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SURVEY AND SUMMARY

A tale of three fingers: the family of mammalian Sp/XKLF transcription factors

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ABSTRACT

One of the most common regulatory elements is the GC box and the related GT/CACC box, which are widely distributed in promoters, enhancers and locus control regions of housekeeping as well as tissue-specific genes. For long it was generally thought that Sp1 is the major factor acting through these motifs. Recent discoveries have shown that Sp1 is only one of many transcription factors binding and acting through these elements. Sp1 simply represents the first identified and cloned protein of a family of transcription factors characterised by a highly conserved DNA-binding domain consisting of three zinc fingers. Currently this new family of transcription factors has at least 16 different mammalian members. Here, we will summarise and discuss recent advances that have been directed towards understanding the biological role of these proteins.

INTRODUCTION

G-rich elements such as GC (GGGGCGGGG) and GT/CACC boxes (GGTGTGGGG) are important *cis*-acting elements required for the appropriate expression of housekeeping as well as many tissue-specific and viral genes. These motifs have been found and functionally analysed in promoters, enhancers and locus control regions (LCRs) of genes that are under different modes of control, such as cell cycle regulation, hormonal activation and developmental patterning. Moreover, GC/GT boxes are commonly found in promoters embedded in CpG-rich methylation-free islands. Maintenance of the appropriate methylation patterns is essential for normal development (1). It has been shown for the APRT gene that the GC/GT motifs are required to maintain the methylation-free active status of the gene (2,3).

For long it has been known that the general transcription factor Sp1 (named according to the original purification scheme that included Sephacryl and phosphocellulose columns) (4) can bind and act through GC/GT boxes, and Sp1 was therefore thought to be an extremely versatile protein essential for many different functions of the mammalian nucleus. More recently, however, it has become clear that Sp1 is not the only transcription factor binding and acting through these elements. It simply represents the first identified and cloned protein of a new and still growing family of transcription factors. Family members contain a highly conserved DNA-binding domain consisting of three zinc fingers. Currently this family of transcription factors comprises at least 16 different mammalian members (Fig. 1). Accordingly, our view on Sp1 recognition elements as well as Sp1 itself has changed dramatically. The identification of all these transcription factors binding to Sp1 sites raises the question that is central to this review: what are the functions of these proteins?

THE ZINC FINGER REGION DEFINES THE FAMILY

The 81 amino acid DNA-binding domain that is found close to the C-termini of all members essentially defines the Sp/XKLF family of transcription factors. It consists of three C2H2-type zinc fingers arranged similar to those found in the *Drosophila melanogaster* regulator protein Krüppel. Accordingly, some of the proteins have been named Krüppel-like factors. The striking similarity of the linker amino acids between the individual fingers as well as the identical length of the DNA-binding domain strongly suggest that the higher order structure of the three fingers is crucial for the biological function of the proteins (Fig. 2). Structural studies on zinc finger peptides bound to DNA have provided information that allows predictions on the DNA sequence recognised by finger domains. The amino acids of the Sp1 zinc fingers that are most likely to make specific contacts with the DNA are the amino acids KHA within the first, RER within the second and RHK within the third zinc finger domain (Fig. 2). These critical amino acids are conserved in Sp3, Sp4, BTEB1, TIEG1 and TIEG2 proteins (Fig. 2). Consistent with this conservation, Sp3, Sp4, BTEB1 and TIEG2 recognise classical Sp1-binding sites (5–7). In addition, the relative affinity for the GC box is very similar, if not identical, between these proteins (5,6). GT or CACCC boxes are also recognised by these proteins but with slightly lower affinities (5). In Sp2, a leucine residue replaces the critical histidine residue within the first zinc finger (Fig. 2). Although not studied in detail, it was shown that Sp2 does not bind to the classical Sp1

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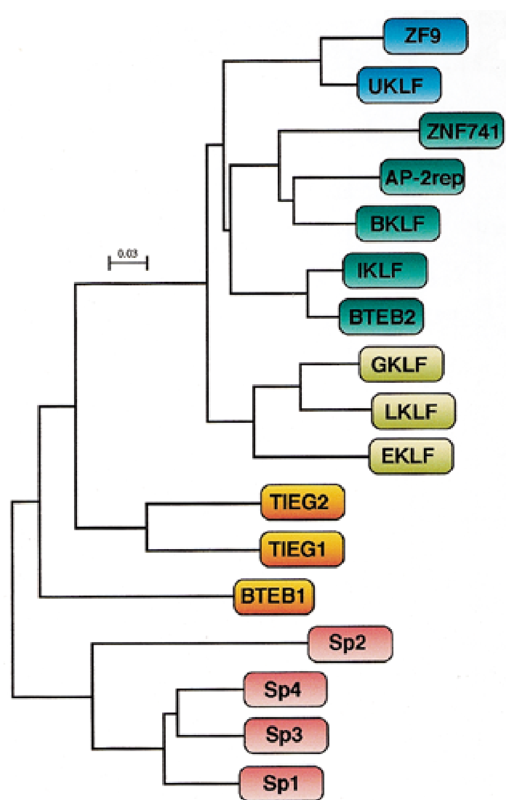

Figure 1. Phylogenetic tree of the Sp/XKLF transcription factors. The tree was generated with the CLUSTAL W Multiple Sequence Alignment Program v.1.7 (June 1997) (90) using the amino acid sequences of the DNA-binding domains of the Sp/XKLF proteins.

