Mutational Analysis of the Yeast DEAH-Box Splicing Factor Prp16

Hans-Rudolf Hotz and Beate Schwer

Department of Microbiology, Cornell University Medical College, New York, New York 10021
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ABSTRACT

Prp16 is an essential yeast splicing factor that catalyzes RNA-dependent hydrolysis of nucleoside triphosphates. Prp16 is a member of the DEAH-box protein family, which is defined by six collinear sequence motifs. The importance of residues within four of the conserved motifs was assessed by alanine-scanning mutagenesis. Mutant alleles of PRP16 were tested for in vivo function by complementation of a Δprp16 null strain. In motif I (GETGSGKT), alanine substitutions at Gly-378, Lys-379, and Thr-380 were lethal, whereas replacement of the amino acids in positions 373-377 were viable. In the signature DEAH-box (motif II), Asp-473 and Glu-474 were essential, whereas the H476A mutant was viable. The S505A and T507A mutants in motif III (SAT) were viable. In motif VI (QRSGRAGRTAPG), mutants Q685A, R686A, G688A, R689A, and R692A were lethal, whereas G691A, P695A, and G696A supported growth. Instructive structure-function relationships were established by conservative substitutions at essential residues identified by alanine scan. Overexpression of nonviable alleles impaired the growth of wild-type PRP16 cells. Deletion analysis of the 1071-amino-acid Prp16 protein revealed that the N-terminal 204 amino acids and the C-terminal 100 residues were dispensable for PRP16 function in vivo. These studies provide an instructive framework for functional analysis of other DEAH-box splicing factors.

Pre-mRNA splicing entails the ordered assembly of U1, U2, U4/U6/U5 snRNPs, and trans-acting proteins onto the pre-mRNA to form a spliceosome. Catalysis occurs by two sequential transesterification reactions. In step 1, the 5′ splice site is cleaved and a branched lariat intermediate is formed. In step 2, the 3′ splice site is cleaved and the two exons are joined (reviewed by Moore et al. 1993). The reaction is driven by conformational rearrangements in the spliceosome that are triggered by nucleoside triphosphate (NTP) hydrolysis (Schwer and Guthrie 1992a; Kim and Lin 1996; reviewed by Madhani and Guthrie 1994; Nilsen 1994). The yeast splicing factors Prp2, Prp16, and Prp22 act at sequential ATP-requiring steps during the splicing reaction (reviewed by Ruby and Abelson 1991; Beggs 1995). Prp2, Prp16, and Prp22 are members of the DExH-box family of nucleic acid-dependent ATPases (Chen and Lin 1990; Burgess et al. 1990; Company et al. 1991). This family is defined by six conserved motifs arrayed in a collinear fashion (Goral et al. 1989; Linder et al. 1989). All three yeast proteins display RNA-dependent ATPase activity (Kim et al. 1992; Schwer and Guthrie 1991; B. Schwer and C. Gross 1998). The 100-kD Prp2 protein functions before the first cleavage/ligation reaction, i.e., it transiently associates with the spliceosome to activate it for the first catalytic step (King and Beggs 1990; Kim and Lin 1993). The 121-kD Prp16 protein is required for the second step. Hydrolysis of NTP by Prp16 elicits a conformational change in the spliceosome, which is seen as protection of the 3′ splice site region in the pre-mRNA from oligo-directed RNAseH cleavage (Schwer and Guthrie 1992a). The 130-kD Prp22 protein is necessary for release of mRNA from the spliceosome (Company et al. 1991; B. Schwer and C. Gross, 1998).

DEAH-box proteins play important roles in a variety of biological processes, including recombination, transcription, and DNA repair. Among the DExH proteins with documented RNA helicase activity are vaccinia virus nucleoside triphosphate phosphohydrolase-II (NPH-II), mammalian helicase A (HuHelmA), Drosophila maleless, and hepatitis C virus NS3 protein (Shuman 1992; Lee and Hurwitz 1993; Lee et al. 1997; Jin and Peterson 1995; Kim et al. 1995). The conserved sequence motifs presumably constitute a component of the active site for nucleic acid-dependent NTP hydrolysis shared by the DExH proteins. However, the structural basis for properties such as cofactor and substrate specificity remains unknown. Segments flanking the highly conserved ATPase domain may contribute to the specific biological functions of individual DExH proteins, e.g., by facilitating essential protein-protein interactions.

