Regulation of genomic imprinting by gametic and embryonic processes

J. Richard Chaillet,¹ Debra S. Bader, and Philip Leder

Department of Genetics, Harvard Medical School, Howard Hughes Medical Institute, Boston, Massachusetts 02115 USA

Parental genomic imprinting refers to the phenomenon by which alleles behave differently depending on the sex of the parent from which they are inherited. In the case of the murine transgene *RSVlgmyc*, imprinting is manifest in two ways: differential DNA methylation and differential expression. In inbred FVB/N mice, a transgene inherited from a male parent is undermethylated and expressed; a transgene inherited from the female parent is overmethylated and silent. Using a series of *RSVlgmyc* constructs and transgenic mice, we show that the imprinting of this transgene requires a *cis*-acting signal that is principally derived from the repeat sequences that make up the 3' portion of the murine immunoglobulin α heavy-chain switch region. Such imprinting is relatively independent of the site of transgene insertion but is influenced by the structure of the transgene itself. Imprinting is also modulated by genetic background. Detailed studies indicate that the paternal allele is undermethylated and expressed in inbred FVB/N mice and in heterozygous F₁ FVB/N/C57Bl/6J mice but is overmethylated and silent in inbred C57Bl/6J mice. Consequently, the FVB/N genome appears to carry alleles of modulating genes that dominantly block methylation and permit expression of the paternally imprinted transgene. Furthermore, our results suggest that overmethylation is the default status of both parental alleles and that the paternal allele can be marked in *trans* by polymorphic factors that act in postblastocyst embryos.

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There is increasing evidence to indicate that apart from obvious sex-chromosome differences, the parental haploid contributions to the developing mammalian embryo are fundamentally unequal (McGrath and Solter 1984; Surani et al. 1986). This difference is manifest as a requirement of both maternal and paternal genomes for development to proceed normally. In nuclear transplanted mouse zygotes composed solely of maternal pronuclei (gynogenones) or solely of paternal pronuclei (androgonones), there is a failure to complete embryogenesis. The genetic causes of this failure are localized to distinct chromosomal regions, identified by the association of phenotypic abnormalities with uniparental disomic inheritance of these regions (Searle and Beechey 1978; Cattanaach and Kirk 1985).

These embryologic and genetic studies predicted the existence of distinct imprinted loci in which the two parental alleles are differentially marked (imprinted) in their respective germ lines. This prediction was realized with the identification of mouse transgenes in which the maternal allele was methylated differently than the paternal allele (Hadchouel et al. 1987; Reik et al. 1987; Sapienza et al. 1987; Swain et al. 1987). For the transgenic line *TG.A*, this methylation imprinting correlated

absolutely with expression imprinting, a difference in expression of the parental transgene alleles (Swain et al. 1987). More recently, a number of endogenous imprinted mouse genes have been identified, each with demonstrable expression differences between the maternal and the paternal alleles (Barlow et al. 1991; Bartolomei et al. 1991; DeChiara et al. 1991; Leff et al. 1992; Hatada et al. 1993; Giddings et al. 1994; Hayashizaki et al. 1994; Villar and Pedersen 1994; Guillemot et al. 1995). Interestingly, for at least four of these genes, associated methylation imprinting has also been demonstrated (Sasaki et al. 1992; Bartolomei et al. 1993; Ferguson-Smith et al. 1993; Hatada et al. 1993; Stoger et al. 1993; Hayashizaki et al. 1994). The common feature of all of these loci is the distinction of the parental alleles at the molecular level, evident primarily as differences in which one of the parental alleles is silent, whereas the other is expressed.

For all imprinted loci, transgenic and endogenous, the underlying molecular imprinting mechanism is not known. Nonetheless, the study of imprinted transgenic mouse strains has provided insights into the role of developmental events (Chaillet et al. 1991; Ueda et al. 1992), as well as the role of DNA methylation in this process (Chaillet 1992; Brandeis et al. 1993; Sasaki et al. 1993). One transgenic strain, *TG.A*, has been particularly informative (Swain et al. 1987; Chaillet et al. 1991). This line was created using the *RSVlgmyc* transgene that ex-

¹Present address: Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260 USA.

presses *c-myc* derived from a murine Burkitt-like immunoglobulin heavy-chain α region/*c-myc* (*IgA/c-myc*) translocation. The line dramatically exhibited both expression and methylation imprinting in which the relatively undermethylated, paternally derived allele is expressed in the heart (and no other tissue), whereas the overmethylated, maternally inherited allele is not expressed at all. The distinctive maternal pattern of overmethylation is established in the female gamete and maintained throughout adult life, whereas the distinctive paternal undermethylated pattern is established in the early embryo and maintained thereafter (Chaillet et al. 1991).

