the HLA-DPB1 Glu[69] marker may be high.

Screening for disease-associated genetic markers may provide new tools for prevention of occupational diseases (22). So far in CBD, blood and lung lymphocyte testing for Be-specific proliferation have been used to identify individuals with Be allergy and disease (23). However, because lymphocyte tests do not predict disease or risk, genetic testing may have a role, in association with lymphocyte Be-specific proliferation testing, in the identification and follow-up of individuals at risk of beryllium disease.

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7. A group of 33 CBD cases (46 ± 16 years of age; 30 males and 3 females; 30 Caucasians, 2 Hispanics, and 1 African American) were retrospectively identified according to established criteria (see below) including (i) a history of exposure; (ii) compatible chest x-ray abnormalities; (iii) abnormal lung function tests [vital capacity (VC) 79 ± 18% of predicted and diffusing capacity (DLCO) 62 ± 21% of predicted]; (iv) a pulmonary biopsy showing noncaseating granulomas; and (v) a positive Be-stimulated lymphocyte proliferation test (LTT) in the blood or bronchoalveolar lavage (the test was performed according to published protocols in each of the contributing institutions) (*2, 3*). A group of 44 Be-exposed unaffected cases [40 ± 9 years of age; 25 males and 19 females; 31 Caucasians, 11 Hispanics, and 2 African Americans ($P > 0.09$ compared with CBD cases)] were identified out of a group of 46 Be workers during a CBD survey at a Be plant (Brush Wellman Medical Department, Tucson, AZ). None had a history of pulmonary disease or abnormal chest x-rays or pulmonary functions. We identified Be-exposed individuals as cases by LTT tests and clinical evaluation, as described above. CBD cases were evaluated at the Pulmonary Branch of NIH, Bethesda, MD; the Cleveland Clinic, Cleveland, OH; the National Jewish Center for Immunology and Respiratory Medicine, Denver, CO; the University of Pennsylvania, Philadelphia, PA; and the University of Wales, Penarth, United Kingdom. Be-exposed individuals were evaluated at the Brush Wellman Medical Department; blood LTTs were performed at Specialty Laboratories (Santa Monica, CA) and at the National Jewish Center for Immunology and Respiratory Medicine according to standard procedures. Informed consent was obtained from all individuals.
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9. The typing of HLA-DPB1 was carried out by gel electrophoresis heteroduplex analysis (*24*). A 311-bp fragment spanning the entire DPB1 exon 2 was generated by PCR with the primers UG19 (GCTGCAGGAGAGTGGCGCCTCCGCTCAT) and UG21 (CGGATGCCGGCCCAAAGCCCTCACTC) using phenol/chloroform-extracted DNA from snap frozen Ficoll-Hypaque–purified peripheral blood mononuclear cells (*3*). The PCR product was further used as a template to obtain DNA from alleles DPB1*0201, *0202, *0401, *0402, *1501, and *1801 (group 1) and DPB1*0101, *0301, *0501, *0601, *0801, *0901, *1001, *1101, *1301, *1401, *1601, *1701, and *1901 (group 2) with the hypervariable region F-complementary oligonucleotides DP1 and DP2 as the 3′ end primers and the oligonucleotide UG19 as the 5′ end primer. The PCR reactions were carried out with standard protocols. Unclear typing results were confirmed by sequence-specific oligonucleotide DNA hybridization and direct sequencing of the PCR product. All data are presented as means ± SDs. Demographic data were compared by variance analysis. We compared the allele and polymorphism frequencies by the χ^2 test with Bonferroni correction according to which the *P* values are multiplied by the number of allelic variants tested for each comparison (J. L. Tiwari and P. I. Terasaki, Eds., *HLA and Disease Associations* (Springer-Verlag, New York, 1985), pp. 18–27].
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13. We carried out analysis of the HLA-DRB4*0101 gene frequencies in the CBD case and Be-exposed unaffected case groups with an ARMS assay with the primers DRw53-1 (TCCTCAATGGGACGGAGCGA) and DRw53-2 (CTCCACAACCCCGTAGTTGTA), specifically amplifying a 239-bp DNA fragment.
14. Analysis of the frequencies of the TNFβ Nco I polymorphism was carried out by DNA amplification of the TNFβ gene with the primers Nco I-1 (CCGTGCTTCGTGCTTTGGACTA) and Nco I-2 (AGAGCTGGTGGGGACATGTCTG) followed by Nco I restriction fragment polymorphism analysis of the 740-bp PCR product, as described by Messer *et al.* (*12*).
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Regional Codon Randomization: Defining a TATA-Binding Protein Surface Required for RNA Polymerase III Transcription

Brendan P. Cormack* and Kevin Struhl†

The TATA-binding protein (TBP) is required for transcription by all three nuclear RNA polymerases. TBP was subjected to regional codon randomization, a codon-based mutagenesis method that generates complex yet compact protein libraries. Analysis of 186 temperature-sensitive TBP mutants yielded 65 specifically defective in transcription by RNA polymerase III (Pol III). These mutants map to a limited TBP surface that may interact with Tds4, a component of the Pol III transcription factor TFIIIB. Strains that contain the Pol III–defective derivatives have increased amounts of messenger RNA, which suggests that competition among TBP-interacting factors for limiting quantities of TBP determines the ratio of Pol II and Pol III transcription in vivo.

