Identification of Two Regulatory Elements within the High Mobility Group Box Transcription Factor XTCF-4*

Some members of the Wnt family of extracellular glycoproteins regulate target gene expression by inducing stabilization and nuclear accumulation of β-catenin, which functions as a transcriptional activator after binding to transcription factors of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family. Three different members of this family have been identified in *Xenopus laevis* thus far that differ in their ability to influence mesodermal differentiation and to activate expression of the Wnt target gene fibronectin. Here we report on the isolation and characterization of additional variants of XTCF-4. We show that the differential ability of these proteins and other members of the TCF family to activate target genes is neither due to preferential interaction with transcriptional cofactors of the groucho family or SMAD4 nor to different DNA binding affinities. Expression of these proteins in an epithelial cell line reveals differences in their ability to form a ternary complex with DNA and β-catenin. Interestingly, formation of this ternary complex was not sufficient to activate target gene expression as previously thought. Our experiments identify two amino acid sequence motifs, LVQP and SFLSS, in the central domain of XTCF-4 that regulate the formation of the DNA-TCF-β-catenin complex or activation of target genes, respectively. Biochemical studies reveal that the phosphorylation state of these XTCF-4 variants correlates with their ability to form a ternary complex with β-catenin and DNA but not to activate target gene expression. The described variants of XTFC-4 with their different properties in complex formation provide strong evidence that in addition to the regulation of β-catenin stability the isoforms of TCF/LEF transcription factors and their posttranslational modifications define the cellular response of a Wnt/wingless signal.

Wnt-1 was originally identified as a proto-oncogene in mice (1) and was found to be a vertebrate homolog of the *Drosophila* segment polarity gene *wingless*. To date at least 15 additional vertebrate homologs have been identified (for review see Ref. 2). Members of the Wnt family of extracellular glycoproteins are able to activate at least two different signaling pathways in vertebrates (reviewed in Refs. 3 and 4). The canonical Wnt pathway is involved in different developmental processes such as cell fate specification and cell migration. The major cytoplasmic effector of this pathway, β-catenin, accumulates in the cytoplasm in response to a Wnt signal. Subsequently, β-catenin can enter the nucleus where it binds to the N terminus of TCF/LEF1 transcription factors to function as a transcriptional coactivator. Dereglulation of the Wnt/β-catenin pathway leads to carcinogenesis. Mutations increasing the stability and thus the cytoplasmic/nuclear pool of β-catenin were found in colon carcinomas and malignant melanomas (4). The identification of c-myc as a target gene of TCF-4/β-catenin links Wnt signaling with cell proliferation and thus with carcinogenesis (5). Another important target gene of TCF/LEF in this context leading to cell cycle deregulation was identified as cyclin D1 (6).

Members of the TCF/LEF family were originally identified as T- and B-lymphocyte-specific transcription factors. Sequence-specific DNA binding is mediated by the HMG box, and the target motif of these factors is given by the sequence (C/G)TT-CN-TG(A/T) (7). Members of this family are unable to activate transcription of reporter gene constructs that carry multiple copies of their minimal binding motifs (8) and thus lack activation properties on their own. This raises the question how these proteins work as transcriptional regulators. One well characterized target gene regulated by LEF-1 is the T-cell receptor α gene, TCRα. Activation of the TCRα promoter by LEF-1 is strictly context-dependent. Under these circumstances LEF-1 functions as an architectural transcription factor by bending DNA, thus facilitating stable interactions of other known transcription factors like CREB/ATF, PEBP2α, and Ets-1 (9).

In addition, LEF-1 interacts with proteins thought to function as transcriptional activators like ALY (10) and SMADs (11, 12). More recently, a functional interaction of LEF-1 as well as XTCF-3 with β-catenin has been demonstrated (13-15). As a major player in canonical Wnt signaling, β-catenin has been shown to function as a transcriptional coactivator directly binding TBP (16), pontin52 (17, 18), and p300/CBP (19, 20). p300/CBP synergizes with β-catenin in activation of target genes. A synergistic effect specific for β-catenin-mediated activation of Xwin was also reported for SMAD4; however, a direct binding between both β-catenin and SMAD4 remained elusive (11).

Despite binding to the same target site, members of the TCF/LEF family differ in their function. When overexpressed on the ventral side of *Xenopus* embryos, mLLEF-1 induces the

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* The abbreviations used are: TCF, T-cell factor; LEF, lymphoid enhancer factor; CKII, casein kinase II; ESG, enhancer of split groucho; grg, groucho; GST, glutathione S-transferase; FN, fibronectin; HMG, high mobility group; TCRα, T-cell receptor α; WntRE, Wnt-responsive element; DTT, dithiothreitol; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.
formation of a secondary body axis via activation of the Wnt target gene *siamois* (13, 14), whereas XTCF-3 fails in this assay (15). In epithelial A6 cells TCF-1 activates the Wnt target gene *fibronectin*, whereas XTCF-3 does not (21). Two hybrid studies demonstrated that unlike TCF-1, XTCF-3 binds transcriptional corepressors of the groucho family (22) as well as CtBP (23). This led to the model that XTCF-3 functions as a transcriptional repressor in the absence of β-catenin by recruiting corepressors, whereas LEF-1 is unable to function as a repressor due to lack of interaction with grouchos or CtBP (23). However, Levanon et al. (24) showed physical and functional interaction of the human groucho homolog TLE-1 with hLEF-1. The contradictory results may be explained by different splice variants of LEF-1 that were used by Roose et al. (22) and Levanon et al. (24), respectively.