In the current work we have approached an understanding of the regulation of *RSVlgmyc* imprinting through a combination of molecular, genetic, and embryologic experiments. We first determined that multiple lines bearing the *RSVlgmyc* transgene and created in the FVB/N inbred background all possess the same imprinting characteristics as the original *TG.A* line (Swain et al. 1987). Thus, the *RSVlgmyc* transgene is imprinted independently of the genomic integration site and maintains this characteristic in an inbred background (see below). Together, these observations indicate that the instructive sequences required for imprinting must be contained within the transgene. Furthermore, these transgenic mice should be contrasted with transgenic strains that appeared to be imprinted only on mixed strain backgrounds (Sapienza et al. 1989; Allen et al. 1990; Engler et al. 1991). In the latter cases, the apparent transgene parent-of-origin effect on methylation was the result of unlinked, strain-specific modifier alleles that were inherited in a parent-specific fashion (Allen and Mooslehner 1992; Sasaki et al. 1993).

We then assessed a series of mouse lines bearing systematically deleted transgene constructs. These enabled us to localize the *cis*-acting region necessary for transgene imprinting with respect to both methylation and expression. We also noted a difference in the penetrance of parental imprinting in two inbred mouse strains, FVB/N and C57BL/6J. Developmental studies in appropriate homozygous and heterozygous mice of these strains allowed us to postulate the existence of *trans*-acting modulating factors that act in the early embryo to block a high level of paternal transgene methylation and thus permit its expression. Taken together, these results are consistent with a model in which a *cis*-acting signal attracts methylation to the maternal and paternal transgenes at different times in development, subject (in the paternal allele) to embryonic modification by the *trans*-acting products of genes that are present in at least two allelic forms in the mouse.

Results

Transgene imprinting is independent of integration site

Because of the potential effects of strain-specific modifier genes on the methylation of transgenes (Allen and Mooslehner 1992; Sasaki et al. 1993), we have continu-

ally bred our transgene-bearing lines, *TG.A* and *TG.B*, into the inbred FVB/N strain background where they display the imprinted phenotype, for example, undermethylation and expression of the paternal allele and overmethylation and silencing of the maternal allele (Chaillet et al. 1991). We have extended this analysis by examining four additional transgenic lines created directly in an FVB/N strain background. As summarized in Table 1, all six lines (*TG.A*, *TG.B*, *TG.C*, *TG.D*, *TG.E*, and *TG.F*) are parentally imprinted with respect to methylation and expression (when tested). For example, in the *TG.C* line (shown in Fig. 1A), the paternal allele is expressed in the heart, which is assayed as a 168-bp RNase-resistant, transgene-specific band. The maternal allele is transcriptionally silent. Using digestion with methylation-sensitive restriction enzymes as an assay of methylation state (Fig. 1B), it can be seen that the maternal allele is highly methylated (overmethylated), whereas the paternal allele is poorly methylated (undermethylated). Similar patterns of sex-of-parent-dependent methylation are also observed in the *TG.D*, *TG.E*, and *TG.F* lines. We therefore conclude that the transgene is a portable imprinted construct that is imprinted independently of the site of genomic integration and contains a *cis*-acting signal needed for expression and methylation imprinting.

Identification of the cis-acting imprint signal

We attempted to locate the *cis*-acting imprinting signal by analyzing a series of transgenic lines containing deletion constructs using expression and methylation assays.

Table 1. Imprinting characteristics of *RSVlgmyc* transgenic lines in an FVB/N background

Line	Allele methylation		Allele expression	
	maternal	paternal	maternal	paternal
<i>TG.A</i>	+	+	–	+
<i>TG.B</i> ^a	+	+	+	+
	+	+	–	+
<i>TG.C</i>	+	+	–	+
<i>TG.D</i>	+	+	N.T.	N.T.
<i>TG.E</i>	+	+	N.T.	N.T.
<i>TG.F</i>	+	+	N.T.	N.T.

The summary of the *TG.A* and *TG.B* lines is from Swain et al. (1987) and Chaillet et al. (1991). *TG.C*, *TG.D*, *TG.E*, and *TG.F* were created directly on an inbred FVB/N genetic background. Lines *TG.D*, *TG.E*, and *TG.F* were not examined for transgene expression. For methylation studies: (+) The poorly methylated pattern in Fig. 1B; (++) the highly methylated pattern in Fig. 1B. For expression studies, + refers to heart expression, and – refers to no detectable expression. (N.T.) Not tested.

^aOf mice inheriting a maternal transgene allele, ~50% had a highly methylated, nonexpressing transgene phenotype, identical to the *TG.A* maternal allele. The remainder of the maternally inherited alleles were poorly methylated, transcriptionally active in the heart, and had a methylation pattern identical to the paternally transmitted *TG.A* allele (Chaillet et al. 1991).