The TATA-binding protein (TBP) is required for transcription by all three nuclear RNA polymerases (*1–4*). TBP interacts with TATA-associated factors (TAFs) to form distinct complexes that are specific for the individual RNA polymerases (Pol). The Pol II–specific complex, TFIID, contains at least 10 TAFs that have been

implicated in the transcription of specific subsets of promoters and in the response to transcriptional activator proteins (*5–11*). For Pol I, TBP associates with three TAFs and forms the selectivity factor SL1 (*1*). The Pol III–specific complex, TFIIIB, contains two TAFs (*12–15*), one of which (Tds4/Brf1/Pcf4) shows a sequence similarity to the Pol II initiation factor TFIIB (*16–18*). Although a few TBP mutations that differentially affect transcription in vitro by the RNA polymerases have been described (*3*), the regions of TBP required for assembly into these polymerase-specific complexes are not known.

To identify surfaces of TBP required for polymerase-specific transcription in vivo, we wished to generate a highly representative library of TBP mutants. In standard oligonucleotide-directed mutagenesis schemes, defined amounts of the three non–wild-type nucleotide precursors are included at each step of oligonucleotide synthesis. This approach avoids bias in nucleotide substitutions at each mutated position, but it strongly favors amino acid changes that correspond to codons with one nucleotide difference from the wild-type codon.

To overcome this problem, we developed a codon-based mutagenesis approach, regional codon randomization (Fig. 1). At the relevant step of oligonucleotide synthesis, the column is dismantled and the silica matrix is split into two portions that are repacked into mutant and wild-type columns. The mutant column is subjected to three rounds of synthesis with an equimolar mixture of the four nucleotide precursors, whereas the wild-type column is subjected to three synthetic cycles with wild-type nucleotides. The two columns are then combined, and the process is repeated for each codon being mutated. The resultant oligonucleotide has a defined mutation frequency, which depends on the ratio of silica beads in the mutant versus that in the wild-type column. Moreover, because all 63 mutant codons are equally probable at each position, the oligonucleotide yields a representative, yet highly compact, library of mutant proteins. For a 20–amino acid region averaging one mutated codon per oligonucleotide, only 3000 clones are necessary to contain all possible single amino acid substitutions; with the use of nucleotide-based mutagenesis, 10^7 clones would be required (*19*). Thus, for a region of this size, all possible amino acid changes are easily sampled by conventional genetic screening.

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With this approach, we generated six mutant TBP libraries (approximately 10^6 independent clones each) that cover residues 117 to 168, which include the basic region between the direct repeats, residues 217 to 240, which include the correspond-

