A New β-Catenin-dependent Activation Domain in T Cell Factor*§

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Transcription of the lymphoid enhancer factor-1 (LEF1) gene is aberrantly activated in sporadic colon cancer, whereas this gene is not expressed in the normal adult colon. We have shown previously that promoter 1 of the LEF1 gene is activated by T cell factor (TCF)-β-catenin complexes in transient transfection assays, suggesting that LEF1 is a target of the Wnt pathway in colon cancer. To further explore the link between LEF1 expression and the Wnt pathway, we studied two response elements in the promoter. Surprisingly we found that the LEF1 promoter is selectively activated by specific isoforms of the LEF/TCF transcription factor family that contain an alternative C-terminal “E” tail. These isoforms, TCF-1E and TCF-4E, activate the promoter in a β-catenin-dependent manner. We show that a complete E-tail domain is necessary for full activity and delimits residues within two highly conserved peptide motifs within the tail that are required (KKCRARFG; WCXX-CRRKKKC). These peptide motifs are not only conserved among the TCF family members but are also found in two newly identified DNA-binding proteins named papillomavirus binding factor and GLUT4 enhancer factor. This study thus identifies a new and unique set of motifs used by the Wnt pathway for target gene regulation.

Wnt ligand-Frizzled receptor interactions at the plasma membrane trigger a cytoplasmic signal transduction cascade that propagates to the nucleus via the stabilization and release of cytoplasmic β-catenin, an armadillo repeat protein with nuclear import/export capabilities (reviewed most recently in Refs. 1 and 2). Nuclear-localized β-catenin forms a transcription regulatory complex with any member of the lymphoid enhancer factor/T cell factor family (LEF/TCF) (LEF-1, TCF-1, TCF-3, and TCF-4) (reviewed recently in Ref. 3). All four LEF/TCFs bind to Wnt target genes through their high mobility group DNA-binding domains and position a potent regulatory domain within β-catenin to activate transcription (4–6). When Wnt signals operate properly, the changes in gene expression directly affect specific cell fates and differentiate or to grow and divide (reviewed in Refs. 3, 7, and 8). Unregulated constitutive Wnt signaling, which is enabled by mutations in cytoplasmic Wnt signaling components, is thought to be an initiating event for many types of cancer, including colon cancer (5, 9, 10). The most common mutations in colon cancer, truncation mutations of adenomatous polyposis coli, disallow β-catenin degradation and increase the concentration of β-catenin protein in cells (9, 11, 12). High levels of β-catenin cause an increase in the formation of LEF/TCF-β-catenin complexes in the nucleus, which leads to inappropriate activation of Wnt target genes. This is proposed to be a key component of Wnt-linked carcinogenesis.

Some of the Wnt target genes encode components of the Wnt signal transduction pathway, and their increased expression may have inhibitory or facilitatory effects on Wnt signaling (13–19). For example, although it is not expressed in the normal adult colon, the LEF1 gene is aberrantly expressed in the majority of sporadic colon cancers, in which Wnt signaling is constitutive (20). Although the underlying mechanism of aberrant LEF1 expression is not fully understood, only the promoter for full-length forms of LEF-1 is activated. A second intronic promoter that produces dominant negative forms of LEF-1 remains silent. We have shown that the promoter for full-length LEF-1 is likely to be a Wnt target gene, because it is activated by TCF-1-β-catenin and TCF-4-β-catenin complexes and is sensitive to cellular levels of β-catenin. Inappropriate LEF1 expression may contribute to increased Wnt signal strength by increasing the concentration of LEF/TCF-β-catenin complexes in the nucleus, and such increases appear to be Wnt pathway-directed. We show in this study that the promoter for full-length LEF-1 is activated by β-catenin through two Wnt response elements, but surprisingly this occurs only when β-catenin is recruited by a specific TCF isoform that contains an alternative C-terminal tail referred to as the E-tail.

