Functional analysis of the transcription factors Sp3 and Sp4

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D r u k w e r k Optima Grafische Communicatie, Rotterdam

C o v e r Edematous Sp3 deficient E13.5 embryo

I S B N 90 77017 39 9

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Functionele analyse van de transcriptie factoren Sp3 en Sp4

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
Rector Magnificus

Prof. dr. ir. J. H. van Bemmel

en volgens besluit van het College voor Promoties
De openbare verdediging zal plaatsvinden op

woensdag 9 januari 2002 om 15.45 uur

door

Roelof Jan Pieter Bouwman

geboren te Groningen

Promotiecommissie

Promotor	Prof. dr. F. G. Grosveld
Overige leden	Prof. dr. E. A. Dzierzak
	Dr. A. O. Brinkmann
	Dr. H. R. Delwel
Co-promotor	Dr. J. N. J. Philipsen

Dit proefschrift kwam tot stand binnen de vakgroep Celbiologie en Genetica aan de faculteit der Geneeskunde en Gezondheidswetenschappen van de Erasmus Universiteit Rotterdam. De vakgroep maakt deel uit van het Medisch Genetisch Centrum Zuid-West Nederland.

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Chapter one - Introduction

Regulation of transcription by the Sp-family of transcription factors

1.1 General introduction and aim of this thesis

Biological processes are largely controlled by the synthesis and the modulation of proteins. For the synthesis of proteins, two steps are of critical importance. Firstly, the genetic information for the protein has to be transcribed from the DNA of the gene encoding the protein to the mRNA intermediate and secondly the mRNA has to be translated into the amino acid sequence that forms the protein.

For the regulation of transcription, control of initiation is of critical importance. In eukaryotes, the transcription of genes to mRNA is accomplished by RNA polymerase II that acts in a complex with a number of multi-subunit accessory factors (reviewed in Lemon and Tjian, 2000). One of these factors, basal transcription factor IID (TFIID), is essential for the initiation of transcription of a gene through its interaction with the core promoter, a regulatory sequence present 30-40 bp upstream or downstream of the transcriptional start site. The interaction of TFIID with the core promoter is often established through the TATA box, an AT-rich sequence 25-30 bp upstream of the start site that can be bound by the TFIID subunit TATA binding protein (TBP). Another motif that can be found in many promoters is the initiator (Inr; Smale and Baltimore, 1989) that encompasses the RNA start site and consists of the sequence Py-Py-A_{+1}-N-(T/A)-Py-Py. Many promoters contain functionally important sequences downstream of the promoter and one of these motifs with the putative consensus sequence (A/G)G(A/T)CGTG and called DPE (downstream promoter element) can interact with Inr to provide a binding site for TFIID in TATA-less promoters (Burke and Kadonaga, 1996; Burke and Kadonaga, 1997). This implicates a role for TFIID in the specificity of transcription initiation, which can also can be deduced from the specific interaction of some TBP associated factors (TAFs) with distinct sequence specific transcription factors (Chen *et al.*, 1994a; reviewed in Verrijzer and Tjian, 1996). In addition, certain TAFs may show a tissue restricted interaction with TFIID as appeared to be the case for TAF(II)105 in B cells (Dikstein *et al.*, 1996).

The basal transcription machinery formed by the RNA polymerase II complex is not sufficient for the adequate initiation of transcription *in vivo*. In addition, sequence specific DNA binding proteins or transcription factors are required that bind to proximal promoter elements within 50-200 bp upstream of the TATA box and stabilize the interactions of the pre-initiation complex with the DNA. Apart from the proximal promoter, transcription factors also bind to so-called enhancer elements that are located more distally from the TATA box, up to several kilobases upstream or downstream. Enhancers regulate transcription independent of their position or orientation relative to the start site (Banerji *et al.*, 1981; Moreau *et al.*, 1981) and can directly interact with the proximal promoter upon transcription factor binding, thus looping out the intervening DNA (Li *et al.*, 1991; Mueller-Storm *et al.*, 1989; Su *et al.*, 1991). Such a looping model is also applicable to another type of distal regulatory element, the locus control region (LCR) that regulates the

expression of a whole gene locus in a position independent manner (reviewed in Grosveld, 1999).

Although transcription factors like Sp1 can directly interact with TBP and TAFs, it has recently become clear that they require additional cofactor complexes to fully potentiate transcription activation (reviewed in Rachez and Freedman, 2001). Interestingly a cofactor complex like CRSP (cofactor required for Sp1 activation; Ryu *et al.*, 1999) shares subunits with other cofactors like the mediator (Kim *et al.*, 1994), the TRAP/DRIP (Fondell *et al.*, 1996; Naar *et al.*, 1999; Rachez *et al.*, 1998) and the NAT (Sun *et al.*, 1998) complex which suggests that they are functionally related.

In eukaryotes, transcription takes place in the nucleus where the DNA is packed into a chromatin structure. At its basic level of organisation, chromatin consists of DNA wrapped around a repetitive array of protein cores or nucleosomes formed by the histone proteins H2A, H2B, H3 and H4 (two copies of each per core). Packaging of DNA into chromatin has important consequences for the regulation of transcription as is exemplified by the inability of proteins like TBP to bind to their consensus site if it is occluded by a nucleosome (Imbalzano *et al.*, 1994). Therefore, transcription activation by sequence specific transcription factors is tightly connected to the action of chromatin modifying complexes. Currently two major types of chromatin modifiers are known, one that modifies histone polypeptides covalently and another that alters nucleosome location or conformation in an ATP-dependent manner (reviewed in e.g. Kadonaga, 1998; Kingston and Narlikar, 1999; Kuo and Allis, 1998; Wu and Grunstein, 2000). The first identified member of the latter category is the yeast SWI/SNF complex, a multiprotein complex that is required for normal mating type switching and/or sucrose fermentation. SWI/SNF related complexes in human include the erythroid Krüppel like factor (EKLF) coactivator remodelling complex 1 (E-RC1), that specifically interacts with transcription factor EKLF in the activation of the β-globin promoter (Armstrong *et al.*, 1998). Possible covalent modifications of histones include phosphorylation, ubiquitination, methylation, and acetylation. Of these, acetylation has been most intensively studied in relation to transcriptional regulation which has lead to the identification of several histone acetyltransferases (HAT) and histone deacetylases (HDACs). HAT activity is often associated with *trans*activation whereas a decreased acetylation via HDACs results in repression of transcription. The interplay with transcription factors is complex which is illustrated by the fact that they can not only target histone acetylation (e.g. Kundu *et al.*, 2000) but also can be regulated by acetylation themselves (e.g. Boyes *et al.*, 1998; Zhang and Bieker, 1998; Zhang *et al.*, 2001).

In summary, the initiation of transcription is accomplished via the interactions of many different proteins with common and gene specific regulatory motifs. Clearly, sequence specific transcription factors play a crucial role in the specificity of transcription initiation.

A group of related sequence specific DNA binding proteins that has been implicated in the regulation of many different processes is the Sp/XKLF family of transcription factors (reviewed in Philipsen and Suske, 1999; Turner and Crossley, 1999). Binding sites for these

transcription factors (GC/GT boxes) are present in the transcription regulatory regions of a diverse set of genes. These include the human β-globin locus control region that consists of 5 erythroid specific *cis*-acting elements of which at least one depends on the integrity of Sp/XKLF DNA motifs (Philipsen *et al.*, 1993).

In the erythroid lineage as well as in other cell types the simultaneous occurrence of several homologous GC/GT box binding factors precludes a readily deduction of their role in transcriptional regulation. The availability of powerful techniques for the genetic modification of mice enables us to overcome this problem. With the objective to analyze the function of Sp/XKLF members Sp3 and Sp4 *in vivo*, we have disrupted their genes in the mouse. In this thesis, an initial description of phenotypical characteristics of both knockout mice is presented.

Outline of this thesis

After an introduction on the Sp-subgroup of Sp/XKLF transcription factors in chapter 1, chapters 2, 3, and 4 describe different aspects of the phenotype of Sp3 knockout mice. Sp3 is essential for survival during late gestation and in the perinatal period. Null mutants are growth retarded and display abnormalities in for instance late tooth development, heart functioning and lymphopoiesis.

Sp4-/- mutants, described in chapter 5, show an apparently normal development *in utero* and during the first week after birth but then become severely growth retarded. A high percentage of Sp4 deficient mice die around weaning and suffer from starvation. Surviving knockout mice remain small and the males display an impaired mounting behaviour and do not reproduce.

Finally, in chapter 6 the phenotypes of Sp knockout mice are discussed in relation to each other.

1.2 The Sp/XKLF family of transcription factors

Many different prokaryotic and eukaryotic proteins use zinc-coordinated motifs to bind to DNA. One common type of these so called zinc fingers consists of a beta sheet and an alpha helix that contain two cysteine and two histidine residues that contact the zinc atom. These C2H2 zinc fingers are often found in clusters that allow each of their alpha helices to tightly interact with the major groove of the double stranded DNA helix (Pavletich and Pabo, 1991).The amino acid composition of the zinc fingers determines their DNA binding specificity and by using them in different arrangements DNA binding proteins can recognize the specific sequences of nucleotides to which they bind.

One particular combination of three conserved Cys2His2 zinc fingers forms the DNA binding motif of the still expanding Sp/XKLF (Specificity Protein/Krüppel Like Factor) family of transcription factors (reviewed in Philipsen and Suske, 1999; Turner and Crossley, 1999). Krüppel like factors have been named after the *Drosophila* segmentation gene Krüppel that shows a similar arrangement of zinc fingers (Schuh *et al.*, 1986). In human, the Krüppel-like three zinc finger motif was first found in Sp1 (Kadonaga *et al.*, 1987). The zinc fingers of the Sp/XKLF family are structurally related to those of the transcription factor Zif268/Egr-1 and therefore are likely to contact the DNA in the same fashion as has been determined for this protein (Kriwacki *et al.*, 1992; Kuwahara *et al.*, 1993; Narayan *et al.*, 1997; Pavletich and

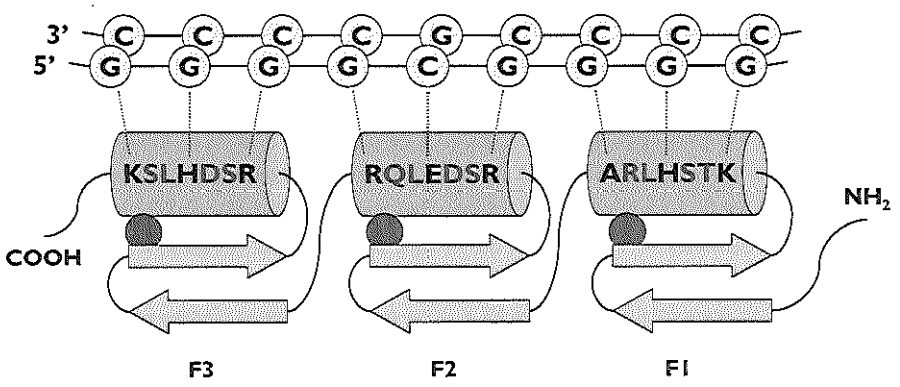

FIGURE 1: **Schematic drawing of the three Sp1 zinc fingers interacting with a classical GC box.**
The individual fingers (F1, F2 and F3) are depicted as a β sheet (arrows) and an α helix (cylinder) held together by a zinc ion (dark grey sphere). DNA containing a classical GC box (5'GGGGCGGGG 3') is shown as a double array of beads on strings with the nucleotide pairs partially overlapping. The amino acids in the α helices are indicated and interactions between critical residues (black) in each finger and a specific triplet of nucleotides of the GC box are shown with dotted lines. Orientations of protein and DNA are marked.

Pabo, 1991). Accordingly family founder Sp1 is thought to contact the DNA with the amino acids KHA in the first, RER in the second and RHK in the third zinc finger (Figure 1). As a consequence of the conserved DNA binding motif, Sp/XKLF members all recognize the same GC- (GGGGCGGGG) and GT- (GGTGTGGGG) boxes albeit with different affinities due to substitutions of critical amino acids in the first (L for H in Sp2) or in the third (K for L in e.g. EKLF, UKLF, BKLF) finger. GC- and GT- boxes are important for the expression of many different ubiquitous as well as specifically regulated cellular and viral genes. In addition, these motifs are required for the maintenance of the methylation-free CpG island that is thought to play a role in transcriptional regulation of the adenine phosphoribosyltransferase (APRT) gene (Brandeis *et al.*, 1994; Macleod *et al.*, 1994).

As the name of the family already indicates, it can be subdivided into at least two major subgroups. Firstly the Sp proteins named after transcription factor Sp1, that are virtually identical in their zinc finger region but also have similar N-terminal motifs and secondly a more heterogeneous group including the KLFs. Although many Krüppel like factors are unified by the aforementioned amino acid substitution in the third zinc finger they can differ substantially at their N-terminus. Nevertheless, a further subdivision in groups with common structural motifs is possible (Philipsen and Suske, 1999).

The Sp/XKLF family comprises a large number of homologous transcription factors of which at the moment already 20 members have been cloned in human. With the completion of the genomic sequencing projects (Lander *et al.*, 2001; Venter *et al.*, 2001), it will be only a matter of time before the full family has been identified. Knowing all the members of the family raises one important question: what determines their specificity? Obviously, the regulation of transcription via GC and GT boxes by these proteins is a complex process that needs to be tightly controlled. Factors like EKLF (erythroid Krüppel like factor) or Sp4 show tissue restricted expression patterns and their *in vivo* requirement in those tissues became apparent after gene targeting experiments in mice (Gollner *et al.*, 2001a; Nguyen-Tran *et al.*, 2000; Nuez *et al.*, 1995; Perkins *et al.*, 1995; Supp *et al.*, 1996, this thesis). Others like Sp1 and Sp3 are ubiquitously expressed but also fulfill distinct functions as has been indicated by various *in vivo* and *in vitro* experiments (chapters 1.4; Bouwman *et al.*, 2000; Gollner *et al.*, 2001b; Marin *et al.*, 1997; this thesis).

Since the work presented in this thesis concerns transcription factors Sp3 and Sp4, the Sp1 subfamily will be discussed in more detail in the next chapter.

1.3 Structural characteristics of the Sp1 subfamily

Sp1, the first cloned member of the Sp1 subfamily (Kadonaga *et al.*, 1987; Kadonaga *et al.*, 1988), was identified as a *trans*activator of the SV40 (Simian Virus 40) early promoter (Dynan and Tjian, 1983a; Dynan and Tjian, 1983b). It was long thought that Sp1 would be

essential for the transcriptional control of many of the genes that are regulated via GC or GT boxes. However, that view changed dramatically when shortly after each other several new members of what was to become the Sp/XKLF family of transcription factors were cloned (e.g. Hagen *et al.*, 1992; Imataka *et al.*, 1992; Kingsley and Winoto, 1992; Miller and Bieker, 1993; Sogawa *et al.*, 1993).

The structural element that defines the relationship between these proteins is the conserved three zinc finger DNA binding motif that has been discussed in the previous chapter. The zinc fingers are not only responsible for the similar binding site specificities of individual members, they are also involved in interactions with other proteins (discussed in chapter 1.5.5).

Within the Sp/XKLF family Sp1, Sp2, Sp3 and Sp4 form a subgroup based on homology in the zinc finger domain and their similar modular structure (Figure 2). Sp3 (SPR-2) and Sp4 (SPR-1) were cloned in a search for GT box binding factors that would be responsible for cell type specific activation of the rabbit uteroglobin promoter (Hagen *et al.*, 1992). Independently, Sp2 and Sp3 were cloned from a human T cell library during a search for proteins containing the Sp1 zinc finger domain (Kingsley and Winoto, 1992).

Sp1, Sp3, and Sp4 contain 2 major glutamine rich *trans*activation domains (A and B, Figure 2) that are essential for transcription activation. Next to these A and B domains, serine/threonine rich sequences are located that may be targets for post-translational modification (chapter 1.5.4). While Sp2 has only one glutamine rich domain, it does share a highly charged domain C and a serine/threonine rich region with the other members of the subfamily. Another connection between the human Sp1-4 genes is that they co-localize with the four HOX gene clusters on chromosomes 12q13 (Sp1/HOX C) (Matera and Ward, 1993), 17q21.3-q22 (Sp2/ HOX B) (Scohy *et al.*, 1998), 2q31 (Sp3/HOX D) (Kalff-Suske *et al.*, 1996) and 17p15 (Sp4/HOX A) (Kalff-Suske *et al.*, 1995) suggesting an evolutionary relationship.

Nevertheless, Sp2 is more distantly related to Sp1 than Sp3 and Sp4. This distant relationship is also reflected in a different consensus binding site affinity due to the substitution of a critical histidine residue 1 to a leucine residue in zinc finger.

Apart from the similar functional domains, Sp1, 3, and 4 also show some unique structural features that have been linked to their *trans*activation potential and that will be discussed in chapter 1.4.

Recently several new Sp/XKLF genes have been cloned and based on the composition of their zinc finger domains two of them, Sp5 (Harrison *et al.*, 2000; Treichel *et al.*, 2001) and Sp6 (Scohy *et al.*, 2000) have been added to the Sp-subfamily.

The DNA binding domain of Sp6 has been described as more closely related to that of Sp2 than that of the other Sp factors (Scohy *et al.*, 2000), although from its amino acid composition it might be better to conclude that both Sp2 and Sp6 are more distantly

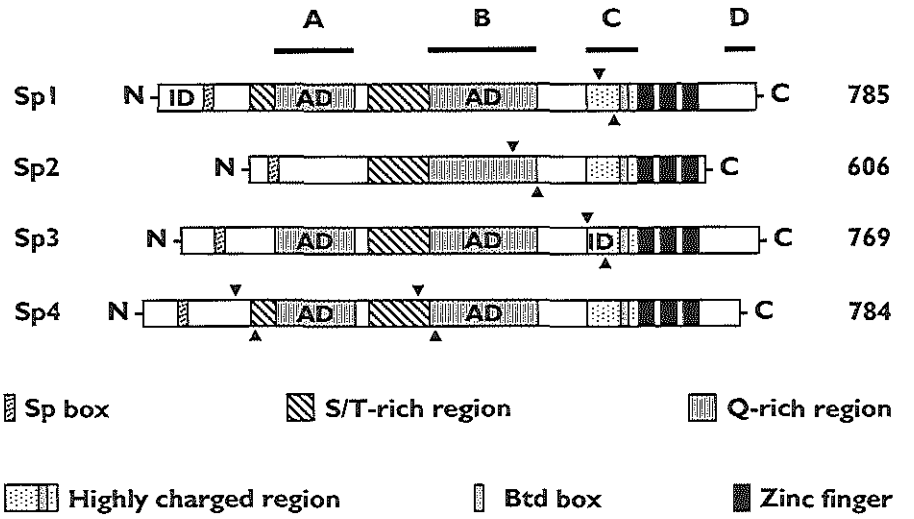

FIGURE 2: **Schematic representation of human Sp1-4 showing their similar modular structure.**
Sp and Btd boxes, serine/threonine-rich, glutamine-rich and highly charged regions and zinc fingers are indicated, as well as activation (AD) and inhibitory (ID) domains. A, B, C and D modules of Sp1 (Courey and Tjian, 1988) are marked with black bars. Each pair of arrow heads points at a PEST domain with a significant PESTfind score (>5.0; Rogers et al., 1986). On the right: lengths in amino acids according to accession numbers P08047 (Sp1), Q02086 (Sp2), CAC34575 and Q02447 (Sp3) and CAA48563 (Sp4).

FIGURE 3: **The Drosophila Btd box is conserved in Sp1-6.**
Alignment of Btd boxes (rectangle; Wimmer et al., 1993) from drosophila Btd (accession number CAA82545) and D-Sp1(CAB55429), human Sp1 (P08047), Sp2 (Q02086), Sp3 (Q02447) and Sp4 (CAA48563), mouse Sp5 (AAF87798), zebrafish "Sp5" (AAK83353) and human Sp6 (AC003665). Strictly and highly (>75%) conserved amino acids are indicated with a dark grey and a light grey background respectively.

Btd	R R S V R C T C P N C
dSp1	. . R A T C D C P N C
hSp1	T R R E A C T C P Y C
hSp2	R R R M A C T C P N C
hSp3	L R R V A C T C P N C
hSp4	L R R V A C S C P N C
mSp5	R R C R R C R C P N C
dSp5	R R C R R C R C P N C
hSp6	T R R E A C R C P N C

related to Sp1 than Sp3 and Sp4. Nevertheless, chromosomal localization revealed that the Sp2 and Sp6 genes are closely linked both in rat (10q31-q32.1) and in human (17q21.3-q22) (Scohy *et al.*, 2000; Scohy *et al.*, 1998) which has led to the suggestion that they share the same ancestral founder gene (Scohy *et al.*, 2000).

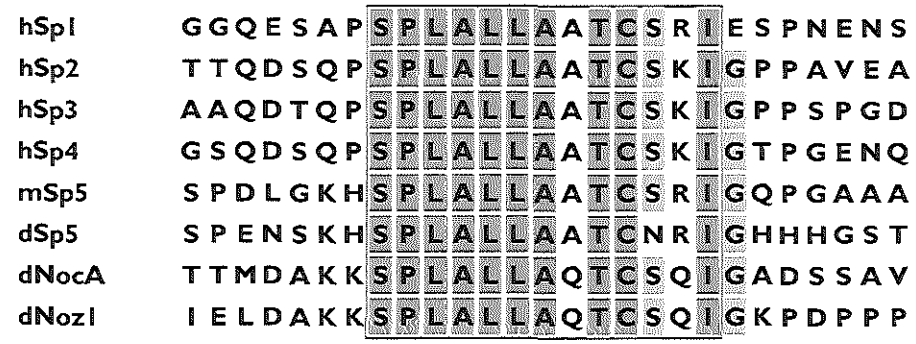

FIGURE 4: **The Sp box is conserved in Sp1-5 and Drosophila and zebrafish NocA-like proteins.**
Alignment of Sp box (rectangle) containing sequences from human Sp1 (accession number P08047), Sp2 (Q02086), Sp3 (CAC34575) and Sp4 (CAA48563), mouse Sp5 (AAF87798), zebrafish "Sp5"(AAK83353), drosophila NocA (A55929) and zebrafish Noz1 (AAK08969). Strictly and highly (>75%) conserved amino acids are indicated with a dark grey and a light grey background respectively.

At the N-terminal site of their Sp-like zinc finger domain, Sp5 contains in addition a so-called Buttonhead box (Harrison *et al.*, 2000; Figure 3). This conserved stretch of 11 amino acids was originally identified in the *Drosophila* Sp1 homologue Buttonhead (Btd; Wimmer *et al.*, 1993). It is also present in Sp1-4 but not in other Sp/XKLF family members and may contribute to the *trans*activation potential, since a deletion of an overlapping region resulted in reduced activity of Sp1 *in vitro* (Courey and Tjian, 1988). Furthermore, domain C (Yieh *et al.*, 1995), and more specifically the Btd element within domain C (Athanikar *et al.*, 1997), is involved in synergistic activation by Sp1 or Sp3 with sterol regulatory element-binding proteins (SREBPs; see also chapter 1.5.5). Interestingly, one proline and three cysteine residues of the Btd domain are also conserved in Sp6 and another *Drosophila* Sp1 related protein D-Sp1 (Wimmer *et al.*, 1996), indicating that they may have a crucial role in the function of this domain. The Btd box is evolutionarily conserved in Sp-factors as it is also present in a recently cloned zebrafish homologue of Buttonhead that is almost identical to Sp5 (accession number AAK83353; Figure 3). Harrison and co-workers (Harrison *et al.*, 2000) identified another stretch of conserved amino acids consisting of the sequence SPLALLAATCSR/KI (Sp box) that is located at the N-terminus of Sp1, 2, 3, 4, and 5 and can also be found in "zebrafish Sp5" (Figure 4). This element contains an endoproteolytic cleavage site and is situated close to a region at the N-terminus of Sp1 that targets proteasome-dependent degradation of C-terminal sequences *in vitro* (Su *et al.*, 1999; see also chapter 1.5). Although it is not required to direct cleavage, the fact that the Sp box is highly conserved in Sp1-5 might indicate that this motif has a function in the regulation of proteolysis. Another possible role for the Sp box may lie in the control of *trans*activation

potential via the interaction with a putative repressor protein that interacts with the N-terminus of Sp1 (Murata *et al.*, 1994). Besides in Sp-proteins, sequences similar to the Sp box can also be found in the *Drosophila* NocA zinc finger protein (Cheah *et al.*, 1994), a zebrafish homologue Noz1 or NocA-like (Andreazzoli *et al.*, 2001; Sagerstrom *et al.*, 2001) and putative human homologues (accession numbers NP 079345, XP 053438; Figure 4). Genetic mutations indicated that NocA is involved in the development of embryonic brain and the adult ocellar structures (Cheah *et al.*, 1994). However, the protein has not been studied in detail and therefore the relevance of its Sp-like box remains to be explored.

Although the function of the Btd and Sp boxes are not clear at the moment, their presence in Sp-proteins but not in the other Sp/XKLF members confirms the relationship between these transcription factors and suggests that they have specific roles in the regulation of gene expression.

With the exception of the Btd and the Sp boxes however, the N-terminal regions of Sp5 and Sp6 are completely different from those of Sp1-4 and therefore Sp5 and Sp6 will not be discussed in the remainder of this chapter.

1.4 Functional analysis of the Sp1 subfamily: transactivation properties

1.4.1 Introduction on transactivation assays in Drosophila Schneider cells

Studies on the *trans*activation potential of Sp1, Sp3 and Sp4 as described below were performed *in vivo* in transiently transfected *Drosophila* Schneider SL2 cells unless indicated otherwise. The ideal host for functional analysis would be a mammalian cell line not expressing these proteins. However, when these experiments were performed, it was not yet known that mammalian cells lacking important transcription factors like Sp1 could be viable (Marin *et al.*, 1997). The reason for the use of SL2 cells was that Sp1-like proteins were thought not to be present in *Drosophila*. Although this has been challenged by the cloning of the *Drosophila* Sp1 homologues Buttonhead (Wimmer *et al.*, 1993) and D-Sp1 (Wimmer *et al.*, 1996), SL2 cell extracts are reportedly devoid of Sp1-like activities (Courey and Tjian, 1988; Santoro *et al.*, 1988). Nonetheless, it has become increasingly clear that there are important differences in the composition of the transcription machinery between different cell types, not to mention those between different species. Therefore, the results regarding *trans*activation potential should always be related to the system in which they were obtained.

1.4.2 Sp1

The presence of an activity in a Hela cell fraction termed Sp1 that could stimulate

transcription in a promoter dependent manner *in vitro* was the first direct evidence of the existence of sequence specific transcription factors (Dynan and Tjian, 1983a). After some initial experiments with this heterogeneous fraction (e.g. Gidoni *et al.*, 1984; Gidoni *et al.*, 1985; Jones and Tjian, 1985), Briggs and co-workers succeeded in further purification, resulting in one protein that could recapitulate this specific *in vitro trans*activation and which was named Sp1 (Briggs *et al.*, 1986). On basis of a partial amino acid sequence of this purified protein, the cDNA encoding Sp1 could be cloned thus allowing a more detailed study of its *trans*activational properties (Kadonaga *et al.*, 1987; Kadonaga *et al.*, 1988).