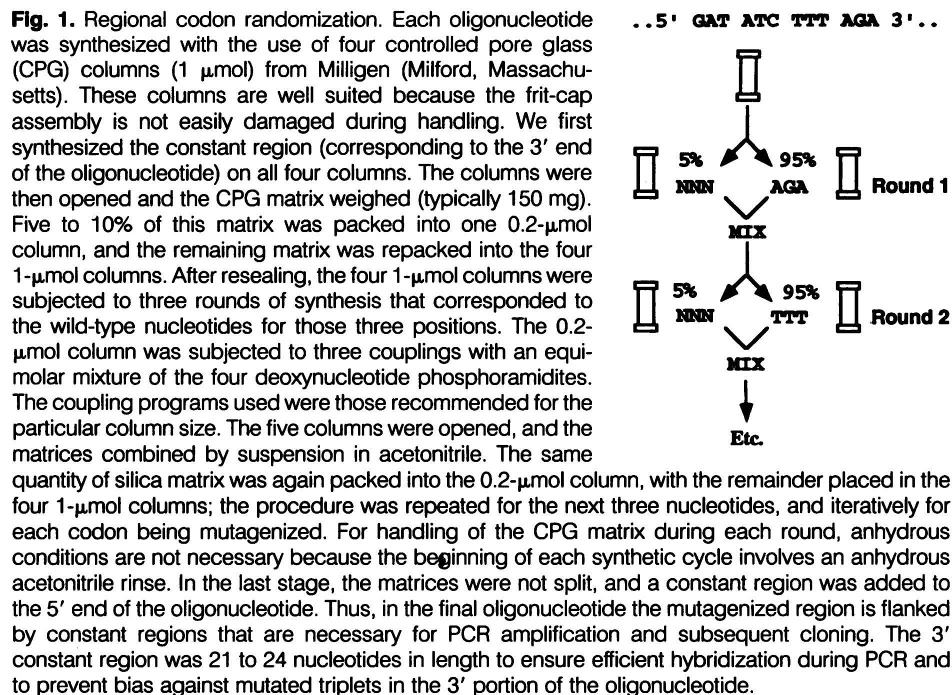

Fig. 1. Regional codon randomization. Each oligonucleotide was synthesized with the use of four controlled pore glass (CPG) columns (1 μmol) from Milligen (Milford, Massachusetts). These columns are well suited because the frit-cap assembly is not easily damaged during handling. We first synthesized the constant region (corresponding to the 3′ end of the oligonucleotide) on all four columns. The columns were then opened and the CPG matrix weighed (typically 150 mg). Five to 10% of this matrix was packed into one 0.2-μmol column, and the remaining matrix was repacked into the four 1-μmol columns. After resealing, the four 1-μmol columns were subjected to three rounds of synthesis that corresponded to the wild-type nucleotides for those three positions. The 0.2-μmol column was subjected to three couplings with an equimolar mixture of the four deoxynucleotide phosphoramidites. The coupling programs used were those recommended for the particular column size. The five columns were opened, and the matrices combined by suspension in acetonitrile. The same quantity of silica matrix was again packed into the 0.2-μmol column, with the remainder placed in the four 1-μmol columns; the procedure was repeated for the next three nucleotides, and iteratively for each codon being mutagenized. For handling of the CPG matrix during each round, anhydrous conditions are not necessary because the beginning of each synthetic cycle involves an anhydrous acetonitrile rinse. In the last stage, the matrices were not split, and a constant region was added to the 5′ end of the oligonucleotide. Thus, in the final oligonucleotide the mutagenized region is flanked by constant regions that are necessary for PCR amplification and subsequent cloning. The 3′ constant region was 21 to 24 nucleotides in length to ensure efficient hybridization during PCR and to prevent bias against mutated triplets in the 3′ portion of the oligonucleotide.

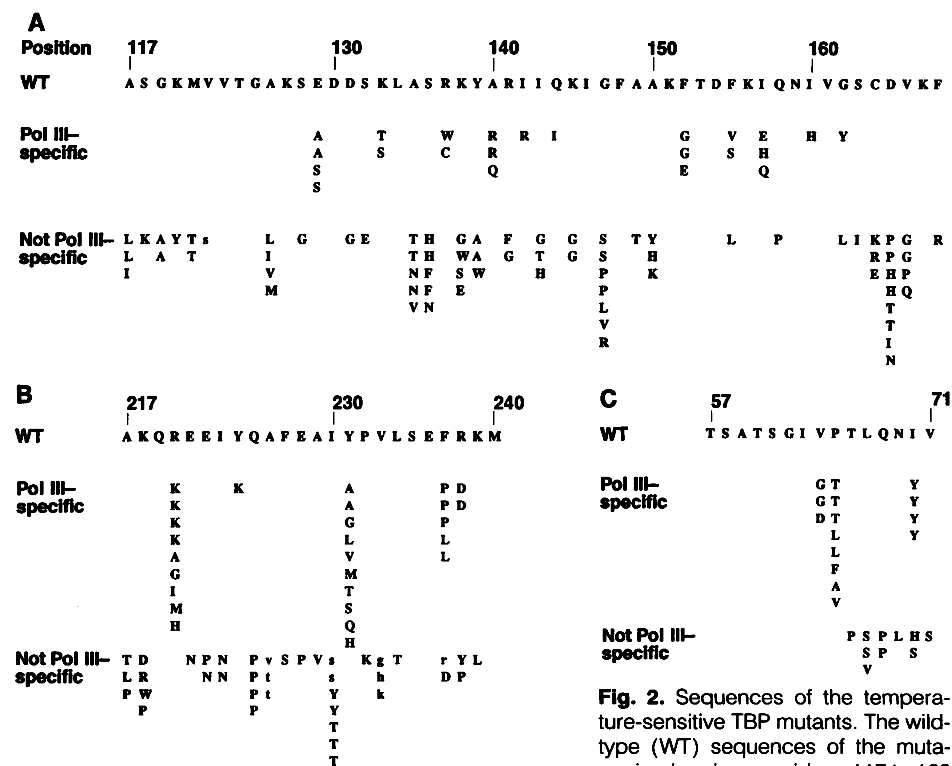

Fig. 2. Sequences of the temperature-sensitive TBP mutants. The wild-type (WT) sequences of the mutagenized regions, residues 117 to 168 (**A**), 217 to 240 (**B**), and 57 to 71 (**C**), are shown (*28*). Indicated below the sequences are the amino acid substitutions that yield a mutant TBP specifically defective in tRNA transcription. For certain positions, the same amino acid change (though not necessarily the same codon change) was independently isolated more than once. Indicated below the Pol III–specific mutations are substitutions that did not result in a specific loss of Pol III transcription. Substitutions that show decreased Pol II transcription and increased Pol III transcription are shown in small letters. The lack of temperature-sensitive mutations between residues 57 to 63 probably reflects the fact that this region is not part of an evolutionarily conserved TBP core.

