Lsh, a member of the SNF2 family, is required for genome-wide methylation

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Methylation patterns of the mammalian genome are thought to be crucial for development. The precise mechanisms designating specific genomic loci for methylation are not known. Targeted deletion of Lsh results in perinatal lethality with a rather normal development. We report here, however, that Lsh−/− mice show substantial loss of methylation throughout the genome. The hypomethylated loci comprise repetitive elements and single copy genes. This suggests that global genomic methylation is not absolutely required for normal embryogenesis. Based on the similarity of Lsh to other SNF2 chromatin remodeling proteins, it suggests that alteration of chromatin affects global methylation patterns in mice.

Received July 18, 2001; revised version accepted September 26, 2001.

Methylation of cytosine residues in the genome is thought to be crucial for normal development in mammals as well as plants, frogs, and fish. DNA methylation is involved in the regulation of a diverse range of biological processes such as genomic imprinting and X-chromosome inactivation (for reviews, see Yelvin and Razin 1993; Razin and Shemer 1995; Robertson and Jones 2000). Establishment of genomic methylation patterns in mammals is a highly orchestrated process, with almost complete erasure during early embryogenesis and a resetting of the pattern subsequent to implantation. In general, genes are relatively unmethylated in expressing tissues and methylated in nonexpressing tissues leading to the hypothesis that methylation controls tissue specific gene expression. Alternatively, genomic methylation may primarily protect against the expression of parasitic sequences (Walsh and Bestor 1999). Deletion of DNA methyltransferase in Xenopus leads to abnormal development of the embryo (Stancheva and Meehan 2000; Stancheva et al. 2001). Reports on mice that are deficient in DNA methyltransferases support also a role for methylation in development as these mice die early during embryogenesis (Li et al. 1992; Lei et al. 1996; Okano et al. 1999).

Lsh [lymphoid specific helicase] belongs to the family of SNF2/helicases. SNF2-like proteins are frequently involved in chromatin remodeling (Jarvis et al. 1996). Lsh shows a preferential lymphoid expression pattern in adult mice and has been shown to be important for normal lymphoid development (Geiman et al. 1998; Geiman and Muegge 2000). However, a low expression of Lsh has been found in multiple embryonal tissues suggesting a broader role for Lsh in development (Geiman et al. 2001). SNF2-containing protein complexes can alter nucleosomal structure in vitro (Peterson and Workman 2000) and recombinant SNF2 homologs on their own can cause nucleosomal sliding along DNA (Hamiche et al. 1999; Langst et al. 1999). This in vitro activity of SNF2 family members is held responsible for altering chromatin accessibility in vivo. A recent study identified the gene Ddm1 [decrease in DNA methylation], another SNF2 family member, as a modulator of genomic methylation in Arabidopsis thaliana (Jeddeloh et al. 1999). The methylation system in plants appears to be highly conserved although homologs for the chromomethylases have not been identified in mammals, possibly because mammals primarily maintain CG methylation instead of methylation at CNG sites (Lindroth et al. 2001). This latter Ddm1 study suggests a relationship between regulation of chromatin structure and genomic methylation in plants. Because Ddm1 shares about 50% identity with Lsh over the region containing the helicase domains (Jeddeloh et al. 1999), we studied the effect of Lsh on genomic methylation patterns in mice.

Results and Discussion

To determine whether Lsh has an effect on DNA methylation we examined genomic DNA derived from Lsh−/− mice (Geiman and Muegge 2000; Geiman et al. 2001) for their methylation status at multiple repetitive sequences that are highly methylated. The minor satellite sequences [50,000–100,000 copies] are located around centromeres. Analysis of genomic DNA from fibroblasts, brain, liver, intestine, heart and lung, and the whole body (Fig. 1A,B) as well as thymus (K. Dennis, T.M. Geiman, and K. Muegge, unpub.) showed increased digestibility with HpaII, a methylation-sensitive enzyme. The pattern of the HpaII digest was comparable with the MspI digest [a methylation-insensitive isoschizomer] suggesting a substantial loss of methyl groups at the minor satellite sequence in the absence of Lsh. The defect in methylation was detectable in newborn tissue (Fig. 1B) as well as fetal tissue (Fig. 1A) as early as day 13.5 of gestation (the earliest time point we have examined).