Analysis of the genetic structure of the human TCF-1 gene and analysis of the detected mRNA transcripts revealed extensive alternative splicing events. These result in a maximum of 96 theoretical splice variants, at least 5 of which have been shown to be expressed in vivo (25). Alternative splice variants also have been reported for TCF-3, TCF-4, and LEF-1. These contradictory results may be explained by different splice variants of the human groucho homolog TLE-1 with hLEF-1. The complications with LEF-1 that were used by Roose et al. (22) and Levanon et al. (24) are due to lack of interaction with grouchos or CtBP (23). However, these motifs neither influence groucho nor SMAD4 binding to the transcription factors. Instead, we found that XTCF-4-β-catenin DNA complex formation was accompanied by a dephosphorylation event observed in XTCF-4 isoforms lacking these motifs.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs Used in This Study**—Additional TCF-4 variants were isolated exactly as described (26). In short, TCF-4 clones were isolated from an A6 λ-ZAP cDNA library. Complete cDNA clones were obtained by 5′-rapid amplification of cDNA ends and combining overlapping fragments. The XTCF-3 clone was isolated from a gastrula cDNA library was screened with a 600-base pair (bp) fragment of the human groucho homolog TLE-1 with hLEF-1. The transcription factors. Instead, we found that XTCF-4-β-catenin DNA complex formation was accompanied by a dephosphorylation event observed in XTCF-4 isoforms lacking these motifs.

**Cell Culture and Reporter Gene Assays**—Transfection of epithelial A6 cells with different expression constructs and luciferase reporter gene assays were performed as described (21), except for the use of Effectene transfection reagent (Qiagen) instead of Lipofectin (Life Technologies, Inc.). Cell extracts were used for PCRs and analyzed using 15 μl × 10−3 M Tris/HCl, pH 7.5, 150 μM NaCl, 0.5% Nonidet P-40, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride. The used phosphatase inhibitors were sodium vanadate (10 μM), sodium fluoride (10 μM), and sodium molybdate (10 μM).

**Bacterial Expression of HMG Box Transcription Factors and In Vitro Transcription**—For expression of His-tagged fusion proteins, cDNAs were evaluated by pGEM or pGEM-T (Promega) or Ncol inserts into the expression vectors. The primers used were 5′-CTTCTTCTCTTCTTCC-3′.

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For the isolation of a full-length Xenopus ESG-1 clone, a ZAP λ-Xenopus cDNA library was screened with a 660-base pair fragment of ESG-1 (28) obtained by PCR amplification of a Xenopus gastrula library. Only partial cDNA clones were isolated, the longest encoding amino acids 34–756. The N-terminal region of ESG-1 (756 base pairs) was amplified by PCR of a Xenopus oocyte cDNA library and subcloned into the BamHI site of ESG-1. The obtained full-length sequence was 95% homologous to the previously isolated ESG-1 and submitted to GenBank (accession number AF289027). For GST pull-down assays with GST-SMAD4, human SMAD4 cloned into pGex4T3 (Amersham Pharmacia Biotech). The truncated version of XTCF-3 lacking the HMG domain and C terminus (amino acids 1–322) which was used as a negative control was prepared by PCR and subcloned into the EcoRI-Xhol sites of pCS2+. The primers used were 5′-CGGGAATT-TCATGCCCCTCCTAAACAGACGGACG-3′ and 5′-CGGCTCGAGACTGGGC-TCCTGTCTCTTCC-3′.

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after 10 min of treatment at 30 °C as longer treatment (30 min) resulted in a nearly complete loss of DNA binding.

**RESULTS**

**Screening for XTCF-4 Reveals Different Splice Variants**—In our attempt to isolate Xenopus members of the TCF/LEF family, we recently identified a Xenopus homolog of TCF-4 (26). In addition to the published XTCF-4 sequence, which we now refer to as XTCF-4B (GenBank™ accession number AF207708), we isolated in this screen two highly identical variants that differ in two short amino acid motifs (Fig. 1A). Whereas XTCF-4A (GenBank™ accession number AF287150) contains both of these stretches, XTCF-B lacks the sequence SFLSS at position 288–292. XTCF-4C (GenBank™ accession number AF287151) additionally lacks 4 amino acids (LVPQ) at position 256–259. In comparison to other known members of the TCF/LEF family, XTCF-4A resembles XTCF-3, whereas XTCF-4C is similar to murine LEF-1 (see Fig. 1A). As identical gaps are present in the mLEF-1 sequence, we exclude the possibility that these sequence variations might be an artifact. Based on the fact that the multiple TCF-1 isoforms are due to alternative splicing (25), we assume that the isolated XTCF-4 variants were also generated by alternative splicing.