ing region following the second direct repeat, and residues 57 to 71, which include the corresponding region preceding the first repeat (*20*). The basic region was chosen for mutagenesis because it has been implicated in protein-protein interactions (*21–23*). Analysis of 25 to 30 unselected clones from each library revealed that the nucleotide usage at mutated codons and the mutation frequency per oligonucleotide and per codon were in accordance with random expectation (*24*).

The TBP mutant libraries were introduced into yeast cells, and upon plasmid shuffling (*25*), individual proteins were screened for their ability to support cell growth at 23°C, 30°C, and 37°C (*25*). Of 40,000 TBP derivatives tested, approximately 30% did not support cell growth at any temperature, 70% behaved indistinguishably from wild-type TBP, and 1% permitted growth at 23°C and 30°C but not at 37°C. The plasmids from 262 of these temperature-sensitive yeast strains were rescued and the mutated region was sequenced; 186 mutant proteins contained single substitutions (Fig. 2).

For each of the 186 mutant proteins, we examined ribosomal RNA (rRNA), mRNA, and transfer RNA (tRNA) transcription upon shifting the strains to the restrictive temperature (Fig. 3). As expected, most of the mutants showed either a general (Fig. 3, lane 7) or minimal decrease in transcription of all three RNA classes. However, 65 mutants (representing 46 different substitutions and 19 residues) showed a large decrease in tRNA transcription; five of these are shown (Fig. 3, lanes 1 through 5). In all 65 cases, rRNA transcription did not appreciably change, whereas the amounts of three mRNAs (*HIS3, TRP3*, and *DED1*) were greater than the amounts observed in strains that contained wild-type TBP. Thus, we have identified a class of TBP mutants that are specifically defective in Pol III transcription. A variety of substitutions yielding the same phenotype was observed at most of the 19 positions (Fig. 2), which suggests that the Pol III–specific phenotype results from the loss of the wild-type residue rather than the presence of a detrimental amino acid.

The Pol III–specific mutations are likely to define a specific TBP function and not to reflect an inherent temperature sensitivity in Pol III transcription. In particular, we also isolated ten TBP mutants (representing eight different substitutions; one mutant is shown in Fig. 3, lane 6) in which transcription of a subset of Pol II genes (*HIS3* and *TRP3*, but not *DED1*) is compromised, whereas Pol III transcription is increased slightly (*26*). The transcriptional effects in this class of TBP mutants are reproducible but clearly less marked than those observed with the Pol III–specific mutants. With one exception (Phe^{237}), the five residues defined by the Pol II–defective mutations differ from the 16 residues identified by the Pol III mutations (Fig. 2).

The crystal structure of *Arabidopsis thaliana* TBP at 2.6 Å resolution indicates that the protein consists of two very similar structural domains related by approximate twofold symmetry (*27*). Although we mutagenized most of the upper surface of the protein, the positions defined by the Pol III–specific mutants are highly clustered (Fig. 4A). These positions are largely confined to the inside faces of helices H2 and H2′, to those residues pointing toward H2 in the connecting region between the two domains extending into strand S1′, and to those residues in S1 pointing toward H2′. No Pol III–defective mutants were obtained in strand S5.

Sixteen of the 19 positions identified by the Pol III–defective mutants define a solvent-exposed and nearly continuous surface that forms a groove across the top of the protein (Fig. 4B). We suspect that the three exceptional positions not exposed to solvent (70, 160, and 162) do not normally directly contribute to the Pol III–specific function; they are defined by individual substitutions of large aromatic residues (I70Y, I160H, and G162Y) (*28*) that are likely to disrupt the local structure. In support of this view, a leucine substitution at position 162 does not cause a Pol III–specific defect but rather causes a small decrease in transcription by all three polymerases. No Pol III–specific mutants were obtained at position 227, even though this residue lies on the same face of helix H2′ as three residues that yielded Pol III–specific mutants. This apparent exception may be explained by the fact that Phe^{227} is largely buried by residues at the NH_2-terminus of the crystallized protein

