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Transcription Factor Sp1 Is Essential for Early Embryonic Development but Dispensable for Cell Growth and Differentiation

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Summary

Transcription factor Sp1 has been implicated in the expression of many genes. Moreover, it has been suggested that Sp1 is linked to the maintenance of methylation-free CpG islands, the cell cycle, and the formation of active chromatin structures. We have inactivated the mouse *Sp1* gene. *Sp1*$^{-/-}$ embryos are retarded in development, show a broad range of abnormalities, and die around day 11 of gestation. In *Sp1*$^{-/-}$ embryos, the expression of many putative target genes, including cell cycle-regulated genes, is not affected, CpG islands remain methylation free, and active chromatin is formed at the globin loci. However, the expression of the methyl-CpG-binding protein MeCP2 is greatly reduced in *Sp1*$^{-/-}$ embryos. MeCP2 is thought to be required for the maintenance of differentiated cells. We suggest that Sp1 is an important regulator of this process.

Introduction

Sp1 was one of the first eukaryotic transcription factors to be identified and cloned as a factor that binds to the SV40 early promoter (Dynan and Tjian, 1983). It contains three zinc finger motifs, Cys-2–His-2, which bind to a consensus GGG GCG GGG (Letovsky and Dynan, 1989). Sp1 is an abundant nuclear protein in most cells, but the level of expression changes during development and varies in different cell types (Saffer et al., 1991). Sp1 is the founding member of a growing family of proteins with highly homologous zinc-finger domains that bind GC or GT boxes (Hagen et al., 1992; Imataka et al., 1992; Kingsley and Winoto, 1992; Miller and Bieker, 1993; Crossley et al., 1996). This has precluded a straightforward interpretation of expression studies of putative Sp1-responsive genes, and hence, little is known about its biological role.

The molecular properties of Sp1 have been studied in vitro in great detail. The protein is phosphorylated (Jackson et al., 1990) and highly glycosylated (Jackson and Tjian, 1988). The N-terminus contains glutamine- and serine/threonine-rich domains that are essential for transcriptional activation (Courey et al., 1989). The C-terminal domain of Sp1 is involved in synergistic activation and interaction with other transcription factors (Li et al., 1991). Sp1 has been shown to interact directly with the TATA-box protein accessory factor $TAF_{II}110$ (Gill et al., 1994). When bound to distant sites in *cis*, it can interact with itself, thus looping out the intervening DNA (Li et al., 1991; Su et al., 1991). This suggests that Sp1 may establish interactions between promoters and distant regulatory elements in vivo through such a looping mechanism (Ptashne, 1986). Sp1 binding sites appear in numerous promoters and other regulatory sequences of tissue-specific and ubiquitous genes (over 1500 citations).

A number of observations imply a wider role for Sp1-like proteins in nuclear processes. Sp1 binding sites are critical for the maintenance of the methylation-free CpG island of the *APRT* gene (Brandeis et al., 1994; Macleod et al., 1994). Since Sp1 binding sites occur very frequently in CpG islands, this suggests that Sp1 prevents methylation of many CpG islands in the genome. Maintenance of the appropriate methylation patterns is essential for normal development (Li et al., 1992). A second link between Sp1 and nuclear architecture is that Sp1 may function in remodeling chromatin structures, as was first observed for the SV40 virus early promoter (Jongstra et al., 1984). Another example is the β-globin locus control region (LCR) (Grosveld et al., 1987) studied extensively in our laboratory. The most important part of the LCR, which can activate chromatin in single-copy transgenic mice (Ellis et al., 1996), is completely dependent on the presence of Sp1 binding sites (Philipsen et al., 1993). Third, Sp1 has been implicated in the expression of cell cycle-regulated genes like thymidine kinase (*TK*) (Karlseder et al., 1996), dihydrofolate reductase (*DHFR*) (Lin et al., 1996) and b-*myb* (Zwicker et al., 1996). It has been suggested that Sp1 activity is modulated by components of the cell cycle machinery like the cyclins (Shao and Robbins, 1995) and Rb-like proteins (Shao and Robbins, 1995), and Sp1 has been shown to interact directly with the growth- and cell cycle-regulated transcription factor E2F in vitro (Karlseder et al., 1996; Lin et al., 1996). Thus, Sp1 may be intricately linked to the regulation of the cell cycle and the establishment of transcriptional competence through the maintenance of methylation-free islands and the organization of chromatin structure, in addition to its direct role in transcriptional activation.

In order to address these issues directly, we have inactivated the mouse *Sp1* gene in ES cells and characterized the phenotype of Sp1-deficient mice. *Sp1*$^{-/-}$ embryos survive until day 9.5 (E9.5) of gestation. They are severely retarded in growth and show a broad range of phenotypic abnormalities. *Sp1*$^{-/-}$ ES cells contribute extensively to every tissue of chimeras until E9.5 but fail to contribute after early embryonic development, thus demonstrating that the defect in *Sp1*$^{-/-}$ cells is cell autonomous. Furthermore, we show that in *Sp1*$^{-/-}$ embryos the expression of many putative Sp1 target genes, including cell cycle-regulated genes, is unaffected, that methylation-free islands are faithfully maintained, and that the globin loci are activated, implying that active chromatin structures can still be formed. However, *Sp1*$^{-/-}$ embryos express the MeCP2 protein at 10-fold-reduced levels. The MeCP2 protein binds to methylated

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Table 1. Genotype Distribution of Sp1 Heterozygous Crossings

Stage	Total	+/+	+/−	−/−
E7.5	75	18 (24%)	43 (57%)	14 (19%)
E8.5	137	35 (26%)	74 (54%)	28 (20%)
E9.5	143	34 (24%)	79 (55%)	30 (21%)
E10.5	78	24 (31%)	36 (46%)	18 (23%)
E11.5	33	11 (33%)	18 (55%)	4 (12%)
Pups	97	37 (38%)	60 (62%)	0 (0%)

The genotype of embryos was determined by PCR analysis and that of pups by Southern blotting, as described in Experimental Procedures. The observed distributions at E7.5–10.5 are not significantly different from the expected Mendelian 1:2:1 ratio (Chi-square test, $p > 0.05$), indicating that no loss of null embros occurs before E10.5.

DNA and is essential for normal embryonic development but not for growth and differentiation of ES cells. Thus, it has been proposed that MeCP2 is required for the maintenance of differentiated cells (Tate et al., 1996). We conclude that Sp1 is dispensable for growth and differentiation of primitive cells and suggest that Sp1 plays a role in the maintenance of differentiated cells through the regulation of genes like *MeCP2*.

