Development 124, 3621-3632 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV4905

Imprinting of *Igf2* and *H19* from a 130 kb YAC transgene

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SUMMARY

A stringent test for imprint control elements is to examine their function at ectopic loci in transgenic experiments. *Igf2* and *H19* are part of a larger imprinting region and as a first step, we examined these reciprocally imprinted genes in transgenic experiments using a 130 kb YAC clone. After paternal inheritance, *H19* was appropriately repressed and *Igf2* was expressed, irrespective of copy number or genetic background. After maternal inheritance *H19* was consistently expressed, albeit with some variability. The levels of *H19* expression per copy of the transgene inversely correlated with *Igf2* (-*lacZ*) expression in *cis*. The consistent imprinting of *H19* from this YAC contrasts with the previously described imprinting of mini-*H19* transgenes, which only occurs at multi-copy loci, is inconsistent, and is prone to genetic background effects. We propose a novel model in which silencing of the *H19* gene is the default state and its activation after maternal inheritance is the key mechanistic event for imprinting in this region. In addition, in situ analysis of the *Igf2-lacZ* reporter indicates that additional mesoderm-specific enhancers are present within the YAC clone. No obvious phenotype was detected from the excess gene dosage of *H19*.

Key words: *Igf2/H19*, genomic imprinting, YAC transgenes, mouse

INTRODUCTION

Genomic imprinting is an epigenetic process which results in parent of origin dependent monoallelic expression of certain mammalian genes (Ainscough and Surani, 1996; Efstratiadis, 1994). Some imprinted genes are clustered in domains that have unique properties, including early replication of the paternal chromosome (Kitsberg et al., 1993). How imprinted domains are regulated is not clear but one possibility is that they are controlled by Imprinting Control Elements (ICE) which interact with a number of genes with additional imprinting *cis* elements in the same region.

Our studies have focused on a cluster of imprinted genes on mouse distal Chr 7, which includes Insulin-like growth factor 2 (*Igf2*) and *H19* (Searle and Beechey, 1990; Ferguson-Smith et al., 1991; Sasaki et al., 1992). *Igf2* is a fetal mitogen while the function of the *H19* RNA, which is apparently not translated, is unclear. These two genes are separated by 70 kb (Zemel et al., 1992; Koide et al., 1994) and have similar expression patterns (Lee et al., 1990; Poirier et al., 1991), but they are only expressed from the paternal or maternal allele, respectively (DeChiara et al., 1991; Bartolomei et al., 1991). Other imprinted genes in this region include *Ins2* (paternally expressed), *Mash2* (maternal) and $p57^{Kip2}$ (maternal; Giddings et al., 1994; Guillemot et al., 1995; Hatada and Mukai, 1995). The syntenic region in humans, Chr 11p15.5, shows similar imprinting of *IGF2, H19* and $p57^{KIP2}$ (Ohlsson et al., 1993; Giannoukakis et al., 1993; Zhang and Tycko, 1992; Zhang et al., 1993; Matsuoka et al., 1996), in addition to the *KVLQT1* gene, which is imprinted in a tissue-specific manner (Lee et al., 1997).

Initial attempts to address the mechanism of genomic imprinting focused on parent of origin dependent methylation differences around *Igf2* and *H19*. Differential methylation was first detected in a region 3 kb upstream of *Igf2* (Sasaki et al., 1992) and later, in the body of the gene (Feil et al., 1994) where the active paternal allele is more methylated. In contrast to *Igf2* the active allele of *H19* is unmethylated while the inactive paternal allele is highly methylated (Ferguson-Smith et al., 1993; Bartolomei et al., 1993; Tremblay et al., 1995). Methylation was shown to play an important role in imprinting by targeted deletion of the DNA methyltransferase gene in mice, as the resulting lack of methylation was associated with biallelic *H19* expression and loss of *Igf2* expression (Li et al., 1992, 1993).

Analysis of chromatin structure within the *Igf2*/*H19* domain by nuclease sensitivity assays showed that the promoter region of *H19* is less accessible on the inactive allele (Ferguson-Smith et al., 1993; Bartolomei et al., 1993; Koide et al., 1994). By contrast, the repressed *Igf2* allele has accessible chromatin and, more significantly, is expressed at low levels (Sasaki et al., 1992). Long range physical analysis detected a region between *Igf2* and *H19* (A6-A4) which is highly sensitive to nuclease on both parental alleles. This region is unmethylated and, furthermore, is conserved between mouse and man, suggesting a possible role in the regulation of *Igf2* and *H19* (Koide et al., 1994).

It has been suggested that reciprocal imprinting of *Igf2* and *H19* is linked by a mechanism which ensures mutually exclusive expression from the same chromosome (Bartolomei et al., 1993). However, in this regard, a number of important

points require careful consideration. In support of a mechanistic link, targeted deletion of a 13 kb region including the *H19* gene caused activation of the *Igf2* gene located 70 kb away, as well as an increase in methylation at the region upstream of *Igf2* (Leighton et al., 1995b). However, in some tissues, *Igf2* was only slightly upregulated, implying that an additional level of control exists. In this context, it is of interest that mutation analysis in some Beckwith-Wiedemann patients is consistent with the notion of an ICE located hundreds of kb upstream of *IGF2* that influences methylation and expression imprinting of *IGF2* and *H19* (Hoovers et al., 1995; Reik et al., 1995; Brown et al., 1996). Secondly, while there is evidence that the endoderm-specific enhancers downstream of *H19* may be shared by *H19* and *Igf2* (Leighton et al., 1995a), enhancers which regulate expression in mesoderm have not yet been identified, so the possibility of co-regulation cannot be fully addressed at present. Most importantly, *H19* and *Igf2* are expressed from the same chromosome in some tissues (Svensson et al., 1995; Sasaki et al., 1995; Mutter et al., 1993; Jinno et al., 1995), suggesting that the two genes may be regulated independently.

One way to directly test whether imprinted genes need to be located within an imprinted domain or whether they are regulated independently is to study them outside of their normal chromosomal context. Previous studies with a 30 kb *Igf2* transgene did not yield significant results on control of its expression or imprinting (Lee et al., 1993), implying that no enhancers or ICEs are located close to this gene. Studies with *H19* transgenes did show imprinting in a few instances on a specific genetic background, suggesting the presence of a gene-specific 'imprinting' element within this region (Bartolomei et al., 1993). However, these *H19* transgenes only function in this way as complex multi-copy loci.

Recently YAC transgenes have been used to analyse expression of a number of genes at ectopic loci and have been found to exhibit more reproducible gene activity compared to smaller transgenic constructs, presumably due to inclusion of additional locus control regions, or alternatively extra insulating effect from flanking DNA sequences (for example see Lee et al., 1996; Schedl et al., 1993, 1996).

We are interested in investigating regulation of the entire imprinting region on distal Chr 7. As a first step, we have investigated the well characterised *Igf2*/*H19* region using a 130 kb YAC clone in transgenic experiments. This YAC extends from promoter 1 of *Igf2* to 35 kb downstream of *H19*. Both genes were expressed and imprinted from 1-2 copy YAC inserts. However the levels of *H19* expression after maternal inheritance were variable, and inversely correlated with expression of *Igf2-lacZ* in *cis*. Additional control elements which may exist further upstream of *Igf2* from Beckwith Wiedemann syndrome studies (Brown et al., 1996), may stabilise the imprint(s) in vivo. From available evidence, we propose a new model in which silencing of *H19* is the most stable state and its activation following maternal inheritance is mechanistically a critical step.

MATERIALS AND METHODS

YAC manipulations and transgenic mice

The YAC ICRFy902HO522 (Koide et al., 1994) was modified by homologous recombination in yeast strain AB1380 using alkali-cation yeast transformation kit (Bio 101), and vectors constructed in this laboratory. The right arm was replaced with an arm containing LYS2 and *Neo* driven by the thymidine kinase promoter, using the vector pLUNET. Next, *lacZ* was introduced into exon 4 of *Igf2* at the *Kpn*I site using pop in-pop out methods with URA3. Finally the fragmentation vector pURAFRAG was used to generate the 130 kb YAC clone YZ, by targeted recombination at the CE22 region 35 kb downstream of *H19* (Koide et al., 1994).

High quality intact YZ DNA was purified for microinjection as described by Schedl et al. (1993). Conditions for preparative pulsed field gel electrophoresis were 195 V, 1% agarose, 0.5× TBE, 9°C, pulse time 40 seconds, for 24 hours (Pharmacia Gene Navigator apparatus). Dialysed YAC DNA was injected at a concentration of 5 ng/μl into fertilised mouse eggs from (C57BL/6 × CBA) F_1 × 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 2-cell stage to oviducts of day 1 pseudopregnant F_1 females.