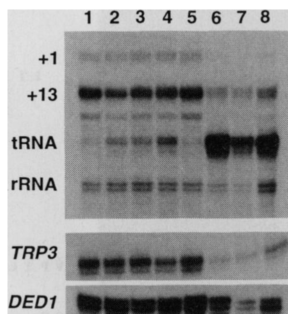

Fig. 3. Transcriptional analysis of strains that carry temperature-sensitive TBP alleles. The TBP derivatives were as follows (*28*): lane 1, E129S; lane 2, R137W; lane 3, F152E; lane 4, R220K; lane 5, Y231V; lane 6, F237R; lane 7, A217P; and lane 8, wild-type. RNAs were prepared from cells grown at the restrictive temperature (37°C) for 1 hour and subjected to nuclease S1 analysis as described (*2*). Equal amounts of RNA (determined by absorbance at 260 nm) were hybridized to completion with a 10- to 100-fold excess of oligonucleotide probes complementary to *TRP3, DED1*, and *HIS3* +1 and +13 transcripts, the junction of the 25*S* rRNA and nontranscribed spacer region, and the intron of the Ile tRNA. Because the half lives of these RNA species are short, the signal at any given time reflects transcription initiation, not accumulated RNA levels (*2*).

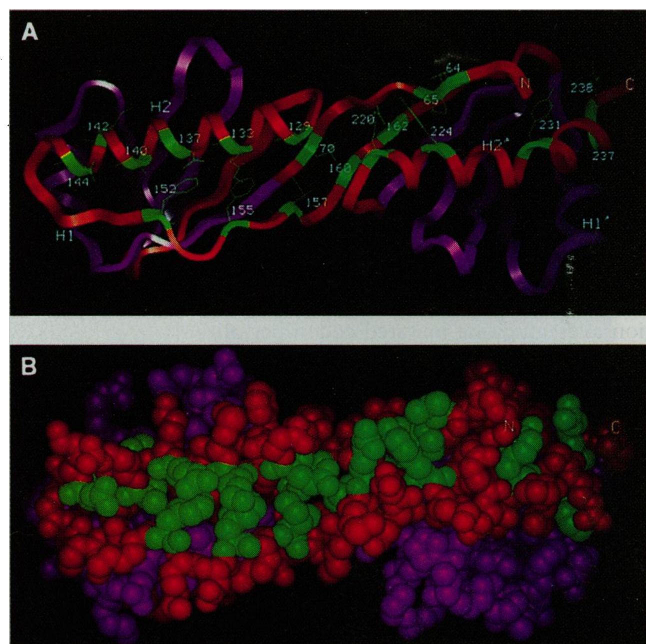

Fig. 4. Mapping the Pol III–specific mutations onto the structure of *A. thaliana* TBP. The *Saccharomyces cerevisiae* and *A. thaliana* TBPs are 85% identical, and therefore the structures are expected to be highly related. (**A**) Ribbon diagram showing residues not mutated in this study (purple), residues that were mutated but did not yield a Pol III–specific phenotype (red), and residues that did mutate to a Pol III–specific phenotype (green). In this view, the protein has been rotated around the *x* axis from the standard view (*27*) such that helices H2 and H2′ are facing forward and the concave surface of the protein and stirrups face into the page. (**B**) Space-filling model of the identical view of TBP. In the same color scheme, the atoms are shown as solid spheres of radius equal to the van der Waals radius.

(corresponding to residue 61 of yeast TBP). These few possible exceptions notwithstanding, the majority of the Pol III–specific mutations are limited to a solvent-exposed surface that is likely to interact with Pol III–specific factors.

The best candidates for a protein that interacts with the surface defined by these mutations are the two TAFs (Tds4/Brf1/Pcf4 and 90K) that associate with TBP to form transcription factor IIIB (TFIIIB). To address this issue, we determined whether overproduction of Tds4 would suppress the temperature-sensitive phenotypes caused by the various TBP derivatives (Fig. 5). For all 19 Pol III–defective strains tested, a partial reversal of the temperature-sensitive phenotype was obtained upon overproduction of Tds4. Conversely, the overexpression of Tds4 did not suppress the phenotype in nine mutant strains that were not specifically defective in Pol III transcription. Indeed, Tds4 overproduction sometimes exacerbated the temperature-sensitive growth defect. These results suggest that the Pol III–specific TBP mutants may be defective in interacting with Tds4.

