Multiple Domains Define the Expression and Regulatory Properties of Foxp1 Forkhead Transcriptional Repressors

Bin Wang‡, Danjuan Lin‡, Chuan Li§, and Philip Tucker‡¶

From the ‡Department of Molecular Genetics and the Institute for Cellular and Molecular Biology, University of Texas, Austin, Texas 78712-0162 and §Expression Technologies, Inc., San Diego, California 92121

The Foxp subfamily of forkhead/HNF3 transcription factors has recently been recognized because of its involvement in autoimmune disease, speech and language disorders, and lung development. Domains unique to this subfamily include a divergent DNA-binding winged helix, a leucine zipper, a zinc finger, and a polyglutamine tract. Little is known about the properties of these proteins that are fundamental to their function as transcription factors nor how the Foxp sequence motifs regulate their transcriptional regulatory properties. We report here a structure/function analysis of the Foxp1 protein. We have analyzed the alternative splice isoforms 1A and 1C and also report the cloning and characterization of a novel isoform Foxp1D that lacks the polyglutamine domain. We have isolated the preferred DNA-binding sites for Foxp1 transcription factors, Foxp1A, C, and D isoforms and the related Foxp2 protein repress gene transcription via binding to this consensus site or to a naturally occurring site within the SV40 and the interleukin-2 promoters. In some cases the strength of Foxp1 repression is mediated by the polyglutamine domain. Unlike previously characterized forkhead factors, Foxp1 proteins can form homodimers or heterodimers with subfamily members. The dimerization domain was localized to an evolutionarily conserved C2H2 zinc finger and leucine zipper motif. Finally, we demonstrate that Foxp1, although broadly expressed, is further regulated by tissue-specific alternative splicing of these functionally important sequence domains. These results suggest that Foxp1 proteins have diverse functional roles in different cell and tissue types.

The Fox gene family encompasses a large number of transcription factors homologous to forkhead and HNF3. Fox transcription factors are identified by a conserved 110-amino acid DNA-binding (winged helix) domain (1, 2). These proteins play important roles in regulating gene transcription during early development and organogenesis and control cell type- and tissue-specific gene expression (1, 2). Among the Fox transcription factors, recent attention has been drawn to members of the Foxp subfamily (Foxp1, Foxp2, and Foxp3). The functional importance of the Foxp proteins is underscored by their spontaneous mutation in mouse and human diseases. Misregulation of Foxp1 expression is seen in a variety of tumors, leading to the speculation that FOXP1 is a tumor suppressor gene (3). Mutations in FOXP2 provided the first incidence for linkage of a single mutant allele to speech and language disorders (4). FOXP3 (JM2) mutations cause X-linked autoimmunity-allergic disregulation syndrome in humans (5), and the mouse ortholog of Foxp3 is responsible for the Scrufty mouse phenotype (6, 7).

Despite the interesting physiological roles that have been uncovered by mutations in Foxp genes, little is understood about how Foxp transcription factors participate in signaling and gene regulation. Several candidate target genes with promoters containing forkhead binding sites have been proposed (8, 9). Identification of a Foxp subfamily-specific DNA binding preference would be useful in validating current candidates and help identify new targets. Clues to the mechanisms by which Foxp proteins regulate transcription may lay in several interesting protein domains unique to the Foxp subfamily. Foxp transcription factors are among the most distantly related Fox subfamily members. Homology is limited to an 80-amino acid region within the DNA-binding domain that is truncated at the carboxyl helices Wing 1 and Wing 2 (5, 6, 8, 9). Such divergence from the HNF3 winged helix structure would predict significant differences in the preferred DNA-binding sequences for Foxp proteins. The HNF3 structure further indicated that Fox proteins bind DNA as monomers (10–12). However, the observation that all characterized Foxp proteins contain a leucine zipper motif that resembles the N-myc zipper dimerization domain questioned the generality of this conclusion. Mouse Foxp3 and Foxp2 and human FOXP1 also include an N-terminal polyglutamine stretch of 37–40 residues (9). Among transcription factor domains, polyglutamine has been identified as a common protein-protein interaction motif that can regulate transcriptional activity (13). The role of the polyglutamine domains in Foxp proteins has not been tested.

In this study, we provide evidence that various Foxp1 isoforms repress transcription via direct binding to Foxp consensus DNA-binding sites present in the promoter regions of SV40 and IL-2.1 We show that the N-terminal polyglutamine domain strongly modulates Foxp1 repression activity on SV40 promoter. We demonstrate that a domain including leucine zipper and zinc finger motifs is both necessary and sufficient for specific dimerization between Foxp proteins. Finally, we show that Foxp isoform expression is highly regulated in tissues and cells.

¶To whom correspondence should be addressed: Dept. of Molecular Genetics and the Institute for Cellular and Molecular Biology, University of Texas at Austin, 1 University Station A5000, Austin, TX 78712-0162. Tel.: 512-475-7705; Fax: 512-475-7707; E-mail: philtucker@mail.utexas.edu.

1The abbreviations used are: IL, interleukin; HA, hemagglutinin; GST, glutathione S-transferase; mAb, monoclonal antibody; CASTing, cyclic amplification and selection of targets; EMSA, electrophoretic mobility gel shift assay.

Received for publication, July 17, 2002, and in revised form, March 18, 2003
Published, JBC Papers in Press, April 10, 2003, DOI 10.1074/jbc.M207174200
MATERIALS AND METHODS

Plasmid Constructions

Expression Vectors—Foxp1A was cloned into pCDNA/Neo (Invitrogen) and is driven by cytomegalovirus immediate-early promoter. In our original DNA cloning effort, we isolated a partial Foxp1A cDNA lacking exon 1. This cDNA encodes the same protein as Foxp1D and was cloned into pCDNA/Neo. 5′-HA-Foxp1A and 3′-HA-Foxp1A were generated by introducing an HA epitope tag in frame of Foxp1A using PCR. Foxp1A mutants were generated using single-stranded site-directed mutagenesis (14). Expression vectors encoding full-length Foxp1C and Foxp2 were described previously (9) and kindly provided by Dr. Ed Morrissey (University of Pennsylvania School of Medicine).