Results

Targeted Disruption of the *Sp1* Gene

The 3′ end of the human *Sp1* cDNA was used to isolate genomic cosmid clones of the mouse *Sp1* gene. Six overlapping cosmids were isolated and used to construct the targeting vector (Figure 1A). The exons encoding the zinc-finger domains and the C-terminus (Kadonaga et al., 1987; Chestier and Charnay, 1992) are replaced by a *lacZ-neomycin* (β-*geo*) fusion gene containing a splice acceptor and the encephalomyelitis virus internal ribosomal entry site (IRES) (Mountford et al., 1994), rendering expression of β-*geo* dependent on the splice acceptor and translation from the IRES. This strategy results in the deletion of two important domains of the Sp1 protein: the DNA-binding zinc fingers and the C-terminus that is required for synergistic activation (Courey et al., 1989). It leaves the 65 kDa N-terminus intact (see below). For clarity, we will refer to this deletion as a null mutation throughout this paper.

E14 ES cells were transfected with XhoI-digested targeting vector, and G418-resistant clones were analyzed for homologous recombination by Southern blotting (Figure 1B). Thirteen correctly targeted clones out of 36 clones analyzed were further characterized by probing with Bluescript to exclude additional random integration events.

Mice Lacking Sp1 Die Early during Embryogenesis

Two $Sp1^{+/-}$ ES cell clones with the correct karyotype were injected into C57BL/6 blastocysts. High percentage chimeras were obtained with both clones. Mating of chimeric males with C57BL/6 and FVB females resulted in germ-line transmission of the *Sp1* knockout allele. Results are shown in the C57BL/6 background, but they have been confirmed in the FVB background. The F1 mice appeared to be normal; the F2 $Sp1^{+/-}$ mice were initially smaller than wild-type littermates, but no other obvious abnormalities were found (data not shown). None of 97 live-born animals from heterozygous matings was homozygous for the *Sp1* knockout (Table 1). Genotyping of embryos during earlier stages of development by PCR (Figure 1C) showed no statistically significant loss of *Sp1*-null embryos up to E10.5–11 (Table 1). Thus, $Sp1^{-/-}$ embryos survive until E9.5–10; by E10.5–11, all of the Sp1-deficient embryos have died.

There is a remarkable heterogeneity in phenotype (Figure 2). Frequently, −/− embryos had developed to the 10–12 somite stage, while others in the same litter looked like a small undifferentiated mass of cells (Figure 2). Many structures and tissues can be recognized in the advanced −/− embryos, e.g., somites, otic vesicle, limb bud, blood, the developing eye, and heart (Figure 2), indicating no specific developmental defect. However, −/− embryos are always much smaller than their littermates, and their gross morphology does not correspond to any specific stage of normal mouse development.

Hence, $Sp1^{-/-}$ embryos survive well beyond preimplantation, but importantly, Sp1 is absolutely essential

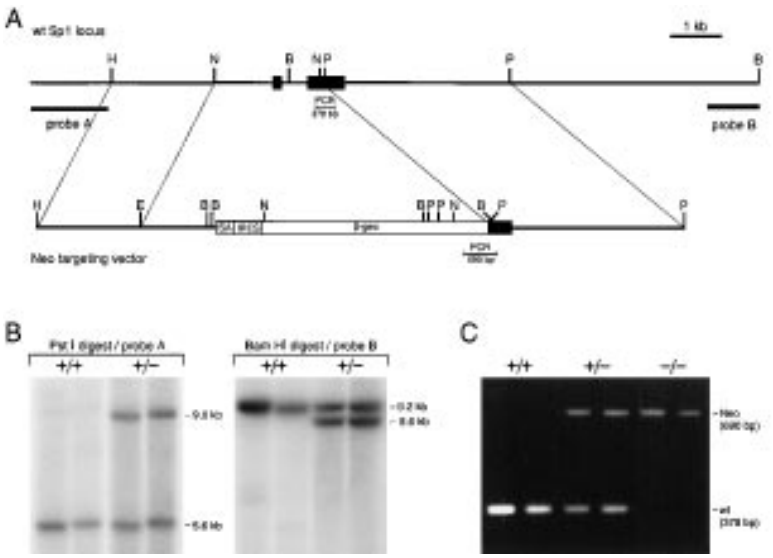

Figure 1. Targeted Disruption of the *Sp1* Gene

(A) The 3′ end of the mouse *Sp1* gene. The two exons (closed boxes) encoding the three zinc fingers and the C-terminus of Sp1 are replaced by a *lacZ-neomycin* fusion gene (β-*geo*) in the targeting construct. The β-*geo* gene contains a splice acceptor (SA), the picornaviral IRES, and SV40 polyadenylation signals (not indicated) (Mountford et al., 1994).

(B) Southern blot analysis of targeted ES cells. DNA isolated from ES cell clones was digested with PstI or BamHI, Southern blotted, and hybridized with probe A or probe B (panel [A]) as indicated. Probe A detects a 5.6 kb band (wild-type allele) or a 9.0 kb band (targeted allele); probe B detects a 9.2 kb band (wild-type allele) or an 8.6 kb band (targeted allele).

(C) PCR analysis of mouse embryos. The primers detect a 378 bp band for the wild-type allele (wt; panel [A]) or a 690 bp band for the targeted allele (Neo; panel [A]).

