Lymphoid enhancer factor/T-cell factor (LEF/TCF) high mobility group box transcription factors are the nuclear transducers of the Wnt/β-catenin signaling cascade. In Xenopus, three members of the LEF/TCF family, XLEF-1, XTCF-3, and XTCF-4, with distinct but partially overlapping expression patterns have been identified. The individual Xenopus LEF/TCF family members differ extremely in their properties of target gene regulation. We observed that in contrast to LEF-1, neither XTCF-3 nor XTCF-4 can induce secondary axis formation upon ventral overexpression in Xenopus embryos. To identify functional motifs within the LEF/TCF transcription factors responsible for target gene activation or repression, we created various mutants and a set of XLEF-1/XTCF-3 chimeras. In overexpression studies, we asked whether these constructs can mimic an activated Wnt/β-catenin pathway and lead to the formation of a secondary body axis. In addition, we examined their capacity to rescue a loss-of-function phenotype given by dominant negative LEF-1 expression. We further analyzed their ability to directly activate target genes in reporter gene assays using the LEF/TCF target promoters, siamois and fibronectin. We found that a region homologous to exon IVa of hTCF-1 is an activating element. This is flanked by two small repressing motifs, LVQ and SXSSS. Our findings implicate that the motifs identified here play an essential role in determining cell type-specific activity of LEF/TCF transcription factors.

The Wnt/β-catenin signaling pathway plays a key role in many important developmental processes such as the limb bud outgrowth (1), neural crest cell induction (2–5), patterning of the central nervous system (6), and body axis induction (7–10). Dorsal enrichment of cytosolic/nuclear β-catenin (11, 12) and subsequent activation of target genes by β-catenin/XTCF-3 is required for establishing the endogenous body axis (13, 14). Inhibition of the Wnt/β-catenin pathway by cadherins (15), conductin/axin (16, 17), duplin (18), dnLEF-1 (19), or XTCF-3 (20) leads to a loss of the endogenous axis. Conversely, activation of the Wnt/β-catenin signaling cascade on the ventral side of Xenopus four-cell stage embryos by overexpression of XWnt-8, β-catenin, and mLEF-1 induces the formation of a secondary body axis (8, 19, 21). Besides the important function in early development, the Wnt/β-catenin pathway has been shown to be involved in cancer formation. Disregulation of this pathway by loss of function mutations in the tumor suppressor APC or stabilizing mutations in the oncogene β-catenin, leading to the up-regulation of the target genes c-myc and cyclin D1, are found in numerous human cancers and cancer-derived cell lines (22–24).

LEF/TCF transcription factors are the nuclear transducers of the Wnt/β-catenin signaling cascade (19, 25). They belong to the family of sequence-specific HMG box transcription factors. They have been originally described as architectural transcription factors that regulate gene expression by binding DNA (26). They have no intrinsic transactivation domain, apart from context-dependent transactivation domains in LEF-1 (27). In the simplest model of the Wnt/β-catenin signaling cascade, they interact with the transactivator β-catenin (19, 28) and regulate the expression of target genes like siamois (13), nodal related-3 (29), Xtwin (30), c-myc (23), fibronectin (31), and many others (see also the Wnt home page on the World Wide Web at www.stanford.edu/~rnusswe/wntwindow.html). This simple model of target gene activation has been complicated by the discovery of a variety of binding partners and modifiers of TCFs that either promote transcriptional activation or repression, such as grouscho (32, 33), CtBP (20), cAMP-response element-binding protein-binding protein (34), Smad 3 and 4 (35, 36), or NLK (37). TCF-driven transcription is further complicated by the fact that they are differentially spliced, resulting in a multiplicity of splice variants as reported for hTCF-1 (38) and hLEF-1 (39). Different splice variants of the same TCF family member (e.g. XTCF-4) either activate or repress the promoter of the Wnt/β-catenin target gene fibronectin (40). Their specific character is not due to variable DNA-binding properties or differences in the recruitment of β-catenin, Smads, or groucho. Instead, phosphorylation regulated by the LVPQ and SFLSS motifs seems to play a crucial role in regulating TCF activity and ternary complex formation (40). Among the TCFs, the exon/intron structure is best characterized in hTCF-1 (38). Sequence comparison revealed that the LVPQ and SFLSS motifs in XTCF-4 flank a sequence motif homolog to exon IVa of hTCF-1. According to the genomic structure of hTCF-1, we hereafter refer to this motif as exon IVa.

Thus far, three different members of the LEF/TCF family with distinct but partially overlapping expression patterns have been described in Xenopus: XTCF-3 (25), XLEF-1 (41), and XTCF-4 (42). Although there is no doubt that they are...
nuclear transducers of Wnt/β-catenin signaling, it has never been shown that a Xenopus-specific LEF/TCF family member can induce a secondary axis upon ventral injection. Instead, the early embryological data presented so far describe a repressive function of XTCF-3 on axis formation (20). To discriminate the functional role of the distinct LEF/TCF family members, it is important to know their common structure and to identify motifs responsible for target gene activation or repression. Xenopus provides a suitable assay system, since capacities of axis induction and target gene activation can be compared. In this study, we focus on the contribution of exon IVa and its flanking regions to the activity of these transcription factors in a detailed functional analysis using all known Xenopus LEF/TCF genes. Our findings demonstrate that exon IVa is an activating element in LEF/TCF transcription factors, whereas the flanking LVPQ and SXXSS motifs together form a repressing element.

**MATERIALS AND METHODS**

**Plasmids**—Wild-type XLEF-1, XTCF-3, and XTCF-4 are the same as described (40). Point mutations in XTCF-3 were constructed with a site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the description of the manufacturer. PCR fragments were amplified using a proofreading polymerase (Peqlab, Erlangen, Germany) and combined to create deletion mutants. The following primers were used: XTCF-3 5′, XTCF-3 3′, and XTCF-3ΔC 3′ as previously described (40). The following were also used: XTCF-3ΔLVPQ forward, 5′-CAGTAGGCCATGGTGACTG-3′; XTCF-3ΔSLVSS reverse, 5′-GACCTCACAGTACTACGAGG-3′; XTCF-3ΔLVPQ SLVSS reverse, 5′-AAACCGGGGACATAGTGTTCCATGGT-3′; XTCF-3 exon IVa forward, 5′-CGTTGGGCTGGGAGGGCCAA-3′; XTCF-3 forward, 5′-CCAGCCCAATGGTGGTGTCAT-3′; XTCF-3 reverse, 5′-TTTTCACTCCATACGTTG-3′; XTCF-3 CLVPQ forward, 5′-AAGTGGAGACGATAGTGTTCCATGGT-3′. Exon IVa from XTCF-3 was amplified using either wild-type XTCF-3 or the ΔLVPQΔSLVSS mutant as template. The constructs were N-terminally fused to six copies of the chicken myc tag, inserted into the Xhol site of pCS2 expression vector (43), and verified by sequencing. Additionally, the LVPQ motif was removed in some constructs and in the SSLVSS motif of XTCF-3 or deleted the entire motif.