Yeast Two-hybrid Constructs—pAS1CYH2-Foxp1FL and pACTII-Foxp1FL′ were generated by subcloning a Foxp1A (V543L,R544E) BstYI fragment encoding amino acids 146–564 into the BamHI sites of pAS1CYH2 and pACTII in translational frame, respectively. Yeast two-hybrid vectors were kindly provided by Clarence Chan (University of Texas at Austin). pAS1CYH2-ab, a and b constructs were made by PCR amplifying the Foxp1A cDNA.

Transcription Reporter Constructs—1xb-pGL3 promoter was generated by subcloning a single Foxp1A DNA-binding site B upstream of the SV40 promoter into the BglII site of pGL3 promoter (Promega, Madison, WI). 3xbp-GL3 control was made by inserting tandemly ligated BstHI sites of pGL3 promoter and pACTII into the transcriptional frame, respectively. Yeast two-hybrid vectors were kindly provided by Clare Chan (University of Texas at Austin). pAS1CYH2-ab, a and b constructs were made by PCR amplifying the Foxp1A cDNA.

Isolation of Foxp1A cDNA Clones

A single Foxp1A cDNA clone was isolated by using a (CAG) 12 repetitive oligonucleotide probe to screen a cDNA library prepared in a Zap from poly(A)+ mRNA isolated from B-cell leukemia (BCL-1) cells. The BCL-1 cells were stimulated in culture by IL-5 and IL-2 to differentiate before harvesting of RNA. Its complete sequence was determined on both strands by the Sequenex method (8). The Foxp1A cDNA sequence was confirmed from independent cDNAs using fragments generated from the initial isolate (fragment 1, nucleotides 1–357; fragment 2, nucleotides 1516–1978) as hybridization probes. Seven randomly picked clones were plaque-purified, and the cDNA inserts were excised as plasmids (λ ZAP protocol, Stratagene).

RNA Analysis

RIP analysis assays were performed using the RPA kit according to manufacturer's protocols (Ambion, Austin, TX). 20 μg of mouse total RNAs were used to assay for Foxp1a isoforms using either “s” probe (nucleotides 923–955) or “p” probe (nucleotides 1978–2122) 2 μg of mouse total RNAs were used for mouse-MPDH control. Either a DNA sequence ladder or radiolabeled PUC19 digested with SalI was used as size marker for protected RNA bands. Mouse tissue Northern blot was hybridized with the 3′ probe. The hybridization and washes were performed under high stringency (14) and exposed for 3 days on a Molecular Dynamics PhosphorImager.

Western Blot Analysis and in Vitro Co-immunoprecipitation

Monoclonal antibody 1G1 was generated against the forkhead DNA-binding domain of Foxp1. In vitro transcription and translation were performed using the TNT-coupled reticulocyte lysate system (Promega). Monoclonal anti-HA asces 16B12 (BABC, Berkeley, CA) was used in 1:1000 dilution, and 1G1 asces were used in 1:5000 dilution in 5% bovine serum albumin, phosphate buffered saline with 0.1% Triton X-100. Horseradish peroxidase-conjugated goat anti-mouse IgG (Amer sham Biosciences) was used as secondary antibody at 1:1,500 dilution in PBS containing 5% bovine serum albumin, phosphate buffered saline with 0.1% Triton X-100. The blots were visualized with the ECL kit (Amer sham Biosciences). In cell line Western blots, 50 μg of protein from various cell lysates were loaded. For immunoprecipitation, one-half of 35S labeled in vitro translation lysates were incubated with 175 μl of RIPA buffer (25 mM Tris, pH 7.8, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 100 μM phenylmethylsulfonyl fluoride) containing 0.1 μl of 16B12 at 4 °C for 1 h. The antibody and lysate mix was incubated with a 50% protein A (Sigma) RIPA slurry for 1 h at 4 °C before being washed in 1 ml of RIPA three times. One-half of the final products were loaded onto SDS-PAGE.

Yeast Two-hybrid Assay

We found that expression of Foxp1FL′ fused to the GAL4 activation domain (pACTII-FL') inhibited cell growth, perhaps because of disregulation of yeast gene. For the purpose of yeast expression, we circumvented this problem by introducing into the forkhead DNA-binding domain two point mutations (V543L,R544E) that had been shown to eliminate DNA binding in other forkhead transcription factors (16, 17). Yeast transformations were performed as described (18). Foxp1a pAS1CYH2-subclones were co-transformed with either pACTII-Foxp1FL or pACTII-Foxp1 and pACTII-T antigen from Clontech) into strain Y190. As a positive control, pV3A-1 (pA5ICYH2-P53) was co-transformed with pTD1-1. Protein-protein interactions were assayed by growth on selective media lacking histidine and confirmed by β-galactosidase activity assays using the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) filter assay (19).

GST Pull-down Assay

Constructs for in vitro transcription/translation of Foxp1C and Foxp3 (JM2) were obtained from Dr. Edward Morrissey (University of Pennsylvania) and Dr. Talal A. Chatila (Washington University School of Medicine, St. Louis, MO), respectively. Luciferase control or Foxp proteins were [35S]Met-labeled by in vitro translation, and equal aliquots of each protein were incubated with 1 μg of GST-Foxp1 (amino acids 378–578) fusion protein in NETN buffer (20 mM Tris·HCl, pH 8.0, 50 mM NaCl, 0.5% Nonidet P-40, 0.5 mM EDTA, pH 8.0) at a final volume of 250 μl at 4 °C for 45 min. The beads were washed three times in NETN buffer and then boiled in 10 μl of gel loading buffer for 10 min prior to loading on SDS-PAGE.

Isolation of Foxp1-binding Sites

Foxp1 CAST experiments were carried out as described (20) with minor modifications. A random oligonucleotide pool (76 nucleotides) containing unique flanking sequences was rendered double-stranded by second strand synthesis. 2 μl of C-terminally HA-tagged Foxp1a in vitro translation product and 0.5 μl of anti-HA antibody 16B12 were used for binding and immunoprecipitation, respectively. PCR was carried out after phenol chloroform extraction and ethanol precipitation of the eluted oligonucleotides. One-fourth of the precipitated was used for subsequent PCR amplification. Four rounds of selection and amplification were carried out, and the final PCR product was digested with EcoRI and BamHI and subcloned into pBluescript II KS (+) for sequencing.