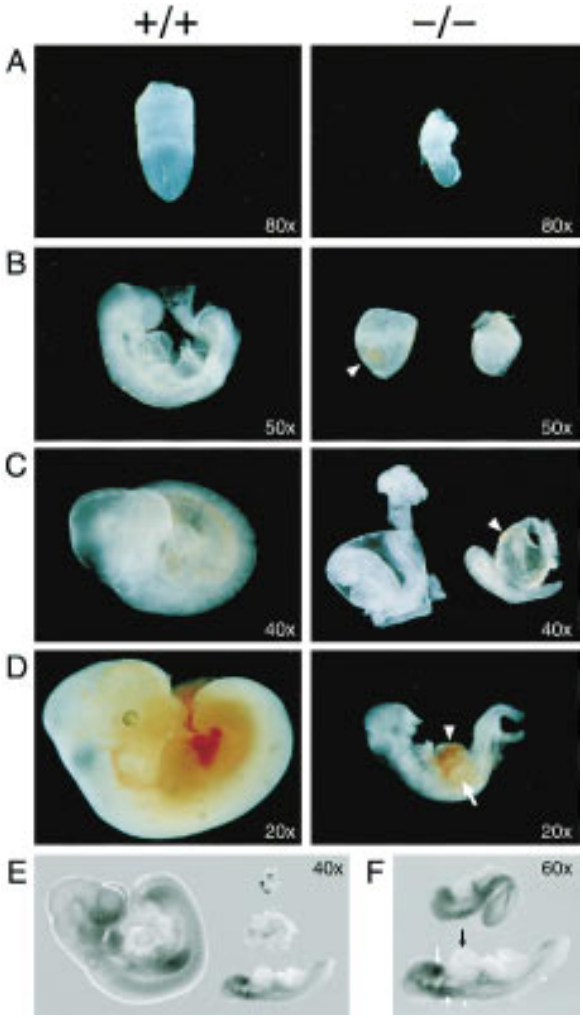

Figure 2. Phenotype of *Sp1*-Null Embryos at Different Developmental Time Points

(A) E7.5 embryos.
(B) E9 embryos.
(C) E10 embryos. Note that the −/− embryo on the right has developed outside the yolk sac.
(D) E11 embryos. Note that the −/− embryo had started to decompose at the time of dissection. Wild-type littermates of the −/− embryos are shown on the left. White arrowheads indicate the presence of erythroid cells in −/− embryos; the arrow points at the limb bud of the E11 −/− embryo (D).
(E and F) Phenotypic heterogeneity of *Sp1*-null embryos. (E) Four embryos from one litter (E10) are shown. Left: heterozygous embryo. Right: three −/− embryos. (F) Heterozygous embryo at E8.5 (top) compared with one of the −/− embryos shown in (E). Indicated are the developing eye (large white arrow), heart (black arrow), otic vesicle (small white arrow) and somites (between arrowheads). Original magnifications are as indicated.

for normal embryogenesis. The broad range of abnormalities suggests that no particular cell lineage or developmental process is affected but that Sp1-deficiency causes a general cellular defect that precludes normal development and survival.

Sp1$^{-/-}$ ES Cells Are Viable and Apparently Normal

Since ES cells are derived from blastocysts and *Sp1* knockout embryos survive beyond this stage, we inactivated the second allele in *Sp1*$^{+/-}$ ES cells. To achieve this, we replaced β-*geo* in the targeting vector with a *hygromycin* gene (Figure 3A). *Sp1*$^{+/-}$ ES cells were transfected with this construct, and clones were picked after hygromycin B selection (Figure 3B). Thirty out of fifty-nine clones analyzed were homologous recombinants, 14 at the wild-type allele and 16 at the already targeted allele. *Sp1*$^{-/-}$ ES cells grew at normal rates in standard ES cell media. Flow cytometry (Vindelov et al., 1983) showed that the percentage of cells in G1, S, and G2+M phase of the cell cycle was the same for wild type and Sp1-deficient ES cells (data not shown). The absence of Sp1 DNA-binding activity was confirmed by a gel-retardation analysis with ES cell protein extracts (Figure 3C). The probe, derived from the SV40 early promoter, bound Sp1 and Sp3 proteins present in nuclear extracts of wild type and +\− ES cells, as identified by the presence of supershifts after the addition of Sp1- or Sp3-specific antibodies. No DNA-binding Sp1 protein is detected in −/− ES cells, while +/− ES cells show reduced levels of Sp1 as compared to wild-type cells. (Figure 3C).

However, the 65 kDa N-terminal domain is detectable in +/− and −/− ES cells by Western blotting, but at levels lower than those of full-length Sp1 (Figure 3D). The N-terminal domain of Sp1 can act as a superactivator in transient transfection assays; this activity is dependent on overexpression of the N-terminus in combination with DNA-binding Sp1. It is not observed when the Antennapedia activation domain is fused to the Sp1 DNA binding domain (Courey et al., 1989) or in combination with the closely related family member Sp3 (Hagen et al., 1994). Therefore, the low expression level and the fact that +/− animals develop normally exclude the likelihood that the N-terminal domain acts as a gain-of-function or interfering mutation.

Thus, we have successfully targeted both alleles of the *Sp1* gene in ES cells. Surprisingly, the −/− cells appear normal under standard tissue-culture conditions, and they can be induced to differentiate and form embryoid bodies as efficiently as control cells (data not shown).

The Defect in *Sp1*$^{-/-}$ Mice Is Cell Autonomous

It remained possible that certain tissues or cell types could develop normally from Sp1-deficient cells. To test this directly, we injected *Sp1*$^{-/-}$ ES cells into C57BL/6 host blastocysts. Chimeric embryos were dissected at stages E9.5–11 (Figure 4). At E9.5, *Sp1*$^{-/-}$ ES cell contribution was detected in four out of five embryos (Figure 4A, middle and right). Note that the E9.5 embryo with the highest contribution of donor ES cells, as judged by X-gal staining (Figure 4A, right), is morphologically similar to the Sp1-deficient embryo shown in Figure 2C, left. Very few *lacZ*-positive cells could be detected in any tissue of four embryos dissected at E10.5 (Figure 4B, left), while in the remaining three there was a modest contribution to random tissues (Figure 4B, middle and right). At E11, a very limited contribution of the donor ES cells was observed in three embryos (Figure 4C), while the remaining four were completely negative. Several mice were analyzed 14 days after birth; they showed no contribution of the *Sp1*$^{-/-}$ ES cells to the coat, nor to any other tissue analyzed by X-gal staining and PCR (data not shown).