Transgene copy number

DNA from tail samples of founder animals was digested with *Pvu*II, the fragments separated through 1.5% agarose, blotted onto hybond N+ membrane and hybridised with a [^{32}P]dCTP-labelled 1 kb *Bam*HI-*Xho*I (BX) probe 3′ to the *lacZ* insert in the *Igf2* gene. This detects a polymorphism between the endogenous *Igf2* gene and the *Igf2-lacZ* fusion. YAC copy number was determined by densitometry of the hybridising bands using Image Quant phosphorimager software, normalised for the endogenous signal as two copies.

DNA and methylation analysis

DNA was prepared, using standard procedures, from: (1) whole embryos at day 13 of gestation to be used for methylation analysis, (2) yolk sacs of embryos to be used for β-gal analysis and RNA preparation, and (3) tail samples of 14-day-old mice to be used for analysing transgenesis.

For methylation analysis 10 mg DNA was digested to completion with appropriate restriction enzymes (Boehringer Mannheim) and the fragments separated through 1.2% agarose. The DNA was transferred to Hybond N+ filters by alkali blotting for 10-16 hours, neutralised for 5 minutes in pH 7.4 neutralising solution, pre-hybridised in Church buffer (Church and Gilbert, 1984) at 65°C for 30 minutes and hybridised in Church buffer with [^{32}P]dCTP-labelled DNA probe for 16-24 hours at 65°C. DNA probes were labelled using Random Primers DNA labelling system (Gibco BRL). Hybridised filters were washed three times in 2 × SSC, 0.1% SDS, and once in 0.2 × SSC, 0.1% SDS. All washes were done for 30 minutes at 65°C. Hybridisation signals were detected using Kodak XAR film.

RNA and expression analysis

RNA was prepared from embryos at day 13 of gestation using Trizol Reagent (Gibco BRL). For allele-specific RT-PCR analysis 10 μg RNA was precipitated and treated with DNase I (Gibco BRL), supplemented with RNase inhibitor (Boehringer Mannheim (BM)) for 1 hour at 37°C followed by 10 minutes at 65°C. 0.4 μg treated RNA was reverse transcribed with Superscript II (RT) (Gibco BRL) for 1 hour at 37°C, using random hexamers (Pharmacia) and RNase inhibitor. Controls in which no RT was added were done in parallel. Enzymes were heat inactivated at 65°C for 10 minutes. PCR amplification of *H19* exon 1 was performed using primers 624, 5′TGGGGGAAGATGGGAGAGCT3′ and 625, 5′ATCCCATGGTCTCTGCCACT3′ which amplify a 634 bp fragment containing two *Msp*I sites in BALB/c DNA (type *b* allele), and a single site in C57BL/6, CBA and C3H DNA (type *c* alleles). PCRs were performed in 50 μl reactions containing 5 μl 10× PCR buffer (BM), 1 μl cDNA, 100 ng each primer, 100 μM dNTPs and 5 units Taq DNA polymerase (BM), for 30 cycles at 94°C, 60°C and 72°C for 1 minute, 0.5 minutes

and 1 minute respectively. 10 μl of each reaction was digested to completion with *Msp*I (BM), the fragments separated through 2% agarose, transferred to Hybond N+ and hybridised as described above.

lacZ expression

Embryos for β-gal analysis using X-Gal were washed twice with cold PBS immediately after dissection from the uterus, and fixed for 1 hour at 4ºC in 5% formaldehyde, 0.8% glutaraldehyde, 0.02% NP40, 1 mM $MgCl_2$, 0.1 mg/ml sodium deoxycholate in PBS. Embryos were then washed twice in PBS and incubated for 48 hours at 30ºC in X-Gal staining solution (1 mg/ml X-Gal, 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM magnesium chloride in PBS). After two further washes in PBS embryos were fixed overnight in 4% formaldehyde in PBS, and stored in 70% ethanol. Photographs of embryos were taken using a Zeiss Stemi SV II microscope and Fujichrome 64T film.

FISH analysis

Primary embryonic fibroblasts (PEFs) were derived from heterozygous day-13 transgenic embryos by standard procedures. Metaphase chromosome spreads were prepared, and FISH analysis performed as described by Pinkel et al. (1986), using the cosmid clone cCH (Koide et al., 1994). FISH signals on metaphase chromosomes were visualised by conventional fluorescence microscopy (Zeiss Axiophot microscope, 100× objective and two filter sets for DAPI and FITC/PI).

Mouse strains

F_1 (C57BL/6 × CBA) and BALB/c mice were used throughout these experiments. The YAC clone was derived from C3H mice. $F_1 \times F_1$ crosses were used for analysis of *lacZ* expression and for methylation analysis. BALB/c mice were crossed with F_1 mice for polymorphic analysis of *H19* expression. In these experiments the BALB/c *H19* allele was denoted $H19^b$, the C57BL/6 and CBA alleles $H19^c$, and the transgenic C3H allele $H19^{c'}$. No polymorphisms were known between the $H19^c$ and the $H19^{c'}$ alleles.

RESULTS

Characterisation of YZ transgenic mice

To study expression and imprinting of *Igf2* and *H19* we constructed a 130 kb YAC transgene, YZ, in which the *Igf2* coding sequence was interrupted with the *lacZ* reporter gene (Fig. 1A). YZ extends from promoter 1 of *Igf2* to 35 kb downstream of *H19*, and includes the differentially methylated region around exons 5 to 6 of *Igf2* (Feil et al., 1994), the unmethylated intergenic hypersensitive region, A6-A4 (Koide et al., 1994), the differentially methylated regions around *H19* (Ferguson-Smith et al., 1993; Bartolomei et al., 1993; Tremblay et al., 1995) and the endoderm-specific enhancers downstream of *H19* (Yoo-Warren et al., 1988) (Fig. 1B).

Six founder mice carrying this transgene (YZ3, 6, 8, 13, 15 and 22) were generated by pronuclear injection of YZ into fertilised (CBA × C57BL/6) F_2 eggs. From 1141 injected eggs, 44 live born animals were obtained. Transgenesis was checked by Southern hybridisation for *lacZ* (Fig. 1B, probe Z); animals lacking *lacZ* were not characterised further. All six founders transmitted the transgene(s) to their offspring and in only one case, YZ6, was segregation of transgenes evident giving rise to lines YZ6A and YZ6B.

YZ integrity was assessed by digesting DNA with informative restriction endonucleases and hybridising with probes specific to different regions of the YAC (Fig. 1B and Table 1).

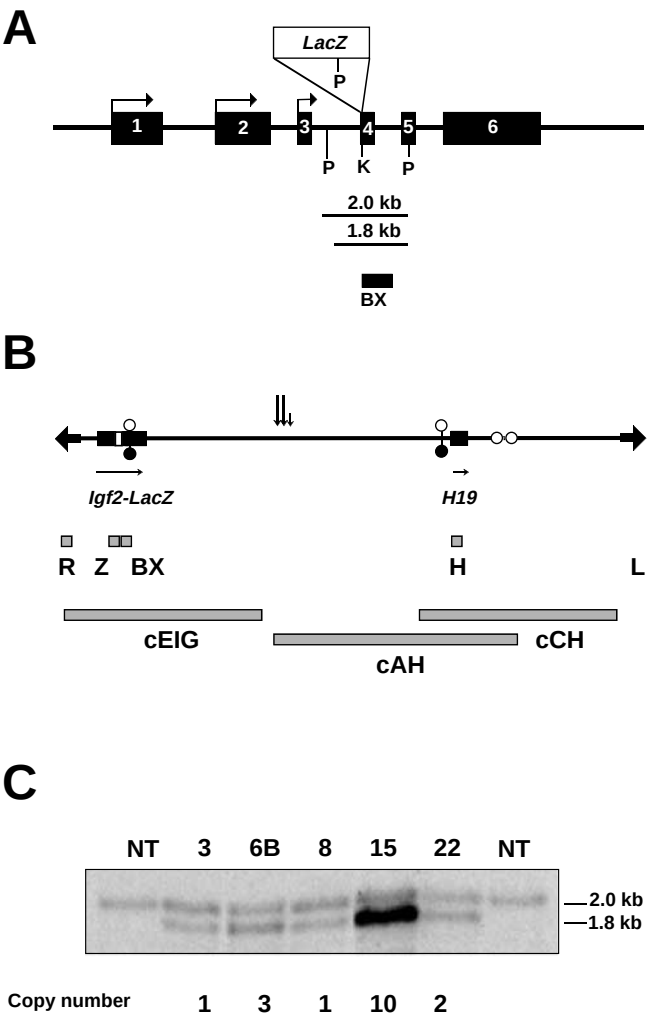


Fig. 1. Structure of YZ transgene and copy number determination in transgenic mice. (A) *Igf2* gene structure and position of insertion of *lacZ*. K, *Kpn*I. P, *Pvu*II. The probe BX detects a *Pvu*II polymorphism between endogenous and transgenic DNA, shown in C. (B) Structure of the 130 kb transgene YZ, showing relative locations of *Igf2-lacZ* and *H19*. The intergenic hypersensitive region, A6-A4 (Koide et al., 1994), is indicated by vertical arrows, and the endoderm-specific enhancers downstream of *H19* are indicated by white circles on the line. Regions of differential methylation between the parental alleles are shown above (maternal) and below (paternal) the line. White circles represent unmethylated regions, black circles are methylated. R, *Neo* probe-specific for the right YAC arm. Z, *lacZ* probe. BX, *Igf2* probe. H, *H19* probe. L, Left YAC arm. Presence or absence of L was detected with primers to URA3. (C) Copy number estimation. Tail DNA from transgenic mice was digested with *Pvu*II and hybridised with probe BX, to show the endogenous 2.0 kb fragment (2 copy) and the transgenic 1.8 kb fragment. Relative intensity of the two bands was used to estimate transgene copy number in each line. NT, non-transgenic.