To analyze whether hypomethylation is a widespread phenomenon in the genome, other methylated sequences with high copy number were examined. The major satellite sequence, with 700,000 copies in centromeric regions, also showed substantial hypomethylation in DNA isolated from fetal brain tissue of Lsh-deleted animals [Fig. 2] as well as from MEF cultures or body tissue of newborn mice [K. Dennis and K. Muegge, unpub.]. Sequences of the intracisternal A-particle retrovirus [IAP] [1000–2000 copies] are heavily methylated in littermate controls but not in Lsh-deleted animals. Line 1, another retroviral element [50,000–100,000 copies], Sine B1, the murine homolog of human Alu repeats, as well as examination of telomeric sequences showed sub-
stantial hypomethylation in the absence of Lsh [Fig. 2].

Moreover, comparison of HpaII and MspI digests of DNA from Lsh-deficient tissue appear almost indistinguishable suggesting substantial loss of methylation at the examined sites.

To determine whether Lsh deficiency selectively leads to hypomethylation of repetitive elements or also effects single copy sequences a number of specific genomic loci were examined. The genes for β-Globin and Pgk-2 (phosphoglycerate kinase) are highly methylated single copy genes with a tissue-specific expression pattern. Pgk-1, another highly methylated gene, is located on the X-chromosome. The methylation sensitive sites examined were located either 5′ of the gene (Pgk-1), within the exon (Pgk-2), or 3′ of the gene (Globin) and did not contain any known repetitive sequences (Singer-Sam et al. 1990; Kafri et al. 1992; Tada et al. 1997). All three examined loci revealed a substantial loss of methylation in newborn tissue as well as embryonic tissue from Lsh−/− mice [Fig. 3A–C]. In addition, the upstream region of the H19 gene was examined comprising the imprinting control region with all four CTCF (CCCTC-binding factor) binding sites [Bell and Felsenfeld 1999]. This region showed substantial hypomethylation in Lsh−/− mice [Fig. 3D]. In contrast the Igf2r gene showed no difference in methylation between Lsh−/− tissue and wild-type tissue [K. Dennis and K. Muegge, unpubl.]. These results demonstrate that loss of the Lsh gene effects methylation of repetitive elements and single-copy sequences including the imprinted region of the H19 gene.

A global defect in methylation was visualized using ethidium bromide stain. Genomic DNA derived from thymus and MEF [Fig. 4A] as well as brain [K. Dennis and K. Muegge, unpubl.] of Lsh-deficient mice was highly digestible with the methylation-sensitive enzyme HpaII. To quantify the extent of hypomethylation, genomic DNA from Lsh−/− mice was tested in vitro for its ability to accept methyl groups and compared with littermates using the SssI methyltransferase. Genomic DNA derived from embryonic liver and body and as well as newborn brain of Lsh-deleted mice accepted two to three times more methylation than controls [Fig. 4B]. Assuming an average methylation level of 60%–70% in control samples, this difference would suggest a genome-wide methylation level of only 13%–32% in genomic DNA from Lsh-deleted mice. In addition, genomic DNA from newborn brain was digested with MspI, radiolabeled at 5′ ends, and degraded to single deoxynucleoside monophosphates. Cytosine as well as methyl-cytosine was sepa-

Figure 1. Hypomethylation of minor satellite sequences in Lsh−/− mice. (A) Southern analysis of genomic DNA derived at day 13.5 of gestation. DNA was digested with HpaII or MspI, blotted, and probed for minor satellite sequences using MR150. (B) Southern analysis of genomic DNA derived from newborn mice within 24 h after birth. Whole body comprises every tissue with the exception of the examined internal organs. DNA was digested with HpaII or MspI, blotted, and probed for minor satellite sequences using MR150.

