Stringent Programming of DNA Methylation in Humans

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We describe a PCR-based method called Amplified Methylation Polymorphism (AMP) for scanning genomes for DNA methylation changes. AMP detects tissue-specific DNA methylation signatures often representing junctions between methylated and unmethylated DNA close to intron-exon junctions and/or associated with CpG islands. Identical AMP profiles are detected for healthy, young, monozygotic twins.

Keywords: DNA methylation, methylation-sensitive PCR, tissue-specific, identical twins

The importance of DNA methylation in mammalian biology has been recently highlighted by its involvement in several human diseases (Hendrich, 2000), and the list of such diseases can be expected to rapidly expand with the discovery of new methods for detecting DNA methylation variation between individuals or tissues. To aid DNA methylation research, we have developed a PCR-based protocol called Amplified Methylation Polymorphism (AMP) that allows the rapid scanning of the methylation status of thousands of CpG and CpNpG sites in mammalian genomes. The detailed AMP protocol is provided as Supplementary Note 1. Briefly, AMP uses a single arbitrary decamer oligonucleotide primer containing the recognition sequence of a methylation-sensitive restriction enzyme (e.g., *Hpa*II, which recognizes CCGG) to generate amplified profiles from both undigested genomic DNA and genomic DNA digested with the same methylation-sensitive enzyme. Amplification from digested template depends on the methylation status of the cytosines within, or closely linked to the amplicon (see later). The recognition sequences for the methylation-sensitive restriction enzyme are located in the middle of the oligonucleotide followed by up to four selective nucleotides extending to the 3' end (see Supplementary Table 1). Changing any of the 3' selective nucleotides results in a completely separate set of AMP markers (AMPs) being amplified from the genome (see Supplementary Note 2). PCR products are radiolabeled, separated on large polyacrylamide sequencing gels, and detected by autoradiography (Figure 1). Paired PCRs on undigested and digested genomic DNA detect the methylation status of more than 75 loci on average for each unique oligonucleotide primer, representing at least 150 CpG or CpNpG sites around the genome. The AMP protocol is very robust, reliable and produces identical profiles on replicate DNA extractions (Figure 1a, 1d). Using as few as 60 unique AMP primers, for example, approximately 4,500 loci around the genome (or 9,000 CpG or CpNpG sites) can be assessed for methylation status. More sensitive fluorescent labeling and detection of AMPs on microarrays of genomic DNA could enable assessment of many more loci per oligonucleotide primer and direct mapping of AMPs on genomes.

Comparison of the profiles from digested and undigested genomic DNA reveals three classes of AMPs (Figure 1a): digestion-resistant, digestion-sensitive, and digestion-dependent. In the case of humans and mice, ~ 90% of AMPs are digestion-resistant (amplified from both undigested and digested template), 7 to 8% are digestion-dependent (amplified from digested but not undigested template) and 1 to 2% are digestion-sensitive (amplified from undigested but not digested template). Digestion-resistant and digestion-sensitive AMPs represent methylated and unmethylated genomic DNA, respectively (Figure 1b). The nature of digestion-dependent AMPs was initially intriguing, but we have strong cooperative genetic and molecular evidence that these markers represent a methylated amplicon sequence flanked by an unmethylated restriction site, followed by a linked sequence that inhibits amplification from undigested template (Figure 1b). The nature of inhibition of amplification is presumably due to tertiary structure

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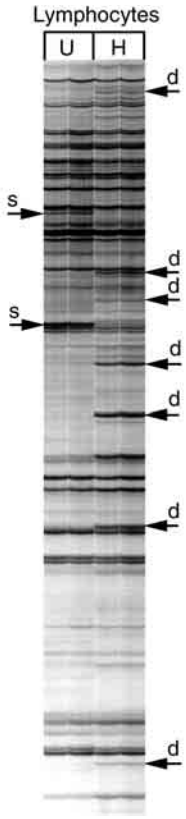

Figure 1a

AMP detects DNA methylation variation in mammalian genomes.

Note: Comparison of AMP profiles generated from undigested (U) and *Hpa*II-digested (H) genomic DNA reveals three classes of AMP markers: digestion-resistant (without arrows), digestion-sensitive (s) and digestion-dependent (d). Only half of the total AMP profile generated using one oligonucleotide primer on human lymphocyte DNA is shown. Identical profiles side-by-side are derived from replicate DNA extractions.