Figure 1. Genomic imprinting in an inbred strain background. The *TG.C* transgenic line was created directly in the inbred FVB/N strain. (A) Ribonuclease protection assay showing *TG.C* expression of *c-myc*. Heart RNA was isolated from adult individuals of *TG.C* and analyzed for transgene-specific *c-myc* expression using a mouse *c-myc* cDNA fragment (365-bp *NotI*–*PstI* restriction fragment). The 168-bp band represents transcription of an *IgA/c-myc* fusion transcript containing the truncated transgene *c-myc* exon 1 (23 bp) and intact exon 2. Endogenous protected fragments of 225 and 365 bp and a nonspecific (transgene or endogenous) fragment of 145 bp are also evident. (B) Southern blots showing methylation patterns of the parental alleles of *TG.C*. Tail DNA was isolated and digested with *HpaII*, and the Southern blot was hybridized with α . (M) Maternal; (P) paternal.

As can be seen in Figure 2A, the transgene is composed of three elements of different biological origins: 440 bp of the Rous sarcoma virus long terminal repeat (RSV LTR), a 2.1-kb portion of pBR322, and a 15-kb *EcoRI* fragment containing the breakpoint region of a Burkitt-like translocation involving the mouse immunoglobulin α heavy-chain region (*IgA*) and the *c-myc* proto-oncogene (Swain et al. 1987; Chaillet et al. 1991). This breakpoint region can also be conveniently divided into three sequence elements: a region comprising coding exons, intervening sequences, and switch repeat elements of *IgA* (α), relatively pure *IgA* repetitive switch recombination sequences (α), and a truncated mouse *c-myc* gene. We arbitrarily define α as the 1756-bp *EcoRI*–*XbaI* restriction fragment between RSV and α in the transgene construct (Fig. 2A). Germ-line α consists of tandemly repeated homology units based on a primordial pentamer motif (Davis et al. 1980; Arakawa et al. 1993). It is important to note that our defined α region also contains copies of the switch repeat motif with homology decreasing with distance from α . Taking note of the above, we constructed a series of transgene deletions, each removing approximately one or more of these five distinct sequence elements. All lines were analyzed for methylation and, when appropriate, for expression. Because transcription of the transgene is initiated at a cryptic promoter site within the α region and because expression depends on the presence of enhancer sequences within the RSV element (D.S. Bader, unpubl.), removal of these elements precludes examination of expression but not methylation. Because *c-myc* is the reporter element, its removal also precludes examination of expression.

The imprinting characteristics of the transgene and several of the deletion lines are summarized in Figure 2B

and are shown in detail in the figures that follow. As would be expected, construct C, in which only the pBR322 sequences have been removed, maintains appropriate imprinted differences with respect to both methylation and expression (Fig. 3A,B). Construct D, deleted for α sequences, also maintains both methylation and expression differences (Fig. 3A,B). Construct B, in which the RSV sequences have been deleted (and thus can only be assayed with respect to methylation), also preserves imprinting (Fig. 3B). Taken together, these analyses localize the required *cis*-acting imprinting signal to either the switch recombination sequences or *c-myc*. Because these regions are required either for transcription of the transgene or for detection of its expression, deletion constructs that lack these regions can be assayed for methylation imprinting only.

Removal of major portions of the *c-myc* gene has no effect on methylation imprinting (construct E, Figs. 2 and 4). When further *c-myc* sequences and the majority of repetitive switch recombination sequences (5' relative to the coding strand of the α gene) are removed, methylation imprinting also persists (construct F, Figs. 2 and 4). Furthermore, the 3' *myc* sequences adjacent to pBR322 are not required because methylation imprinting is maintained despite their removal (construct G, Figs. 2 and 4). Only when a small (206 bp) region containing the junction of the switch recombination repeats and the α intron is deleted from construct F (construct H, Figs. 2 and 4) is methylation imprinting lost. This loss of imprinting is also observed in a more extensive deletion that includes this region (construct I, Figs. 2 and 4). In each of these cases, both parental alleles are highly and equally methylated, a situation also seen with the deletion of all mouse sequences, that is, pBR and RSV alone