Cosmid clones cAH, cCH and cEIG, which span the entire YAC (Koide et al., 1994), were also used as probes to screen for rearrangements within the inserts (Fig. 1B and Table 1). No rearrangements were detected in lines YZ3, 6B, 8, 13, 15 or 22. However, in line YZ6A an additional hybridising band was detected by probe cAH suggesting that the YAC was rearranged within this region. YZ6A was therefore excluded

Table 1. Summary of transgenic lines

Founder	Sex		Copy number	Probe L	R	Z	BX	H	cCH	cAH	cEIG
3	F		1	+	+	+	+	+	+	+	+
6	F	A	nd	nd	nd	+	+	nd	+	§	+
		B	3	+	+	+	+	+	+	+	+
8	M		1	+	+	+	+	+	+	+	+
13	M		nd	+	+	+	+	nd	+	+	+
15	M		10	+	+	+	+	+	+	+	+
22	M		2	+	+	+	+	+	+	+	+

F, female, M, male. +, sequences are present based on Southern analysis for probes R (*Neo* resistance gene); Z (*lacZ* gene); BX (*Igf2* gene); H (*H19* gene); cCH, cAH and cEIG (cosmids spanning entire YAC construct) and by PCR analysis of L (*URA3* gene). nd, not determined. §, rearrangement detected.

from further study. In preliminary experiments line YZ13 showed little or no expression of the *Igf2-lacZ* gene and therefore this line was also not analysed further.

Copy number of the remaining *lacZ*-positive transgenes was estimated using probe BX (Fig. 1C). YZ3 and YZ8 had single copy inserts, YZ22 had two copies, YZ6B had three copies and YZ15 had ten copies. Full imprinting and gene expression pattern analysis was completed in lines YZ3, YZ8, YZ15 and YZ22. *H19* expression was not studied in line YZ6B, since derivation of this line was hindered by characterisation of segregating alleles.

The YAC inserts in lines YZ3, 8, 15 and 22 were localised by fluorescence in situ hybridisation (FISH) on primary embryonic fibroblasts (PEFs). Fig. 2 shows that the integrations occurred at single loci, at different autosomal locations in the mouse genome. Signal intensity supported the copy number assessment shown in Fig. 1C in each case. Transgenes YZ3 and YZ15 were found to be close to a genetically defined imprinted region on proximal Chr 7 (Cattanach and Kirk, 1985; Beechey and Cattanach, 1995). However, YZ8 and YZ22 had integrated into chromosomes (15 and 4 respectively) that are not expected to contain imprinted genes. The latter were therefore ideal for analysing the regulation of *Igf2* and *H19* by elements contained on the YAC, outside an imprinted chromosomal context.

Transgenic *H19* expression is imprinted at ectopic sites in the mouse genome

To analyse parental origin-specific expression of *H19* from the YAC, a polymorphic *Msp*I site was used which is present in *H19* exon 1 in BALB/c DNA. In our RT-PCR assay this gives rise to a 321 bp fragment (referred to as $H19^b$ allele). This site is not present in the YAC transgene (from strain C3H) or at the

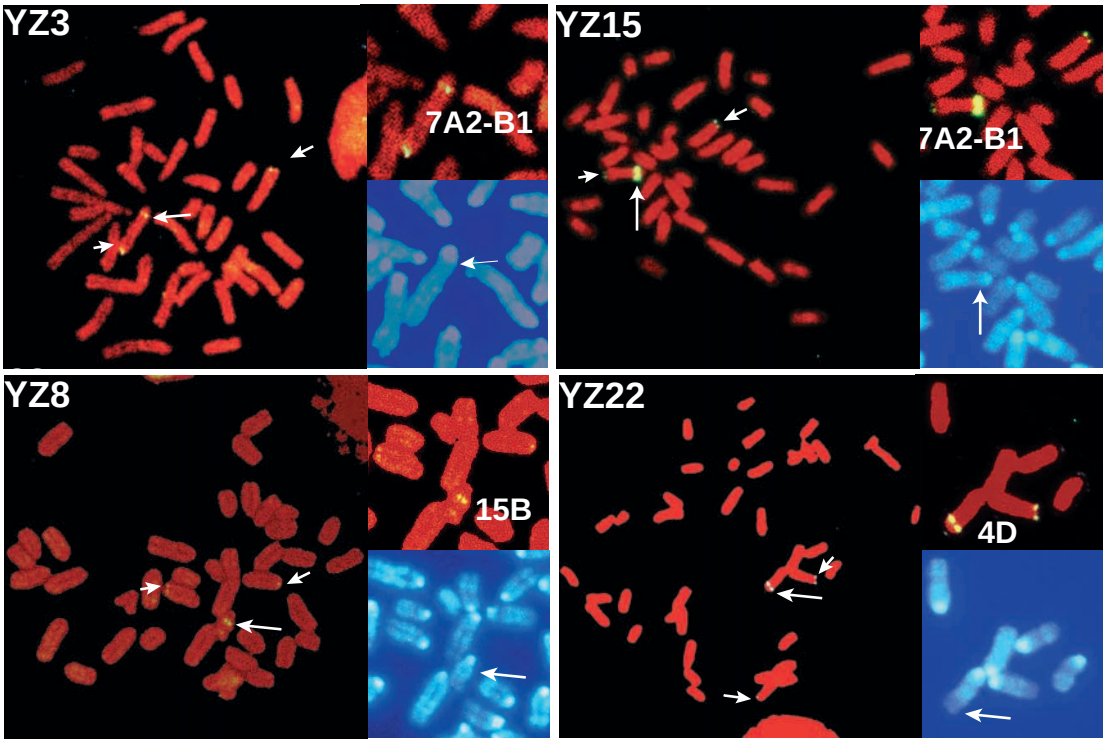


Fig. 2. Detection of YAC integration sites in lines YZ3, 8, 15 and 22 by FISH analysis. Representative chromosome spreads show single insertion sites in each line, following hybridisation with probe cCH to the *H19* region. The YAC inserts are indicated by long arrows, and are also shown in the inset, upper panel. Chromosomal locations are also indicated (inset-upper panel), as determined by G-banding (inset-lower panel). The endogenous *H19* signals on distal Chr 7 are also shown (short arrows).

endogenous alleles of the transgenic founder strain (C57BL/6, CBA), giving rise to a 440 bp fragment (referred to as $H19^{c}$ for endogenous, and $H19^{c'}$ for transgenic allele) (Fig. 3A).

To study paternal transmission, $H19^{c/c/c'}$ males from lines YZ3, YZ8, YZ15 and YZ22 were first mated with $H19^{b/b}$ females. RNA was prepared from transgenic embryos at day 13 of gestation and used for RT-PCR analysis of *H19* exon 1 followed by hybridisation of the *Msp*I digested products with the 321 bp probe shown in Fig. 3A. Clear expression from the maternally inherited $H19^{b}$ allele was observed in each line. In contrast, little or no *H19* transcript was detected from the paternally inherited $H19^{c}$ or $H19^{c'}$ alleles (Fig. 3B, lanes 4 to 7).

To see if *H19* was expressed from the transgenes after maternal transmission we first generated $H19^{b/c/c'}$ female mice for each YZ line. These females were mated with $H19^{c/c}$ males and embryos containing both the YAC and the $H19^{b}$ allele were selected for RT-PCR analysis. In all cases expression from the $H19^{c'}$ allele was detected in addition to the endogenous maternal $H19^{b}$ allele (Fig. 3B, lanes 8 to 11). These results demonstrate that *H19* expression from the YAC only occurred after maternal transmission in all four lines.