E-tail isoforms of TCF-1, TCF-3, and TCF-4 are generated from alternative splicing at the 3’-end of the pre-mRNA to encode a longer alternative C-terminal region (21–23). The LEF1 locus does not produce an E-tail isoform, because it is missing an E-tail-specific exon. Instead, the most abundant isoform of LEF-1 carries an “N”-tail. Neither the expression pattern nor the function of TCF E-tail isoforms is very well characterized except that they are detected as one of the most abundant isoforms in cell lines, and the TCF-4E and TCF-3 E-tails are able to bind CtBP, a transcription repressor. We show here for the first time that the E-tail of TCF-1 carries a novel transcription-activating function with respect to the...
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LEF1 promoter. This activity is not present in TCF-3E or LEF-1N but remains dependent on β-catenin recruitment.

EXPERIMENTAL PROCEDURES

Transient Transfection Assay—COS cells were transiently transfected with Effectene reagent according to the manufacturer’s protocol (Qiagen). COS-7 cells were plated at a density of 150,000 cells/well in six-well plates 20 h before transfection. Luciferase reporter constructs (LEF1 promoter, −672, +314, or thymidine kinase (TK) promoter, −64, +46) (0.5 µg) were co-transfected with β-catenin (0.4 µg), LEF/TCF (0.2 µg), and β-galactosidase (0.1 µg) expression constructs. The TOPFLASH luciferase reporter plasmid (0.4 µg) was co-transfected with 0.02 µg of LEF/TCF expression vector. Cells were harvested 18–20 h after transfection, and β-galactosidase activity was determined using the Galacton-Plus substrate (Applied Biosystems) to normalize luciferase activity for each point (each point was performed in duplicate). The fold induction was calculated relative to the luciferase reporter plasmid alone. Results were repeated from 4 to 11 times independently.

Western Blot Analysis—COS cells were transiently transfected with either 0.5 µg of the indicated TCF constructs using the Effectene reagent (Fig. 2) or 10 µg using a BTX 600 electroporator (Fig. 1, Ref. 20). 3% (Fig. 2C) or 20% (Fig. 1C) of whole cell lysates from transfected cells were separated by electrophoresis on SDS-10% polyacrylamide gels. Blots of these gels were probed with either TCF-1 monoclonal antibody (anti-TCF-1, Upstate Biotechnology) or LEF-1N polyclonal rabbit antisera at a 1:1000 dilution (20).

Immunofluorescence—Immunofluorescence detection of TCF-1E proteins in transiently transfected COS cells was performed as described previously with the following modifications (24). Cells were transfected by electroporation and plated on coverslips. After 24 h, cells were fixed with 3.7% formaldehyde, washed three times in 1× phosphate-buffered saline, and permeabilized with 0.5% Nonidet P-40. Cells were blocked with horse serum (1:100) and hybridized with rabbit polyclonal LEF-1 antiserum (1:1000), which detects all LEF/TCFs, for 1 h at room temperature. Cells were washed and hybridized to goat anti-rabbit-conjugated fluorescein isothiocyanate serum (1:1000, Amersham Biosciences) for 1 h at room temperature. Cells were dipped in 4',6-diamidino-2-phenylindole solution for 10 s, washed, and mounted on slides for viewing.

DNase I Footprinting—Partially purified recombinant LEF-1N and TCF-1E were used in standard DNase I footprinting assays as described previously (20). Equivalent amounts of protein were judged by SDS-PAGE and Coomassie staining.

Plasmid Construction—The LEF1 promoter luciferase reporter plasmid has been described previously (20). The EYR eukaryotic expression plasmid was used to express LEF and TCF coding sequences (34). An overlap extension PCR method was used to generate nucleotide substitutions in the two LEF/TCF response elements (+283) (35, 36). Complete details of plasmid construction and mutational analysis are described in the Supplemental Material.