Figure 2. Summary of genomic imprinting of transgenic lines. All constructs are portrayed as the linearized versions injected into pronuclei of mouse zygotes. (A) Physical characteristics of the transgene. *MspI* restriction endonuclease sites are shown as vertical marks at the top. The horizontally hatched area is the 440-bp *PvuII*–*EcoRI* fragment of the LTR of the Schmidt–Ruppin strain (subgroup D) of RSV (Yamamoto et al. 1980; Gorman et al. 1982). pBR is the *EcoRI*–*AccI* fragment of pBR322 containing *Amp^R*. The large *EcoRI* fragment of the circularized construct (bisected by the unique *KpnI* site) contains the breakpoint region of a Burkitt-like immunoglobulin α /c-myc translocation from the S107 plasmacytoma in which the 5' region of the switch recombination sequences of IgA ($S\alpha$) has been translocated into the 5' region of c-myc (Kirsch et al. 1981). Exon 1 of c-myc is truncated (and not shown to scale), leaving 23 bp of the 3' end intact (corresponds to bp 968–bp 990 of GenBank locus *Muscm1*). The arrow represents the approximate start of transcription in $S\alpha$ of the IgA/c-myc fusion transcript (D.S. Bader, unpubl.). $C\alpha$ is a 1756-bp *EcoRI*–*XbaI* fragment (with an internal *XbaI* site) containing intervening sequences and constant region coding exons (corresponds to bp 6374–bp 4620 of GenBank locus *Musialpha*, excluding internal sequence conflicts). Relevant methylation-sensitive *Bst*UI sites (not shown) for the methylation patterns of constructs C and G are clustered in RSV (four sites) and the first intron of c-myc (eight sites). (1, 2, and 3) Exons 1–3 of mouse c-myc. Restriction sites: (B) *Bam*HI; (X) *Xba*I; (R) *Eco*RI; (K) *Kpn*I. The density of the stippling and size of the bar above the IgA portion of the transgene correspond to the degree of homology of the repeated units, which decreases with distance from $S\alpha$. (B) Imprinting characteristics of modified versions of the transgene. For construct H, the last 206 bp of $C\alpha$ has been deleted (corresponds to bp 4825–bp 4620 of *Musialpha*). For construct I, the last 355 bp of $C\alpha$ have been deleted (corresponds to bp 4973–bp 4620 of *Musialpha*). The transgenic lines created with these constructs are listed. Results are described as a difference or equivalence between maternal and paternal methylation or as expression in the heart. (–) No detectable expression from either maternal or paternal transgene alleles. (M) Maternal; (P) paternal; (NT) not tested.

(construct J, Figs. 2 and 4). Evidently, maternal-type overmethylation is the default methylation pattern.

Is the endogenous IgA locus imprinted?

Because a segment of the IgA locus is required for transgene imprinting, it is logical to ask whether the IgA locus itself is subject to parental imprinting. To test this possibility, it was necessary to detect an mRNA polymorphism between two inbred mouse strains so that products of each parental allele in an interspecific cross can be distinguished using an RNase protection assay. Fortunately, there are sequence polymorphisms in the 3'-untranslated regions of immunoglobulin α heavy-chain mRNAs from inbred C57Bl/6J and *Mus spretus* mice that allow us to distinguish them from each other. The results of an RNase protection experiment using splenic RNA derived from mice in which the C57Bl/6J and *M. spretus* alleles were reciprocally inherited from

the male and female parents are shown in Figure 5. Very clearly, both alleles are expressed in the hybrid mice and the locus is evidently not subject to expression imprinting in this genetic background. Because the immunoglobulin loci undergo random allelic exclusion, this bi-allelic IgA expression must represent a mixture of monoallelically expressing cells (Alt et al. 1980). Consistent with the absence of expression imprinting, the IgA locus also does not appear to be subject to methylation imprinting. Using a variety of methylation-sensitive restriction endonucleases, we were unable to detect methylation imprinting within the $C\alpha$ and $S\alpha$ sequences in the same interspecific crosses (data not shown).

Penetrance of parental imprinting is influenced by genetic background

In addition to the *cis*-acting requirement for genomic imprinting, *trans*-acting factors must also be involved in

Figure 3. Imprinting analysis of transgene constructs. (A) Ribonuclease protection assay examining expression in transgenic lines *TG.KD* (created with construct C; lanes 1,2) and *TG.QT* (created with construct D, lanes 3,4). The relationships of the probe to protected fragments are shown in Fig. 1A. (B) Southern blots of methylation-sensitive endonuclease digests showing methylation patterns of imprinted transgenes. (Lanes 1,2) Alleles of *TG.ID* (created with construct B) digested with *HpaII*. (Lanes 3,4) Alleles of *TG.KD* (created with construct C) digested with *BstUI*. (Lanes 5,6) Alleles of *TG.QT* (created with construct D) digested with *HpaII*. All DNA samples were obtained from tails of adult mice and hybridized with either the probe Ca (*TG.ID*, *TG.KD*) or RSV (*TG.QT*). The parental source of the transgene is indicated at the bottom of each lane.

creating an imprinted locus. In the case of our transgene, these factors presumably act directly or indirectly through the imprint signal that we have identified. The fact that imprinting of endogenous genes (Babinet et al. 1990; Latham and Solter 1991; Forejt and Gregorova 1992; Reik et al. 1993) and transgene methylation and expression (Engler et al. 1991; Allen et al. 1990) can be modified by strain background provided a genetic tool

with which to study the putative *trans*-acting elements that influence parental imprinting. We noticed that as we passed our original imprinted transgene, *TG.A* (Swain et al. 1987; Chaillet et al. 1991), into the C57Bl/6J inbred strain, after eight generations, the paternally inherited transgene acquired the heavily methylated pattern normally associated with the maternally inherited allele (data not shown). These findings suggested that the paternal allele might be silenced in the inbred C57Bl/6J background.

