Investigation of Elements Sufficient To Imprint the Mouse *Air* Promoter

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Imprinted maternal-allele-specific expression of the mouse insulin-like growth-factor type 2 receptor (*Igf2r*) gene depends on a 3.7-kb element named region 2, located in the second intron of the gene. Region 2 carries a maternal-allele-specific methylation imprint and contains an imprinted CpG island promoter (*Air*) that expresses a noncoding antisense RNA from the paternal inherited allele only. Here, we use transgenes to test the minimal requirements for imprinting of *Air* and to test if the action of region 2 is restricted to *Igf2r*. Transgenes up to 9 kb with *Air* as a single promoter are expressed but not imprinted. When coupled to the *Igf2r* CpG island promoter on a 44-kb transgene, *Air* was imprinted in one of three lines. However, *Air* on a 4.6-kb fragment is also imprinted in 2 of 14 lines when inserted in an intron of an adenine phosphoribosyltransferase (*Aprt*) transgene, and in one line, the imprinted methylation and expression of *Air* have been transferred onto the *Aprt* CpG island promoter. These data suggest that a dual CpG island promoter setting may facilitate *Air* imprinting as a short transgene and also show that *Air* can transfer imprinting onto other genes. However, for reliable *Air* imprinting, elements are necessary that are located outside a 44-kb region spanning the *Air*-*Igf2r* promoters.

Within the mammalian genome, more than 40 imprinted genes have been identified (for an up-to-date list, see www.mgu.har.mrc.ac.uk/imprinting/). Imprinted genes show parental-allele-specific expression such that only one of the two parental copies present in a diploid cell is expressed. The hallmark of an imprinted gene is the presence of an imprint that ultimately allows the cellular transcription machinery to discriminate between the two parental alleles. Because imprinting can occur in inbred mice with genetically identical parental alleles, imprints cannot be acquired in diploid cells but instead must be set when the parental alleles are in physically separate compartments, during germ cell development or before nuclear fusion in the zygote. In addition, the imprint must be epigenetic, stably inherited through mitosis in embryonic development, and reset upon germ line formation (for a review, see references 7, 28, and 35). Several possibilities exist for an imprint mark, such as chromatin composition, organization, and histone acetylation or methylation state (21, 24). However, DNA methylation is by far the best candidate as it fulfills all the desired criteria; in particular, de novo methylation patterns that are differently acquired by the male and female gamete could function as the imprinting mark. Support for a role of DNA methylation comes from the demonstration that mice lacking a functional maintenance methyltransferase gene (*Dnmt1*) show a loss of imprinted expression, observed as either biallelic silencing or biallelic expression (13, 16, 25, 26). All tested imprinted genes have been associated with allelic methylation differences in somatic tissue, and some also show gamete-specific methylation (26, 31, 36). Finally, for three imprinted genes, deletion of the sequences carrying the methyl-

ation imprint resulted in loss of imprinting, thereby identifying these methylated sequences as imprint control elements (8, 29, 34, 39, 40). Methylation in the mammalian genome is present on CpG dinucleotides, and DNA elements that carry a methylation imprint can be defined as CpG islands, since they contain clustered CpGs and are associated with active transcription. However, methylated CpG island promoters are unusual, as the majority of CpG island promoters are unmethylated regardless of expression state (3).

The reason why imprinted CpG islands attract a methylation imprint during gametogenesis has not yet been resolved, but two models have been proposed. First, specialized sequences are proposed to exist within the imprinted CpG island that either prevent or attract de novo methylation activity in one gamete. An allele discrimination signal (ADS) has been proposed to exist in the insulin-like growth factor type 2 receptor gene (*Igf2r*) imprinted intron 2 CpG island that attracts gamete-specific shielding factors, thereby protecting a nearby de novo methylation signal (DNS) from methylation. In this model, the primary allele discrimination is not de novo methylation in the oocyte, but the shielding that prevents methylation in spermatozoa. De novo methylation can subsequently occur later in gametogenesis or even after fertilization (9). Second, an alternative sequence-independent model proposed that the presence of direct repeats that have also been found in several imprinted CpG islands attracts methylation in the gamete as a response to unusual secondary structure formation. The ability of methylation to target these repeats in imprinted CpG islands was suggested to have evolved from a host defense response to suppress genomic parasitic DNA elements (5, 20, 38).

In order to identify genes involved in the imprint methylation pathway, the DNA sequences necessary and sufficient to attract and maintain the methylation imprint need to be characterized. Targeted deletions from the endogenous locus can

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FIG. 1. The maternal and paternal endogenous (wt) *Air-Igf2r* loci are represented by the first two lines. The *Air* and *Igf2r* promoters are CpG island promoters (ellipses), and the arrows indicate the transcription starts. The methylated CpG islands are shown as stippled ellipses. Region 2 is presented as a line with the ADS-DNS motif depicted with an asterisk. The *Igf2r* exons are shown as numbered black boxes. Transgenic constructs REP, LAI, SAI, RAA, and FAA are derived from the endogenous *Air-Igf2r* locus. Construct REP contains a 9-kb *Air* fragment (including *Igf2r* exons 3 through 5) coupled to an EGFP reporter (stippled box), SV40 intron, and polyadenylation cassette (black triangle). The LAI construct spans 44 kb, from the *Igf2r* promoter up to *Igf2r* exon 7, and contains an EGFP-tagged (stippled box) *Igf2r* exon 1. 5' SV40 polyadenylation cassette and 3' rabbit β -globin polyadenylation cassettes are shown as black triangles. The small *Air-Igf2r* SAI construct is similar to LAI, but lacks *Igf2r* intron 1 and is shorter at the 3' end, lacking *Igf2r* exons 6 and 7. Constructs RAA and FAA contain a complete mouse *Aprt* gene with a rabbit β -globin polyadenylation cassette inserted at the 5' end (black triangles), the endogenous *Aprt* polyadenylation signal is used 3'. The *Aprt* exons are shown as open boxes. In construct RAA, the *Air* promoter is inserted in an antisense orientation with respect to the *Aprt* promoter, whereas in construct FAA the *Air* promoter is inserted in a sense orientation. The probes used for methylation analyses (LA, MC, MS, SB, and SE) and for expression analyses (SVA, GFPAIR, GFPIGF2R, MIMs1, PS, and DE) are shown. For details of the constructs and the probes, see Materials and Methods.