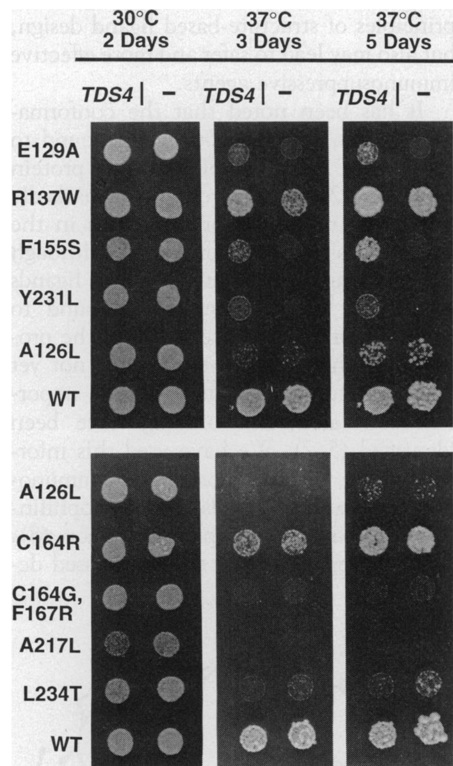

Fig. 5. Suppression of the growth defect of various TBP strains by overexpression of the TFIIIB component Tds4. The *TDS4* gene (*19*) was cloned into an *ADE2* 2μ vector. The resulting molecule and the parental vector were introduced into temperature-sensitive strains that carried the indicated TBP alleles. Approximately 10^5 cells were spotted onto synthetic plates lacking adenine, and these were grown at 30°C or 37°C for the indicated times. The E129A, R137W, F155S, and Y231L mutants (*28*) are Pol III–specific TBP derivatives; the others are not. Only a subset of strains subjected to this assay are shown; these were chosen to indicate the range of growth and suppression phenotypes. WT, wild type.

Our genetic analysis of TBP defines a surface of TBP that is required for Pol III transcription, probably through its interaction with Tds4. However, the results do not exclude the possibility that this region may also be important for interaction with Pol I, Pol II, or other Pol III factors. Indeed, although both F237P and F237L (*28*) are Pol III–specific mutations, F237R decreases Pol II transcription (Fig. 3). The residue at position 237 and three of the four other residues identified by Pol II–defective mutations define a small patch that is distinct from, but partially overlaps, the surface defined by the Pol III–specific mutations (*29*). We note that our analysis is limited by the initial selection for temperature-sensitive TBP mutants. Mutations that affect a particular TBP function might not be temperature-sensitive or might be so compromised for another function that the strain would be inviable at all temperatures.

The 65 strains that contain the Pol III–defective derivatives show increased Pol II transcription; conversely, some TBP mutants that are defective for Pol II transcription show increased amounts of Pol III transcription. This suggests that the Pol II– and Pol III–specific factors compete for limiting amounts of TBP in vivo. This competition likely reflects overlapping recognition sites on TBP for polymerase-specific factors, consistent with the proximity of the Pol III– and Pol II–specific mutations. In light of the Tds4 overexpression results and the marked similarity of Tds4 with the Pol II factor TFIIB, Tds4 and TFIIB association might commit TBP to Pol III and Pol II transcription, respectively. Alternatively, the competition for TBP might involve Tds4 and a Pol II–specific TAF. We do not know why in the Pol III–defective mutants there is no observable increase in Pol I transcription; perhaps the Pol I–specific TAFs interact with a different surface of TBP or perhaps sequestering of Pol I transcription to the nucleolus effectively creates two separate pools of TBP. Our experiments show that mutations in TBP can alter the relative amounts of transcription by the nuclear polymerases and prompt the speculation that cells might regulate the balance of the various classes of RNAs by modifying TBP.

Note added in proof: After this paper was submitted, we were made aware of previous work (*30*) describing a codon-based mutagenesis method related to that presented here.