RESULTS

Isoform-specific Activation of the LEF1 Promoter—In a previous study we showed that a fragment of the LEF1 promoter was activated 7-fold by TCF-1-β-catenin and TCF-4-β-catenin complexes in a transient transfection assay (20). Activation was dependent on β-catenin, because no activation was seen when a mutant β-catenin that could not bind to LEF/TCF was co-expressed. Two putative response elements were localized by DNaseI footprinting with recombinant LEF-1 protein at +190 and +283, and deletion of this downstream region abrogated activation by TCF-β-catenin. Furthermore the depletion of β-catenin protein in a colon cancer cell line by the reintroduction of wild type adenomatous polyposis coli lowered both the activity of the LEF1 promoter in a transient co-transfection assay and also the levels of endogenous LEF-1 protein (20). These data suggested that LEF1 is a target of the Wnt pathway and that the response elements for LEF/TCF-β-catenin complexes are located in a position ~200 nucleotides downstream of the start site of transcription. These data also suggested that LEF1 is subject to autoregulation by LEF-1-β-catenin complexes, because all LEF/TCF family members recognize the same DNA consensus sequence. To test for this possibility, we co-transfected LEF-1 and β-catenin expression vectors with the LEF1 promoter reporter plasmid (Fig. 1A). Surprisingly, we were unable to observe activation of the promoter (1.5-fold). In contrast, co-expression of full-length TCF-1 with β-catenin enabled gene activation similar to that we had observed previously with TCF-1 and TCF-4 (20-fold; Fig. 1A) (20). TCF-3 was also unable to activate the LEF1 promoter.

That LEF/TCFs should differ so dramatically in their ability to activate transcription is surprising, because although they are encoded by separate genes, they are highly homologous. They share a highly similar DNA-binding and β-catenin-binding domain, and thus they both bind β-catenin and recognize the identical DNA consensus sequence. Nevertheless, regions of LEF/TCFs exist that are not highly conserved among the four family members. One of the most notable differences occurs between the alternative C-terminal tails for each protein. Previously we reported that both TCF-1 and TCF-4 were able to activate the LEF1 promoter (20). The forms of the two proteins expressed in those assays contained full-length E-tails (TCF-1E and TCF-4E). In this study, full-length TCF-1E was...
used because it is the most active of the two (13). The LEF1 gene does not encode an E-tail isoform because it does not have the E-tail-specific exon (22). Instead the most common isoform of LEF-1 that is expressed contains an N-tail, and this isoform was used in our assays. TCF-3E was not very active either, because only a low level of expression of the ΔC376 and ΔC388 truncations made it difficult to conclude whether the CR1 and CR2 peptide motifs are important for E-tail activity. Therefore, to more directly test whether the CR motifs are necessary for activation, a more targeted set of mutations was designed. These mutations consisted of 5-amino acid substitution of CR1 (CR1mt) or CR2 (CR2mt) within the context of the full-length TCF-1E protein (Fig. 2, A and B). To minimize general disruption of protein folding, the pentapeptide VALAL was used in the substitution. This sequence is similar to VAHAL, which adopts β-sheet or α-helical structures depending on the surrounding context of the sequence (25). These full-length mutant proteins were compared with wild type full-length TCF-1E in the transient transfection assay with the LEF1 promoter reporter plasmid. Both TCF-1E and LEF-1E were able to activate expression ~8-fold, whereas wild type LEF-1N was again inactive in the assay (Fig. 1C). Western blot analysis of the expression of these three proteins showed that protein levels are equivalent. Thus the dramatic differences in activity are not caused by a change in expression level or stability; rather the results show that sequences in the E-tail and not other unique sequences within TCF-1 are sufficient to convert LEF-1 into an activator of its own promoter.

**Mutational Analysis of the E-tail Activation Domain**—To identify the sequences within the long E-tail that are necessary for LEF1 promoter activation, a set of C-terminal TCF-1E truncations were created (Fig. 2A). Four C-terminal truncations were designed with end points at amino acid 376 (ΔC376), amino acid 388 (ΔC388), amino acid 440 (ΔC440), and amino acid 457 (ΔC457). At the beginning of the E-tail are three tandemly conserved regions (CRs) found in TCF orthologs and homologs (CR1, CR2, and CR3, Fig. 2B) (22). The first two of these peptide motifs are enriched in cysteines, basic residues, and aromatic amino acids. These motifs also share similarities with nuclear localization signals. The end points of the largest C-terminal truncations were designed to end near these conserved motifs, with the largest deletion, ΔC376, eliminating CR2 but not CR1. Each of these truncation mutants was tested in the transient transfection assay with the LEF1 promoter reporter plasmid. Fig. 3A shows that only full-length TCF-1E was capable of robust LEF1 promoter activation (14-fold); all C-terminal truncations were compromised in their ability to activate the reporter (4-fold). Expression analysis of the mutant TCF-1E proteins showed that although most were expressed to levels similar to wild type TCF-1E, ΔC376 and ΔC388 were either not expressed very well or were not stable. An intact E-tail may be important for overall structure and activity.