To examine this possibility, we directly created a number of transgenic lines in an inbred C57Bl/6J back-

Figure 4. Localization of *cis*-acting imprinting signal. Southern blots of *HpaII* (lanes 1–4,7–12) or *BstUI* (lanes 5,6) digests showing methylation patterns of the parental alleles of transgenic lines *TG.LU* (created with construct E, lanes 1,2), *TG.MI* (created with construct F, lanes 3,4), *TG.OW* (created with construct G, lanes 5,6), *TG.QI* (created with construct H, lanes 7,8), *TG.QE* (created with construct I, lanes 9,10), and *TG.OD* (created with construct J, lanes 11,12). All DNA samples were obtained from tails of adult mice and hybridized with either Ca (lanes 1–10) or RSV (lanes 11,12).

Figure 5. Biallelic expression of the endogenous *IgA* gene. Total spleen RNA was isolated from adult individuals and assayed for *IgA* expression using a subcloned mouse *IgA* genomic DNA fragment. The species-specific protected antisense fragments correspond to differences within the 3' UTR detected in a ribonuclease protection assay. (Lanes 1,2) Uniquely sized protected bands corresponding to *M. spretus* or C57Bl/6J alleles. (Lanes 3,4) Biallelic *IgA* expression in interspecific crosses made between these species. (Lane 3) An F₁ hybrid between a C57Bl/6J female and a *M. spretus* male; (lane 4) a reciprocal cross to that shown in lane 3 such that the *M. spretus* allele is now maternally inherited. This F₂ animal was created by mating an F₁ female to a C57Bl/6J male.

ground by using fertilized C57Bl/6J oocytes as targets for injection of the *RSVlgmyc* construct. One of these lines, *TG.NL*, demonstrated a complete loss of paternal allele expression that was associated with a high level of DNA methylation (Fig. 6A,B, lanes 1), a behavior that was identical to that of the maternal allele (Fig. 6A,B, lanes 2). If this aberrant paternal behavior is determined by modifier alleles present (or absent) in the C57Bl/6J genome, movement of the transgene to the FVB/N background might restore the imprinted state. Accordingly, a wild-type FVB/N female was bred to a *TG.NL* (C57Bl/6J) male to produce F₁ offspring that inherit the transgene from their C57Bl/6J father. As shown in Figure 6, A and B, lanes 3, the paternally inherited transgene is transcriptionally active in the F₁ background and the transgene displays the characteristic undermethylated pattern associated with an imprinted paternal allele. Alternatively, in F₁ offspring created by crossing a *TG.NL* (C57Bl/6J) female with a wild-type FVB/N male, the maternal transgene remains silent and displays the overmethylated pattern of an imprinted maternal allele (Fig. 6A,B, lanes 4). These results indicate that the presence of the FVB/N haplotype exerts a dominant effect that restores the activating imprint. Moreover, because in one cross the "silent" transgene is inherited from the C57Bl/6J father and activated by modifying alleles provided in trans from the FVB/N mother, these factors must have established the paternal imprint by acting after fertilization. Because our earlier results established that the maternal overmethylated state is acquired during oogenesis and is maintained thereafter (Chaillet et al. 1991), the

current results indicate that paternal imprinting occurs later, after zygote formation, and requires *trans*-acting factors, in this case, provided by the FVB/N genome.

Paternal methylation pattern is acquired at a postblastocyst stage

Because the paternal imprint is determined by events that take place after fertilization, we can begin to ask when during embryonic development this occurs. Formally, this question can only be answered in terms of transgene methylation as the expression of the transgene is limited to cardiac tissue and appears only after this organ forms (Swain et al. 1987; Jackson et al. 1990). Two observations indicate that paternal methylation occurs after the blastocyst stage of embryogenesis. First, previous developmental studies in FVB/N mice indicated that the paternal transgene is unmethylated in the blastocyst (Chaillet et al. 1991). The paternal transgene is also unmethylated in the C57Bl/6J blastocysts (Fig. 6B, lane 5), indicating that its adult methylation pattern is acquired after blastocyst formation. Second, founder FVB/N transgenic mice of the *TG.C*, *TG.D*, *TG.E*, and *TG.F* lines, created by injecting an unmethylated DNA construct into a fertilized oocyte, acquire adult methylation patterns identical to those characteristic of paternal imprinting (data not shown). Likewise, the transgenic founder (*TG.NL*) created in the C57Bl/6J background by injecting an unmethylated construct displays an overmethylated pattern (Fig. 6B, lane 6) that must have been acquired during embryogenesis. These findings are con-