It was discovered that Sp1 can directly interact with itself which has important implications for its *trans*activation potential (Pascal and Tjian, 1991). Sp1 is not only able to stimulate transcription from proximal promoters but also from distal enhancers (Courey *et al.*, 1989). *In vitro* experiments suggest that Sp1 tetramers are involved in the synergistic activation via distant sites (Mastrangelo *et al.*, 1991) that enable them to interact with each other, thus looping out the intervening DNA (Li *et al.*, 1991; Mastrangelo *et al.*, 1991; Mueller-Storm *et al.*, 1989; Su *et al.*, 1991). For the multimerisation, activation domain B appeared to be of critical importance (Pascal and Tjian, 1991). Together with domain A, domain B also mediates superactivation of Sp1 dependent transcription which can be achieved by non-DNA binding mutants in case of multiple binding sites (Courey *et al.*, 1989; Hagen *et al.*, 1995; Pascal and Tjian, 1991). For synergistic activation via binding to multiple sites, domain D is required in addition to both *trans*activation domains (Pascal and Tjian, 1991).

1.4.3 Sp2

Since the binding site specificity of Sp2 differs from that of the other Sp proteins (Kingsley and Winoto, 1992), the inability of Sp2 to activate promoters containing GC boxes (Chen *et al.*, 1998; Rotheneder *et al.*, 1999; Zhao and Chang, 1997) can be readily explained. Data from the only report of a promoter that is affected by co-transfected Sp2 indicate that this transcription factor may function in a cell type dependent manner. Sp2 represses Sp1 or Sp3 driven activation of a construct containing the murine CTP:phosphocholine cytidylyltransferase α promoter in Drosophila cells but activates the same construct in C3H10T1/2 mammalian cells (Bakovic *et al.*, 2000). It seems likely that Sp2 has different characteristics than Sp1,3, and 4 since it has only one glutamine rich *trans*activation domain whereas two domains are required for superactivation and synergistic activation by Sp1 (Pascal and Tjian, 1991).

1.4.4 Sp3

Although Sp3 was found to be highly homologous to Sp1 with similar affinities for GC and GT boxes, it soon became clear that there are some striking functional differences. Sp3 can

activate transcription from different promoters in SL2 cells as well as in certain mammalian cell lines (e.g. Ding *et al.*, 1999; Galvagni *et al.*, 2001; Ihn and Trojanowska, 1997; Liang *et al.*, 1996; Udvadia *et al.*, 1995; Zhao and Chang, 1997) and upon cotransfection with Sp1 additive (e.g. Ihn and Trojanowska, 1997; Ko *et al.*, 1998) and synergistic (e.g. Bigger *et al.*, 1997; Netzker *et al.*, 1997) effects were noticed. However, under other circumstances Sp3 is at most weakly active and in case of promoters containing multiple adjacent binding sites, it can repress transcription driven by Sp1 or other transcription factors (Birnbaum *et al.*, 1995; De Luca *et al.*, 1996; Dennig *et al.*, 1996; Majello *et al.*, 1997). Intriguingly, there is also a report that shows that Sp1 can inhibit the Sp3-mediated *trans*activation of the mouse growth hormone L2 promoter in Schneider cells (Yu *et al.*, 1999). It should be noted that some promoters could be activated by Sp3 in *Drosophila* cells but not in certain mammalian cells (Hansen *et al.*, 1999) and vice versa (Sjottem *et al.*, 1996) which further demonstrates the complex nature of this transcription factor.

1.4.5 Sp4

As was shown for Sp3, the functional properties of Sp4 turned out to be different from those of Sp1 despite obvious structural similarities. Compared with Sp1, Sp4 shows similar *trans*activation potential through its glutamine rich activation domains. In addition, Sp4 can be superactivated by fingerless Sp1 and repressed by Sp3 (Hagen *et al.*, 1992). However, whereas Sp1 can synergistically activate promoters containing multiple binding sites (Courey *et al.*, 1989; Pascal and Tjian, 1991), *trans*activation by Sp4 only occurs in an additive manner (Hagen *et al.*, 1995). The *trans*activation potential of Sp4 with respect to different promoters and cell types has not been studied as intensively as in the case of Sp1 or Sp3. Several promoters could be activated by Sp4 in mammalian cell lines as well as in *Drosophila* cells (Ahlgren *et al.*, 1999; Hagen *et al.*, 1995; Hagen *et al.*, 1994; Majello *et al.*, 1994; Rotheneder *et al.*, 1999; Wong *et al.*, 2001) but others only seemed to respond to different family members (Kwon *et al.*, 1999; Yan *et al.*, 2000).

1.5 Specificity in the regulation of transcription by Sp1 family members

1.5.1 Introduction

As was shown in the previous chapter, Sp1 family members have different functional properties and most likely fulfill specific roles in the regulation of biological processes. Unique functions *in vivo* are demonstrated by the clear and different phenotypes of Sp1, Sp3, and Sp4 knockout mice (Bouwman *et al.*, 2000; Gollner *et al.*, 2001a; Gollner *et al.*, 2001b; Marin *et al.*, 1997; Nguyen-Tran *et al.*, 2000; Supp *et al.*, 1996; this thesis). On the other

hand, under certain circumstances apparently depending on cellular conditions and promoter context, Sp-factors seem to be at least partly redundant. This chapter focuses on various control mechanisms that could determine the combined effect of these highly homologous proteins on transcription regulation.

1.5.2 Expression

The presence of Sp1, Sp3 and perhaps also Sp2, in many if not all different cell types does not imply that the levels of these proteins are not subject to specific regulation. Whereas there are no published data for Sp2 with respect to this topic, there are several reports on the control of Sp1 and Sp3 expression.

Expression of Sp1 differs between cell types and during development and is down regulated in many fully differentiated cells. (Saffer *et al.*, 1991). Murine Sp1 mRNA can undergo alternative splicing (Persengiev *et al.*, 1995; Takahara *et al.*, 2000) which during spermatogenesis leads to an N-terminally truncated protein that lacks the first glutamine rich *trans*activation domain and both serine/threonine rich domains (Persengiev *et al.*, 1995). This protein is severely impaired in its potential to *trans*activate from single binding sites but can act synergistically with full length Sp1 in *Drosophila* Schneider cells. It is not clear whether it has a function *in vivo* since it normally seems to be expressed at relatively low levels.

Although the available amount of Sp1 protein appears to be regulated to a large extent at the mRNA level (Saffer *et al.*, 1991), there are also other control mechanisms. As mentioned before (chapter 1.3), Sp1 undergoes proteasome dependent degradation under conditions of nutrient starvation and adenylate cyclase stimulation (Han and Kudlow, 1997). Initiation of this process is thought to be determined by a low glycosylation state of Sp1 (Han and Kudlow, 1997); see also chapter 1.5.4) and consists of an endoproteolytic cleavage triggered by an N-terminal region of Sp1 (Su *et al.*, 1999).The cleavage site is situated in the Sp-box that is conserved in Sp1-5 (chapter 1.3), which may indicate that proteolysis of other Sp-proteins can be regulated in a similar fashion.

The N-terminal peptide that directs cleavage remains stable and might have a functional role, for instance via interactions with the proteasome-dependent system (Su *et al.*, 1999) or with other proteins (Murata *et al.*, 1994; chapter 1.5.5). Interestingly, under other circumstances C-terminal Sp1 peptides have been found. Despite the presence of both ends of Sp1 mRNA, in naive human myeloid leukemia cells the GC box is bound by a truncated, approximately 30 kDa DNA binding Sp1 isoform and full length Sp1 protein can only be found after differentiation (Rao *et al.*, 1998). Treatment of full length Sp1 with the serine protease myeloblastin, that is downregulated during differentiation, yields a fast migrating binding activity similar to that in undifferentiated cells. Therefore, myeloblastin or a related protease might provide a switch that regulates Sp1 dependent transcription through limited proteolysis. In such a scenario, the small C-terminal peptide could act as a transcription inhibitor since it lacks the *trans*activation domains but does contain the DNA binding zinc

finger region. Apoptosis also coincides with Sp1 proteolysis (Piedrafita and Pfahl, 1997; Rickers *et al.*, 1999) and the action of a caspase-3 like protease in B cells can produce a similarly truncated DNA binding Sp1 isoform (Rickers *et al.*, 1999). The caspase-3 cleavage site is contained in a region of domain C (Figure 2) that has earlier been identified as a PEST sequence, a putative target motif for inducible proteolysis (Mortensen *et al.*, 1997; Rechsteiner and Rogers, 1996; Rogers *et al.*, 1986). Although this specific sequence is not conserved in the other Sp proteins, they all contain a PEST motif at a different positions (Figure 2). Sp1 is also degraded by the cathepsin-like protease termed SPase that is expressed in the green monkey kidney cell line CV-1 (Nishinaka *et al.*, 1997). SPase displays some specificity in its action since it targets Sp1 and the phosphorylated retinoblastoma susceptibility gene product (Rb) but not other nuclear factors such as c-Jun or c-Fos. Although not assessed directly, it is likely that SPase also degrades Sp3 - and perhaps other Sp/XKLF factors - given the complete depletion of different GC-box binding proteins from extracts treated with this protease (Chen *et al.*, 1994b; Nishinaka *et al.*, 1997). Interestingly, in the context of known interactions of Sp1 with cell cycle regulators (e.g. Datta *et al.*, 1995; Karlseder *et al.*, 1996; Lin *et al.*, 1996), SPase expression is regulated through the cell-cycle with high levels at the G_1/S transition (Fu *et al.*, 1998).

Often, for several cell types and promoters, Sp1 and Sp3 have been identified as the major GC/GT box binding activities. Variation in especially the expression of Sp1 and Sp3 may have important consequences for transcription activation given the dual nature of Sp3 that can function as an activator as well as an inhibitor.

There are a number of reports that show variations in the ratio Sp1/Sp3 under different cellular conditions. Sp3 was originally cloned as one of the factors binding to the GT box of the uteroglobin promoter that is active in endothelial cells from lung and endometrium (Hagen *et al.*, 1992). Interestingly, a recent report suggests that the Sp1/Sp3 ratio is involved in the up-regulation of tissue factor expression upon progestin-induced decidualization of human endometrial stromal cells (HESCs, Krikun *et al.*, 2000). Progestin treatment of HESCs results in an up-regulation of the *trans*activator Sp1 whereas Sp3 which is unable to enhance activation of the tissue factor promoter, is down-regulated.

In another endothelial cell line, human umbilical vein endothelial cells (HUVECs), hypoxia enhances the amount of Sp1 protein while Sp3 levels remain unaltered (Xu *et al.*, 2000). Hypoxia induces a similar change in the Sp1/Sp3 ratio in myoblasts, albeit in these cells this is achieved via the post-transcriptional downregulation of Sp3 protein levels (Discher *et al.*, 1998).

In addition, relatively high Sp1/Sp3 ratios are also seen in epithelial cells compared to fibroblasts (Apt *et al.*, 1996) and in endothelial cells compared to non-endothelial cells (Hata *et al.*, 1998). It should be noted that the expression of both Sp1 and Sp3 was found to be much higher in the endothelial than in the non-endothelial cell lines (Hata *et al.*, 1998).

In most cases, the increase of the Sp1/Sp3 ratio has been correlated with the increased expression of responsive genes. In their respective cellular contexts, these genes can be

activated by Sp1 and repressed by Sp3 which suggests that transcription is regulated via the cooperative action of both transcription factors.

*Trans*activation by Sp3 may also be dependent upon the regulation of alternative translation initiation. The nucleotides surrounding the first two internal AUG codons allow reasonably efficient translation initiation (Kozak, 1986; Kozak, 1987) giving rise to ± 70 kDa N-terminal truncated Sp3 isoforms (Kennett *et al.*, 1997). It has been found that the activation of a reporter gene by Sp1 or full length Sp3 is inhibited by the smaller isoforms (Kennett *et al.*, 1997; Whetstine and Matherly, 2001). Since these truncated Sp3 molecules lack part of the *trans*activation domains, it seems likely that they are less potent activators and repress transcription by competing for binding sites. It was then suggested that the contradictory results obtained with Sp3 in *trans*activation assays are a consequence of differences in Sp3 expression constructs leading to different ratios of full length versus truncated isoforms (Kennett *et al.*, 1997). This may indeed explain some results, as has been indicated in experiments with specifically designed constructs (Pan *et al.*, 2000; Whetstine and Matherly, 2001), but nto all since full length Sp3 can also repress Sp1-mediated transcription (Dennig *et al.*, 1996; Fandos *et al.*, 1999).

Nevertheless, regulation of alternative translation initiation probably provides an extra level of control in the regulation of Sp3-dependent transcription. Although the functional relevance of such a mechanism in biological processes still needs to be explored, it has recently been shown that the ratio between full length protein and isoforms of the expected size can indeed be subject to regulation. Glucose treatment of human hepatoma cells results in a increased level of full length protein versus reduced amounts of the smaller isoforms (Moon *et al.*, 1999). A different process takes place during early myoblast differentiation when there is a specifically pronounced up-regulation of the ± 70 kDa isoforms (Fandos *et al.*, 1999). There is also a report of a 28 kDa protein specifically detected with Sp3 antibodies in decidualized endometrial stromal cells (Gao and Tseng, 1996) but the nature of this protein is unknown. The same holds true for a fast migrating Sp3 complex that seems to replace the alternatively transcribed proteins in the uterus upon pregnancy (Tu *et al.*, 1998). Possibly small DNA binding isoforms of Sp3 are a result of proteolysis as has been shown for Sp1 (Rao *et al.*, 1998).

Whereas Sp1 and Sp3 are ubiquitously expressed and Sp2 mRNA has been detected in various different cell lines, Sp4 shows a more restricted expression pattern (Hagen *et al.*, 1992; Kingsley and Winoto, 1992; Saffer *et al.*, 1991; Supp *et al.*, 1996; chapter 5 of this thesis). Throughout murine development, high levels of Sp4 mRNA have predominantly been found in the brain (Supp *et al.*, 1996). The robust expression of Sp4 in the central nervous system correlates with the impaired mounting behaviour of Sp4-/- mutant males (Gollner *et al.*, 2001a; Nguyen-Tran *et al.*, 2000; Supp *et al.*, 1996; this thesis). Another aspect of the Sp4 knockout phenotype is sudden cardiac arrest and this could be linked to the specific expression of Sp4 in the conductive system of the heart (Nguyen-Tran *et al.*, 2000). The recent identification of the murine Sp4 gene will be helpful in the further

characterization of its regulation (Song *et al.*, 2001). Preliminary experiments indicate that Sp1 but not Sp3 has a repressive effect on its proximal promoter (Song *et al.*, 2001).

1.5.3 Binding site specificity

Obviously, Sp2 is the least homologous member of the subfamily since it lacks a second glutamine-rich *trans*activation domain and, perhaps more importantly, because it has a different binding site specificity. *In vitro* translated Sp2 binds weakly to a critical GT box of the T cell antigen receptor variable gene segment Vα11.1 promoter and not at all to an Sp1 consensus GC box (Kingsley and Winoto, 1992). Based on these data, it seems likely that Sp2 is involved in the regulation of a different set of genes than Sp1, Sp3 and Sp4 that all bind to GC and GT boxes with similar affinities (Hagen *et al.*, 1992). However, binding affinities determined *in vitro* can not always be extrapolated to the *in vivo* situation where events like post-translational modifications and interactions with chromatin and other proteins play a role. There are several examples of promoter or enhancer sequences that are specifically bound by distinct members of the Sp1 family *in vivo*. For instance, Sp2 has been shown to bind to a GC box containing region that appears to be essential for the activity of the methionine adenosyltransferase II (MATII) promoter in Jurkat cell extracts (Halim *et al.*, 2001). Strikingly, whereas Sp3 and Sp4 were also part of the complexes bound to this region, Sp1 could not be detected. Another example is a GT motif in the neuronal nicotinic acetylcholine receptor β4 promoter that specifically interacts with Sp1 and Sp3 in extracts from a neuronal cell line and from rat brain (Bigger *et al.*, 1997). Although Sp2 and Sp4 are present in these cells, there was no evidence that they could bind to this motif while Sp4 antibodies did show binding of this factor to a consensus GC box.

1.5.4 Post-translational modifications

Glycosylation

Like many other transcription factors, Sp1 is subject to post-translational modifications which can influence its activity. Early evidence for the significance of these processes was obtained when *Escherichia coli*-synthezised human Sp1 turned out to be a less effective *trans*activator *in vitro* than HeLa cell derived protein (Kadonaga *et al.*, 1988). The two major types of post-translational modification that are thought to be involved in transcription regulation by Sp1 are glycosylation and phosphorylation.

There are no published data that show glycosylation of the other Sp-proteins, but they do contain putative glycosylation sites (Hagen *et al.*, 1992; Kingsley and Winoto, 1992). This may indicate that glycosylation plays no role in the specificity of different Sp-subfamily members in transcription regulation. Nevertheless, because of the lack of experimental evidence for such an assumption, the data on glycosylation of Sp1will be discussed here.

O-glycosylation has been related to the nuclear localisation, the stability and/or the *trans*activation potential of Sp1, which is repressed in the recombinant protein (Kadonaga *et al.*, 1988) and when the O-linked N-Acetylglucosamine (GlcNAc) monosaccharide residues are bound by lectin (Jackson and Tjian, 1988). However, direct evidence for the effect on Sp1 function had not been obtained and with respect to *trans*activation potential, it has recently been shown that glycosylation can have adverse effects (Roos *et al.*, 1997; Yang *et al.*, 2001). One of the explanations suggested for an increased *trans*activation was that GlcNAc residues might act as ligands for recognition by components of the transcription machinery (Jackson and Tjian, 1988). In contrast, the opposite of such a mechanism has been observed for the glycosylation of the carboxy-terminal part of Sp1 activation domain B (Roos *et al.*, 1997). Interactions between this domain and dTAF(II)110 and full-length Sp1 are markedly decreased upon glycosylation which correlates with a decreased *trans*activation potential *in vitro* (Roos *et al.*, 1997) and *in vivo* (Yang *et al.*, 2001).

Glucose deprivation in combination with adenylate cyclase stimulation results in reduced glycosylation of Sp1, associated with an increased susceptibility to proteasome dependent degradation (Han and Kudlow, 1997). It has recently been shown that the ATPase activity of 26S proteasome subunit Sug-1 (also known as p45 or TRIP1) is involved in this degradation and that Sug-1 can directly interact with Sp1 (Su *et al.*, 2000). The process is blocked in cells treated with glucosamine, a metabolic derivative of glucose that is used primarily as a substrate for protein glycosylation (Han and Kudlow, 1997). Interestingly, the majority of the GlcNAc residues lie within the N-terminal part of Sp1 (Jackson and Tjian, 1988), the first 54 amino acids of which trigger the endoproteolytic cleavage that forms the first step in the degradation of the protein (Su *et al.*, 1999). It has been suggested that glycosylation blocks protein interactions and prevents Sp1 from entering into protein complexes that are readily degraded by proteasomes (Roos *et al.*, 1997).

Phosphorylation

O-glycosylated proteins are also phosphoproteins and there is some evidence indicating that both types of Sp1-modification may be reciprocally regulated (Du *et al.*, 2000; Haltiwanger *et al.*, 1998). Upon electrophoresis of mammalian cell extracts, two variants of Sp1 can be distinguished with apparent masses of 95 and 105 kDa that arise by differential phosphorylation. It has been shown that Sp1 becomes phosphorylated at its N-terminus by DNA-dependent protein kinase upon binding to DNA (Gottlieb and Jackson, 1993; Jackson *et al.*, 1990). Also the C-terminus of Sp1 can be phosphorylated which has been linked to cell cycle progression from G_0 to G_1. *In vitro* data suggest that the unknown kinase that mediates this phosphorylation specifically targets serine residues in the most N-terminal zinc fingers 1 and 2 (Black *et al.*, 1999) . Whereas these types of phosphorylation do not seem to affect DNA binding activity, threonine phosphorylation of the zinc finger domain of Sp1 by casein kinase II does result in a reduced affinity for the Sp1 consensus binding site

(Armstrong *et al.*, 1997). Although there are several additional reports that implicate Sp1 phosphorylation with decreased binding activity (e.g. Borellini *et al.*, 1990; Leggett *et al.*, 1995; Schafer *et al.*, 1997; Zhu and Liao, 2000), phosphorylation can also result in increased binding (e.g. Haidweger *et al.*, 2001; Kumar and Butler, 1998; Merchant *et al.*, 1999; Rafty and Khachigian, 2001; Rohlff *et al.*, 1997). In some cases, increased binding through phosphorylation has been correlated with enhanced *trans*activation (e.g. Merchant *et al.*, 1999; Pal *et al.*, 1998; Rafty and Khachigian, 2001; Zheng *et al.*, 2001). For instance, protein kinase c-ζ (PKC-ζ) mediated phosphorylation of Sp1 in smooth muscle cells stimulates platelet-derived growth factor β-chain (PDGF-β) expression (Rafty and Khachigian, 2001). Interestingly, PKC-ζ, that can directly interact with the Sp1 zinc finger region (Pal *et al.*, 1998), has no apparent effect on two other factors that bind the PDGF-β promoter, Egr-1and Sp3 (Rafty and Khachigian, 2001).

Apparently, certain kinases can specifically regulate the transcriptional activity of distinct Sp-proteins. Even in the highly conserved zinc finger region there are differences in the localisation of serine or threonine residues that enable specificity in the modification by kinases and phosphatases. Nevertheless, it is likely that there are also forms of phosphorylation that effect different Sp-factors in an equal manner. Sp1 for instance interacts with cyclin A and can be phosphorylated via a cyclin A associated kinase (Haidweger *et al.*, 2001). As a result, DNA binding is increased concomitantly with *trans*activation mediated via Sp1 binding sites. However, Sp3 DNA binding is similarly affected and Sp3 as well as Sp4 have also been reported to interact with cyclin A, suggesting a common regulatory pathway (Haidweger *et al.*, 2001).

1.5.5 Cooperative interactions with other proteins

There are few data on functional interactions between Sp-factors and proteins other than those directly involved in the transcription machinery. Sp1, Sp2 and perhaps the other Sp's as well may be bound by proteins that repress their *trans*activation potential, but the nature of these putative inhibitors is not known (Murata *et al.*, 1994). In another report, it has been suggested that the physical association of Sp1 with the nuclear pore protein p62 reflects a role for this protein in nuclear translocation or in the spatial organisation of gene transcription (Han *et al.*, 1998). The specific repressive interaction of the retinoblastoma-related protein p107 with Sp1 indicates that Sp-proteins may be differentially regulated through the cell cycle (Datta *et al.*, 1995).

Sequence specific transcription factors

Most data on proteins that functionally interact with Sp1 involve other sequence specific transcription factors. Examples include ubiquitous factors like Oct-1(Strom *et al.*, 1996) NF-κB (Pazin *et al.*, 1996; Perkins *et al.*, 1994; Perkins *et al.*, 1993) and E2F-1(Karlseder *et*

al., 1996; Lin *et al.*, 1996) but also tissue specific regulators like MEF-2 (Grayson *et al.*, 1998) and GATA proteins (Merika and Orkin, 1995). Together with Sp1, these transcription factors can synergistically activate transcription of various target genes. It is not clear to what extent these interactions provide specificity in Sp-mediated transcription, since often the highly conserved zinc finger region of Sp1 is involved in direct physical interactions. Indeed, it has recently been shown that not only Sp1 but also Sp2, Sp3 and Sp4 can physically interact with E2F-1 (Rotheneder *et al.*, 1999). On the other hand, apart from the zinc fingers also non-conserved domains can play a role as has been shown for Sp1 and NF-κB in case of the HIV-1 promoter (Majello *et al.*, 1994). The interaction of Smad3 with Sp1 but not with Sp3 demonstrates that distinct Sp-proteins can specifically cooperate with other transcription factors (Inagaki *et al.*, 2001).

Sequence specific transcription factors can also repress each other's activity which can be achieved via several different mechanisms. The pro-myelocytic leukemia protein (PML) associates with the zinc fingers containing C-terminal region of Sp1 and prevents it from binding to DNA (Vallian *et al.*, 1998). Interestingly, PML does not affect other complexes associated with the same DNA motif which may indicate that it possesses a specificity for Sp1 and not for other Sp/XKLF factors. Often inhibition of transcription occurs via competitive binding of transcriptional repressors to or nearby activator binding sites. Because of its dual nature especially Sp3 seems an attractive candidate to act as a negative switch in Sp-mediated transcription regulation. In those cases that an inactive Sp3 inhibits *trans*activation mediated by other Sp/XKLF members, it seems likely that this is a result of competition for the same binding sites. In support of such a scenario, it has been shown that fingerless Sp3 mutants are not able to suppress Sp1 or Sp4 mediated *trans*activation (Hagen *et al.*, 1995; Hagen *et al.*, 1994). However, fingerless Sp3 can also repress transcription induced by Sp1 or unrelated transcription factors if tethered to a promoter via a heterologous DNA binding domain (De Luca *et al.*, 1996; Majello *et al.*, 1994) Moreover, repression is independent of the distance to the transcription start site (Majello *et al.*, 1994) and can even be achieved from a nascent RNA target in case of the human immunodeficiency virus type 1 long terminal repeat (HIV-1 LTR) promoter (De Luca *et al.*, 1996).

*Trans*activation assays with mutant proteins reveal that the *trans*activation potential of Sp3 is influenced by an inhibitory domain (Dennig *et al.*, 1996; Majello *et al.*, 1997). This domain resides in a highly charged stretch of amino acids that is not present in the comparable region of Sp1 (domain C, Figure 2) and which resembles repressor domains present in C/EBPβ (Williams *et al.*, 1995), C/EBPε(Angerer *et al.*, 1999), c-Fos and FosB (Brown *et al.*, 1995). The presence of a repressive module explains the earlier observed inactivity of the N-terminal region of Sp3 despite the presence of glutamine-rich domains that resemble the Sp1 *trans*activation domains A and B (Hagen *et al.*, 1994). Mutation of a critical KEE amino acid triplet results in relief of repression and potentiates Sp3 *trans*activation, especially of promoters containing multiple binding sites (Dennig *et al.*, 1996).

Exactly how the inhibitory domain exerts its function remains to be resolved. However, it has been shown to function autonomously, independent of its context or position (Dennig *et al.*, 1996). Therefore, it seems likely that it functions in *trans* via interactions with other proteins. Supporting this hypothesis, recently the cloning of a protein termed SIF-1 (Sp3-interacting factor) has been reported that specifically interacts with the intact Sp3 inhibitory domain (Suske, 1999). Overexpression of the free inhibitory domain has no effect on Sp3 dependent transcription (Dennig *et al.*, 1996) which is in accordance with the experiments that show that Sp3 can only repress transcription when tethered to a promoter (De Luca *et al.*, 1996; Dennig *et al.*, 1996; Hagen *et al.*, 1994). Therefore, it is tempting to speculate that the interaction with a protein like SIF-1 somehow affects the basal transcription machinery. So far, there is no direct evidence for such a mechanism, but further experiments with SIF-1 might clarify this issue. The physical and functional interactions of dTAF110 with Sp3, the only component of the basal transcription machinery that was tested, are not affected after deletion of the inhibitory domain (Dennig *et al.*, 1996).

The basal transcription machinery

A central theme in the regulation of gene expression by transcription factors is the communication with the basal transcription machinery. Together with other complexes like the Sp1 cofactor CRSP, the TFIID subunit of the RNA polymerase II holocomplex plays a critical role in these interactions. Sp1 can directly interact with TBP (Emili *et al.*, 1994) and dTAF(II)110/hTAF(II)130 via the glutamine rich activation domains A and B (Gill *et al.*, 1994; Hoey *et al.*, 1993; Tanese *et al.*, 1996) and with hTAF(II)55 through the C-terminal domain (Chiang and Roeder, 1995). Interestingly TAF(II)250 plays a important role in the frequently observed stimulation of Sp1 transcriptional activity by Rb (Shao *et al.*, 1995). Apart from the cooperation between Sp3 and dTAF(II)110 (Dennig *et al.*, 1996), that is required for Sp1 mediated *trans*activation *in vitro* (Chen *et al.*, 1994a), nothing is known about the interactions of other Sp's with the basal transcription machinery. Therefore, it remains to be resolved whether basal factors confer any specificity on transcription regulation by different Sp-proteins.