GC box but to a GT-rich element in the TCR Vα promoter (8). In all the other family members, the DNA-contacting amino acids of the first and second fingers are conserved. In the third finger, however, the critical lysine is replaced in some members by a leucine (Fig. 2). Thus, one would expect slight differences in the DNA-binding site preferences of the members of this subfamily. In agreement with this structural determinant it was shown that EKLF, UKLF and BKLF have a binding preference for GT boxes over classical GC boxes (9–12).

NOMENCLATURE AND CLASSIFICATION

Based on the structural relationships between the proteins (Figs 1 and 2), we suggest dividing the Sp/XKLF family into three subgroups: (i) the four Sp transcription factors; (ii) the close relatives, which currently consist of BTEB1, TIEG1 and TIEG2; (iii) the Krüppel-like factors (XKLFs). The latter subfamily also includes BTEB2, GBF/ZF9, ZNF741 and AP-2rep. Features of individual family members are summarised in Table 1.

To date, four Sp genes have been identified in the mammalian genome. The overall domain structure of these factors is very similar (Fig. 3). In addition to the highly conserved C-terminal zinc finger region, they contain glutamine-rich activation domains flanked by serine/threonine-rich stretches. Their evolutionary relationship is well documented by the co-localisation of the four human Sp genes with the four HOX gene clusters on chromosomes 12q13 (Sp1/HOX C) (13), 17q21.3–q22 (Sp2/HOX B) (14), 2q31 (Sp3/HOX D) (15) and 7p15 (Sp4/HOX A) (16). Sp1, Sp3 and Sp4 are more closely related to each other than to Sp2. This is especially evident on comparison of the three zinc fingers, which reveal significant differences between Sp2 and the other three factors (Fig. 2).

As outlined above, in BTEB1, TIEG1 and TIEG2 all amino acids that are believed to specifically contact the DNA are conserved and these proteins consistently recognise the classical GC box. However, the domains N-terminal to the zinc fingers do not share any similarity to the Sp factors. BTEB1, originally named simply BTEB (basic transcription element binding protein), has been identified and cloned along with rat Sp1 by virtue of its binding to the basal transcriptional element (GA-GAAGGAGGCGTGGCCAAC) of the cytochrome P-4501A1

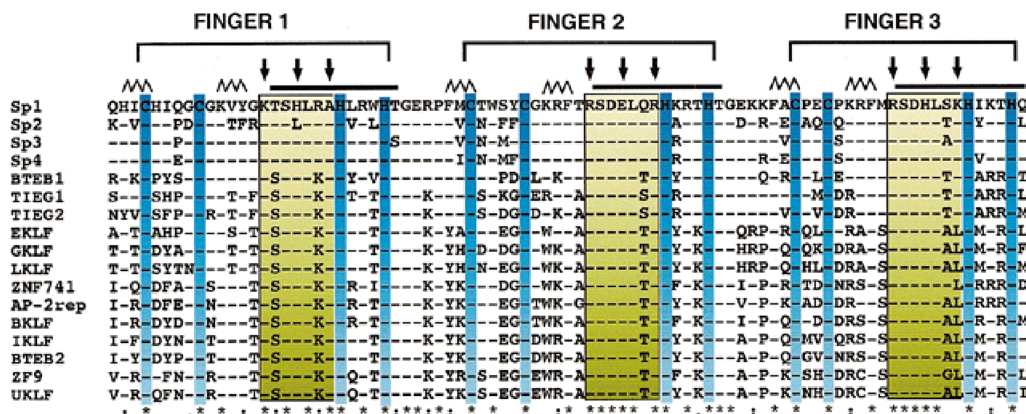

Figure 2. Protein sequence alignment of the zinc finger domains of mammalian Sp/XKLF family members. All sequences are human sequences with the exception of LKLF, AP-2rep, BKLF and IKLF, which are of mouse origin. The cysteine and histidine residues that are involved in zinc coordination are in blue. Arrows point to the amino acid positions that probably determine the recognition specificity of the fingers by contacting specific bases of the DNA. Black lines indicate α-helices; β-sheets are shown as zig-zag lines. The amino acids that are thought to make base contacts are boxed (adapted from 69). Residues conserved between all family members are indicated (*).