be used to identify essential elements necessary for methylation, but only transgenes can identify the minimal configuration sufficient for the imprint. Such a transgenic approach has been successful in the identification of the minimally imprinted configuration for the maternally expressed *H19* and paternally expressed insulin-like growth factor type 2 (*Igf2*) neighboring loci. Results have shown that large 130-kb transgenes that contain both the *H19* and *Igf2* genes as well as 140-kb transgenes that contain only the *H19* gene are reliably imprinted (1, 15). *H19* transgenes as small as 10 kb can be imprinted, but mostly as multicopy transgenes and with less than 100% frequency (12, 14, 23). These results indicate that an imprinted *H19* transgene demands multiple elements in addition to the sequences that carry the methylation imprint, the *H19* promoter, and the downstream situated enhancers. The G-rich repetitive element located upstream of the *H19* promoter is, however, dispensable (12, 14, 23, 30). A transgenic approach

has also been applied to the paternally expressed *Snrpn* gene, and results show that 75- to 85-kb multicopy transgenes containing the entire *Snrpn* gene and flanking DNA can be imprinted (10, 27). A minimal imprinted configuration has also been delineated on multicopy transgenes as a dual element of 1.2 kb composed of a 200-bp minimal *Snrpn* promoter coupled to a 1.0-kb human imprint regulatory element located 35 kb upstream of the human *SNRPN* promoter (27).

The maternally expressed *Igf2r* gene differs from the above imprinted genes, since it contains, in intron 2, a promoter generating a paternally expressed imprinted antisense RNA (*Air*) (17) that overlaps the *Igf2r* promoter (see Fig. 1 for an overview of the imprinted *Air-Igf2r* locus). This *Air* RNA is an unusual genomic transcript that is noncoding with no or few introns. An imprinted configuration for *Igf2r* has been identified on 300-kb transgenes that contain the whole 87-kb *Igf2r* gene and the 108-kb *Air* RNA plus additional flanking genes.

Igf2r contains a methylation imprint inherited from the oocyte on a 3.7-kb fragment known as region 2 that is located within intron 2 (31). Region 2 contains direct repeats (20), an ADS-DNS motif (9), and the CpG island *Air* promoter (17). Region 2 is necessary for *Igf2r* imprinting in a transgene situation, as deletion results in *Igf2r* expression from both parental alleles (39), and is also necessary for imprinting the endogenous locus (40). In contrast, region 2 is not sufficient for *Air* imprinting, because short multicopy *Air* transgenes that contain all or part of region 2 are not imprinted by methylation (39). Imprinting of small *Air* transgenes might therefore demand multiple elements, similar to *H19* and *Snrpn* transgenes.

Here, we have tested whether stable *Air* expression and/or the presence of the *Igf2r* promoter and *Igf2r* expression are sufficient for imprinting of small *Air* transgenes. Our results show that in contrast to multicopy *H19* transgenes, multicopy *Air*-expressing transgenes are not imprinted. However, the *Air* promoter can, albeit at a low frequency, recapitulate imprinting on a fragment as small as 4.6 kb when embedded in an adenine phosphoribosyltransferase (*Aprt*) transgene and can, in one instance, transfer imprinting onto the *Aprt* gene. Interestingly, including the *Igf2r* CpG island promoter and all flanking sequences up to the *Air* promoter does not increase the imprinting frequency for *Air* above that seen in combination with the *Aprt* CpG island promoter. Thus, this study indicates that a dual-promoter setting may facilitate *Air* imprinting and that elements for reliable *Air* imprinting are contained outside a 44-kb region spanning the *Air*-*Igf2r* promoters but inside the previously defined 300-kb region.

MATERIALS AND METHODS

DNA constructs. (i) **REP.** Construct REP (for reporter) consists of a 9.1-kb *SpeI* fragment (nucleotide bp 120364 to 132177, GenBank accession number AJ249895) containing region 2 isolated from cosmid 940PS (31) ligated into the *BglII* site of reporter plasmid pEGFP-1 (Clontech). The pEGFP-1 reporter has two modifications. An internal ribosome entry site sequence was introduced into the *SmaI* site, and a simian virus 40 (SV40) intron obtained as a 179-bp *XhoI*/*NotI* fragment from plasmid pCMV β (Clontech) was inserted into the *NotI* site of pEGFP-1. The 10.8-kb construct was excised from the vector by *SpeI*/*SspI* digestion.

(ii) **LAI.** Construct LAI (for large *Air*-*Igf2r* construct) was built in a modified pBeloBAC-II vector (Research Genetics) that allowed excision of the final 44-kb LAI construct with *RsrII*/*BsiWI* digestion. LAI consists of a 40-kb *NotI* fragment containing region 2 (bp 98069 to 138490; AJ249895) from cosmid OT1 (17) ligated to a 4.0-kb *EcoRI*/*NotI* fragment (bp 94101 to 98069; AJ249895) containing the *Igf2r* promoter isolated from cosmid 3L (31). A 59-bp *AgeI*/*NotI* fragment (bp 98010 to 98069; AJ249895) from *Igf2r* exon 1 has been replaced in frame by a 735-bp *AgeI*/*NotI* fragment of reporter pEGFP-1 (Clontech). In a *BglII* site (bp 94139; AJ249895) upstream of the *Igf2r* promoter, a 198-bp *BamHI* fragment from pCMV β (Clontech) containing the SV40 polyadenylation signal was introduced in an antisense orientation to terminate the *Air* transcript. The 3' end of the construct contains a 1-kb *HincII* fragment from the rabbit β -globin gene, which includes part of intron 2, and complete exon 3 with its polyadenylation signal to terminate the *Igf2r* transcript.

(iii) **SAI.** Construct SAI (for small *Air*-*Igf2r* construct) was built in the modified pBeloBAC-II vector and excised as a 20-kb *RsrII*/*BsiWI* fragment. It consists of the H/X-14 construct (39), which is a *HindII*/*XhoI* fragment containing region 2 (bp 133005 to 118712; AJ249895), and is ligated to the same enhanced green fluorescent protein (EGFP)-tagged *EcoRI*/*NotI* *Igf2r* promoter fragment that was used in construct LAI. The 5' and 3' polyadenylation cassettes were identical to that in the LAI construct.