Figure 6. Examination of the imprinting characteristics of transgenic lines created on a C57Bl/6J background. (A) Ribonuclease protection assay examining transgene expression in the *TG.NL* C57Bl/6J transgenic line (lanes 1,2) and F₁ hybrids between *TG.NL* and wild-type FVB/N mice (lanes 3,4). (Lane 3) An F₁ hybrid between a wild-type FVB/N female and a *TG.NL* male; (lane 4) an F₁ hybrid between a *TG.NL* female and a wild-type FVB/N male. (B) Southern blots of *HpaII* digests showing methylation patterns of the parental alleles of *TG.NL* mice (lanes 1,2,5,6,7) and F₁ hybrids between *TG.NL* and wild-type FVB/N mice (lanes 3,4). (Lane 3) An F₁ hybrid between a wild-type FVB/N female and a *TG.NL* male; (lane 4) an F₁ hybrid between a *TG.NL* female and a wild-type FVB/N male; (lane 5) blastocyst DNA carrying the paternal allele of *TG.NL*. Thirty-nine blastocysts were obtained from matings between a C57Bl/6J *TG.NL* male and wild-type C57Bl/6J females, and the DNA was extracted and digested with *HpaII* for this experiment. (Lane 6) The *TG.NL* founder transgenic mouse; (lane 7) day-7.5 *TG.NL* embryos containing a paternally derived transgene. Eight day-7.5 embryos from a mating between a *TG.NL* male and a wild-type C57Bl/6J female were pooled, and the DNA was extracted and digested with *HpaII* for this experiment. All DNA samples except that of lanes 5 and 7 were derived from tails of adult mice. The Southern blots were hybridized with the α probe.

sistent with a highly methylated transgene in the adult arising from an unmethylated transgene in C57Bl/6J blastocysts. Because day-7.5 C57Bl/6J embryos with a paternal *TG.NL* transgene exhibit the fully methylated pattern of the adult transgenic carrier (Fig. 6B, lane 7), the “adult” pattern must be acquired around the time of embryonic implantation. This timing coincides roughly with the onset of de novo methylation in the embryo (Monk et al. 1987).

Discussion

The cis-acting imprint signal

The fact that parental imprinting can be conveyed relatively independently of the genomic integration site by the *RSVlgmyc* transgene provides strong evidence that a *cis*-acting region within the transgene is necessary for its imprinted behavior. Using deletion analysis, we have systematically examined the transgene and identified within it a region that fulfills this requirement. The maximum size of the signal that is required to establish and/or maintain parental imprinting corresponds to our smallest loss-of-imprinting deletion, or 206 bp. It would be an oversimplification to assume that this region is sufficient to convey the imprint signal. Rather, it is subject to the influence of its immediate transgenic context and may require specific relationships to other sequences within the transgene. For example, we have shown that the endogenous *IgA* locus of the mouse is not imprinted, although it carries this same 206-bp region. Obviously, the endogenous region resides in a very different immediate sequence context than does the transgenic copy. Furthermore, constructs in which the *lacZ* gene is substituted for *c-myc* do not display a methylation or expression imprint (construct *K*, Fig. 2B; data not shown), suggesting an interaction between separate elements of a more complex and more complete imprinting signal.

A number of mutation-associated imprinting phenomena in humans also emphasize the importance of subtle sequence relationships in genomic imprinting. Balanced germ-line translocations of chromosome 11 in the Beckwith–Weidemann syndrome (BWS) are always maternally derived (Pueschel and Padre-Mendoza 1984; Tommerup et al. 1993). This suggests that BWS translocations disrupt a normal imprinting mechanism or induce an abnormal mechanism. A recent report (Haas et al. 1992) suggests that the Philadelphia chromosome in patients with chronic myelogenous leukemia (CML) is derived from a balanced translocation of paternal chromosome 9 and maternal chromosome 22, creating a fusion of the *bcr* and *abl* genes (Shtivelman et al. 1985; Grosfeld et al. 1986). *bcr* and *abl* are not likely to be imprinted in their germ-line configuration (Schwartzberg et al. 1991; Tybulewicz et al. 1991; Reik 1992; Melo et al. 1994; Riggins et al. 1994). Therefore, the somatically derived Philadelphia chromosomes in CML, if imprinted, must utilize “cryptic” germ-line imprint signals near *bcr* and/or *abl* to either direct the translocation or to select for an imprinted translocation in leukemic cells.

Interestingly, the region required for imprinting corresponds to the 3' border of the *IgA* switch recombination signal and contains the repeated primordial pentamers [CTG(A/G)G] and related sequences that characterize this region. Repetitive DNA sequences are a recurrent theme among imprinted genes, and such sequences are associated with regions of allelic differences in DNA methylation (Neumann et al. 1995). For example, a 700-bp cluster of direct repeats (with a 27-bp unit homology) are present in the mouse *Igf2* gene, just upstream of the region where methylation differences between the parental alleles are apparent (Sasaki et al. 1992). Moreover, the differentially methylated region 2 of the imprinted *Igf2/mpr* gene (Stoger et al. 1993) also contains internally repeated DNA sequences. Taken together with the fact that repeated sequences can be regions of genetic instability and can influence chromosomal structure and, hence, gene expression [as in position effect variegation exhibited by arrays of repeated transgenes in *Drosophila* (Dorer and Henikoff 1994)], it is possible that repeated sequences in the appropriate context are a generic feature of imprint signals.

