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 (Dyman 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 TAF110 (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) (Karseder et al., 1996), dihydrofolate reductase (DHFR) (Lin et al., 1996) and b-myb (Zwickert 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 (Karseder 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 CpG islands, this suggests that Sp1 prevents 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.
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 (Kadowaga 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 littersmates, 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

<table>
<thead>
<tr>
<th>Stage</th>
<th>Total</th>
<th>×/+</th>
<th>×/-</th>
<th>×−/−</th>
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<tr>
<td>E7.5</td>
<td>75</td>
<td>18 (24%)</td>
<td>43 (57%)</td>
<td>14 (19%)</td>
</tr>
<tr>
<td>E8.5</td>
<td>137</td>
<td>35 (26%)</td>
<td>74 (54%)</td>
<td>28 (20%)</td>
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<tr>
<td>E9.5</td>
<td>143</td>
<td>34 (24%)</td>
<td>79 (55%)</td>
<td>30 (21%)</td>
</tr>
<tr>
<td>E10.5</td>
<td>78</td>
<td>24 (31%)</td>
<td>36 (46%)</td>
<td>18 (23%)</td>
</tr>
<tr>
<td>E11.5</td>
<td>33</td>
<td>11 (33%)</td>
<td>18 (55%)</td>
<td>4 (12%)</td>
</tr>
<tr>
<td>Pups</td>
<td>97</td>
<td>37 (38%)</td>
<td>60 (62%)</td>
<td>0 (0%)</td>
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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 embryos occurs before E10.5.
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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 Sp**1**+/− 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).
We conclude that the defect in Sp1<sup>−/−</sup> 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<sup>−/−</sup> 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-idiuromidase, α-N-acetylgalactosaminidase, β-glucuronidase, 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 (Koivistö 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<sup>−/−</sup> 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 e') are
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Figure 4. Contribution of Sp1<sup>−/−</sup> ES Cells to Chimeric Embryos

CS7BL/6 host blastocysts were injected with 12-14 Sp1<sup>−/−</sup> 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<sup>−/−</sup> 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.

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 Smal and HpaII, end labeled, size fractionated, and autoradiographed (Figure 6A). Because the bulk DNA is heavily methylated and contains relatively few HpaII and Smal sites, it is cut infrequently. However, CpG islands are normally unmethylated, contain many HpaII and Smal 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 Smal, HpaII, and the methylation-insensitive HpaII isoschizomer MspI (Figure 6B). No significant difference in the methylation of the HpaII and Smal sites in the APRT CpG island was observed between +/+, +/−, and −/− Sp1 ES cells. In fact, the Smal 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<sup>−/−</sup> 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<sup>−/−</sup> mice. In addition, we checked the methylation status of the CpG islands in the DHFR (Kafri et al., 1992) and ApoA1 (Shemeret 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
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+/− genes is not apparent 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; Ima-taka 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
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Figure 7. Expression of DNA Methyltransferase and MeCP2 in Sp1<sup>−/−</sup> 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 1/1, 1/2, and 2/2 E9.5 mouse embryos. The top part of the gel is shown. Extracts were made of three combined 2/2 embryos or individual 1/1 and +/+ embryos as indicated. Ten percent of the −/− extracts was used per reaction; the amount of extract was calibrated using probes (A) DNA was isolated from wild-type, 1/+, and 2/2 ES cells, digested with HpaII or SmaI, and electrophoresed on a 2% agarose gel. The fraction containing the methylation-free islands, known as MeCP2 through (i) competition with a 100-fold molar excess of 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 HpaII or SmaI, and with the methylation-insensitive HpaII isoschizomere 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 −/− 9.5 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 DNA 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 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, and labeled with [α-<sup>32</sup>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 HpaII or SmaI, or with the methylation-insensitive HpaII isoschizomere 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 −/− 9.5 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 DNA 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).

The LCR, which contains 6 functional Sp1 binding sites (Philipersen 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 Sp1−/− mice is extensive to every tissue of E9.5 chimeras but not newborn mice.

*Experimental Procedures*

**Construction of the Targeting Vectors**
A 129 mouse cosmide 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 K5. 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-Smal 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 (Nue et al., 1995). Clones were selected, after electroporation with 15 μg of Xhol-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, (Gurten 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′-AATTTCCTGGCTTTGAGTG-3′; 378 bp product), and a sense primer in the Neo gene amplifying the targeted allele (5′-GGGATCCTGGCCCTATCGTG-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 (Maarsen 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. J ean-Marc Egly, Strasbourg). 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, Smal, HpalI, orMspI. ES cell DNA was end labeled with [α-32P]CTP and run on a 2% agarose gel to reveal the fraction containing the methylated- 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 out 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 ES5 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 recognition site screening of two novel GT box binding proteins: a

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