Gel Mobility Shift Assays

For DNA binding assays, oligonucleotides A (CAAGTTAAAAAGA- CAACAAATAA), B (AGGAATACCAACATACATAATG), V1P (TTCACTTTTACATTAGGAGGG), V2P (TTTATTGTAGGACCCGAGGGG), 1L-2 wt (ATCCGGAATTTGT-TCTAACAT), and 1L-2 mut (ATCGAAAAACTGTTACTAACAT) were annealed to their complementary strands and then gel-purified to eliminate nonhybridized oligonucleotides. Either in vitro translated protein, Escherichia coli expressed proteins or nuclear extracts were used as a source of Foxp1. Construction and expression of Foxp1 GST fusion proteins and electrophoretic mobility shift assays were previously described (8). Nuclear extracts of various cell lines were isolated by the method of Lee et al. (21). For competition experiments, competitors were allowed to bind with the GST fusion proteins for 5 min at room temperature before the labeled probe was added. The supershift assay was done by the method of Neufeld (22).

Dual Luciferase Assays in HEK 293T

Cells in a 6-well plate were co-transfected with 100 ng of pRL-TK (Promega) as a normalization control, and 800 ng of each eukaryotic expression vector using FuGENE 6 reagent (Roche Molecular Biochemicals). Dual luciferase assays were performed 48 h after transfection using a dual luciferase assay kit (Promega) according to the manufacturer's protocols.

Induction and Luciferase Assays in Jurkat

Cells grown in RPMI complete medium plus HEPES, pH 7.2, were harvested, washed with medium, and resuspended at 1.5 × 10^7/0.3 ml into electroporation cuvettes. IL-2-pGL3 (15 μg), pRL-TK (5 μg), and each expression vector DNA (G5–320 μg) or DNA of its empty vector (to normalize each sample to 40 μg) were added. Following 10 min at room temperature, the DNAs were electroporated (Bio-Rad) at 240 V, 950 microfarads. The samples were left at room temperature for 10 min before being resuspended in 10 ml of Jurkat medium (RPMI with
complete medium) and transferred to T25 flasks. 24 h after electroporation, the cells were treated with either phorbal ester phorbol 12,13-dibutyrate (50 nM) and ionomycin (1 μM) (induced) or Me₂SO (uninduced). Following an 8-h incubation, whole cell lysates were prepared for dual luciferase assays and Western analysis.

RESULTS
Foxp1 Has at Least Four Splice Isoforms That Display Cell/Tissue-specific Expression Patterns—Foxp1A, originally named glutamine-rich factor-1, was first isolated in a search for polyglutamine-containing transcription factors in B cell cDNA libraries (8). The cDNA isolated is identical to the reported Foxp1 isoform A (9) except that our cDNA encodes two additional glutamines at positions 172 and 203. The Foxp1 gene, assembled from our genomic clones of the locus and the public data bases, has 16 coding exons (Fig. 1A, top panel). Based on the Foxp1 genomic sequence and several unique Foxp1 expressed sequence tag clones (GenBankTM accession numbers BI649134, BG65862, BB858702, and BI697624), we have identified a novel Foxp1 isoform, Foxp1D (Fig. 1A, bottom panel). The Foxp1D cDNA contains a unique 5′-untranslated exon (exon 2b) and two in-frame stop codons that precede its putative initiating methionine in exon 3 (methionine 131 of Foxp1A). Foxp1D mRNA encodes a 576-amino acid protein that lacks the polyglutamine domain (Fig. 1B).

Previously, two additional isoforms, Foxp1B and Foxp1C, were isolated from mouse lung tissues (9) (Fig. 1B). Although their unique sequences suggest that different Foxp1 isoforms may possess distinct transcription regulatory properties, essentially nothing is known about how each isoform differs in their expression pattern and functional properties.

First we attempted to characterize tissue-specific Foxp1 isoform expression in mouse by Northern blotting with a probe (Fig. 1B, 3′ probe) common to all Foxp cDNAs (Fig. 1C). Foxp1 messages of 3.2, 2.4, and 1.6 kb were detected. In brain and
RNase protection assays using two unique probes: 5' and 3' probe (Fig. 1B). Our approach was most effective in distinguishing Foxp1B (protected band size, 72 nucleotides) from other Foxp1 isoforms (145 nucleotides), because the sequence of the 3' probe (Fig. 1B) is partially spliced out in Foxp1B. As shown in Fig. 1 (D and E), Foxp1B is weakly expressed in most tissues surveyed, with the highest expression observed in spleen, WEHI 231, and 70Z B cell lines. Based on the known expression level of Foxp1B, we can deduce specific expression of Foxp1A using the 5' probe. This probe overlaps a region that is shared by Foxp1A and Foxp1B but is missing in Foxp1C and 1D (Fig. 1B). Foxp1A expression can be estimated based on the signal differences of Foxp1A + 1B (Fig. 1E, middle panel) and Foxp1B (Fig. 1E, top panel). High levels of Foxp1A were detected in 70Z, BCL1, EL4, C2C12, fibroblasts, brain, and spleen. Finally, although individual expression levels of Foxp1C and Foxp1D cannot be distinguished by our assay, these two isoforms add up to high abundance in WEHI 231 and testis (Fig. 1E).

To study Foxp1 protein expression, we generated a monoclonal antibody (1G1) against the Foxp1 forkhead domain. The ability of 1G1 to detect Foxp1 proteins on Western blots was confirmed using a GST-Foxp1 forkhead fusion protein (data not shown) and in vitro translated Foxp1A (Fig. 2A). Both 1G1 and the anti-HA mAb 16B12 recognized the full-length in vitro translated Foxp1A (~95 kDa) with HA epitope-tagged either on its N-(5'HA) or C-terminal (3'HA) (~70 kDa). Consistently, in vitro translation of Foxp1A produced a shorter translation product that is in frame with the full-length product (recognizable by 1G1). Because anti-HA mAb recognizes the truncated protein produced by 3'HA but not 5'HA, the truncation must occur at the N terminus. This property was later used in demonstrating that Foxp1 self-associates in vitro (Fig. 3B) and discussed below in “Results”. 1G1 showed no reactivity on Western blots toward Foxp1 proteins except Foxp1A. Foxp1A produces a full-length protein and a truncated protein (~50 kDa). The truncated protein is recognized by anti-HA mAb 16B12 but not 1G1. Therefore, 1G1 can be used in Western blots to detect full-length Foxp1A but not its truncated forkhead domain.