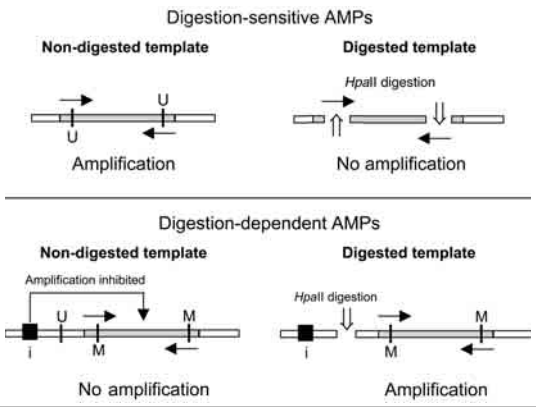

Figure 1b

AMP detects DNA methylation variation in mammalian genomes.

Note: Models for digestion-sensitive and digestion-dependent AMPs. A single oligonucleotide primer (arrow) is used in paired PCRs on non-digested and HpaII-digested genomic DNA. Digestion-sensitive AMPs (upper panel) are amplified from undigested template, but digestion of unmethylated restriction sites (U) prevents primer annealing resulting in no amplification from digested template. Digestion-dependent AMPs (lower panel) represent a methylated amplicon sequence flanked by an unmethylated restriction site, followed by a linked sequence (i) that inhibits amplification from undigested template. Cleavage of the unmethylated restriction site (U) but not the methylated primer-annealing sequences (M) allows amplification of digestion-dependent AMPs from digested template.

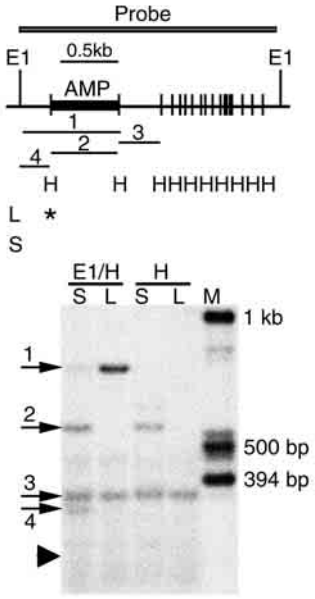

Figure 1c

AMP detects DNA methylation variation in mammalian genomes.

Note: Southern analysis confirms tissue-specific DNA methylation polymorphisms between sperm (S) and lymphocytes (L) and that digestion-dependent AMP represent junctions between methylated and unmethylated DNA in the genome. E1, *Eco*RI site; H, *Hpa*II site (short vertical lines on map) or *Hpa*II digest; E1/H, *Eco*RI plus *Hpa*II double digest. Ts1 (thick solid line on map) is a tissue-specific digestion-dependent AMP amplified only from *Hpa*II-digested lymphocyte, but not sperm DNA, and it is located on the border of a CpG island. Methylation at *Hpa*II sites was interpreted from restriction fragment sizes (1-4). The *Hpa*II site on the left side of the amplicon (*) is methylated in lymphocytes but not in sperm, and other *Hpa*II sites moving towards the centre of the CpG island are unmethylated in both lymphocytes and sperm (see arrow head indicating low molecular weight smear of *Hpa*II fragments). A 10 kb fragment was also detected in lymphocyte DNA digested with *Hpa*II but is not shown. For further information see Supplementary Note 3.

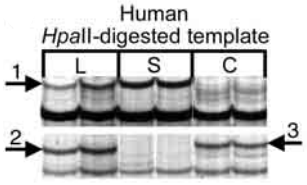

Figure 1d

AMP detects DNA methylation variation in mammalian genomes.

Note: Three tissue-specific digestion-dependent human AMPs amplified with the Operon oligonucleotide primer I-08. Only one individual is shown for each tissue (replicate DNA extractions); however, all seven individuals analyzed for each tissue displayed these three tissue-specific DNA methylation signatures. L, lymphocytes; S, sperm, C; cerebellum.

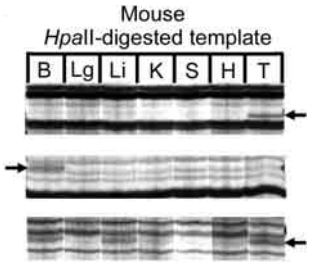

Figure 1e

AMP detects DNA methylation variation in mammalian genomes.