Chromatin modifier complexes

Transcription regulation *in vivo* takes place in a chromatin environment which may affect the activity of individual transcription factors in a differential manner. Synergistic activation by Sp1 and SREBP-1a *in vitro* requires chromatin and a multiprotein coactivator complex that includes CREB binding protein (CBP; Naar *et al.*, 1998). In transient transfection assays, as mentioned before, the Btd box conserved in Sp1-5 is needed for synergistic activation of the low-density lipoprotein (LDL) promoter that contains a single SREBP binding site

(Athanikar *et al.*, 1997). Although this might suggest some degree of redundancy in the Sp-family, Sp2 can not substitute for Sp1 indicating that other domains are also necessary. Moreover, deletion of the Btd box has no effect on other promoters that contain multiple SREBP binding sites. Nevertheless Sp3 can replace Sp1 in synergistic activation with SREBP-1 and could possibly equally well cooperate equally well with the coactivator. As expected from a CBP containing complex, the coactivator harbors HAT activity but it is not clear whether histone acetylation has any functional relevance for SREBP-1a/Sp1 synergy (Naar *et al.*, 1998).

Sp1 can also be a direct target for histone deacetylase 1 (HDAC1)-mediated transcriptional repression (Doetzlhofer *et al.*, 1999). However, binding involves the carboxy-terminal domain including the conserved zinc fingers and the observed repression is not likely to be specific for Sp1. Indeed, Sp3 has also been reported to interact with HDAC1 (Doetzlhofer *et al.*, 1999).

In summary, the functional characteristics of individual Sp-factors are only partly overlapping despite the high degree of homology. Their activity in transcriptional regulation can be tightly controlled and is dependent upon the cellular conditions and promoter context.

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Chapter two

Transcription factor Sp3 is essential for post-natal survival and late tooth development

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The EMBO Journal **19**, 655-661, 2000.

Transcription factor Sp3 is essential for post-natal survival and late tooth development

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Sp3 is a ubiquitously expressed transcription factor closely related to Sp1 (specificity protein 1). We have disrupted the mouse Sp3 gene by homologous recombination. Sp3-deficient embryos are growth retarded and invariably die at birth of respiratory failure. The cause for the observed breathing defect remains obscure since only minor morphological alterations were observed in the lung, and surfactant protein expression is indistinguishable from that in wild-type mice. Histological examinations of individual organs in $Sp3^{-/-}$ mice show a pronounced defect in late tooth formation. In Sp3 *null* mice, the dentin/enamel layer of the developing teeth is impaired due to the lack of ameloblast-specific gene products. Comparison of the Sp1 and Sp3 knockout phenotype shows that Sp1 and Sp3 have distinct functions *in vivo*, but also suggests a degree of functional redundancy.

Keywords: knockout/post-natal death/Sp1/Sp3/tooth development

Introduction

Many housekeeping, tissue-specific and viral genes contain functionally important GC and related GT/CACC boxes. It has been known for some time that the general transcription factor Sp1 (specificity protein 1) (Kadonaga *et al.*, 1987) can bind to and act through these elements, and it was generally accepted that Sp1 is involved in the expression of many different genes. More recently, however, it became clear that Sp1 is not the only protein acting through 'Sp1-binding sites' but represents the first identified and cloned protein of a large, still growing family of transcription factors united by the presence of a highly conserved DNA-binding domain consisting of three zinc fingers. This Sp/XKLF superfamily of proteins includes the Sp transcription factors, their close relatives BTEB1, TIEG1 and TIEG2, and the Krüppel-like factors (reviewed in Philipsen and Suske, 1999).

The Sp subfamily of transcription factors is composed of four proteins (Sp1, Sp2, Sp3 and Sp4) characterized by very similar structural features (Suske, 1999). In addition to the highly conserved DNA-binding domain, all four proteins contain glutamine-rich activation domains adjacent to serine/threonine-rich stretches in their N-terminal region. The linkage to the four human Hox gene clusters also documents their close evolutionary relationship (Kalff-Suske *et al.*, 1995, 1996; Scohy *et al.*, 1998). Sp1, Sp3 and Sp4 are more closely related to each other than to Sp2 (Philipsen and Suske, 1999; Suske, 1999). Consistently, Sp1, Sp3 and Sp4 recognize the classical GC box and the related GT/CACC box with identical affinity (Hagen *et al.*, 1992, 1994). Sp3 and Sp1 are ubiquitously expressed, unlike Sp4, which shows a complex expression pattern but is most abundant in neuronal tissues (Supp *et al.*, 1996).

A large variety of biological functions have been assigned to Sp1-binding sites. This raises the question of which of the tasks are performed by which protein *in vivo*. This question is particularly interesting for Sp1 and Sp3 because both proteins are ubiquitously expressed. Mice lacking Sp1 and Sp4 have been reported (Supp *et al.*, 1996; Marin *et al.*, 1997). The most interesting aspect of the Sp4 phenotype is the complete absence of mating behavior in Sp4 *null* males. Since their reproductive organs are fully developed and apparently normal, the most likely cause of this behavioral abnormality is a neurological defect.

Sp1 *null* embryos are severely growth retarded, and die after day 10 of embryonic development (E10). They display a wide range of abnormalities, but all characteristic hallmarks up to this developmental stage are present. Blastocyst injections of Sp1 *null* embryonic stem (ES) cells showed that these cells contribute efficiently to early chimeric embryos, but after embryonic day 11 (E11) this declines very rapidly with no detectable contribution to any tissue of newborn animals. Thus, Sp1 deficiency causes a cell-autonomous defect, and it appears that Sp1 function is generally required for cellular survival after E10 (Marin *et al.*, 1997).

It was surprising that the Sp1 *null* embryos survive until the post-implantation stage. More than 3000 publications have implicated this factor in the activation of a very large number of genes and in cellular processes such as cell cycle regulation, chromatin remodeling and the propagation of methylation-free CpG islands. Thus, a cell lacking Sp1 would be predicted to stand little chance of surviving, but surprisingly Sp1 *null* ES cells have normal growth characteristics and survival rates. It has been suggested that this might be due to the presence of Sp3, the most closely related Sp family member (Marin *et al.*, 1997).

Sp3 has been shown to act as a transcriptional activator on many promoters similarly to Sp1 (e.g. Udvadia *et al.*, 1995; Liang *et al.*, 1996; Ihn and Trojanowska, 1997;

Zhao and Chang, 1997). In other promoter settings, however, Sp3 remained inactive or acted only as a weak activator (e.g. Hagen *et al.*, 1994; Majello *et al.*, 1994; Dennig *et al.*, 1995; Kumar and Butler, 1997). Biochemically, the most obvious differences between Sp3 and Sp1 are the presence of a potent inhibitory domain in Sp3 (Dennig *et al.*, 1996) and the existence of three Sp3 isoforms (Kennett *et al.*, 1997; Suske, 1999). However, the significance of these differences for *in vivo* functions remains an open question.

Here we describe the targeted disruption of the mouse Sp3 gene by homologous recombination and analysis of Sp3 *null* mice. We find that the absence of Sp3 has consequences for mouse development that are very different from those observed in Sp1 knockout mice. Sp3 *null* mice develop until birth with no obvious gross abnormalities other than a reduction in body weight. After natural birth or Caesarian section, the knockout mice invariably die within 10 min, apparently of respiratory failure. Histological examination revealed minor structural abnormalities in the lungs. Furthermore, the mice show a pronounced defect in dentin/enamel layer formation of the developing teeth. We conclude that Sp1 and Sp3 may have similar, and therefore redundant, functions during early development but exert distinct and highly specific functions in later developmental stages.

Results

Isolation of mouse Sp3 gene fragments

Primers derived from the human Sp3 cDNA were used to amplify a fragment of the mouse Sp3 gene by PCR. The primers A3/K (5′-CAGATCATTCCTGGCTCT-3′) and A3/L (5′-TCTAGATCGACACTATTGAT-3′) that produced a unique 210 bp fragment with human and mouse genomic DNA were used to screen a genomic mouse ES cell P1 phage library (Genome Systems Inc.). A single P1 clone of ~80 kbp was mapped and several fragments were subcloned into the Bluescript KS vector. Sequence analyses revealed that both activation domains of Sp3 are encoded by a single large exon (Figure 1A).

Targeted disruption of the mouse Sp3 gene

The target vector pBS-P-B-Δ-lacZ-neo-5′-Sp3 (Materials and methods) was designed to substitute exon 2 [amino acids 38–490 according to Yajima *et al.* (1998)] of the murine Sp3 gene for IRES-LacZ-neo-polyA sequences (Figure 1A). The *lacZ–neo* fusion gene is expressed under the control of the endogenous Sp3 promoter. The deleted sequences encode both glutamine-rich activation domains of Sp3 (Dennig *et al.*, 1996). Thus, the disruption should result in a *null* allele.

Plasmid pBS-P-B-Δ-lacZ-neo-5′-Sp3 was linearized at a unique *Not*I site present in the vector for transfection into E14 ES cells. Cells were maintained subsequently under G418 selection. A total of 36 G418-resistant colonies were analyzed by Southern blotting for the homologous recombination event. Hybridization of *Eco*RI-restricted DNA from individual clones with an *Eco*RI–*Pst*I intron fragment was predicted to show a >11 kbp fragment from the wild-type locus and a 5.4 kbp fragment from a correctly targeted locus (Figure 1A). Fourteen clones showed the predicted mutant fragment of 5.4 kbp (Figure 1B). In addition, PCR analysis with a set of primers specific for the wild-type and the targeted Sp3 gene (Figure 1C) confirmed the targeted disruption of the mouse Sp3 gene.

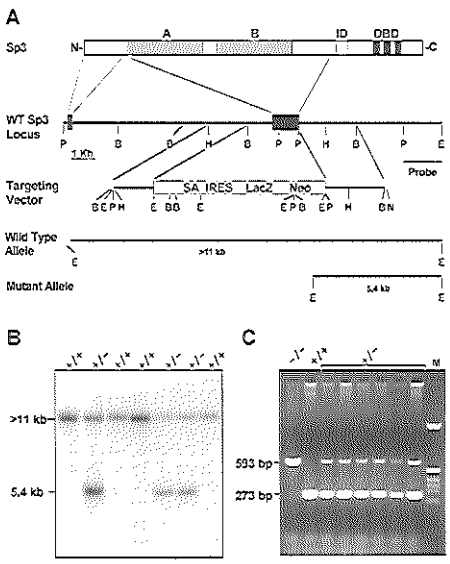

Fig. 1. Targeted disruption of the mouse Sp3 gene. (A) Schematic representation of the Sp3 protein structure. The glutamine-rich activation domains A and B, the inhibitory domain (ID) and the zinc fingers (black bars) of the DNA-binding domain (DBD) are indicated. Connecting lines with the corresponding murine Sp3 gene regions indicate the derivation of the N-terminal part of the Sp3 protein. Both glutamine-rich activation domains (A and B) are encoded by a single large exon. In the targeting vector, this exon was replaced by a cassette containing a splice acceptor site (SA), an internal ribosomal entry site (IRES) and a *lacZ–neo* fusion gene (LacZ Neo) (Mountford *et al.*, 1994). The positions of relevant restriction sites (B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; N, *Not*I) and the probe used for Southern blotting are indicated. Restriction of genomic DNA with *Eco*RI and hybridization with the indicated probe detects a >11 kbp fragment of the wild-type allele and a 5.4 kbp fragment of the mutated allele. (B) Southern blot analysis of targeted ES cells. (C) PCR analysis of mouse embryos. The primers produce a 273 bp DNA fragment from the wild-type and a 593 bp fragment from the targeted allele.

Two of the targeted ES clones with the correct karyotype were injected into C57BL/6 blastocysts. Breeding of the chimeras revealed germline transmission of the mutated Sp3 allele. Mating of heterozygous animals resulted in embryos deficient in all three Sp3 isoforms (Figure 2).

Mice lacking Sp3 die immediately after birth due to respiratory failure

Heterozygous $Sp3^{+/-}$ mice derived from male germline chimeras exhibited no discernible phenotype. Sp3-deficient mice derived from heterozygous matings were not viable (Table I). Genotyping of embryos obtained by Caesarian section shortly before the parturition date (E18.5) showed no statistically significant loss of $Sp3^{-/-}$ mice up to birth (Table I). The weight of these pups was ~25% lower than that of their wild-type littermates (Figure 3; Table II).

Close monitoring of E18.5 embryos after Caesarian section and of litters after birth revealed that $Sp3^{-/-}$ mice

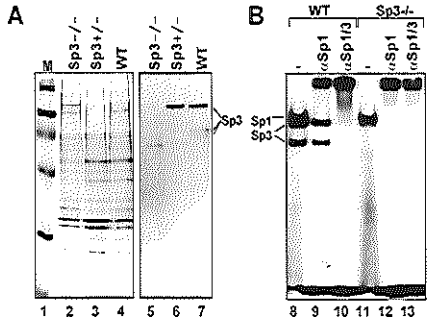

Fig. 2. Sp3 protein expression in wild-type and Sp3 mutant mice. (A) Western blot analysis of wild-type, $Sp3^{+/-}$ and $Sp3^{-/-}$ animals. Nuclear extracts (6 μg of protein) from brains of wild-type (WT), heterozygous ($Sp3^{+/-}$) and Sp3-deficient ($Sp3^{-/-}$) mice were fractionated through 7.5% SDS–polyacrylamide gels, stained with Coomassie Blue (lanes 1–4; M, marker lane) or blotted on nitrocellulose filter and incubated with Sp3-specific antibodies (lanes 5–7). (B) Electrophoretic mobility shift assay of GC box binding activity in wild-type (WT) and Sp3-deficient ($Sp3^{-/-}$) mouse embryonic fibroblasts. Crude nuclear extracts (1.25 μg of protein) were incubated with ^{32}P-labeled GC box oligonucleotide in the absence (lanes 8 and 11) or presence of antisera against Sp1 (lanes 9 and 12, αSp1) or a mixture of antisera against Sp1 and Sp3 (lanes 10 and 13, αSp1/3).

Table 1. Genotype distribution of Sp3 heterozygous crossings

	Total	+/+	+/–	–/–
E18.5	242	70 (28.8%)	115 (47.1%)	57 (23.4%)
Day 10	68	24 (35.3%)	44 (64.7%)	0 (0%)

The genotype was determined by PCR analysis as described in Materials and methods.

were born alive and made visible efforts to breathe. In contrast to their wild-type and heterozygous littermates, the Sp3 *null* mice died within 10 min post-partum. The duration of the pregnancy was prolonged by up to 2 days with progesterone injections in an attempt to promote the maturation of the Sp3 *null* fetuses. However, this did not rescue the neonatal lethality of the knockout mice.

Expression of lung-specific genes is not affected in $Sp3^{-/-}$ mice

Lung tissue dissected from several mutant neonates obtained from independent litters failed to float on water, indicating that the alveoli were never filled with air. Histopathological examination of the lung (Figure 4) revealed that lung tissue of Sp3 *null* mice is more compact, featuring a smaller mean alveolar space diameter and thicker septa between the alveoli. In addition, fewer capillaries are observed in $Sp3^{-/-}$ lung tissue, but instead abnormal cubical pale cells are present at the inner surface of the alveoli. Ultrastructural analysis of these cells shows disruption of the apical membrane along with a small rim of cytoplasm leading to an artificial intracellular space filled with slightly electron-dense material (Figure 4).

The histopathological examination suggests that the inability of the $Sp3^{-/-}$ mice to breathe might be attributed to an intrinsic inability of the lungs to expand. We have examined the presence of transcripts encoding surfactant and other lung-specific proteins involved in lung development and function. Northern blot analyses of RNA from E18.5 mice revealed that expression of surfactant proteins A, B, C and D, and the transcription factors, thyroid transcription factor 1 (TTF-1), which is highly expressed in lung Clara cells, and lung Krüppel-like factor (LKLF), was not affected in lungs of $Sp3^{-/-}$ embryos (Figure 5). In addition, elastin and p21 mRNAs were expressed at normal levels (data not shown). The only transcript that was slightly reduced (2-fold) was uteroglobin/Clara cell secretory protein (UG/CCSP). However, uteroglobin/CCSP-deficient mice develop normally and are apparently healthy (Stripp *et al.*, 1996). Thus, the 2-fold lower expression of uteroglobin/CCSP cannot account for the breathing failure. In conclusion, the molecular cause of the respiratory defect remains unknown at present.

Impact on tooth development

In the course of analyzing histological sections of mouse E18.5 embryos, we observed a morphological deviation in the developing teeth of $Sp3^{-/-}$ mice. In our sections, the dentin/enamel layer of the teeth is generally disrupted from the ameloblast cells in wild-type mice, leading to the creation of an artificial empty space (Figure 6). This was not observed in Sp3 *null* mice. At late embryonic stages, the processes of dentinogenesis (dentine formation) and amelogenesis (enamel formation) take place. Close inspection of the sections revealed that the developing teeth of wild-type mice contain a continuous row of ameloblasts and a row of odontoblasts interrupted by small vessels (Figure 6A). Two layers of differentially stained pink and purple material, consisting of predentin, dentin and enamel, are visible between the ameloblasts and odontoblasts (Figure 6C). In $Sp3^{-/-}$ mice, only a few vessels are observed and the odontoblasts are arranged in a continuous row. Most significantly, teeth of $Sp3^{-/-}$ mice contain only the pink layer and lack the purple layer (Figure 6D).

Lack of ameloblast-specific transcripts in $Sp3^{-/-}$ mice

Odontoblasts and ameloblasts express genes that are required to form the dentin and the enamel extracellular matrix, respectively. We asked whether the altered dentin/enamel layers in sections of teeth from $Sp3^{-/-}$ mice might reflect the lack of odontoblast- and/or ameloblast-specific gene products. Amelogenin (Snead *et al.*, 1983; Couwenhoven and Snead, 1994) and ameloblastin (Krebsbach *et al.*, 1996; Lee *et al.*, 1996) are ameloblast-specific gene products involved in enamel formation. The dentin-matrix protein 1 (DMP1) is expressed specifically in odontoblasts (MacDougall *et al.*, 1998a). Tuftelin, yet another specialized protein secreted into the developing enamel matrix, is also synthesized in odontoblasts at this stage of development (Diekwisch *et al.*, 1997). Northern blot analyses were performed with RNA extracted from jaws or heads of newborn mice and of E18.5 embryos (Figure 7). Amelogenin and ameloblastin mRNA was detectable at birth (Figure 7A and B) and at E18.5 (Figure 7E and F) in wild-type and heterozygous mice but not in $Sp3^{-/-}$ mice. In accordance with published data, hybridization with the ameloblastin-specific probe detected

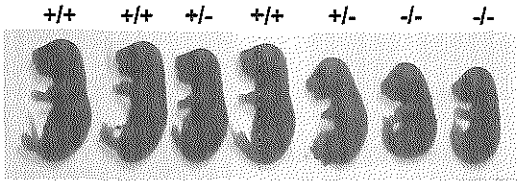

Fig. 3. Sp3 mutant E18.5 embryos. Genotyped E18.5 embryos of one litter that are wild-type (+/+), heterozygous (+/–) or homozygous (–/–) for the targeted Sp3 gene locus. Homozygous mutants are ~25% smaller than wild-type and heterozygous embryos.

Table II. Weight of embryos (g) of Sp3 heterozygous crossings

	+/+	+/–	–/–
E13.5	0.16, SD = 0.02, n = 19	0.15, SD = 0.02, n = 31	0.12, SD = 0.02, n = 15
E18.5	1.10, SD = 0.16, n = 18	1.05, SD = 0.14, n = 39	0.72, SD = 0.08, n = 17

two distinct transcripts that were both expressed only in teeth (Krebsbach *et al.*, 1996). The odontoblast-specific transcript encoding DMP1 (MacDougall *et al.*, 1998a) as well as transcripts encoding tuftelin (Diekwisch *et al.*, 1997; Zeichner-David *et al.*, 1997; MacDougall *et al.*, 1998b) were detectable at similar intensities in wild-type, $Sp3^{+/-}$ and $Sp3^{-/-}$ mice (Figure 7C and D). Thus, ameloblast-specific but not odontoblast-specific gene products are missing in Sp3-deficient mice. These results strongly suggest that the absence of ameloblast-specific proteins contributes to the observed histological tooth phenotype in $Sp3^{-/-}$ mice.

Discussion

Sp3-deficient mice are not viable

Our results demonstrate that the ubiquitous transcription factor Sp3 is essential for immediate post-natal survival of mice. $Sp3^{-/-}$ mice were found in expected Mendelian ratios until birth, but they all died within a few minutes post-partum due to respiratory failure. The molecular cause for the observed breathing defect of the $Sp3^{-/-}$ mutants remains obscure. Only slight morphological alterations were observed in the lung that we attributed initially to developmental delay. However, a prolonged pregnancy did not rescue the lethal phenotype. In addition, surfactant protein gene expression in lung of Sp3 mutant mice was apparently indistinguishable from that in wild-type mice. Thus, undiscovered physiological alterations, such as in the enervation of respiratory muscles, are more likely to be responsible for the respiratory failure of Sp3 *null* mice.

Tooth development is impaired in Sp3-deficient mice

Our analyses of Sp3-deficient embryos provide evidence that Sp3 plays an essential role in late tooth development. Newborn $Sp3^{-/-}$ embryos show only partial formation of the dentin/enamel layer, indicating that dentinogenesis or amelogenesis (dentin and enamel formation) is impaired. A consequence of epithelial cell differentiation into ameloblasts is the expression of proteins that form the enamel extracellular matrix, a scaffold required to nucleate calcium phosphate salts to form calcium hydroxylapatite. Although

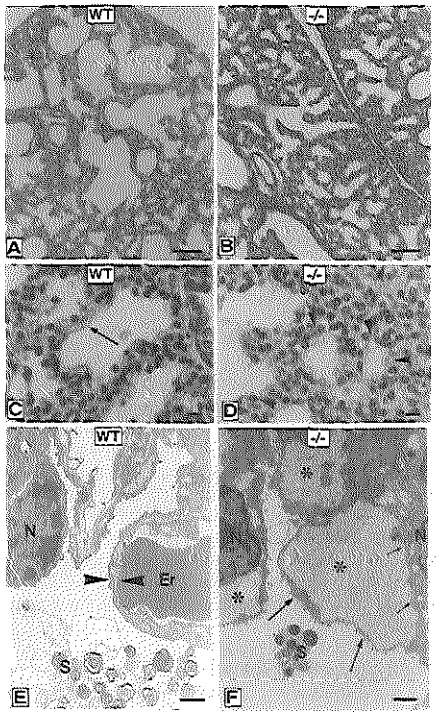

Fig. 4. Histological and ultrastructural analyses of lung tissue in wild-type and $Sp3^{-/-}$ mice. Lung tissue at E18.5 of wild-type (WT) (A, C and E) and Sp3 knockout mice (–/–) (B, D and F) is shown in histological sections stained with hematoxylin and eosin (A–D), or in ultrastructural sections (E and F). At low magnification (A and B), lung tissue from $Sp3^{-/-}$ mice is more compact, with a smaller mean diameter of alveolar spaces and a thicker septum between individual alveoli. At higher magnification (C and D), capillaries are regularly seen at the inner surface of the alveoli in wild-type mice (C, arrow). Lung tissue from Sp3-deficient mice exhibits small alveoli with the inner surface lined with cuboid pale cells (D, arrowheads). Ultrastructural analysis revealed that the apical membranes of these cells together with a small rim of cytoplasm (F, large arrows) are disrupted from their original location near the nucleus (N, small arrows in F). These lead to artificial intracellular spaces (asterisks) filled with slightly electron-dense material. In wild-type lung tissue, a regular air–blood boundary (between arrowheads in E) consisting of an endothelial cell, a basement membrane and a pneumocyte type I is observed. An erythrocyte ('Er' in E) filling the space of a capillary and surfactant ('S' in E and F) are indicated. Bar in (A) and (B), 100 μm; in (C) and (D), 10 μm; in (E) and (F), 0.5 μm.

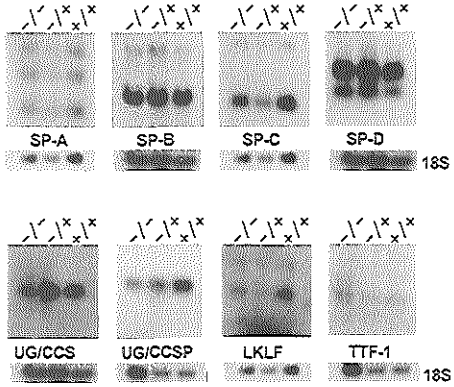

Fig. 5. Expression of putative Sp3 target genes in the lung. RNA was extracted from lungs of wild-type (+/+), $Sp3^{+/-}$ (+/–) and Sp3-deficient (–/–) E18.5 embryos, subjected to electrophoresis through 1.2 or 0.8% formaldehyde–agarose gels and transferred to nylon membranes. The filters were hybridized with cDNA fragments of surfactant proteins A (SP-A), B (SP-B), C (SP-C) and D (SP-D), uteroglobin/Clara cell secretory protein (UG/CCSP; two different membranes), lung Krüppel-like factor (LKLF) and thyroid transcription factor (TTF-1). As a control, the filters were probed with an 18S rRNA-specific oligonucleotide.

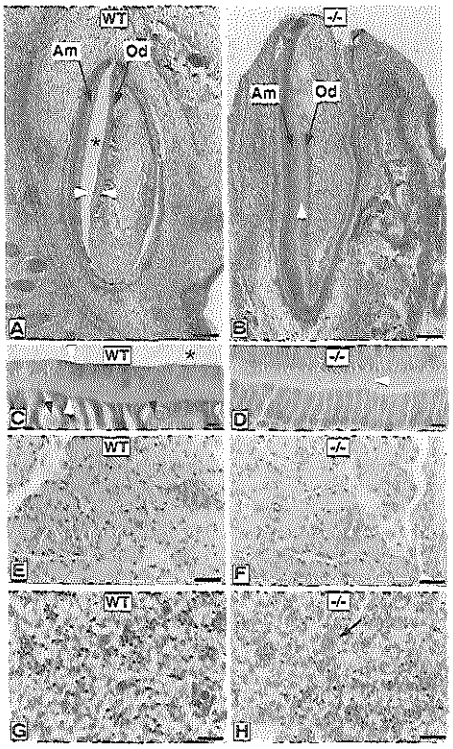

Fig. 6. Histological analysis of developing teeth in wild-type and $Sp3^{-/-}$ mice. Teeth at E18.5 of wild-type (A and C) and Sp3-deficient mice (B and D) are shown in histological sections stained with PAS. At low power magnification, the developing tooth of wild-type mice shows a continuous row of ameloblasts ('Am', arrow) and odontoblasts ('Od', arrow) interrupted by small vessels interspersed between the odontoblasts (black arrowheads in C). Between the ameloblasts and odontoblasts, a sheet of pink and purple stained material that contains dentin and enamel is visible (marked in A and C by two white arrowheads). This layer of secretory products has been disrupted from the ameloblasts, leaving an artificial space (asterisks in A and C) that is regularly observed in wild-type mice of this age, but not in Sp3 *null* mice. In contrast, Sp3 knockout mice show only a pink layer (white arrowheads in B and D). In addition, only a few vessels are observed in the dental papilla, leaving the odontoblasts in a continuous row ('Od' with arrow in B and bottom cell layer in D). (E–H) PAS-stained histological sections of E18.5 tissues with no obvious morphological alterations in knockout mice. (E and F) Pancreas. (G and H) Liver: the arrow in (H) points to a megakaryocyte. Liver cells have a pale cytoplasm and are interspersed by small clusters of hematopoetic cells. Bar in (A) and (B), 100 μm; in (C) and (D), 10 μm; in (E–H), 30 μm.

the ameloblast cell layer is present in $Sp3^{-/-}$ mice, the ameloblast-specific products amelogenin and ameloblastin are absent. In contrast, the odontoblast-specific gene products DMP1 and tuftelin are present in Sp3 *null* embryos. At this stage, we do not know whether Sp3 functions as a direct regulator of ameloblast-specific genes. Recently, the cloning and preliminary characterization of the murine ameloblastin (Dhamija *et al.*, 1999) and the amelogenin promoter (Chen *et al.*, 1998) have been reported. The ameloblastin promoter contains a potential Sp3-binding site (CACCC box) in a promoter region that contributes to its cell type-specific expression. Thus, the amelogenin and ameloblastin genes might be direct targets of Sp3.