In various B and T cell lines (Fig. 2B) and in tissues (Fig. 2C), 1G1 detected at varying intensities three predominant proteins whose sizes (~95, 70, and 50 kDa) were indistinguishable from those of in vitro translated Foxp1A, D, and C, respectively (Fig. 2E). Foxp1A is widely expressed, with highest levels in lung and B cell line 70Z and with moderate levels in brain, spleen, and other lymphoid lines (J558, WEHI 231, 33BTE, and 70 BET). Foxp1D expression is also tissue- and cell-type specific, with the highest levels in WEHI 231, 70Z, and lung. Moderate Foxp1D levels accumulate in brain, spleen, and testis and lymphoid lines M12.4, 33BTE, and 70 BET. Foxp1C protein is widely expressed in tissues, but its expression is somewhat limited in lymphocytes. Foxp1B was not observed in our Western blotting (with the possible exception of spleen), either because its expression level is low (consistent with our RPA results) or because 1G1 does not recognize its truncated forkhead domain with equivalent sensitivity.

**Foxp1 Undergoes Self-association via a Zinc Finger and Leucine Zipper-containing Domain—Forkhead transcription factors were not known to self-associate. However, the ability of FOXP3 to form homodimers was anticipated because it contains a leucine zipper domain (5). The importance of the leucine zipper domain was highlighted in some of the X-linked autoimmune-allergic deregulation syndrome patients, who had developed this lethal disease simply because their FOXP3 alleles contained a single amino acid deletion within the leucine zipper domain.**

The leucine zipper domain is shared by all three members of...
the Foxp family (Fig. 3A). To test whether Foxp1 self-associates, we took advantage of the previous observation (Fig. 2A) that in vitro translation of HA-tagged Foxp1A consistently produced both a full-length and an N-terminal truncated protein. For 3′HA in vitro translation products, anti-HA monoclonal antibody 16B12 recognized both the full-length protein (95 kDa) and the N-terminal truncation (70 kDa) on Western (Fig. 2A) and efficiently immunoprecipitated both forms of proteins (Fig. 3B). For 5′HA in vitro translation products, the full-length protein (95 kDa) but not the N-terminal truncation (70 kDa) was recognized by 16B12 on Western (Fig. 2A). However, 16B12 immunoprecipitation of full-length 5′HA co-immunoprecipitated the nontagged truncated form (Fig. 3B), indicating that Foxp1A self-associates in vitro. A parallel 16B12 immunoprecipitation with a large excess of in vitro translated luciferase did not precipitate significant amounts of protein (Fig. 3B).

We next verified the self-association property of Foxp1 using the yeast two-hybrid system. Foxp1A lacking both its N-terminal polyglutamine and C-terminal acidic-rich domains (termed Foxp1FL; Fig. 3C) was subcloned into GAL4-based activation and DNA-binding domain vectors. Co-transformation of yeast with pASICYH2-Foxp1FL' and pACTII-Foxp1FL' activated reporter expression, both for His complementation and β-galactosidase (Fig. 3D), indicating self-association of the protein. Co-transformation of pASICYH2-Foxp1FL' and pTD1-1 (large T antigen fused to the GAL4 activation domain) did not activate reporter expression (Fig. 3D), indicating that the observed Foxp1 self-interaction was specific.

To map the self-association domain, the GAL4 DNA-binding domain was fused to various Foxp1A deletions (Fig. 3C) and co-transformed with pACTII-Foxp1FL' into yeast. The Foxp1 self-association domain corresponds to an evolutionarily conserved region (amino acids 338–426; Fig. 3C, bar ab), including a C2H2 zinc finger and the leucine zipper motif. The leucine zipper motif alone (amino acids 362–426; Fig. 3C, bar b) is capable of mediating Foxp1 homodimerization (Fig. 3D). The C2H2 zinc finger (amino acids 338–361; Fig. 3C, bar a) does not directly mediate Foxp1 homodimerization (Fig. 3D). However, weak interaction between the leucine zipper and T-antigen (Fig. 3D) suggests that the zinc finger contributes specificity to the Foxp1 self-interaction.

To test whether different Foxp family members interact physically via the conserved leucine zipper domain, a Foxp1A GST fusion protein of this region was used to pull down 35S-labeled in vitro translated Foxp proteins. All of the Foxp proteins tested (Foxp1A, 1C, 1D, and JM2/FOXP3) but not the luciferase control were pulled down. We suggest that all Foxp proteins bearing the domain could interact physically with each other, although the extent to which this occurs in cells remains to be determined. These results suggest that homodimerization and heterodimerization may provide an additional means to diversify Foxp1 regulation.

Selection of Foxp1 DNA Targets and Analysis of Binding—DNA binding of Foxp proteins had been previously performed on several promoters containing forkhead consensus sites (8, 23). Because Foxp proteins have the most divergent forkhead domains within the family, we performed a CASTing experiment to determine the site-specific DNA binding preferences of Foxp1A. PCR primers with random 26-base core sequences were used for the first round of selection. In vitro translated C-terminally HA-tagged Foxp1A was used as a source of Foxp1A protein, and the 16B12 antibody was used to pull down the protein-oligonucleotide complex. The sequences of individual isolates cloned following four cycles of CASTing were determined and organized into groups (Fig. 4A). From 76 inde-
dependent isolates, three unique sequences were isolated 25, 21, and 26 times, respectively, and the other four unique sequences were isolated once. The resulting Foxp1 consensus, TTATT-TRTRTTWKYKWYTWT, contains a 7-nucleotide (underlined) core, that is a subset of the 7-nucleotide core sequence shared among all forkhead binding sites: 5'H11032-TRTTKRY (1). There are an additional 13 flanking bases preferred by Foxp1A. Because our CASTing only resulted in seven unique sequences from 76 independent isolates, all individual bases within the current consensus site might not be required for Foxp1 binding.