Note: Tissue-specific AMPs in a mouse. B, brain; Lg, lung; Li, liver; K, kidney; S, spleen; H, heart; and T, testis.

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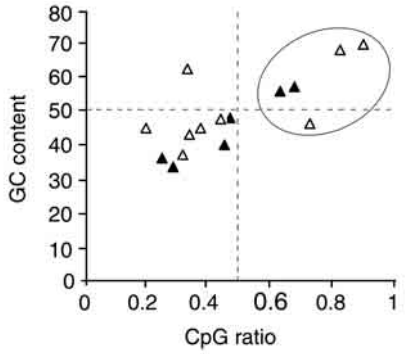

Figure 1f

AMP detects DNA methylation variation in mammalian genomes.

Note: Five out of 15 tissue-specific AMPs mapped to classical CpG islands (circled) with a GC content of about 50 % or higher and a CpG ratio greater than 0.5. Open triangles, digestion-dependent AMPs; closed triangles, digestion-sensitive AMPs. CpG ratio = [number of CpGs / (number of Cs × number of Gs)] / total number of base pairs, and GC content = [(number of Cs + number of Gs) × 100] / total number of base pairs (Gardiner-Garden & Frommer, 1987).

Figure 1g

AMP detects DNA methylation variation in mammalian genomes.

Note: Identical AMP profiles were detected for identical twins (T1 and T2). Only half of the AMP profile from one oligonucleotide on *Hpa*II-digested template is shown, however, these twins had identical AMP profiles over ~ 8,000 CpG sites in the genome. Identical profiles side-by-side are derived from lymphocyte DNA extractions at 14 and 16 years of age.

involving the amplicon and flanking DNA. Cleavage of the unmethylated restriction site removes the amplicon from the inhibitory sequence and permits amplification of digestion-dependent AMP markers. Inheritance analysis of digestion-dependent AMPs in plant mapping populations showed that the inhibitory sequence is always co-inherited with the amplicon (D.K.H., H.T.A. and B.J.C., manuscript in preparation), and Southern analysis with cloned AMPs confirmed that digestion-dependent amplicons are methylated and linked to unmethylated restriction sites (Figure 1c). Thus, digestion-dependent AMPs detect the junctions of methylated and unmethylated DNA in the genome. AMP is more efficient than other methylation-sensitive PCR protocols (Liang et al., 2002) and non-PCR-based restriction landmark genome scanning (RLGS) (Costello et al., 2000), but more importantly, unique features of AMP are that ~ 7 to 8 % of amplified fragments (i.e. digestion-dependent AMPs) represent the junction between methylated and unmethylated DNA in the genome, and AMPs often map close to coding regions of genes (Table 1; see Supplementary Table 2).

AMP detects abundant and distinct tissue-specific DNA methylation patterns in mammalian tissues (Figure 1d, 1e). For humans, 21 separate individuals were sampled for lymphocytes, cerebellum or sperm (seven individuals for each tissue type). To assess the extent of tissue-specific DNA methylation within one mammalian individual, a mouse of the inbred strain BALBc was analyzed on replicate DNA extractions over a greater range of tissues including brain, lung, liver, kidney, spleen, heart and testis. For the human tissues, while 17 out of a total of 450 AMP markers (3%) were tissue-specific, 13 out of 32 digestion-dependent AMPs (40%) varied depending on the tissue type (see Supplementary Table 3). Similarly, for the mouse tissues, 15 out of a total of 600 (2.5%) and 13 out of 35 digestion-dependent AMPs (31%) were tissue-specific. About the same frequency of tissue-specific variation was observed for digestion-sensitive AMPs (30–40%), although this marker type is much less common in AMP profiles (see Supplementary Table 3).

Cloning, sequencing and mapping (see Supplementary Note 2) of nine digestion-dependent and six digestion-sensitive tissue-specific human AMPs showed that ~ 50 % map close to coding regions of genes (Table 1). Five of the 15 tissue-specific AMPs mapped across or close to predicted intron-exon junctions, and others mapped close to 5' and 3' regions of genes. About 30% of tissue-specific AMPs also mapped to CpG islands (Figure. 1f), some of which were associated with predicted intron-exon junctions (Table 1). CpG islands are regions of mammalian genomes that are usually close to or within genes, and are enriched for GC content and CpG frequency (Antequera & Bird, 1993; Bird, 1986; Gardiner-Garden & Frommer, 1987). Except for CpG islands associated with inactive genes on the X chromo-