The low level of expression of the ΔC376 and ΔC388 truncations made it difficult to conclude whether the CR1 and CR2 peptide motifs are important for E-tail activity. Therefore, to more directly test whether the CR motifs are necessary for activation, a more targeted set of mutations was designed. These mutations consisted of a 5-amino acid substitution of CR1 (CR1mt) or CR2 (CR2mt) within the context of the full-length TCF-1E protein (Fig. 2, A and B). To minimize general disruption of protein folding, the pentapeptide VALAL was used in the substitution. This sequence is similar to VAHAL, which adopts β-sheet or α-helical structures depending on the surrounding context of the sequence (25). These full-length mutant proteins were compared with wild type full-length TCF-1E in the transient transfection assay, and their subcellular localization was examined by immunofluorescence (Fig. 3, A and B). Western analysis showed that CR1mt and CR2mt were expressed as well as wild type TCF-1E protein (inset, Fig. 3A). Amino acid substitutions in either of the conserved CR motifs abrogated TCF-1E activity; CR1mt expression exhibited a 1.8-fold activation of the reporter plasmid, and CR2mt exhibited a 2.5-fold activation. Immunofluorescence detection of CR1mt and CR2mt protein showed that both mutants were localized to the nucleus as is true of the wild type TCF-1E protein (Fig. 3B). Therefore, mislocalization of the protein cannot account for the dramatic loss of activity. The ability of CR1mt and CR2mt to activate the TOPFLASH reporter plasmid was also assessed (Fig. 3C). Each mutant was able to elicit

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### Table: Isoform-specific Activation of Transcription

| Isoform  | E-tail   | promoter activation (fold) |
|----------|----------|-----------------------------|
| TCF-1E   |          | 14                         |
| ΔC457    |          | 1.8                        |
| ΔC440    |          | 2.5                        |
| ΔC388    |          | 4                          |
| CR1mt    |          | 1.8                        |
| CR2mt    |          | 2.5                        |

**Fig. 2. Conserved sequences in the E-tail of TCF-1E.** A. TCF-1E is a 524-amino acid protein; the last amino acid of each C-terminal truncation is shown. CR1mt and CR2mt are full-length TCF-1E proteins with a 5-amino acid substitution mutation in a CR motif (indicated by an X); the sequence of the CR mutations is shown in B. B. E-tail homologous sequences from the human LEF/TCF family are aligned with the TCF orthologs. Amino acid sequences for TCF orthologs were derived from the following GenBank entries: Drosophila melanogaster (dTCF, accession numbers AAC47464 and CAA70343); and C. elegans (Pop-1, accession number AAC05308); and Prf-1/PBF (accession number AAF73483), and G4EF (accession number AAF975160). A consensus CR1 and CR2 is shown above the alignment. The sequence of the CR1 and CR2 mutations are shown (CR1mt, CR2mt). The asterisk after the PBF and G4EF proteins indicates the end of the protein.
between 20- and 30-fold activation of the luciferase reporter plasmid. This is in dramatic contrast to the pattern observed with the \( \text{LEF1} \) promoter reporter in which CR1mt and CR2mt are inactive (Fig. 3A). The deletions ΔC440 and ΔC457 were also tested for their ability to activate TOPFLASH. Each deletion mutant was able to activate TOPFLASH in a \( \beta \)-catenin-dependent manner, however, only to half the level of those observed with wild type TCF-1E (data not shown). We conclude that transcription activation of the \( \text{LEF1} \) promoter requires an intact E-tail, whereas a standard Wnt-responsive reporter plasmid such as TOPFLASH is not heavily dependent on those sequences. Indeed, LEF/TCF family members that do not contain an E-tail such as LEF-1N and TCF-1B can activate TOPFLASH as well as TCF-1E (Fig. 1B). We conclude that the CR1 and CR2 motifs are not involved in nuclear localization or basic activities associated with \( \beta \)-catenin recruitment and transcription activation. Rather these unique C-terminal peptide motifs as well as an intact E-tail appear to be required for activation of certain promoters or Wnt response elements.