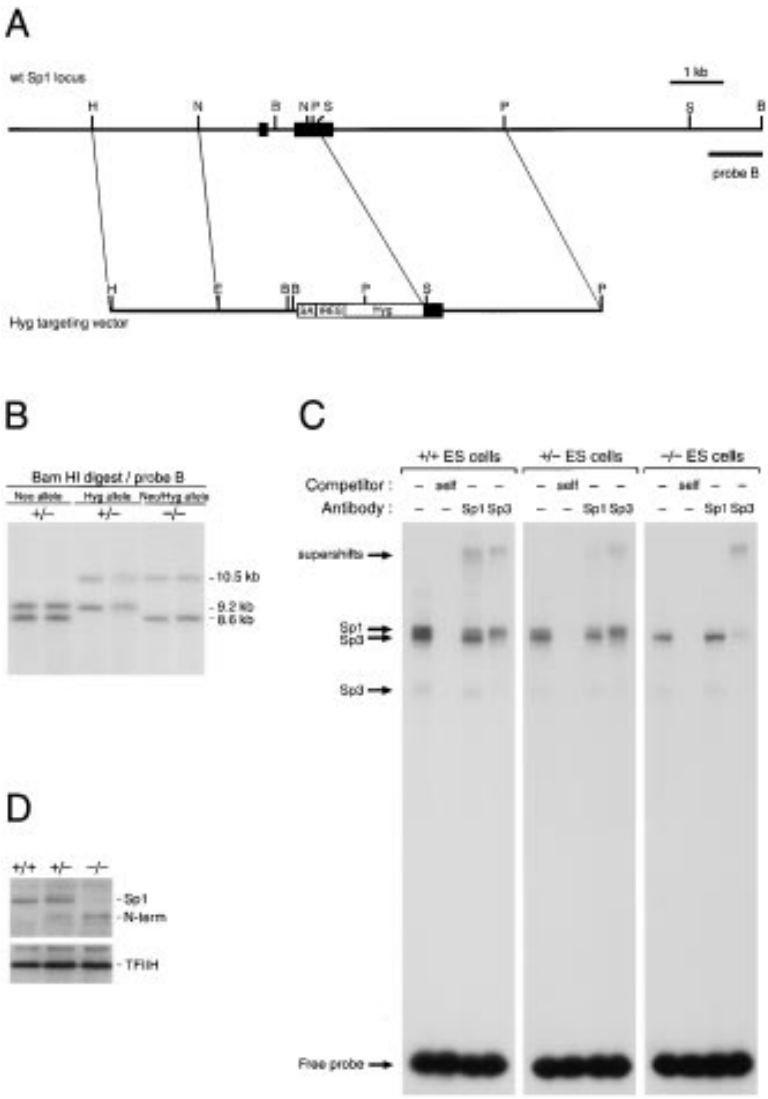

Figure 3. Generation of *Sp1*-Null ES Cells

(A) *Sp1*$^{+/-}$ ES cells were transfected with a targeting vector similar to that described in Figure 1A, except that the β-*geo* gene was replaced with a hygromycin resistance gene (*hyg*).

(B) Analysis of hygromycin-resistant ES cell clones. The DNA was digested with BamHI, Southern blotted, and hybridized with probe B (Figure 1A). Random integrations (+/−, Neo mutant), targeting at the already targeted allele of the *Sp1* gene (+/−, Hyg mutant), and targeting at the remaining wild-type allele of the *Sp1* gene (−/−, Neo/Hyg mutant) are shown. Fragment sizes are 8.6 kb for the "Neo" allele, 9.2 kb for the wild-type allele, and 10.5 kb for the "Hyg" allele.

(C) Gel-retardation analysis of Sp1 DNA-binding activity. Two micrograms of protein from +/+, +/−, or −/− ES cells was incubated with an oligonucleotide probe containing canonical Sp1 binding sites (Philipsen et al., 1990). The reaction products were electrophoresed through a 4% acrylamide/0.5 × TBE gel. The specificity of protein–DNA complexes is demonstrated by self competition with a 100-fold molar excess of unlabeled oligonucleotide as indicated. The identity of complexes is revealed by the addition of antibodies specific to Sp1 or Sp3, resulting in slower migration of bands containing either Sp1 or Sp3 (supershifts).

(D) Western blot analysis of +/+, +/−, and −/− ES cells. The positions of wild-type 90 kDa Sp1 and the 65 kDa N-terminus (N-term) are indicated (top). The blot was reprobed with an antibody recognizing the 89 kDa TFIIH subunit to control for loading differences (bottom).

We conclude that the defect in *Sp1*$^{-/-}$ cells is cell autonomous. The cells are intrinsically unable to contribute to any tissue of the mouse after early embryonic development, even when supported by wild-type cells from the C57BL/6 host blastocysts. The results also exclude that the primary defect is in the extraembryonic tissues, since in chimeras these are derived from the host blastocysts.

Expression of Putative Sp1 Target Genes

Preliminary data obtained with electron microscopy suggested that *Sp1*$^{-/-}$ embryos might suffer from a defect in lipid metabolism or the lysosomal compartment (data not shown). Therefore, we checked the expression of proteins involved in lipid metabolism (ALDR, ApoE, ApoAII, CAT, ScoAD, HMG-CoA reductase) and lysosomal enzymes (β-galactosidase, α-fucosidase, α-galactosidase, α-iduromidase, α-N-acetylgalsctosaminidase, β-glucuromidase, β-glucosidase, β-hexosaminidaseA, β-mannosidase), but none of these was affected (data not shown).

A large number of ubiquitous and tissue-specific genes have been suggested to be regulated by Sp1 (over 1500 publications). We analyzed the expression of such potential target genes in ES cells (data not shown) and E8.5 mouse embryos with RT–PCR. Expression analysis included cell cycle-regulated genes like *APRT* (Macleod et al., 1994), *HPRT* (Hornstra and Yang, 1992), DHFR (Birnbaum et al., 1995), and *TK* (Karlseder et al., 1996); tissue-restricted genes like the *LDL receptor* (Koivisto et al., 1994) and Apolipoprotein A1 (*ApoA1*) (Lamon−Fava et al., 1992); and the tissue-specific α- and β-*like globins* (Yu et al., 1991; Philipsen et al., 1993). *collagen αIV* was used as a control, since Sp1 has not been implicated in the regulation of this gene. As shown in Figure 5, the expression of most of these genes is not affected in *Sp1*$^{-/-}$ embryos with the exception of the *TK* gene, which is 2- to 3-fold reduced in the −/− embryos (Figure 5A).

The expression levels of the *LDL receptor* and *ApoA1* genes are indistinguishable between the wild-type, heterozygous, and homozygous mice (Figure 5B). The embryonic α-globin (Zeta) and β-globins (βH1 and ϵ^{Y}) are

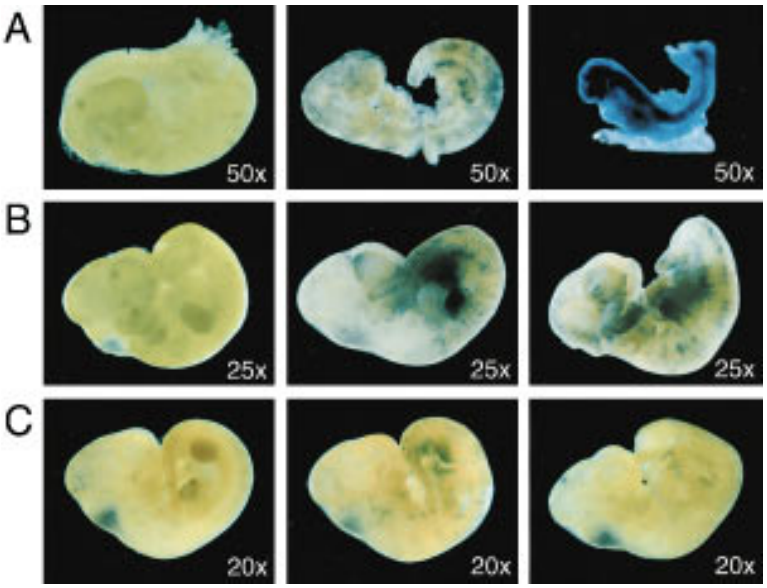

Figure 4. Contribution of *Sp1*$^{-/-}$ ES Cells to Chimeric Embryos

C57BL/6 host blastocysts were injected with 12–14 *Sp1*$^{-/-}$ ES cells and transferred into foster mothers. Embryos were dissected at stages E9.5, E10.5, and E11, fixed, and stained with X-gal to reveal the presence of *Sp1*$^{-/-}$ cells.