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Table 1

Tissue-Specific Human AMPs Often Map Close to Junctions of Coding and Non-Coding Regions of Genes

Marker	Marker type	Tissue specificity			Distance to nearest gene (kb)	Location of AMP relative to gene	Distance to nearest coding sequence (kb)	Nearest coding sequence	Accession number
		L	C	S					
Ts1[a,b]	d	+	+	—	0	Intron/Exon	0	Hypothetical protein	NM_024617
Ts2.1[b]	s	+	+	—	0	Intron	1	Dystrophin	NM_004006
Ts2.2	s	+	+	—	177	5′ region	177	Golgi vesicular membrane trafficking protein	NM_005868
Ts3	d	+	+	—	1	5′ region	1	Fibroblast growth factor 17	NM_003867
Ts4[a]	s	—	—	+	0	3′ UTR	3	Hypothetical protein	XM_088632
Ts5	d	+	+	—	170	5′ region	170	Spinal cord growth factor B	AB033832
Ts6[a]	s	+	+	—	8	5′ region	8	Zinc finger protein 195	NM_007152
Ts7.1	d	+	+	—	69	3′ region	69	Hypothetical protein	XM_098000
Ts7.2[a]	d	+	+	—	160	5′ region	160	Transcription factor 8	NM_030751
Ts8.1	d	—	+	—	13	5′ region	13	Ran binding protein 1	XM_018400
Ts8.2[b]	d	—	+	—	0	Intron/Exon	0	Poly rC-binding protein 2	NM_005016
Ts9.1[b]	s	+	+	—	0	Intron/ Exon	0	Onstatin M receptor	NM_003999
Ts 9.2	s	+	+	—	0	Intron	17	Hypothetical protein	NM_145280
Ts10	d	—	+	—	52	5′ region	52	Hypothetical protein	XM_078163
Ts11[a,b]	d	+	—	—	0	Intron	1	Protease	NM_002775

Note: [a] Five out of 15 tissue-specific AMPs mapped to classical CpG islands with a GC content of about 50% or higher and a CpG ratio greater than 0.5[5].

[b] AMPs mapping to within 1 kb of intron-exon junctions. d, digestion-dependent AMP; s, digestion-sensitive AMP; L, human lymphocyte DNA; C, human cerebellum DNA; S, human sperm DNA; +, DNA fragment amplified; -, DNA fragment not amplified.

some and imprinted genes (Panning & Jaenisch, 1996), they have been generally assumed to be unmethylated in normal healthy tissues (Antequera & Bird, 1993; Costello & Vertino, 2002; Futscher et al., 2002).

Southern analysis confirmed the reliability of the AMP protocol for detecting tissue-specific DNA methylation profiles at the junction of methylated and unmethylated DNA (Figure 1c). Southern analysis also demonstrated that depending on each individual AMP marker, either or both of the primer annealing sites need(s) to be methylated in order for amplification to occur (Figure 1c).

We also used the AMP protocol to investigate the degree of concordance of DNA methylation patterns in lymphocyte DNA from three pairs of healthy, young, monozygotic dichorionic human twins (see Supplementary Note 4). The first pair of twins was sampled for DNA at 14 and 16 years of age, and at both ages, the twins had identical AMP profiles (including observed intensity of amplified fragments) over ~ 8,000 CpG sites. An example of AMP profiles generated from lymphocytes for this set of identical twins is shown in Figure 1g. The other two pairs of twins were only sampled at 14 years of age, and each pair of twins also had identical AMP profiles over 2,200 CpG sites.

In summary, our findings indicate stringent programming of DNA methylation patterns close to coding regions and/or in CpG islands to produce tissue-specific 'methylomes'. A future priority for our research will be to determine the relevance of our discovery to developmental regulation of transcription and alternative splicing. Despite demonstrations that defects in DNA methylation lead to developmental abnormalities and diseases (Hendrich, 2000), the relationship between DNA methylation and developmental regulation of gene expression has been a subject of controversy (Costello & Vertino, 2002; Futscher et al., 2002; Holliday & Pugh, 1975; Riggs, 1975; Walsh and Bestor, 1999; Warnecke & Clark, 1999). Perhaps the position of DNA methylation within CpG islands and/or close to intron-exon junctions in mammalian genomes are crucial in determining gene expression or some other important aspect of chromatin organization, in a tissue-specific manner.