(iv) **RAA and FAA.** Constructs RAA (for reverse *Air*-*Aprt*) and FAA (for forward *Air*-*Aprt*) contain a 5-kb *NcoI* fragment with the entire mouse *Aprt* gene (a gift from Harry Vrieling, Leiden University). A 1.2-kb *EcoRI* fragment from rabbit β -globin, including part of exon 2, complete intron 2, and exon 3 containing a polyadenylation signal, was inserted in an antisense orientation into the

EcoRV site (bp 108; M11310) upstream of the *Aprt* promoter. A 4.6-kb *SpeI*/*BglII* fragment (bp 120364 to 127575; AJ249895) from cosmid 940PS containing region 2 was inserted into the *HindIII* site (bp 1694; M11310) of *Aprt* intron 2 in an antisense (in construct RAA) or sense (in construct FAA) orientation with respect to the *Aprt* promoter. Both constructs were released from the pBluescript II vector (Stratagene) by *Bss*HII/*NotI* digestion.

Transgenic mice. Transgenic mice were generated by injecting DNA into FVB/N-fertilized oocytes by approved procedures (2). Approximately 300 oocytes injected with the REP construct generated 11 founder transgenes, 550 oocytes injected with the LAI construct generated 3 founder transgenes, 450 oocytes injected with the SAI construct generated 2 founder transgenes, 300 oocytes injected with the RAA construct generated 5 founder transgenes, and 200 oocytes injected with the FAA construct generated 9 founder transgenes. Transgenes were bred hemizygotously onto an FVB/N background and identified by Southern blotting or PCR on genomic tail DNA.

DNA and methylation analyses. Genomic DNA was isolated by the sodium dodecyl sulfate-proteinase K procedure (2). The following oligonucleotides were used: for typing construct REP, GFPF (5'-CTGGTGAACCGCATCGAGC TGAA-3') and GPFAR (5'-ACCTCTACAAATGTGGTATGGCTG-3'); for constructs LAI and SAI, EFPF (5'-GTAAACGCCACAAGTTCAGC-3') and EFP2R (5'-GGTGTCTCTGCTGGTAGTGTC-3'); for construct FAA, APRTX2F (5'-TATCTCGCCCTCTTGAAAGACC-3') and M404F (5'-GT GACTCACTTTTGAGAAC-3'); for construct RAA, APRTX3R (5'-CCATA CTCAGAGAATAGGAGGC-3') and M404F. Methylation analysis probes (see also Fig. 1) were as follows: for SB, a 723-bp *HindIII*/*BglII* fragment (bp 971 to 1694; M11310) from the *Aprt* gene; for SE, a 700-bp *EcoRV*/*SmaI* fragment (bp 108 to 808; M11310) upstream from the *Aprt* promoter; for LA, a 984-bp *SacI*/*AgeI* fragment (bp 97026 to 98010; AJ249895) from the *Igf2r* promoter; for MS, *SfuI*/*MluI* (bp 124992 to 126086; AJ249895); for MC, *MluI*/*BglII* (bp 126086 to 127575; AJ249895). The digestion of methylation-sensitive enzymes was regularly monitored by hybridization to mitochondrial DNA as previously described (38). Methylation was quantified with a PhosphorImager (Fujix) and expressed as a percentage in multiples of 10. Transgene copy numbers were determined by comparing the transgene to the endogenous signal in a Southern blot with a PhosphorImager (Fujix). Single-copy lines were confirmed by hybridizing with an end fragment from the transgene.

RNA analyses. Total RNA was isolated with Tri Reagent (Molecular Research Center) according to the manufacturer's protocol. For the RNase protection assay (RPA) the RPAIII kit (Ambion) was used, and for the Northern blot assay the formaldehyde method was used (2). Expression analysis probes were as follows: SVA, a 350-bp *HpaI* fragment from construct REP, protects a 240-bp fragment from the SV40 polyadenylation signal; A3, a 252-bp *XhoI*/*XbaI* fragment (bp 2165 to 2417; M11310) protects *Aprt* exon 3 (134 bp); GFPF2R and GPFAR, templates made by PCR with T3IGGFP (5'-AATTAACCTCCTCACTA AAGGGAAAACCTTGTCGCCGTTTAC-3') and T7IGEX1 (5'-TAATACGAC TCATATAGGGAGTCACGGAGCGCCTCTC-3') on construct LAI (281 bp); GPFAR detects endogenous *Air* (185 bp) and transgenic *Air* (275 bp). GFPF2R detects endogenous (188, 163, 143, and 132 bp) and transgenic (278, 253, 233, and 222 bp) *Igf2r* RNA; MIMs1 is a *MluI*/*MseI* fragment (bp 126086 to 126293; AJ249895) (17) that detects unspliced (207, 171, and 148 bp) or spliced (112, 76, and 53 bp) *Air* RNA; *Gapdh*, templates generated by PCR with GAP-DHF (5'-CGGGGTACACAGCGCATCTTCTTGTGCAG-3') and GAP-DHR (5'-CGTCTAGATGGGTGGTCCAGGGTTTCTTAC-3'); PS, a 1.5-kb *EcoRI*/*HindIII* fragment (bp 1 to 1694; M11310) containing *Aprt* exons 1 and 2 (19); DE, a 1.3-kb *HindIII* fragment (bp 1694 to 3066; M11310) containing *Aprt* exons 3 to 5 (19).