**Functional Diversity of LEF/TCFs**

*In Xenopus,* three members of the LEF/TCF family, XLEF-1, XTCF-3, and XTCF-4, have been identified. They are highly homologous in the N-terminal β-catenin binding site (48, 49) and in the HMG box, which mediates DNA binding. Lower homology between the different LEF/TCF family members is found in the core region between the β-catenin binding site and the HMG box and within the C terminus (elements A, B, and C in Figs. 1A and 7).

We have isolated three variants of XTCF-4, XTCF-4A, XTCF-4B, and XTCF-4C, which differ in the presence or absence of the two small motifs, LVPQ and SLVSS, flanking exon IVa according to hTCF-1 (Refs. 38 and 40; Fig. 1A). Recently, we have shown that XTCF-4A and -4B act as repressors of the Wnt/β-catenin target gene fibronectin, whereas XTCF-4C activates the fibronectin promoter (40). This prompted us to identify regulatory motifs within the TCF-4 subgroup that are responsible for the subtype-specific gene control. Evidence for a physiological role of these isoforms is given by their temporal expression profile in early embryogenesis.

We found by restriction pattern analysis of RT-PCR fragments that XTCF-4C, the activating form, is expressed first, starting at neurula stage 16 (46). As ontogenesis proceeds, the repressing XTCF-4 variants appear, XTCF-4A at stage 18 and XTCF-4B at stage 21. Coexpression of all three variants was observed from stage 21 until stage 35 (Fig. 1B). Although similar variants in hTCF-4 have not been identified yet, the genomic structure of hTCF-4 reveals putative splice acceptor and donor sites, which lead to variants containing or missing the LVPQ and SLVSS motifs (50).

In Xenopus, XTCF-3 also contains these short motifs that are flanking exon IVa, whereas XLEF-1 possesses neither the short motifs nor an exon IVa-corresponding insert (25, 40, 41). XTCF-3 behaves as repressor in promoter reporter assays comparable with XTCF-4A and -4B (40), whereas XLEF-1 is an activator (40).

To elucidate the relevance of the two short motifs and exon IVa flanked by them in TCF function, we generated several mutants of XTCF-3 and XLEF-1 affecting this domain and compared their activities with those of the XTCF-4 isoforms. We exchanged, for example, the serine residues with alanines in the SSLVSS motif of XTCF-3 or deleted the entire motif. Additionally, the LVPQ motif was removed in some constructs (Fig. 1A). To exclude the influence of the co-repressor C/EBP and XTCF-3 function, we also generated XTCF-3 mutants lacking murine leukemia virus reverse transcriptase (Invitrogen). cDNA according to 25 ng of RNA was amplified using the following primers: H4 forward (5′-C CGGGATACACGTTTACATGCT-3′) and H4 reverse (5′-GGATGCGGTAACGCUATCGTTTACATGCT-3′), 30 cycles; siamois forward (5′-CTCCTCACTCGTGGTAAGCAGTCGACG-3′) and siamois reverse (5′-GGGACGAGTAAGGTGGT-3′), 34 cycles.

To discriminate between the XTCF-4 isoforms, cDNA of different XTCF-3 stages was amplified with the following primers: XTCF-4 forward (5′-CCCTCGAGGCCGCTTATTACCTACAGCAAC-3′) and XTCF-4 reverse (5′-CTTCTCGGAGCAGTCAACGGCGGTTC-3′).

**RESULTS**

*Mutants and Chimera of Xenopus LEF/TCF Members*—In Xenopus, Plasmids function of LEF/TCF family members was studied. The following were used: XTCF-3A, XTCF-3B, and XTCF-3C, the activating forms, are the same as described (40). Point mutations in XTCF-3 were constructed with a site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the description of the manufacturer. PCR fragments were amplified using a proofreading polymerase (Peqlab, Erlangen, Germany) and combined to create deletion mutants.

**Immunoblotting and Immunoprecipitation**—Radioimmunoprecipitation of protein from Xenopus egg extracts was performed as described (46). The antibody was a goat anti-mouse antibody coupled with horseradish peroxidase. Visualization was performed using ECL substrate (Amersham Biosciences). Nonidet P-40 lysis buffer lysates of 106 cells were precipitated with 1 μg of protein A-purified 9E10 antibody. One-twentieth of the precipitate was loaded on 7.5% SDS-PAGE, transferred to nitrocellulose, incubated with the 9E10 anti-myc antibody. Secondary antibody was a goat anti-mouse antibody coupled with horseradish peroxidase. Visualization was performed using ECL substrate (Amersham Biosciences). Nonidet P-40 lysis buffer lysates of 106 cells were precipitated with 1 μg of protein A-purified 9E10 antibody. One-twentieth of the precipitate was loaded on 7.5% SDS-PAGE. The transfectected constructs were detected as described above.
the C terminus. As a well characterized activating XTCF-3 construct, which was found to activate the TOPFLASH reporter in colon carcinoma cells (32) and to activate the fibronectin promoter in Xenopus A6 cells (31), we included in our analyses the XTCF-3Δgrg mutant. To avoid recruitment of the corepressor CtBP, we additionally truncated the XTCF-3Δgrg construct at the C terminus (XTCF-3ΔgrgαC).

One of the main differences between XLEF-1 and the other Xenopus TCFs is the lack of exon IVa. In this context, it is remarkable that the corresponding exon in hLEF-1 has been shown to activate transcription in A6 cells (32). In murine LEF-1, we additionally truncated the XTCF-3Δgrg construct (Fig. 1A).