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References

Antequera, F., & Bird, A. (1993). CpG islands. In J. P. Jost & H. P. Saluz (Eds.). *DNA Methylation: Molecular biology and biological significance* (pp. 169–185). Basel, Switzerland: Birkhauser Verlag.

Bird, A. (2002). DNA methylation patterns and epigenetic memory. *Genes and Development, 16,* 6–21.

Bird, A. P. (1986). CpG-rich islands and the function of DNA methylation. Nature, 321, 209–213.

Costello, J. F., & Vertino, P. M. (2002). Methylation matters: A new spin on maspin. *Nature Genetics, 31*, 123–124.

Costello, J. F., Frühwald, M. C., Smiraglia, D. J., Rush, L. J., Robertson, G. P., Gao, X., Wright, F. A., Feramisco, J. D., Peltomäki, P., Lang, J. C., Schuller, D. E., Yu, L., Bloomfield, C. D., Caligiuri, M. A., Yates, A., Nishikawa, R., Su Huang, H., Petrelli, N. J., Zhang, X., O'Dorisio, M. S., Held, W. A., Cavenee, W. K., & Plass, C. (2000). Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. *Nature Genetics, 24,* 132–138.

Futscher, B. W., Oshiro, M. M., Wozniak, R. J., Holtan, N., Hanigan, C. L., Duan, H., & Domann, F. E. (2002). Role for DNA methylation in the control of cell type specific maspin expression. *Nature Genetics, 31,* 175–179.

Gardiner-Garden, M., & Frommer, M. (1987). CpG islands in vertebrate genomes. *Journal of Molecular Biology, 196,* 261–282.

Hendrich, B. (2000). Methylation moves into medicine. Current Biology, *10*, R60-63.

Holliday, R., & Pugh, J. E. (1975). DNA modification mechanisms and gene activity during development. *Science, 187*, 226–232.

Liang, G., Gonzalgo, M. L., Salem, C., & Jones, P. A. (2002). Identification of DNA methylation differences during tumorigenesis by methylation-sensitive arbitrarily primed polymerase chain reaction. *Methods, 27*, 150–155.

Panning, B., & Jaenisch, R. (1996). DNA hypomethylation can activate Xist expression and silence X-linked genes. *Genes and Development, 10*, 1991–2002.

Riggs, A. D. (1975). X inactivation, differentiation, and DNA methylation. *Cytogenetics and Cell Genetics, 14*, 9–25.

Walsh, C. P., & Bestor, T. H. (1999). Cytosine methylation and mammalian development. *Genes and Development, 13,* 26–34.

Warnecke, P. M., & Clark, S. J. (1999). DNA Methylation Profile of the Mouse Skeletal — Actin Promoter during Development and Differentiation. *Molecular and Cellular Biology, 19,* 164–172.

Supplementary Note 1: Amplified Methylation Polymorphism (AMP) Protocol

AMP is a PCR-based protocol for detecting DNA methylation polymorphisms in genomic DNA. DNA methylation polymorphisms are detected by using oligonucleotide primers carrying recognition sequences for methylation-sensitive restriction endonucleases on genomic template undigested or digested with the corresponding enzyme. The procedure listed below is for use of the methylation-sensitive enzyme *Hpa*II and corresponding *Hpa*II oligonucleotide primers, but the protocol can be modified accordingly for all other methylation-sensitive enzymes with a four base pair recognition sequence (e.g. *Hha*I, *Aci*I, *Acc*II and *Mae*II). The *Hpa*II recognition sequence is 5'-CCGG-3', but digestion only occurs if both cytosines are unmethylated (McCleland & Nelson, 1992).

AMP Template Preparation

Genomic DNA was prepared from human lymphocytes and spermatozoa by using QIAamp DNA mini-kit (QIAGEN) and from human cerebellum and mouse tissues using the protocol described by Ausubel et al. (1998). *Hpa*II-digested template was then prepared by overnight digestion of 5.0 µg of high-quality genomic DNA using a ten-fold excess (i.e. 50 units) of methylation sensitive enzyme (e.g. *Hpa*II). Digestion of the DNA was confirmed by separation and detection of 250 ng of digested DNA in a 0.7 % agarose gel. Digested DNA was precipitated, washed(Sambrook et al., 1989) and resuspended at 50 ng/µL in sterile milliQ water.