To confirm binding of Foxp1A and 1D to the isolates, electrophoretic mobility gel shift assays (EMSA)s of two of the most common sequences (Fig. 4, A and B) were performed. Corresponding sense and antisense oligonucleotides were synthesized, annealed, and end-labeled. Duplexes were gel-purified for EMSA as previously described (8). In vitro translated Foxp1A and Foxp1D bound specifically to the two consensus oligonucleotides (Fig. 4B). Routinely we observed sharp yet nonspecific gel shifts (caused by binding of a protein(s) in the in vitro translation reticulocyte lysate), whereas the specific shifts corresponding to Foxp1A and 1D binding consistently gave multiple sizes that were difficult to resolve. This suggested that Foxp1A in vitro translation products included both full-length and N-terminally truncated products, both of which are capable of forming homo- and heterodimers. Foxp1 did not bind to an HNF3-specific binding site (V1P) contained in the immunoglobulin heavy chain variable gene (VH) promoter V1P (8). The diffuse Foxp1A-DNA complexes, when bound by the 1G1 antibody, produced supershifts that were much sharper (Fig. 4D).

Several lines of evidence indicate that the interaction between the isolates and Foxp1 forkhead domain is specific. First, Foxp1A forkhead point mutants W539L and V543L,R544E did not bind the isolated oligonucleotides (Fig. 4B), although similar amounts of wild-type and mutant protein were used in the assay, as indicated by Western blot using anti-Foxp1A monoclonal antibody (Fig. 4C). Similar mutations within the forkhead domain of HNF proteins have been shown to abolish DNA binding (16, 17). Second, double-stranded oligonucleotides A and B can compete for the binding of a GST-fused Foxp1A to radiolabeled oligonucleotide A, but no com-

---

**Fig. 4. Foxp1 binds to its consensus DNA sequences with specificity.** A. derived Foxp1 consensus binding sites. The sequence of 76 cloned Foxp1-binding sites derived by four rounds of selection are aligned about the TATTTRT core binding consensus sequence. Seven different sequences were obtained, and the number of times the oligonucleotide was cloned as an insert is indicated. B. Foxp1 interacts with its consensus binding sites, and the interaction requires the forkhead domain. Gel mobility shift of 32P-labeled selected sites A, B, and V1P (a forkhead consensus site within the immunoglobulin heavy chain variable gene (VH) promoter V1P) (8) with *in vitro* translated Foxp1A, Foxp1D, luciferase, and mutant Foxp1A (V543L,R544E and W539L) proteins. C. confirmation of protein integrity and loading of *in vitro* translated HSV-labeled luciferase, Foxp1A, and Foxp1A forkhead mutants (V543L,R544E and W539L) by Western blot. D. anti-Foxp1 mAb 1G1 but not a control antibody at equivalent concentrations interacts with and supershifts Foxp1A/DNA (probe B) complexes. 1 μl of 1G1 ascites was added increasingly at 1:20, 1:10, and 1:5 dilutions or undiluted. E. gel mobility shift of selected Foxp1A site A by a GST-Foxp1 forkhead domain fusion protein containing the forkhead domains (8) is competed by selected sites A and B but not by V1P. Each competitor was added increasingly at 0.2-, 1-, 5-, and 25-pmol quantities.
A. **Fig. 5.** Foxp1 family members repress transcription through Foxp1-binding sites. A, Foxp1A and Foxp1D repress transcription through binding sites within and appended to the SV40 early region promoter. Transcription reporter constructs are pGL-3 promoter, a SV40 firefly luciferase reporter vector; 1xb-pGL3 promoter, pGL3 promoter with inserted Foxp1-binding site; pGL3-control, SV40 firefly luciferase reporter vector with SV40 enhancer sequences; and 3xb-pGL3-control, pGL3 control plasmid with Foxp1-binding sites. Co-transfections of 293 cells were performed with the empty mammalian expression vector pCEP4L or with pCEP4L expressing Foxp1A or Foxp1D. Firefly luciferase activities were measured and normalized to Renilla luciferase activities 48 h after transfection. All of the experiments were performed in triplicate. B, Western blot analysis of exogenous Foxp1 protein expression levels. Left panel, transfected 293 cells in the experiment of A expressed similar levels of Foxp1A and Foxp1D. Right panel, transfected 293 cells in the experiment of Fig. 6B expressed similar levels of wild type and forkhead mutated Foxp1A. C, Foxp1C and Foxp2 strongly repress transcription through binding sites within and appended to the SV40 early region promoter. Shown are summaries of normalized firefly luciferase activity (%) derived identically as in A.

petition was observed by V1P (Fig. 4E). Third, single-stranded oligonucleotides A and B were not bound by wild type Foxp1, nor did they compete with double-stranded annealed versions for binding (data not shown).

**Foxp1 Isoforms Repress Transcription through Binding Sites within and Appended to the SV40 Early Promoter**—To test whether Foxp1 can regulate transcription, we co-transfected Foxp1A or Foxp1D with a firefly luciferase reporter driven by the SV40 promoter, with or without the Foxp1-binding site B inserted upstream of the promoter (Fig. 5A; pGL3 promoter versus 1xb-pGL3 promoter). We employed a recipient cell line, human embryonic kidney cells (293T), which expressed no 1G1-detectable levels of endogenous Foxp1A or Foxp1D (Fig. 5B). As shown in Fig. 5A (left panel), both Foxp1A and Foxp1D repressed transcription regardless of whether a Foxp1 consensus DNA-binding site was appended to the SV40 promoter. For Foxp1A, a 3–4-fold repression of transcription was observed with or without the addition of a synthetic binding site. However, for Foxp1D, repression was stronger than Foxp1A on the SV40 promoter alone (~5-fold) and was significantly enhanced (~25-fold) when a consensus Foxp1 site was placed upstream. The same effects with essentially identical magnitudes of repression were observed in co-transfections utilizing a reporter with the same SV40 promoter but bearing a trimerized consensus Foxp1-binding site and an SV40 enhancer in a remote position (Fig. 5A, right panel). Foxp1A and Foxp1D were expressed about equally, as indicated by Western blots of transfected lysates (Fig. 5B, left panel). Thus, we conclude that the polyglutamine-devoid Foxp1D is a stronger repressor and that a synthetic Foxp1-binding site is not fully required to repress SV40 transcription (see below).