Physiological functions of Sp1 and Sp3

Sp3 is a ubiquitously expressed transcription factor with structural features similar to Sp1. Sp1 and Sp3 recognize the same DNA elements and, in many reports, Sp3 has been shown to act as a potent transcriptional activator similar to Sp1 (Udvadia *et al.*, 1995; Liang *et al.*, 1996; Ihn and Trojanowska, 1997; Zhao and Chang, 1997). The physiological function of Sp1 and Sp3, however, appears to be significantly different. In contrast to Sp3 *null* mice, Sp1-deficient embryos are already severely retarded at early embryonic stages and die around day 10–11 of gestation (Marin *et al.*, 1997). Thus, the obvious structural similarity, the common DNA recognition sites and the ubiquitous expression of Sp1 and Sp3 do not reflect identical physiological functions. Nevertheless, there still might be many overlapping functions of Sp1 and Sp3 *in vivo*. The Sp1 and Sp3 knockouts demonstrate that each protein on its own is not responsible for the expression of a very large number of essential genes, such as housekeeping, tissue-specific and cell cycle-regulated genes. This suggests that Sp1 and Sp3 have a wide range of redundant functions that can compensate for each other in Sp1 and Sp3 knockout mice.

Another, tissue-restricted member of the Sp family of transcription factors, Sp4, might also contribute to redundancy *in vivo*. Sp4, although expressed predominantly in the brain, is also detectable in epithelial tissues, testis and developing teeth (Supp *et al.*, 1996). Disruption of the mouse Sp4 gene revealed that Sp4 is also important

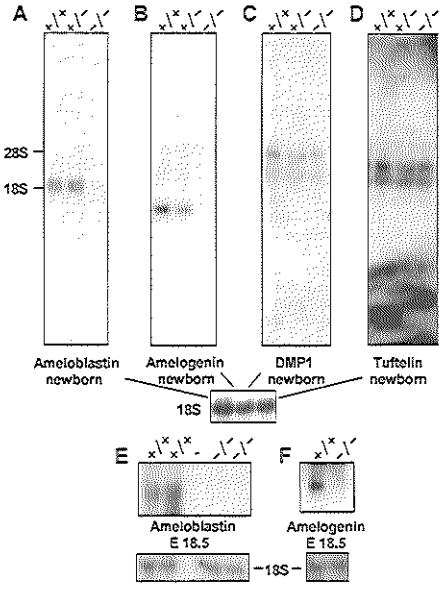

Fig. 7. Expression of putative Sp3 target genes in teeth. RNA was extracted from head or jaws of wild-type (+/+), $Sp3^{+/-}$ (+/–) and Sp3-deficient (–/–) newborn or E18.5 embryos, subjected to electrophoresis through 1.2% formaldehyde–agarose gels and transferred to nylon membranes. The filters were hybridized with cDNA fragments encoding ameloblastin (A and E), amelogenin (B and F), DMP1 (C) and tuftelin (D). As a control, the filters were probed with an 18S rRNA-specific oligonucleotide.

for early post-natal survival since approximately two-thirds of the $Sp4^{-/-}$ mice die within a few days after birth for unknown reasons (S.Philipsen and G.Suske, unpublished data). So far, essential target genes for Sp4 have not been identified.

On many reporter constructs containing multiple Sp-binding sites, Sp3, unlike Sp1, is inactive or acts only as a weak activator. The molecular basis for the inactivity of Sp3 under these conditions has been mapped to an inhibitory domain located between the second glutamine-rich activation domain and the zinc finger region (Dennig *et al.*, 1996). The integrity of a charged amino acid triplet (KEE) within this domain is absolutely essential for inhibitor function. Mutation of these amino acids converts Sp3 to a strong activator (Dennig *et al.*, 1996) that is almost indistinguishable from Sp1. A most intriguing possibility would be that this molecular difference between Sp1 and Sp3 reflects the differences in their physiological functions. Can a mutant of Sp3 lacking the inhibitory domain rescue the Sp1 knockout? The generation of transgenic lines expressing different isoforms and mutants of Sp3 and intercrossings with heterozygous Sp1, Sp3 and Sp4 mice could provide new insight into the physiological and biochemical relationships between these three transcription factors.

The early embryonic lethality of Sp1 knockout mice and the post-natal lethality of Sp3 knockout mice preclude the analysis of later developmental stages. In order to achieve this, conditional disruption of the Sp1 and Sp3 genes in specific tissues at any given stage of development, as well as the generation of compound knockouts of Sp1, Sp3 and Sp4 will be an important step to unravel further the physiological roles of these proteins *in vivo*.

Materials and methods

Generation of the Sp3 homologous recombination construct

As starting plasmid, we chose the Bluescript derivative pBS4.5*Bam*HI that contains a 4.5 kb *Bam*HI fragment encoding both glutamine-rich activation domains on a single exon and ~1 kbp upstream and 2.5 kbp downstream intron sequences of the Sp3 gene locus (see Figure 1A). We removed the exon and the 5′ intron sequences by restriction with *Pst*I and re-ligation, leading to pBS-P-B. β-galactosidase sequences within the Bluescript vector that may interfere with the *lacZ* gene (see below) were removed by *Nae*I and *Kpn*I restriction, Klenow filling and re-ligation (plasmid name: pBS-P-B-Δ). Next, we introduced an IRES-LacZ-neo cassette obtained as a 7.3 kbp *Sal*I fragment from the plasmid pGT1.8Iresβgeo (Mountford *et al.*, 1994) into the *Sal*I polylinker site of pBS-*Pst*I-*Bam*HIΔlacZ, leading to pBS-P-B-Δ-lacZ-neo. In a final step, we cloned 5′ intron sequences of the Sp3 gene as a 1.7 kbp *Hin*dIII–*Bam*HI fragment into the *Xho*I polylinker site of pBS-P-B-Δ-lacZ-neo using *Xho*I linkers, leading to the knockout construct pBS-P-B-Δ-lacZ-neo-5′-Sp3. For transfection into ES cells, the plasmid was linearized with *Not*I.

Transfection and selection of ES cells

E14 ES cells were electroporated with 15 μg of *Not*I-linearized targeting vector pBS-P-B-Δ-lacZ-neo-5′-Sp3. Clones were selected with G418 (200 μg/ml) and homologous recombination was analyzed by Southern blotting of *Eco*RI-restricted genomic DNA with the probe indicated in Figure 1. Unwanted random integrations were detected by hybridizing these blots with a Bluescript vector-specific probe.

Generation of chimeric and Sp3-deficient mice

$Sp3^{+/-}$ ES cell clones were karyotyped, and two clones (#10 and #38) were microinjected in C57BL/6 host blastocysts. Chimeric males were mated to C57BL/6 females. Germline transmission was obtained with both clones. The F_1 offspring were interbred to expand the stocks. No viable Sp3 *null* animals were obtained with mice derived from clones #10 and #38. Preliminary *in utero* analysis of Sp3 *null* fetuses revealed no obvious differences between the two lines. For the analyses presented herein, we have used line #10. To extend the duration of pregnancy, pregnant females received daily subcutaneous injections of progesterone (5 mg/kg body weight) from day 16 after discovery of the vaginal plug. Pregnancies extended 2 days beyond their normal duration were delivered by Caesarian section.

Genotyping of embryos by PCR

DNA was prepared from tail snips and analyzed for the presence of the wild-type and knockout Sp3 allele by PCR. Three primers were used: a sense primer in the Sp3 gene amplifying the wild-type allele (5′-ACTACTAGTGGGCAAGTCCA-3′), a sense primer in the *neo* gene amplifying the knockout allele (5′-AGCGCATCGCCTTCTATCG-3′) and an antisense primer in the Sp3 gene (5′-TACCATTGCACA-TTTAATGA-3′). PCR conditions were 94°C, 1 min; 60°C, 1 min; 72°C, 1 min; for 30 cycles.

Nuclear extracts and gel retardation assays

Nuclear extracts were prepared according to Andrew and Faller (1991). Electrophoretic mobility shift assays (EMSAs) were performed by pre-incubating 1–3 μl of nuclear extract with 1.5 μg of unspecific competitor poly(dI–dC) in a buffer containing 10 mM HEPES pH 7.9, 150 mM KCl, 1 mM dithiothreitol (DTT), 0.5 mM $MgCl_2$, 0.1 mM EDTA, 8.5% glycerol for 10 min on ice. Subsequently, 0.1 ng of ^{32}P-labeled double-stranded GC box oligonucleotide was added to a final volume of 20 μl, and samples were incubated for another 20 min on ice. Antisera against Sp1 and Sp3 used for supershift assays were described previously (Hagen *et al.*, 1994). Usually, 1 μl of the appropriate antiserum was added to the binding reaction, and incubation was continued at room temperature for another 20 min. Samples were analyzed on 4% native polyacrylamide gels in 45 mM Tris, 45 mM boric acid, 1.6 mM EDTA. Gels were transferred to Whatman 3MM paper, dried under heat and vacuum, and exposed to X-ray films overnight.

Western blotting

Nuclear extracts (6 μg of protein) were prepared from mouse embryonic E18.5 brains according to Gorski *et al.* (1986), separated on 10% SDS–polyacrylamide gels, blotted on nitrocellulose or PVDF membranes, and probed with a rabbit αSp3 serum (Hagen *et al.*, 1994). Primary antibodies were visualized using the Amersham ECL kit.

Northern blot analyses

Total RNA from embryonic mouse tissues and cell lines was extracted by the guanidinium/isothiocyanate procedure using the Qiagen kit. RNA was separated through 0.8 and 1.2% agarose gels containing 2.2 M formaldehyde and blotted to nylon membranes. Pre-hybridization and hybridization were carried out as described (Braun and Suske, 1998). Gene-specific probes were obtained from appropriate plasmids or primer sets. Detailed information is available upon request.

Morphological analysis

Embryos were dissected at day 18.5 post-coitum, and a small tail snip was removed for genotyping. Fetuses were cut in half longitudinally and fixed in Carnoy's solution (60% ethanol, 30% chloroform, 10% acetic acid) at 4°C overnight and embedded in paraffin according to standard procedures. Sections were stained with hemalaun and either eosin (HE) or periodic acid–Schiff (PAS) reagent. For electron microscopy, lung tissue samples were cut into small pieces of ~1 mm^3 and immersion fixed with 2.5% paraformaldehyde, 2.5% glutaraldehyde and 0.05% picrinic acid in 0.067 M cacodylate buffer pH 7.4 for 2 h at 4°C. Standard procedures for dehydration and embedding in Epon were employed. Thin sections were stained with uranyl acetate and lead citrate, and examined in an EM 109 electron microscope (Zeiss).

Acknowledgements

We thank Iris Rohner and Waltraud Sperling for excellent technical assistance, Drs Martha Kalff-Suske and Jörg Klug are gratefully acknowledged for critically reading the manuscript. This work was supported by grants of the Deutsche Forschungsgemeinschaft to G.S. and the NOW to P.G.

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Received November 17, 1999; revised and accepted December 16, 1999

Chapter three

Genetic background dependent late gestational lethality of Sp3 deficient mice and Sp1/Sp3 compound heterozygous mice: indications of heart failure

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Abstract

Targeted deletion of the Sp1-related transcription factor Sp3 in the mouse is incompatible with post-natal survival. Mutant newborns are growth retarded and, although they make visible efforts to breathe, fail to inflate their lungs. However, lung development is not dramatically affected in the absence of Sp3. We have further investigated the lethal phenotype of Sp3 knockout mice. A possible cause of their impaired growth may lie in circulatory problems, signs of which become apparent during late gestation. The severity of the knockout phenotype increased upon backcrossing to the C57BL/6 inbred strain, resulting in prenatal lethality. Mutant hearts appear underdeveloped and display a thin myocardium and ventricular septal defects, suggesting that *in utero* lethality can be attributed to impaired heart functioning. We propose that, in combination with a developmental delay, the improper development of the heart also contributes to lethality after birth.

In addition we describe the phenotype of Sp1/Sp3 compound heterozygous mice that appear to share phenotypical characteristics with Sp3-/- mice. This indicates that Sp1 and Sp3 may be at least partly fulfill similar functions *in vivo*.

Introduction

Transcription factor Sp3 belongs to the Sp/XKLF (Specificity Protein/X Krüppel Like Factor) family of transcription factors (Philipsen and Suske, 1999; Suske, 1999; Turner and Crossley, 1999). Members of this family all share a DNA binding domain that has first been identified in Sp1 (Kadonaga *et al.*, 1987). This motif contains 3 Cys_2His_2 zinc fingers that specifically bind to so-called GT- and GC-boxes which can be found in numerous enhancer and promoter elements. Therefore, Sp/XKLF proteins have been implicated in the transcriptional regulation of many different genes. Within the family, Sp3 is most closely related to Sp1 and Sp4 that have a similar modular structure of serine/threonine rich regions and glutamine rich *trans*activation domains. Like Sp1, Sp3 is ubiquitously expressed whereas Sp4 is predominantly expressed in the brain and in the conductive system of the heart (Hagen *et al.*, 1992; Kingsley and Winoto, 1992; Nguyen-Tran *et al.*, 2000; Saffer *et al.*, 1991; Supp *et al.*, 1996).

The successive targeted inactivation of the murine Sp4, Sp1 and Sp3 genes has revealed that each of these transcription factors has a specific function *in vivo*. Sp4 knockout mice display impaired male sexual behavior, growth retardation and pronounced lethality in the first 3-4 weeks after birth (Gollner *et al.*, 2001a; Nguyen-Tran *et al.*, 2000; Supp *et al.*, 1996). Surviving mutants suffer from cardiac rhythm disturbances and some of them die from sudden cardiac arrest at the age of 6-8 months (Nguyen-Tran *et al.*, 2000). Sp1 deficient embryos survive until day 10 of embryonic development (E10), after which they die of cell autonomous defects (Marin *et al.*, 1997). Sp3 is essential for post-natal survival and late tooth and bone development (Bouwman *et al.*, 2000; Gollner *et al.*, 2001b). Sp3 deficient newborns are growth retarded, unable to breathe and die within a few minutes after birth. Apart from the growth retardation, no gross morphological defects were found that could account for the phenotype.

To find an explanation for the neo-natal lethality of the Sp3 knockout mice, morphology of organs critical for survival was analyzed in more detail. No structural abnormalities could be identified in the brain, but Sp3 deficient embryos display a heart defect that becomes more apparent after crossing back to the C57BL/6 genetic background. Both atria and ventricles of mutant hearts have a very thin myocardium and ventricular septal defects (VSDs) are found often. We conclude that, in combination with the generally delayed development, impaired heart functioning is one of the factors that contribute to the lethal phenotype of the Sp3 knockout mouse.

This chapter also includes a initial description of the phenotype of Sp1/Sp3 compound heterozygous mice. Of the Sp-proteins, especially Sp1 and Sp3 are likely to be cooperatively involved in transcriptional regulation of a large number of genes. Not only are these transcriptional factors highly homologous, they also share their binding site preference and an ubiquitous expression pattern. We have found that Sp1/Sp3 compound heterozygous mice are growth retarded and suffer from perinatal lethality in a manner similar to Sp3

deficient mice which indicates that the *in vivo* functions of Sp1 and Sp3 are at least partly connected.

Material and Methods

Mice. Embryos derived from timed matings of Sp3+/- x (Sp3+/- or Sp1+/-) mice were genotyped by PCR on yolk sac DNA as described (Bouwman *et al.*, 2000; Marin *et al.*, 1997) but using the following oligonucleotides for the Sp3 alleles: 5' gcgtgcaagccagtggtc 3' (wild type Sp3 allele sense primer), 5' agcgcatcgccttctatcg 3' (targeted Sp3 allele sense primer) and 5'ggacgattctatgatgcctcc 3' (common antisense primer).

Morphology and histology. ***(A) newborn brain.*** For the study of newborn brains, E20.5 fetuses were anaesthetized with Nembutal and perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) / 0.1 % picric acid in PBS (solutions on ice). Brains were isolated, post-fixed in PFA for 2 hours at 4 °C, incubated overnight at 4 °C in 10 % sucrose / 0.1 M phosphate buffer (PB) and embedded in 11 % gelatin /10 % sucrose. After post-fixation in 10% formaline / 30% sucrose for 2 hours at room temperature and overnight incubation at 4 °C in 30% sucrose / 0.1 M PB, samples were frozen and 40 µm cryostat sections were made. Sections were stained with histological dyes according to standard protocols or using X-gal as a substrate for β-galactosidase activity. X-gal staining was performed overnight at 30°C in PBS containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6.3H_2O$, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40 and 1mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). For immunostaining of calbindin (CaBP), sections were washed 4 times 10 minutes with 0.1 M Tris buffered saline pH 7.6 (TBS) and blocked for 1 hour at 4 °C with 10% normal goat serum in TBS containing 0.5% Triton X-100. Following incubation with 1:10000 rabbit anti CaBP in 2% normal goat serum / 0.4% Triton X-100 in TBS (TBS+) for 2 days at 4°C, sections were washed 4 times for 10 minutes with TBS and incubated for 2 hours with biotinylated goat anti rabbit diluted 1:200 in TBS+. Finally, sections were washed 4 times for 10 minutes with TBS and stained using the avidin/biotin-peroxidase-complex (ABC) method (Vector Laboratories, Burlingame, CA, USA) and 0.05% diaminobenzidine. For microscopic analysis, gelatin sections were mounted on glass slides using a gelatin/chrome alum solution (3 mg/ml gelatin, 0.15 mg/ml $KCr_2(SO_4)_2.12H_2O$ in H_2O). ***(B) embryonic and fetal hearts.*** Hearts of embryos or fetuses of different days of gestation were isolated and fixed overnight with 4% PFA at 4 °C. After fixation, hearts were photographed using a Leica Wild M10 microscope (Leica Camera AG, Solms, Germany) equipped with a JVC KY-F55B 3-ccd videocamera (JVC Ltd., Yokohama, Japan), dehydrated and embedded in paraffin. Subsequently, 5 µm sections were made that were HE stained and mounted on glass slides.

All sections were photographed using an Olympus BX40 microscope equipped with the DP50 digital camera system (Olympus Optical Corporation, Tokyo, Japan). Digital images were captured and resampled using a combination of Pixera Viewfinder (Pixera Corporation, Los Gatos, C.A., U.S.A.) and Adobe Photoshop (Adobe, San Jose, C.A., U.S.A.) software.

RT- PCR on newborn hearts. E18.5 hearts were snap frozen in liquid nitrogen and stored at -80°C until RNA was isolated using lithium-chloride urea precipitation (Auffray and Rougeon, 1980). After Dnase I treatment, cDNA was synthesized by reverse transcription using SuperRT reverse transcriptase (H.T. Biotechnology, Cambridge, UK).

RT-PCR was performed using the following sets of oligonucleotides: glyceraldehyde-3-phosphate-dehydrogenase (gapdh) sense 5' gtcgtggagtctactggtgt 3' and antisense 5' gttcagctctgggatgacct 3'; myosin heavy chain (mhc) α antisense 5' agggtctgctggagaggta 3', mhc β antisense 5' tctgcttccacctaaagggc 3' and mhc common sense 5'aatgagctggaggctgagca 3'; myosin light chain (mlc) 1A sense 5' ttctctctcacgagccacct 3', mlc 1V sense 5' cttcttccctcctctttgtg 3' and mlc common antisense 5' cactgcccgtaggtgatctt 3'.

Results

Breeding of Sp3 knockout mice. The initial characterization of Sp3 knockout mice was largely done using a colony of F2 129xC57BL/6 mice that was maintained by brother-sister matings (Bouwman *et al.*, 2000). The results described in this chapter were obtained with mice that were crossed back to C57BL/6 an average of three generations more. Currently we are crossing Sp3 heterozygous males with C57BL/6 and FVB females to obtain inbred strains of Sp3 mutant mice in two different genetic backgrounds.

Targeted deletion of the ubiquitously expressed transcription factor Sp3 does not affect morphology of the newborn brain. Previously we have shown that targeted deletion of transcription factor Sp3 in the mouse results in perinatal lethality, most likely due to respiratory failure (Bouwman *et al.*, 2000). Although it was observed that Sp3 deficient newborns make visible efforts to breathe, a diminished control of respiration in the brain could not be ruled out. Therefore, we decided to analyze the morphology of the Sp3 mutant brain in more detail. Following perfusion fixation we carefully analyzed gelatin sections of newborn brains, thereby especially focusing on the brain stem, where the respiration center is located (reviewed in Guz, 1997). However, no morphological differences in Sp3 knockout brains were found and immunohistochemistry on the calcium binding protein calbindin-D28K revealed normal neuronal expression patterns. In addition, we performed an X-gal staining of Sp3+/- and Sp3-/- brains using the β geo lacZ-neomycin

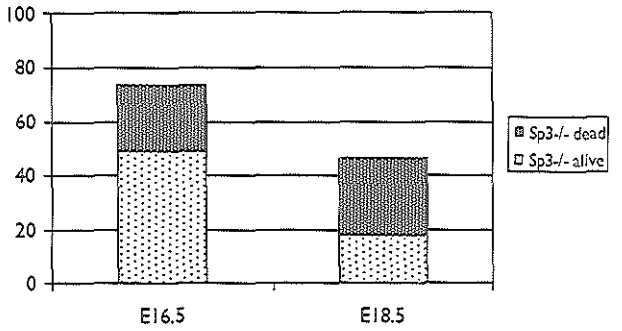

FIGURE 1: **Lethality of Sp3-/- mutants during late gestation upon backcrossing to the C57/BL6 inbred strain.**
Schematic representation of the amount of Sp3-/- embryos relative to the value expected according to Mendelian law (100%) from Sp3+/- x Sp3+/- matings at E16.5 (49 embryos out of 9 litters analyzed) and at E18.5 (113 embryos out of 17 litters analyzed).

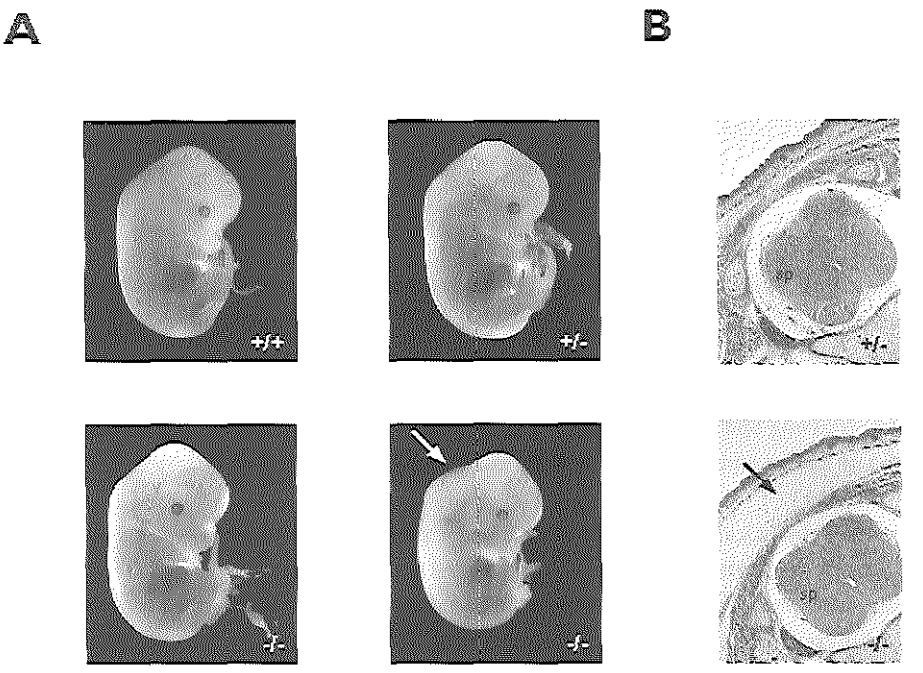

FIGURE 2: **Subcutaneous edema in Sp3-/- fetuses during late gestation.**
(*A*) Two different E13.5 Sp3-/- embryos compared to an Sp3+/+ and Sp3+/- littermate. (*B*) Transversal sections of E16.5 Sp3+/- and Sp3-/- littermates. Arrows point at subcutaneous edema, sp = spinal cord.

fusion gene inserted in the Sp3 locus (Bouwman *et al.*, 2000). Staining patterns appeared to be identical in the presence or absence of Sp3 and only reflected its ubiquitous expression in transcriptionally active cells (not shown).

The Sp3 knockout phenotype becomes more severe upon crossing to the C57BL/6 genetic background: evidence of heart failure during late gestation. In a mixed 129-C57BL/6 genetic background, heart function of Sp3-/- mutants was sufficient to allow survival until birth. However, after further backcrossing to the C57BL/6 strain, the number of living E18.5 Sp3-/-mice dropped to less than one fifth of the amount expected according to Mendelian law (Figure 1). The presence of partially resorbed Sp3-/- fetuses at E16.5 and E18.5 indicates lethality during late gestation that appears to be gender-independent. Between E13.5 and E16.5 some Sp3-/- embryos display subcutaneous edema and later during development peripheral hemorrhages can be observed (Figure 2). Both phenomena are well known signs of circulatory failure. From approximately E15.5 onwards a dilation of the atria becomes apparent and also the right ventricle of Sp3 deficient fetuses is relatively large (Figure 3). Accordingly, the myocardium of especially the atria was found to be extremely thin. In addition, sections of mutant hearts revealed a ventricular septal defect (Figure 4).

At the molecular level, E18.5 Sp3 deficient hearts contain a reduced amount of the adult cardiac myosin heavy chain I isoform (mhc1-α) relative to Sp3+/- and wt hearts (Figure 5). This may indicate that mhc1-α is a direct target gene of Sp3. Yet, the expression of other myosin I isoforms including the atrial and ventricle specific light chains is normal and it is known that mhc1-α is up-regulated around birth (Lyons *et al.*, 1990). Therefore, it might very well be that the relatively low expression of mhc1-α reflects the general delay in development and is only indirectly caused by the absence of Sp3-mediated transcriptional regulation. Importantly, Sp3-/- embryonic stem cells have a normal capability to form beating heart colonies *in vitro* which argues against an intrinsic defect in cardiac myocyte differentiation (Gollner *et al.*, 2001b). Nevertheless, heart development *in vivo* is clearly perturbed in the absence of Sp3 and does not depend on the myocyte lineage alone (e.g. Chen *et al.*, 1998; Subbarayan *et al.*, 2000; Tran and Sucov, 1998). At present, we are measuring the expression levels of a set of genes that are known to be important for normal embryonic heart development. Preliminary data indicate an increased expression of Erbb2 in E18.5 mutant hearts, whereas other genes like N-myc and TEF-1 are transcribed at normal levels (not shown). However, the effects of an impaired circulation are already visible around E13.5 and therefore more relevant differences in expression are likely to be found at earlier stages of embryonic development.