Table 1. Classification and function of Sp/XKLF proteins

Factor	Accession numbers	Known expression sites	Function / knockout phenotype	Features / remarks	H. chrom.	Ref.
Sp1	H: J03133 M: AF062566 R: D12768	Ubiquitous.	Knockout: transcriptional activator required for early mouse development and the maintenance of differentiated cells.	Glutamine-rich transcriptional activation domains. Phosphorylated, glycosylated (O-linked).	12q13	(4,13,51,79, 80,91-93)
Sp2	H: M97190, D28588	Various cell lines. Tissues: unknown.		Does not bind to GC-boxes. Full length Sp2 recently cloned from myeloblast cell line KG1 D28588.	17q21.3-q22	(8)
Sp3	H: X68560 S52144 M: AF062567	Ubiquitous.	Transcriptional repressor or activator, depending on promoter context and cell type.	Glutamine-rich transcriptional activation domains. KEE sequence required for repressor function.	2q31	(5,8,15,37,4 0,44)
Sp4	H: X68561 S50516 M: U62522 R: U07610 (HF-1b)	Predominantly in brain; also in testis, epithelial tissues, developing teeth.	Knockout: transcriptional activator required for murine growth and male reproductive behaviour.	Glutamine-rich transcriptional activation domains. Human genomic sequence AC004595.	7p15.3-p21	(5,16,53,94, 95)
BTEB1	H: D31716 M: Y14296 R: D12769 S: U57346 X: U35409	mRNA: ubiquitous. Protein: Liver, brain.	Positive regulation of AP-2α expression. Binds to the Basic Transcription element in the promoter of the rat P-4501A1 gene.	Protein levels controlled post-transcriptionally. Up-regulated by thyroid hormone in tadpoles.	9q13	(17-19,72,96-98)
TIEG1	H: U21847; S82439 (EGRα) M: AF049880 (GCBIPR, GCBP-23b, GIF, ZF10) R: U78875 (CPG20)	H: High in osteoblasts, muscle and pancreas. M.: high in brain, liver, heart, kidney, lung and testis.	TGFβ-inducible early protein. Transcriptional activator. Overexpression induces apoptosis in pancreatic epithelial cells; inhibitor of DNA synthesis.	Proline-rich N-terminal domain; potential SH3-binding regions.	8q22.2	(20,22,23,25 ,74,75,99-102)
TIEG2	H: AF028008	Ubiquitous, high in pancreas and muscle.	TGFβ-inducible early protein involved in the regulation of cell growth. Repressor.	Homologous to TIEG1.		(7)
EKLF	H: U65404 M: M97200	Erythroid cells.	Knockout: transcriptional activator.essential for the expression of the adult β-globin gene.	Proline-rich N-terminal activation domain. Phosphorylated and acetylated. Human genomic sequence AD000092.	19p13.2	(12,26,43,54 ,55,82,103-106)
GKLF	H: U70663 (EZF) M: U20344 (EZF)	Gut, epithelium, placenta, fibroblasts.	Growth arrest. Negative regulator of cell division in the gut. Activator/repressor.	Downregulated during intestinal tumorigenesis.	9q31	(27,28,107-110)
LKLF	M: U25096	Lung, blood vessels, haematopoietic cells.	Knockout: essential for tunica media formation in blood vessels. Required for the survival of resting T-cells, and possibly for fetal liver haematopoiesis.		19p13.11-p13.13	(62-64,111,112)
ZNF741	H: U28282				Xp11.21	
AP-2rep	M: Y14295	Brain, kidney, liver, lung.	Negative regulation of AP-2α expression. Upregulated during kidney development.			(18)
BKLF	M: U36340	Ubiquitous, high in brain and haematopoietic cells.	Activator / repressor. Knockout: required for murine growth; negative regulator of proliferation of myeloid cells.	Interacts with the co-repressor mCtBP2.		(9,38,65)
IKLF/ BTEB2	H: D14520 (BTEB2) M: AF079852 (IKLF)	Placenta, epithelial lining of the intestine.	High levels in proliferating cells in the intestine (mouse). Transcriptional activator.			(29,33)
ZF9	H: AB017493 (GBF) M: AF072403 (CPBP) R: AF001417 U (ZF9)	Ubiquitous; high in liver, lymphocytes and placenta.	Up-regulated during early hepatic fibrosis in rats. Transcriptional activator.	Homology with the proto-oncogene Bcl (U51869), on chromosome 10.	10p15	(34-36,113)
UKLF	H: AB015132	Ubiquitous.	Transcriptional activator.		2q32	(10)

Species: H, human; M, mouse; R, rat; S, pig; X, *Xenopus*.