The goals of this study were as follows: (1) to define the minimal domain of Prp16 capable of sustaining cell growth and (2) to identify individual amino-acid residues within the ATPase domain essential for Prp16 function. Our mutagenesis strategy was guided by sequence similarity between Prp16 and other DExH- and DEAD-box proteins, particularly NPH-II, hepatitis C virus NS3,
and eIF-4A (Figure 1), for which NTPase and RNA helicase activity have been demonstrated and extensive mutational and biochemical data are available (Shuman 1992; Gross and Shuman 1995, 1996; Heil ek and Peter son 1997; Pause et al. 1993; Pause and Sonenberg 1992; Ray et al. 1985; Rozen et al. 1989; Schmid and Linder 1991). Using those studies as a framework, we have identified 10 residues that are crucial for Prp16 function and determined the essential features of the individual amino acid side chains by conservative replacements. We show that overexpression of defective prp16 alleles in a wild-type PRP16 strain elicits a dominant negative growth phenotype.

MATERIALS AND METHODS

Deletion mutants of PRP16: N-terminal deletion variants were generated by PCR amplification using oligonucleotide primers that introduced NdeI restriction sites at the codons for Ser136, Ile205, and Met226, substituting the respective amino acid by methionine. NdeI/Xhol fragments of the PCR-amplified DNA (490, 283, and 220 bp, respectively) were inserted into p358-PRP16 (CEN TRP1) in lieu of the wild-type NdeI/Xhol fragment (Schwer and Guthrie 1991). C-terminal truncations were created using oligonucleotide primers that introduced a stop codon at residues 922, 972, or 1022, respectively. ClaI/SphI fragments (1030, 880, and 730 bp, respectively) were inserted into p358-PRP16 in place of the wild-type fragment. We sequenced the entire fragments that were replaced in each case to exclude the occurrence of unwanted, PCR-generated mutations.

Test of PRP16 function by plasmid shuffle: Strain YXP16 (MATα ura3 trp1 his7 prp16::LYS2 p360-PRP16-1) was transformed with p358 plasmids (CEN TRP1) bearing wild-type PRP16 (p358-PRP16) or mutant alleles as specified. Trp+ transformants were selected and plated on medium containing 5-FOA to select against retention of p360-PRP16-1 (URA3 CEN; Boeke et al. 1987). Selection was performed at 25° and 30°.

Overexpression of PRP16 alleles: Plasmid pGAL10-PRP16 (2μ LEU2) contains wild-type PRP16 under the control of the GAL10 promoter. pGAL10-PRP16 was digested with Xhol/SphI and the corresponding fragments from the mutant alleles were inserted. YXP17 (MATα ura3 trp1 his7 leu2 prp16::LYS2 p360-PRP16-1) was transformed with plasmids pGAL10-PRP16 and the mutant alleles as specified. To test if overexpression of these plasmids could support growth of the Δprp16 null strain, Leu+ cells were streaked to 5-FOA plates containing 2% galactose. To assess the effect of overexpression in a PRP16 wild-type strain, YHR1 (MATα ura3 trp1 his7 leu2 prp16::LYS2 p358-PRP16-1) was transformed with plasmids pGAL10-PRP16 and the mutant alleles as specified. Leu+ transformants were grown in liquid culture (SD-Leu medium containing 2% glucose) to late logarithmic growth phase and diluted to an OD600 of 0.1. Serial dilutions (10-fold) were spotted in parallel to glucose and galactose-containing SD-Leu medium.

RESULTS

Truncation mutants: To define the minimal functional domain of Prp16, a series of N- and C-terminal deletion mutations was designed to progressively truncate the 1071-amino-acid Prp16 protein (Figure 2). The in vivo function of the truncated genes was tested by using the plasmid shuffle procedure (Boeke et al. 1987). Growth of the Δprp16 null strain YXP16 depends on an extrachromosomal copy of PRP16 on a CEN URA3 plasmid. Wild-type and truncated PRP16 alleles on CEN
Mutational Analysis of Prp16

TRP1 plasmids were introduced into YXP16 cells. Trp+ transformants were plated on medium containing 5-FOA to select against retention of the URA3 plasmid. As expected, cells containing the vector were incapable of growth on 5-FOA, whereas cells bearing the PRP16 plasmid grew readily. Alleles that did not support growth on 5-FOA are marked as “lethal” in Figure 2A. Mutants that survived under counterselective conditions were tested for complementation of a Δprp16 null strain using the plasmid shuffle technique. The mutated genes were named according to the amino acid coordinates of their polypeptide products. (A) The bars represent the size of the truncated Prp16 proteins with the NTPase domain indicated by the darker shading. Alleles that did not support growth on 5-FOA plates are marked “lethal.”