Different assays were performed to compare the activating or repressing properties of our constructs. To study whether these transcription factors can mimic an activated Wnt/β-catenin signal, we injected their RNA into the ventral side of Xenopus four-cell stage embryos and screened for the induction of a secondary axis. We further tested whether coinjection of these constructs together with dominant negative mLEF-1 at the dorsal side rescues the formation of the endogenous axis. To analyze the repressing function of the constructs in axis induction, we coinjected them ventrally with mLEF-1 and proved whether they suppress the formation of a secondary axis. Finally, to demonstrate that the activation via the LEF/TCF constructs is direct and due to target gene activation, we examined their effects on the activity of target gene promoters in reporter gene assays.

Exon IVa is necessary for the induction of a secondary axis, and the LVPPQ and SLVSS motifs counteract its function—Injection of the LEF/TCF constructs into the ventral blastomeres of Xenopus 4-cell stage embryos revealed that, in addition to the β-catenin binding site and the HMG box, further elements are required for the induction of a secondary axis. Unlike murine LEF-1, none of the Xenopus TCFs were able to induce a secondary axis upon ventral injection (Fig. 2B and Table I). Even the XTCF-3Δgrg mutant, which does not interact with groucho corepressors (32), and the ΔgrgΔC mutant of XTCF-3, which additionally lacks the CtBP binding motif, could not induce the formation of a secondary axis (Fig. 2B and Table I). The failure of this ΔgrgΔC mutant in axis formation indicates that the DNA- and β-catenin-binding sites alone are not sufficient for the induction of a secondary axis.

In contrast to XTCF-3 and XTCF-4, XLEF-1 was able to induce a secondary axis, but in comparison with its murine ortholog, the ectopic axis appeared at much lower frequency and was less complete (Fig. 2, A and B, and Table I). The late neurula stage was the optimal stage to monitor the weak ectopic axis formation by XLEF-1 (Fig. 2A). Since the most obvious difference between mLEF-1 and XLEF-1 is the lack of exon IVa in XLEF-1, we inserted the exon IVa of XTCF-3 into XLEF-1. Most strikingly, the chimera XLEF-1 + exon IVa induced a secondary axis with higher frequency than mLEF-1 (Fig. 2B and Table I). This indicates that exon IVa is essential for the induction of an ectopic axis. Additional insertion of the

**Fig. 1.** Schematic representation of Xenopus LEF/TCF transcription factors. A, the β-catenin binding site, the HMG box, exon IVa and the flanking LVPPQ and SLVSS motifs are as indicated. Regions A, B, and C are of lower homology. Exon IVa in XLEF-1 + exon IVa and in XLEF-1 + exon IVa − LVPPQ − SLVSS is derived from XTCF-3. All constructs were inserted into the pCS2 vector. The lower part shows the sequence of exon IVa of XTCF-3 and of XTCF-3 mutants with four serine residues mutated into alanines (XTCF-3 258,259,263,263SA) or lacking the LVPPQ motif (XTCF-3ΔLVPPQ) or with deleted LVPPQ motif and two or three serine residues mutated into alanines (XTCF-3ΔLVPPQ 258,259,263SA) and the mutant lacking both the LVPPQ and the SLVSS motif (XTCF-3ΔLVPPQΔSLVSS). The calculated molecular weights of the proteins are given.

B, identification of the XTCF-4 variants in Xenopus embryogenesis by specific enzymatic digestion of RT-PCR fragments. The upper panel shows the characteristic restriction pattern of the amplification products of XTCF-4A, XTCF-4B, and XTCF-4C plasmids after digestion with XbaI, RsaI, and a combination of XbaI and RsaI. XTCF-4A is positively identified by the appearance of a 291-bp fragment and a 213-bp fragment following digestion with XbaI as well as by a 104-bp fragment after digestion with XbaI and RsaI. XTCF-4B is identified by a 305-bp fragment following digestion with XbaI and RsaI. The amplification product of XTCF-4C is cut neither by XbaI nor by RsaI. Thus, XTCF-4C is identified by a 477-bp fragment after digestion with XbaI and RsaI. The lower panel shows the same restriction analysis of the amplification products of Xenopus cDNAs at different stages. At stage 16, the restriction analysis reveals that only XTCF-4C is expressed. At stage 18, the appearance of the 104-bp fragment in the XbaI/RsaI digest indicates the expression of XTCF-4A. The 477-bp fragment in the XbaI/RsaI digest indicates the expression of XTCF-4C. XTCF-4B is not expressed at this stage. From stage 21 onward, all three variants are expressed.

Functional Diversity of LEF/TCFs
FIG. 2. Ventral injection of LEF/TCF constructs. A, unlike its murine ortholog, XLEF-1 did not induce a well defined secondary axis. The axis-inducing capacity of XLEF-1 was monitored at the late neurula stage (stage 19). Insertion of the exon IVa from XTCF-3 in XLEF-1 (XLEF-1 + exon IVa) led to the formation of a secondary axis indistinguishable from those induced by mLEF-1. Further insertion of the LVPQ and SLVSS (XLEF-1 + exon IVa + LVPQ + SLVSS) motifs reverted the axis-inducing capacity to wild-type XLEF-1 levels. 500 pg of the indicated mRNA were injected into both ventral blastomeres at four-cell stage embryos. B, calculation of secondary axis formation upon ventral injection of 0.5 ng of mRNA of the LEF/TCF constructs revealed that none of the XTCF-3 or XTCF-4 constructs is able to induce an ectopic axis. MLEF-1 injection led to secondary axis formation in 59.0% of the injected embryos (n = 133); the values for XLEF-1, XLEF-1 + exon IVa, and XLEF-1 + exon IVa + LVPQ + SLVSS injection were 32.9% (n = 160), 64.5% (n = 107), and 26.8% (n = 181), respectively. The complete set of injections, number of embryos, and number of independent experiments are given in Table I. C, activation of siamois expression upon LEF-1 injection. RT-PCR of stage 10 Xenopus embryos revealed that siamois expression is increased upon ventral injection of 0.5 ng of mLEF-1 or XLEF-1 + exon IVa. Upon injection of wild-type XLEF-1 or XLEF-1 + exon IVa + LVPQ + SLVSS, only a slight increase was observed in siamois expression. The control lane shows siamois expression in uninjected embryos. H4 shows the amplification of the loading control histone 4. D, detection of the injected constructs. 0.5 ng of the indicated myc-tagged construct was injected at the ventral blastomeres of Xenopus 4-cell stage embryos. Radioimmune precipitation assay lysates according to one embryonic equivalent of stage 14 embryos were loaded on a 7.5% SDS-PAGE and detected with the monoclonal antibody 9E10 against the myc epitope. The asterisks indicate the specific band of the injected LEF/TCF construct. All XTCF-3 constructs with an integer C terminus give rise to three specific bands. The size of the upper band, which is marked by an asterisk, corresponds to the expected molecular weight. The bars indicate the molecular masses of 67 and 45 kDa, respectively.
Induction of ectopic axes by LEF/TCF constructs