RESULTS

Single-promoter *Air* transgenes are expressed but not imprinted. Previously, it has been shown that short transgenes from 1 to 14 kb containing the intronic CpG island known as region 2 (see Fig. 1) do not carry a methylation imprint (39). These transgenes were not characterized for expression and may have lacked sequences needed for *Air* promoter activity and termination of the *Air* transcript. To test whether imprinting of *Air* requires stable *Air* transcription, we generated the REP construct that contains 9 kb, including region 2, coupled to an EGFP reporter, as described in Materials and Methods (Fig. 1). Five REP transgenic lines were generated and ana-

TABLE 1. Imprinting status of *Air* transgenes^a

Construct	Line	Copy no.	<i>Air</i>				<i>Aprt-Igf2r</i>			
			Methylation		Expression		Methylation		Expression	
			p	m	p	m	p	m	p	m
REP	26	2	0	0	+	+				
	27	2	0	0	+	+				
	30	10–15	50	50	+	+				
	65	4	0	0	+	+				
	90	2	0	0	+	+				
	13	2	30	ND	ND	ND				
	24	3	ND	0	ND	ND				
	36	10–15	40	ND	ND	ND				
	77	3	ND	40	ND	ND				
	78	3	20	ND	ND	ND				
	87	5	30	ND	ND	ND				
LAI	46	1	0	100	+	–	100	70	–	+
	48	2	0	0	+	+	80	80	+	+
	313	>30	0	0	+	+	80	80	+	+
SAI	112	2	0	0	+	+	100	100	–	–
	119	2	0	0	+	+	50	50	+	+
RAA	14	1	0	100	+	–	0	0	+	+
	21	2	0	0	+	+	0	0	+	+
	27	10–15	40	40	+	+	ND	ND	ND	ND
	30	2	0	0	+	+	ND	ND	ND	ND
	58	10–15	50	50	+	+	ND	ND	ND	ND
FAA	3	3	0	0	+	+	ND	ND	ND	ND
	4	1	0	100	+	–	0	100	+	–
	8	2	0	0	+	+	ND	ND	ND	ND
	21	1	0	0	+	+	ND	ND	ND	ND
	26	3	10	10	+	+	ND	ND	ND	ND
	30	4	20	20	+	+	ND	ND	ND	ND
	37	1	0	0	+	+	0	0	ND	ND
	57	2	50	50	+	+	ND	ND	ND	ND
	67	2	0	0	+	+	ND	ND	ND	ND

^a The methylation and expression status of the *Air* promoter on all transgenes is shown. Methylation and expression of either the *Igf2r* promoter (in LAI and SAI constructs) or the *Aprt* promoter (in RAA and FAA constructs) were determined only for transgenes linked to an imprinted *Air* promoter and for some representative transgenes linked to a nonimprinted *Air* promoter. Methylation is given as a percentage. The data for imprinted transgenes generated in this study are shown in boldface. Construct, abbreviated transgene name, as described in Materials and Methods; line, the number assigned to the independent integration sites of each transgene; p and m, paternal and maternal expression of the transgene, respectively; ND, not determined.

lyzed for *Air* imprinting upon hemizygous transmission through the paternal and maternal germ lines.

Methylation of the *Air* promoter was analyzed using the methylation-sensitive restriction enzymes on genomic DNA from adult tail and spleen and embryo 13.5 days postconception (dpc). These enzymes can only digest unmethylated DNA and are consequently informative for the methylation status of the recognition site. For all transgenes, we used the methylation-sensitive enzymes *Mlu*I and *Sfu*I that cut within the core of the *Air* CpG island. The methylation status of these restriction sites is representative for the whole CpG island and identical for tail, spleen, and embryo DNA (references 31 and 39 and data not shown). Imprinted methylation was absent in all REP lines (Table 1). Four lines had no methylation on the *Air* promoter following transmission from either parent; one line, REP-30, showed 50% methylation that was unchanged following maternal and paternal transmission (Fig. 2A; Table 1).

Transgenic *Air* expression was then analyzed in cardiac and 13.5-dpc embryo tissue, as these samples have a high level of endogenous *Air* expression (17, 39). All the REP transgenes

expressed high levels of *Air* (data not shown), thus confirming that sequences needed for *Air* promoter activity are contained on the 9-kb fragment. When analyzing the REP lines for *Air* expression, we found that in contrast to the endogenous locus, the transgenic *Air* RNA is spliced. A reverse transcription (RT)-PCR product was obtained using primers present in the polyadenylation cassette of the EGFP reporter and the mapped *Air* transcription start. The sequence of the RT-PCR product identified the splice donor GAACTGAG-GTAAGC located 53 bp downstream of the major transcription start for the *Air* RNA (17). The acceptor (GTCCCGGA) is part of an SV40 intron, located between the EGFP reporter and the polyadenylation cassette. An RPA with a transgene-specific probe (SVA) from the polyadenylation cassette showed an absence of imprinted expression for the transgenic *Air* RNA, as all five REP lines have equal expression levels for both parental transmissions (Fig. 2B; Table 1). An additional six REP lines were generated and analyzed for methylation differences by comparing the founder to its progeny with the *Mlu*I and *Sfu*I sites in the *Air* CpG island promoter. *Air* methylation was

FIG. 2. Imprinting analyses of transgenic lines REP-65 and REP-30. (A) Methylation analyses of the *Air* promoter. A Southern blot of tail genomic DNA digested with *Sac*I (– lanes) or *Sac*I and *Mlu*I (+ lanes) and hybridized with probe MS detecting endogenous (wt) and transgenic (tg) bands (sizes in kilobases are indicated) is shown. (B) Expression analyses of the transgenic *Air* promoter. An RPA was carried out of adult cardiac tissue total RNA with the transgene-specific probe (SVA) from the polyadenylation cassette in combination with an *Aprt* exon 3 probe (A3) as loading control. Lanes: i, input probes; c, tRNA hybridization to the probes; p and m, paternal and maternal transmission of the transgene, respectively; wt, a nontransgenic sample.

unchanged in offspring, following germ line transmission from four male founders and two female founders, similar to all other REP lines and nonimprinted lines (Table 1 and data not shown). Based on the absence of a methylation change upon germ line transmission, these lines were excluded from detailed expression analyses. Thus, a total of 11 REP lines lack imprinted characteristics for *Air*.

Low-frequency imprinting of *Air-Igf2r* transgenes. Previously, it has been shown that large, 300-kb transgenes that contain both the *Air* and *Igf2r* promoters were successfully imprinted in three of three sites when integrated into autosomes (39). During the same experiment, a related transgene with no *Igf2r* expression was inadvertently derived that failed to be imprinted. This result, combined with the failure of imprinting of small *Air* transgenes, suggested the possibility that normal *Igf2r* expression is a requirement for *Air* imprinting. To test if *Igf2r* expression is sufficient for imprinting of *Air* on short transgenes, we generated two differently sized constructs that contain both the *Air* and *Igf2r* promoters. The LAI construct is 44 kb and spans from 3.8 kb upstream of the *Igf2r* promoter up to *Igf2r* exon 7 and includes an EGFP-tagged *Igf2r* exon 1 (Fig. 1). The 20-kb SAI construct is similar to LAI but lacks the 20-kb *Igf2r* intron 1 and only extends up to *Igf2r* exon 5 (Fig. 1). Both constructs are flanked 5' and 3' by polyadenylation signals to terminate the transcripts from the *Air* and *Igf2r* promoters. Three LAI and two SAI transgenic lines were generated.