The growth phenotypes for the functional alleles are marked as follows: ++, growth was indistinguishable from that of wild-type cells; +, cells grew slower than wild type; -, no colonies were formed after 3 days of incubation at 25°C, 30°C, and 37°C (or 6 days at 15°C). (B) Temperature-sensitive growth of PRP16 deletion mutants. Strains containing full-length or deletion alleles were streaked to YPD medium. The plates were incubated at the indicated temperatures and photographed after 3 days (6 days in case of 15°C incubation).

### Table: Mutations of PRP16 Tested for Complementation

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<tr>
<th>Allele</th>
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<th>Growth at 30°C</th>
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Figure 2.—Truncation mutations of PRP16 were tested for complementation of a Δprp16 null strain using the plasmid shuffle technique. The mutated genes were named according to the amino acid coordinates of their polypeptide products. (A) The bars represent the size of the truncated Prp16 proteins with the NTPase domain indicated by the darker shading. Alleles that did not support growth on 5-FOA plates are marked “lethal.”

The growth phenotypes for the functional alleles are marked as follows: ++, growth was indistinguishable from that of wild-type cells; +, cells grew slower than wild type; -, no colonies were formed after 3 days of incubation at 25°C, 30°C, and 37°C (or 6 days at 15°C). (B) Temperature-sensitive growth of PRP16 deletion mutants. Strains containing full-length or deletion alleles were streaked to YPD medium. The plates were incubated at the indicated temperatures and photographed after 3 days (6 days in case of 15°C incubation).
A  

**motif I**

<table>
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<th>lethal</th>
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<th>E374</th>
<th>T375</th>
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</tbody>
</table>

**viable**

| 15°   | ++   | ++   | ++   | ++   | +    | ++   |
| 25°   | ++   | ++   | ++   | ++   | +    | ++   |
| 30°   | ++   | ++   | ++   | ++   | +    | ++   |
| 37°   | ++   | ++   | ++   | ++   | +    | ++   |

**Growth**

(B) Temperature-sensitive growth of PRP16 alleles compared with wild-type PRP16 (wt).

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**motif II**

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<th>A476</th>
<th>H476</th>
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<td>✗</td>
<td>✗</td>
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</table>

**viable**

| 15°   | ++   | ++   | ++   | ++   | ++   |
| 25°   | ++   | ++   | ++   | ++   | ++   |
| 30°   | ++   | ++   | ++   | ++   | ++   |
| 37°   | ++   | ++   | ++   | ++   | ++   |

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**motif III**

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<th>T507</th>
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<td>↑</td>
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</table>

**Figure 3.—Point mutations in motifs I–III.**  
(A) The wild-type sequences of the motifs are indicated (the numbers specify the position of the respective residue in the wild-type sequence). The lethal substitutions are listed above the sequence; viable mutants are listed below. 5-FOA survivors were tested for growth at different temperatures, and the growth phenotypes were scored as follows: ++, growth was indistinguishable from that of wild-type cells; +, cells grew slower than wild-type; +/−, only very small colonies were formed; −, no colonies were formed after 3 days of incubation at 25°, 30°, and 37° (or 6 days at 15°).
subsequently streaked to YPD plates and incubated at 15°, 25°, 30°, and 37° (Figure 2, A and B). The deletion of 135 and 204 amino acids from the N terminus of Prp16 did not affect the ability of the PRP16(136-1071) or PRP16(205-1071) allele to support growth at all temperatures tested. However, deleting 225 amino acids from the N terminus of Prp16 resulted in a nonfunctional protein. Prp16 was less tolerant of C-terminal deletions. Whereas 50 amino acids could be removed without affecting cell growth, deleting 100 amino acids resulted in a temperature-sensitive growth phenotype; i.e., cells bearing the PRP16(1-971) allele did not grow at 37°. Deletion of 150 amino acids from the C terminus was lethal. We conclude that the N-terminal 204 and the C-terminal 100 residues in the Prp16 protein are not essential for in vivo function.

We created alleles that were truncated from both the C and N termini. Strains carrying PRP16(136-971) and PRP16(1-971) were not viable, whereas cells carrying the PRP16(136-1021) and PRP16(205-1021) alleles grew at 30° but were temperature sensitive (Figure 2B). Thus, although the C-terminal region of Prp16, from amino acids 972 to 1021, is not essential per se, it becomes important when the N terminus is deleted. This may result from misfolding of the mutant proteins or, alternatively, the segment from amino acids 972–1021 may be functionally redundant to the nonessential N terminus of Prp16.