Although not strictly quantitative, the levels of transgenic *H19* expression seen were similar in at least three independent embryos from each line. Also, the 321 bp probe used was internal to both allele specific fragments so was expected to hybridise to each fragment with the same affinity, giving an approximate measure of the relative amount of transcript from each allele. We therefore estimated the level of expression from the maternally inherited transgenes relative to endogenous levels of *H19* transcript by phosphorimager densitometry. This analysis showed that transgenic *H19* expression was high in line YZ8 (approx. 80% of the endogenous level) and relatively low in line YZ3 (approx. 40%). The level of activity seen in the ten copy line, YZ15, was also low (approx. 20%) when

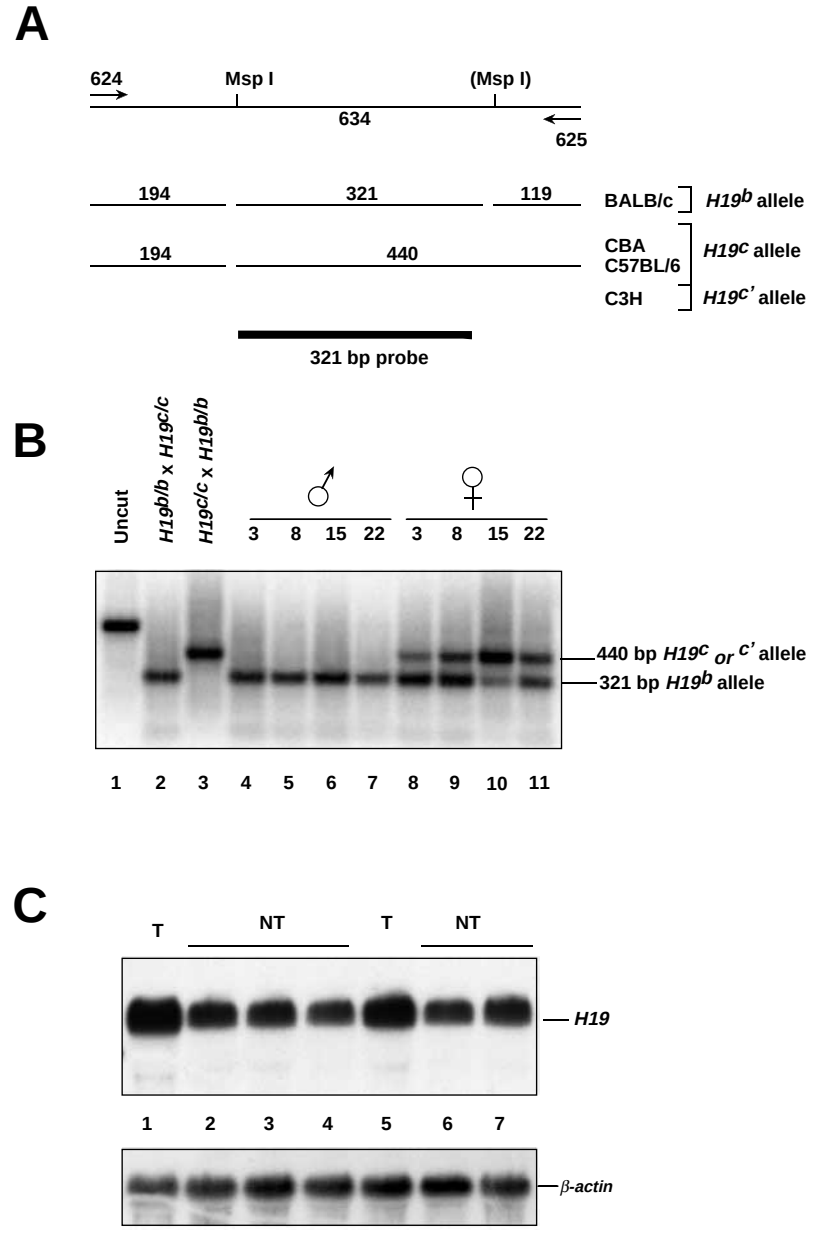
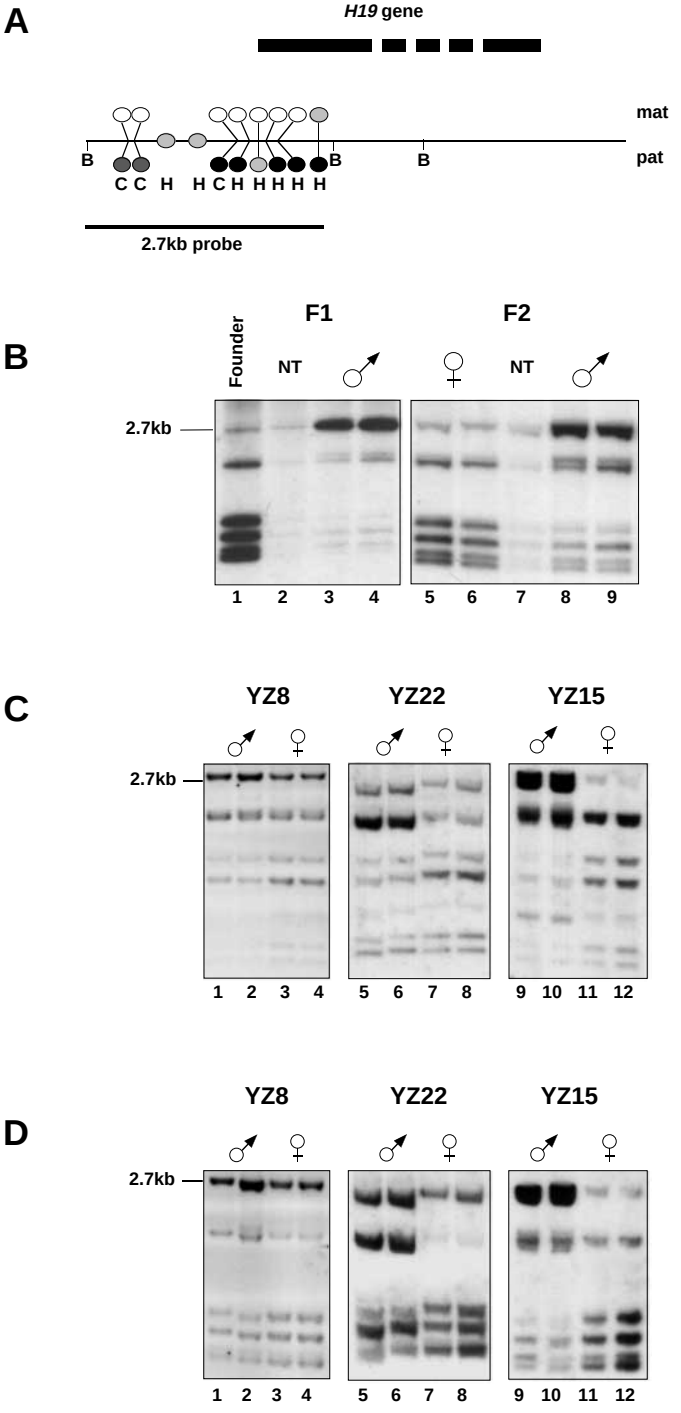


Fig. 3. Transgenic *H19* expression is consistently imprinted. (A) Diagram of the 634 bp region of *H19* exon 1 which is amplified by primers 624 and 625, showing the relative position(s) of *Msp*I site(s) in four mouse strains. This region in BALB/c DNA (referred to as $H19^{b}$ allele) contains an additional *Msp*I site (shown in brackets) which is not present in the founder strains (CBA, C57BL/6) referred to as $H19^{c}$ allele, or the transgene strain (C3H) referred to as $H19^{c'}$. (B) RT-PCR analysis of *H19* exon 1 in heterozygous day 13 embryos derived from mating $H19^{b/b}$ females with $H19^{c/c/c'}$ males (lanes 4-7), and $H19^{b/c/c'}$ females with $H19^{c/c}$ males (lanes 8-11). Female transmission of the $H19^{b}$ allele was selected for in each case so that *H19* expression from the transgene ($H19^{c'}$ allele) could be detected separately from the endogenous maternal transcript. *Msp*I digested reaction products were hybridised with the 321 bp probe shown in A. One representative sample is shown for male and female transmission from each line tested (YZ3, 8, 15 and 22). Transgenic *H19* expression was only detected after maternal transmission in all lines. Controls show that no expression was detected from the endogenous paternal allele under the reaction conditions used in this assay: Lane 1, undigested PCR product. Lane 2, $H19^{b/b}$ female × $H19^{c/c}$ male, only the $H19^{b}$ allele was detected. Lane 3, $H19^{c/c}$ female × $H19^{b/b}$ male, only the $H19^{c}$ allele was detected. (C) Northern analysis of *H19* in heterozygous day 13 sibling embryos derived from mating a heterozygous transgenic YZ15 female with a non-transgenic male. The two embryos which inherited YZ15 from their mother (lanes 1 and 5, T) are expressing *H19* at approximately two times the level seen in non-transgenic littermates (NT, lanes 2-4, and 6, 7), as measured by phosphorimager densitometry: Lane (1) 1.96. (2) 1.03. (3) 1.03. (4) 0.99. (5) 1.87. (6) 0.93. (7) 1.02. *β-actin* analysis was included as a loading control. 10 μg total RNA was loaded per lane, and the *H19* and *β-actin* transcripts detected with [^{32}P]dCTP labelled cDNA probes.

adjusted for transgene copy number. This was supported by northern analysis which indicated that transgenic YZ15 embryos expressed *H19* at approximately two times that seen in non-transgenic littermates, instead of eleven times as would be expected if each transgene copy was fully active (Fig. 3C). In line YZ22, *H19* activity was about 50% of endogenous levels per transgene copy. These data are discussed in relation to activity of the *Igf2-lacZ* gene fusion located in *cis* in each line (see Discussion).