Finally, there is one example that draws together the joint themes of repeated sequences, recombination, and sensitivity to subtle alterations of gene structure. There is a strong parental inheritance preference in the fragile X syndrome (Laird 1987), a disorder associated with an expansion of a CGG trinucleotide repeat (Fu et al. 1991; Verkerk et al. 1991). At least an element of this preference may involve the induction of a maternal germ-line imprint. Such an imprint may in turn stimulate a series of postzygotic effects exemplified by trinucleotide repeat expansion, DNA methylation, and gene inactivation (Fu et al. 1991; Verkerk et al. 1991; Reyniers et al. 1993; Wohrle et al. 1993). In view of the foregoing, it is possible that potentially imprintable sequences are abundant and that their properties are only realized when the appropriate sequence context has been created by subtle somatic recombinatorial events.

Trans-acting control and timing of the imprinting mark

We have already noted that *trans*-acting factors must be involved in creating an imprinted transgenic locus. Genetic evidence for this emerges from experiments in which we have shown that the imprinted properties of a transgene are lost in one genetic background (C57Bl/6J) but can be restored by breeding the transgene into another genetic background (FVB/N). Furthermore, because the imprint is restored in an F₁ hybrid formed between permissive and nonpermissive strains, we know that the responsible genetic modifiers act in a dominant fashion. Moreover, because the transgene can be donated by a male parent of the nonpermissive strain to be activated in an F₁ hybrid formed between that male and a female from the permissive strain, we conclude that the dominant modifier genes must act after fertilization.

The time and the molecular basis for marking the imprinted allele can be detailed further. Using methylation as our imprint marker, earlier results established that

the (maternal) overmethylated state is acquired during oogenesis and is maintained thereafter (Chaillet et al. 1991). Our current results indicate that in contrast, the paternal allele does not acquire its distinctive methylation pattern until sometime after blastocyst formation but before day 7.5 of embryonic development. Furthermore, the distinctive paternal imprint occurs only in the presence of the products of the modifying genes supplied by the FVB/N genome. In its simplest form, this product would be a *trans*-acting factor supplied by the FVB/N genome that prevents hypermethylation of the paternal allele at the time of extensive embryonic de novo methylation (Monk et al. 1987).

It is worth mentioning that three other transgenic lines created directly in a C57Bl/6J background displayed a somewhat weaker, variably imprinted phenotype (data not shown). Their paternal alleles were transcriptionally active at a level less than that observed in FVB/N, and their transgenes displayed a weakly maternal pattern of methylation. When these C57Bl/6J transgenic strains were bred with FVB/N, the F_1 offspring displayed the strong methylation and expression phenotype characteristic of the FVB/N background. Variation in imprinting behavior in the C57Bl/6J background suggests that factors in addition to the strain-related modifying genes influence the extent of paternal de novo methylation during embryogenesis. It is possible that this property is influenced by the site or manner of genomic integration.

Endogenous imprinted loci also exhibit variability in genomic imprinting. For example, only the paternal allele of the *Igf2* gene is normally active. However, in selected CNS regions (DeChiara et al. 1991) and certain tumors of somatic origin (Ogawa et al. 1993; Rainer et al. 1993), both parental alleles of *Igf2* are active and thus not imprinted. It is not difficult to imagine that this variability in *Igf2* imprinting could develop in an analogous fashion to the methylation of both alleles of the *RSVlgmyc* transgene. The normally active paternal *Igf2* allele is more methylated than the maternal allele. If the relatively poorly methylated maternal allele gains methylation in a directed (CNS) or sporadic (tumor) fashion following fertilization, it will become activated and result in biallelic *Igf2* expression.

The role of methylation

Virtually every discussion of imprinting includes a consideration of the role that DNA methylation might play in establishing an imprinted pattern of gene expression. Early consideration of the *RSVlgmyc* transgene pointed out the clear correlation between the imprinted methylation and expression patterns (Swain et al. 1987; Chaillet et al. 1991). In support of this, strong correlations have also been observed between the levels of DNA methylation and transcription for endogenous imprinted genes (Sasaki et al. 1992; Bartolomei et al. 1993; Ferguson-Smith et al. 1993; Hatada et al. 1993; Stoger et al. 1993; Hayashizaki et al. 1994). The strongest evidence thus far

comes from the observation that expression imprinting of three of these genes (*Igf2*, *Igf2/mpr*, and *H19*) is abolished on a cytosine methyltransferase-deficient mouse background, either because the normally silent allele is activated (*H19*) or because the normally active allele is silenced (*Igf2* and *Igf2/mpr*) (Li et al. 1993).