(CYP1A1) gene promoter (17). More recently, BTEB1 has also been cloned from mouse (18) and man (19). Human TIEG1 (TGFβ-inducible early gene), also named EGRα (early growth response gene α), has been cloned independently by differential display PCR from a human fetal osteoblast cell line after TGFβ treatment (TIEG1; 20) and an androgen-dependent prostate cell line (EGRα; 21). Both proteins are expressed from alternative promoters of the same gene. TIEG1 differs from EGRα by the presence of 11 additional amino acids at its N-terminus (22). The mouse orthologue of human TIEG1 has been termed mGIF (mouse glial cell-derived neurotrophic factor inducible factor) (23). The recently cloned human TIEG2 protein (7) is distinct from TIEG1/EGRα/mGIF, although structurally and functionally closely related to TIEG1. The 512 amino acid protein contains several proline-rich regions and shares 44% similarity to TIEG1 outside the DNA-binding domain (Fig. 3) (7). The expression of TIEG proteins is up-regulated in response to TGFβ, by the glial cell-derived neurotrophic factor (GDNF) (23), a distant member of the TGFβ family of proteins, and by other hormones like EGF and oestrogens (24,25).

The third group of proteins have been dubbed XKLFs (pronounce X-klefs) or 'Krüppel-like factors' after the *D.melanogaster* zinc finger protein Krüppel. The 'X' usually indicates the major expression site of the factor, i.e. erythroid cells (EKLF) (12,26), gut (GKLF) (27), also called epithelial zinc finger protein (EZF) (28), intestine (IKLF) (29), lung (LKLF) (30), ubiquitous (UKLF) (10) or fetal (FKLF) (31). However, these names are somewhat misleading because expression is not always restricted to these tissues. Another ubiquitously expressed factor has been named basic (BKLF) because its N-terminus is rich in basic residues (9). BKLF is probably identical to TEF-2, a protein that binds to the GT-I motif of the SV40 enhancer (32). Furthermore, four additional members of this subfamily have been reported, including basic transcription element binding protein 2 (BTEB2) (33), zinc finger protein 741 (ZNF741) (only sequence in database), repressor of AP-2 transcription (AP-2rep) (18) and zinc finger protein 9 (Zf9) (34). The latter is also known as core promoter binding protein (CPBP) (35) and GC-rich binding factor (GBF) (36).

Zf9/CPBP/GBF is distinct from but closely related to UKLF (10). Similarities are not only seen in the C-terminal zinc finger region but also in the N-terminal part of the protein. Most significantly, the first N-terminal 47 amino acids of UKLF and Zf9/CPBP/GBF are highly conserved (Fig. 3). This conserved domain is rich in acidic amino acids and lies within the trans-activation domain of the proteins (10).

BTEB2 has been originally cloned from a human placenta cDNA library using rat BTEB1 as a probe (33). However, BTEB2 is more related to the Krüppel-like factors than to BTEB1. BTEB2 is probably an N-terminal truncated human orthologue of murine IKLF, since the designated 5'-untranslated region of human BTEB2 is highly conserved within the coding region of IKLF. It remains to be clarified whether the published BTEB2 cDNA sequence is incomplete or might contain mutations in the assigned 5'-untranslated region that would lead to an N-terminal truncated protein.

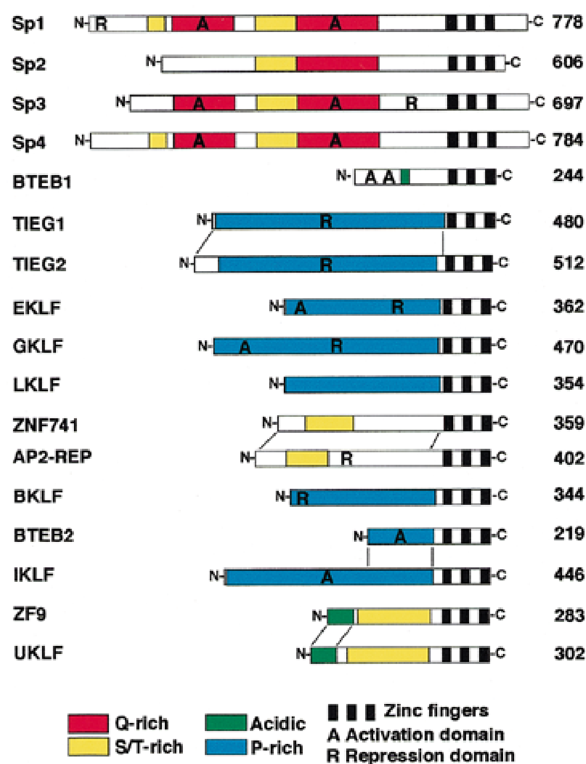

Figure 3. Structural features of Sp/XKLF family members. Each coloured box refers to the region containing a preponderance of one or several types of amino acids. The black boxes represent the zinc fingers. Activation (A) and repression (R) domains are indicated. Connecting lines depict similarities of the transcription factors outside the zinc fingers. The sizes (amino acids) of the proteins are indicated on the right and refer to the human proteins with the exception of LKLF, AP-2rep, BKLF and IKLF, which refer to mouse proteins.