The role of the apparently non-transcribed *H19* RNA is unclear, although it has been suggested to have tumour suppressor activity (Hao et al., 1993), or that it may directly or indirectly control *Igf2* expression (Leighton et al., 1995b). Previous experiments in which *H19* was over-expressed from a small transgene, controlled only by the downstream endoderm-specific enhancers, resulted in embryonic lethality (Brunkow and Tilghman, 1991). However, the lethal phenotype has since been attributed to the presence of a synthetic linker sequence in the transgene, as unmodified *H19* transgenes driven by the same downstream enhancers had no effect (Pfeifer et al., 1996). In our experiments all four transgenic lines tested over-expressed the unmodified *H19* from the YAC after maternal transmission. We also detected no obvious phenotypic effects at any stage of development, suggesting that the dosage of *H19* is not critical during embryonic development. However, the maximium level of *H19* overexpression seen in our transgenic lines was two fold, which may be due to selection against embryos with much higher levels of *H19* expression. It is possible that such embryos were eliminated in utero during the derivation of founder transgenic animals.



Parental-specific methylation of *H19* is maintained on the YAC

We next investigated whether the methylation patterns of transgenic *H19* reflected the imprinted expression described earlier. Methylation sensitive restriction enzymes *Cfo*I and *Hpa*II were used to analyse specific CpG residues within the 2.7 kb *Bam*HI fragment as described previously (Ferguson-Smith et al., 1993), (Fig. 4A). Complete methylation at the *Cfo*I sites results in a hybridising fragment of 2.7 kb. When these sites are unmethylated three small hybridising fragments are produced. Additional intermediate sized fragments are generated when the sites are partially methylated.

We first exploited the high transgene copy number in line YZ15, since this allowed us to address the methylation status of the transgene DNA with minimal interfering signal from the endogenous alleles. Fig. 4B shows that the YAC was largely unmethylated at these *Cfo*I sites in tail DNA from the founder in line YZ15 prior to passage through the germline (lane 1,

Fig. 4. Imprinted methylation at *H19* is maintained on the YAC transgene. (A) *H19* gene structure and location of methylation sensitive *Cfo*I and *Hpa*II sites known to show differential methylation between parental alleles. Bold lines are *H19* exons 1-5. C, *Cfo*I. H, *Hpa*II. B, *Bam*HI. White circles represent unmethylated sites, black circles are methylated. Grey circles represent partial methylation. Circles above the line show methylation on the maternal allele (mat), below the line is paternal specific (pat). Circles on the line show no difference between the parental alleles. (B) Methylation analysis of *Cfo*I sites in tail DNA from line YZ15 which contains ten copies of YZ. Lane 1, DNA from male founder. Lanes 2-4, F_1 progeny derived from mating founder YZ15 with a non-transgenic (C57BL/6 × CBA) female. Lanes 5-9, F_2 progeny derived from mating transgenic F_1 progeny with non-transgenic (C57BL/6 × CBA) mice. NT, non-transgenic littermate. The position of a fully methylated 2.7 kb band is indicated. Following paternal transmission the YAC is methylated in this region. After maternal transmission the YAC becomes de-methylated. (C, D) Methylation analysis of *Hpa*II sites (**C**) and *Cfo*I sites (D) in day 13 embryos derived from mating transgenic lines YZ8, (lanes 1-4), YZ22, (lanes 5-8) and YZ15, (lanes 9-12) with non-transgenic (C57BL/6 × CBA) mice, showing patterns of differential methylation similar to that described in B. Two independent samples are shown from maternal and paternal transmission for each line.

Founder). However, after transmission through the male germline, most of the DNA in this region became methylated (lanes 3 and 4) and the intensity of unmethylated fragments correlated well with the endogenous maternal allele (lane 2, NT). Subsequent passage through the female germline resulted in demethylation of most transgene copies (compare the 2.7 kb band in lanes 5 and 6 with the endogenous paternal 2.7 kb band in a non-transgenic littermate, lane 7). Conversely, the region remained methylated after a second passage through the male germline (lanes 8 and 9).

These results demonstrate that the *cis* elements required for establishing and maintaining paternal-specific methylation of this region are present within the transgene, and are subject to the same imprinting modifications seen at the endogenous locus upon passage through male and female germlines.

We next analysed methylation of this region in embryos at day 13 of gestation, at *Hpa*II (Fig. 4C) and *Cfo*I sites (Fig. 4D). As expected, in lines YZ22 and YZ15 significantly more methylation was observed after paternal inheritance (lanes 5, 6 and 9, 10) than after maternal inheritance (lanes 7, 8 and 11, 12). A similar pattern of differential methylation was seen in the 3 copy line YZ6B (not shown). Methylation analysis in the single copy lines YZ3 (not shown) and YZ8 was less reliable due to co-migration of bands from the endogenous *H19* allele (lanes 1 to 4).

We previously showed that the A6-A4 region located between *Igf2* and *H19* is unmethylated in embryos on both parental alleles (Koide et al., 1994). Analysis of A6 in lines YZ15 and YZ22 showed that this region was also unmethylated on the transgenes (not shown). We also examined the differentially methylated region in the gene body of *Igf2* in line YZ15. This region was more methylated after paternal transmission, consistent with the pattern at the endogenous locus (not shown). This analysis was not extended to lower copy lines since the methylation differences in this region were too subtle (Feil et al., 1994).

The expression and methylation data described are consistent with the imprinting seen at the endogenous *H19* locus. This demonstrates that elements required for imprinting of *H19* are located within the 130 kb YAC.

Mesodermal enhancers for both *Igf2* and *H19* are located in the 130 kb domain

Previous experiments have localised two enhancers within 10 kb downstream of *H19* which activate both *H19* and *Igf2* in endodermal cells (Yoo-Warren et al., 1988; Leighton et al., 1995a). However, enhancers for expression of either gene in mesoderm remain to be discovered. We have asked whether the mesodermal enhancers are present on the YAC transgene.

As mentioned previously the YAC construct carries a *lacZ* reporter inserted into the *Igf2* gene, allowing us to make clear comparisons between expression pattern from the YAC and from the endogenous gene. The pattern of *Igf2* expression in the normal mouse and rat embryo has been well characterised previously (Stylianopoulou et al., 1988; Lee et al., 1990). β-gal staining patterns were similar in all lines tested (YZ3, 6B, 8, 15 and 22), and were consistent with the endogenous pattern, both spatially and temporally.

Fig. 5 shows the results of staining for β-gal activity in embryos derived from male founders YZ8 or YZ15 between day 11 and day 15 of gestation, the period of development when the most extensive *Igf2* expression is seen. Before day 11 strongest β-gal activity was seen in the somites, and around the branchial arches (not shown). At day 11 staining was seen in both the sclerotome and dermomyotome parts of the somites, with strongest activity in myotomes, liver, and the developing limb buds (Fig. 5A). By day 12 staining was clearly seen in developing vertebrae and ribs which are derived from the sclerotomes. Strong activity was still evident in the limb buds, and notably in the apical ectodermal ridge (Fig. 5B). After day 12 of gestation embryos were bisected to allow staining of internal organs. Strong staining was seen in cartilaginous tissues, but in the limbs expression was now restricted to the forming cartilage and muscles. Strong staining was also seen in muscular tissues such as the tongue, and in the developing pituitary gland (Rathkes pocket). From day 13 of gestation staining was also seen in layers of the meninges covering the neural tissues, and in the choroid plexus (Fig. 5C). After day 13 activity in the developing muscles increased strongly (Fig. 5D), while ossifying cartilage stopped expressing. At no stage was any staining evident in neural tissue. These results demonstrate that the enhancer elements responsible for controlling expression of *Igf2* in tissues of mesodermal origin are present on the YAC.