| Construct            | Double axis % | Total no. of experiments | Total no. of embryos |
|----------------------|--------------|--------------------------|----------------------|
| Control              | 0            | 11                       | 225                  |
| mLEF-1               | 59.0         | 7                        | 133                  |
| XTCF-4A              | 0            | 2                        | 50                   |
| XTCF-4C              | 0            | 2                        | 50                   |
| XTCF-3               | 0            | 2                        | 50                   |
| XTCF-3ΔC             | 0            | 2                        | 50                   |
| XTCF3ΔLVPQ           | 0            | 2                        | 50                   |
| 259,263SA            | 0            | 3                        | 67                   |
| XTCF3ΔLVPQ           | 0            | 3                        | 67                   |
| 259,263SAΔC          | 0            | 2                        | 50                   |

The secondary axes induced by these constructs are much less complete than the axes induced by mLEF-1 and XLEF-1 + exon IVa (see Fig. 2B).

Functional Diversity of LEF/TCFs

Frequency of ectopic axis induced by injection of 500 pg of RNA of the indicated construct into the ventral blastomeres of Xenopus four-cell stage embryos is shown. The number of independent experiments and total number of embryos (at least 50) are given.

LVPG and SLVSS motifs in the XLEF-1 + exon IVa chimera reversed the effect of the exon IVa completely. The XLEF-1 + exon IVa + LVPG + SLVSS construct induced ectopic axis in similar frequency and incomplete shape as XLEF-1 wild-type. This demonstrates that the LVPG and SLVSS motifs repress the formation of an ectopic axis. The induction of a secondary axis upon ventral activation of the Wnt/β-catenin pathway is due to the up-regulation of the Wnt/β-catenin target gene siamois. Therefore, we tested in RT-PCR the ability of the different LEF-1 constructs to induce siamois expression (Fig. 2C). We found that the constructs with the highest capacity in axis formation (mLEF-1 and XLEF-1 + exon IVa) strongly increased siamois expression, whereas those constructs with a low capacity in axis formation (XLEF-1) only slightly enhanced siamois expression.

To rule out the possibility that the differences in secondary axis formation were caused by different amounts of the ectopically expressed construct, their protein products were detected by immunoblotting using an antibody (9E10) against the MYC tag. All constructs were expressed in comparable amounts (Fig. 2D).

Next we asked if the TCF mutants suppress the formation of mLEF-1-induced secondary axis. Injection of 500 pg of mLEF-1 mRNA resulted in 59.0% secondary axis formation. This ratio allowed the detection of synergistic or repressive effects if one of our LEF/TCF mutants was coexpressed.

Knowing that XTCF-3 is able to recruit the corepressors groucho and CBP (20, 32), we expected that the XTCF-3 constructs counteract the activity of mLEF-1 in this assay. Indeed, all XTCF-3 constructs with the exception of XTCF-3ΔgrgΔC, which consists only of the β-catenin binding site and the HMG box, suppressed the formation of the secondary axis induced by mLEF-1 (Fig. 3 and Table II). Surprisingly, XTCF-3Δgrg, a well defined activating construct in reporter gene assays in cell culture (31, 32), suppressed secondary axis formation. Most likely, this suppression is due to target gene repression by the co-repressor CBP. The C-terminally truncated XTCFs (XTCF-3ΔC, XTCF-3ΔLVPQ 259,263SAΔC, XTCF-4A, and XTCF-4C), which possess no binding motif for CBP but contain the core region and thus the groucho binding site, all suppressed the induction of a secondary axis. Taken together, the ability of Xenopus TCFs to suppress mLEF-1-induced secondary axis requires the presence of either the central region between the β-catenin binding site and the HMG box consisting of the region A, exon IVa, and region B (see Figs. 1A and 7) or the C terminus. Not surprisingly, XLEF-1, having a weak axis-inducing activity on its own, did not suppress the mLEF-1 phenotype (Fig. 3 and Table II). We did not observe an additive effect of mLEF-1 and XLEF-1. This is in line with the observation that the axis-inducing capacity of XLEF-1 is very weak. Coinjection of mLEF-1 with the chimeric XLEF-1 containing the exon IVa, which can induce a secondary axis, increased the frequency of an ectopic axis formation (Table II). Decreasing the amount of injected RNA revealed that the effect was additive to mLEF-1 (not shown). Again, insertion of the flanking motifs reversed the effect of exon IVa (Fig. 3 and Table II).

In summary, at the ventral side, XTCF-3 and XTCF-4 suppressed the induction of a secondary axis by mLEF-1, whereas XLEF-1 did not. Exon IVa is an activating element that promotes the induction of a secondary axis. The flanking LVPQ and SLVSS motifs act as repressors, abolishing the effect of exon IVa.

The Presence of LVPG and SXXSS in LEF/TCFs Prevents Rescue of Ventralization by Dominant Negative LEF-1—Injection of dominant negative mLEF-1 (ΔHMGLEF-1, dnLEF-1) into the dorsal blastomeres of four-cell stage Xenopus embryos led to a ventralized phenotype (Fig. 4A). The ventralized phenotype was calculated by determining the average dorso-antior index (DAI) (47), a quantitative scale ranging from 0 (fully ventralized) over 5 (normal) to 10 (fully dorsalized). Dorsal injection of 700 pg of ΔHMGLEF-1 mRNA led to an average DAI of 2.63 ± 0.08 (Fig. 4B and Table III), indicating a partial loss of dorsal structures. This moderate phenotype allowed the detection of synergistic or antagonistic effects of LEF/TCF constructs upon coinjection with ΔHMGLEF-1.