Figure 2. Hypomethylation of repetitive sequences in Lsh−/− mice. Southern analysis for repetitive sequences. Genomic DNA derived from Lsh−/− or littermate controls was digested with HpaII or MspI, blotted, and probed for major satellite sequences, or digested with HpaII and MspI (M) and probed for IAP, Sine B1, Line 1, or telomeric sequences.

Figure 3. Hypomethylation of single copy sequences in Lsh−/− mice. (A) Southern analysis of the β-Globin gene. Genomic DNA was derived from newborn mice or embryos at day 13.5 gestation. DNA was digested with BamHI with or without the methylation sensitive restriction enzyme HhaI, blotted, and probed for β-Globin. (B) Southern analysis of the Pgk-2 gene. (C) Southern analysis of the Pgk-1 gene. (D) Southern analysis of the H19 upstream imprinted region.
DNA derived from brain samples of methyl-cytosine in genomic DNA. Equal amounts of genomic DNA derived from day 13.5 embryonic body, embryonic liver, or from newborn brain were methylated in vitro by SssI CG methylase using radiolabeled S-adenosyl-methionine as donor. This approach allows determination of the amount of unmethylated CG sites in the genome and serves as an indirect measurement of genomic methylation levels. The amount of incorporated radiolabeled methyl groups on cytosines per microgram of DNA was measured using radiolabeled S-adenosyl-methionine as donor. This approach allows determination of the amount of unmethylated CG sites in the genome and serves as an indirect measurement of genomic methylation levels. The amount of incorporated radiolabeled methyl groups on cytosines per microgram of DNA was measured in Lsh−/− deleted samples and control littermates as described previously [Antoun et al. 2000]. (C) Direct measurement of methyl-cytosine in genomic DNA. Equal amounts of genomic DNA derived from brain samples of Lsh−/− mice and littermate controls were digested with MspI, radiolabeled at the 5′-ends, and digested with nuclease P1 to generate 5′-deoxynucleotides. Cytosine and methyl-cytosine were separated by thin layer chromatography [Cedar et al. 1979] and quantified using PhosphorImager analysis. The ratio of methyl-cytosine to total cytosine indicates the level of methylation at all CCGG sites. HpaII digests should not generate methyl-cytosine spots and serve as controls, indicating the specificity of the assay.

We hypothesize that Lsh protein may participate in the process of de novo or maintenance DNA methylation rather than simply altering the level of DNA methyltransferase mRNA and protein for several reasons: (1) mRNA levels for DNA methyltransferases were comparable between Lsh-deficient samples and control samples in embryonic bodies [Fig. 5A], embryonic liver, or MEF cultures [T. Fan and K. Muegge, unpubl.]. (2) The level of Dnmt1 protein (whose targeted deletion results in a 95% reduction of the Dnmt1 protein) is a targeted Dnmt1 allele that leads to a 95% reduction of the Dnmt1 protein. Mutant embryos develop to day 10.5 and show 30% reduction of genome methylation levels [Li et al. 1992]. The S and C alleles of Dnmt1 are more severe and lead to an arrest in embryonic development around day 8.5 with an almost undetectable genomic methylation level. In contrast, conditional mutants that lack Dnmt1 from day 12 of gestation in neuroblasts die shortly after birth from respiratory stress without any obvious defects in the brain structure [Fan et al. 2001]. The methyltransferases Dnmt3a and Dnmt3b are thought to be involved in de novo methylation. Dnmt3a−/− mice look normal at birth and die runted by 4 wk of age with no apparent change in global methylation levels [Okano et al. 1999]. Dnmt3b−/− could not be recovered at birth and showed developmen-