Although we could not examine the pattern of *H19* expression from the YAC in similar detail, RT-PCR analysis on muscle from mice inheriting the transgenes maternally clearly showed that *H19* is expressed from the YAC in this mesodermal tissue (not shown). This demonstrates that enhancers for expression of *H19* in tissues of mesodermal origin are also present within this 130 kb domain.

Imprinting of *Igf2-lacZ*

Endogenous *Igf2* is expressed predominantly from the paternal allele (DeChiara et al., 1991). However, the maternal allele has *potentially* active chromatin and shows a low level of expression (Sasaki et al., 1992). In the choroid plexus and the leptomeninges *Igf2* is not imprinted (DeChiara et al., 1991). It is not known how tissue-specific repression of the maternal allele is achieved.

In order to test whether the sequences required for appropriate imprinting of *Igf2* are located on the YAC, β-gal activity was analysed following both paternal and maternal transmission. The frequency of imprinted *Igf2-lacZ* expression from the YAC transgene was greater than that seen for other transgenes inserted at random locations in the genome (reviewed by Reik et al., 1990), and it always occurred in the expected direction, suggesting that an important part of the *Igf2* imprinting mechanism is located within the 130 kb domain.

Fig. 6A shows representative embryos at day 12 of gestation, from the low copy lines YZ3, YZ8 and YZ22, and the ten copy line YZ15. Each of these lines was previously fully characterised for, and shown to imprint, the *H19* gene. Lines containing one or two copies of YZ also showed clear imprinting of *Igf2-lacZ*. Line YZ8 showed strong X-Gal staining after paternal transmission, so for the purpose of comparison to other lines (based on visual observations of at least 20 embryos in each line) we have taken this to represent maximum activity (High). Visual observations took account of both the time taken for initial staining to appear, and the final intensity of staining

after full reaction time. In line YZ22 β-gal activity after paternal transmission was also clear, but it was not as strong (Medium activity). No staining was ever seen in these lines after maternal transmission. Imprinted activity was also observed in line YZ3. After paternal transmission the full pattern of staining was always observed at consistent high levels. After maternal transmission, some weak staining was seen, most significantly in the developing forelimbs (Low activity, see Fig. 6A).

Strikingly, and in contrast to the well maintained imprinting of *H19*, the ten copy line YZ15 showed similar levels of *Igf2-lacZ* activity after both maternal and paternal transmission (Fig. 6A). The 3 copy line, YZ6B, also showed biallelic β-gal activity (not shown).

To investigate whether the lack of *Igf2-lacZ* imprint in some lines is actually related to overall copy number of the YAC in the mouse genome, we tested the effect of transmitting two of the low copy YACs, which showed appropriate imprinting, through the maternal germ line at the same time. YZ8 (one copy) and YZ22 (two copy) were used since *Igf2-lacZ* expression was never observed from either line individually after maternal inheritance (see Fig. 6A). Strikingly we found that when the two YAC inserts were co-transmitted maternally, *Igf2-lacZ* was activated at low levels (Fig. 6B), demonstrating that the presence of three copies of the YAC at independent loci is sufficient to up-regulate expression of *Igf2-lacZ*. We suggest that this up-regulation is related to reduced *H19* expression per transgene copy, as a direct consequence of increased copy number of the transgene (see below).

DISCUSSION

We have addressed the regulation of genomic imprinting using a 130 kb YAC transgene containing the *Igf2* and *H19* genes. This study demonstrates that key elements involved in regulating reciprocal *Igf2* and *H19* gene activity in *cis* are present on the transgene as well as the elusive enhancers responsible for expression in mesodermal tissues. The high frequency of imprinting of both *Igf2* and *H19* was relatively more consistent for both genes after paternal transmission. The expected expression of *H19* also occurred consistently after maternal transmission but at variable levels which inversely correlated with relaxation of *Igf2* repression. The extent of this may be directly regulated by the relative activity of the *H19* locus in *cis*. We propose a novel model in which the *H19* gene is most stably maintained in a repressed state, and this has to be relieved following maternal inheritance as the key mechanistic event of imprinting the *Igf2/H19* region. These points are discussed below.

Expression pattern of *Igf2* and *H19*

In situ analysis of reporter gene activity showed appropriate expression of *Igf2* from a transgene for the first time, demonstrating that, in addition to the endodermal enhancers, the elusive mesodermal enhancers are contained within our construct. If *Igf2* and *H19* are regulated by identical control elements in mesoderm, as in endodermal tissues (Leighton et al., 1995a), then *H19* should also be expressed appropriately from the YAC. In this study we could not visualise the trans-

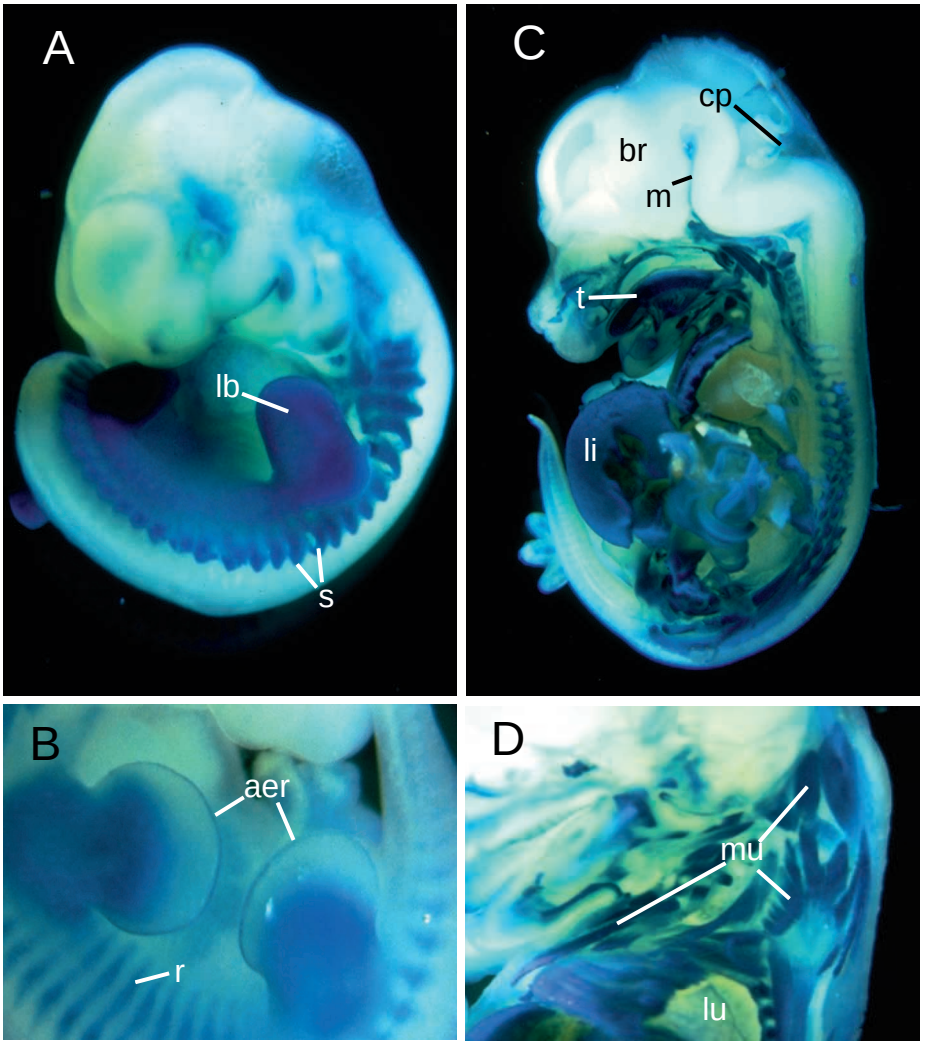


Fig. 5. Expression analysis of *Igf2-lacZ*. (A) Day 11 embryo derived from line YZ15, showing strong expression in the developing limb buds (lb) and somites (s). (B) X-Gal staining at day 12 in an embryo derived from line YZ8 clearly shows activity in the developing ribs (r) and in the apical ectodermal ridge (aer) of the limbs. (C) Saggital section of a day 15 embryo from line YZ15 shows widespread β-gal activity in tissues of mesodermal and endodermal origin, and in tissues of ectodermal origin known to express *Igf2*. (li), liver. (t), tongue. (br), brain. (m), meninges. (cp), choroid plexus. (D) At embryonic day 15 *Igf2-lacZ* is strongly expressed in developing muscles (mu). Expression can also be seen in the bronchial epithelium of the lung (lu).

genic *H19* expression pattern in the embryo since this gene was not disrupted with reporter sequences. However, enhancers for mesodermal *H19* expression must also be present on the YAC, since RT-PCR analysis showed that transgenic *H19* was expressed in skeletal muscle. Although this does not necessarily mean that *H19* and *Igf2* utilise the same mesodermal enhancers, it remains a distinct possibility.