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19. In codon-based mutagenesis, there are 64 codons per position and 20 positions, which together yield 1260 possible outcomes. At a mutation rate of one codon change per oligo, the frequency of a particular change is 1/1280. In nucleotide-based mutagenesis, there are three non–wild-type nucleotides per position and 60 positions, which together yield 180 possible outcomes. At a mutation rate of one nucleotide change per oligo, the frequency of a particular change is 1/180. However, the frequency of obtaining an amino acid substitution that requires three specific nucleotide changes is $(1/180)^3 = 2 \times 10^{-7}$. Assuming a Poisson distribution, approximately a third of the molecules that contain a particular codon change will contain a mutation at another codon or codons. Thus, to achieve onefold coverage of the complete spectrum of single amino acid substitutions, codon-based mutagenesis requires approximately 3000 molecules and nucleotide-based mutagenesis requires approximately 10^7 molecules.
20. The sequences of the six oligonucleotides (bold represents mutagenized codons and italics represents sites used for cloning) are as follows: N1: CCA.AAA.ACT.ACA.GCT.TA*G.ATC.T*TT.**GCA.TCA.GGG.AAA.ATG.GTT.GTT.ACC.GGT.GCA.AAA.AGT.GAG.GAT.GAC.TCA.AAG.CTT.GCC.AGT**.AGA.AAA.TAT.GCA.AGA.ATT.ATC. N2: GAT.GAC.TCA.*AAG.CTT*.**GCC.AGT.AGA.AAA.TAT.GCA.AGA.ATT.ATC.CAA.AAA.ATC.GGA**.TTT.GCT.GCT.AAA.TTC.ACA.GAC. N3: TAA.CCC.T*TC.TAG.A*CG.TAT.AGG.**GAA.TTT.AAC.GTC.ACA.CGA.ACC.GAC.AAT.ATT.TTG.TAT.TTT.GAA.GTC.TGT.GAA.TTT.AGC.AGC.AAA**.TCC.GAT.TTT.TTG.GAT.AAT.TCT. N4: AAA.TTC.ACT.TAG.CAC.AGG.GTA.TAT.**AGC.TTC.AAA.AGC.TTG.GTA.AAT.TTC.TTC.CCT.TTG.CTT.TGC**.ACC.AGT.AAG.AAC.AAT.CTT. N5: TCG.TCT.ACT.*GGT.ACC*.CCA.TCA.**CAT.TTT.TCT.AAA.TTC.ACT.TAG.CAC.AGG.GTA.TAT.AGC**.TTC.AAA.AGC.TTG.GTA.AAT.TTC.TTC.CCT. N6: AAC.TGT.TTT.CGG.*ATC.C*AA.CCT.GCA.CCC.CAA.AGT.CAC.AGT.TGC.**CAC.AAT.GTT.CTG.CAG.TGT.TGG.AAC.AAT.ACC.TGA.TGT.GGC.GGA.GGT**.GTC.TTT.TTC.AGA.TTC.TGG.GG.
These six oligos were used as primers in polymerase chain reaction (PCR) amplifications of a wild-type TBP template. PCR was carried out for 25 cycles of 1 min at 93°C, 3 min at 42°C, and 1 min at 72°C. The amplified fragments were digested with appropriate restriction enzymes and then

subcloned into TBP derivatives into which we had inserted the relevant restriction sites. For oligo N4, the Kpn I site used for cloning was introduced by a second round of PCR with the following primer that overlaps the 5' constant region of the mutated oligonucleotide (N4Kpn): TCT.ACT.*GGT.ACC*.-CCA.TCA.CAT.TTT.TCT.AAA.TTC.ACT.TAG.CAC.-AGG. None of the introduced sites changed the amino acid sequence of TBP. For oligo N1, the PCR fragment was digested with Bgl II; because the Bgl II site would have altered the protein sequence, we introduced a compatible Bcl I site into the TBP gene. The ligation product does not regenerate either site, and the amino acid sequence is not changed. For oligo N6, the PCR fragment was digested with Bam HI and cloned into the naturally occurring Bgl II site in TBP. All six libraries were constructed in a previously described *TRP1* centromeric vector that carried TBP and its own promoter on a 2.4-kb genomic fragment (*31*).

21. W. S. Lee, C. C. Kao, G. O. Bryant, X. Liu, A. J. Berk, *Cell* **67**, 367 (1991).
22. S. Buratowski and H. Zhou, *Science* **255**, 1130 (1992).
23. D. K. Lee, J. Dejong, S. Hashimoto, M. Horikoshi, R. G. Roeder, *Mol. Cell. Biol.* **12**, 5189 (1992).
24. Oligonucleotides 1 through 3 and 4 and 5 averaged 0.85 and 0.5 mutated codons per molecule, respectively. Nucleotide usage at the mutated codons for all six oligonucleotides was 28% for G; 23% for A; 22% for T; and 27% for C.
25. Each library was transformed into yeast strain BYΔ2 (*31*), which contains a deleted TBP locus and a *URA3* centromeric plasmid that carried wild-type TBP; the plasmid encoding wild-type TBP can be removed from the resulting transformants by growth on 5-fluoro-orotic acid, which is selectively toxic to *URA3*+ cells. To screen for temperature-sensitive mutants, we replica-plated transformants on medium that contained 5-fluoro-orotic acid at 23°C, 30°C, and 37°C.
26. The transcriptional patterns of A226T, V122S, and three double mutants were indistinguishable from that of F237R, which is shown in Fig. 3. Similar but less pronounced transcriptional patterns were observed for A226V, I230S, V232H, V232G, and V232K (*28*).
27. D. B. Nikolov *et al.*, *Nature* **360**, 40 (1992).
28. Mutants are referred to by the single-letter code; thus, $Ile^{70} \rightarrow Tyr$ is given as I70Y. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
29. The patch is defined by residues 226, 230, 232, and 237. Residue 122 (defined by V122S) (*28*) maps in the DNA binding region of TBP. The Pol II–specific phenotype of V122S may reflect a DNA binding defect similar to that observed in other TBP derivatives (*3*).
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31. B. P. Cormack, M. Strubin, A. S. Ponticelli, K. Struhl, *Cell* **65**, 341 (1991).
32. We thank R. Green for expert advice and help in synthesizing the mutant oligonucleotides, J. Szostak for generously allowing us free access to his oligonucleotide synthesizers, S. Buratowski for providing the *TDS4* gene, S. Mian for help with Fig. 4, and S. Burley for providing the atomic coordinates from the *A. thaliana* TBP crystal structure. We thank members of the Struhl laboratory for helpful discussions and M. Oettinger and R. Green for critical review of the manuscript. Supported by a predoctoral fellowship from the Howard Hughes Medical Institute (B.P.C.) and a research grant to K.S. from NIH (GM 30186).