AMP PCR and Detection

Each AMP PCR contains 1x DAF buffer (10mM Tris pH8, 10m KCl, 5mM $MgCl_2$) (Caetano-Anolles et al., 1991; Waldron et al., 2002), 1.5 units of DNA polymerase Stoffel Fragment (Applied Biosystems), 20 µM dNTPs, 1 µCi α-labelled ^{33}P-dATP and 5 µM of a single arbitrary decamer oligonucleotide primer containing the recognition sequences of a methylation sensitive restriction enzyme (e.g., Supplementary Table 1). AMP profiles are generated from 50 ng of genomic DNA (undigested and digested with the same methylation-sensitive enzyme). PCR cycling conditions involve a hot start (85°C), followed by denaturation at 94°C for 5 min, then 30 cycles of: 94°C for 30 secs, 60 sec at each of 57°C, 56°C, 55°C, 54°C and 53°C[19]. The PCR was concluded with a

final extension step at 72°C for 5 min. Analysis on replicate DNA extractions yield identical AMP profiles, thereby confirming the reliability of the AMP protocol (Figure 1a, 1d).

AMP profiles are separated in sequencing gels (4% polyacrylamide, 7.5 M urea, 1 × TBE; 100 W for 2hr 15 mins) and detected by overnight exposure to X-ray film (Kodak Biomax MR). Using radiolabelling of AMP PCR products, each paired PCR (on undigested and digested genomic DNA) with a unique primer detects the methylation status of more than 75 loci, representing at least 150 CpG or CpNpG sites in the genome.

Supplementary Note 2: Cloning, Sequencing and Mapping AMP DNA Fragments

Cloning of DNA fragments from polyacrylamide gels was performed as described by Thomas et al. (Thomas et al., 1995). The authenticity of clones was demonstrated by differential hybridisation with AMP profiles containing and not containing the target DNA fragment for cloning. Clones were further confirmed by amplification of the insert with the corresponding AMP primer, followed by sizing on large polyacrylamide gels. Authenticated clones were sequenced and used in Blastn searches of the NCBI and Ensembl genome databases to locate the AMP loci in the human genome. Cloning, sequencing and mapping of more than 20 AMPs confirmed that amplification was completely dependent on the 3' sequence of the AMP oligonucleotide primer (data not shown).

Supplementary Note 3: Southern Analysis of Tissue-Specific AMP Ts1

Southern Hybridization was performed as described by Sambrook et al. (1989). Approximately 12 μg of genomic DNA was digested overnight with restriction enzymes before agarose gel separation and blotting on Hybond N^+ membranes (Amersham). Nested PCR was used to amplify probes spanning the tissue-specific AMP Ts1. The oligonucleotide primers used to amplify the Ts1 probe were 5'-GGGATTGCTGACTCTGG GGTTGTG–3' and 5'-GAGTTGGGCGGATGGCTTGAGG-3' in the primary PCR, and 5'- GGGACGTG-GTTTCTAGGCTTGTTAGGT-3' and 5'- GATGGCTTGAGGTCAGGAATTGGAGAG-3' in the nested PCR. PCR amplification with oligonucleotides listed above to produce radiolabelled products, restriction digest and sizing on polyacrylamide sequencing gels confirmed all restriction sites were present in genomic DNA used in Southern analysis.

Supplementary Note 4: AMP Analysis of Lymphocytes From Monozygotic Human Twins

Monozygosity was confirmed by identity at nine microsatellite markers (ABI Profiler™ kit). The twins were selected as dichorionic (based on their mother's report that there were two placentas at birth) because monochorionic twins frequently share placental vasculature and may exchange stem cells.

References

Ausubel, F. M. (Ed.). (1998). *Current protocols in molecular biology*. New York: John Wiley & Sons.

Caetano-Anolles, G., Bassam, B. J., & Gresshoff, P. M. (1991). DNA amplification fingerprinting: a strategy for genome analysis. *Bio-Technology, 9*, 553–557.

McCleland, M., & Nelson, M. (1992). Effect of site-specific methylation on DNA modification methyltransferases and restriction endonucleases. *Nucleic Acids Research, 20*, 2145-2157.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: A laboratory manual*. New York: Cold Spring Harbour Press.

Thomas, C. M., Vos, P., Zabeau, M., Jones, D. A., Norcott, K. A., Chadwick, B. P., & Jones. J. D. (1995). Identification of amplified restriction fragment polymorphism (AFLP) markers tightly linked to the tomato Cf-9 gene for resistance to Cladosporium fulvum. *Plant Journal, 8,* 785–794.