Previous transgenic experiments showed that the mesoderm-specific enhancers are not located within 14.5 kb upstream to 6.5 kb downstream of *Igf2* (Kou and Rotwein, 1993; Lee et al., 1993), or within 11 kb upstream to 12 kb downstream of *H19* (Brunkow and Tilghman, 1991; Yoo-Warren et al., 1988). In addition we have found that a 3 kb region containing the intergenic DNase I hypersensitive sites (A6-A4) (Koide et al., 1994) does not have this activity (T. Koide and J. D. Brenton, unpublished). This leaves three broad regions on the YAC in which the mesodermal enhancer(s) may be located which are currently under investigation.

Imprinting of the *H19* gene

Imprinted *H19* expression was observed in all four of the transgenic lines tested, irrespective of the YZ integration site, or copy number, which ranged from one to ten. Methylation and expression of the *H19* gene after maternal and paternal transmission were consistent with the patterns seen at the endogenous locus. These results demonstrate that elements which control imprinting of *H19* are located on the YAC.

Transgenic experiments by others have suggested that the DNA around *H19* contains element(s) which are important for imprinting. *H19* transgenes containing 3.7 kb of upstream sequence and the downstream endoderm-specific enhancers, were found to imprint in some cases, but only as multi-copy loci on the DBA genetic background (Bartolomei et al., 1993; Pfeifer et al., 1996; Elson and Bartolomei, 1997). More recently, *H19-lacZ* reporter genes with 10.5 kb of upstream flanking sequence have also shown such imprinting (J. D. Brenton, personal communication). However, the element(s) located near *H19* are not sufficient to confer *consistent* imprinting, either between, or even within established transgenic lines. Imprinting of *H19* on the YAC transgene, in contrast, occurred in all cases and was not affected by either copy number or genetic background. While this could be accomplished by specific elements, it may simply

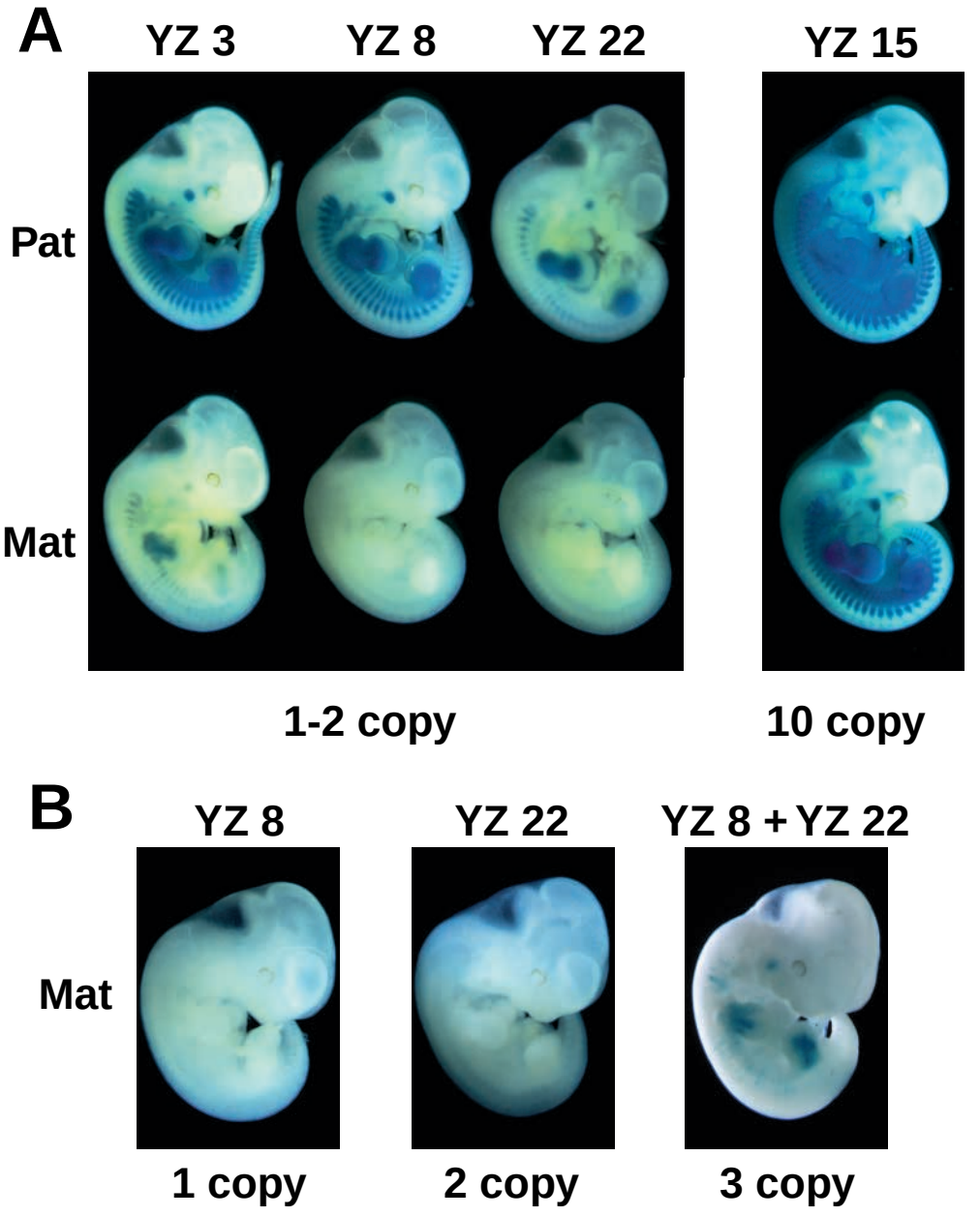


Fig. 6. *Igf2-lacZ* expression is imprinted in low copy number transgenic lines. (A) Embryos at day 12 of gestation from transgenic lines, YZ3, 8, 22 and 15 following transmission of the YAC inserts through the paternal (Pat) and maternal (Mat) germlines. β-gal activity was clearly greater when the transgenes were paternally inherited in one or two copies (lines YZ3, 8 and 22). When multiple copies of the YAC were integrated no parental-specific difference was seen in the level of β-gal activity (line YZ15). (B) Day 12 embryos from a single litter derived from mating a female containing both YZ8 and YZ22 with a non-transgenic male, demonstrating segregation of the two inserts. All embryos were stained simultaneously for the same duration under identical conditions. Note that X-Gal staining was only seen in embryos where the two inserts had been co-transmitted, demonstrating a copy number dependent *trans* effect on *lacZ* activation.

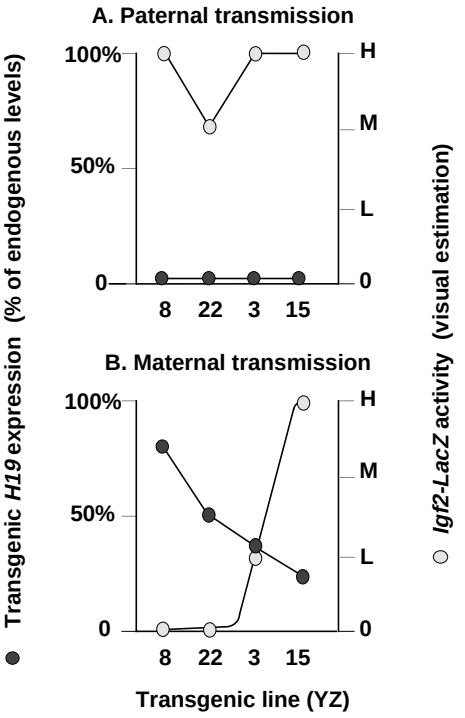


Fig. 7. The level of *Igf2-lacZ* expression inversely correlates with the level of *H19* expression per transgene copy. L, low activity. M, medium activity. H, high activity. (A) After paternal transmission little, or no transgenic *H19* expression was detected in any line. Conversely, *Igf2-lacZ* was expressed at medium to high levels. (B) After maternal transmission no *Igf2-lacZ* expression was seen in lines that express transgenic *H19* at approx. 50% or more per transgene copy relative to endogenous *H19* expression (lines YZ8 and YZ22). Where the relative *H19* level was approximately 40% *Igf2-lacZ* was weakly expressed (line YZ3) whereas at *H19* levels of 20% *Igf2-lacZ* was highly expressed (line YZ15).

be a function of transgene size since the *H19* gene was surrounded by 90 kb of upstream and 35 kb of downstream sequence on the YAC. This contrasts with the *Igf2-lacZ* gene which was 'protected' by only 1-2 kb of upstream and 120 kb of downstream sequence. In this respect it is noteworthy that a boundary element may exist downstream of the human *H19* gene since three downstream genes are all biallelically expressed in multiple tissues (Yuan et al., 1996).