ACTIVATION VERSUS REPRESSION

The majority of the Sp/XKLF transcription factors have been reported to act in a positive manner. Some factors, however, exert an inhibitory effect on transcription. Sp3 was originally found to repress Sp1-mediated activation by binding to the same site and thereby preventing Sp1 binding and activation (37). Similarly, BTEB1 activated the AP2 promoter while AP-2rep repressed it by competing for the same site (18). In addition, BKLF has been shown to compete with EKLF thereby silencing EKLF-mediated activation (38). These examples suggest mechanisms by which the expression of target genes might be regulated by competition between repressors and activators. However, the attractive simplicity of such models is already challenged by the observation that repressors can also function as activators, as has been demonstrated for Sp3 and BKLF (9,39,40). In addition, TIEG1/EGRα was reported to be an activator (24), yet in other experiments it acted as a repressor (7,23). The features determining whether these proteins act as repressors or activators are not well understood. In the case of Sp3 and BTEB1, the structure and the arrangement of the recognition sites appear to determine the effect on transcription. Promoters containing a single binding site are activated, whereas promoters with multiple binding sites often do not or only weakly respond to Sp3 (40). Vice versa, BTEB1 activated promoter constructs with multiple GC boxes but repressed transcription of constructs containing a single GC box (17). Whether Sp3 acts as an activator or as a repressor of Sp1-mediated activation might also depend on the cellular context (41).

Discrepant reports on activation/repression functions are likely due to the use of different experimental systems and conditions, but might also reflect the modular structure of these transcription factors. Both activation and repression domains have been identified in Sp1 (42), Sp3 (40), EKLF (43) and GKLF/EZF (28). The molecular basis for the inhibitory activity of Sp3 has been mapped to a transferable repression domain located between the second glutamine-rich activation domain and the first zinc finger function (Fig. 3) (40). The amino acid triplet KEE within this domain is absolutely essential for repressor function (40). Unraveling the transcriptional role of Sp3 is further complicated by the fact that three Sp3 isoforms exist. The full-length 115 kDa Sp3 is initiated at a non-AUG codon (8) whereas two smaller ~70 kDa Sp3 species arise from internal translation initiation sites (44). All three isoforms contain the inhibitory domain. The full-length Sp3 carries both glutamine-rich activation domains whereas the smaller species contain only the second activation domain B and thus can act only as very weak activators. So far, it is not known whether the relative abundance of the different Sp3 isoforms varies under certain conditions. We can only assume that such modular structures would allow regulation of the transcriptional activity of these factors.

PHYSIOLOGICAL FUNCTION OF Sp/XKLF PROTEINS

Although it is satisfactory that there are at least 16 different proteins that can serve as effectors of the large variety of biological functions that have been assigned to Sp1 binding sites, the downside is that it raises the question which tasks are performed by which family members *in vivo*? This question is particularly complicated because different factors are often present in the same cells and hence the prospect of overlapping functions is always looming. Gene inactivation in mice has been the most powerful tool to elucidate specific functions of individual family members. Knockouts of Sp1, Sp4, EKLF, LKLF and BKLF have been described, all of them resulting in surprisingly different phenotypes. This is consistent with the idea that the number of zinc finger genes has increased during evolution to fulfil the requirement for specialised functions in multicellular organisms.

Sp1: a complex puzzle with few easy pieces

The ubiquitously expressed Sp1 protein has been implicated in the activation of a very large number of genes and is thought to be involved in cellular processes such as cell cycle regulation, chromatin remodelling and the propagation of methylation-free islands (2,3,45–50). Thus, a cell lacking Sp1 would be predicted to stand little chance of survival. Surprisingly, Sp1 null ES cells could be generated without any difficulty and these cells showed normal growth characteristics, maintained their methylation-free islands and could differentiate *in vitro* to form embryoid bodies with similar efficiencies to control ES cells (51; U.Jägle, unpublished results). However, all Sp1 null embryos are severely retarded in growth and die after day 10 of development. They display a wide range of abnormalities at

this stage. Some embryos appear as an undifferentiated mass of cells, while in others all the typical hallmarks of early embryogenesis are present, such as the developing heart, eyes, otic vesicles, somites, erythroid cells and extra-embryonic tissues. Blastocyst injections with Sp1 null ES cells showed that these cells initially contribute efficiently to chimaeric embryos, but after day 11 of development 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 day 10 of mouse development (51). The Sp1 null phenotype is remarkably similar to the phenotypes described for two proteins involved in DNA methylation: the DNA methyltransferase enzyme, Dnmt1 (1), and the methyl-CpG-binding protein, MeCP2 (52). Expression levels of Dnmt1 were normal in the Sp1 knockout embryos and no changes in DNA methylation patterns could be detected. However, MeCP2 protein levels were reduced 20-fold in the absence of Sp1. Like Sp1, MeCP2 is dispensable for growth and differentiation of ES cells, but MeCP2-deficient cells are unable to contribute to mouse tissues after early embryogenesis. Thus, it has been proposed that MeCP2 is required for the maintenance of differentiated cells. The phenotype of the Sp1 knockout suggests that Sp1 has an essential role in cellular survival during mouse development by acting as a regulator of MeCP2 expression.