We attempted to rescue the loss-of-function phenotype by coinjection of the LEF/TCF constructs and found that XTCF-4C rescued the endogenous axis formation (DAI = 4.34 ± 0.10), whereas XTCF-4A did not (DAI = 2.75 ± 0.10; Fig. 4 and Table III), indicating that the short motifs LVPG and SXXSS confer repressive function on XTCF-4. Coinjection of XTCF-3 and XTCF-3-mutants revealed that its functional properties are different. XTCF-3 enhanced the effect of dnLEF-1, since the embryos were more ventralized as seen by a decrease in the DAI (1.72 ± 0.16, Fig. 4 and Table III). This is in line with the reports that overexpression of XTCF-3 at the dorsal side leads to a partial loss of dorsal structures (20, 25). Neither deletion nor mutation of the LVPG and SLVSS motifs in XTCF-3 (XTCF-3ΔLVPG 259,263SA or XTCF-3ΔLVPGΔSLVSS) had any effect on its activity in this assay. The DAI of <2 was similar to the coinjection of ΔHMGLEF-1 with wild-type XTCF-3 (1.72). In either case, the injected XTCF-3 construct enhanced the effect of ΔHMGLEF-1. Deletion of the core region (XTCF-3Δgrg), however, led to a mutant that rescued the dnLEF-1 phenotype (DAI = 3.75 ± 0.24). This indicates that additional repressive elements are localized in the core region between the β-catenin binding site and the HMG box in XTCF-3, preventing the rescue of the dnLEF-1 phenotype. The C terminus of XTCF-3 did not play a role in this assay, since no differences in activity were observed when the C terminus was present or absent (XTCF-3 versus XTCF-3ΔC, XTCF-3ΔLVPG 259,263SA versus XTCF-3ΔLVPG 259,263SAΔC or XTCF-3Δgrg versus XTCF-3ΔgrgΔC). Wild-type XLEF-1 was able to rescue the formation of the endogenous axis (DAI = 3.52 ± 0.24). Again, insertion of the exon IVAs enhanced the rescue activity (for XLEF + exon IVa, DAI = 4.43 ± 0.08). Further
A, mLEF-1-induced secondary axis formation was abolished by XTCF-4A and XTCF-3 constructs (XTCF-3 Δgrrg) but not by XLEF-1. 0.5 ng of mLEF-1 was ventrally coinjected with 0.5 ng of the indicated LEF/TCF. B, calculation of secondary axis formation upon ventral coinjection of 0.5 ng of mRNA of the LEF/TCF constructs together with 0.5 ng of mLEF-1 mRNA. All XTCF-3 and XTCF-4 constructs except the XTCF-3 ΔgrrgΔC mutant suppressed the mLEF-1-induced secondary axis formation, whereas the XLEF-1 constructs did not. Instead, upon coinjection of the XLEF-1 + exon IVa construct, the frequency of secondary axis formation was increased. The exact values and the number of injected embryos and independent experiments as well as the S.E. values are given in Table II.
insertion of the two repressive flanking motifs had dramatic effects on the activity of the endogenous axis. The XLEF-1 + exon IVa + LVPQ + SLVSS construct did not rescue the formation of the endogenous axis (DAI = 2.91 ± 0.17). Since the two short repressing motifs even override the activating XLEF-1 at the dorsal side in an exon IVa-independent manner, they may have a general repressive function. The underlying molecular mechanism remains to be clarified. It could include changes in protein.

In summary, dorsally, XLEF-1 rescues the formation of the endogenous axis, whereas XTCF-3 suppresses axis formation. The ability of XTCF-4 to rescue the endogenous axis depends on the absence of LVPQ and SXXSS, which flank the activating exon IVa.

**Exon IVa and the Flanking LVPQ and SXXSS Motifs Are Important for Target Gene Activation**—To further characterize the activity of the LEF/TCF constructs, we performed reporter gene assays with the promoters of the Wnt/β-catenin target genes siamois and fibronectin. We transiently transfected two cell lines, the Xenopus A6 cells and the human 293 cells, with the LEF/TCF expression constructs together with one of the target gene promoters. A cytomegalovirus-β-galactosidase construct was cotransfected to normalize for transfection efficiency. These transfection studies confirmed the importance of both exon IVa and the LVPQ and SXXSS flanking regions for activation of the reporter genes. The LVPQ and SXXSS motifs repressed target gene promoter activation, while the exon IVa enhanced promoter activity. Nevertheless, the effects were TCF-, promoter-, and cell type-specific.

First, we used the Xenopus fibronectin promoter as a target. With this target gene promoter, we observed that in the two different epithelial cell lines, the Xenopus A6 cells (Fig. 5A) and the h293 cells (Fig. 5B), deletion of the LVPQ and SFLSS flanking motifs in XTCF-4 (XTCF-4C) turned this transcription factor into an activator. In XTCF-3, deletion of the LVPQ motif (XTCF-3ΔLVPQ) or mutation of all serines in the SLVSS motif (XTCF-3 258,259,263,263SA) had no effect on target promoter activity. Similar to wild-type XTCF-3, these mutants did not significantly activate the fibronectin promoter. The activity of the fibronectin promoter was increased 1.6-fold in h293 cells and increased 1.7- or 1.9-fold, respectively, in A6 cells if both motifs were destroyed (XTCF-3ΔLVPQ 259,263SA or XTCF-3ΔLVPQ 258,259,263SA in Fig. 5, A and B). The 4grg mutant of XTCF-3 (XTCF-3Δ4grg) was the most potent activator of the fibronectin promoter among the XTCFs, indicating additional repressive elements in XTCF-3 apart from the LVPQ and SLVSS motifs. Deletion of the C termini of any of the XTCF-3 constructs did not alter the activation of the target gene promoter (XTCF-3 versus XTCF-3ΔC and data not shown). Wild-type XLEF-1 activated the fibronectin promoter in both cell lines approximately 2-fold. Insertion of exon IVa into XLEF-1 led to an enhanced activity (XLEF-1 + exon IVa). Again, this effect was abolished by insertion of the LVPQ and SLVSS motifs (XLEF-1 + exon IVa + LVPQ + SLVSS).