Evidence of heart failure has also been obtained in case of one Sp3 deficient mouse that survived birth. To investigate the Sp3 knockout phenotype in a different genetic background, we crossed the mixed 129-C57BL/6 strain with FVB mice. Mating of F2xFVB Sp3+/- mice resulted in an Sp3-/- mutant that survived for one day after caesarian section at E19.5. Although still smaller than its littermates, this pup was relatively large compared to newborn Sp3-/- mice of the 129-C57BL/6 background. Respiration seemed normal after a critical phase immediately after birth and apart from the smaller size no abnormalities were noticed.

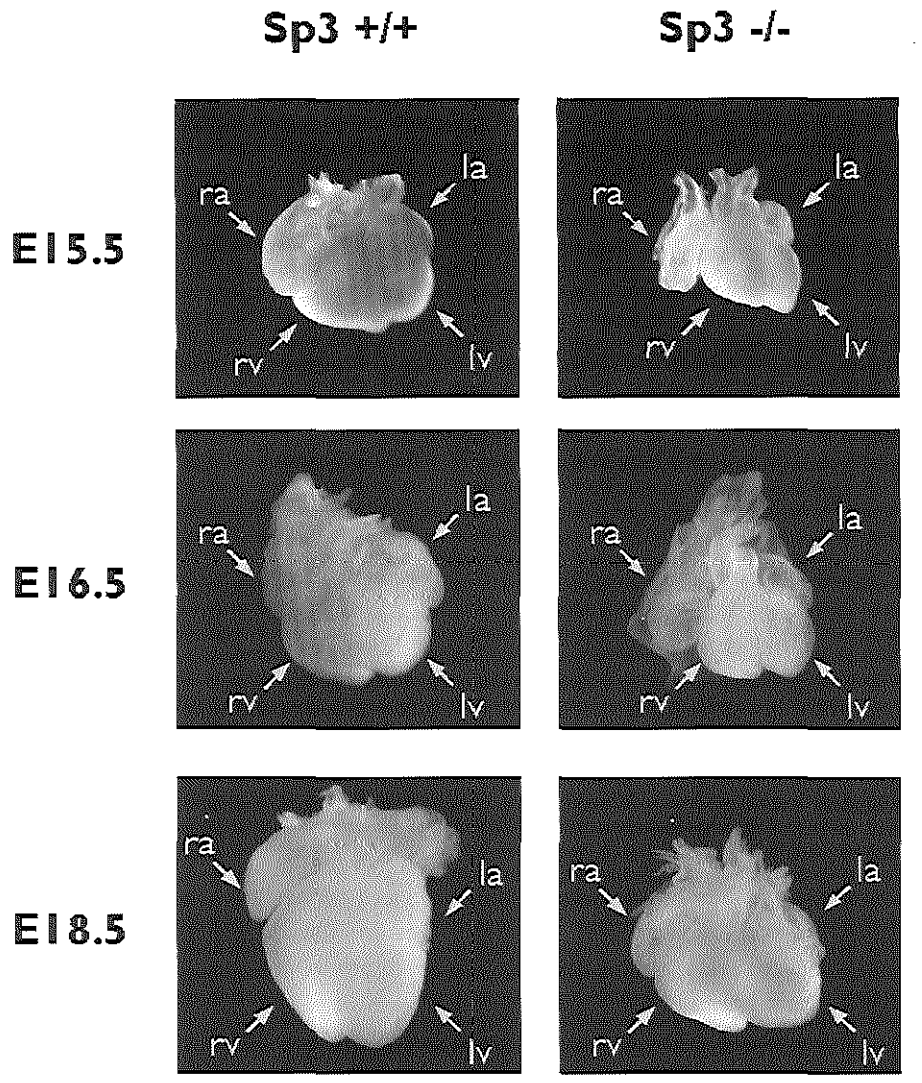

FIGURE 3: **Sp3-/- hearts at late gestation have a thin, translucent myocardium and an underdeveloped appearance.**
Sp3-/- hearts compared to Sp3+/+ hearts at E15.5 (B and A), E16.5 (D and E) and E18.5 (F and E) all photographed at the same magnification (ra = right atrium, la = left atrium, rv = right ventricle, lv = left ventricle).

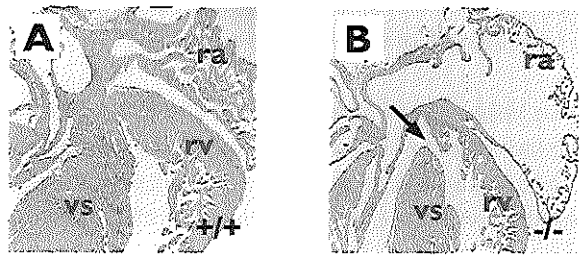

FIGURE 4 (ABOVE): **Ventricular septum defect in an E18.5 Sp3-/- newborn.**
Coronal 5 µm HE stained sections through the heart of an (*A*) E18.5 Sp3+/+ and (*B*) E18.5 Sp3-/- newborn, both photographed at the same magnification. Arrow indicates VSD, rv = right ventricle, ra = right atrium, vs = ventricular septum. Also note the thin atrial and ventricular myocardium of the Sp3-/- heart.

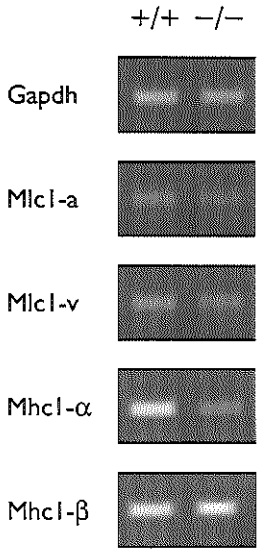

FIGURE 5 (ABOVE TO RIGHT): **Reduced expression of myosin heavy chain I α in newborn Sp3-/- hearts.**
RT-PCR on total heart RNA of E18.5 wild type (+/+) and Sp3 knockout (-/-) littermates using specific primers for myosin I isoforms mlc1-a, mlc1-v, mhc1-α and mhc1-β and GAPDH as a control. Densitometric analysis indicates an approximately 2-fold lower expression of mhc1-α in E18.5 knockout hearts relative to wild type and heterozygous (not shown) littermates.

The Sp3-/- pup was transferred to a foster mother together with an Sp3+/- littermate and both survived until the next day. However, the knockout was found dead a few hours later that day, whereas the heterozygous littermate was still alive and had been nursed. Upon dissection, the heart of the Sp3-/- pup showed grossly enlarged atria that were filled with blood (Figure 6) whereas the lungs were still filled with air. So far, no other Sp3-/- mice have

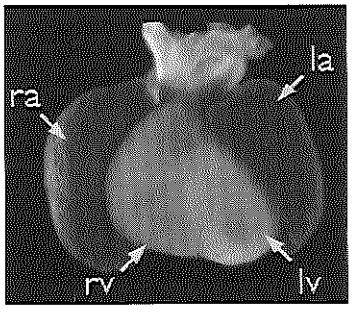

FIGURE 6: **Abnormal heart morphology in an Sp3-/- pup that survived for 1 day after birth.**
Immature appearance of the heart of an Sp3-/- pup that died at 1 day post-partum with dilated, blood-filled atria (compare E18.5 wild type in figure 4, photographed at the same magnification; ra = right atrium, la = left atrium, rv = right ventricle, lv = left ventricle).

managed to survive the respiratory distress immediately after birth which prevents us from drawing conclusions regarding post-natal aspects of the phenotype.

Sp1/Sp3 compound heterozygous mice show phenotypical characteristics of Sp3-/- mice. The fact that targeted deletion of each of the highly homologous transcription factors Sp1 and Sp3 is incompatible with mouse survival indicates that they both have an essential function *in vivo* (Bouwman *et al.*, 2000; Marin *et al.*, 1997). However, it is also evident that Sp1 and Sp3 are not absolutely required for cellular survival and many putative target genes are normally expressed in their absence (Bouwman *et al.*, 2000; Marin *et al.*, 1997; this thesis). It seems likely that other Sp/XKLF members can, to some extent, take over their function as transcription regulators. Since Sp1 and Sp3 can both activate transcription, and share the same consensus binding site and ubiquitous expression pattern they might partially substitute for each other in some cases.

On the other hand, under certain circumstances Sp3 can also function as a repressor of Sp1-mediated *trans*activation, which has led to the suggestion that the Sp1/Sp3 ratio is an important determinant for the regulation of gene expression (Philipsen and Suske, 1999; Suske, 1999).

One way to assess the functional relationship between different proteins is through the use of compound knockout mice. Because of the early lethality of Sp1-/- and Sp3-/- mice we started to set up matings between heterozygous mice of both lines. So far, out of the more than 50 pups genotyped around post-natal day 10 only 2 were Sp1+/-Sp3+/- mice. After a critical period around weaning these females seemed to develop normally, apart from a generally reduced size and an eye defect in one of them that has frequently been observed in Sp1+/- mice (Ulrike Jägle, unpublished data).

Apparently, most Sp1+/-Sp3+/- mice do not survive after birth and therefore we decided to investigate the genotypical distribution *in utero*. Interestingly, caesarean sections at E18.5 revealed a phenotype similar to that of Sp3 deficient mice. All 7 E18.5 Sp1+/-Sp3+/- pups examined were approximately 30-40 % smaller than their wild type littermates and died because of respiratory distress

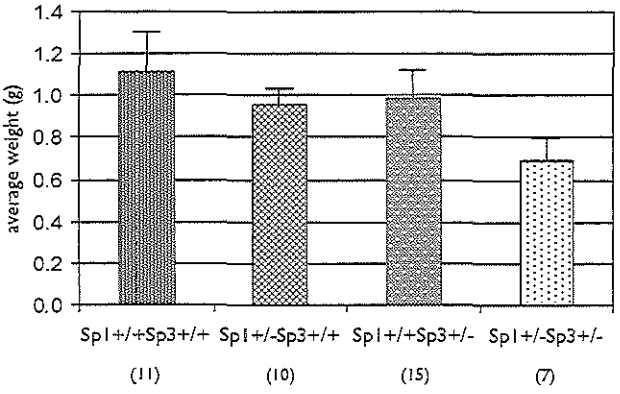

FIGURE 7: **Sp1+/-Sp3+/- E18.5 newborns are under-represented and smaller than their Sp1+/-, Sp3+/- and wild type littermates.** Graphic representation of the average weights of E18.5 Sp1+/- x Sp3+/- offspring. Results were obtained from 8 different litters, the number of pups per genotype are indicated between brackets.

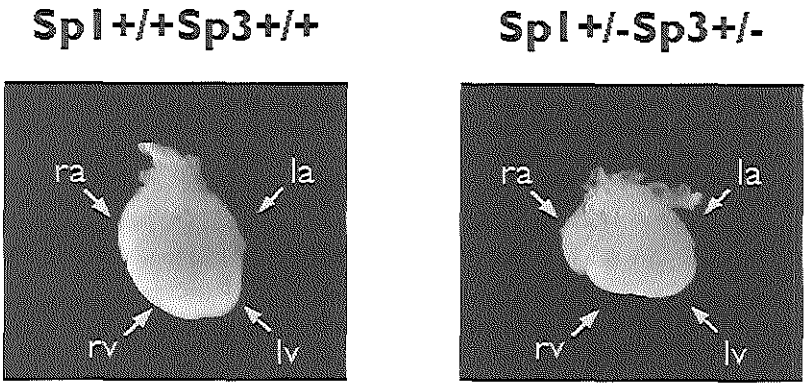

FIGURE 8: **Sp1+/-Sp3+/- newborn hearts have a premature appearance.** Compared to hearts of wild type littermates (*left*), E18.5 Sp1+/-Sp3+/- (*right*) hearts have abnormal, compacted ventricles resembling an earlier developmental stage (see Figure 3, photographed at the same magnification; ra = right atrium, la = left atrium, rv = right ventricle, lv = left ventricle).

(Figure 7). In addition, one or both eyes had often developed incompletely or were absent (not shown). Compound heterozygous newborn hearts have an premature appearance (Figure 8) resembling to what is seen in the Sp3 knockout and currently their morphological characteristics are being investigated.

Whether all compound heterozygous mice survive until birth remains to be determined; given the distribution of genotypes in the litters examined so far it is possible that there is some degree of *in utero* lethality.

Although the phenotype of the Sp1+/-Sp3+/- mice needs to be explored further, it does not support the hypothesis that the ratio between both transcription factors is a major determinant in transcription regulation. Rather, it seems that Sp1 and Sp3 may at least partly take over each others function *in vivo*. The high mortality rate of compound heterozygous mice precluded a systematical analysis of the phenotypes of Sp1-/-Sp3+/- or Sp1+/-Sp3-/- mice *in utero* which could further clarify the issue of their genetic interaction.

Discussion

The ubiquitous transcription factor Sp3 is essential for post-natal survival since Sp3 knockout mice die from respiratory distress. Despite the distinct phenotype, gross morphological abnormalities were initially not found making it difficult to asses the underlying defects (Bouwman *et al.*, 2000). Therefore, we decided to study the anatomy of some organs of vital importance in more detail.

The time immediately after birth is one of the most critical phases in development. The transition from fetal to neonatal life requires elimination of the placental circulation, lung expansion and an increase in lung blood flow. Neurological or muscular defects, abnormalities in the circulation, morphological obstructions of respiration or impaired lung development can all result in respiratory failure. There are many examples of mouse mutants that do not survive the perinatal period. In some cases, structural defects were found that are likely to inhibit breathing like the absence of the distal parts of the ribs in the Myf5 knockout (Braun *et al.*, 1992; Kaul *et al.*, 2000) or improper tracheal and lung morphogenesis in Hox5a deficient mice (Aubin *et al.*, 1997). In other cases, such as in the DNA topoisomerase II knockout mouse (Yang *et al.*, 2000), the lack of both spontaneous and tactile stimulated movements pointed at a defect in neuromuscular function. In various mouse mutants, malformations of the heart may contribute to neonatal lethality (Ma *et al.*, 1998; Nagasawa *et al.*, 1996; Tachibana *et al.*, 1998; Takeuchi *et al.*, 2000). Often however the cause of death remained obscure (Gerard *et al.*, 1999; Li *et al.*, 1994; Rudolph *et al.*, 1998; Schweizer *et al.*, 1999).

Compared to wild type littermates, Sp3-/- lungs appear to be more compact and flattening of the type I cells is found less frequently in the Sp3-/- newborn (Bouwman *et al.*, 2000 and unpublished data). Both phenomena are consistent with a relatively immature status (Ten Have-Opbroek, 1981). At present, it is not clear whether the observed differences can account for the severe respiratory distress of Sp3 knockout mice. The fact that mutant lungs show normal transcription of surfactant proteins (Bouwman *et al.*, 2000) indicates that there is no dramatic delay in development.

Moreover, the observation that one knockout pup survived birth implicates that Sp3 may not be absolutely essential for lung functioning. Although a diminished neuromuscular function can not be excluded, the fact that Sp3 knockout newborns do attempt to breathe suggests that there is another reason for the observed neonatal lethality. In addition, studies of newborn brain, where Sp3 is expressed ubiquitously, did not reveal any morphological abnormalities.

The extremely thin myocardium of especially the atria prompted us to examine also the Sp3-/- heart more closely. In a mixed 129xC57BL/6 genetic background, heart function in Sp3 deficient fetuses was apparently sufficient to allow development until term, However, after back crossing to C57BL/6 for a few generations more we noted a lower number of Sp3-/- pups at E18.5 than expected according to Mendelian law. Also heterozygous Sp3 mutants appear to be affected in a C57BL/6 genetic background resulting in a gender-independent lethality before weaning. During late gestation numerous resorptions were found, most of which were homozygous Sp3 knockout mutants. Lethality during mid- and late- gestation can often be attributed to insufficient oxygen supply caused by improper cardiac, erythroid or vascular development (reviewed in Copp, 1995). From E13.5 onwards, Sp3-/- embryos occasionally displayed subcutaneous edema and peripheral hemorrhages, which has been indicative of heart failure in several other mouse mutants (e.g. Mesaeli *et al.*,

1999; Schilham *et al.*, 1996; Svensson *et al.*, 2000; Tevosian *et al.*, 2000). In addition to these observations, Sp3 mutant hearts show a defective closure of the ventricular septum. Interestingly, similar heart defects have been described in other mouse mutants, including mice deficient for Jumonji (Lee *et al.*, 2000; Takeuchi *et al.*, 1999) and retinoic acid receptor RXRα (Kastner *et al.*, 1994; Sucov *et al.*, 1994) which also share other aspects of their phenotype with the Sp3 knockout (Kitajima *et al.*, 1999; Makita *et al.*, 2001; chapter 6 of this thesis). The myocardial defects of RXRα-/- mice appear to be non cell-autonomous (Chen *et al.*, 1998; Subbarayan *et al.*, 2000; Tran and Sucov, 1998) and since Sp3-/- ES cell derived cardiac myocytes display normal *in vitro* differentiation (Gollner *et al.*, 2001b), this may also be the case for Sp3-/- mice. It was suggested that impaired signalling from outside the myocyte lineage is responsible for the heart phenotype of the RXRα mutant mice. An attractive candidate effector region would be the endocardium where growth factors like neuregulin-1 are secreted, that are necessary for the formation of myocardial trabeculae (Kramer *et al.*, 1996; Meyer and Birchmeier, 1995). Analysis of genes that are differentially expressed in (hearts of) E13. 5 RXRα-/- embryos suggested that part of the phenotype can be explained by a deficiency in cardiac metabolism (Ruiz-Lozano *et al.*, 1998). Although we could not establish a link between Sp3 and metabolism using a comparable approach (unpublished data), it will be interesting to examine the expression of RXRα target genes in Sp3 mutant mice. If Sp3 and RXRα are part of the same genetic pathway, similarities in expression profiles of both knockouts may be found. In that respect, it should be noted that the Sp3 homologue Sp1 can structurally and functionally interact with RXRα (Suzuki *et al.*, 1999) and that Sp1/Sp3 compound heterozygous mice show phenotypical characteristics of Sp3 deficient mice including growth retardation and perinatal lethality. Strikingly it has recently been shown that Sp4, the other known close relative of Sp3, is important for normal heart function (Nguyen-Tran *et al.*, 2000). Sp4 knockout mice that survive after weaning display a sudden cardiac arrest syndrome due to a disturbed heart rhythm, which may also explain the unanticipated lethality we observe in adult Sp4 deficient mice that were independently generated in our laboratory (Gollner *et al.*, 2001a; chapter 5 of this thesis). It will be interesting to investigate whether Sp3 and Sp4 play an independent role in heart development or if they can at least partly take over each other's function.

Considering the apparent effect of the genetic background on the Sp3 knockout phenotype: similar observations have been reported before. Disruption of, for instance, the cell growth inhibiting transcription factor Necdin results in neo-natal lethality after crossing with C57BL/6 mice, whereas crossing with C57BL/6xC3H or C57BL/6xCBA mice reduces lethality to wild type levels (Gerard *et al.*, 1999). Differences in the genetic background might also explain the absence of an overt phenotype in another Necdin knockout model (Tsai *et al.*, 1999). Intriguingly, Necdin has recently been shown to specifically repress Sp1 dependent activation of the c-Myc P1 promoter via a so called GN box, a motif resembling multiple aligned GC boxes (Matsumoto *et al.*, 2001) . Since Sp3 can also function as a repressor of Sp1 in case of promoters containing multiple binding sites (Birnbaum *et al.*,

1995; Dennig *et al.*, 1996; Majello *et al.*, 1997) the phenotypes of mice that lack Necdin or Sp3 may both be related to a perturbed regulation of Sp1 mediated transcription.

The increased severity of Sp3 knockout mice upon backcrossing to the C57BL/6 genetic background emphasizes the role of Sp3 in heart development. On the other hand, a possible decrease in severity in a mixed background may result in post-natal survival of the Sp3-/- mice, which would permit us to examine the role of Sp3 during adult life. Therefore, it remains important to further investigate the Sp3 knockout phenotype in different genetic backgrounds.

In summary, we have shown that Sp3 deficient embryos suffer from heart defects which - depending on the genetic background - may lead to prenatal lethality. It seems likely that these heart defects also contribute to the earlier observed neonatal lethality of Sp3-/- mutants.

Acknowledgements

We thank Thijs van Aken, Elize Haasdijk and Bert Wisse for expert technical assistance and Drs. Adriana Gittenberger-de Groot, Ank ten Have-Opbroek and Chris de Zeeuw for specialist advice. This work was supported by a NWO grant to F.G.

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Chapter four

Impaired B- and T- lymphoid development in Sp3-/- mice

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Abstract

The Sp/Krüppel like factor (Sp/XKLF) Sp3 plays a role in the development of multiple murine organs and tissues. Like its close homologue Sp1, Sp3 is expressed in many - if not all - different cell types. We have studied the expression of Sp3 in cells of the hemapoietic system using a lacZ-neomycin fusion gene inserted in the mouse Sp3 locus. In lymphocytes, Sp3 appears to be down regulated upon terminal differentiation. To investigate the function of Sp3 in lymphopoiesis, the B and T cell populations of Sp3 deficient mice were analyzed at embryonic day 18.5 (E18.5). In line with their generally retarded growth, spleens of E18.5 Sp3-/- mutants are small and contain relatively low numbers of especially the most developed B cells at that stage which are IgM^+. T cell development is also affected in the absence of Sp3 resulting in 2-fold higher percentages of $CD4^-CD8^-$ double negative- and $CD4^{low/-}$ $CD8^+$ immature single positive- T cell precursors in Sp3-/- E18.5 thymi.

Introduction

Sp3, an Sp/Krüppel like factor (Sp/XKLF; Philipsen and Suske, 1999) family member, is essential for normal late gestational growth and post-natal survival in the mouse (Bouwman *et al.*, 2000; this thesis). In the absence of Sp3 several developmental processes are affected, varying from tooth morphogenesis and bone formation to heart development (Bouwman *et al.*, 2000; Gollner *et al.*, 2001; this thesis). This wide range of defects correlates with the ubiquitous expression pattern of Sp3 and the fact that it has a similar preference for GC (GGGGCGGGG) and GT (GGTGTGGGG) boxes as its close homologue Sp1. GC and GT boxes are known to be important for the transcription regulatory regions of many different genes, varying from housekeeping- to tissue-specific- and viral- genes (Philipsen and Suske, 1999; Turner and Crossley, 1999). Among these are genes that play a role in B lymphocyte development and function, like the non-receptor protein tyrosine kinase Bruton's agammaglobulinemia tyrosine kinase (Btk) (Himmelmann *et al.*, 1996; Muller *et al.*, 1996; Rohrer and Conley, 1998). Also some genes involved in T cell development are known to be regulated through Sp1 binding sites. In fact, Sp3 has originally been cloned from an αβ T cell line in a search for Sp1 related factors that bind to a GT box required for the *trans*activation of the T cell antigen receptor (TCR) variable gene segment Vα11.1 (Kingsley and Winoto, 1992).

Lymphopoiesis can be divided into antigen-independent development, when different antigen receptors are formed via V-D-J recombination, and selective differentiation upon antigen recognition. Specific cell surface markers allow the identification of distinct stages during these processes. Whereas T cell development takes place in the thymus, B cells are formed in bone marrow or in the fetal liver. In contrast to the well-balanced gradual process in bone marrow, B lymphopoiesis in the fetal liver takes place in a wave, with increased percentages of more mature cells towards birth (Hardy *et al.*, 2000). Compared to their adult counterparts, fetal liver derived B cell precursors show a relatively high proliferation rate. In addition, cells of the first category can be induced to clonal expansion by pre-B cell antigen receptor (BCR) mediated signalling, while fetal cells are driven out of the cell cycle after μ heavy chain introduction.

We have found that Sp3 expression is down-regulated upon differentiation of B- and T-lymphocyte precursors in bone marrow and thymi respectively of young adult mice. To gain more insight in the role of Sp3 in lymphopoiesis we have analyzed distinct B and T cell populations of Sp3 E18.5 knockout mice. Fetal B cell development is impaired in the absence of Sp3, resulting in low percentages of $B220^{+}IgM^{-}$ pro- and pre- B lymphocytes and immature IgM^{+} B cells. Cultured Sp3-/- pre-B cells are able to develop to a mature $IgM^{high}IgD^{high}$ stage, indicating that there is not an absolute block in differentiation.

Not only B cell differentiation, but also T cell development is affected in the Sp3 knockout. Flow cytometric analysis of E18.5 Sp3-/-thymocytes revealed a relatively high percentage of precursor cells before the $CD4^{+}CD8^{+}$ double positive (DP) stage. In contrast,

the relative amount of $CD4^+$ single positive (SP) cells in Sp3-/- thymi is lower than in those of wild type littermates.

In light of the fact that Sp3 expression is down regulated upon terminal differentiation of lymphoid cells, these findings indicate a stimulatory role for Sp3 in both B and T cell development.

Materials and Methods

Knockout mice. The generation of Sp1 and Sp3 knockout mice has been described before (Bouwman *et al.*, 2000; Marin *et al.*, 1997). Sp3-/- and Sp1+/-Sp3+/- fetuses were obtained from timed matings of heterozygous mice and genotyped by PCR (Marin *et al.*, 1997; Chapter 3).

Flow cytometric analysis and ex vivo culturing of fetal liver derived B cells. Upon dissection, single cell suspensions were prepared (Slieker *et al.*, 1993) and directly analyzed by flow cytometry (2×10^4- 1×10^5 cells; Hendriks *et al.*, 1996) or cultured as described (Dingjan *et al.*, 2001; Rolink *et al.*, 1991). Fluorescein-di-β-D-galactopyranoside from Molecular Probes Europe (Leiden, The Netherlands) was used as a substrate for β-galactosidase activity. The following monoclonal antibodies were obtained from Pharmingen (San Diego, C.A., U.S.A.): PE-conjugated anti-CD4, anti-CD5/Ly-1, anti-CD11b/Mac1, anti CD43/S7 and anti-Ter119; biotinylated anti-IgM; Cy-Chrome-conjugated anti B220/RA-6B2 and anti-CD8; FITC-conjugated anti B220/RA3-6B2 and anti-CD3. Southern Biotechnology Associates (Birmingham, A.L., U.S.A.) supplied PE-conjugated anti-IgD. Anti-CD8/53-6.7, anti-Gr1/RB6-8C5 and ER-MP20 were purified monoclonal antibodies conjugated to biotin according to standard procedures. Secondary antibodies used were TriColor- or PE-conjugated streptavidin (Caltag Laboratories, Burlingame, C.A., U.S.A.) or streptavidin-APC (Pharmingen).

Results

Sp3 expression is down regulated upon lymphocyte differentiation. Similar to what has been described for the closely related transcription factor Sp1, the presence of Sp3 in many different tissues and cell types does not exclude the possibility that its expression is tightly regulated. Accordingly, a role for Sp3 in lymphopoiesis may be indicated by alterations in the transcriptional activation of the Sp3 gene during differentiation. To determine whether such regulation occurs, we have analyzed Sp3 expression in different types of blood cells. This was accomplished via flow cytometric analysis, using the β-galactosidase activity of the lacZ-neomycin fusion gene (β geo; Friedrich and Soriano,

1991) present in the targeted allele of Sp3 heterozygous mice. Samples from spleen, thymus, bone marrow and peritoneal cavity of five week old mice were labelled with antibodies specific for the individual hematopoietic lineages and assayed for expression of the Sp3 gene with fluorescein-di-β-D-galactopyranoside (FDG) as a fluorogenic β geo substrate.

Fluorescence intensity of lacZ expressing cells is comparable in all different cell types and high percentages of cells with β-galactosidase activity were especially observed in the myeloid lineage, for instance in granulocytes and in peritoneal macrophages (not shown). Significant numbers of lacZ expressing cells during B lymphopoiesis were only found in $B220^+CD43^+IgM^-$ pro- and $B220^+CD43^-IgM^-$ pre-B cell precursors in the bone marrow (approximately 20% in both; Table 1.1). Mature B cells hardly show any lacZ expression (Table 1.1).