Sp4: is it all between the ears?

Sp4 is highly expressed in the developing central nervous system, but it is also abundant in epithelial tissues, developing teeth and testes. Sp4 knockout mice are born at normal Mendelian ratios but two-thirds of these mice die within a few days after birth (53). Reduced body weight and a slightly stunted appearance are features of Sp4 null mice (our own unpublished observations). The most interesting aspect of the phenotype is the complete absence of mating behaviour in Sp4 null males. Since their reproductive organs are fully developed and apparently normal, with the testes containing motile mature sperm, the most likely cause of this behavioural abnormality is a neurological defect. Surgical removal of the vomeronasal organ in newborn mice results in the absence of mating behaviour later in life and the hypothalamus is also known to play an important role in reproductive behaviour. However, both the vomeronasal organ and the hypothalamus have a histologically normal appearance in Sp4 null mice (53). It remains possible that a specific subset of cells is missing in either of these organs. The vomeronasal organ is primarily important for the detection of pheromones and a family of putative pheromone receptors expressed in this organ has been described. It is conceivable that the subset of neurons expressing the pheromone receptors that elicit reproductive behaviour are missing in the Sp4 null males, but this awaits further investigation.

EKLF: one factor to activate one gene?

Since EKLF is expressed specifically in erythroid cells it was not surprising that EKLF null mutants die *in utero* due to severe anaemia (54,55). The production of the first, nucleated red cells in the yolk sac (primitive erythropoiesis) was not affected but the generation of enucleated erythrocytes in the fetal liver (definitive erythropoiesis) was severely impaired. EKLF null mice fail to express adult-type β-like globin genes in definitive erythroid cells, but do express all the other erythroid-specific genes examined at normal levels, including the embryonic β-like globins in primitive cells (54,55).

Unlike humans, mice do not possess fetal-specific β-globin genes. Nevertheless, the switch from fetal (γ) to adult (β) gene expression is reproduced in definitive erythropoiesis of transgenic mice harbouring the complete human β-globin locus (56). This switching process was studied in EKLF knockouts, revealing that the γ-globin genes were fully activated as opposed to a complete failure to express the β-globin gene. Furthermore, the kinetics of switching correlates with EKLF levels. It is delayed in EKLF heterozygotes, but expedited by transgenic overexpression of EKLF. This has identified EKLF as the first factor that is directly involved in γ to β switching (57). Since the γ genes are still silenced after the switching period, it is unlikely that EKLF has a role to play in γ gene silencing. Thus, EKLF is an adult-specific factor required for activation of the β gene promoter in definitive erythropoiesis. It is noteworthy that mutations in the EKLF-binding site of the β promoter have been described in certain β-thalassaemia patients (58).

Erythroid precursors are found in normal numbers in EKLF null mutants and erythroid maturation *per se* is thought to ensue normally (54,55,59). This leaves us with an uncomfortable question: does EKLF activate only one particular gene in one particular locus? This would be a very cumbersome mechanism to regulate gene expression. Fortunately, there exist some data suggesting additional roles for EKLF. One piece of data suggests that EKLF function is required for red cell stability (60). This suggestion is underpinned by the observation that forced expression of γ-globin or β-globin chains under the control of EKLF-independent promoters does not alleviate the fatal anaemia of EKLF null mutants (61; M.Wijgerde, unpublished data).

LKLF: a survival and maturation factor?

Analysis of the LKLF knockout has revealed several interesting aspects of its function in the development and physiology of mice. LKLF null fetuses die *in utero* as a result of exsanguination due to intra-embryonic and intra-amniotic bleeding. Impaired fetal liver haematopoiesis has been observed (62), but the primary cause of lethality is a defect in blood vessel morphology (63). The mechanism of action of LKLF is unknown. LKLF might be required for the survival of endothelial cells after completion of the primitive vasculature. Alternatively, LKLF null vessels might fail to produce growth factors that serve as signals to attract and organise the cells required for tunica media formation, an essential process for vessel wall stabilisation. Expression of the prospective factors (angiopoietins 1 and 2, TGFβ, Tie 1 and Tie 2, epidermal growth factor and platelet-derived growth factor B) has been assessed by *in situ* hybridisation and was found to be normal (63). Therefore, the downstream effector molecules of LKLF function in vessel wall stabilisation remain elusive.