Second, we used the Xenopus siamois promoter as a target. We observed that the same XTCF constructs that activated the fibronectin promoter also activated the siamois promoter (XTCF-4C, XTCF-3ΔLVPQ 259,263SA, XTCF-3ΔLVPQ 258,259,263SA) in A6 cells (Fig. 5C). This underlines the general importance of the LVPQ and SXXSS motifs in target gene regulation. However, a different situation was observed in h293 cells (Fig. 5D). Here, the siamois promoter was activated by XTCF-4C but not by a corresponding XTCF-3 mutant (XTCF-3ΔLVPQ 259,263SA or XTCF-3ΔLVPQ 258,259,263SA). The only XTCF-3 construct that activated the siamois promoter in h293 cells was the XTCF-3Δ4grg mutant. These findings indicate that apart from the C terminus and the LVPQ and SXSS motifs, additional repressive elements define the differences between XTCF-3 and XTCF-4. Importantly, these elements in the XTCFs display their repressive function depending on the cellular context (compare siamois promoter in A6 and h293 cells; Fig. 5, C and D) and target gene promoter (compare siamois and fibronectin promoters in 293 cells; Fig. 5, D and B, respectively). Deletion of the C terminus of any XTCF-3 mutant tested had no effect on the activity of target gene promoters (XTCF-3 versus XTCF-3ΔC and data not shown).

The most dramatic effect on reporter gene activity was observed upon transfection of the XLEF-1 construct containing exon IVa. In A6 cells, this XLEF-1 + exon IVa construct led to a 9.1-fold increase in siamois promoter activity compared with a 3-fold increase with wild-type XLEF-1 (Fig. 5C). The activation via exon IVa was suppressed by the flanking motifs (XLEF-1 + exon IVa + LVPQ + SLVSS, 5.6-fold activation). The effect was similar in h293 cells (Fig. 5D); the chimera XLEF-1 + exon IVa increased the promoter activity (5.8-fold versus 2.2-fold without exon IVa), while the LVPQ and SLVSS
motifs counteracted the activity of exon IVa (XLEF-1 + exon IVa + LVPQ + SLVSS, 3-fold activation). In either case, reporter gene constructs with mutated LEF/TCF binding sites did not respond to the transfected transcription factors (not shown).

To demonstrate the expression of the transfected LEF/TCF constructs, the myc-tagged proteins were immunoprecipitated using the 9E10 myc antibody (Fig. 6, A and B). Indeed, the myc antibody recognizes a band of correct size and comparable signal intensity in the immunoprecipitates of both cell lines. Since immunoprecipitates of single transfection experiments are shown in Fig. 6, slight differences in the amount of the ectopically expressed proteins reflect the variability of the transfection efficiency. Since the values of the reporter gene assays were normalized on β-galactosidase activity and represent the average of at least seven independent transfections, differences in the expression level of the constructs are not responsible for the differences in their transactivation properties.

To rule out the possibility that the activating or repressing effects of our LEF/TCF constructs were caused by incorrect subcellular localization, the transfected cells were immunostained for TCFs. Each of the transfected TCF splice variants or mutants was located in the nucleus (Fig. 6C) independent of the presence of the C terminus or the LVPQ or SXXSS motif. The differences in reporter gene activation, therefore, are not due to inappropriate subcellular localization of the transfected TCF. Untransfected cells show a weak nuclear staining with TCF3/4 antibody (Fig. 6C, long exposure) due to the endogenously expressed XTCF-3 and XTCF-4 (31).
In the present study, we provide evidence that exon IVa in LEF/TCF transcription factors is an activating element, whereas the LVPG and SXXSS flanking motifs are repressing elements. XTCF-3 reveals the strongest repressive character among the family members, most likely due to specific elements within regions A and B.

**The Activating Exon IVa—**We have shown for the first time that exon IVa represents a crucial activating element in LEF/TCF transcription factors and is required for the induction of *siamois* expression and the formation of an ectopic axis. The presence of the exon IVa in mLEF-1 (also called exon VI according to the genomic structure of hLEF-1; Ref. 39) and absence of this exon in XLEF-1 is the most obvious difference between these orthologs. Exon IVa is a highly conserved region with 70% amino acid identity between mLEF-1, XTCF-3, and XTCF-4. Here we show that this particular exon is important for LEF function. Insertion of exon IVa increased the activity of XLEF-1 during secondary axis induction upon ventral overexpression, rescue of the endogenous axis upon dorsal overexpression, and activation of target gene promoters. This is in line with the findings of Carlsson et al. (27), who showed that Gal4 fusions with hLEF-1 containing exon IVa were much stronger transactivators than those lacking exon IVa. Activation via exon IVa could be due to the recruitment of an additional activating cofactor or, alternatively, to changes in LEF protein structure. We observed activation via exon IVa in *Xenopus* and human cell lines as measured by promoter assays as well as in axis formation and *siamois* induction in *Xenopus* embryos. If the activation is achieved via the recruitment of a coactivator, this cofactor should be ubiquitously expressed. A likely candidate for such a cofactor is ALY (51), although a *Xenopus* homolog has not yet been identified. The fact that in hLEF-1 and in hTCF-1 exon IVa is naturally differentially spliced (38, 39) implicates a physiological relevance of this element. Since the presence of LEF-1 splice variants in *Xenopus* remains elusive, we are currently screening different embryonic stages and tissues for XLEF-1-exon IVa activity.

The finding that all other XTCF-3 and XTCF-4 variants contain exon IVa but are unable to induce a secondary axis indicates that additional inhibitory elements dominate the activating exon IVa. These elements are not the groucho binding elements of the Smad 4 binding site, since we have recently shown that XLEF-1 without exon IVa as well as XTCF-3 and XTCF-4 bind these cofactors (40). We also exclude the CtBP binding site, because XTCF-4 does not contain this motif, and deletion of the C terminus in our experiments had no effect on XTCF-3 function. The XTCF-3agrg and XTCF-3agrgAC mutants activate target gene promoters (Refs. 31 and 32 and this study), indicating that most of the repressive motifs are deleted. To our surprise, these mutants were unable to induce a secondary axis. Based on the results presented here, this can now be explained by the fact that these mutants lack not only the repressive motifs but also the activating element, exon IVa.