Methylation of the *Air* promoter was investigated using the methylation-sensitive restriction enzymes *Mlu*I and *Sfi*I. Of five lines, only line LAI-46 revealed a difference in methylation of *Air* on the 40-kb transgene-specific fragment (Fig. 3A; Table 1). The maternal transmission of LAI-46 has a 100% methylated *Air* promoter whereas the paternal transmission has no methylation. LAI-48, LAI-31, and the two SAI lines have an unmethylated or hypomethylated *Air* promoter that was un-

changed on maternal and paternal transmission (Fig. 3A; Table 1).

The LAI and SAI lines were then investigated for *Air* expression by RPA using a probe (GFP-*Air*) that spans part of the EGFP-tagged *Igf2r* exon 1. The endogenous *Air* RNA runs through the *Igf2r* promoter, and this probe can detect endogenous and transgenic *Air* RNA as differently sized fragments. Both parental transmissions of the LAI-313 and LAI-48 lines that lack a methylation imprint express equal levels of transgenic *Air* RNA (Fig. 3C; Table 1). However, for the LAI-46 line and the two SAI lines no *Air* RNA could be detected with the GFP-*Air* probe (Fig. 3C and data not shown). Based on the splicing of *Air* RNA that occurs in the REP lines, we investigated whether the *Air* RNA in the LAI and SAI lines is also spliced by using RPA with probe MIMs1 that covers the splice donor and the *Air* transcription starts (17). The two SAI lines have equal spliced *Air* expression upon both parental transmissions (Table 1). Line LAI-46 produces spliced *Air* RNA upon paternal transmission but no *Air* RNA upon maternal transmission (Fig. 3D). Thus, LAI-46 has imprinted *Air* expression. Splicing of the *Air* RNA is similar (i.e., with the same splice donor) to the REP-27 line that was used as a splicing control (Fig. 3D) and explains why no *Air* RNA could be detected through the *Igf2r* promoter by probe GFP-*Air* (Fig. 3C).

The relationship between the methylation-expression status of the *Air* promoter and that of the linked *Igf2r* promoter in LAI and SAI transgenes was then investigated (Table 1). Methylation of the *Igf2r* promoter was analyzed for a *Sal*I (for LAI) or *Not*I (for SAI) and two *Sma*I (for LAI and SAI) sites that are located within the *Igf2r* promoter CpG island. For all lines, the *Not*I and *Sal*I sites have a degree of methylation identical to that of the *Sma*I sites (data not shown). The *Igf2r* promoter in line LAI-46 has 70 and 100% methylation after

FIG. 3. Imprinting analyses of transgenic lines LAI-48 and LAI-46. (A) Methylation analyses of the *Air* promoter by Southern blotting of adult spleen genomic DNA cut with *Hind*III (– lanes) or *Hind*III and *Sfu*I (+ lanes) hybridized with probe MC detecting both endogenous (wt) and transgenic (tg) fragments (sizes in kilobases are indicated). Note that *Hind*III cuts inside the LAI construct and that LAI-46 shows an aberrant fragment due to a deletion of the 3' polyadenylation cassette. (B) Methylation analyses of the *Igf2r* promoter. A Southern blot of adult tail genomic DNA cut with *Ssp*I (– lanes) or with *Ssp*I and *Sal*I (+ lanes) hybridized with probe LA detecting endogenous (wt) and transgenic fragments (tg) is shown (sizes in kilobases are indicated). (C) Expression analyses of endogenous (wt) and transgenic (tg) *Air* RNA in cardiac tissue by RPA with probe GFPAIR that covers part of the EGFP-tagged *Igf2r* exon 1. (D) Expression of *Air* RNA in cardiac tissue with RPA probe MIMs1 that covers multiple transcription start sites at the *Air* promoter, yielding multiple protected bands for both spliced and unspliced *Air* RNA. The transgenic line REP-27 is used as a control for spliced *Air* RNA. (E) Expression of *Igf2r* RNA detected by RPA with probe GFPIGF2R on cardiac (LAI-48) or embryonic 12.5-dpc (LAI-46) total RNA. Note that this probe covers multiple transcription starts of the *Igf2r* promoter and part of the EGFP tag, resulting in multiple protected fragments for both the endogenous (wt) and transgenic (tg) *Igf2r* RNA. The part of the LAI-46 line showing transgenic *Igf2r* RNA (top) has been overexposed compared to the bottom panel, showing endogenous *Igf2r* RNA. Lanes: i, input probe; c, tRNA hybridization to the probe; p and m, paternal and maternal transmission of the transgene, respectively; wt, nontransgenic sample.

maternal and paternal transmission, respectively (Fig. 3B). In lines LAI-48 and LAI-313, the *Igf2r* promoter has 80% methylation for both parental transmissions; in the SAI-112 and SAI-119 lines, the *Igf2r* promoter has 100 and 50% methylation, respectively, for both transmissions (Fig. 3B and Table 1 and data not shown).

Expression of the *Igf2r* promoter in the LAI and SAI transgenic lines in cardiac tissue was analyzed by RPA with probe GFPIGF2R. This probe covers multiple *Igf2r* transcription start sites (31, 33) and part of the EGFP tag, thereby detecting both endogenous and transgenic *Igf2r* RNA as differently sized fragments. Transgenes LAI-48, LAI-313, and SAI-119 do not have imprinted *Igf2r* expression and express similar levels of *Igf2r* RNA upon both parental transmissions (Fig. 3E and Table 1 and data not shown). SAI-112 has no detectable *Igf2r* expression on any transmission (Table 1 and data not shown).

Neither of the two parental transmissions for LAI-46 had any detectable *Igf2r* expression in cardiac tissue, despite the fact that maternal transmission showed 30% reduction in *Igf2r* methylation (Fig. 3B and data not shown). We therefore analyzed *Igf2r* expression of line LAI-46 in 12.5-dpc embryos, as these embryos have higher levels of *Igf2r* RNA and reduced methylation on the *Igf2r* promoter (31). The *Igf2r* promoter of LAI-46 12.5-dpc embryos is 80% methylated on paternal transmission and 50% methylated on maternal transmission (data not shown) and shows weak but maternal-allele-specific *Igf2r* expression as analyzed by RPA (Fig. 3E; Table 1). So in summary, of five lines that contain both the *Air* and *Igf2r* promoters, only line LAI-46 is imprinted for *Air*, and this correlates with *Igf2r* imprinting.