A first glimpse of LKLF functions at later developmental stages has been provided by the analysis of chimaeric mice made with LKLF null ES cells. These data have demonstrated a role for LKLF in the maintenance of mature T cells (64). Mature T cells are present in the circulation in a resting state and become activated if their T cell receptors bind the appropriate peptide antigens presented by major histocompatibility molecules. Activated T cells start expressing a large number of

new genes and this is followed by cell cycle entry and cell division. LKLF protein levels are significantly reduced upon activation of mature T cells. Activated T cells are more prone to undergo apoptosis in the peripheral lymphoid organs. This is thought to reflect a host defence mechanism against autoimmune reactions. In chimaeric mice, LKLF null T cells are in a spontaneously activated state, triggering their removal from the circulation through apoptosis in the peripheral lymphoid organs. Thus, these data demonstrate that LKLF regulates the resting state and survival of mature T cells. The link between proliferation, differentiation and survival of highly specialised cell types could very well serve as a paradigm for the other Sp/XKLF family members.

BKLF: a proliferation switch?

Only a preliminary report on the phenotype of the BKLF knockout has appeared (65). BKLF null mice come to term and develop normally. However, they develop a chronic myeloproliferative disease during adulthood. An excess of myeloid cells is already found *in utero* and BKLF null myeloid cells have an increased proliferative capacity *in vitro* (65). A detailed description of the phenotype would be of interest, in particular in relation to the activated phenotype of LKLF null T cells. Are similar switches controlling cellular proliferation operated by BKLF in myeloid and by LKLF in T cells? Furthermore, BKLF levels are reduced in fetal livers of EKLF knockout mice (55) and it would therefore be interesting to know what happens to EKLF levels in BKLF null mice.

Who's done it?

To understand regulation by Sp/XKLF family members it is of pivotal importance to determine the binding site–transcription factor relationships that are relevant under physiological conditions. Knockouts may provide the first clues to answer this question. However, these data are always indirect because the effects on gene transcription may be due to secondary effects of the knockout phenotype. A systematic approach to tackle this problem for Sp/XKLF proteins has recently been described. It is based on earlier work in which a DNA-binding specificity mutant was used to show a direct *in vivo* interaction between a DNA target site and a transcription factor in *D.melanogaster* (66). This protocol was adapted to a functionally important GT box motif in the β-globin LCR that interacts with Sp/XKLF factors. Sp1, Sp3, BKLF, EKLF and possibly other Sp factors are present in erythroid cells (50,67). Which of these factors is directly acting through the GT box motif in the β-globin LCR? To address this issue, the GT box of the β-globin LCR was first mutated to abolish binding of wild-type Sp/XKLF factors. The amino acids in EKLF and Sp1 involved in DNA recognition were changed such that the mutated zinc finger domains would recognise the mutated element (68,69). The capacity of the mutated Sp1 and EKLF factors to activate β-globin gene transcription through the mutant GT box was then assayed in transgenic mice. The analysis showed that EKLF acts *in vivo* through this element, resulting in a stronger DNase I hypersensitive site over the LCR fragment and activation of β-globin gene transcription. No such effect was observed with Sp1, emphasising the specific role of EKLF in this context (67). Thus, in addition to the β-globin promoter site revealed by the knockout (see above), EKLF has a functional target site in the main regulatory element of the β-globin locus, the LCR. These data do not exclude a functional role for other Sp factors in LCR activity. It would be of particular interest to find EKLF relatives that activate other erythroid-specific genes through GT boxes and to extend this analysis to primitive erythroid cells, since these are unaffected by the EKLF knockout. In this regard, the recently reported FKLF protein is an exciting candidate (31).

HOW TO GET SPECIFICITY?

The initial characterisation of the different Sp/XKLF family members provides much information about the potential functions and activities of these proteins. The most obvious question, however, concerns the specificity of the individual family members. That specificity exists is illustrated by the distinct knockout phenotypes of the different family members. The question remains: how is it obtained? Binding site preferences confer a certain level of specificity. Although all proteins recognise very similar DNA target sites, the relative affinities for specific sequences differ. For instance, Sp factors bind more tightly to GC boxes than to GT boxes (70) while XKLF bind preferentially to GT boxes (9–11,71).

Another level of specificity is obtained by the expression patterns of individual family members. Some factors, like Sp1 and Sp3, are ubiquitously expressed, and others, like the TIEGs, have a quite widespread distribution (Table 1). A third group is restricted to certain cell types, for instance EKLF to erythroid cells. However, it should be mentioned that in most cases only the mRNA distribution has been analysed. This does not necessarily reflect the distribution and the level of the protein. BTEB1 mRNA is expressed ubiquitously, but the protein was found in only a few tissues (72). A careful analysis of the expression pattern of the protein rather than the mRNA will yield new insights into the real distribution of the other family members that might be helpful in understanding their function.