**The Repressing Elements LVPG and SXXSS—**During early embryogenesis, the three XTCF-4 variants are differentially expressed, implicating that alternative splicing of the LVPG and SFLSS motifs is physiologically important. The results provided here demonstrate that the LVPG and SXXSS motifs repress the activity of all *Xenopus* LEF/TCF transcription factors. Insertion of these two motifs into an activator abolishes its activating properties (e.g. XTCF-4C → XTCF-4A), and deletion of these motifs within a repressor converts it into an activator (e.g. XTCF-3 → XTCF-3LVPG 258,259,263SA). At the ventral side of *Xenopus* embryos and in reporter gene assays, these repressing elements dominate the activating exon IVa. The frequency and completeness of the secondary axis induced by XLEF-1, as well as reporter gene activity, were increased by insertion of the exon IVa. This increase was abolished and dropped down to wild-type XLEF-1 activity by further insertion of the flankng motifs (XLEF-1 + exon IVa + LVPG + SLLVSS). In this context, it is remarkable that murine LEF-1, which is known to induce ectopic axis (Ref. 19 and this study) and to activate the fibronectin promoter (31), contains the activating exon IVa but does not contain the LVPG and SXXSS flanking motifs. At the dorsal side of *Xenopus* embryos, these repressing elements dominate the activating exon IVa and the remaining activating region of XLEF-1 (XLEF-1 and XLEF-1 + exon IVa rescued dsLEF-1-ventralized embryos; XLEF-1 + exon IVa + LVPG + SLVSS did not rescue). In addition to the recruitment of the corepressors groucho (32, 33) and CtBP (20), we identified the additional repressing elements LVPG and SXXSS, which prevent gene activation by XTCF-3 or XTCF-4. Recently, it has been described that phosphorylation of LEF/TCF transcription factors by the NLK leads to target gene repression (37). To examine this, we cotransfected the upstream regulator of the NLK, TAK-1, as well as a kinase-dead construct of TAK-1 together with the XTCF-4 variants and found no significant changes in target gene promoter activity (not shown).

We propose three possible modes of repression via the LVPG

| Average DAI | S.E. | Total no. of experiments | Total no. of embryos |
|-------------|------|--------------------------|----------------------|
| ΔHMGLEF-1   | 2.63 | 0.08                     | 18                   | 380                   |
| + XTCF-4C   | 2.75 | 0.10                     | 5                    | 105                   |
| + XTCF-3    | 4.34 | 0.10                     | 5                    | 102                   |
| + XTCF-3ΔC  | 1.72 | 0.16                     | 6                    | 135                   |
| + XTCF3LVPG | 1.88 | 0.08                     | 2                    | 52                    |
| + XTCF3LVPG 259,263SA | 1.67 | 0.09                     | 4                    | 82                    |
| + XTCF3LVPG 259,263SAΔC | 1.42 | 0.35                     | 4                    | 90                    |
| + XTCF3LVPG 259,263SAΔC, 300 pg | 2.27 | 0.05                     | 2                    | 51                    |
| + XTCF3LVPGΔLVPQ | 1.05 | 0.05                     | 3                    | 64                    |
| + XTCF-3agrg | 3.75 | 0.24                     | 4                    | 76                    |
| + XTCF-3agrgΔC | 3.83 | 0.05                     | 4                    | 82                    |
| + XTCF3agrgΔC, 150 pg | 3.26 | 0.13                     | 2                    | 51                    |
| + XLEF-1   | 3.52 | 0.24                     | 4                    | 87                    |
| + XLEF-1 + exon IVa | 4.43 | 0.08                     | 4                    | 85                    |
| + XLEF-1 + exon IVa + LVPG + SLVSS | 2.91 | 0.17                     | 3                    | 67                    |
FIG. 5. **Reporter gene assays.** Exon IVa contributes to target gene activation, whereas the flanking LVPQ and SXXSS motifs are repressive elements. Reporter gene assays were performed using the *Xenopus* fibronectin and *stamosis* promoters as target gene promoters in *Xenopus* A6 cells and in h293 cells. Normalized luciferase activity is shown. Each bar represents the average of 7–18 independent transfections. The error bar indicates the S.E. A, activation of the *Xenopus* fibronectin promoter in A6 cells. XTCF-4A did not activate the fibronectin promoter, whereas XTCF-4C activated it more than 2-fold. In XTCF-3, both LVPQ and SLVSS motifs had to be mutated or deleted to transform XTCF-3 into a slight activator (XTCF-3ΔLVPQ 259,263SA and XTCF-3ΔSLVPP 258,259,263SA 1.7- and 1.9-fold, respectively). Destroying only one of the motifs had no effect on the activity of the target gene promoter (XTCF-3ΔLVPQ, XTCF-3 258,259,262,263SA). The XTCF-3Δgrg mutant was more potent in activating the fibronectin promoter (more than 3-fold) than any of the other XTCF-constructs. XLEF-1 wild type activated the fibronectin promoter about 2-fold. Insertion of exon IVa raised the activity (2.7-fold activation). Further insertion of the LVFPQ and SLVSS motifs abolished the effect of exon IVa (XLEF-1 + exon IVa + LVPQ + SLVSS 2-fold activation). B, activation of the *Xenopus* fibronectin promoter in h293 cells. While XTCF-4A did not activate the fibronectin promoter, XTCF-4C activated it 2-fold. In XTCF-3, both LVPQ and SLVSS motifs had to be disturbed to
FIG. 6. Expression of the transfected LEF/TCF constructs. A, immunoprecipitation of the transfected A6 cells with a monoclonal antibody against the c-my c tag followed by Western blotting with the same antibody revealed that all transfected LEF/TCF constructs are expressed in comparable amounts. The bars indicate the 45- and 67-kDa marker bands. B, immunoprecipitation of the transfected 293 cells with a monoclonal antibody against the c-my c tag followed by Western blotting with the same antibody revealed that all transfected LEF/TCF constructs are expressed in comparable amounts. The bars indicate the 45- and 67-kDa marker bands. C, immunostaining with a monoclonal antibody against TCF3/4 demonstrates that all transfected XTCF constructs are localized in the nucleus. The antibody recognizes endogenous TCF3-3 and -4 in A6 cells. Colocalization with 4',6-diamidino-2-phenylindole (DAPI) staining demonstrates the nuclear localization of the endogenous XTCFs (long exposure). Upon transfection with any of the XTCF constructs, some of the cells (the transfected ones) were stained much more intensively. The staining overlaps with the 4',6-diamidino-2-phenylindole staining, indicating nuclear localization of the transfected XTCF.

and SXXSS motifs: 1) the LVPQ and SXXSS motifs could change the overall structure of LEF/TCFs in such a manner that transactivation via β-catenin cannot take place; 2) a corepressor could specifically bind to these motifs; 3) alternatively, phosphorylation regulated by the SXXSS serine-rich motif could prevent the formation of a ternary complex between DNA, TCF, and β-catenin as we have shown for XTCF-4 (40). Indeed, it is sufficient to mutate two serine residues of the SLVSS motif in XTCF-3LVPQ to alter its repressive behavior.