Low-frequency imprinting of *Air*-*Aprt* transgenes. The observation that *Air* can be imprinted when combined with the

FIG. 4. Imprinting analyses of transgenes RAA-21 and RAA-14. (A) Methylation analyses of the *Air* promoter. A Southern blot is shown of genomic spleen DNA digested with *EcoRV* (– lanes) or *EcoRV* and *MluI* (+ lanes) and hybridized with probe MS detecting both endogenous (wt) and transgenic (tg) *Air* fragments (sizes in kilobases are indicated). (B) Methylation analyses of the *Apert* promoter. A Southern blot is shown of spleen DNA cut with *NcoI* (– lanes) or *NcoI* and *SmaI* (+ lanes) and hybridized with probe SE, which detects both endogenous (wt) and transgenic (tg) *Apert* fragments (sizes in kilobases are indicated). (C) Expression of the *Apert* and *Air* promoters by Northern blot analyses of adult cardiac tissue total RNA. Probe PS consists of *Apert* exon 1 and 2 (top) and detects both endogenous and transgenic *Apert* RNA and transgenic *Air* RNA due to the reverse orientation of the *Air* promoter (see Fig. 1). The signal from transgenic *Apert* is not visible at this exposure with this short probe. Probe DE consists of *Apert* exon 3 to 5 (middle) and detects endogenous and transgenic *Apert* RNA as indistinguishable bands. Transgenic *Apert* expression from line RAA-14 was analyzed in an *Apert*^{–/–} background. *Gapdh* was used as a loading control (bottom). Lanes: p and m, paternal and maternal transmission of the transgene, respectively; wt, nontransgenic sample.

Igf2r promoter prompted us to test if nonimprinted CpG island promoters could also facilitate *Air* imprinting. Two constructs were generated that positioned the *Air* promoter in opposite orientations into a mouse *Apert* transgene. Construct RAA contains the complete *Apert* gene, polyadenylation signal, and promoter, plus a 4.6-kb fragment containing region 2 inserted into intron 2 of the *Apert* gene so that the *Air* promoter faces the *Apert* promoter and potentially generates an antisense RNA. Upstream of the *Apert* promoter, a polyadenylation cassette was introduced to terminate *Air* RNA (Fig. 1). Five transgenic RAA lines were generated and investigated for imprinting of *Air* and *Apert*.

Methylation analyses of the *Air* promoter showed no methylation for low-copy-number lines RAA-21 and RAA-30 and a low level of methylation (40 to 50%) for high-copy-number lines RAA-27 and RAA-58 (Table 1). The methylation status was unchanged on parental transmission with the exception of one line, RAA-14. This line showed imprinted methylation for *Air*, gaining 100% methylation upon maternal transmission and losing all methylation upon paternal transmission (Fig. 4A). Methylation of the maternally derived RAA-14 transgene was, however, inconsistent between individuals from one litter. For example, of 15 offspring with a maternally inherited transgene, 12 had a methylated *Air* promoter but 3 were unmethylated. Female offspring with a methylated or unmethylated transgenic *Air* promoter subsequently produced offspring with the same variability of methylated transgenes (data not shown). Paternal transmission showed no variability and always resulted in an unmethylated *Air* promoter (Fig. 4A).

The expression of the RAA transgenes was investigated by Northern blot analyses. Two probes, PS (covering *Apert* exons 1 and 2) and DE (covering *Apert* exons 3 to 5), were used to discriminate between the *Air* and *Apert* RNA, respectively. Note that the *Air* RNA in the RAA lines was spliced, using the same

donor identified in the REP lines, but spliced to a novel acceptor (GTGAGTGG) located in antisense orientation within *Apert* exon 2. The *Air* RNA had equal levels of expression upon both transmissions in all lines, except in RAA-14 (Fig. 4C and Table 1). Line RAA-14 showed *Air* expression upon paternal transmission, which corresponds to the unmethylated status of the *Air* promoter. Maternally transmitted RAA-14 transgenes with a methylated *Air* promoter (80% of offspring) completely lacked *Air* expression, whereas the 20% of offspring with an unmethylated *Air* promoter expressed spliced *Air* (Fig. 4C).

To address whether the imprinted *Air* promoter of RAA-14 is able to imprint the *Apert* promoter, methylation and expression analyses of *Apert* were performed. Methylation of the *Apert* promoter for lines RAA-21 and RAA-14 was analyzed for two *SmaI* sites located within the *Apert* CpG island promoter. For both transgenic lines, both sites are unmethylated on maternal and paternal transmission, as is the endogenous *Apert* promoter (Fig. 4B; Table 1). *Apert* expression was analyzed by RT-PCR. Both RAA-21 and RAA-14 lines produce transgenic *Apert* RNA upon both transmissions, as detected by RT-PCR (Table 1). This transgenic *Apert* RNA includes the 125-bp *Igf2r* exon 3, due to the orientation of region 2 (data not shown). However, this RNA overlaps with endogenous *Apert* on a Northern blot, so we analyzed *Apert* expression of line RAA-14 in an *Apert*^{–/–} background (Fig. 4C). The paternal and maternal transmissions express equal levels of the transgenic *Apert* RNA when compared to *Gapdh*, indicating that the *Apert* promoter has no imprinted expression (Fig. 4C; Table 1).