A comparable pattern was seen during T cell development. In the thymus $CD4^-CD8^-$ double negative (DN) precursors develop via an intermediate $CD8^+TCR^-$ immature single positive (ISP) stage to $CD4^+CD8^+$ double positive (DP) cells that form the most abundant group of thymocytes. In most T cell populations, the percentage of Sp3 expressing cells becomes virtually undetectable after the DN (13 ± 2 %) and DP (5 ± 0.5 %) precursor stages (Table 1.2 and data not shown). The 12.2 % of $CD8^+$ SP thymocytes that is lacZ positive may consist to a large extent of ISP intermediates between the DN and the DP stage. Alternatively, Sp3 is specifically expressed only in thymic $CD8^+$ cells but not in others such as those from the spleen or the peritoneum (Table 1.2 and data not shown).

TABEL 1 : **Percentage of lacZ expressing cells in lymphoid populations of young adult (bone marrow, thymus, spleen; n = 3) and newborn (fetal liver, spleen; n = 2) Sp3+/- mice.**

1.1: B cell populations

	Bone Marrow	Fetal Liver	Fetal Spleen
$B220^+CD43^+IgM^-$	23.4 ± 17.5	79.5 ± 7.8	84.5 ± 3.5
$B220^+CD43^-IgM^-$	21.5 ± 6.3		
$B220^+IgM^+IgD^-$	2.2 ± 0.8	86.0 ± 2.8	90.0 ± 4.2
$B220^+IgM^+IgD^+$	4.1 ± 0.6		

1.2: T cell populations

	Thymus	Spleen
$CD4^-CD8^-$	12.6 ± 2.2	
$CD4^+CD8^+$	5.0 ± 0.5	
$CD4^-CD8^+$	12.4 ± 1.8	1.7 ± 0.6
$CD4^+CD8^-$	0.2 ± 0.1	1.8 ± 0.2

Because of the difference between bone marrow and in fetal liver derived B cells, we have also examined Sp3 expression in E18.5 mice. In contrast to what was found in young adult mice, more than 80% of all lineage B cells are lacZ positive at E18.5 (Table 1.1; Figure 1). Moreover, there is no decrease in the relative amount of Sp3 expressing cells upon differentiation to the stage of IgM^+ immature B cells.

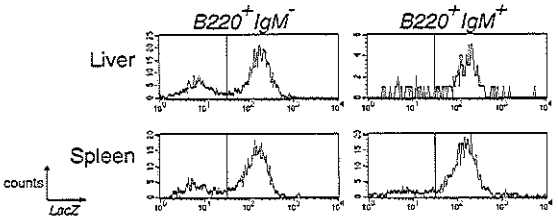

FIGURE 1: **Sp3 is abundant in both IgM^- and IgM^+ B lymphoid populations of late mouse fetuses.**
LacZ expression was analyzed in fetal liver and spleen of two E18.5 Sp3+/- littermates and depicted as a histogram that has been divided in expressing (*right panel*) and non-expressing cells (*left panel*). The percentages of lacZ positive cells are shown in Table 1.1.

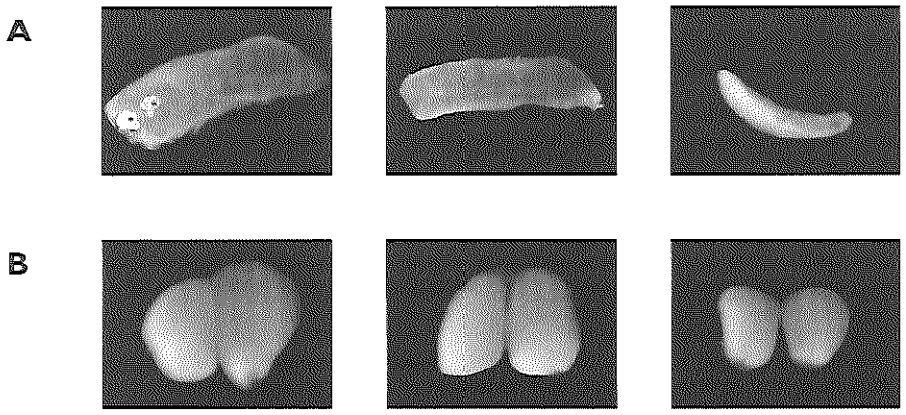

FIGURE 2: **Morphological characteristics of E18.5 Sp3+/+, Sp3+/- and Sp3-/- spleens and thymi.**
(*A*) Spleens of E18.5 Sp3+/+ (+/+), Sp3+/- (+/-) and Sp3-/- (+/-) littermates photographed at the same magnification. The knockout spleen is relatively small and pale (* = remnants of pancreatic tissue). (*B*) Thymi of the same mice as in panel *A* at an equal magnification for the three indicated genotypes. Like the spleen, also the thymus of the knockout is small compared the normal E18.5 size.

Impaired lymphoid development in Sp3 deficient newborn mice. The differential regulation of Sp3 expression during lymphopoiesis prompted us to examine the B and T cell populations in Sp3 deficient mice. Consistent with the generally retarded growth of Sp3-/- newborn mice (Bouwman *et al.*, 2000[1]; chapters 2 and 3), their spleens are relatively small when compared to those of wild type littermates (Figure 2A). Not only the absolute number of splenic B cells is low, there are also relatively few $B220^+$ lineage B cells present in Sp3 knockouts. Flow cytometric analysis of lymphoid cells from E18.5 liver and spleen indicates a 2-fold lower percentage of $B220^+IgM^-$ B cell precursors and a 6-fold lower percentage of more mature $B220^+IgM^+$ B cells than normally found at this age (Figure 3). In addition, heterozygous Sp3 knockout mice show a slightly but significantly retarded B cell development, indicating a gene dosage effect of Sp3 on B lymphopoiesis.

Similar to spleens, thymi of newborn Sp3 deficient mice are smaller than those of wild type littermates (Figure 2B). Flow cytometric analysis demonstrated that the relative numbers of the $CD4^+CD8^+$ DP or common thymocytes are normal or near normal in the absence of Sp3 (Figure 3). However, in Sp3 knockouts the percentage of mature $CD4^+$ T_H cells is lower than in wild type littermates whereas there are approximately twice as many DN and ISP thymocyte precursors (Figure 4).

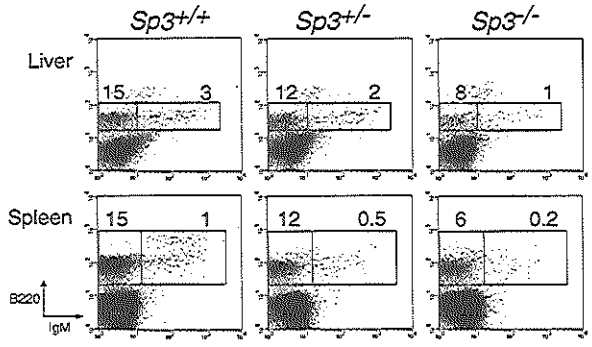

FIGURE 3: **A lower percentage of mature B cells in Sp3 knockout mice.** Representative flow cytometric analysis plots of E18.5 Sp3+/+, Sp3+/- and Sp3-/- livers and spleens, gated for lymphocytes and screened for B cells using B220 and IgM markers. Indicated are the percentages of IgM^- and IgM^+ B cells.

Fetal liver derived Sp3-/- pre-B cells can differentiate to the mature IgD^+ stage in culture. The perinatal lethality of the knockout mice precluded the analysis of more mature stages of B lymphoid differentiation in the absence of Sp3 *in vivo*. We therefore used a culture system of fetal liver derived pre-B cells to study differentiation beyond the $B220^+IgM^+IgD^-$ immature B cell stage (Rolink *et al.*, 1991). Fetal liver cells were cultured in the presence of the cytokine interleukin-7 (IL-7), thereby specifically inducing the

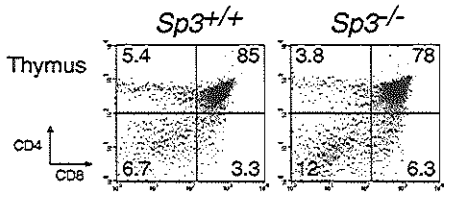

FIGURE 4: **CD4/CD8 analysis of thymocytes in Sp3 knockout mice.**
Representative flow cytometric diagrams of T cell populations in Sp3+/+ and Sp3-/- E18.5 thymi. Lymphocytes were gated on the basis of forward and side scatter characteristics and analyzed with anti-CD4 and anti-CD8. Indicated are the percentages of $CD4^-CD8^-$ DN, $CD8^+$ ISP, $CD4^+CD8^+$ and $CD4^+$ SP cells.

proliferation of μ heavy chain positive pre-B cells. After 5 days of culturing IL-7 was removed from the medium and the cells were placed on S17 stroma cells for 48 h, to allow further differentiation. Flow cytometric analysis demonstrated that Sp3-/- B cell progenitors can differentiate into $B220^+$ IgM^+ and surface IgD^+ cells albeit possibly with a reduced efficiency (Figure 5). Currently more experiments are underway to enable a quantitative analysis of B cell differentiation in the absence of Sp3.

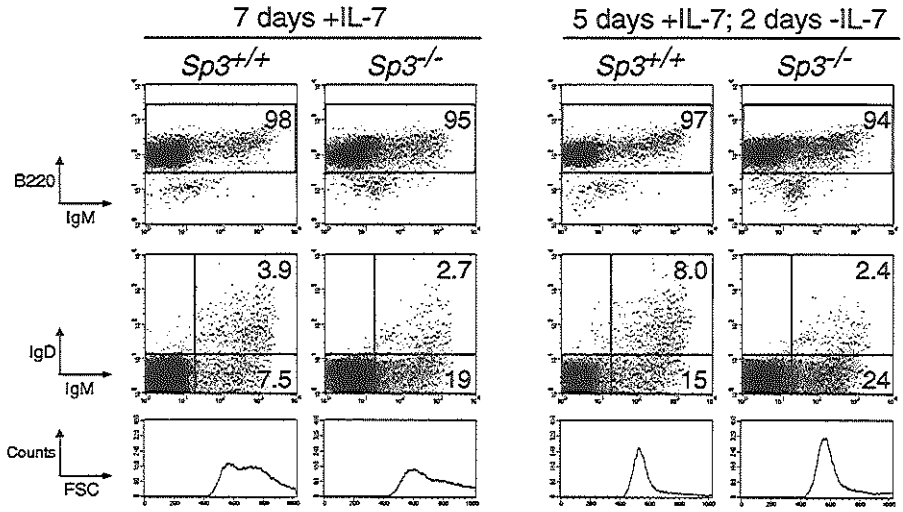

FIGURE 5: **Sp3-/- B cells can differentiate to the IgD^+ stage. Flow cytometric analysis of B cell differentiation in fetal liver derived cells that were cultured in the presence of IL-7.**
$B220^+$ B cells were gated and analyzed for IgM and IgD expression. The percentages of $B220^+$, IgM^+IgD^- and IgM^+IgD^+ cells are indicated. Removal of IL-7 after five days of culture further stimulated differentiation of Sp3+/+ cells and of Sp3-/- cells (right part) although the percentage of IgD^+ did not increase in the knockout culture. Nevertheless expression of surface IgD is possible in the absence of Sp3 indicating that there is no absolute block in differentiation. In addition, forward scatter histograms show differentiation towards smaller cells after the removal of IL-7 from the medium.

Discussion

Based on their ubiquitous expression and a large number of *in vitro* promoter analyses, transcription factor Sp3 and its close homologue Sp1 are likely to be involved in the transcription regulation of many different genes. To study the role of Sp3 in the development of the lymphoid lineage, we have compared the populations of B and T cells in Sp3 deficient E18.5 late fetuses and wild type littermates. At birth, Sp3-/- mutants can easily be distinguished from their littermates because of their generally retarded growth which is also reflected in the small sizes of thymus and spleen.

Compared to the wild type situation, the E18.5 Sp3-/- thymus contains nearly normal numbers of $CD4^+CD8^+$ DP intermediate cells but a 2-fold higher percentage of both DN and ISP progenitor thymocytes. In addition, less DP cells have matured to the $CD4^+$ T_H stage. Since transcription of the Sp3 gene appears to be down regulated after the $CD8^+$ ISP stage, Sp3 may predominantly be functional in early T cell development. At present, it remains to be determined what is the cause of the relatively high numbers of DN and ISP cells in the absence of Sp3. Contrary to other thymocytes, the wild type ISP population mainly consists of cycling cells (MacDonald *et al.*, 1988) and a role for Sp3 in driving expansion toward the DP stage may be reflected in a reduced proliferation capacity of Sp3-/- ISP cells. Increased percentages of progenitor thymocytes have also been found in mice deficient for the bHLH (basic helix loop helix) Hela E-box binding protein (HEB; Barndt *et al.*, 1999). Indeed, the number of HEB-/- ISP cells that are found in G_1 is relatively high. With respect to the ISP population, similar results have been reported for high mobility group protein (HMG) TCF1 mutants (Verbeek *et al.*, 1995). Unlike Sp3- and HEB-knockout mice however, these mice show normal percentages of DN cells. Currently, we are using CD44 (hyaluronic acid receptor) and CD25 (IL-2Rα) markers for a further subdivision of DN thymocytes (Godfrey *et al.*, 1993) which will be helpful to identify possible partial blocks in differentiation of these cells.

The effect of Sp3 deficiency on B cell development is more pronounced, resulting in approximately 6-fold lower amounts of mature $B220^+IgM^+$ cells in the newborn spleen. Although late B cell differentiation is possible in *ex vivo* cell cultures, it may proceed at a lower rate in the absence of Sp3. This would mean that there is a B cell autonomous defect, since the cultured cells were allowed to differentiate in the presence of stromal cells. Further experiments are required to address this question. Another indication that B cell development is specifically affected by the absence of Sp3 is that it appears to proceed normally in Sp1/Sp3 compound heterozygous newborns which show a growth retardation comparable to that of the Sp3 knockouts (chapter 3 of this thesis; our own unpublished data). Similar to what was seen in T cell development, Sp3 is predominantly present in rapidly proliferating precursor cells during B lymphopoiesis. Again, this could be indicative of a role for Sp3 during specific stages of differentiation.

It is not clear which Sp3 target genes are responsible for the B cell phenotype. Btk seemed an attractive candidate given the importance of GC boxes for its promoter function and the relatively small and immature B cell population in Btk mutant mice (Hendriks *et al.*, 1996; Kerner *et al.*, 1995; Khan *et al.*, 1995; Rawlings *et al.*, 1993; Thomas *et al.*, 1993). In addition, Sp1 and Sp3 are able to *trans*activate the Btk promoter synergistically with PU.1 through adjacent binding sites. However, Western blotting experiments indicate that the overall Btk expression in E18.5 Sp3-/- spleens is not affected which implies that Sp3 is not essential for the transcriptional regulation of this gene (unpublished data). Likewise, Sp1 was found to be dispensable for Btk expression which may indicate that Sp1 and Sp3 are redundant with respect to Btk *trans*activation or that other related factors such as Sp4 are more important *in vivo* (Muller *et al.*, 1999).

Several other mouse mutants have been described with impaired B cell development including mice deficient for transcription factors like Pax5, E2A, E2.2, HEB, Sox-4 and NF-κB (reviewed in: Reya and Grosschedl, 1998) and mice lacking stromal cell-derived factor-1 (SDF-1; Nagasawa *et al.*, 1996) or its receptor CXCR4 (Zou *et al.*, 1998). In addition to the aforementioned T cell phenotype of the HEB knockout (Barndt *et al.*, 1999) some of the other mutants share aspects of their phenotype with the Sp3 knockout. For example perinatal lethality and ventricular septal defects have also been found in SDF-1-/- and CXCR4-/- mice. Although SDF-1 and CXCR4 are expressed at normal levels in E13.5 Sp3-/- embryos (G. Suske, unpublished data) it can not be excluded that Sp3 is involved in their transcriptional regulation in specific cell types or at other stages of development.

In summary, we have performed a first analysis of the role of Sp3 in murine lymphopoiesis. Sp3 expression appears to be regulated during lymphoid differentiation and decreases at later stages. In the absence of Sp3, both B- and T-cell development are hampered, leading to relatively low percentages of mature cells and accumulation of DN and ISP precursor thymocytes. Although at least in lineage B cells Sp3 is not absolutely required for terminal differentiation, our results suggest a specific function for Sp3 in B- and T-lymphoid development.

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Chapter five

Complex phenotype of mice homozygous for a null mutation in the Sp4 transcription factor gene

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Genes to Cells **6**, 689-697, 2001.

Complex phenotype of mice homozygous for a null mutation in the *Sp4* transcription factor gene

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Abstract

Background: **Sp4 is a zinc finger transcription factor which is closely related to Sp1 and Sp3. All three proteins recognize the same DNA elements and can act as transcriptional activators through glutamine-rich activation domains. Unlike Sp1 and Sp3, which are ubiquitous proteins, Sp4 is highly abundant in the central nervous system, but also detectable in many other tissues.**

Results: **We have disrupted the mouse *Sp4* gene by a targeted deletion of the exons encoding the N-terminal activation domains. *Sp4* knockout mice show a complete absence of *Sp4* expression. They develop until birth without obvious abnormalities. After birth, two-thirds die within 4 weeks. Surviving mice are growth retarded. Male $Sp4^{null}$ mice do not breed. The cause for the breeding defect remains obscure since they show complete spermatogenesis. In addition, pheromone receptor genes in the vomeronasal organ appear unaffected. Female $Sp4^{null}$ mice have a smaller thymus, spleen and uterus. In addition, they exhibit a pronounced delay in sexual maturation.**

Conclusions: **The phenotype of the $Sp4^{null}$ mice differs significantly from those described for $Sp1^{-/-}$ and $Sp3^{-/-}$ mice. Thus, the structural similarities, the common recognition motif and the overlapping expression pattern of these three transcription factors do not reflect similar physiological functions.**

Introduction

The Sp family of transcription factors is composed of five proteins (Sp1, Sp2, Sp3, Sp4 and Sp5) characterized by a highly conserved DNA-binding domain at the C-terminus (Harrison *et al.* 2000; Philipsen & Suske 1999; Suske 1999; Treichel *et al.* 2001). In addition, Sp1, Sp2, Sp3 and Sp4 show similarities in their glutamine-rich N-terminal region (Suske 1999). The linkage of these four *Sp* genes to the four human *Hox* gene clusters also documents their close evolutionary relationship (Kalff-Suske *et al.* 1995, 1996; Scohy *et al.* 1998). Sequence alignments revealed that Sp1, Sp3 and Sp4 are more closely related to each other than to Sp2 (Philipsen & Suske 1999; Suske 1999). Consistently, Sp1, Sp3 and Sp4 recognize the classical GC-box and the related GT/CACC-box with identical affinity (Hagen *et al.* 1992, 1994). Sp1 and Sp3 are ubiquitously expressed, contrary to Sp4, which shows a complex expression pattern but is most abundant in neuronal tissues (Hagen *et al.* 1992; Supp *et al.* 1996).

A large variety of biological functions have been assigned to *Sp* factor-binding sites. This raises the question of which of these functions are performed by which Sp protein *in vivo*. Gene ablation studies in mice have provided important clues to the answer to this question.

Sp1 targeted embryos are severely retarded in growth, and die after day 10 of embryonic development (E10). They display a wide range of abnormalities, but all characteristic hallmarks of this developmental stage are

Communicated by: Shinichi Aizawa

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present. Blastocyst injections of Sp1-deficient embryonic stem cells showed that these cells contribute efficiently to early chimeric embryos, but after E11, this declines very rapidly with no detectable contribution to any tissue of newborn animals. Thus, Sp1 deficiency causes a cell-autonomous defect, and it appears that Sp1 function is generally required for cellular survival after E10 (Marin *et al.* 1997). Sp3-deficient embryos are growth retarded and invariably die at birth of respiratory failure. The cause for the observed breathing defect is not clear. Only minor morphological alterations were observed in the lung, and surfactant protein expression is indistinguishable from wild-type mice. In addition, $Sp3^{-/-}$ mice show a pronounced defect in late tooth formation. The development of the dentin/enamel layer is impaired due to a strongly reduced expression of ameloblast-specific gene products (Bouwman *et al.* 2000). A mutation of the *Sp4* gene has been reported previously (Supp *et al.* 1996). However, the mice in this study still expressed a truncated *Sp4* mRNA fragment encoding the two strong activation domains at a high level. Thus, the question remains whether the phenotype of these *Sp4* mutant mice reflects the physiological consequences of a complete *Sp4* knockout. $Sp5^{-/-}$ mice show no overt phenotype (Harrison *et al.* 2000).

Here we describe the targeted disruption of the mouse *Sp4* gene by deleting the exons that encode for the N-terminal activation domains. We found that the complete absence of Sp4 has severe consequences for postnatal mouse development. $Sp4^{null}$ mice develop until birth without obvious abnormalities. After birth, approximately two-thirds of the knockout mice die within 4 weeks. Those that survive are size retarded. Male $Sp4^{null}$ mice do not breed and have a slightly reduced testis size. However, they show complete spermatogenesis. In addition, the pheromone receptor genes in the vomeronasal organ that are essential for mating behaviour appear to be unaffected. Female $Sp4^{-/-}$ mice have a small thymus, spleen and uterus, and they reach puberty with a pronounced delay.

Results

Targeted disruption of the mouse *Sp4* gene

A targeting vector was designed to replace sequences that encode the N-terminal activation domains of Sp4 protein (amino acids 4–557). This was obtained by replacing exons 2 and 3 of the mouse *Sp4* gene (Song *et al.* 2001) by IRES-*LacZ*-polyA/PGK-*neo* sequences in the targeting vector (Fig. 1). The *lacZ* gene is expressed under control of the endogenous Sp4 promoter. The *phosphoglycerate kinase* (PGK) promoter controls the *neomycin resistance* (*neo*) gene and the *herpes simplex virus thymidine kinase* (*hsvtk*) gene to ensure expression in embryonic stem cells.

The targeting plasmid pPNT*Sp4i/e*/IRES-*LacZ* (Fig. 1B) was linearized at a unique *Not*I site present in the vector for transfection into E14 ES cells. Cells were subsequently maintained under G418 and gancyclovir selection. A total of 200 G418 resistant colonies were analysed by Southern blotting for the homologous recombination event. Hybridization of *Eco*RV restricted DNA from individual clones with a *Bgl*II-*Bam*HI intron fragment was predicted to show a > 10 kb fragment from the wild-type locus and a 6 kb fragment from a correctly targeted locus (Fig. 1B). One clone showed the predicted mutant fragment. In addition, PCR analysis with a set of primers specific for the wild-type and the targeted *Sp4* gene confirmed the expected targeted disruption of the mouse *Sp4* gene. Hybridization with a Bluescript vector probe showed no evidence for additional unwanted random integration events in this clone. The integrity of this clone was further confirmed by karyotyping. The targeted ES clone was injected into C57BL/6 blastocysts. Breeding of male chimeras resulted in germ-line transmission of the targeted *Sp4* allele.

Complete absence of Sp4

Matings of heterozygous $Sp4^{+/-}$ animals were set up to obtain embryos deficient in both wild-type *Sp4* alleles (Fig. 1C,D). To test whether this resulted in the complete loss of *Sp4* gene expression, we performed Northern blot analyses of RNA from brain and heart of both control and $Sp4^{-/-}$ embryos (Fig. 2A). Consistent with previous results, a probe coding for an N-terminal activation region of Sp4 detected two transcripts larger than 28S RNA in wild-type and $Sp4^{+/-}$ mice. These transcripts were undetectable in $Sp4^{-/-}$ mice (Fig. 2A, lanes 1–3). In addition, a probe encoding for the zinc finger region also shows no signal in $Sp4^{-/-}$ mice (Fig. 2A, lanes 10 and 11). The absence of the Sp4 protein in homozygous $Sp4^{-/-}$ mice was confirmed by Western blot analyses with an antiserum directed against the C-terminal domain of Sp4 (Fig. 2B). From these data we conclude that the *Sp4* knockout mice lack any residual *Sp4*-specific transcripts and protein.

Expression of Sp1 and Sp3 in Sp4 null mice

To analyse whether the expression of other members of the Sp-family of transcription factors is altered in

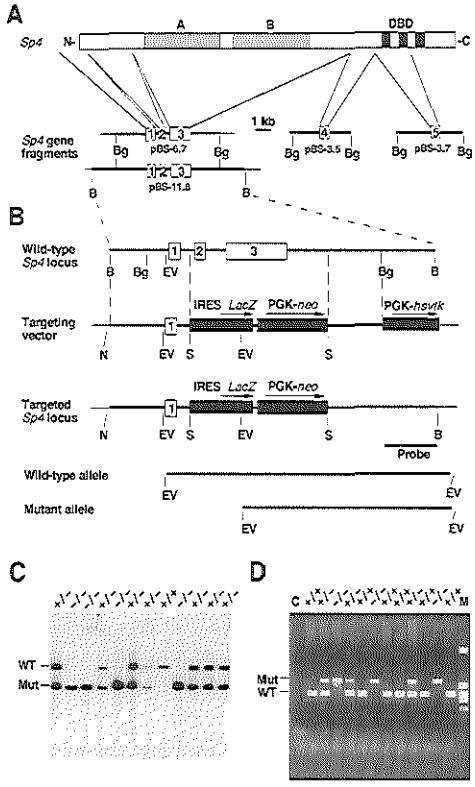

Figure 1 Targeted disruption of the mouse *Sp4* gene. (A) Schematic representation of the Sp4 protein structure and *Sp4* gene fragments. The glutamine-rich activation domains A and B and the zinc fingers (black bars) of the DNA binding domain (DBD) are indicated. Connecting lines with corresponding murine *Sp4* gene fragments indicate the derivation of individual Sp4 domains from different exons. Exons 1, 2 and 3 are present on a 6.7 kb *Bgl*II and an 11.8 kb *Bam*HI fragment. Exon 4 coding for 76 amino acids preceding the DNA-binding domain, and exon 5 coding for the first two zinc fingers are present on a 3.5-kb *Bgl*II and a 3.7-kb *Bgl*II fragment, respectively. (B) Schematic presentation of the knockout strategy. In the targeted *Sp4* locus, a cassette containing IRES-*lacZ* sequences and the *neomycin resistance* gene driven by the PGK promoter (PGK-*neo*) replaces exons 2 and 3. The targeting vector also contains a positive selection marker (PGK-*hsvtk*). Expected fragments of the wild-type and the mutant allele after restriction with *Eco*RV, and the probe used for Southern blotting are indicated. B, *Bam*HI; Bg, *Bgl*II; EV, *Eco*RV; S, *Sal*I and N, *Not*I. (C) Southern blot analysis of mouse embryos. Restriction of genomic DNA with *Eco*RV and hybridization with the probe indicated in Fig. 1B detected a > 10 kb fragment of the wild-type allele (WT) and a 6 kb fragment of the mutated allele (Mut). (D) PCR analysis of mouse embryos. The primers described in Experimental procedures produced a 390 bp DNA fragment from the wild-type allele (WT) and an approximately 600 bp fragment from the targeted allele. +/+, wild-type; +/−, $Sp4^{+/-}$; −/−, $Sp4^{-/-}$; C, negative control; M, size marker.

$Sp4^{-/-}$ mice, we performed Northern analyses with *Sp1*- and *Sp3*-specific probes. These experiments revealed that the abundance of *Sp1* transcripts was unchanged (Fig. 2A, lanes 4–6 and 12–13) whereas *Sp3* transcripts were slightly (approximately twofold) enhanced in $Sp4^{-/-}$ mice (Fig. 2A, lanes 7–9). Thus, the absence of Sp4 might be partially compensated by enhanced transcription of the *Sp3* gene.