Waldron, J., Peace, C. P., Searle, I. R., Furtado, A., Wade, N., Finlay, I., Graham, M. W., & Carroll, B. J. (2002). Randomly amplified DNA fingerprinting: A culmination of DNA marker technologies based on arbitrarily-primed PCR amplification. *Journal of Biomedicine and Biotechnology, 2,* 141–150.

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Supplementary Table 1

Examples of *Hpa*II AMP Oligonucleotide Primers; *Hpa*II Sites are Underlined (These particular primers can be purchased from Operon Technologies (Alameda, CA, USA))

5′-TTTGCCCGGA-3′	5′-TGGACCGGTG-3′	5′-ACCCGGTCAC-3′
5′-AACCCGGGAA-3′	5′-TTCCCGGGTT-3′	5′-TTTGCCCGGT-3′
5′-CACCCGGATG-3′	5′-TCAGTCCGGG-3′	5′-TGCCGGCTTG-3′
5′-CCCCGGTAAC-3′	5′-CAGTGCCGGT-3′	5′-GTCCGGAGTG-3′
5′-ACACCGGAAC-3′	5′-AAGACCGGGA-3′	5′-TCCCGGTGAG-3′
5′-GAATCCGGCA-3′	5′-ACCCGGAAAC-3′	5′-TGCCGGTTCA-3′
5′-AGCCGGGTAA-3′	5′-CTACCGGCAC-3′	5′-ACCTCCGGTC-3′
5′-CTCCGGATCA-3′	5′-TTTCCGGGAG-3′	5′-AGGCCGGTCA-3′
5′-CAACCGGTCT-3′	5′-CCGCCGGTAA-3′	

Supplementary Table 2

Human AMPs Often Map Close to Junctions of Coding and Non-Coding Regions of Genes

Marker	Marker type	Distance to nearest gene (kb)	Location of AMP relative to gene	Distance to nearest coding sequence (kb)	Nearest coding sequence	Accession number
R1	r	0	Intron	4	Candidate tumour suppressor gene	NM_020381
R2	r	0	Intron	8	Tyrosine protein kinase LYN	M16038
R3	r	0.2	3′ end	0.2	Pregnancy-associated plasma protein A	NM_002581
R4	r	7	5′ end	7	Hypothetical protein	AK096991
R5	r	0	Intron	4	Blood vessel epicardial substance	NM_007073
R6	r	440	5′ end	440	Protein tyrosine phosphatase, receptor-type, G	NM_002841
R7	r	65	3′ end	65	Cysteine- and tyrosine-rich protein 1	NM_052954
D1[a]	d	0.3	5′ end	0.3	Pleckstrin homology domain containing protein	NM_021622
D2[b]	d	0	Intron	0.6	Neuropilin 2	NM_003872
D3	d	2	3′ end	2	Hypothetical protein	XM_171149
D4[a,b]	d	0	Intron	0.1	Camp-specific 3′,5′-cyclic phosphodiesterase 4D	NM_006203

Note: AMP markers listed in this table did not show tissue-specificity between lymphocytes, cerebellum and sperm.

[a] mapped to CpG islands.

[b] AMPs mapping to within 1 kb of intron-exon junctions. r, digestion-resistant AMP; d, digestion-dependent AMP. For details of cloning, sequencing and mapping AMPs see Supplementary Note 2).

Supplementary Table 3

Summary of Type and Frequency of AMP Markers Detected in Human and Mouse Tissues

	Total Number of AMP Markers Detected	
AMP Marker Type	Human	Mouse
Total	450 (13)*	600 (15)*
Digestion-Dependent	32 (13)*	35 (11)*
Digestion-Sensitive	10 (4)*	11 (4)*

Note: For the human analysis, 21 separate individuals were sampled for lymphocyte, cerebellum or sperm (seven individuals for each tissue type), and tissue-specific AMPs were defined as showing tissue-to-tissue variation, but no variation over the seven individuals for each tissue type. The AMP protocol also detected nucleotide polymorphism between the human individuals. To assess the extent of tissue-specific DNA methylation within one mammalian individual, a mouse of the inbred strain BALBc was analyzed for replicate DNA extractions over a greater range of tissues including brain, lung, liver, kidney, spleen, heart and testis.

* number of markers that were tissue-specific are shown in brackets.

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