(A) Embryos at E9.5. Five embryos were analyzed, of which one had no detectable contribution of the donor ES cells (left). Note the correlation between the severity of the phenotype and the contribution of −/− ES cells to the embryo as revealed by X-gal staining (middle and right).

(B) Embryos at E10.5. Seven embryos were analyzed, four of which contained hardly any *LacZ*-positive cells (left). Embryos containing detectable amounts of X-gal-positive cells (middle and right) are retarded in development relative to those showing no staining (left).

(C) Embryos at E11. Contribution of the donor ES cells could not be detected in four of the seven embryos analyzed; a small contribution was observed in the remaining three (shown). Magnifications are as indicated.

less abundant in the knockout mice than in wild-type or heterozygous mice (Figure 5C), but they are still expressed at significant levels, suggesting that the chromatin of the globin loci is activated in *Sp1*$^{-/-}$ erythroid cells. Hence, Sp1 does not appear to be essential for expression of these genes in E8.5 mouse embryos.

Taken together, the data show that many putative Sp1 target genes are expressed normally in *Sp1*$^{-/-}$ mice, implying that the number of genes critically dependent on the presence of Sp1 is much smaller than expected.

Methylation Status of CpG Islands

Recent transgenic mice experiments have shown that Sp1 binding sites are essential to maintain the methylation-free status of the CpG island of the *APRT* gene, suggesting that Sp1 is required to prevent methylation of CpG islands in mammals (Brandeis et al., 1994; Macleod et al., 1994). We tested this hypothesis in *Sp1*-null mutant ES cells and mice. DNA from wild-type and single- and double-knockout ES cells was digested with the methylation-sensitive enzymes SmaI and HpaII, end labeled, size fractionated, and autoradiographed (Figure 6A). Because the bulk DNA is heavily methylated and contains relatively few HpaII and SmaI sites, it is cut infrequently. However, CpG islands are normally unmethylated, contain many HpaII and SmaI sites, and therefore are cut frequently by these enzymes. This fraction of DNA, known as the HpaII tiny fragments (HTF) (Cooper et al., 1983), is seen as a low molecular weight smear on an agarose gel after end labeling of the digest (Figure 6A). Since we observe no difference in the HTF fraction of DNA from +/+, +/−, and −/− cells (Figure 6A), we conclude that many, if not all, methylation-free islands are faithfully maintained in Sp1-deficient ES cells. In addition, we examined the methylation status of the CpG island for the *APRT* gene (Brandeis et al., 1994; Macleod et al., 1994) using SmaI, HpaII, and the methylation-insensitive HpaII isoschizomer MspI (Figure 6B). No significant difference in the methylation of the HpaII and SmaI sites in the *APRT* CpG island was observed between +/+, +/−, and −/− Sp1 ES cells. In fact, the SmaI digest reveals some methylation of the island in the control cells but not in +/− or −/− cells. Since the *APRT* gene is dispensable under tissue-culture conditions (She and Taylor, 1995), it is unlikely that these results can be simply explained by positive selection for APRT-expressing ES cells.

Next, we examined CpG islands in E9.5 Sp1-deficient embryos. Since only very small quantities of DNA could be obtained from individual *Sp1*$^{-/-}$ embryos, we pooled these samples and performed a methylation analysis of the *APRT* CpG island by PCR (Kafri et al., 1992) with HpaII- or MspI-digested DNA samples from +/+, +/−, and −/− embryos. As shown in Figure 6C, the fragment corresponding to the methylation-free island could only be amplified in undigested DNA. These results are consistent with those obtained in ES cells (Figure 6B) and show that the methylation-free island of the *APRT* gene is not affected by the knockout. As a control, we amplified a region of the *APRT* gene that is known to be methylated (Macleod et al., 1994). An *APRT*-specific band was found in the HpaII-digested DNA samples but not in the samples that had been cut with MspI. Hence, CpG methylation outside the island region appears to be normal in the *Sp1*$^{-/-}$ mice. In addition, we checked the methylation status of the CpG islands in the *DHFR* (Kafri et al., 1992) and *ApoA1* (Shemer et al., 1991) genes. As shown in Figure 6C, there was no amplification of island-specific PCR products except in the uncut DNA, indicating that the HpaII sites in the *DHFR* CpG island and the HhaI sites in the *ApoA1* CpG island of +/+, +/−, and −/− embryos are not methylated. From these results we conclude that Sp1 is not generally required to maintain the methylation-free status of mammalian CpG islands.

To investigate further the potential link between Sp1

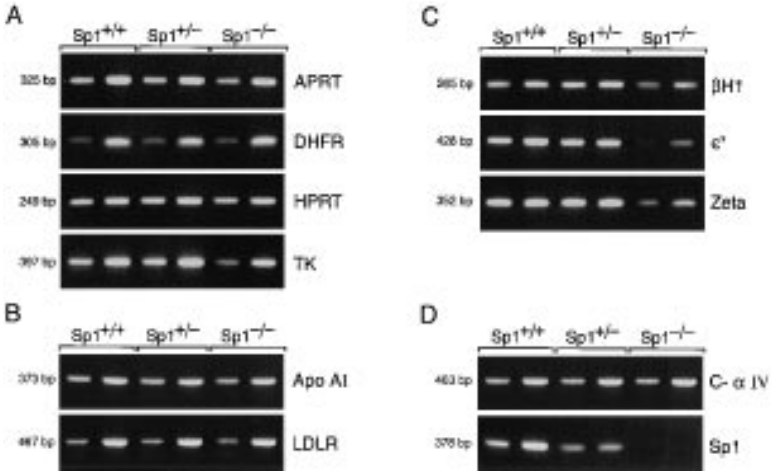

Figure 5. Expression of Putative Sp1 Target Genes

RNA was isolated from individual E8.5 embryos, cDNAs were synthesized, and the expression of putative target genes was analyzed by PCR. Samples were taken after variable numbers of cycles and analyzed by electrophoresis on 1.5% agarose gels. cDNAs were analyzed in duplicate for three different embryos of each genotype (wild type, +/−, or −/−).

(A) Expression of cell cycle-regulated genes (*APRT*, *DHFR*, *TK*, *HPRT*). These genes are also known as typical "housekeeping" genes. Expression of the *TK* gene is reduced in −/− embryos, while expression of the other genes is not affected.

(B) Expression of the tissue-restricted *ApoAI* and *LDLR* genes. No significant differences are detected between the samples.

(C) Expression of embryonic globin genes. The β-*like* (β*H1* and ϵ^{Y}) and α-*like* (*Zeta*) globin genes are expressed at lower levels in the *Sp1*$^{-/-}$ embryos.