Figure 4. Global hypomethylation in Lsh−/− mice. (A) Genomic DNA from embryonal fibroblasts (MEF) or adult thymus from radiation chimeras [Geiman and Muegge 2000] was digested with the methylation-sensitive enzyme HpaII and the nonsensitive enzyme MspI, subjected to agarose gel electrophoresis, and visualized by ethidium bromide stain. (B) Methyl acceptance assay. Equal amounts of genomic DNA derived from day 13.5 embryonic body, embryonic liver, or from newborn brain were methylated in vitro by SsI CG methylase using radiolabeled S-adenosyl-methionine as donor. This approach allows determination of the amount of unmethylated CG sites in the genome and serves as an indirect measurement of genomic methylation levels. The amount of incorporated radiolabeled methyl groups on cytosines per microgram of DNA was measured in Lsh−/− deleted samples and control littermates as described previously [Antoun et al. 2000]. (C) Direct measurement of methyl-cytosine in genomic DNA. Equal amounts of genomic DNA derived from brain samples of Lsh−/− mice and littermate controls were digested with MspI, radiolabeled at the 5′-ends, and digested with nuclease P1 to generate 5′-deoxynucleotides. Cytosine and methyl-cytosine were separated by thin layer chromatography [Cedar et al. 1979] and quantified using PhosphorImager analysis. The ratio of methyl-cytosine to total cytosine indicates the level of methylation at all CCGG sites. HpaII digests should not generate methyl-cytosine spots and serve as controls, indicating the specificity of the assay.

Figure 5. Expression of DNA methyltransferases and measurement of Mtase activity in Lsh−/− mice. (A) RT–PCR analysis. Total RNA of embryonic body [2 wild type, 1 heterozygote, and 3 knockout] derived from day 17.5 gestation was reverse transcribed and subjected to real-time PCR analysis for measurement of Dnmt1, Dnmt3a, or Dnmt3b or Gapdh transcripts as control. (CT) Cycle threshold, cycle number at which each PCR reaction reaches a predetermined fluorescence threshold, set within the linear range of all reactions. (B) Western analysis. Cellular extracts derived from fetal brain tissue were analyzed using specific antiserum against murine Dnmt1, Dnmt3a [Imgenex], Dnmt3b [Affinity Bioreagents], β-Actin [Sigma], or PCNA (Santa Cruz) as control. A similar result was obtained using lysates derived from embryonic body of day 17.5 gestation. (C) Mtase activity. Cellular extracts were prepared from indicated tissues and examined in vitro for their ability to transfer radio-labeled methyl-groups onto synthetic template poly[d(I–C)]·poly[d(I–C)] [Li et al. 1992]. Embryonic bodies are from day 13.5 gestation.
Materials and methods

Southern analysis
Genomic DNA was prepared from the indicated tissues, digested, separated by electrophoresis on 1% agarose gels, and transferred by blotting on Nytran plus membranes [Schleicher & Schuell]. Membranes were hybridized overnight at 42°C in hybridization buffer [Amersham] with 32P-labeled probes and washed twice in 2xSSC/0.1%SDS at 65°C or 42°C for 30 min and twice in 0.2xSSC/0.1%SDS at 65°C or 42°C for 30 min. The following oligonucleotide probes were used for detection of repetitive sequences: Major satellite [60-mer GenBank accession no. X06899, base pair 1–60], L1 (60-mer accession no. D84391, base pair 5670–5729), Sine B1 [27-mer accession no. AC002121, base pair 13464–13490], telomeric probe [42-mer, 5′-TTAGGG-3′]. The minor satellite probe was a 66-mer oligonucleotide 5′-GACTGAAAAACACATTCGTTG-3′. The major satellite probe and the minor satellite probe were labeled with [γ-32P]dCTP and used for Southern analysis. DNA was digested with restriction enzymes and electrophoresed to resolve DNA fragments of different sizes. Southern blots were hybridized with [32P]labeled probes, washed, and exposed to x-ray film. The intensity of the bands was quantified by phosphorimager analysis.