It is intriguing that expression or imprinting has rarely, if ever been observed from single copy mini-*H19* transgenes (Brunkow and Tilghman, 1991; Bartolomei et al., 1993; Pfeifer et al., 1996; Elson and Bartolomei, 1997 and J. F-X. A., J. D. Brenton and F. Shamanski, unpublished). In contrast the single copy YAC transgenes can clearly express and imprint *H19*. The reasons for these differences between the small *H19* transgenes and the YAC are unclear at present but it suggests the presence of additional sequences on the YAC that allow for appropriate activity of low copy loci.

From the available evidence, we propose an alternative model for imprinting of this region in which repression of the *H19* gene is the most stable state. In this context, the proposed 'imprinting element' near *H19* (Bartolomei et al., 1993) may in fact function as a silencer element rendering repression of *H19* the default state, whose influence must be overcome following maternal inheritance. This key and potentially complex mechanistic event may be the reason for variable expression of maternally inherited transgenic *H19*. Additional *cis* control elements on the YAC may overcome the effects of the silencer resulting in the relatively consistent imprinting of *H19* from the YAC. The intergenic region, A6-A4, that we previously identified (Koide et al., 1994) may serve this critical function and we are currently testing this possibility. This model could also explain why single copy *H19* transgenes are seldom if ever expressed. The variable activation of mini-*H19* transgenes (after maternal inheritance) in complex multi-copy loci could be an artefact due to the presence of multiple copies of the *H19* downstream enhancers; these enhancers may mimic, albeit inefficiently, functions of specific *cis* elements postulated to be present on the YAC which counteract the silencer element proximate to *H19*. Further support for this model is provided by our recent analysis of *H19* reporter constructs in *Drosophila*, investigating any underlying conserved epigenetic mechanisms (Lyko et al., 1997). These studies revealed a striking effect whereby a 1.2 kb sequence upstream of *H19* functions as a silencing element. The silencer is apparently located within a region that is necessary for *H19* imprinting in mice (Elson and Bartolomei, 1997), suggesting an evolutionarily conserved mechanism for gene silencing in *Drosophila* and imprinting in mice. Our proposal that repression of *H19* is the key mechanistic event for imprinting in this domain can be tested in mice using further modifications of the YAC.

Imprinting of the *Igf2-lacZ* gene

Igf2-lacZ was imprinted *consistently* in lines which contain one or two copies of the YAC. In lines containing three or more copies at a single locus, however, *Igf2-lacZ* was expressed from both parental chromosomes. This contrasts with the more reliable imprinting of *H19* discussed above.

In the ten copy line, YZ15, biallelic *Igf2-lacZ* expression was observed despite differential methylation in the body of the gene, suggesting that methylation of this region does not play a key role in *Igf2* imprinting. Importantly, imprinted expression was seen in other transgenic lines, and this was in the absence of the differentially methylated region located 3 kb upstream of *Igf2* (Sasaki et al., 1992), suggesting that the region is functionally not essential for appropriate expression or imprinting of *Igf2*.

Intriguingly the presence of three copies of the YAC at different loci resulted in some degree of maternal *Igf2-lacZ* activation, suggesting a *trans* activation effect in response to increased copy number. The correlation between transgene copy number, level of *Igf2-lacZ* activity, and level of *H19* expression led us to propose that reduction in the amount of promoter-enhancer interaction at the *H19* locus to below a critical threshold level at any one locus leads to activation of the *Igf2-lacZ* gene on the same chromosome (see below and Fig. 7). Reduced activity of the *H19* gene could be caused by competition for a limited pool of *trans* acting factors when multiple copies are present in the same nucleus. Alternatively *trans* sensing between the increased number of *H19* alleles present could result in transfer of the methylation pattern from the inactive endogenous paternal allele to one or more copies of the transgene via transient DNA heteroduplexes or by some other pairing dependent mechanism (Bestor and Tycko, 1996; LaSalle and Lalande, 1996).

Relationship between *Igf2* and *H19* expression

After paternal transmission *Igf2-lacZ* was expressed at medium to high levels in all lines, while no *H19* expression was detected (Fig. 7A). This is consistent with activity of the genes at the endogenous locus. However, expression of both genes was observed from a single locus after maternal transmission under certain circumstances (Fig. 7B). There are indeed precedents for such expression of endogenous *Igf2* and *H19* from the maternal chromosome at the same time, as seen in the choroid plexus/meninges (Svensson et al., 1995), and in trophoblast cells (Sasaki et al., 1995; Mutter et al., 1993; Jinno et al., 1995).

In lines where the level of maternal *H19* expression per transgene copy was greater than ~50% relative to endogenous levels, *Igf2-lacZ* was repressed (Fig. 7B, lines YZ8 and YZ22). However, when the level of maternal *H19* per transgene copy was less than 50% of endogenous levels, *Igf2-lacZ* was active (Fig. 7B, lines YZ3 and YZ15). Therefore, our data suggests that there may be a critical threshold for *H19* transcription which is necessary to keep *Igf2* repressed. It is important to remember in this context that the maternal endogenous *Igf2* allele is never completely repressed and its chromatin structure is *potentially* active on both parental chromosomes (Sasaki et al., 1992).

One possibility is that repression of *Igf2* is directly controlled by high levels of the *H19* RNA. However, a direct role of the RNA seems unlikely since imprinting has been observed in the endogenous domain in the absence of any *H19* product (Ripoche et al., 1997). We favour an alternative explanation where the level of *Igf2* expression from the maternal chromosome is inversely related to the rate of maternal *H19* transcription and not the amount of *H19* transcript itself. Indeed, it is possible that the act of transcribing the *H19* gene is not required at all; interaction between the *H19* locus and sequences (enhancers) that are essential for *Igf2* transcription would be sufficient.

Co-expression of *Igf2* and *H19* from the same chromosome could be accomplished by a 'flip-flop' mechanism of promoter-enhancer interaction, as seen at the *β-globin* locus (Wijgerde et al., 1995). Thus, when enhancer activity at *H19* is reduced, for example, by limited availability of *H19*-specific transcription factors, *Igf2* expression is enhanced. In this way *Igf2* and *H19* may be transcribed alternately to generate both messages from the same chromosome. Such a mechanism would explain why multi-copy YZ transgenes escape *Igf2* imprinting while maintaining the *H19* imprint, and also allow for expression of both *Igf2* and *H19* from the maternal chromosome in some endogenous tissues (Svensson et al., 1995; Sasaki et al., 1995; Mutter et al., 1993; Jinno et al., 1995).

In this study we have demonstrated that the transgene YZ contains elements required for appropriate expression and imprinting of *Igf2* and *H19*, which function most effectively when 1-2 copies of the YAC were inserted at individual loci. The differentially methylated region upstream of *Igf2* (Sasaki et al., 1992) is apparently not essential for expression or imprinting of these genes. In addition, a putative imprint control element (ICE) which may exist further upstream of *IGF2* from analysis of Beckwith Wiedemann (BWS) patients (Hoovers et al., 1995; Reik et al., 1995; Brown et al., 1996; Lee et al., 1997) may not play a key role in initiation of *Igf2* or *H19* imprinting. It is however possible that one or both of these regions may serve to stabilise the imprinted expression. This is consistent with some leaky expression of maternally inherited *Igf2-lacZ* in a single copy YAC transgenic line that mirrors relaxation of imprinting seen in some BWS patients. We have also postulated that the 'imprinting' *cis* element proximate to *H19* may in fact be a silencer element, since the available data suggests that repression of *H19* is the default state. The critical mechanistic step for imprinting in the *H19/Igf2* domain requires the effects of this silencer to be overcome following maternal inheritance. The intergenic region (A6-A4) on the YAC transgene is a candidate 'activator' *cis* element that may counteract the influence of the silencer resulting in the relatively efficient imprinted expression of the 1-2 copy ectopic YAC loci. In this context, it is particularly interesting that there are imprinting mutations inherited from the maternal germ line which result in biallelic repression of *H19* and biallelic expression of *IGF2* (Reik et al., 1995) suggesting that this may, in some instances, be due to mutations in an activator element.

We thank A. Schedl and C. Huxley for useful advice on YAC DNA preparation; W. Reik for communicating details of the *Msp*I polymorphism in *H19* exon 1; A. Ferguson-Smith for primers spanning this region; and T. Tada for assistance with FISH. This work was supported by grants from the Wellcome Trust (036481) and the International Human Frontiers Science Programme. T.K. is supported by fellowships from the Wellcome Trust and the Uehara Memorial Foundation.

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(Accepted 11 July 1997)