(D) Expression of the *collagen αIV* gene was used to standardize the PCR assay. In agreement with the data shown in Figure 3C, expression of the C-terminal part of the *Sp1* gene is reduced in +/− and undetectable in −/− embryos.

and DNA methylation, we analyzed the expression of two genes associated with DNA methylation, *DNA methyltransferase* (Li et al., 1992) and *MeCP2* (Lewis et al., 1992). It should be noted that these genes are essential for embryonic development and that the null phenotypes are reminiscent of the *Sp1*$^{-/-}$ phenotype (Li et al., 1992; Tate et al., 1996). The DNA methyltransferase protein methylates C residues at CpG dinucleotides. As shown in Figure 7A, this gene is expressed at normal levels in *Sp1*$^{-/-}$ mice, consistent with the DNA methylation analysis shown in Figure 6, which suggested that overall DNA methylation is not affected by the *Sp1* knockout. While the MeCP2 protein has no known enzymatic activity, it binds specifically to methylated CpGs without any other apparent sequence specificity and is mainly localized to heterochromatin, which is known to be highly methylated in the mouse (Lewis et al., 1992); it might function as a transcriptional repressor (Nan et al., 1997). Interestingly, we find that the expression level of the *MeCP2* gene is 2-fold lower in heterozygous and hardly detectable in homozygous *Sp1* knockout embryos (Figure 7B). Since the RT–PCR assay is indirect, we performed a gel-retardation analysis to quantitate the levels of MeCP2 protein (Figure 7C). This analysis shows that the level of MeCP2 protein is reduced 2- to 3-fold in heterozygous and more than 20-fold in −/− embryos. Together, these data suggest that MeCP2-deficiency contributes significantly to the *Sp1*$^{-/-}$ phenotype.

Discussion

Targeted Disruption of the *Sp1* Gene

In this paper, we describe the targeted inactivation of the mouse gene encoding transcription factor Sp1. Our results demonstrate that Sp1 is essential for normal mouse embryogenesis. *Sp1*$^{-/-}$ embryos are found in normal numbers during early embryonic development, but they all die around day 11 of gestation. The knockout embryos are severely retarded in development and display a marked heterogeneity in phenotype. This might reflect a general function for Sp1 in every cell type, consistent with its ubiquitous expression pattern (Saffer et al., 1991). Frequently observed abnormalities in *Sp1*$^{-/-}$ embryos are asymmetry along the body axis, incomplete turning, and growth outside the yolk sac. Interestingly, the differentiative capacity of *Sp1*$^{-/-}$ cells does not appear to be severely impeded, since we observe many of the structural hallmarks of normal E8.5–9.5 embryos in the best-developed −/− embryos.

From the large number of putative Sp1 target genes, it might have been predicted that inactivation of Sp1 would be incompatible with cellular growth and differentiation and thus result in very early preimplantation lethality. However, we show that many putative target genes are expressed at normal levels in *Sp1*$^{-/-}$ mice. We suggest that other members of the Sp1 family, like the closely related Sp3 protein (Hagen et al., 1992; Imataka et al., 1992; Kingsley and Winoto, 1992), may compensate at least in part for the loss of Sp1 activity, ameliorating the effect of the *Sp1* knockout. Despite this potential for overlapping or compensatory functions early in embryogenesis, our data demonstrate that Sp1-dependent gene activation is absolutely essential for normal mouse development.

Sp1 and Nuclear Architecture

Transgenic mouse experiments investigating the CpG island of the *APRT* gene have suggested that Sp1 plays a role in the maintenance of methylation-free islands (Brandeis et al., 1994; Macleod et al., 1994). We have tested this hypothesis in *Sp1*$^{-/-}$ ES cells and embryos. Our analyses show that the methylation-free status of CpG islands in general, and that of the *APRT* island in particular, are faithfully maintained. Since integrity of Sp1 binding sites is required to keep the *APRT* island unmethylated (Brandeis et al., 1994; Macleod et al., 1994), we suggest that the binding of other family members may be sufficient to maintain the methylation-free status. The Sp3 protein would be an attractive candidate because of its ubiquitous expression pattern and its

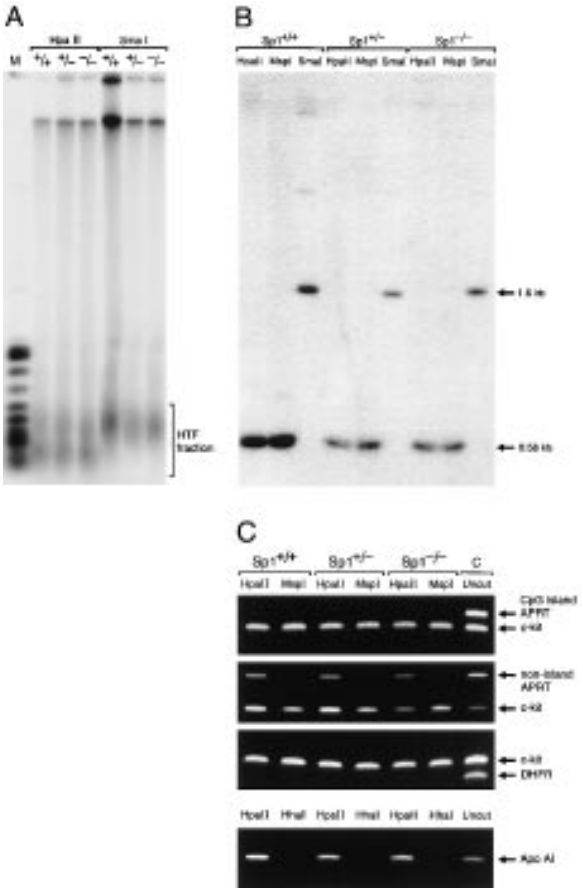

Figure 6. DNA Methylation in *Sp1*-Null ES Cells and Embryos

(A) DNA was isolated from wild-type, +/−, and −/− ES cells, digested with the methylation-sensitive enzymes HpaII or SmaI, end labeled with [α-^{32}P]dCTP, and electrophoresed on a 2% agarose gel. The fraction containing the methylation-free islands, known as the HpaII tiny fragments (HTF) fraction (Cooper et al., 1983), is indicated. The marker is pUC19 digested with MspI.

(B) DNA isolated from wild-type, +/−, and −/− ES cells was digested with the methylation-sensitive enzymes HpaII or SmaI, or with the methylation-insensitive HpaII isoschizomer MspI. The digested samples were run on a 1% agarose gel, Southern blotted, and hybridized to detect the methylation-free island of the *APRT* gene (Macleod et al., 1994). Fragment sizes are indicated on the right.