If the polyglutamine tract is inhibitory to repression, we would anticipate that Foxp1C, which eliminates this motif via alternative splicing, might also be a stronger repressor than Foxp1A. As summarized in Fig. 5C, each of the four reporters were repressed to nearly background levels in 293 cells in which the Foxp1C isoform was transiently overexpressed (data not shown). However, the polyglutamine inhibitory effect is not observed for the highly Foxp1-related (~87% identical forkhead domain) polyglutamine-containing Foxp2 protein. Foxp2 repressed in our assay as strongly as Foxp1C (Fig. 5C) under conditions where both proteins were overexpressed equivalently to Foxp1A and D (data not shown). Because Foxp2 binds to the reporter consensus and SV40 sites with affinity similar to the Foxp1 proteins (data not shown), factors other than the polyglutamine track (e.g. posttranslational modifications; heteromerization) likely contribute.

The data of Fig. 5 also suggest that at least a component of Foxp1 repression may be mediated through a Foxp1-specific binding site within the SV40 promoter that had not been previously characterized. Experiments of Fig. 6 support this hypothesis. Scanning the sequence of the minimal SV40 promoter revealed a putative Foxp1 consensus TTATTTATG within the TATA-associated AT block (13) of the early region (Fig. 6A). Mutation of this site reduced luciferase activity of the resulting reporter about 30–50-fold relative to wild type SV40 when transfected alone into 293 cells (data not shown). Co-transfection with full-length Foxp1A had no significant effect, whereas Foxp1D modestly activated the mutant promoter (Fig. 6B, left panels). When a trimerized consensus Foxp1-binding site was appended to the mutant promoter, co-transfection with either form of Foxp1 repressed transcription 2–3-fold (Fig. 6B, right panels). Given the magnitude of the repression observed by Foxp1D on 3Xb-pGL3 transcription (Fig. 5B), this relatively modest rescue suggests that the two sites might act synergistically in that configuration. No repression was observed for Foxp1A with a mutated forkhead domain (V543L,R544E) (Fig. 6B), even though the mutant protein expression was equivalent to that of wild type protein (Fig. 5B, right panel).

To test whether Foxp1 directly interacts with the putative forkhead site in the wild type SV40 promoter, EMSA analysis was performed, initially with in vitro translated proteins. Wild type Foxp1A interacts with the SV40 promoter, but no shift was observed when Foxp1A with point mutations in the forkhead domain (W539L) was employed (Fig. 6C). In whole cell lysates produced from J558 or M12.4 B cell lines, complexes formed with the Foxp1-binding site within the SV40 promoter were supershifted by the anti-Foxp1 monoclonal 1G1 (Fig. 6D), indicating that the SV40 promoter might be a natural target for Foxp1 proteins.

**Molecular Analysis of Foxp1**

---

Molecular Analysis of Foxp1

---

The data of Fig. 5 also suggest that at least a component of Foxp1 repression may be mediated through a Foxp1-specific binding site within the SV40 promoter that had not been previously characterized. Experiments of Fig. 6 support this hypothesis. Scanning the sequence of the minimal SV40 promoter revealed a putative Foxp1 consensus TTATTTATG within the TATA-associated AT block (13) of the early region (Fig. 6A). Mutation of this site reduced luciferase activity of the resulting reporter about 30–50-fold relative to wild type SV40 when transfected alone into 293 cells (data not shown). Co-transfection with full-length Foxp1A had no significant effect, whereas Foxp1D modestly activated the mutant promoter (Fig. 6B, left panels). When a trimerized consensus Foxp1-binding site was appended to the mutant promoter, co-transfection with either form of Foxp1 repressed transcription 2–3-fold (Fig. 6B, right panels). Given the magnitude of the repression observed by Foxp1D on 3Xb-pGL3 transcription (Fig. 5B), this relatively modest rescue suggests that the two sites might act synergistically in that configuration. No repression was observed for Foxp1A with a mutated forkhead domain (V543L,R544E) (Fig. 6B), even though the mutant protein expression was equivalent to that of wild type protein (Fig. 5B, right panel).

To test whether Foxp1 directly interacts with the putative forkhead site in the wild type SV40 promoter, EMSA analysis was performed, initially with in vitro translated proteins. Wild type Foxp1A interacts with the SV40 promoter, but no shift was observed when Foxp1A with point mutations in the forkhead domain (W539L) was employed (Fig. 6C). In whole cell lysates produced from J558 or M12.4 B cell lines, complexes formed with the Foxp1-binding site within the SV40 promoter were supershifted by the anti-Foxp1 monoclonal 1G1 (Fig. 6D), indicating that the SV40 promoter might be a natural target for Foxp1 proteins.

Foxp1 Isoforms Repress Transcription through Binding Sites within the IL-2 Promoter—We sought to extend the above results on synthetic and SV40 promoter repression to a more biologically relevant system. The importance of the Foxp family in immune regulation has been recently documented (24–26). We noticed that the IL-2 promoter contains an uncharacterized sequence that can be aligned with our Foxp1 consensus (Fig.
In EMSA experiments, a specific interaction (as judged by 1G1 supershift and data not shown) was observed between this IL-2 promoter Foxp1-binding site and the SV40 promoter. Foxp1 repression of SV40 translation was induced by co-transfection of Foxp1 and SV40 reporter constructs (Fig. 7). Foxp1 repression of SV40 translation was induced in a dose-dependent manner by co-transfection of Foxp1 and SV40 reporter constructs (Fig. 7). Lack of repression by the Foxp1A forkhead mutant W539L indicated that the activity was sequence specific. This was confirmed by the experiment of Fig. 7D, in which mutations of the IL-2 promoter that eliminated Foxp1 binding in EMSAs (Fig. 7B) also eliminated Foxp1-mediated repression of IL-2 promoter.