Embryonic expression of the $Sp4^{LacZ}$ allele in heterozygous $Sp4^{+/-}$ mice

Expression of *Sp4* mRNA during embryonic development was previously monitored by *in situ* hybridization (Supp *et al.* 1996). Using the $Sp4^{IRES-lacZ}$ allele driven by the *Sp4* promoter, we were able to recapitulate the endogenous expression pattern of the

Sp4 gene. In whole mounts of heterozygous E12.5 embryos, β-galactosidase activity was highest in the central nervous system but also detectable in many other tissues (Fig. 3).

Survival rates and growth of $Sp4^{-/-}$ mice

Heterozygous $Sp4^{+/-}$ mice exhibited no discernible phenotype and were able to breed. Genotyping of embryos obtained by Caesarean section shortly before the parturition date (E18.5) showed no loss of $Sp4^{-/-}$ mice up to birth (Table 1). $Sp4^{-/-}$ newborn mice showed no obvious abnormalities at birth. However, approximately half of them died within 10 days of birth (Table 1). An additional 30% died within the following 3 weeks. Their cause of death remains undetermined. A comparison of growth rates (Fig. 4) revealed that within the first 4 weeks the gain of weight of the $Sp4^{-/-}$ mice was almost arrested. After this period their growth appeared relatively normal. However, the body weight of the surviving $Sp4^{-/-}$ mice never reached that of their wild-type littermates (Fig. 4).

Table 1 Genotype distribution of *Sp4* heterozygous crossings

	Total	+/+	+ −	−/−
E18.5	22	4 (18.2%)	11 (50.0%)	7 (31.8%)
Day 10	149	50 (33.6%)	79 (53.0%)	20 (13.4%)

The genotype was determined by PCR analysis as described in Experimental procedures.

We determined standard blood parameters (red cells (haematocrit, cell volume, haemoglobin per cell), white cells, platelets) of the $Sp4^{-/-}$ mice. These fell within normal ranges. Furthermore, we determined alkaline phosphatase, aspartate aminotransferase, albumin, creatinin, lactate dehydrogenase 1, bilirubin, and urea levels in the circulation. Of these, aspartate aminotransferase and lactate dehydrogenease 1 were slightly elevated (20% increase) in the knockout mice, but this difference was not significant. Thus, the circulation is apparently normal and these data provide no clues to the physiological problems underlying the growth retardation of $Sp4^{null}$ mice.

Male $Sp4^{-/-}$ mice do not breed

Breeding of $Sp4^{-/-}$ females with wild-type male mice

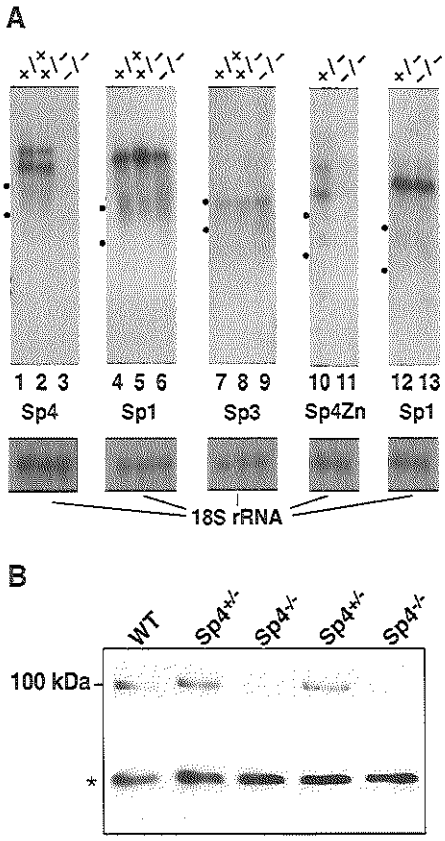

Figure 2 Complete absence of Sp4 in $Sp4^{null}$ mice. (A) Northern analyses of *Sp1*, *Sp3* and *Sp4* transcripts. RNA was extracted from heart (lanes 1–9) and brain (lanes 10–13) of wild-type (+/+), $Sp4^{+/-}$ (+/−) and $Sp4^{-/-}$ (−/−) E18.5 embryos, subjected to electrophoresis through 0.8% formaldehyde/agarose gels, and transferred to nylon membranes. The filters were hybridized with DNA-fragments encoding *Sp4* (lanes 1–3 and 10–11), *Sp1* (lanes 4–6, and 12–13) and *Sp3* (lanes 7–9). Two different *Sp4*-specific probes encoding the glutamine-rich activation domain (lanes 1–3), and the DNA-binding domain (lanes 10–11) were used to detect *Sp4* mRNA. The dots indicate the migration of 28S and 18S ribosomal RNA. As a control, the filters were probed with an 18S ribosomal RNA specific oligonucleotide. (B) Western blot analysis. Nuclear extracts (50 μg of protein) from brains of adult female wild-type (WT), heterozygous ($Sp4^{+/-}$) and *Sp4*-deficient ($Sp4^{-/-}$) mice were fractionated through 7.5% SDS–polyacrylamide gels, blotted on nitrocellulose filter and incubated with an Sp4-specific antiserum. Sp4 migrates as a 100 kDa protein. The asterisks depict an unspecific cross-reacting protein.

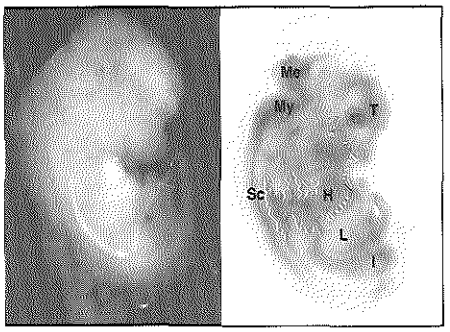

Figure 3 *LacZ* expression in heterozygous $Sp4^{+/-}$ embryos. Lateral view of E12.5 WT (left) and $Sp4^{+/-}$ (right) embryos stained for β-galactosidase activity. H, heart; L, liver; I, intestinal tract; Me, metencephalon; My, myelencephalon; T, telencephalon; Sc, spinal cord.

was sporadically successful. However, all attempts to breed adult $Sp4^{-/-}$ males with female wild-type mice were unsuccessful. This prompted us to examine whether a failure of spermatogenesis might be responsible for the observed infertility of $Sp4^{-/-}$ males. Histological analyses of testicular and epididymal cross-sections revealed complete spermatogenesis in the seminiferous epithelium (Fig. 5). In addition, luminal spermatozoa in the epididymis of $Sp4^{-/-}$ males were indistinguishable in numbers and shape from those in wild-type litter mates.

The highest expression of *Sp4* is found in the central nervous system (Hagen *et al.* 1992). Thus, Sp4 might exert its essential functions primarily in the brain. Wild-type females that were mated with $Sp4^{-/-}$ males did not contain copulation plugs, indicating an altered

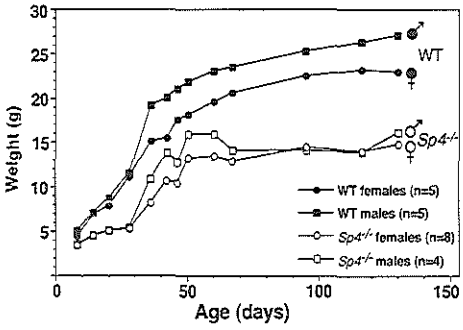

Figure 4 Growth curve (body weight vs. age) of male and female wild-type (WT) and $Sp4^{-/-}$ mice.

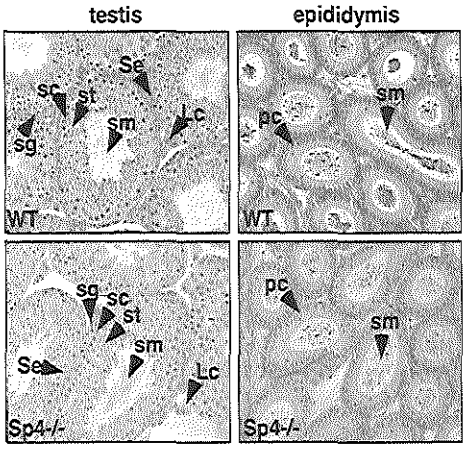

Figure 5 Histological analyses of testis (left) and epididymis (right). Haematoxylin staining revealed complete spermatogenesis in $Sp4^{-/-}$ mice. Abbreviations are: Se, Sertoli cells; Lc, Leydig cells; sg, spermatogonia; sc, primary spermatocytes; st, spermatides; sm, spermatozoa; pc, principle cell. Original magnification: × 200.

reproductive behaviour of $Sp4^{-/-}$ males. Since the hypothalamus and the vomeronasal organ (VNO) play important roles in reproductive behaviour (Buck 1995), we examined the VNO and the hypothalamus in sections, but no gross abnormalities were found in these tissues (data not shown).

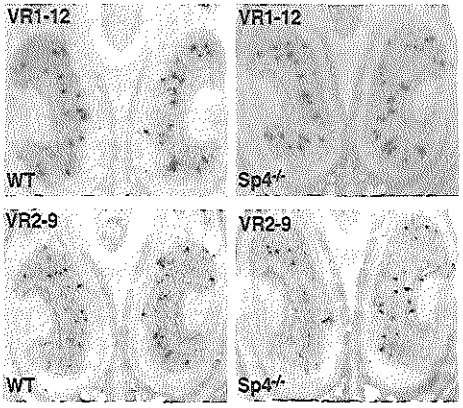

Figure 6 Analysis of VR expression by *in situ* hybridization. Cross-sections of vomero nasal organs dissected from neonatal WT and $Sp4^{-/-}$ mice were hybridized with Digoxigenin-labelled anti-sense RNA probes for the VNO receptor genes VR-12 and VR2-9.

Sexual behavioural alterations may reflect an altered gene expression pattern in specific neuronal cells of the VNO. VNO neurones express pheromone receptors of different classes (Belluscio *et al.* 1999; Herrada & Dulac 1997; Matsunami & Buck 1997). Therefore, we asked whether the expression of a subset of pheromone receptors might be altered in $Sp4^{-/-}$ mice. We analysed the expression of two different receptor genes in the VNO of $Sp4^{-/-}$ neonatal mice by *in situ* hybridization (VR1-12 from the apical zone and VR2-9 from the basal zone). The expression of both genes was unaffected in $Sp4^{-/-}$ mice (Fig. 6). Thus, we consider it unlikely that the function of the VNO is impaired in $Sp4^{-/-}$ mice.

The onset of puberty is impaired in female $Sp4^{-/-}$ mice

We determined the organ/body weight ratio of individual organs and found a significantly smaller thymus, spleen, uterus and testis in $Sp4^{-/-}$ mice compared to control mice ($P < 0.05$) (Fig. 7A). Other organs like the heart or kidney had a normal organ/body weight ratio. A reduced uterus size was also observed in heterozygous $Sp4^{+/-}$ females (Fig. 7B). The very small uterus in $Sp4^{-/-}$ females prompted us to examine the onset of puberty by determining vaginal opening time (VOT). We found that $Sp4^{-/-}$ females have a pronounced delay in vaginal opening time (VOT > 200 days) compared with wild-type and heterozygous $Sp4^{+/-}$ mice (VOT < 40 days). The delayed puberty is in agreement with the small uteri of $Sp4^{-/-}$ females, which is characteristic for prepubertal immaturity.

Discussion

Phenotype of $Sp4^{null}$ mice

Our results demonstrate that the transcription factor Sp4 is important for the early postnatal survival of mice, since two-thirds of the newborn die within 4 weeks post-partum. The molecular cause for the observed death, however, remains unclear.

Sp4-deficient mice that survive are impaired in their reproduction. Adult $Sp4^{-/-}$ did not breed at all and the mating of $Sp4^{-/-}$ females was only sporadically successful. The reduced testis and uterus weights of *Sp4-/-* mice indicated that Sp4 plays an important role for the function of these organs. A direct action of Sp4 in these organs seems plausible, since *Sp4* mRNA is expressed in testis and uterus (unpublished data).

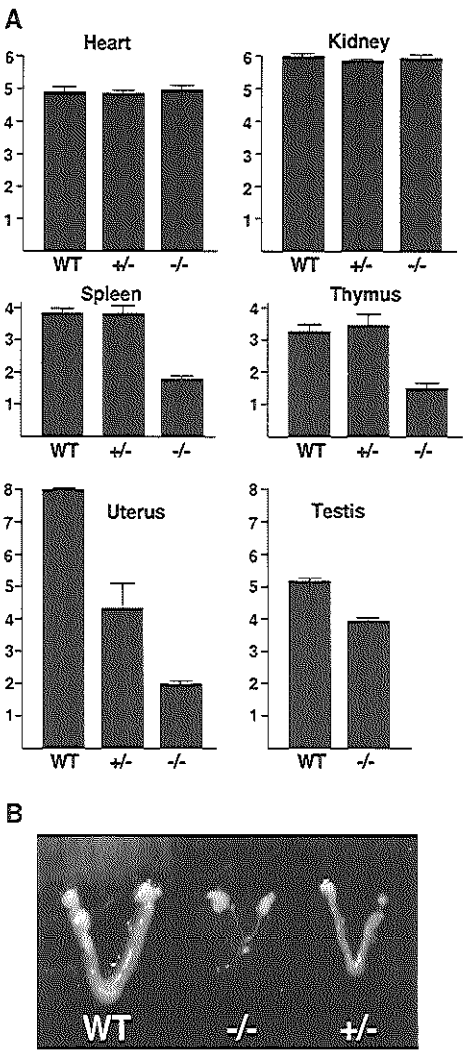

Figure 7 Organ sizes in $Sp4^{null}$ mice. (A) Relative weight of various organs. The values represent the ratio of organ mass (in mg) per body mass (in grams). (B) Uteri of 62-day-old female wild-type (WT), $Sp4^{+/-}$ (+/–) and $Sp4^{-/-}$ (–/–) littermates.

However, all attempts to detect a morphological alteration of the testis failed.

One could nevertheless speculate that the smaller reproductive organs are linked to the maturation of sexual functions, as reflected by the delay in the onset of puberty of $Sp4^{-/-}$ females. It is noteworthy that these animals also have a smaller thymus. The reduced

thymus size and the delay in the onset of puberty may be physiologically linked. It is known that the thymus is involved in female sexual maturation, since neonatal thymectomized mice also show a significant delay in the onset of puberty (Besedovsky & Sorkin 1974).

Sp4^{null} mice vs. *Sp4* mutant mice

A targeted mutation of the *Sp4* gene has been reported previously (Supp *et al.* 1996). Two exons coding for the three zinc fingers of the Sp4 DNA-binding domain were replaced by a targeting vector resulting in a 19 kb deletion of mouse genomic DNA. Such a large deletion, however, raises the possibility that the expression of an additional gene was disturbed. In addition, the *Sp4* gene was not completely inactivated. A truncated *Sp4* mRNA fragment encoding the two strong activation domains was still expressed at a high level (Supp *et al.* 1996). The expression of the activation domains of Sp4, however, might gain new functions or might interfere with other Sp-family members. It might act as a superactivator by interacting with glutamine-rich activation domains of other transcription factors. Indeed, such a mechanism has been demonstrated for the N-termini of Sp1 and Sp4. Both truncated proteins strongly enhance the transcriptional activity of Sp1 and Sp4 (Pascal & Tjian 1991; Hagen *et al.* 1995, and unpublished data). Therefore, it seems likely that the activation domains of Sp4 expressed *in vivo* also interfere with Sp1 or other transcription factors.

The *Sp4* knockout mice described here do not express domains of Sp4 that might interfere with other transcription factors. Neither the activation domains nor the DNA-binding domain were expressed. Nevertheless, the *Sp4*^{null} mice exhibit at least partial similarities with *Sp4* mutant mice lacking only the DNA-binding domain. Both the *Sp4*^{null} and the *Sp4* mutant strains have a high mortality rate after birth and males do not breed. Body weight/organ ratios, or the onset of the puberty were not reported for the *Sp4* mutant mice lacking only the DNA-binding domain. However, the extremely late onset of puberty is a phenotypic manifestation that would hardly have been missed by propagating this mouse strain. Thus, it seems likely that not all characteristic hallmarks of a *Sp4*^{null} mutation are detectable in mice lacking only the Sp4 DNA-binding domain.

Function of Sp-family members *in vivo*

Sp4 is closely related to the two ubiquitously expressed transcription factors Sp1 and Sp3. All three proteins recognize the same DNA elements and can act as transcriptional activators through glutamine-rich activation domains (Dennig *et al.* 1996; Hagen *et al.* 1995, 1994). The essential physiological functions, however, appear to be significantly different. In contrast to *Sp4*^{null} mice, *Sp1* mutant embryos are already severely retarded at early embryonic stages and die around day 10 of gestation (Marin *et al.* 1997). *Sp3*$^{-/-}$ mice develop until birth but die a few minutes post-partum due to respiratory failure (Bouwman *et al.* 2000). Thus, the obvious structural similarity, the common DNA recognition motif and the overlapping expression pattern do not reflect similar physiological functions. Nevertheless, there might be many overlapping functions *in vivo*. Since *Sp3* mRNA is up-regulated in *Sp4*^{null} mice, one could suggest that it might compensate at least partially for the loss of *Sp4* in *Sp4*^{null} mice. To unravel precisely the degree of redundancy between the individual Sp transcription factors, it might be necessary to generate mice deficient for two or all three GC-box binding Sp factors.

Experimental procedures

Cloning and mapping of the mouse *Sp4* gene

The cloning of the mouse *Sp4* gene has recently been described (Song *et al.* 2001). A 11.8 kb *Bam*HI fragment and three *Bgl*II fragments of 6.7 kb, 3.7 kb and 3.5 kb in length (Fig. 1A) that hybridized with the human *Sp4* cDNA were subcloned into the *Bam*HI site of the pBluescript KS vector leading to the plasmids pBS-11.5 (11.5 kb *Bam*HI fragment), pBS-6.7 (6.7 kb *Bgl*II fragment), pBS-3.7 (3.7 kb *Bgl*II fragment) and pBS-3.5 (3.5 kb *Bgl*II fragment). Subsequently the plasmids clones were mapped by Southern blotting and partially sequenced.

Generation of the *Sp4* homologous recombination construct

As a starting plasmid we chose the 7.2 kb pPNT vector (Tybulewicz *et al.* 1991) that contains PGK*neo* and PGK*hsvtk* cassettes separated and flanked by a number of unique cloning sites. We first introduced a part of the third intron of the *Sp4* gene into the multiple cloning site of the pPNT vector that separates the PGK*neo* and the PGK*hsvtk* cassettes. The 1.8 kb *Sp4* intron fragment was obtained from plasmid pBS-6.7 (Fig. 1A) as a [*Ecl*136II]/*Xba*I-*Eco*RI fragment and cloned into *Xba*I-*Eco*RI restricted pPNT plasmid leading to pPNT*Sp4*i. 5′-flanking sequences and the first exon of the *Sp4* gene were obtained from the plasmid pBS-11.5 (Fig. 1A) as a 4 kb *Not*I-[*Alw*44I]/*Sal*I-*Xho*I fragment and cloned into the *Not*I and *Xho*I (adjacent sites flanking the PGK*neo* cassette) restricted pPNT*Sp4*i plasmid. The resulting 13 kb plasmid was named

pPNT*Sp4i/e*. In a final step we introduced an IRES-*LacZ* cassette obtained as a 5.7 kb *SalI* fragment from the plasmid pGT1.8IRESβ*gal* into the *SalI* site of pPNT*Sp4i/e* leading to the knockout construct pPNT*Sp4i/e*/IRES-*LacZ*. The plasmid pGT1.8IRESβ*gal* is a derivative of pGT1.8IRESβ*geo* (Mountford *et al.* 1994) lacking the *neomycin resistance* gene. It was constructed by cloning a 3.3-kb [*Bam*HI]/*Xba*I-*Xba*I fragment of pGT1.8IRESβ*geo* back into the backbone of *Xba*I restricted pGT1.8IRESβ*geo*.

Transfection and selection of ES cells

E14 ES cells were electroporated with 15 μg of *Not*I-linearized targeting vector pPNT*Sp4i/e*/IRES-*LacZ*. Clones were selected with G418 (200 μg/mL) and gancyclovir. Homologous recombination was analysed by Southern blotting of *Eco*RV-restricted genomic DNA with the probe indicated in Fig. 1. Unwanted random integrations were detected by hybridizing the blots with a Bluescript vector-specific probe.

Generation of chimeric and Sp4-deficient mice

The $Sp4^{+/-}$ ES cell clone was karyotyped and microinjected in C57BL/6 host blastocysts. Chimeric males were mated to C57BL/6 females, and germ-line transmission was obtained. The F1 offspring were interbred to expand the stocks and to obtain $Sp4^{null}$ mice.

Genotyping of mice by Southern blotting and PCR

DNA was prepared from tail snips and analysed for the presence of wild-type and targeted *Sp4* alleles by Southern blotting or PCR. Southern blots were performed under standard conditions using the 1.8 kb *Bgl*II-*Bam*HI fragment of the third *Sp4* intron as a probe. For PCR analyses, three primers were used, a sense primer in the *Sp4* gene amplifying the wild-type allele (5′-CCAGTAACAATCACTAGTGTTGCA-3′), a sense primer in the *neomycin resistance* gene amplifying the targeted allele (5′-CATCGCCTTCTATCGCCTTCTTGA-3′) and an anti-sense primer in the *Sp4* gene (5′-CTCACAACCATATACCAATGC AAG-3′). PCR conditions were 94 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min for 30 cycles.

Northern blot analyses

Total RNA of mouse organs was extracted by the guadinium/isothiocyanate procedure using the Qiagen kit. RNA was separated through 0.8% agarose gels containing 2.2 M formaldehyde and blotted on to nylon membranes. Prehybridization and hybridization was carried out as described (Braun & Suske 1998). Gene-specific probes were obtained from appropriate plasmids or primer sets as follows. *Sp4*: 420 bp PCR-fragment amplified with A8-3 and A8-6 primers on plasmid pBS-6.7 (see above). *Sp4*Zn encoding the *Sp4* zinc finger region: 340 bp PCR fragment (nucleotides 2074–2414 of the murine Sp4 cDNA) obtained by RT-PCR using mouse RNA as template. *Sp1*: 311-bp fragment containing nucleotides 1371–1682 of the rat Sp1 cDNA (Imataka *et al.* 1992) obtained by RT-PCR using mouse RNA as template. *Sp3*: 800 bp *Pst*I fragment obtained from plasmid pBS4.6*Hin*dIII (Bouwman *et al.* 2000). The hybridization reaction with the Sp4 zinc finger encoding region as a probe contained 1 μg each of plasmids encoding *Sp1 and Sp3* to avoid cross-hybridization of the *Sp4* zinc finger probe with *Sp1 and Sp3* transcripts.

Western blotting

Nuclear extracts (50 μg of protein) were prepared from adult female brains according to Gorski *et al.* (1986), separated on 7.5% SDS-polyacrylamide gels, blotted on nylon membranes, and probed with a rabbit anti-*Sp4* serum (Santa Cruz sc-645). Primary antibodies were visualized using the Amersham ECL kit.

Whole mount LacZ staining

E12.5 embryos were dissected from uteri and fixed in 4% paraformaldehyde for 30–45 min and subsequently rinsed three times with LacZ rinse-buffer (0.1 M phosphate buffer, pH 7.3, 2 mM $MgCl_2$, 0.01% sodium desoxycholate, 0.02% Nonidet P-40) for 30 min. LacZ staining was performed with 1 mg/mL of X-gal solution in LacZ rinse-buffer supplemented with 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide over night at 37 °C. Embryos were post-fixed in 95% ethanol over night and stored in ethanol/glycerol (1 : 1).

Organ weights

Animals were sacrificed by cervical dislocation. Organs were collected and immediately frozen on dry ice prior to weight determination.

Acknowledgements

Dr M. Kalff-Suske is gratefully acknowledged for critically reading the manuscript. The pPNT vector was kindly provided by R. Klein with permission of R. C. Mulligan. This work was supported by grants of the Deutsche Forschungsgemeinschaft (SFB397) to G.S. and the NOW to F.G.

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Received: 22 March 2001
Accepted: 31 May 2001

Chapter six - Discussion

Functional analysis of the transcription factors Sp3 and Sp4 - discussion and future prospects

6.1 The physiological role of Sp-factors

Many functional aspects of Sp-transcription factors have been investigated using transient transfections in combination with reporter plasmids. Additional approaches included the specific inactivation of Sp1 and Sp3 using antibodies (Bevilacqua *et al.*, 1997; Bevilacqua et *al.*, 2000) or antisense RNA (Hata et *al.*, 1998; Noti, 1997; Noti et *al.*, 2000; Park et *al.*, 2000; Verrecchia et *al.*, 2001; Xiao et *al.*, 1999). However, all these methods are of limited value for the assessment of the physiological role of proteins. A more powerful way to determine the role of Sp transcription factors *in vivo* is through gene inactivation in the mouse.

As was already suggested by their unique *trans*activational properties (chapter 1), Sp1, Sp3 and Sp4 each appear to have a distinct function *in vivo*. Gene targeting experiments in the mouse yielded clear and different phenotypes that in addition seem to be consistent with expression patterns of the individual Sp family members. Disruption of the ubiquitously expressed Sp1 (Marin *et al.*, 1997) and Sp3 (Bouwman *et al.*, 2000; this thesis) genes results in a general growth retardation and becomes incompatible with survival at mid- and late-gestation, respectively. The lethality of Sp1 deficient mice around E11 precluded a systematic analysis of the role of Sp1 in different organs and biological processes. However, mutant embryos show a broad range of defects and Sp1-/- embryonic stem (ES) cells are unable to contribute to any tissue of chimeric mice beyond mid-gestation. There is a marked heterogeneity of the knockout phenotype, with some embryos developing to the 10-12 somite stage and others that remain an undifferentiated mass of cells. Apparently, many early processes are not blocked but slowed down in the absence of Sp1 and subtle differences for instance between genetic backgrounds may have a dramatic impact on the phenotype of Sp1-/- embryos. Interestingly, there are also variations in the severity of Sp3-/- phenotypes. Backcrossing of Sp3 deficient mice in the C57/BL6 inbred strain results in pronounced lethality during late gestation whereas most Sp3 knockout mice in a mixed 129xC57/BL6 background survive until birth (this thesis). The establishment of mutant strains in different genetic backgrounds may provide valuable information about the roles of Sp-proteins *in vivo*. For instance, impaired heart functioning in Sp3 knockout mice became especially apparent in C57/BL6 mice. In contrast to Sp1 deficient embryos, Sp3 mutants are able to survive during organogenesis which enabled us to investigate several biological processes. Initial experiments, most of which are described in this thesis, have uncovered a role for Sp3 in erythropoiesis (preliminary data), lymphopoiesis, heart, bone, (Gollner et *al.*, 2001b) and tooth-development. With respect to ossification and B cell development there are indications of cell autonomous defects, but the direct involvement of Sp3 in the course of the other processes has not yet been determined. In all cases additional research is required to fully apprehend the nature of the disturbances. Part of these experiments can be done using the Sp3-/- mice we have generated. For example, differentiation and proliferation of erythroid and lymphoid cells can be investigated in *ex vivo* colony assays. With respect to the impaired heart development, it is important to determine at which stage of gestation abnormalities are appearing. Such knowledge will be useful for the identification of the underlying defects at the physiological but also at the molecular level (see below). In addition, the appearance of phenotypical defects in knockout mice may

coincide with alterations in Sp3 expression in the wild type situation. Comparison of Sp3 expression between all tissues and organs during development as has been performed for Sp1 (Saffer *et al.*, 1991) may be informative. The β geo marker inserted in the targeted Sp3 allele is of limited use for such experiments since it only indicates the transcriptional activation of the Sp3 gene, which does not necessarily correlate with protein levels. Furthermore, in some organs like the fetal liver we have not been able to detect lacZ staining despite the fact that Sp3 is expressed (unpublished data). The reason for this discrepancy is not clear but it has been observed before in Sp1 knockout mice suggesting that it is caused by a common element in the inserted DNA like the IRES (internal ribosome entry site). Nevertheless, the β geo marker can be easily detected in most other tissues and allows a first estimation of expression patterns. For example, pilot experiments on E18.5 (Sp1+/- x Sp3+/-) litters have revealed differences in the intensity of lacZ staining between different organs of Sp1- and Sp3- heterozygous pups (unpublished data).