(C) DNA was isolated from wild-type, +/−, or −/− E9.5 embryos and digested with HpaII, HhaI, or MspI. The samples were subjected to PCR amplification to reveal the methylation status of individual CpG islands (*APRT*, *DHFR*, *ApoAI*) (Kafri et al., 1992). A fragment of the c-*kit* gene lacking HpaII and HhaI sites was used as an internal control for the amplification reaction. Note that for the *ApoAI* gene this control was done in a separate reaction because the c-*kit* and *ApoAI* fragments comigrate; the *ApoAI* island does not contain HpaII sites. A region of the *APRT* gene containing three partially methylated HpaII sites was amplified as a control for the detection of methylation (nonisland APRT) (Macleod et al., 1994). All the samples were redigested with the appropriate enzymes to show that the digests were complete (data not shown).

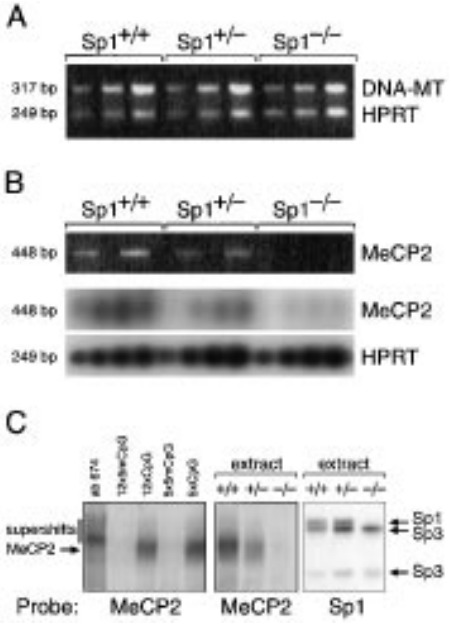

Figure 7. Expression of DNA Methyltransferase and MeCP2 in *Sp1*$^{-/-}$ Embryos

RT–PCR was performed as described for Figure 5. HPRT served as an internal control in the amplification reactions.

(A) Expression of DNA-methyltransferase.

(B) Expression of MeCP2. The amplification products were separated on 1.5% agarose gels, Southern blotted, and hybridized with the oligonucleotides used for PCR (bottom).

(C) Gel-retardation analysis of MeCP2 protein in +/+, +/−, and −/− E9.5 mouse embryos. The top part of the gel is shown. Extracts were made of three combined −/− embryos or individual +/+ and +/− embryos as indicated. Ten percent of the −/− extracts was used per reaction; the amount of extract was calibrated using probes binding Sp3 or Oct-1 (data not shown). Probes shown are an oligonucleotide containing five symmetrically methylated CpGs (MeCP2) and the Sp1 binding site (Figure 3C). The complex was identified as MeCP2 through (i) competition with a 100-fold molar excess of oligonucleotides with 5 or 12 symmetrically methylated CpGs but not with nonmethylated versions of the same oligonucleotides (left); (ii) competition with eukaryotic DNA containing methylated CpGs but not with E. coli DNA lacking methylated CpGs (data not shown); and (iii) supershifts with the MeCP2 antibody #674 (left) (no supershifts are observed with the methylated probe alone or in unrelated bandshift reactions [data not shown]). Quantization of MeCP2-binding activity is as follows (the wild-type level is arbitrarily set at 1): +/+, 1.00; +/−, 0.34; −/−, 0.04.

structural similarity to Sp1 (Hagen et al., 1992; Kingsley and Winoto, 1992).

Our work on the human β-globin LCR has shown that Sp1 binding sites are essential for LCR activity (Philipsen et al., 1990; 1993). This is best illustrated by 5′HS3 of the LCR, which contains 6 functional Sp1 binding sites (Philipsen et al., 1990). Importantly, 5′HS3 is the only LCR element capable of activating single-copy transgenes in mice (Ellis et al., 1996). The chromatin structure at 5′HS3 and the β-*globin* promoter is correctly remodeled in erythroid cells of these mice, suggesting that Sp1-like proteins are directly involved in chromatin opening. The phenotype of mice null for the erythroid-specific Sp1 family member EKLF (Nuez et al., 1995; Perkins et al., 1995) is consistent with this notion. These mice die of failure to express the β-*major* gene, and the DNase I hypersensitive site at the promoter is not formed (Nuez et al., 1995; Perkins et al., 1995; Wijgerde et al., 1996). Interestingly, the embryonic β- and α-*globin* genes are expressed normally, suggesting that other

Sp1-family members are involved. Since erythropoiesis in *Sp1*$^{-/-}$ mice is not severely affected, Sp1 does not appear to be a key activator of the erythroid program, at least in the embryonic stage.

Thus, our data do not support models in which Sp1 plays a pivotal role in the organization and maintenance of nuclear architecture.

Sp1 May Be Required for the Maintenance of Differentiated Cells

We have shown that *Sp1*$^{-/-}$ ES cells can contribute extensively to every tissue of E9.5 chimeras but not to later embryonic time points or newborn mice. This suggests that Sp1-deficiency causes a cellular defect and cell death only after commitment and/or differentiation have occurred. Thus, it could be predicted that the expression of genes linked to cellular maintenance would be reduced in the knockout.

We have identified two genes that are expressed at a lower level in *Sp1*$^{-/-}$ embryos, the *TK* gene and the *MeCP2* gene. The significance of the lower expression of the *TK* gene is not clear at the moment, in particular since other cell cycle-regulated genes are not affected, and *Sp1*$^{-/-}$ ES cells have normal growth rates and cell cycle distribution. In contrast, the reduced expression of the MeCP2 protein is very likely to contribute directly to the phenotype of *Sp1*$^{-/-}$ mice. The *MeCP2* gene is X-linked, and for this reason, the analysis of the phenotype of the *MeCP2* knockout has been limited to studies in ES cell-differentiation assays and chimeric embryos (Tate et al., 1996). ES cells lacking MeCP2 grow and differentiate normally. They contribute to every tissue of the early mouse embryo but fail to make a significant contribution to the newborn animal. Moreover, embryos with a high proportion of *MeCP2*$^{-}$ cells are retarded and unable to develop normally (Tate et al., 1996), as is found for embryos with a high contribution of *Sp1*$^{-/-}$ cells (Figure 4A). Thus, there are striking similarities between the phenotypes of the *MeCP2* and *Sp1* knockouts. MeCP2 is not a transcription factor in the classical sense, but it might perform a structural role in the nucleus and act as a transcriptional repressor. In wild-type mice, the amount of MeCP2 is limiting relative to the number of binding sites (Nan et al., 1997). Hence, the much lower level of MeCP2 found in *Sp1*$^{-/-}$ mice could be predicted to have an effect similar to the MeCP2 knockout. Interestingly, the levels of Sp1 (Saffer et al., 1991) and MeCP2 (Tate et al., 1996) increase during development, and there is a significant (2- to 3-fold) reduction of MeCP2 protein in heterozygous embryos, consistent with the notion of a close genetic interaction between Sp1 and MeCP2. It has been proposed that MeCP2 is required for the survival of differentiated cells (Tate et al., 1996), and thus we suggest that MeCP2-deficiency contributes significantly to the phenotype of *Sp1*$^{-/-}$ embryos.