We also isolated Xenopus homologs of TCF-3 (GenBank™ accession number AF287149) and mLEF-1 (GenBank™ accession numbers AF287147 and AF287148) that are almost identical to the published sequences (15, 27). The isolated Xenopus homologs of mLEF-1 not only lack the two described short amino acid stretches but also the region between (Fig. 1A). Comparison with the recently characterized genomic structure of the human TCF-1 gene (25) reveals that this region is encoded by the alternatively used exon IVA of hTCF-1 (underlined in Fig. 1A). So far we have not been able to detect a Xenopus homolog of XLEF-1 or TCF-1 encompassing this exon. For functional studies, these splice variants were subcloned into pCS2+ containing a cytomegalovirus promoter allowing expression of the constructs in culture cell lines.

**Differential Ability of TCF/LEF Transcription Factors to Activate Target Gene Expression**—To compare the transactivation activities of these HMG box transcription factors, we used the Xenopus fibronectin gene as a direct target for the classical Wnt/β-catenin pathway in Xenopus. As shown previously, in epithelial A6 cells mLEF-1 activates a −499/+20 fibronectin (FN) reporter gene construct significantly stronger than XTCF-3 (Ref. 21 and Fig. 1C). We therefore asked whether the differential potential of TCF/LEF members to activate the fibronectin promoter might be due to differences in their ability to interact with transcriptional repressors of the groucho family. For this purpose, a far Western was performed (9) with purified His-tagged fusion proteins of mLEF-1, XLEF-1, XTCF-3, XTFC-4A, and XTCF-4C using SDS-PAGE. After electrophoresis, proteins were transferred onto a nitrocellulose membrane and overlaid with radioactively labeled Xenopus ESG-1, a member of the groucho family of transcriptional repressors. In contrast to Roose et al. (22), but supporting Levanon et al. (24), we found that all members of the TCF/LEF family can interact with ESG-1 in vitro (Fig. 2). The cytoplasmic domain of Xenopus cell-cell adhesion molecule cadherin-11 was used as negative control demonstrating the specificity of interaction between ESG-1 and TCF/LEF factors. Based on these results, we conclude that all members of the TCF/LEF family have the potential to interact with members of the groucho family and that there is no simple correlation between the ability of a TCF/LEF factor to bind groucho members and their role as transcriptional activators or repressors, respectively.

Another transcriptional corepressor that had been shown to interact with the C-terminal region of XTCF-3 is CtBP (23).
Binding of CtBP to XTCF-3 depends on two PLSL(T/V) motifs in the C terminus of XTCF-3 that are also present in hTCF-4 but not XTCF-4. Since this region is missing in mLEF-1, this opens the possibility that CtBP might be involved in repressing fibronectin expression by XTCF-3. We therefore constructed a C-terminal deletion construct of XTCF-3 (Fig. 1B) that has been shown to be unable to bind CtBP (23), and we tested the ability of this construct to activate the FN-promoter in A6 cells. In several independent experiments XTCF-3D was unable to activate transcription from the −499/+20 construct (Fig. 1C).
**Fig. 2.** Interaction of TCF/LEF transcription factors with ESG-1 group. A, fusion proteins were purified on nickel-agarose and separated on polyacrylamide gels. XLEF-1 and mLLEF-1 were stained by Coomassie (left), whereas the other proteins (right) were detected by an anti-His tag antibody (Qiagen). The asterisk labels the band for XLEF-1. Molecular weights are indicated. Xcad-11 is a His-tagged fusion protein of the intracellular domain of Xenopus cadherin-11 that served as a negative control (29). B, overlay blot with \(^{35}S\)-labeled Xenopus ESG-1, a member of the groucho family. Lanes were as in A. Note that all proteins are interacting with ESG-1 the exception of Xenopus cadherin-11. C, SMAD4 binds directly to TCF/LEF transcription factors. In vitro translated \(^{35}S\)-labeled TCF/LEF proteins (lanes 1–6) were precipitated with GST (lanes 7–12) or SMAD4-GST (lanes 13–18). All tested factors bound GST-SMAD4 except TCF-3 lacking the HMG domain and C terminus.