Construct FAA is the same basic construct as RAA but has the 4.6-kb region 2-containing fragment inserted in a forward orientation, so that both the *Apert* and *Air* promoters have the same direction (Fig. 1). Nine FAA lines were generated and analyzed for *Air* imprinting. One line, FAA-4, had imprinted methylation of the *Air* promoter, being unmethylated on pa-

FIG. 5. Imprinting analyses of transgenes FAA-37 and FAA-4. (A) Methylation of the *Air* promoter was analyzed by Southern blotting with enzymes *Hinc*II (– lanes) or *Hinc*II and *Mlu*I (+ lanes) and hybridized with probe MC, which detects both endogenous (wt) and transgenic (tg) *Air* fragments (sizes are indicated in kilobases). (B) Methylation analyses of the transgenic (tg) and endogenous (wt) *Aprt* promoters. A Southern blot of spleen DNA cut with *Pst*I (– lanes) or *Pst*I and *Sma*I (+ lanes) and hybridized with probe SB is shown. Because *Pst*I cuts outside the transgene at the 5' end, it generates differently sized fragments for each transgenic line and confirms the single-copy integration site. (C) Expression analyses of the *Aprt* and *Air* promoters by Northern blotting of total embryo (14.5 dpc) RNA. Note that due to the orientation of the *Air* promoter, probe PS consisting of *Aprt* exons 1 and 2 (top) only detects endogenous and transgenic *Aprt* RNA, whereas probe DE consisting of *Aprt* exons 3 to 5 (middle) now detects both transgenic and endogenous *Aprt* RNA and transgenic *Air* RNA. Line FAA-4 was analyzed in an *Aprt*^{–/–} background. *Gapdh* was used as a loading control (bottom). Lanes: p and m, paternal and maternal transmission of the transgene, respectively; wt, nontransgenic sample.

ternal transmission and methylated on maternal transmission (Fig. 5A; Table 1). The remaining eight lines had no or low-level *Air* methylation, which was identical upon both parental transmissions (Fig. 5A; Table 1). Expression, analyzed by Northern blotting in an *Aprt*^{–/–} background, shows that line FAA-4 has paternal-allele-specific *Air* expression that inversely correlates with methylation status (Fig. 5C). The transgenic *Air* RNA was again spliced, using the same donor as the REP and RAA lines, but spliced to the normal *Aprt* exon 3 acceptor (GTCTAGAC). Remarkably, Northern blotting also showed that the *Aprt* promoter has paternal-allele-specific expression similar to that of the *Air* promoter on the same transgene (Fig. 5C; Table 1).

Methylation analyses for the *Aprt* promoter performed as for the RAA lines indicated that the *Aprt* promoter of FAA-4 acquired a maternal-allele-specific methylation imprint, as did the *Air* promoter on this transgene (Fig. 5B; Table 1). In contrast to the RAA lines where the *Igf2r* exon 3 is spliced into the transgenic *Aprt* RNA, the *Aprt* RNA produced from FAA-4 is functional due to the orientation of the region 2 fragment. This allowed us to rescue the phenotype from *Aprt*^{–/–} mice (37) in an imprinted manner by crossing in the FAA-4 line. *Aprt*^{–/–} mice (six of six) and *Aprt*^{–/–} mice with a maternally transmitted FAA-4 transgene (four out of four) have up to 50% less body weight than *Aprt*^{+/–} (eight of eight) littermates and died within 4 months (37). However, upon paternal transmission of FAA-4, *Aprt*^{–/–} mice (nine of nine) were indistinguishable from *Aprt*^{+/–} mice and had normal body weight, indicating functional *Aprt* expression from the transgene. In summary, 2 of 14 *Air-Aprt* transgenes have an imprinted *Air* promoter which is maternally methylated and silent and paternally unmethylated and expressed. In one of these lines, the *Aprt* promoter has the same imprinted and

paternal specific expression as the *Air* promoter and rescued the *Aprt*^{–/–} phenotype upon paternal transmission.

DISCUSSION

***Air* behaves as a nonimprinted CpG island promoter by default.** Exploiting transgenes, we have investigated the minimal setting sufficient to imprint the *Air* promoter. Although all transgenes carried the ADS, the DNS, and the direct repeat array that have all been postulated to be involved in attracting the methylation imprint (9, 20), in 27 of 30 transgenic lines *Air* is not imprinted and has no or low-level methylation. The results presented here that examined transgene methylation either in mid-gestation embryos or in adult tissues do not discriminate between a failure to gain a methylation imprint and a failure to maintain the imprint. Despite this, these results may indicate that the default state for the *Air* CpG island is absence of methylation. If so, this contrasts with other experiments (9) that examined transgenic founders as pre- and postimplantation embryos and showed that a multimerized 113-bp fragment from the core of the *Air* promoter attracts and maintains a maternal-allele-specific methylation imprint up to blastocyst stages. Results from these early-embryo founder transgenes indicated that methylation was the default state of the maternal allele and that the paternal allele was protected from methylation. Our results that examined adult founders, F₁-F₂ embryos, and adults containing low- to high-copy-number transgenes offer support for the opposite proposal—that absence of methylation is the default state and that the maternal allele actively acquires its methylation imprint.

***Air* is not imprinted in a single-promoter setting.** We have tested whether imprinting of *Air* requires stable *Air* expression with the REP reporter construct that contains the *Air* promoter

on a 9-kb fragment in a single-promoter setting. This 9 kb is sufficient for *Air* to function as a promoter in an integration-independent manner but not enough for *Air* imprinting, as none of the 11 lines are imprinted. Considering seven additional transgenic lines ranging from 3 to 14 kb that contain the *Air* promoter but do not recapitulate the methylation imprint (39), we conclude that, as distinct from *H19* and *Snrpn* (10, 12, 27), *Air* as a single expressed promoter in low- to high-copy-number transgenes is not sufficient to be imprinted.