The events we have discussed in terms of our imprinted transgene can be considered in the light of a model in which DNA methylation directly mediates one or more steps in the genomic imprinting process. The presence of different methylation patterns on the transgene in the mature male and female gametes establishes a chemical basis that allows one allele to be distinguished from the other. The highly methylated pattern of the oocyte-derived allele is propagated unchanged through subsequent stages of development. Because the paternal transgene is not highly methylated during spermatogenesis, it is unable to maintain its methylation through preimplantation embryogenesis. Subsequently, during postblastocyst embryogenesis, possibly using the system of methylation utilized by other imprinted genes (Li et al. 1993), the paternal allele acquires either a low level (FVB/N background) or a high level (C57Bl/6J background) of methylation. In such a methylation-dependent model, the maternal allele is silenced as a result of the highly methylated pattern it has acquired from the oocyte. The activity of the paternal allele, on the other hand, is regulated by the amount of embryonic de novo methylation acquired during the postblastocyst stage of embryogenesis, which depends on the *trans*-acting factors that are present.

Materials and methods

Transgene DNA constructs

DNA fragments injected into mouse zygotes for the creation of transgenic mice were either the original *RSVlgmyc* plasmid construct (Swain et al. 1987) or modified versions. Constructs containing pBR322 sequences were linearized prior to injection: for constructs A, B, and D at the unique *KpnI* site; for construct E at the *BamHI* site of the circularized plasmid; for construct F at the *XbaI* site; and for construct I at the *EcoRI* site. Constructs with pBR322 sequences deleted (constructs C and G) were constructed by placing plasmid vector sequences (pKS+, Strata-gene) into the unique *KpnI* site of *RSVlgmyc* (Fig. 2). These vector sequences were removed by gel isolation prior to injection of the transgene fragment.

Constructs with partial deletions of $C\alpha$ (constructs H and I) were constructed using PCR and plasmid cloning techniques. The appropriate portion of $C\alpha$ was constructed as an *EcoRI*–*Sall* PCR fragment to replace the entire $C\alpha$ region of construct F. The *XbaI*–*EcoRI* 3' *myc* fragment was constructed as a *Sall*–*EcoRI* PCR fragment. The two PCR fragments were then cloned into the pBR/RSV *EcoRI* vector, and the resultant plasmid was linearized with *Sall* prior to injection into fertilized oocytes.

For construct K, the bacterial *lacZ* gene has been substituted for *c-myc*, leaving 19 bp of 5' *c-myc* and 186 bp of 3' *c-myc* (defined by *MspI* and *BglII* sites near the 5' and 3' ends of the *c-myc* sequences, respectively). The *lacZ* gene contains the SV40 nuclear transport signal, as well as a polyadenylation signal.

Animals

Transgenic mice were created either in an inbred FVB/N or in an inbred C57Bl/6J genetic background. Founder transgenic mice were made by injection of the pronucleus of a fertilized egg (Palmiter and Brinster 1986). Transgenic lines were established from these founders, and the imprinting characteristics of the line were evaluated by passage through the maternal and paternal germ lines.

Tissue and cell collection

Transgene methylation was analyzed in blastocysts collected on day 3.5, postimplantation embryos on day 7.5 and adult tails. Blastocysts were collected from natural pregnancies on day 3.5 (day 0.5 is defined as the day that a vaginal plug was identified) by flushing the uterine horns and collecting the blastocysts free of contaminating maternal tissue (Hogan et al. 1986). Embryos from day 7.5 were collected by removal of the surrounding decidua and extraembryonic membranes.

DNA analysis

DNA from adult tails and embryos was isolated by ethanol precipitation after digestion of the tissue with proteinase K (Boehringer Mannheim, Indianapolis, IN), followed by phenol-chloroform extraction. DNA was digested at the appropriate temperature with a 10-fold excess (U/mg) of restriction endonuclease, electrophoresed on 1% agarose, and transferred to nylon filters (GeneScreen, DuPont) by the method of Southern (1975). The filters were hybridized at 42°C in 40% formamide and washed in $0.1 \times$ SSC, 0.1% SDS, at 65°C. The filters were hybridized with the probe C α , which is the 1756-bp *EcoRI*–*XbaI* fragment of C α . In constructs in which C α was deleted, the 440-bp RSV fragment was used.

RNA analysis

Heart RNA was isolated by homogenizing tissue in a Polytron homogenizer (Brinkman Instruments, Westbury, NY) using a buffer containing 4 M guanidine isothiocyanate, 2.5 mM sodium citrate (pH 7), and 0.1 M β -mercaptoethanol. Total RNA was isolated by the method of Chirgwin et al. (1979) by centrifugation through a 5.7 M cesium chloride, 25 mM sodium acetate cushion. RNA was analyzed for transgene expression by ribonuclease protection assay (Melton et al. 1984). The probe used was a 365-bp *NotI*–*PstI* fragment of the mouse *c-myc* cDNA that includes the 3' portion of exon 1 and the 5' portion of exon 2. Spleen RNA was isolated and analyzed for *IgA* expression by ribonuclease protection assay (see above). The probe used to detect biallelic *IgA* expression was a subcloned mouse *IgA* genomic DNA fragment that includes the 3' portion of the *IgA* gene. This fragment is an ~900-bp *EcoRI*–*HindIII* restriction fragment that was cloned into pKS+ (Stratagene) and linearized with *ScaI*.

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J R Chaillet, D S Bader and P Leder

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