**Fig. 3.** Sequences in the E-tail are necessary for \( \text{LEF1} \) promoter activation. A, COS cells were transiently transfected as described in Fig. 1A. Whole cell extracts from transfected COS cells were analyzed by Western blotting with monoclonal TCF-1 antibody (Upstate Biotech, see inset). B, immunofluorescence detection of full-length wild type (WT) TCF-1E and CR1mt and CR2mt proteins (transiently transfected as in A) shows that all are localized to the nucleus of COS cells. C, COS cells transfected with the TOPFLASH reporter and the indicated expression plasmids show that both E-tail mutants are able to work with \( \beta \)-catenin to activate the reporter.

**Fig. 4.** Comparative DNase I footprint analysis of LEF-1N and TCF-1E. Partially purified recombinant LEF-1N extracts (0.6, 3.0, and 6.0 μg of protein) and TCF-1E (0.1, 0.3, 0.6, 1.5, 3.0, and 4.5 μg of protein) were used in DNaseI footprint analysis with a \( ^{32} \text{P} \)-labeled \( \text{LEF1} \) promoter fragment (−64, +317). Maxim-Gilbert reactions were used to map the protected regions indicated by the solid bars (not shown). Arrows denote differences in DNase I hypersensitivities induced by the two proteins. The footprint that protects +190 is not visible in this figure, but the protection by LEF-1N and TCF-1E is the same. The relative position, orientation, and core sequences of the +190 and +283 response elements are shown in the bottom section of the figure.

**TCF-1E and LEF-1N Bind to the Same Wnt Response Elements in the \( \text{LEF1} \) Promoter**—The CR peptide motifs are highly conserved among the TCF homologs, are located near the high mobility group DNA-binding domain, and are highly enriched in basic residues. This result suggests that these CR motifs might function as independent or auxiliary DNA-binding domains and thus modify the ability of TCF-1E to recognize alternative DNA sequences. Alternative sequence recognition could explain why TCF-1E and TCF-4E and not LEF-1N, TCF-1B, or TCF-3E are able to activate the promoter. To test for alternative DNA sequence recognition, we produced recombinant LEF-1N and TCF-1E protein in bacteria. Partially purified preparations of both proteins were used in a DNase I footprint assay with a fragment from the \( \text{LEF1} \) promoter (−64, −317) spanning the region that contains both previously identified LEF-1N binding sites (Fig. 4). The results show that both LEF-1N and TCF-1E proteins generate footprints over the same sequence in the downstream region including the +190 site (not shown). No additional footprints exist over any other portion of the fragment. The borders of the LEF-1N and TCF-1E footprints are the same, showing that the E-tail region does not make extended contacts with the DNA such that an additional region is protected from DNaseI digestion. Slight differences exist in the DNaseI hypersensitivities surrounding the LEF-1N and TCF-1E footprints (Fig. 4, arrows). Because LEF/TCF proteins induce a strong bend in the DNA on binding, such small differences in the DNaseI digestion pattern may indicate differences in bending. We conclude that LEF-1N and
TCF-1E recognize the same DNA response elements, but minor differences in the DNaseI footprint pattern suggest that the conformation of each protein bound to these sequences may differ.

Transfer of the E-tail-sensitive Wnt Response Elements to a Heterologous Promoter—To test whether this downstream region of the LEF1 promoter contains the TCF-1E-specific response element(s), we transferred a fragment containing the footprints (nucleotides +104 to +308) to a position (−46) relative to the start site of transcription of the minimal Herpesvirus TK promoter in a luciferase reporter plasmid (TK2FP/WT, Fig. 5, Ref. 26). This promoter differs significantly from the LEF1 promoter in that it has a well defined TATA box, a single start site of transcription, and is not GC-rich. In a parallel construct, we introduced nucleotide substitution mutations in each of the two TCF-1E footprints to destroy binding (Fig. 5, TK2FP/MT).