This failure in activation is not due to a nonfunctional protein given the following two observations. First, XTCF-3C is able to bind the Wnt-RE (see Fig. 5A below) and thus is functional with respect to DNA binding. Second, in XTC cells this construct was still able to repress FN expression (Fig. 1E) showing that this construct is still functional with respect to target gene regulation. This experiment together with the ability of XTCF-3\(\text{agg}\) to activate fibronectin expression (21) demonstrate that CtBP is not the major corepressor component of the XTCF-3-mediated transcription factor complex on the FN promoter.

A complex of SMAD4, LEF-1, and \(\beta\)-catenin has recently been shown to regulate the transcriptional activation of the Xenopus homeobox transcription factor twin (11, 12). Subsequent in vitro precipitation experiments demonstrated that SMAD4 binds directly to the HMG box of LEF-1 (11). In the context of this study we therefore aimed to analyze this interaction with respect to other members of the TCF/LEF family. To determine whether SMAD4 preferentially binds to members of this family, GST pull-down experiments were performed. In vitro translated \(^{35}S\)-labeled TCF/LEF proteins were precipitated with GST-SMAD4 or with GST alone. As shown in Fig. 2C, a strong interaction with SMAD4 was observed for all TCF/LEF factors tested, none of them interacting with GST alone. As a negative control, XTCF-3 lacking the HMG domain and the C terminus was used (XTCF3 (amino acids 1–322)). We conclude from these experiments that all Xenopus members of the TCF/LEF family can interact with SMAD4 thereby excluding the possibility that differential complex formation with SMAD4 accounts for the observed differences in transactivation of HMG box transcription factors.

**Different Members of the TCF/LEF Family Bind to Their Target Sequence with Different Affinity**—We next reasoned that the differences in transactivation activity of TCF/LEF members might be due to differences in DNA binding affinity. Different TCF/LEF fusion proteins were purified as judged by Coomassie staining (Fig. 3A), and binding affinities toward two well defined WntRE in Xenopus were determined. The DNA duplexes were derived from the TCF/LEF-binding sites found in the Xenopus fibronectin and siamois promoters (21, 30) (Fig. 3B). We recently reported that mLLEF-1 specifically binds to the WntRE of the fibronectin promoter (21). Here we demonstrate that XTCF-3, XTCF-4A, and-4C are also able to bind specifically to the FN-WntRE (Fig. 3C). Whereas addition in excess of unlabeled oligonucleotides containing the FN-WntRE prevented binding of the fusion proteins to the labeled fragment, addition of an unspecific competitor did not (Fig. 3C). Identical results were obtained with the sia-WntRE indicating the specific binding of TCF/LEF factors (not shown).

To determine the apparent dissociation constant to the used oligonucleotides derived from the siamois or the FN promoter, respectively, we performed quantitative bandshift studies with variable amounts of TCF/LEF fusion proteins (Fig. 4, A and B). The percentage of bound oligonucleotide was plotted as a function of the fusion protein concentration, and the protein concentration that resulted in a binding of 50% of the oligonucleotide was estimated as the apparent \(K_D\) value. The \(K_D\) values observed in our experiments were within a range of 0.3 and 4.6 \(\mu\)M and thus within the same order of magnitude as recently determined for the murine LEF-1 HMG box only (31). In addition, to verify these constants by a different plotting procedure we performed classical Scatchard plot analysis. For this purpose, the ratio of bound protein to free protein concentration (bound/free) is plotted versus the concentration of bound protein. By using this procedure, the \(K_D\) value can be estimated by using the slope of the obtained line. Additionally, the total number of binding sites represented by the used oligonucleotide can also be estimated by the abscessa intercept (for a more detailed description of Scatchard plots see Ref. 32).

The most obvious outcome of these experiments is the fact that all tested fusion proteins have a significant higher affinity toward the sia-WntRE than the FN-WntRE. However, the absolute difference in binding affinity was dependent on the fusion protein used. We found for mLLEF-1 a 2-fold higher affinity toward the siamois-derived target site in comparison to the FN-derived sequence, whereas the fold difference in binding affinity for the others were as follows: XTCF-4A, 2-fold higher; XTCF-4C, 5-fold higher; and XTCF-3, 5-fold higher (Fig. 4, A and B). One major difference between the two target oligonu-
cleotides became obvious by comparing the binding affinities for XTCF-3. Whereas in case of the FN-WntRE XTCF-3 displays sigmoidal binding with comparable low affinity, it exhibits a hyperbolic binding to the sia-WntRE with a significantly higher affinity. A similar behavior was observed for XTCF-4C. XTCF-4A showed sigmoidal binding to both oligonucleotides. In these cases we were not able to plot linear Scatchard plots but obtained graphs with a maximum, which is an indication for positive cooperative binding behavior (32). This lower binding affinity toward the FN-WntRE is most likely not due to protein misfolding or degradation as this experiment was done with different protein preparations, and the same batches of proteins showed a higher binding affinity toward the sia-WntRE. These data raise the possibility that the two different bases in the core sequence as well as the flanking regions influence the binding behavior of a given HMG box transcription factor. In addition, the extended C termini of XTCF-3 and XTCF-4 might influence the binding characteristics on the FN-derived oligonucleotide. Indeed, we found that truncation of the C terminus of XTCF-3 significantly shifts the binding behavior toward the mLEF-1-derived one (Fig. 4C).