Sp3-/- ES cells may also be used to assess the role of Sp3 in biological processes, as has been shown by their reduced capacity to undergo osteogenic differentiation (Gollner *et al.*, 2001b). Unfortunately, efforts to generate chimeric mice with Sp3-/- ES cells have failed to produce viable offspring containing these cells (unpublished data). Upon transfer of injected blastocysts to recipient females there were signs of pregnancy that disappeared before term, suggesting *in utero* lethality similar to what was observed after backcrossing to the C57BL/6 inbred strain. It may be informative to determine the consequences of the contribution of these cells to different organs and tissues during prenatal development. However, the generation of conditional knockout mice (Gu *et al.*, 1994; Kilby *et al.*, 1993; O'Gorman *et al.*, 1991; Porter, 1998) would provide a more straightforward approach to gather such data. Restricted expression of the bacteriophage Cre- or the yeast FLP- recombinase can delete genes that are flanked by their respective loxP or FRT recognition sites in a cell type specific manner. In this way an unbiased understanding can be gained of the role of widely expressed genes like Sp1 and Sp3 in distinct tissues and organs. An even higher level of control of gene targeting may be achieved through the use of ligand inducible recombinases (Feil *et al.*, 1996; Logie and Stewart, 1995; Porter, 1998; Zhang *et al.*, 1996).

Although the Sp4 knockout also displays several different phenotypical characteristics, at least in some instances there is a correlation with the more restricted expression of this transcription factor. Potentially fatal disturbances of heart rhythm can be traced back to specific expression in the cardiac conductive system (Nguyen-Tran *et al.*, 2000) and there might also be a connection between impaired male sexual behaviour and the high levels of Sp4 in the central nervous system (Gollner *et al.*, 2001a; Hagen *et al.*, 1992; Supp *et al.*, 1996; this thesis) . Although the vomeronasal organ (VNO) is essential for the perception of female pheromones that trigger a sexual response in male mice (Dulac, 2000; Keverne, 1999), we have not found abnormalities in the expression of two different types of VNO receptor genes (Gollner *et al.*, 2001a; this thesis). To identify a defect in one or more systems that are responsible for normal male mounting behaviour, a detailed analysis of Sp4 protein expression during development may be helpful. Subsequently the molecular basis of this aspect of the Sp4 phenotype may be resolved (see below).

6.2 Sp-mediated transcription regulation: target gene detection

The physiological abnormalities of Sp-knockout mice are directly or indirectly caused by the aberrant transcription of genes that are regulated by these factors. Previous work in our laboratory has demonstrated that the expression of many putative Sp1 target genes is not dependent on the presence of Sp1 (Marin *et al.*, 1997). Only the expression of genes for the cell-cycle regulated thymidine kinase (TK) and the methylated DNA binding protein MeCP2 is reduced in Sp1-/- embryos. Whereas growth rates and cell cycle distribution of Sp1-/- ES cells was found to be normal, the low levels of MeCP2 could be linked to similarities in phenotype between Sp1 knockouts and chimeric embryos containing a high percentage of MeCP2 deficient cells (Tate *et al.*, 1996). However, the recent generation of MeCP2 deficient mice by different means did not result in lethality during *in utero* development and revealed that MeCP2 is mainly required for normal neurological function and post-natal viability (Chen *et al.*, 2001; Guy *et al.*, 2001). Most likely, the earlier described chimeric phenotype was due to a clonal artefact, and thus the functional significance of lower MeCP2 expression in Sp1-/- embryos remains to be determined.

Impaired tooth and bone development in Sp3 knockout mice correlates with a diminished expression of ameloblast specific genes (Bouwman *et al.*, 2000) and osteoblast restricted osteocalcin, respectively (Gollner *et al.*, 2001b). Yet, transcriptional disorders that underlie the general growth retardation and perinatal lethality of Sp3 null mutants remains to be determined. We have used a combination of cDNA arrays and a method for the amplification of differentially expressed transcripts (selective amplification via biotin- and restriction mediated enrichment or SABRE; Lavery *et al.*, 1997) to compare gene expression levels in E13.5 Sp3 deficient embryos with those in wild type littermates. Knockout embryos contain relatively low amounts of alpha globin, coincident with a temporarily delayed development of erythropoiesis during late gestation (unpublished data). Strikingly, similar to what was found in Sp1 null mutants, also in E13.5 Sp3 knockouts many genes retain a normal expression level. The decision to use total embryos of this age was based on the ubiquitous expression of Sp3 and on the observation that knockout embryos are generally growth retarded from approximately E13.5 onwards. However, the increased severity of the knockout phenotype upon backcrossing to the C57/BL6 inbred strain points at a more important role for Sp3 in circulation than had previously been assumed. Since edematous embryos were already found at E13.5, circulatory problems may be explained by events that occur earlier during organogenesis. When the critical timepoint has been determined, organs that are specifically important for circulation can be used for cDNA array analysis experiments. It should be noted that the thin myocardium and ventricular septal defects of Sp3 knockouts do not necessarily originate in the myocyte lineage. RXRα deficient mice suffer from comparable cardiac abnormalities despite the fact that RXRα is dispensable for the development of ventricular myocytes (Chen *et al.*, 1998; Subbarayan *et al.*, 2000; Tran and Sucov, 1998). Nevertheless, also in case the myocytes are non-autonomously affected alterations in their expression profile are to be expected as is illustrated by the downregulation of metabolic pathways in the absence of RXRα (Ruiz-Lozano *et al.*, 1998).

Sp3 target genes may also be detected using *in vitro* differentiated Sp3-/- ES cells that were found to express high levels of Delta like (Dlk; unpublished data). However, expression differences were minimal in knockout embryos and therefore the physiological significance of this finding remains to be determined.

For Sp4 some interesting potential target genes have been identified in relation to the disturbed control of cardiac rhythm in knockout mice (Nguyen-Tran *et al.*, 2000). The number of connexin 40 (Cx40) expressing distal Purkinje cells of the cardiac conduction system is decreased in the absence of Sp4. Furthermore, the cellular distribution of Cx40 and Cx43 is altered, with less immunostaining at the cellular borders where the gap junction plaques are located. Another marker of the conduction system, the potassium channel protein minK is also expressed aberrantly in Sp4 deficient mice.

Although Sp4 is highly abundant in the adult central nervous system, we have not been able to detect abnormalities in the expression profile of young adult Sp4-/- male brains (unpublished data). It is possible that differentially expressed genes were not represented on the cDNA arrays that have been used or that their relative abundance was too low to allow detection.

The combination of gene knockout strategies in the mouse with expression profile scanning using cDNA micro arrays enables the simultaneous identification of a large number of potential transcription factor target genes. In the light of accumulating evidence for cell type specific regulation of Sp-factors (chapter 1), it will be of critical importance to dissect the sample that is to be investigated as precise as possible from surrounding tissues to avoid quenching of differentially expressed genes.

Since cDNA hybridizations will predominantly allow the detection of the more abundant transcripts, additional procedures like SABRE may be useful to enhance the signals of low expressing genes.

Another way to unravel the function of the Sp family at the molecular level involves the immune precipitation of these proteins when bound to their regulatory sequences (Gould *et al.*, 1990). This method, termed ChIP (chromatin immune precipitation), may be used complementary to the approach described above. After cloning and sequencing of precipitated control elements, the availability of genome databases allows the identification of their target genes. Accordingly, evidence can be provided for the direct involvement of the transcription factor in question in the regulation of these genes.

6.3 Cooperation between Sp/XKLF factors

How do Sp-factors function together in the regulation of transcription? Although its binding site preference is somewhat different from that of the other Sp-proteins, Sp2 can recognize at least some Sp-motifs. In addition, Sp1-4 share glutamine- and serine/threonine- rich domains and the conserved Sp- and Btd- boxes and are frequently co-expressed. It is therefore conceivable that these factors cooperate in transcriptional regulation on several occasions and it will be interesting to compare the phenotype of Sp2 deficient mice with that of the other three knockouts.

The conserved DNA binding domain also implies other Sp/XKLF factors in Sp-mediated transcription and the *in vivo* function of a number of them has been assessed through the generation of knockout mice. Of these proteins, Sp5 is most closely related to Sp1-4 and could be considered as a member of the Sp-subfamily despite the absence of glutamine- or serine/threonine rich domains. Targeted deletion of Sp5 did not result in an overt phenotype but there may be subtle effects on the Brachyury genetic pathway since Sp5-/- T/+ double mutants show an enhancement of the frequency of taillessness (Harrison *et al.*, 2000).

At present, it is largely unknown to what extent different Sp/XKLF factors work together in the regulation of transcription. Despite similar binding site preferences and overlapping expression patterns, unique *trans*activating functions have been described for two family members. Experiments performed in our laboratory indicate that EKLF but not Sp1 activates footprint 2 of one of the Dnase I hypersensitive sites (5'HS3) of the human β-globin locus control region *in vivo* (Gillemans *et al.*, 1998). These data were obtained using transgenic mice harbouring mutant GT boxes that could be specifically recognized by Sp1 and EKLF proteins with the same altered zinc finger region. The targeted deletion of several Sp/XKLF members in the mouse enables an additional approach to uncover interactions between these factors. As has been demonstrated for Sp5 and Brachyury, crossing of mouse mutants may provide evidence for genetic interactions. The phenotype of Sp1/Sp3 compound heterozygous mice resembles that of the Sp3 knockouts with respect to growth retardation and neonatal lethality (chapter 3). Since the targeting of one allele of Sp1 or Sp3 alone does not have a strong effect on mouse development, this suggests that the in *vivo* function of both proteins is interconnected. It will be important to know at what level Sp1 and Sp3 influence each others action. So far there have been no indications of significantly altered levels of one of these transcription factors in the absence of the other (e.g. Bouwman *et al.*, 2000; Marin *et al.*, 1997). However, it remains to be determined whether there are circumstances under which they are involved in mutual transcriptional regulation. Very likely, both factors are at least partly redundant and regulate an overlapping set of target genes. Compound heterozygous mice for Sp4 and Sp1 or Sp3 have also been generated but do not show an obvious phenotype (unpublished data). However, it is likely that Sp4-mediated transcription is influenced by the amount of Sp1 or Sp3 present because preliminary data indicate that effects of Sp1 or Sp3 heterozygosity may be more obvious in the complete absence of Sp4 (unpublished data). Moreover, the Sp4 promoter may be directly regulated by other Sp/XKLF members (Song *et al.*, 2001) and conversely, the levels of Sp3 were found to be 2-fold increased in Sp4-/- brains (Gollner *et al.*, 2001a).

To investigate a possible functional redundancy, similar strategies may be followed as have been described for other closely related transcription factors. Knock-in experiments of myogenin (Wang and Jaenisch, 1997; Wang *et al.*, 1996) and En(grailed)-2 (Hanks *et al.*, 1995) targeted to the loci of Myf5 and En-1 respectively have demonstrated functional similarity between these factors. In these cases, important aspects of knockout phenotypes disappeared after the insertion of the homologous genes. In an analogous setting, GATA-3 and GATA-2 are at most only partially able to replace GATA-1 in prenatal erythroid differentiation (Takahashi *et al.*, 2000; Tsai *et al.*, 1998). However, the impaired

erythropoiesis and consequent embryonic lethality of GATA-1 mutants could be rescued by GATA-2 and GATA-3 transgenes under control of GATA-1 regulatory sequences. It has been suggested that the knock-in approach may not provide adequate levels of GATA-2 or GATA-3 to compensate for GATA-1 function due to differential post-transcriptional regulation (Takahashi *et al.*, 2000; Tsai *et al.*, 1998). Although this argues in favour of a transgenic approach, it may be complicated to faithfully reproduce endogenous expression patterns and therefore, also knock-in experiments should be considered to investigate whether Sp-factors are functionally interchangeable.

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Chapter seven - Samenvatting

Biologische ontwikkelingen vinden plaatst door veranderingen in de beschikbare hoeveelheid of de functionaliteit van eiwitten. In principe bevatten de kleinste bouwstenen van een organisme, cellen, de informatie die nodig is voor de aanmaak van alle eiwitten waarover dat organisme kan beschikken. Het is daarom nodig dat de aanmaak (expressie) van eiwitten zorgvuldig gereguleerd wordt waarbij twee fases van essentieel belang zijn. In de eerste fase wordt de genetische code voor een eiwit die ligt opgeslagen in het DNA omgezet naar RNA in een proces dat transcriptie wordt genoemd. Daarna wordt dit RNA in een zogenaamde translatie stap vertaald naar de aminozuren volgorde waaruit het eiwit bestaat.
Een groot deel van de regulatie van eiwit synthese vindt plaats op het transcriptie niveau waarbij specifieke transcriptie factoren een belangrijke rol spelen. Specifieke transcriptie factoren zijn eiwitten die in staat zijn om aan bepaalde motieven in het DNA te binden om vervolgens de initiatie van transcriptie te stimuleren of te onderdrukken. Door een variatie in transcriptie factor bindingsplaatsen kan het afschrijven van eiwit coderende DNA sequenties of genen worden beïnvloed door verschillende combinaties van transcriptie factoren.

In de experimenten beschreven in dit proefschrift werden de *in vivo* functies van de nauw verwante transcriptie factoren Sp3 en Sp4 onderzocht. Hiervoor werd gebruik gemaakt van de mogelijkheid om genen uit te schakelen in de muis. Wanneer er in deze knockout muizen vervolgens een verstoring van bepaalde processen optreedt, is dat een indicatie voor de rol die de door deze genen gecodeerde eiwitten hebben in levende organismen.

In **hoofdstuk 1** wordt een overzicht gegeven van de familie van transcriptie factoren waartoe Sp3 en Sp4 behoren. Hierbij komen met name de eigenschappen van de Sp factoren Sp1-4 ter sprake waarvan Sp1 het meest is onderzocht. Hoewel Sp1-4 erg op elkaar lijken en aan dezelfde DNA motieven binden (waarbij Sp2 iets afwijkt), zijn er toch ook belangrijke verschillen die een specifieke regulatie van transcriptie mogelijk maken. Sp4 onderscheidt zich door een meer afgebakend expressie patroon dan Sp1, Sp3 en mogelijk ook Sp2. In volwassen muizen komt Sp4 met name voor in neuronaal weefsel maar het wordt ook aangetroffen in het ontwikkelende geleidingssysteem van het hart. Post-translationele modificaties, gereguleerde afbraak en interacties met andere eiwitten vormen andere controle mechanismen voor de activiteit van de diverse Sp's.

Hoofdstuk 2 bestaat uit een eerste beschrijving van het fenotype van de Sp3 knockout. Sp3 blijkt essentieel te zijn voor overleving na de geboorte en voor een normale ontwikkeling van de tanden. Voor de geboorte zijn knockouts vanaf ongeveer dag 13.5 na bevruchting (E13.5) van normale embryonen te onderscheiden door een algemene groeiachterstand. Wanneer er kort voor het einde van de draagtijd op E18.5 een keizersnede wordt gedaan blijken ze, ondanks pogingen daartoe, niet in staat om adem te halen. Behalve hun kleinere postuur werden er geen opvallende morfologische afwijkingen gevonden, zodat niet duidelijk was waardoor de ademhaling belemmerd is in de afwezigheid van Sp3. Ook bleek de expressie van de surfactant eiwitten, van belang voor een goede gaswisseling in de longen, onaangetast te zijn. Dit in tegenstelling tot enkele eiwitten die

specifiek voorkomen in de ameloblast laag van de tanden. Amelogenine en ameloblastine RNA kon niet worden gedetecteerd in de pasgeboren Sp3 knockout wat samen bleek te hangen met een onvolledig gevormde dentine/enamel laag.

Aanvullend onderzoek naar defecten die ten grondslag zouden kunnen liggen aan het fenotype van muizen zonder Sp3 is opgenomen in **hoofdstuk 3**. Terwijl de hersenen en de longen respectievelijk niet en in beperkte mate af blijken te wijken van het normale stadium van ontwikkeling op E18.5 maakt het hart een onvolgroeide indruk. Niet alleen is het myocardium extreem dun, ook worden er gaten in het ventriculair septum aangetroffen. Naarmate de muizen verder worden terug gekruist naar de C57/BL6 stam blijkt het fenotype ernstiger te worden waardoor veel knockouts al tijdens de laatste fase van de ontwikkeling *in utero* sterven.

Vanaf E13.5 zijn duidelijke kenmerken van circulatoire problemen waarneembaar zoals subcutane oedeem vorming. Het lijkt dan ook aannemelijk dat een verminderde hartfunctie bijdraagt aan zowel de sterfte van Sp3 knockouts *in utero* als na de geboorte. Deze laatste aanname wordt onderstreept door het feit dat een Sp3 knockout van een gemengde genetische achtergrond de eerste dag na de geboorte kon overleven maar daarna dood werd aangetroffen met abnormaal vergrootte atria. Verder is er in dit hoofdstuk een korte beschrijving van het fenotype van zogenaamde Sp1/Sp3 dubbel heterozygoten opgenomen. In elke cel van deze dieren is maar een kopie van zowel het Sp1 als het Sp3 gen aanwezig terwijl dat er normaal gesproken (net als van de meeste andere genen) twee zijn. Het gevolg hiervan is een algemene groeiachterstand en een hoog sterfte cijfer vlak na de geboorte. Bovendien is er een nog hogere incidentie van oogafwijkingen in de dubbel mutanten dan er bekend is van muizen die alleen heterozygoot voor Sp1 zijn. Aangezien Sp1 en Sp3 heterozygoten verder niet veel verschillen van niet gemuteerde nestgenoten geven deze resultaten aan dat Sp1 en Sp3 gedeeltelijk een gelijkwaardige functie hebben.

De rol van Sp3 in lymfocyten, een specifiek soort bloedcellen die onderdeel vormen van het immuunsysteem, komt aan de orde in **hoofdstuk 4**. Lymfocyten zijn op te delen in B lymfocyten die adapter eiwitten (antilichamen) maken tegen de lichaamsvreemde eiwitten van ziekteverwekkers en T cellen die onder andere helpen bij de vernietiging van met antilichamen gemarkeerde cellen. In pasgeboren Sp3 knockout muizen blijkt de ontwikkeling van B- en T-cellen verstoord te zijn. Hierdoor hebben de populaties van beide cel soorten een relatief onvolgroeid profiel. Samen met het feit dat Sp3 expressie normaal gesproken minder wordt naarmate lymfocyten zich verder ontwikkelen duiden de resultaten op een rol voor Sp3 in de onvolwassen stadia van deze cellen. Cel kweek experimenten tonen aan dat B cellen in afwezigheid van Sp3 wel in staat zijn om volledig te differentiëren maar dat dit mogelijk minder efficiënt verloopt.

In **hoofdstuk 5** wordt het fenotype van de Sp4 knockout beschreven. De gevolgen van uitschakeling van Sp4 in de muis blijken pas na de geboorte. Aanvankelijk zijn Sp4-/- muizen niet zichtbaar te onderscheiden van hun nestgenoten maar als ze ongeveer een week oud zijn beginnen ze een groeiachterstand op te lopen. Een flink aantal Sp4 knockouts sterft

rond een leeftijd van drie weken wanneer ze normaal gesproken van de moeder gespeend zouden worden. De achterliggende oorzaak is niet precies bekend maar het lijkt er op dat ze zich niet voldoende kunnen voeden. De overlevende Sp4-/- muizen blijven enigszins kleiner dan normaal en de mannetjes planten zich niet voort. Opvallend hierbij is dat het normale seksuele gedrag van Sp4-/- mannetjes verstoord is waardoor ze niet op vrouwtjes reageren. Tenslotte worden volwassen Sp4 knockout muizen buitensporig vaak onverwacht dood aangetroffen. Andere onderzoekers hebben aangetoond dat dit te wijten is aan hartritme stoornissen.

Hoofdstuk 6 bevat een discussie van de bovengenoemde resultaten. Hierin worden suggesties gedaan voor verder onderzoek naar aanleiding van de fenotypes van de Sp3 en Sp4 knockout muizen.

Wanneer de fysiologische rollen van Sp3 en Sp4 precies zijn vastgesteld kan er ook op moleculair niveau onderzoek worden gedaan. Aangezien transcriptie factoren de expressie van andere eiwitten reguleren, liggen de oorzaken van de Sp3 en Sp4 knockout fenotypes in verstoringen van dergelijke processen. In experimenten die niet in dit proefschrift zijn opgenomen werd onderzocht welke genen afhankelijk zijn van Sp3 voor een normaal expressie niveau. Hoewel E13.5 knockout embryonen al in grootte te onderscheiden zijn van hun niet gemuteerde nestgenoten kon dat niet worden herleid tot duidelijke verschillen in RNA populaties. Desalniettemin werd er een correlatie gevonden tussen een verminderde expressie van alfa globine en een vertraagde ontwikkeling van de erythropoiese (vorming van rode bloed cellen). Dit kon met name een dag later tijdens de ontwikkeling, op E14.5, worden waargenomen. Behalve van embryonen is er voor onderzoek naar door Sp3 gereguleerde genen ook gebruik gemaakt van embryonale stamcellen (ES cellen) waarin beide kopieën van het Sp3 gen uitgeschakeld zijn (Sp3-/-). ES cellen zijn in staat om tot elke mogelijke celsoort (zoals bijvoorbeeld bot vormende cellen of spier cellen) te differentiëren. Wanneer Sp3-/- ES cellen er toe worden aangezet om zich willekeurig te ontwikkelen, blijken ze veel meer Delta like (Dlk) RNA aan te maken dan niet gemuteerde ES cellen. De fysiologische significantie van deze bevinding is echter niet duidelijk aangezien er in Sp3 knockout embryonen geen grote verschillen in Dlk expressie kunnen worden waargenomen.

De hartritme stoornissen van Sp4 knockout muizen konden door andere onderzoekers gerelateerd konden worden aan de verstoorde expressie van bepaalde genen. Wij hebben echter nog geen veranderingen in genexpressie kunnen vinden die een verklaring kunnen bieden voor andere aspecten van het Sp4 knockout fenotype zoals het ontbreken van normaal mannelijk seksueel gedrag.

Omdat duidelijk is geworden dat Sp-factoren specifiek gereguleerd kunnen worden in verschillende weefsels en celtypes is het belangrijk om hun *in vivo* functie te bestuderen in meer afgebakende systemen dan in een complete muis. Hierbij kan de mogelijkheid om genen alleen onder bepaalde condities uit te schakelen behulpzaam zijn. Een interessant

aspect aan nauw aan elkaar verwante transcriptie factoren is de vraag of ze in staat zijn elkaars functie (gedeeltelijk) over te nemen. Kruisingen tussen verschillende knockout lijnen zoals beschreven voor Sp1 en Sp3 kunnen daar het genetisch bewijs voor leveren. Ook kan er getracht worden om een uitgeschakeld gen te vervangen door een mogelijk redundant gen waarvan de transcriptie op eenzelfde wijze gereguleerd wordt.

Hoewel er dus een aantal andere benaderingen mogelijk is voor onderzoek naar de rol van Sp3 en Sp4 in levende organismen, kunnen er nog verschillende vraagstukken worden opgelost met behulp van de knockout muizen die wij hebben gegenereerd. Er is met name meer gedetailleerd onderzoek mogelijk naar de hartafwijking en de vorming van bloedcellen in de Sp3 knockout en naar de oorzaak van uithongering en afwijkende seksueel gedrag van de Sp4 knockout.

Abbreviations

ATP	Adenosine triphosphate
BKLF	Basic Krüppel like factor
Bp	Base pair
Btd	Buttonhead
BTEB	Basic transcription element binding protein
C-terminal	Carboxy terminal
CREB	Cyclic AMP responsive element binding protein
DN	$CD4^-CD8^-$ double negative
(c)DNA	(complementary) Deoxyribonucleic acid
DP	$CD4^+CD8^+$ double positive
EGR	Early growth response gene
EKLF	Erythroid Krüppel-like factor
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
Ig	Immunoglobulin
Inr	Initiator
IRES	Internal ribosome entry site
ISP	$CD8^+$ TCR^- immature single positive
kB	Kilo base
kDa	Kilo Dalton
lacZ	β-galactosidase
LCR	Locus control region
N-terminal	Amino terminal
PCR	Polymerase chain reaction
Py	Pyrimidine, base
Rb	Retinoblastoma susceptibility gene product
(m)RNA	(messenger) Ribonucleic acid
RXR	Retinoid X receptor
SP	$CD4^+$ or $CD8^+$ single positive
Sp/XKLF	Specificity protein / x Krüppel like factor
SREBP	Sterol regulatory element-binding protein
TAF	TBP associated factor
TBP	TATA box binding protein
TF	Transcription factor
TIEG	TGFβ-inducible early gene
UKLF	Ubiquitous Krüppel like factor
VNO	Vomeronasal organ
VSD	Ventricular septal defect

Curriculum vitae

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Dankwoord

'Iedereen bedankt' is hetzelfde als 'niemand bedankt' en daarom:

Mijn promotor Frank Grosveld en co-promotor Sjaak Philipsen: bedankt voor de uitdaging van het Sp3/Sp4 knockout project, ik heb veel van jullie geleerd.

De groep van Guntram Suske uit Marburg waar ik nauw mee heb samengewerkt. Guntram und Heike: recht herzlichen Dank für die Zusammenarbeitung (ich habe es oft bedauert das Marburg so weit von Rotterdam enfernt ist).

De naaste collega's op het lab spelen een belangrijke rol in het leven van een AIO. Fokke, Gaetano, Marisol, Nynke, Pieter-Fokko, Rita, Rita, Roy, R^2 en Uli uit de groep van Sjaak, Dave en Laura en de Oct-6ers Aysel, Dies, Ludo, Marko, Martine, Mehrnaz, Smiriti en Wim bedankt voor de goede sfeer, barbeques, Schwarzwalder taarten en Turkse ... (wat waren dat eigenlijk?).

Zonder secretaresses, muizenverzorgers, histologen, computer ondersteuning, keuken en inkoop was het allemaal natuurlijk niet gelukt. Marike en Rita: nog bedankt voor jullie kaartje toen ik in de kreukels lag.

Alar, Rien en Mannoussos, bedankt voor het injecteren.

Verschillende mensen van buiten de afdelingen Celbiologie en Genetica hebben een belangrijke bijdrage geleverd aan m'n promotie onderzoek. Rudi Hendriks en Sabine Middendorp van de Immunologie, Chris de Zeeuw en Elize Haasdijk van de Anatomie, Thijs van Aken van het EDC en Ank ten Have-Opbroek, Adriana Gittenberger-de Groot en Bert Wisse van de afdeling Anatomie en Embryologie van het LUMC bedankt voor jullie tijd en adviezen.

Soms was er ook nog tijd voor iets anders dan werken zoals bijvoorbeeld familie en vrienden. Pa & ma, Frank, Martijn & Marieke, 'schoonouders', Bert & Meike, Frank, Harm: ik ben blij dat er straks weer eens echt vrije weekenden en vakanties zijn zodat soms wat vaker kan worden.

Lieve Son, jou kan ik natuurlijk niet genoeg bedanken. Twee keer promotie stress wens je niemand toe... Zullen we ons nu maar eens over andere dingen druk gaan maken?

Peter