**XTCF-3 Contains an Additional Repressive Element**—In addition to exon IVa and its flanking LVPQ and SXXSS regions, we suggest that a further repressive element is present in XTCF-3, whereas XLEF-1 contains an additional activating element. XTCF-4 reveals chameleon-like properties, since it behaves like XTCF-3 in secondary axis formation assays but like XLEF-1 in our target gene promoter assays. Thus, the specific function of the individual LEF/TCF family member derives from elements in the A and/or B regions (Fig. 7).

We suggest that the XTCF-3-specific element is situated within the in XTCF-3Δgrg deleted region (elements A and B in Figs. 1A and 7) and confers a repressing function on the dorsal side, because the XTCF-3Δgrg mutant rescued the dnLEF-1 phenotype at the dorsal side, whereas the XTCF-3 constructs with or without mutations in the repressing flanking regions transform it into a slight activator (XTCF-3LVPQ 259,263SA). Insertion of exon IVa raised the activity (9.1-fold activation). Further insertion of the LVPQ and SLVSS motifs partially inhibited the effect of exon IVa (XLEF-1 + exon IVa + LVPQ + SLVSS, 2.2-fold activation). C, activation of the Xenopus siamois promoter in A6 cells. XTCF-4C is a stronger activator than XTCF-4A (2.9- and 1.5-fold, respectively). The only XTCF-3 mutant that activated the siamois promoter in these cells was the XTCF-3Δgrg mutant. All other XTCF-3 mutants as well as wild-type XTCF-3 did not activate the siamois promoter. XLEF-1 wild type activated the siamois promoter about 2-fold. Insertion of exon IVa raised the activity (9.1-fold activation). Further insertion of the LVPQ and SLVSS motifs partially inhibited the effect of exon IVa (XLEF-1 + exon IVa + LVPQ + SLVSS, 5.6-fold activation). D, activation of the Xenopus siamois promoter in 293 cells. XTCF-4C is a stronger activator than XTCF-4A (2.3- versus 1.5-fold). The only XTCF-3 mutant that activated the siamois promoter in these cells was the XTCF-3Δgrg mutant. All other XTCF-3 mutants as well as wild-type XTCF-3 did not activate the siamois promoter. XLEF-1 wild type activated the siamois promoter about 2-fold. Insertion of exon IVa raised the activity (5.8-fold activation). Further insertion of the LVPQ and SLVSS motifs denied the effect of exon IVa (XLEF-1 + exon IVa + LVPQ + SLVSS, 3-fold activation).
further ventralized the embryo (see Table III). This suggestion is further supported by the finding that the XTCF-3 grg mutant was the most potent activator among the XTCF-3 constructs used in reporter gene studies. At the ventral side of Xenopus embryos, the XTCF-3 grg mutant suppressed the induction of a secondary axis. The only XTCF-3 mutant not suppressing ectopic axis induction was the XTCF-3 grg C mutant. Taken together, the XTCF-3-specific elements in regions A and B cooperate with the LVPQ and SXXSS flanking regions and the C terminus to suppress the induction of a secondary axis.

Comparing the similarity of the regions A and B between XTCF-3 and XTCF-4 reveals that their amino acid sequences are up to 60% identical. Nevertheless, nonhomologous regions of about 20 amino acids are present within regions A and B. These motifs are likely candidates to provide specific properties to the individual LEF/TCF family member.

In contrast to XTCF-3, we observed that in XTCF-4, deletion of the LVPQ and SFLSS motifs (XTCF-4C) was sufficient to rescue the dnLEF-1 phenotype at the dorsal side. At the ventral side, both XTCF-4 variants (XTCF-4A and XTCF-4C) suppressed the induction of a secondary axis. This indicates that a XTCF-4-specific element allows the rescue of the endogenous axis at the dorsal side upon coinjection with dnLEF-1 but is not sufficient to suppress the induction of a secondary axis upon ventral coinjection with mLEF-1.

Finally, XLEF-1 and a chimera of XLEF-1 containing exon IVa of XTCF-3 without the LVPQ and SXXSS motifs rescued the formation of the endogenous axis upon dorsal coinjection with dnLEF-1. Therefore, as in XTCF-4, a XLEF-1-specific element allows the rescue of the endogenous axis when overexpressed on the dorsal side. Furthermore, the observation that among the Xenopus LEF/TCFs only XLEF-1 was able to induce a secondary axis upon ventral injection indicates the existence of an additional LEF-1-specific activating element. The transactivation domain A of LEF described by Carlsson et al. (27), which consists of amino acids 80–135 of hLEF-1 (part of element A in Figs. 1A and 7), could fulfill this function.

In summary, subtype-specific elements are located in regions A and B (Fig. 7) and confer individual functional properties to the different LEF/TCF family members. Future studies will focus on identifying possible cofactors that bind to these subtype-specific elements and elicit how they contribute to regulate the outcome of Wnt/β-catenin signals.

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**Fig. 7.** Overall structure of LEF/TCF transcription factors. All LEF/TCFs share highly homologous β-catenin binding and DNA binding sites, whereas domains A and B reveal lower homology. The activating exon IVa is flanked by the repressing elements LVPQ and SXXSS. The C terminus (domain C) is TCF-specific. The lower part lists the elements involved in axis formation.
Functional Diversity of Xenopus Lymphoid Enhancer Factor/T-cell Factor Transcription Factors Relies on Combinations of Activating and Repressing Elements

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