cleft of the Tex S1 domain. The Tex structure provides a model for the core of the eukaryotic transcription factor Spt6 and raises the possibility that the N-terminal portion of Spt6 constitutes a nucleosome-binding domain that evolved from an HtH domain.

Materials and Methods

Tex protein expression and purification

Full-length Tex from *P. aeruginosa* strain PAO1 was cloned into a pET24 *kan* expression vector containing a C-terminal hexahistidine tag. The plasmid was transformed into cells of *E. coli* BL21-codonplus-(DE3)-RP (Stratagene). Cells were grown in LB media and induced with 1 mg/ml IPTG at an OD_{600} of 0.6 or alternatively grown using an autoinduction method as described in Ref. 41. In both cases, cells were grown at 37 °C for 5 h and then transferred to 20 °C and grown to saturation. Harvested cells were stored at −80 °C.

Cells were thawed and resuspended in lysis buffer [50 mM Tris, pH 7.5, 500 mM NaCl, 10% glycerol, 10 mM imidazole, and 2 mM β-mercaptoethanol (BME)] in the presence of lysozyme and protease inhibitors. Following sonication and centrifugation (25,000*g*), the soluble fraction was applied to Ni-NTA agarose resin (Qiagen) and eluted with 300 mM imidazole. Protein was dialyzed (50 mM Tris, pH 7.0, 10% glycerol, 2 mM BME, and 35 mM NaCl), applied to a heparin column (5 ml HiTrap Heparin HP, GE Healthcare), and eluted over a NaCl gradient. ΔS1 Tex was purified in the same way, but a Q ion-exchange column (5 ml HiTrap Q HP, GE Healthcare) was used instead of the heparin column as the ΔS1 construct did not bind heparin. Peak fractions from either heparin or Q columns were pooled, dialyzed (50 mM Tris, pH 7.5, 5% glycerol, 100 mM NaCl, and 2 mM BME), and run over a size-exclusion column (Superdex 200 26/70; GE Healthcare). Se-Met-substituted Tex protein was expressed[42] and purified using the same protocol as native protein.

Tex crystallization and structure determination

Crystals (Se-Met and crystal form I) were grown by sitting drop vapor diffusion in 19% (w/v) polyethylene glycol 3350, 0.1 M Bis–Tris, pH 5.5, 0.17 M ammonium sulfate, and 10% (v/v) glycerol. A second crystal form (crystal form II) was grown in 18% w/v polyethylene glycol 4000 and 100 mM sodium acetate, pH 4.6. Data were collected at the National Synchrotron Light Source (NSLS) at Brookhaven National Lab for crystal form I and on a home source (Rigaku Raxis IV) for crystal form II (Table 1). X-ray diffraction data were processed using HKL2000.[43]

Phases were determined for the form I crystals by the single-wavelength anomalous dispersion method using Se-Met-substituted Tex. The programs SOLVE[44] and RESOLVE[45] were used to identify selenium positions (12 out of 13 potential sites were identified) to calculate initial maps to 3.4 Å resolution followed by phase extension to 2.7 Å. Data from native crystals grown in the same conditions (crystal form I) were subsequently collected to extend the resolution of the Tex structure to 2.5 Å. A 2.3-Å structure (crystal form II) was determined using crystal form I as a model for molecular replacement in PHASER.[46] Refinement was performed using CNS,[47] Phenix,[48] and Refmac.[49] O,[50] Coot,[51] and MolProbity[15] were used for model building and validation. PyMOL[52] was used to prepare figures, superimpose the Tex structures, and perform electrostatic calculations (APBS tools). The ConSurf server[19] was used to calculate evolutionary-based conservation scores.

Nucleic acid binding assays

Oligonucleotides were designed using random sequence and purchased from the University of Utah DNA/Peptide Core facility. Oligonucleotide sequences used in this study are the following (5′ to 3′): UCUUUUCCUGUGUUUUUCCGCAAUC (25 nt ssRNA and 25 bp dsRNA, sense), GATTGCGGAAAAACACAGGAAAAGA (25 nt ssDNA), CGCAGGCCCGGCGCGAGGCCGAGGG (25 bp dsDNA, sense), UCCUGUGUUUUUCCGCAAUC (20 nt ssRNA), UUGUUUUUCCGCAAUC (16 nt ssRNA), UUUUUCCGCAAUC (13 nt ssRNA), and UUCCGCAAUC (10 nt ssRNA). Prior to binding studies, oligonucleotides were gel purified on a 20% acrylamide/7 M urea denaturing gel. Double-stranded substrates were mixed in equimolar amounts in 10 mM Tris–HCl, pH 7.5, and 40 mM KCl with one strand being end labeled with fluorescein. Samples were annealed by boiling (5 min) and slow (2 h) cooling to room temperature and gel purified using a nondenaturing 20% acrylamide gel.

Gel mobility shift assays were performed by mixing varying concentrations of protein with nucleic acid substrate in binding buffer [15 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10% glycerol, and 1 U/μl RNasin (Promega) or 1 U/μl RNaseOUT (Invitrogen)]. The final concentration of nucleic acid used in each experiment was at least 20-fold below the K_d for the respective substrate/protein. Reactions were incubated at room temperature for 30 min and then assayed by electrophoresis using 4–20% TBE native gels (Bio-Rad Laboratories) at room temperature. Gels were imaged and quantified using a TYPHOON imaging system with ImageQuant software (GE Health Sciences). The fraction bound was calculated by quantifying the RNA/DNA_{total} (total fluorescence in entire lane) and RNA/DNA_{free}. All RNAs/DNAs of slower mobility than the RNA/DNA_{free} were considered bound. The fraction bound = $1-([RNA/DNA]_{free}/[RNA/DNA]_{total})$. Dissociation constants (K_d values) were calculated by plotting data points and curve fitting using the Hill formalism where fraction bound = $1/(1+(K_d^n/[P]^n))$. Average K_d values were determined by fitting data points from individual experiments and then averaging the calculated dissociation constants. All plots and curve fits were performed using the program KaleidaGraph (Synergy Software).

FP

FP was performed in 96-well format using a Tecan fluorimeter with the same fluorescein-labeled substrates and buffer conditions as the shift analysis. Tex concentrations were varied in individual wells and mixed with RNA at a final concentration that was at least 10-fold below the K_d. Samples were incubated at room temperature for at least 30 min prior to measuring polarization. Polarization values (P) were measured and plotted as a function of Tex concentration. Data points were fit using $P=((P_{bound}-P_{free})\ [Tex]/K_d+[Tex])+P_{free}$. The free and total protein concentrations are assumed to be equal because the RNA concentration is at least 10-fold lower than the K_d.

When using FP to evaluate binding stoichiometry, polarization measurements were performed following the procedure described in Ref. 53. RNA was mixed in