***Air* can be imprinted in a dual-promoter setting.** The hypothesis that *Air* imprinting might need an additional CpG island promoter arose from an observation that 300-kb *Air-Igf2r* transgenes that were inadvertently derived with an inactive *Igf2r* promoter also lacked imprinting for both *Air* and *Igf2r* (39). Considering these transgenes, we tested whether *Igf2r* promoter activity would be sufficient for *Air* imprinting with the LAI and SAI lines that contain both the *Air* and *Igf2r* CpG island promoters. In addition, we tested whether any nonimprinted CpG island promoter could establish an imprinted configuration for *Air* with the RAA and FAA lines that contain the *Air* promoter coupled to the *Aprt* promoter. We chose the mouse *Aprt* gene, as the *Aprt* promoter is a CpG island with a broad expression profile and thus similar to the *Igf2r* promoter. In addition, transgenic studies have shown that *Aprt* is unmethylated as a transgene but can be methylated and silent under some circumstances (11, 18, 19). In a dual-promoter setting, *Air* imprinting was established in 3 of 19 lines (16%), indicating that a second CpG island promoter may facilitate *Air* imprinting. The frequency of *Air* imprinting when coupled to the *Igf2r* promoter (1 in 5 lines) is comparable to the situation when the *Air* promoter is coupled to the *Aprt* promoter (2 in 14 lines). This shows that *Air* on a 4.6-kb fragment can be imprinted in the absence of the *Igf2r* promoter and also indicates that the *Aprt* promoter is as good at facilitating *Air* imprinting as an *Igf2r* promoter. Moreover, the orientation of the *Air* promoter with respect to the *Aprt* promoter does not influence the result, since one in five lines in which both promoters face each other (the RAA lines) and one in nine lines with both promoters in the same orientation (the FAA lines) are imprinted for *Air*. Imprinting of the RAA-14 line is stable with paternal transmission but less robust with maternal transmission, adding further support to the proposal that the maternal methylation is the limiting step in imprinting and that the paternal absence of methylation is the default state. Comparable imprinted behavior has also been observed for a 300-kb *Igf2r-Air* transgene that integrated on the X chromosome (39) and for some *H19* transgenes (12, 14).

An additional point is that the three imprinted dual promoter lines generated in this study are all single copies (as shown by end fragment analysis) (Table 1). No multicopy-imprinted *Air* transgenes were identified. The numbers of transgenes generated here are small but may indicate that two heterologous promoters in a single-copy setting may facilitate imprinting of short transgenes. This study and a previous study (39) did not generate any single-copy transgenes with *Air* as a single promoter (e.g., all REP transgenes were multicopy transgenes). It therefore remains a possibility that single copies of the *Air* promoter are permissive for imprinting in the absence of a dual-promoter setting. Based on a comparative analysis of 1 Mbp spanning the homologous human- and

mouse-imprinted domains containing *Igf2-H19* and many other imprinted genes, Onyango et al. (22) postulated the “two CpG island rule” for imprinted genes. They observed that imprinted genes are associated with more than one CpG island, whereas nonimprinted genes harbor one or no CpG island. The results here support this hypothesis.

A low level of background imprinting has been reported for randomly integrated transgenes (reviewed in Surani et al. [32]). Despite the low frequency of *Air* imprinting, even in a dual-promoter setting (16%), we consider this to recapitulate imprinting of the endogenous locus, since neither the 11 short single-promoter transgenes described here nor any of the 12 short transgenes containing all or part of the *Air* promoter previously described (39) are imprinted. However, compared to *H19* transgenes from 10 to 18 kb which are imprinted with a frequency between 43 to 80%, depending on size and type of construct (12, 14, 23, 30), and *Snrpn* (exon 1 plus upstream sequences) transgenes of 1.2 kb, which are imprinted with an 85% frequency (27), the imprinted frequency for *Air* transgenes (16%) is relatively low. The basis of this difference is not clear. It may result from a difference in the mechanism that imprints *Igf2r* compared to that imprinting *H19* and *Snrpn*. Alternatively, more-consistent imprinting of *Air* transgenes may demand additional elements besides the two CpG island promoters. These elements may include enhancers for either *Igf2r* or *Air* expression. Epigenetic regulation of access to shared enhancers has been shown to play an essential role in imprinting the *Igf2-H19* gene pair (reviewed in reference 6). We argue, however, that the high level of *Air* expression in comparison to that of the endogenous *Air* locus shown by all 30 transgenes described in this study indicates that *Air* enhancers are not absent. Elements that influence the gain or maintenance of the maternal methylation imprint may also be missing. The LAI transgene that spans 44 kb of the endogenous *Igf2r* locus is imprinted in only one of three cases, whereas 300-kb transgenes are imprinted in four of four cases on three autosomal locations and one X-linked location (39). Thus, the missing sequences for reliable imprinting reside outside the 44 kb but within 300 kb. However, it also remains possible that the difference in transgene size is sufficient to shield *Air* from position effects that influence the gain or maintenance of the methylated state.

Silencing of linked promoters by region 2. Imprinting of the *Igf2r* promoter depends on the presence of region 2 as deletion of the paternal region 2, including the unmethylated and expressed *Air* promoter, derepresses the paternal *Igf2r* promoter on transgenes (39) and in the endogenous locus (40). Because region 2 is present in all our transgenes, we investigated whether it is capable of silencing the linked *Igf2r* and *Aprt* promoters in the LAI-SAI and RAA-FAA lines. With line LAI-46, we have shown that imprinted expression of *Air* correlates with reciprocal expression of the linked *Igf2r* gene. Note that in contrast to the endogenous locus, the *Air* RNA in line LAI-46 is spliced. The functional significance of the absence of splicing at the endogenous locus is unclear, since at least for line LAI-46 splicing of *Air* is compatible with silencing of the linked *Igf2r* promoter. However, it remains unclear why in the LAI-313, LAI-48, and the SAI-119 lines an expressed unmethylated *Air* promoter does not silence the *cis*-linked *Igf2r* promoter as predicted by the expression-competition models pro-

posed for sense-antisense pairs of imprinted genes (4). In the two cases of *Air* imprinting in an *Apvt* transgene, neither showed reciprocal effects on the *cis*-linked *Apvt* promoter. Although these numbers are too small for conclusions, they may indicate that not all CpG island promoters can be imprinted by region 2. In one *Apvt* transgene (line FAA-4), the linked *Air* and *Apvt* promoters have the same imprinted methylation and expression, in contrast to the endogenous locus, where *Air* and *Igf2r* are reciprocally expressed and methylated. Although the *Air* promoter in FAA-4 has imprinted *Apvt*, we argue that it has arisen from a mechanism different from that seen at the *Air-Igf2r* locus. A possible explanation is that the methylation imprint of the *Air* promoter has spread onto the *Apvt* promoter because the two CpG island promoters are closely located within a 2-kb fragment, due to the forward orientation of the region 2 insert in these FAA transgenes. Despite the fact that this transgene may not have recapitulated the mechanism that imprints *Igf2r*, it has generated an imprinted and paternally expressed *Apvt* transgene that rescues an *Apvt*-deficient phenotype in a paternal-allele-specific manner. This result indicates a possibility of imprinting other nonimprinted genes by proximity to the *Air* CpG island with potential application in creating parent-specific phenotypes.

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