We conclude that Foxp1A, and likely the other forkhead-containing Foxp members, bind specifically to a heretofore uncharacterized Foxp1 forkhead site within the IL-2 promoter. The data suggest that our isolated Foxp1 preferred binding sites of Fig. 4A can interact and mediate transcription repression by Foxp1 in a native promoter. These results further indicate that Foxp1 isoforms and Foxp2 may display different promoter-specific repression activities in the context of T cell activation.

**DISCUSSION**

In contrast with other forkhead subfamilies, domain homology within Foxp proteins extend beyond their forkhead DNA-binding domain. Distinguishing features of Foxp are the C2H2 zinc finger and leucine zipper motifs as well as a DNA-binding region truncated at the “wing” portions of the forkhead/winged helix domain. Foxp1A, Foxp2, and human FOXP1 also share a polyglutamine stretch at their N termini. Our functional studies demonstrated that each of these domains has an important role in defining the transcriptional regulatory properties of Foxp1.

To fully understand Foxp1 function and the mechanisms that underlie it, identification of physiological target genes would be required. Although previous studies (8, 23) demonstrated interaction between Foxp and target sites identified for other forkhead proteins, isolation of the preferred DNA-binding sequence of Foxp1 constrains the search for target genes. In EMSA experiments, Foxp1 favors the isolated sites strongly over V1P (a prototypic HNF-3-binding site), indicating that the sequences we obtained are indeed highly preferred binding sites. Our casting results also support the proposed role for Foxp1 in regulation of mouse lung-specific CC10 promoter (9), because a Foxp1-binding site is contained within the 165-bp cis-regulatory region upstream of this promoter (TTATTT-GCTT). Despite the fact that Foxp forkhead domains are the most divergent among all family members, our isolated Foxp1-binding sites, nonetheless, contain the conserved HNF core nucleotides. Thus, from a structure-function point of view, it appears that helices 1–3 of the Foxp1 forkhead domain are both necessary and sufficient for the direct base contacts within the core sequence (10–12).

Ectopic overexpression of Foxp1 represses transcription of the SV40 promoter, an SV40 promoter bearing a preferred Foxp1-binding site, and the IL-2 promoter. Foxp1 repression of the SV40 promoter is mediated by binding to a region spanning its TATA box, which contains 9 bases of the Foxp1 consensus. When 3 base pairs of this sequence were mutated, transcription repression by Foxp1 was abolished. In contrast, previous studies showed that whereas deletion of this AT block interfered with the precision of the transcriptional initiation start site, the level of basal transcription was not affected (28, 29). Several possibilities might explain the apparent discrepancy. First
and foremost, the Benoist and Chambon (28) results were obtained in vitro. Second, because the AT block lies within the replication origin for SV40, our point mutation, in addition to eliminating a Foxp1-binding site, might have decreased replication of the reporter plasmids in 293T cells. Third, by mutating the AT block, we might have introduced a new binding site for an unknown factor that strongly represses reporter transcription. However, our detection of a Foxp1-specific protein complex with the SV40 promoter sequence within nuclear extracts of J558 and M12.4 B lymphocyte lines supports our contention that SV40 is a bona fide target of Foxp1.

We have also identified IL-2 as a putative physiological target for Foxp1. Several lines of evidence support this possibility, including direct and specific Foxp1-IL-2 promoter interaction, detection of Foxp1 mRNA and protein expression in T lymphocytes, and detection of a Foxp1-specific protein complex with the IL-2 promoter sequence in nuclear extracts of Jurkat T cells. Our data are consistent with previous observations that Foxp3 can also repress the IL-2 promoter (23) and that X-linked autoimmunity-allergic disregulation syndrome patients produce high levels of IL-2 in their peripheral blood (5).

What is the mechanism for Foxp-mediated transcriptional repression? Previously it was shown that regions within Foxp1 and Foxp2 that span the zinc finger and leucine zipper domains repressed transcription when fused to the GAL4 DNA-binding domain (9). Here, we confirmed experimentally that the leucine zipper, in combination with the zinc finger domain, promotes self-association among different isoforms and subfamily members of Foxp proteins. Because both forkhead truncation and a single amino acid deletion within the leucine zipper domain cause loss of function for FOXP3 (5), Foxp-mediated repression may require both target DNA binding and dimerization. Whether specific dimerization partners are used to "fine tune" Foxp protein in their selectivity of target sites or whether dimerization is required for Foxp to recruit general transcriptional repressor(s) remains to be understood.

Although Foxp1A and Foxp1D repress the IL-2 promoter at similar levels in Jurkat cells, Foxp1D is significantly stronger than Foxp1A in 293T cells. Both isoforms are localized to the nucleus (data not shown), and their in vitro translated products appear to bind Foxp1 consensus DNA sites with comparable affinities. Differential interaction with common cellular proteins or with different cell type-restricted proteins could explain these differences. Even stronger repression in 293 cells was engendered by the Foxp1C isoform. Because the polyglutamine tract is missing from Foxp1C and 1D, it is an obvious candidate for a modulator of Foxp1A repression activity. In support of this idea, polyglutamine tracts are known to mediate...
protein interactions, either by forming multimers (30, 31) or by interacting with polar amino acid-rich sequences (32). Selective expression of the Foxp1 isoform and/or its interaction partner(s) could regulate Foxp1 transcription activity as needed.

The reason(s) for the differential transcriptional activity of Foxp1 and Foxp2 is not structurally obvious. Foxp2 and Foxp1A are highly similar throughout and have conserved all functional domains, including the polyglutamine. Yet Foxp2 repressed at levels equivalent to those of Foxp1C in 293 cells but failed to induce significant repression in Jurkat cells. As with the case of Foxp1A and D, Foxp2 binds strongly in vitro to both the preferred Foxp1 casting sites and to the IL-2 site (data not shown). These differences underscore the functional complexities afforded by these proteins even when analyzed under controlled expression conditions in cultured cell lines.