Most strikingly, although bacterially expressed XTCF-4C binds to the FN-WntRE in vitro with much lower affinity than mLEF-1, it is a more potent in vivo activator of the FN promoter than mLEF-1. We conclude that although members of the TCF/LEF family differ in their in vitro DNA binding affinities toward different promoter target sites, this difference does not correlate with their transactivation potentials.

Members of the TCF/LEF Family Differentially Bind \(\beta\)-Catenin When Bound to DNA—We next asked whether there might be differences in complex formation mediated by the analyzed TCF/LEF factors in vivo. We therefore transfected epithelial A6 cells with expression constructs encoding for the transcription factors. After 3 days we prepared cell lysates under mild conditions and analyzed the behavior of the overexpressed proteins in gel shift assays. Western blot analysis of these lysates revealed that all overexpressed proteins were expressed at comparable levels (Fig. 5C). Although XTCF-4A and XTCF-4C are of nearly identical size, the formed DNA-protein complexes showed different migrating behavior (Fig. 5A). The XTCF-4C-induced DNA-protein complex was of larger size compared with the XTCF-4A one. Whereas the mLEF-1-DNA complex behaved similar to the XTCF-4C-DNA complex, the XTCF-3-induced complex migrates as fast as the XTCF-4A one. Thus, the ability of these transcription factors to trigger target gene activation is paralleled by the migration behavior of the corresponding DNA-protein complexes.

These differences are probably not due to differences in DNA bending as we never observed such differences when using bacterially expressed fusion proteins (see Fig. 3 as example). This raises the possibility that the observed differences in migration behavior reflect differential complex formation with transcriptional coactivators, namely \(\beta\)-catenin, rather than corepressors. We aimed to study this possibility by supershift analyses. We focused on the different TCF-4 variants as antibodies are commercially available that specifically recognize XTCF-4 and \(\beta\)-catenin. Our attempt to include mLEF-1 and XTCF-3 into this study was hampered by the lack of antibodies.
against these proteins that function under native conditions.

For the supershift experiments the buffer and gel conditions were slightly altered compared with the experiments shown in Fig. 5A to achieve a higher resolution for the slower migrating complexes (Fig. 5B). This led to the detection of a faster migrating complex in XTCF-4B- and XTCF-4C-transfected cells that runs at the level of the complex also found in parental and XTCF-4A-transfected cells (for reasons described below and labeled as TCF/DNA in Fig. 5B). Whereas upon XTCF-4A transfection and in untransfected cells only this faster migrating complex was seen, XTCF-4B and XTCF-4C transfection revealed an additional slower migrating complex that corresponds to the main signal seen in Fig. 5A. One additional faster migrating band was also observable in untransfected cells indicating that this signal is of endogenous origin and not due to the overexpressed TCFs (labeled as non TCF in Fig. 5B).

For all tested XTCF-4 variants, addition of an antibody against TCF-4 supershifted the faster migrating band, indicating

**Fig. 4. Determination of binding constants of TCF/LEF factors.** A, quantitative bandshift studies were done with the proteins as indicated on the siamois-derived oligonucleotide, and the resulting shift in mobility was quantified by a PhosphorImager analysis. The results are represented in two different ways. First, the percentage of bound oligonucleotide is given as a function of the concentration of protein added (left side). \(K_d\) is estimated by the protein concentration with 50% occupancy of binding sites. Second, results are shown as Scatchard plots (right side), confirming the \(K_d\) values as well as the theoretical concentration of binding sites (13 nM). The linear slope to determine \(K_d\) was calculated using Cricket Graph III software (Computer Associates International, Inc., Islandia, NY). The regression coefficient \(R\) indicates the performance of regression analyses. Values close to 1 indicate a good correlation; values close to 0 indicate that there is no correlation. The values were obtained from 3 to 5 experiments per protein concentrations. Error bars indicate S.E. B, quantitative bandshift studies were done as described in A except for using an oligonucleotide derived from the fibronectin promoter shown in Fig. 3B. Note the different binding behavior of XTCF-3 and XTCF-4C with respect to the siamois oligonucleotide. In these cases, \(K_d\) is estimated only by the first plotting procedure. C, truncation of the C-terminal domain of XTCF-3 shifts the binding behavior from XTCF-3 on the FN-WntRE toward the one observed in case of mLEF-1.
ing that this band represents an XTCF-4-DNA complex. However, the slower migrating upper band was not supershifted by addition of TCF-4 antibody indicating that most probably the binding epitope for the TCF-4 antibody may be blocked by an additional protein. On the other site, adding an antibody against β-catenin interfered with formation of the upper band and resulted in a strong increase in the TCF-4/DNA band (Fig. 5B) identifying β-catenin as the additional protein in the slower migrating band. Thus, the slower migrating band that we observed in XTCF-4B and XTCF-4C cells, but not in those transfected with XTCF-4A, indicates the presence of a XTCF-4-β-catenin-DNA trimer. This difference in complex formation with β-catenin is not due to an up-regulation of the overall amount of β-catenin as judged by Western blot studies of transfected cells (Fig. 5D). As the TCF-4 variants used differ in the LVPQ and SFLSS motifs, this indicates that the complex formation of the analyzed XTCF-4 variants with β-catenin is directly or indirectly dependent on the two described amino acid motifs. Another striking observation obtained by these experiments using cell lysates was that in vivo expressed XTCF-4A apparently binds with lower affinity to the FN-WnRE than XTCF-4B and -C, although all proteins were expressed at comparable levels (see Fig. 5, B and C).