DNaseI footprint analysis with LEF-1N and TCF-1E recombinant protein was used to confirm that the LEF/TCF binding sites were completely destroyed. The results from the transient transfection analysis are shown in Fig. 5A. Both TCF-1E-β-catenin and LEF-1E-β-catenin complexes were able to activate transcription from the TK2FP/WT reporter, whereas LEF-1N was inactive in the assay. Mutation of the two LEF/TCF footprints in TK2FP/MT abrogated activation by TCF-1E-β-catenin, and control transfections with the parent TK promoter reporter plasmid showed that none of these factors activates the minimal promoter alone (data not shown). Thus, E-tail-dependent activation of the LEF1 promoter occurs via TCF-1E binding to the two previously identified Wnt response elements. We also tested the E-tail deletion and substitution mutations for their effect on the TK2FP/WT reporter to determine whether the same residues of the E-tail were involved in activation of the TATA-containing promoter. The results are identical to that of the LEF1 promoter (Fig. 5B). C-terminal deletions of the E-tail compromise activity, and mutation of either CR motif abrogates activity. Thus, the same residues are involved in activation of the LEF1 and TK promoters through the Wnt response elements identified with DNase I footprinting. Activation is the same whether from a downstream or an upstream location.

DISCUSSION

We have described a new activity of the E-tail isoform of TCF-1 that enables β-catenin to activate transcription of the LEF1 promoter in transient transfection assays. The E-tail isoform of TCF-4 is also able to activate the LEF1 promoter (shown previously in Ref. 20). Other isoforms of TCF-1 as well as LEF-1N and the disparate E-tail isoform of TCF-3 do not function with β-catenin at this promoter. This is true despite the fact that all LEF/TCF isoforms are equally active with the standard TOPFLASCH reporter plasmid. These data suggest that expression of the LEF1 locus may be particularly sensitive to regulation by β-catenin complexes containing TCF-E-tail isoforms. Such forms are detected in colon cancer cell lines that aberrantly express LEF1 (23, 27).

Several possible mechanisms may enable activation of the LEF1 promoter by the E-tail. Such possibilities include the location of response elements relative to the start site of transcription, regulation of different types of basal promoters, differences in DNA sequence recognition, or interactions with other regulatory proteins. The first two possible mechanisms, the downstream location of the Wnt response elements or the type of basal promoter, are unlikely to affect activation because movement of the response elements to a position 5' of the start site of transcription of the heterologous TATA-containing Herpesvirus TK promoter does not change the patterns of activity that we observed (Fig. 5). We conclude that the E-tail activity is not likely to subserve promoter-specific or location-specific regulation. The third mechanism, conformation of DNA sequence recognition or affinity, remains a possibility because the DNase I footprinting experiments with recombinant LEF-1N and TCF-1E protein show minor differences in DNaseI hypersensitivities (Fig. 4). Difficulties with the purification of full-length TCF-1E have prevented us from directly comparing DNA-binding affinities with LEF-1N protein. However, to address whether different DNA binding affinities could account for the disparate activities of TCF-1E and LEF-1N, we created a mutant LEF1 promoter reporter plasmid in which the sequences of the two Wnt response elements at +190 and +283 were changed to the high affinity LEF/TCF response sequences present in the TOPFLASH reporter plasmid. Again only

