these enhancers on the YAC protects *Igf2-lacZ* from LOI effects in this tissue. We previously demonstrated that imprinting of the *H19* gene from the YAC is also position and copy number independent, in all tissues. These observations therefore demonstrate the complexity involved in regulating imprinted expression in this domain, some aspects of which are apparently both tissue and gene specific.

To begin to understand the role that individual elements serve within this domain, we also studied the role of the 1 kb intergenic region which is evolutionarily conserved among mammalian species, and hypersensitive to DNase I enzyme. A Cre-loxP-mediated deletion of this region was induced from the YAC. This analysis was not possible at the endogenous locus as the cloning of flanking DNA has, to date, proven impossible in vectors other than YACs (Greally et al., 1997; Koide et al., 1994). By employing Cre-loxP-mediated excision, we were able to generate and characterise intact transgenic lines prior to deletion, and then selected appropriate lines for further analysis. In this way our investigations were made locus specific, and therefore independent of position effects.

Our initial hypothesis was that the region under investigation may harbour enhancers for expression in skeletal muscle, as the YAC expresses *Igf2* at high levels in these cells. To date, however, the exact location of these enhancers has proven elusive. Indeed, the only enhancers in the entire *Igf2-H19* domain, which have been localised and characterised in any detail, lie within 10 kb downstream of the *H19* gene. These enhancers are tissue specific, directing limited expression of both genes in the liver, gut endoderm and the sclerotome (Brenton et al., 1999; Leighton et al., 1995a). However, it is not likely that the deleted region has a major enhancer-type role as no obvious loss of paternal *Igf2-lacZ* activity was detected in any tissue following the deletion. In contrast, the maternal *Igf2-lacZ* allele was activated in skeletal muscles, most strikingly in the intrinsic muscle cells of the tongue, from a late stage in embryogenesis. No reactivation was detected in other tissues, and no effect was found on the *H19* gene. Thus, our results strongly implicate this region in the temporal control of repression of the maternal *Igf2* allele in skeletal muscle cells.

Recent evidence indicates the presence of additional elements that contribute to the allele specific repression of *Igf2* and *H19*. These elements also act in a tissue-, and gene-, specific manner. One of these elements is located upstream of *H19*, which acts to repress the paternal *H19* allele in some tissues (Brenton et al., 1999; Drewell et al., 2000). By contrast, a region upstream of *Igf2* represses the maternal *Igf2* allele in a subset of mesodermal tissues (Constancia et al., 2000). With the results presented here, these observations strongly suggest that some regulatory aspects occur independently for the *Igf2* and *H19* genes. This is consistent with the reports of loss of *IGF2* imprinting in the human genetic disorder, Beckwith-Wiedemann syndrome (BWS), which can occur independently of *H19* (Brown et al., 1996; Joyce et al., 1997). It is noteworthy that deletion of the intergenic region presented here led to strong reactivation of the normally silent maternal *Igf2-lacZ* allele in tongue, as macroglossia is the most consistent and prominent feature observed in individuals with BWS.

In conclusion, cumulative evidence shows that multiple control elements exist for both activation and silencing of *Igf2* and *H19*. There also appears to be a level of control over expression that occurs independently for the two genes. The elements involved act in a tissue-specific manner. This mechanism has the advantage that it allows for accurate control of the level of activity within individual cells. This may be particularly important for a potent growth factor such as *Igf2*, which may require elaborate control of temporal and tissue specific expression. In addition, mutations within individual control elements would not give rise to widespread disruption of imprinted gene expression. Deletion of these regions from the endogenous locus, or from large YAC transgenes as in this study, will help to further elucidate their function in the complex regulatory mechanism within this domain.

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