**TCF/LEF-β-Catenin Complex Formation Is Dependent on the Phosphorylation of TCF**—By comparing the expression level of TCF-4 variants in cell lysates, we made the observation that XTCF-4B and XTCF-4C appeared in Western blots as a double band (Fig. 5C). This raises the question whether there are differentially phosphorylated forms of XTCF-4. Indeed, treating the lysates with calf intestine alkaline phosphatase, as well as acidic potato phosphatase, resulted in an increased appearance of a faster migrating band in all XTCF-4 variants (Fig. 6A). We conclude from these data that members of the TCF family are the subject of phosphorylation *in vitro*. A never observed an XTCF-4A-β-catenin-DNA complex in our bandshift experiments (Fig. 5B) or the faster migrating form of XTCF-4A in the absence of phosphatase activities (Fig. 6A), we reasoned that the complex formation of XTCF-4 with β-catenin might be dependent on the existence of a dephosphorylated form. By having established the identity of different retarded

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**Fig. 5.** TCF-β-catenin complex formation on the fibronectin oligonucleotide. 

**A**, epithelial A6 cells were transfected with expression constructs as indicated, and cell lysates prepared in the presence of phosphatase inhibitors were assayed for their ability to bind specifically the fibronectin-derived oligonucleotide. The unspecific competition was performed as described under “Experimental Procedures.” The data for XTCF-3C are from an independent experiment in comparison to the other shown results. **B**, bandshifts were done as in A in presence of phosphatase inhibitors, but antibodies against TCF-4 or β-catenin were added. Note that XTCF-4B and XTCF-4C display two distinct signals in the absence of antibodies. The faster migrating signal can be supershifted by adding TCF-4-specific antibodies. Formation of the slower migrating complex is prevented by adding β-catenin antibodies indicating the presence of β-catenin in the slower migrating complex. XTCF-4A transfectants never showed this slower migrating complex: C, immunoblot of A6 cells transfected as indicated and probed for expression of TCF-4. All constructs are expressed at similar levels. Note that XTCF-4B and XTCF-4C clearly show two distinct bands (arrowheads), whereas the second signal is barely visible for XTCF-4A. D, immunoblot for β-catenin protein (arrowhead) in transfected A6 cells showing no significant differences in expression level of β-catenin after transfection.
bands in our mobility shift studies, we therefore repeated these studies with XTCF-4A transfectants in the presence or absence of exogenously added phosphatase activity. These experiments revealed two important observations.

First, the overall binding affinity of XTCF-4A decreased after phosphatase treatment because we needed comparable longer exposure times to detect signals. This loss in binding is most probably not due to any protease contaminations since all experiments were done in the presence of protease inhibitors. Furthermore, we tested the added phosphatases negative for any protease contaminations by treating purified TCF/LEF fusion proteins under identical experimental conditions (inset, Fig. 6B). Therefore, this observation suggests that the binding behavior of TCF/LEF factors is dependent on pre-phosphoryla-