3. T. Li and F. Atcha, data not shown.
TCF-1E was able to work with β-catenin to activate this reporter. Thus if the E-tail modifies DNA sequence affinity, such differences may only be revealed with reporters containing one or two separated response elements and not the closely apposed multiple elements that are found in the TOPFLASH reporter. Current efforts are under way to more carefully analyze DNA binding affinity, DNA bending, and protein-DNA conformations of the LEF1 promoter response sites by LEF-1N and TCF-1E. A fourth possible mechanism involves the engagement of E-tail motifs in protein interactions as opposed to direct DNA binding. The absolute conservation of the cysteine residues in each motif among TCF orthologs suggests a cysteine-based tertiary structure but not one that matches any known metal-chelating domain (Fig. 2B). Co-activators of transcription are obvious candidates if protein interaction is involved, but one direct possibility could be that the E-tail enables a better and more stable interaction with β-catenin. This possibility is unlikely, given that CR1mt and CR2mt are as active as wild type TCF-1E for β-catenin-dependent activation of TOPFLASH and that LEF/TCF without an E-tail efficiently co-immunoprecipitate with β-catenin in COS-7 cells (28). However, multimerized response elements may mask differences between family members in their ability to recruit β-catenin to target promoters, and only careful assessment of β-catenin-binding affinities to DNA-bound LEF/TCF’s can resolve this issue. If co-regulators are recruited through interactions with the E-tail domain, then such regulators are most likely to be ubiquitously expressed, because we have observed E-tail-specific activation of the LEF1 promoter in different cell types including mature and immature human T lymphocyte lines (Jurkat and 2017) and COS-1 cells, a monkey kidney cell line.5 If the E-tail modifies target gene recognition through nucleic acid interactions or protein interactions, it does so via a novel independent mechanism with the CR1 and CR2 motifs playing an important role. The TCF-1 E-tail is a 137-amino acid domain, and for most of this domain, there is little sequence conservation with other TCF E-tails. The exception is the high level of sequence conservation in the most N-terminal part centered over the CR motifs (Fig. 2B). The CR1 and CR2 peptide motifs of 10 and 15 amino acids, respectively, show the highest conservation between TCF-1 and TCF-4, with TCF-3 having the greatest sequence variance and missing the 9 amino acid CR1 motif completely (Fig. 2B). Drosophila TCF and the Caenorhabditis elegans ortholog, Pop-1, have nearly identical CR1 and CR2 motifs. We have introduced amino acid substitution mutations in CR1 and CR2, and E-tail activity is abolished in our assays (Fig. 2B). Interestingly, van de Wetering et al. (4) identified a mutation in the Drosophila TCF/pangolin locus that causes lethality. This mutant allele, dTCF1 locus that causes lethality. This mutant allele, dTCF1, carries a single alanine-to-valine substitution in the CR1 motif, under scoring the functional importance of this region. Although a detailed understanding of the function of the CR region (also referred to as the CRAR region (3)) is lacking, its activities may be likely to be independent and not intrinsically associated with LEF/TCF proteins only. A search of GenBank™ proteins containing similar CR1 and CR2 motifs identified two additional DNA-binding proteins (Fig. 2B). The first, a 513-amino acid protein called papillomavirus regulatory factor (Prf-1) and papillomavirus binding factor (PBF) was identified in a yeast one-hybrid assay for cellular proteins binding to the E2 binding site, P2, of human papillomavirus type 8 (29). The second factor, GLUT4-enhancer factor (G4EF), is highly similar to Prf-1/PBF and was identified as a 387-amino acid DNA-binding protein that recognizes the enhancer for the human GLUT4 transporter gene (30). On the basis of a CX2C-H-X2C-H sequence in the middle portion of each protein, both Prf-1/PBF and G4EF are proposed to contain a TFIIA-like zinc finger DNA-binding domain. Indeed Prf-1/PBF has been shown to bind specifically to DNA sequences with CCGG at the core (29). Unlike the closely juxtaposed high mobility group DNA-binding domain and CR1/2 motifs of the TCFs, the homologous CR1/2 motifs in Prf-1/PBF lie at the extreme C-terminal end of the protein, 139 residues away from the putative zinc finger DNA-binding domain. Thus, if CR1/2 motifs influence DNA binding for all these different proteins, they must do so as independent and auxiliary motifs able to cope with variable placement relative to different types of DNA-binding domains.

The results that we report in this study support the intriguing possibility that not all LEF/TCFs are functionally equivalent and that different isoforms may preferentially regulate different subsets of Wnt target genes. Such a possibility is consistent with genetic data from knock-out and transgenic mice experiments in which the removal of the LEF1 gene or TCF1 gene generated partially non-redundant phenotypes in tissues where their expression patterns overlapped or where overexpression of dominant negative forms of LEF/TCFs produced non-identical phenotypes (31–33). Whether a portion of these non-redundant activities is a result of the expression of E-tail isoforms and whether such specialized functions play a role in tumor development are unknowns that are important to address if we are to understand the role that Wnt signals play in promoting cancer.

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Addendum—While this manuscript was under review, similar observations for TCF-4E regulation of the Cdx1 promoter and the importance of CR1 and CR2 motifs (referred to as CRARP) were published by Drs. Hecht and Stemmler (37).

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