It should be emphasized that there have to be many more Sp1 target genes than the *MeCP2* and *TK* genes identified in this paper. The early embryonic lethality of the mice described here, long before organogenesis has been completed, precludes an exhaustive survey of these target genes. Conditional inactivation (Kilby et al., 1993; Gu et al., 1994) of the *Sp1* gene will be required to address the role of Sp1 in fully developed tissues. In addition, such mice will be a valuable tool to establish the role of Sp1 in the maintenance of terminally differentiated cells.

Experimental Procedures

Construction of the Targeting Vectors

A 129 mouse cosmid genomic DNA library was screened with a probe spanning nucleotides 1820–2103 of pSp1–778c (Kadonaga et al., 1988) corresponding to the C-terminal end of Sp1. Six overlapping cosmids were isolated containing two exons encoding the zinc fingers and the C-terminus of Sp1. The neomycin targeting vector (pBSp1/*Neo*) is a promoterless construct containing 2 kb 5′ and 3.7 kb 3′ regions of homology to the *Sp1* gene in pBS II KS. The β-*geo* cassette containing a splice acceptor and the picornaviral IRES (Mountford et al., 1994) replaces the 2 kb NcoI fragment with the two exons encoding the zinc fingers and C-terminus of Sp1 (Figure 1). The hygromycin targeting vector (pBSp1/*hyg*) was constructed from pBSp1/*Neo* by replacing the NcoI-SmaI fragment containing the β-*geo* gene with a *hygromycin* gene (Figure 3).

Transfection and Analysis of ES Cells

E14 ES cells were cultured as described (Nuez et al., 1995). Clones were selected, after electroporation with 15 μg of XhoI-linearized targeting vector pBSp1/*Neo* or pBSp1/*hyg*, with G418 (200 μg/ml) or with hygromycin B (160 μg/ml) as appropriate. Homologous recombination was analyzed by Southern blotting using two external probes flanking the left and right homologous arms (Figure 1). A pBS probe was used to detect random integrations in the genome.

Generation of Chimeric and Sp1-Deficient Mice

Two independent *Sp1*$^{+/-}$ targeted clones were microinjected in C57BL/6 host blastocysts, and male chimeras with more than 80% contribution of ES cells to the coat were mated with C57BL/6 and FVB females. Germline transmission was analyzed by Southern blot analysis of tail DNA. *Sp1*$^{-/-}$ ES clones were injected in C57BL/6 blastocysts. Embryos were dissected at E9.5–11, fixed in 0.5% glutaraldehyde/1% formaldehyde in PBS, and stained with X-gal to identify *lacZ*-positive cells derived from the −/− ES cells (Bonnerot and Nicolas, 1993).

Genotyping of Embryos

Embryos from heterozygous matings were dissected, and a small part of the embryo was used for PCR analysis, as described, (Gurtner et al., 1995) using three primers: an antisense primer in the *Sp1* gene (5′-TTGGACCCATGCTACCTTGC-3′), a sense primer amplifying the wild-type allele (5′-AATTTGCCTGCCCTGAGTGC-3′; 378 bp product), and a sense primer in the *Neo* gene amplifying the targeted allele (5′-GCGCATCGCCTTCTATCG-3′; 690 bp product). PCR was done for 30 cycles (94°C, 1 min; 60°C, 1 min; 72°C, 1 min).

Gel Mobility-Shift Assays

Two micrograms of nuclear protein extracts from wild type and mutant ES cells or E9.5 embryos (Andrews and Faller, 1991) was used per reaction in gel mobility shifts (Wall et al., 1988) using the dimer Sp1 binding site from the SV40 early promoter (Philipsen et al., 1990). Sp1- and Sp3-specific antibodies were kindly provided by Dr. Guntram Suske (Marburg). Gel mobility-shift assays for MeCP2 (Lewis et al., 1992) contained 0.5 ng of labeled oligonucleotide with 5 symmetrically methylated CpGs, 50 ng of cold nonmethylated version of the oligonucleotide, and protein extract. The gels were quantitated by PhosphorImage analysis. MeCP2-specific antibody #674 was a kind gift of Drs. Xinsheng Nan and Adrian Bird (Edinburgh). Methylated oligonucleotides were synthesized by Isogen BV (Maarssen NL).

Western Blotting

Thirty micrograms of protein per lane was separated on a 10% SDS–PAGE gel, blotted on a PVDF membrane, and probed with a rabbit polyclonal antibody recognizing Sp1 or a mouse monoclonal

antibody recognizing the 89 kDa subunit of TFIIH (a kind gift of Dr. Jean-Marc Egly, Strassbourg). The Sp1 antibody recognizes the N-terminus of Sp1 that has not been deleted in the knockout. Bands were visualized using peroxidase-conjugated secondary antibodies.

DNA Methylation Analysis

DNA from ES cells or pooled embryos was digested with HhaI, SmaI, HpaII, or MspI. ES cell DNA was end labeled with [α-^{32}P]dCTP and run on a 2% agarose gel to reveal the fraction containing the methylation-free islands (Cooper et al., 1983). The methylation status of the *APRT* methylation-free island was analyzed as described (Macleod et al., 1994). In DNA from mouse embryos, the methylation status of the *APRT*, *DHFR*, and *ApoAI* islands was analyzed by PCR (Kafri et al., 1992). As a control for DNA methylation, we amplified a region in the *APRT* gene containing three HpaII sites that are known to be partially methylated (Macleod et al., 1994). As a control for the PCR reactions, we amplified a fragment of the c-*kit* gene lacking HpaII and HhaI sites. PCR reactions were carried our for 30 cycles (94°C, 1 min; 55°C, 1 min; 72°C, 1 min) with $<$100 ng of genomic mouse DNA.

Gene-Expression Analysis by RT–PCR

Total RNA was isolated from E8.5 embryos and used as a template for reverse transcription primed by oligo(dT). One-fiftieth of the synthesized cDNA was used for PCR (94°C, 30 s; 55°C, 30 s; 72°C, 45 s), and aliquots were taken after variable numbers of cycles. The amplified fragments were visualized on 1.5% agarose gels, Southern blotted, and hybridized with the oligonucleotides used in the PCR for quantization by PhosphorImage analysis.

The primer sets used to amplify CpG islands and cDNAs, the sizes of the amplification products, gel-retardation probes, and more detailed experimental procedures are available upon request via e-mail (philipsen@CH1.Fgg.EUR.NL).

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