**Fig. 6. XTCF-4 variants are differentially phosphorylated in A6 cells.**

A, treatment of TCF-4 transfectants with exogenous phosphatase. In the absence of phosphatase activity, XTCF-4B and -C appear as a double band, whereas XTCF-4A appears as a single slower migrating band. The faster migrating signal is strongly enhanced by phosphatase treatment in all three samples. B, lanes 1 and 2, addition of exogenous phosphatase to lysates prepared from XTCF-4A-transfected A6 cells results in the appearance of the slower migrating band (arrowhead) identified in Fig. 5B to represent a TCF-DNA-β-catenin trimer. Lanes 3–5, formation of the slower migrating band can also be triggered by endogenous phosphatases. Addition of phosphatase inhibitors as described under “Experimental Procedure” prevents formation (lane 3), whereas this complex is formed in absence of phosphatase inhibitors (lane 5). In lane 4 only sodium orthovanadate and sodium fluoride were added. Inset in B, reduced binding affinity of XCF-4A is not due to reduced protein amounts as a result of protease contaminations. Treatment of bacterially expressed XLEF-1 or XTCF-4A with phosphatases (lanes 4) did not result in protein degradation in comparison to protein samples that were similar treated in the absence of phosphatases (lanes 3). Lanes 1 are buffer only samples and lanes 2 are buffer including phosphatases but in the absence of fusion proteins. Detection of proteins was either by Coomassie staining (XLEF-1) or immunoblot against the His tag (XTCF-4A). C, bacterially expressed fusion proteins of XTCF-3, XTCF-4C, and mLef-1 are phosphorylated in the presence of casein kinase II. D, phosphorylation of bacterially expressed TCF/LEF transcription factors by casein kinase II enhances the DNA binding affinity. Suboptimal amounts of proteins were used in quantitative bandshift studies, and the results are presented as bars. For each protein equal amounts of protein were used in the presence or absence of CKII. Three independent experiments were performed for each sample, and error bars indicate S.E.
tion. A similar effect has recently been described for *Drosophila* HMGI proteins, non-sequence-specific, DNA-binding proteins containing several HMG boxes (33). Pre-phosphorylation has been shown to be casein kinase II (CKII)-dependent for HMGI proteins. As all TCF/LEF transcription factors have multiple CKII target sites and CKII is a ubiquitously found enzyme, we tested whether these factors are phosphorylated by CKII and whether phosphorylation influences the binding behavior of TCF/LEF factors. In fact, all tested TCF/LEF factors were found to be phosphorylated by CKII (Fig. 6C), and this phosphorylation was accompanied by an increased binding affinity (Fig. 6D).

Second and more important, these experiments indicate that the formation of a TCF-4-β-catenin-DNA complex is dependent on the presence of a partially dephosphorylated form of the transcription factor (Fig. 6B). Treatment of cell lysates with phosphatase inhibitors did not only result in dephosphorylation of the proteins but also in formation of the slower migrating complex indicating entrance of β-catenin into the complex.

To compare the results shown in Fig. 6B and in Fig. 5B, and to test whether this dephosphorylation might also occur in vivo, we prepared lysates of TCF-4A transfectants in the presence or absence of added phosphatase inhibitors. As shown in Fig. 6B, addition of a phosphatase inhibitor mixture to the lysis buffer (as in Fig. 5B) prevented formation of TCF-4-β-catenin-DNA complexes, whereas a lack of phosphatase inhibitors resulted in formation of the slower migrating band. This experiment clearly indicates that endogenous phosphatases are able to dephosphorylate TCF/LEF factors and that most probably this dephosphorylation results in the recruitment of β-catenin into the TCF-DNA complex.

**DISCUSSION**

We report herein that different, highly similar XTCF-4 variants show divergent behavior in target gene activation assays. By using different biochemical approaches, we provide evidence that these differences are neither due to preferential interaction with corepressors of the groucho family or CtBP nor to differential binding of the transcriptional activator SMAD4. We demonstrate that the formation of a TCF-β-catenin-DNA complex and the activation of the target gene fibronectin depends on two short amino acid motifs. These motifs, LVPF and SFLSS, are involved in regulating posttranslational modifications of XTCF-4. Since they are not present within all variants of TCF/LEF factors (e.g., mLEF-1 versus XTCF-3), and since cofactors of the TCF/LEF complex are expressed in a tissue-specific manner, a complex pattern of regulation is emerging that has to be considered when studying Wnt signaling.

**Implications on TCF/LEF-mediated Complex Formation**—In our attempts to elucidate the molecular mechanism responsible for the different transactivation properties of TCF/LEF factors, we here present data that support multiple levels of regulation of TCF/LEF-mediated transcription factor complex formation.

First, there is no correlation between DNA binding affinities of TCF/LEF factors and target gene activation. The transcriptional activator XTCF-4 C has a significantly lower DNA binding affinity than mLEF-1 but activates the expression from the FN promoter stronger than mLEF-1.

Second, although the oligonucleotides used for quantitative band shift analyses both contain the canonical TCF/LEF target site, (C/G)T/TGG/(A/T) (AT), they show significant differences in complex formation with TCF/LEF. All studied transcription factors showed a higher affinity toward the sia-WntRE than toward the FN-WntRE. These differences in binding affinity and behavior of TCF/LEF transcription factors might indicate that the nucleotides flanking the conserved core region of the target site influence the binding affinity by providing additional contacts. These putative interactions do not only modify DNA binding strength but also the type of binding behavior. XTCF-3 displays hyperbolic binding behavior on the sia-WntRE but sigmoidal binding behavior on the FN-WntRE indicating additional protein/DNA interactions. In accordance with this hypothesis is the observation that deleting the C-terminal domain of XTCF-3 shifts the DNA binding curve of this mutant toward the mLEF-1-derived one. Further experiments have to confirm whether these additional, promoter-specific protein/DNA interactions mediated by the C-terminal part of XTCF-3 can be proven and, if so, whether this holds true for XTCF-4C as well. This is of general interest, since it has recently been shown that LEF-β-catenin-SMAD4 complexes are involved in regulating the twin promoter (11, 12), although the same factors are not involved in regulating the c-myc gene (11). Thus, with respect to activation of Wnt target genes for future promoter analysis, the fine structure of the analyzed promoter region has to be taken into account.