Foxp1 expression shows tissue preference in human adults, accumulating to highest levels in peripheral blood lymphocytes and the caudate nucleus of the brain. However, it is broadly expressed at moderate to low levels in many other adult tissues. The broad distribution of Foxp1 suggests it may have functional roles in diverse cell and tissue types. The combination of tissue-specific alternative splicing and subunit-subunit interaction may be important to tailor Foxp1 properties to the needs of individual cell types. During embryonic development, co-expression of Foxp1 and Foxp2 is found in lung epithelia and the developing nervous system (9). Foxp1 expression is detected in every lymphocyte cell line that we have analyzed as well as in spleen and thymus. Several of these lymphoid cells co-express Foxp3 (5). It is possible that one Foxp protein might affect the activity of the other by competing for target gene promoter binding or by acting either as a partner or as a negative regulator via direct protein interaction. Although redundancy among conserved family members often exists for transcription factors, Foxp1-targeted disruption in mice leads to an embryonic lethal phenotype. Therefore, the potential redundancy apparent in the Foxp1 subfamily is insufficient to rescue Foxp1 loss, and at least in one or more of these tissues, Foxp1 has a critical and unique role.

Acknowledgments—We thank Dr. Robert Brenner, Dr. Jiang Wu, Dr. Xueqing Xie, and Riina Luik for helpful discussions and critical reading of the manuscript; Dr. William Bargman for suggestions in generating the monoclonal antibody; Shan Maika, Loren Probst, and Dr. Xueqing Xie, and Riina Luik for helpful discussions and critical reading of the manuscript.

2 B. Wang and P. Tucker, unpublished data.

REFERENCES

1. Carlsson, P., and Mahlapuu, M. (2002) Dev. Biol. 250, 1–23
2. Kaufmann, E., and Knechel, W. (1996) Mech. Dev. 57, 3–20
3. Banham, A. H., Beasley, N., Campo, E., Fernandez, P. L., Fidler, C., Gatter, K., Jones, M., Mason, D. Y., Prime, J. E., Truongbouff, P., Wood, K., and Byrne, G., McEuen, M., Proll, S., Appleby, M., and Brunkow, M. E. (2001) Nat. Genet. 27, 68–73
4. Enard, W., Przeworski, M., Fisher, S. E., Lai, C. S., Wiebe, V., Kietz, T., Monaco, A. P., and Paabo, S. (2002) Nature 418, 869–872
5. Chakravarti, T. A., Blasser, F., Ho, N., Lederer, H. M., Voulgaropoulos, C., Helms, C., and Bowcock, A. M. (2000) J. Clin. Invest. 106, 875–B81
6. Brunkef, M. E., Jeffery, E. W., Hjerrild, K. A., Paaper, B., Clark, L. B., Yassenko, S. A., Wilkinson, J. E., Gulas, D., Ziegler, S. F., and Rammelsd, M. (2001) Nat. Genet. 27, 18–20
7. Wildin, R. S., Ramesdell, F., Peake, J., Faravelli, L., Casanova, J. L., Buist, N., Levy-Lahad, E., Mazzella, M., Goulet, O., Perroni, L., Bricearelli, F. D., Byerse, G., McEuen, M., Proll, S., Appleby, M., and Brunkef, M. E. (2001) Nat. Genet. 27, 18–20
8. Li, C., and Tucker, P. W. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11583–11587
9. Shu, W., Yang, H., Zhang, L., Lu, M. M., and Morrisey, E. E. (2001) J. Biol. Chem. 276, 27488–27497
10. Clark, K. L., Halay, E. D., Lai, E., and Burley, S. K. (1993) Nature 364, 412–420
11. van Dungen, M. J., Cederberg, A., Carlsson, P., Enerback, S., and Wikstrom, M. (2000) J. Mol. Biol. 296, 351–359
12. Weigelt, J., Ciment, L., Dahlman-Wright, K., and Wikstrom, M. (2001) Biochemistry 40, 5866–5869
13. Chamberlain, N. L., Driver, E. D., and Miesfeld, R. L. (1994) Nucleic Acids Res. 22, 3181–3186
14. Sambrook, J., Frisch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., pp. 181–187, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
15. Emmel, E. A., Verweij, C. L., Durand, D. B., Higgins, K. M., Lacy, E., and Crabtree, G. R. (1989) Science 246, 1617–1629
16. Clevidence, D., Overdier, D. G., Tao, W., Qian, X., Pani, L., Xei, E., and Costa, R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3948–3952
17. Khater, U., Kaufman, E., Hartmann, C., Jurgens, G., Knechel, W., and Jackle, H. (1995) EMBO J. 14, 5306–5317
18. Rose, M. D., Winston, F., and Hieter, P. (1990) in Methods in Yeast Genetics (Guthrie, C., and Fink, G., eds) pp. 178–179, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
19. Breeden, L., and Nasmyth, K. (1985) Cold Spring Harb. Symp. Quant. Biol. 50, 645–650
20. Pulcke, R., and Treisman, R. (1990) Nucleic Acids Res. 18, 6197–6204
21. Lee, K. A., Daimon, A., and Green, M. R. (1988) Gene Anal. Tech. 5, 22–31
22. Neufeld, E. J., Skalnik, D. G., Lievens, P. M., and Orkin, S. H. (1992) Nat. Genet. 1, 50–55
23. Schubert, L. A., Jeffery, E. Y., Zhang, Y., Ramesdell, F., and Ziegler, S. F. (2001) J. Biol. Chem. 276, 57672–57679
24. Khattari, R., Cox, T., Yasukyo, S. A., and Ramesdell, F. (2003) Nat. Immunol. 4, 337–342
25. Fontenot, J. D., Gavin, M. A., and Rudensky, A. Y. (2003) Nat. Immunol. 4, 330–336
26. Hori, S., Nomura, T., and Sakaguchi, S. (2003) Science 299, 1057–1061
27. Fiering, S., Northrop, J. P., Nol, G. P., Mattila, P. S., Crabtree, G. R., and Herzenberg, L. A. (1990) Genes Dev. 4, 1823–1834
28. Benoist, C., and Chambon, P. (1981) Nature 290, 304–310
29. Mathis, D. J., and Chambon, P. (1981) Nature 290, 310–315
30. Perutz, M. (1994) Protein Sci. 3, 1629–1637
31. Stett, K., Blackburn, J. M., Butler, P. J., and Perutz, M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6509–6513
32. Imafuku, I., Waragai, M., Takeuchi, S., Kanazawa, I., Kawahata, M., Mouradian, M. M., and Okazawa, H. (1998) Biochem. Biophys. Res. Commun. 253, 16–20