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Structure-Based Design of a Cyclophilin-Calcineurin Bridging Ligand

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The affinity of a flexible ligand that adopts a specific conformation when bound to its receptor should be increased with the appropriate use of conformational restraints. By determining the structure of protein-ligand complexes, such restraints can in principle be designed into the bound ligand in a rational way. A tricyclic variant (TCsA) of the immunosuppressant cyclosporin A (CsA), which inhibits the proliferation of T lymphocytes by forming a cyclophilin-CsA-calcineurin complex, was designed with the known three-dimensional structure of a cyclophilin-CsA complex. The conformational restraints in TCsA appear to be responsible for its greater affinity for cyclophilin and calcineurin relative to CsA.

Realizing the full impact of structural biology on basic research and medicine will require the ability to design high-affinity ligands to biological receptors with the use of structural information (*1*). One approach is to constrain the bound conformation of inherently flexible ligands by introducing structure-guided restraints into the ligand. In this way, greater thermodynamic stability of the receptor-ligand complex can be anticipated because the conformational entropy is reduced in the formation of the complex. A particularly challenging problem is posed by the immunosuppressive agents cyclosporin A (CsA), FK506, and rapamycin. These compounds are comprised of two distinct protein-binding surfaces that allow them to bind two proteins simultaneously. By enabling these multimeric complexes to form, the immunosuppressants inhibit specific signaling pathways that control the cell cycle (*2*). In the case of CsA, this property is the basis for the drug's revolutionary impact on clinical organ transplantation. Therefore, efforts to design novel "bridging" ligands not only serve to advance our understanding of the principles of structure-based ligand design, but also may lead to safer and more effective immunosuppressive agents.

It has been noted that the conformations of CsA and FK506, when bound to cyclophilin A and FK506-binding protein 12 (FKBP12), respectively, differ markedly from their unbound conformations in the solid state and in solution (*3, 4*). Although the conformations adopted by these ligands while they are simultaneously bound to their immunophilin receptors and the protein phosphatase calcineurin have not yet been determined, residues that are important for calcineurin binding have been identified (*5–7*). We have used this information to prepare nonnatural immunophilin ligands that mediate immunophilin-ligand-calcineurin complex formation (*8*). We now report on the structure-based de-

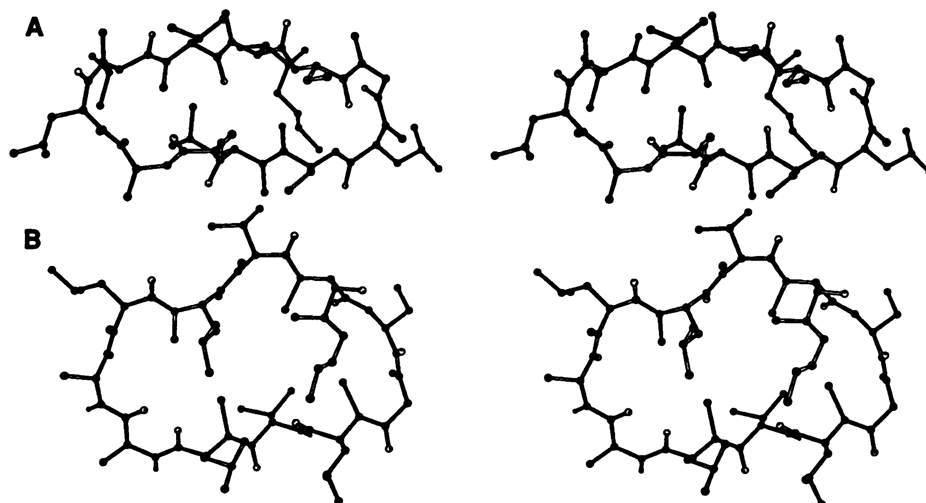

Fig. 1. (**A**) Stereodiagram of the unbound conformation of CsA in the solid state. (**B**) Stereodiagram of the cyclophilin A–bound conformation of CsA.

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