Third, we found, that at least in vitro, all TCF/LEF transcription factors are able to interact with ESG-1, a member of the groucho family. This is of general interest as it is in contrast to an earlier hypothesis suggesting that differences in TCF/LEF-mediated transactivation is a biased ability of these factors to interact with transcriptional corepressors. Although we acknowledge that there still might be differences in the ability to interact in vivo, our results clearly show that additional levels of complexity with respect to TCF/LEF-mediated complex formation have to be postulated. The only tested TCF/LEF-groucho protein interaction using *Xenopus* proteins described so far showed that XTCF-3 can interact with Xgrg-5. However, this result was only based on two-hybrid analyses (21). The same study further provides indirect evidence for an interaction of XTCF-3 with Xgrg-5 and Xgrg-4, since both are translocated to the nucleus in XTCF-3-transfected COS-1 cells. Further experiments are required to analyze the in vivo interactions of all known TCF/LEF and groucho proteins from one species (e.g. *Xenopus* or mouse) under different cellular conditions.

Fourth, another important and striking observation is the fact that complex formation of XTCF-4B with DNA and β-catenin is not sufficient for target gene activation. This is in agreement with a previous observation in a different cell culture system (34). In this case, β-catenin and LEF-1 can activate target gene expression in transformed Jurkat T-cells but not in normal T-lymphocytes despite the nuclear localization of both factors. Thus, additional tissue-specific components of TCF/LEF-mediated transcription factor complexes or additional posttranslational modifications have to be postulated. Of the known components of TCF/LEF-mediated transcription factor complexes, neither p300/CBP nor TBP are expressed in a tissue-specific manner. However, in *Xenopus* we recently described that Xpontin and Xreptin are expressed in a tissue-specific manner (18). Also some groucho members show a distinct expression pattern (35). Beside the tissue-specific expression of cofactors, tissue-specific posttranslational modifications have to be taken into account.

**Protein Phosphorylation as an Additional Level of Regulation in TCF/LEF-mediated Complex Formation**—The experiments described here clearly identify phosphorylation as an additional level of TCF/LEF regulation. First, we found a strict correlation between the presence of a faster migrating dephosphorylated band of XTCF-4B and XTCF-4C and the formation of a ternary complex of DNA, TCF-4, and β-catenin. A dephosphorylated band in Western blots was never observed for XTCF-4A which was paralleled by the absence of a DNA su-
pershift. However, treatment of cell lysates derived from XTCF-4A-transfected cells with different phosphatases resulted in the appearance of the dephosphorylated form and, in parallel, in the formation of a slower migrating band in electrophoretic mobility studies. In other words, dephosphorylation of XTCF-4 most probably at one or more specific sites allows complex formation with β-catenin, whereas phosphorylation of XTCF-4 prevents complex formation. Since complex formation between TCF/LEF transcription factors and β-catenin is a prerequisite for target gene activation, signal transduction pathways that lead to a phosphorylation of TCF/LEF factors thus might function as inhibitors of Wnt signaling. One candidate kinase that might be responsible for this effect is NLK (nemo-like kinase) as it phosphorylates TCF-4 and TCF-3 and inhibits TCF-β-catenin complex formation (36, 37). With respect to these previous publications our results offer an attractive hypothesis. As deletion of SFLSS results in the appearance of the dephosphorylated form of XTCF-4, this sequence motif might either represent the target site of NLK phosphorylation or might be involved in TCF-4/NLK interaction. The observation that the phosphorylated form of XTCF-4 is also present in those variants that lack the SFLSS motif excludes the first possibility and strengthens the latter one. We also note that this motif is serine-rich and thus might also be subject to phosphorylation events that regulate DNA/protein interactions. Further supporting the idea that posttranslational modifications regulate complex formation of TCF/LEF factors with β-catenin in vivo, we found no differences in interaction when using bacterially expressed fusion proteins in coimmunoprecipitation or pull-down studies (not shown). Although the absence of this SFLSS motif allows dephosphorylation and complex formation with β-catenin, it is not sufficient to turn XTCF-4B into an efficient β-catenin complex formation (36, 37). With respect to TCF/LEF-mediated complex formation, it is not sufficient to turn XTCF-4B into an efficient β-catenin complex formation (36, 37). With respect to TCF/LEF-mediated complex formation, it is not sufficient to turn XTCF-4B into an efficient β-catenin complex formation (36, 37).

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TCF/LEF Transcription Factors