RT-PCR analysis
RT-PCR was performed as described previously (Antoun et al. 2001). The expression levels of the Lsh transcripts were quantified by real-time PCR using the ABI PRISM 7500 Real-Time PCR System [Applied Biosystems]. The PCR was performed using SYBR Green I as a double-strand DNA-specific binding dye and continuous fluorescence monitoring. Each reaction contained 100 ng of cDNA template and primers at a concentration of 400 nM in a final volume of 25 µL in SYBR Green PCR Master Mix [Applied Biosystems] containing AmpliTaq Gold. PCR was initiated with one cycle 50°C for 2 min and one cycle 94°C for 10 min followed by 40 cycles: 94°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec. Cycle threshold, the cycle number at which each PCR reaction reaches a predetermined fluorescence threshold, was set within the linear range of all reactions. Melting curve analysis of amplification products was performed at the end of each PCR. As an additional control, PCR products were separated by electrophoresis and detected by hybridization with an internal oligonucleotide probe. The R1 propogol nucleotides were used as primers: dmnt1 [GenBank accession no. X14805: sense base pair 310–330, antisense base pair 681–701], dmnt3a [accession no. AF068625: sense base pair 363–382, antisense base pair 759–779], dmnt3b [accession no. AF068628: sense base pair 305–328, antisense base pair 818–838].

Quantitative measurement of global methylation
Measurement of methylated cytosine at CCGG sites was determined as described previously (Cedar et al. 1979; Li et al. 1992). Purified genomic DNA was digested with HpaII or MspI, treated with alkaline phosphatase and radiolabeled with [α-32P]dCTP at the 5′-termini using polynucleotide kinase. Phenol-chloroform-purified DNA was digested with nuclease P1 and the 5′-deoxyxymononucleotides were separated by thin-layer chromatography on cellulose plates. The radioactivity over methyl-cytosine and total cytosine was scanned by PhosphorImager analysis. The ratio was formed between methyl-cytosine versus total cytosine indicating the percentage of methylation at CCGG sites in the genome. HpaII was used as control digest indicating the specificity of the assay, as HpaII does not recognize methylated CCGG sites and only cleaves unmethylated sites the HpaII digest should only generate cytosine and not methyl-cytosine spots. The methyl acceptor assay was performed as described previously (Antoun et al. 2000). Purified genomic DNA (200 ng) was incubated for 4 h at 37°C with 4 units of M.SssI CpG methylase, 3 µCi [methyl-3H]HJ-adenosyl L-methionine, and 1.5 µM nonradioactive AdoMet. After termination the mixture was spotted on Whatman glass filters washed with 5% trichloroacetic acid, followed by 70% ethanol and the incorporated radioactivity was quantified by liquid scintillation counting.

Muntase activity
Measurement of DNA methyltransferase activity was performed as described previously (Li et al. 1992). Protein extract (20 µg) was added to 5 µCi of [methyl-3H]HJ-adenosyl L-methionine and 4 µg of poly[dI–dC]–poly[dI–dC]. After incubation for 2 h at 37°C, followed by phenol-chloroform extraction the nucleic acids in the aqueous phase were denatured, neutralized, and precipitated and the radioactivity that was incorporated into DNA was quantified by scintillation counting.

Lsh is required for genome-wide methylation
Aberrant methylation patterns have been suspected to promote tumorigenesis [Jones and Gonzalez 1997; Baylin and Herman 2000] to be involved in the process of aging [Lisa 2000] or to be causative for inherited diseases such as the ICF syndrome [Okano et al. 1999; Xu et al. 1999]. The observed defect in global methylation in Lsh−/− mice will be a helpful tool to increase our understanding of the mechanism and consequences of specific genomic methylation patterns in disease.
Acknowledgments

We thank Drs. Scott Durum, Howard Young, Joost Oppenheim, and Timothy Bestor for their suggestions on the manuscript and helpful discussions. We thank Dr. Narayan Bhat and James Cherry for their help on using real-time PCR analysis. We thank Dr. Timothy Bestor for the generous gift of the murine Dnmt1 antisera. We thank Dr. Chun Wun for his generous gift of the murine Dnmt3a antisera. We are grateful to the technical assistance of Rodney Wiles and Terry Stull. This project has been funded in whole or part with Federal funds from the National Cancer Institute, National Institutes of Health, under contract No. N01-CP-65000.

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*Genes Dev.* 2001, 15:
Access the most recent version at doi:10.1101/gad.929101

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