It is clear that in any given cell type co-expression of several family members occurs and we have to assume that these proteins will compete for the same binding sites. The abundance of factors may vary among different cell types and under certain conditions. Alterations in relative abundance have been reported in some cases. In primary keratinocytes, Sp3 levels exceed those of Sp1. This Sp3:Sp1 ratio becomes inverted if the cells are allowed to differentiate (73). In NIH 3T3 cells, GKLF mRNA is high in quiescent cells, but almost undetectable in proliferating cells (27). TIEG1 is induced by TGFβ, EGF and oestrogens in several cell types (24,25,74,75).

DNA-binding studies are usually performed *in vitro* on isolated recognition sequences. However, promoters contain recognition sequences for many different transcription factors and full activation requires simultaneous binding of a whole set of transcription factors that might bind cooperatively to their sites or act synergistically by other mechanisms. So far, little is known of how the different Sp/XKLFs proteins act on natural promoters in combination with other transcription factors *in vivo*.

That different Sp/XKLF proteins might act in a highly specific manner with other transcription factors would be expected since the domains N-terminal of the zinc finger region have little homology between individual family members. Evidence for promoter specificity came from experiments with the EKLF and Sp1 activation domains. In Gal4 fusions, the EKLF activation domain activated the appropriate β-globin promoter

constructs better than the Sp1 activation region (76). That EKLF provides crucial transactivation functions for β-globin expression was also demonstrated in transgenic mice experiments (67).

A further level of specificity could be obtained by interaction with specific co-activators or co-repressors. In the case of BKLF, a protein termed mCtBP2 (murine C-terminal binding protein 2) that binds to the repression domain of BKLF and that could act as a co-repressor has been cloned (38). EKLF requires a SWI/SNF-related chromatin remodelling complex, the so-called EKLF co-activator remodelling complex 1 (E-RC1), to generate a DNase I hypersensitive, transcriptionally active β-globin promoter on chromatin templates *in vitro* (77). In addition, the co-activator complex CRSP (cofactor required for Sp1 activation) is required for transcriptional activation by Sp1 *in vitro* (78). Whether CRSP is specific for Sp1 or whether it could also cooperate with other Sp family members remains to be established.

One could speculate that protein modifications control the interaction with cofactors that would generate specificity. Sp1 for instance is phosphorylated (79) and glycosylated (80). EKLF is phosphorylated at serine and threonine residues *in vivo* and the minimal 40 amino acid transactivation domain contains a casein kinase II phosphorylation site (81). Mutation of this site abolished the activity of this domain in transactivation assays. Furthermore, EKLF undergoes post-translational acetylation within its inhibitory domain and co-transfection with the acetyltransferases CBP and p300, but not P/CAF, enhances the transactivation potential of EKLF (82). Altogether, unravelling the molecular basis for the specificity of individual Sp/XKLF family members represents a formidable scientific challenge.

FUTURE DIRECTIONS

Research on Sp/XKLF proteins has made rapid advances in the last few years, with more and more family members being identified. Most of the knowledge is derived from biochemical analyses, *in vitro* protein–DNA interaction studies and transfection experiments. The knockouts published to date show that individual family members do have specific functions in the mouse. However, even knockout mice may not reflect the whole truth because so little is known about the extent of overlapping functions. For instance, inactivation of Sp1 was expected to have dramatic effects at the cellular level since it was generally believed that Sp1 is the essential transcription factor for expression of housekeeping genes. Possibly, Sp1 is indeed engaged in the expression of these genes *in vivo*, but functionally replaced by Sp3 under knockout conditions. In addition, since deletion of the Sp1 gene leads to lethality early in development, the role of Sp1 in fully developed tissues remains an open question. In this regard, it is interesting to note that mutations of Sp1-binding sites in the LDL receptor and collagen type Iα1 genes have been associated with hypercholesterolaemia and osteoporosis in humans (83–85).

Conditional disruption (86–88) of the Sp1 gene in specific tissues at any given stage of development will be an important step to further unravel the physiological role of Sp1 *in vivo*. The identification of target genes presents another formidable challenge. Recent advances in the detection of differentially expressed genes and the development of DNA microchip arrays provide very useful tools for high throughput analysis of target genes (89). This should be of great help in understanding the downstream effects that result in the knockout phenotypes.

To fully understand the physiological function of the Sp/XKLF proteins, it will be equally important to understand the mechanisms of their mode of action. Detailed characterisation of the individual transcription factors, identification of specific interaction partners, careful analysis of protein modifications and the identification of signals and transduction pathways by which these proteins are regulated will be essential for a mechanistic understanding of transcriptional control by this growing family of transcription factors. With the steadily increasing collection of knockout mice and the large arsenal of *in vitro* and *in vivo* analysis systems available to the scientific community, many more exciting results can be expected in the near future.

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