Missense mutations in motifs I–III: We identified essential amino acids in motifs I–III by alanine-scanning mutagenesis. In motif I (GETGSGKT), residues Gly-373, Glu-374, Thr-375, Gly-376, and Ser-377 could be replaced without affecting the in vivo function of Prp16. However, alanine substitutions at Gly-378, Lys-379, and Thr-380 were lethal (Figure 3A). To establish a structure-function relationship at Lys-379, we introduced conservative (arginine) and nonconservative (glutamate) changes. K379E was lethal, whereas K379R could sustain growth, although the colony size was much smaller than that of wild-type cells (Figure 3B). This suggests that a basic side chain is important at this position. The essential feature of Thr-380 appears to be the hydroxyl group rather than the size of the side chain because T380S was viable, whereas T380V was lethal.

In motif II (DEAHE), alanine substitutions for Asp-473 and Glu-474 were lethal, whereas the H476A and E477A were viable. We examined the important features of the side chains at positions 473 and 474 by introducing conservative changes. Asp-473 and Glu-474 could be replaced by glutamate and aspartate, respectively, but not by asparagine and glutamine. We surmise that at each of these positions an acidic side chain is essential for Prp16 function. We also replaced His-476 by aspartic acid, the signature residue in DEAD-box proteins. The H476D mutant was lethal at low temperatures (25° and 15°), and growth was impaired even at 30° or 37° (Figure 3B).

In motif III (SAT), the S505A and T507A mutants were viable at 30°; however, T507A was cold sensitive (Figure 3B). Therefore, the side chains at these positions are not essential for Prp16 function.

Structure-function relationships in motif VI: Alanine substitutions in motif VI (QRSGRAGRTAPG) of Prp16 established that Gln-685, Arg-686, Gly-688, Arg-689, and Arg-692 were essential, whereas Ser-687, Gly-691, Thr-693, Pro-695, and Gly-696 were not (Figure 4A). We introduced conservative and nonconservative substitutions for the essential residues. Arg-686 could be substituted by lysine, glutamine, and iso-leucine, although R686Q and R686I mutants were temperature sensitive for growth (Ts: C, Cs: Cs−, respectively; Figure 4B). In contrast, Arg-689 and Arg-692 were strictly essential, as substitutions by either lysine or glutamine were lethal (Figure 4A). We also substituted the essential glutamine at position 685 with histidine. (eIF-4A contains a histidine in this position in lieu of a glutamine in DExH box proteins.) The Q685H mutant of Prp16 was viable at 30° and 37°, but showed a cold-sensitive growth defect.

Nonfunctional Prp16 mutants exert dominant negative effects: Previous studies had demonstrated that Prp16 binds to the spliceosome in the absence of ATP, and that a mutant version of Prp16 with reduced ATPase activity (Prp16-1) competed for spliceosome binding with the wild-type protein (Schwer and Guthrie 1992b). This raised the question of whether the nonfunctional Prp16 mutants identified in this study might retain the ability to compete with wild-type protein for binding to the spliceosome and, thus, inhibit growth of a wild-type strain. Mutant alleles G378A, K379A, T380A, and D473A (motifs I and II), as well as Q685A, G688A, R689A, R689Q, R689K, R692A, R692Q, and R692K (motif VI), were placed under the transcriptional control of a GAL10 promoter on a 2μ LEU2 plasmid. The 2μ plasmids were transformed into a PRP16 wild-type strain. Leu+ transformants were selected, and serial dilutions were subsequently spotted to plates containing glucose or galactose as a carbon source (Figure 5). Whereas all the strains grew readily on glucose-containing medium, growth was inhibited on galactose-containing medium (Figure 5 and data not shown). Overexpression of wild-type PRP16 had no effect. As reported for the prp16-1 allele (Schwer and Guthrie 1992b), overexpression of the H476A allele was deleterious to growth only at low temperature (not shown). In summary, mutations of conserved essential residues within the NTPase domain of Prp16 elicited a dominant negative growth defect when overexpressed in an otherwise wild-type cell. We also assessed the effects of overexpression of nonfunctional N- and C-terminal truncation mutants PRP16(226-1071), PRP16(1-921), and PRP16(205-971). We found that growth of wild-type cells was not affected when the truncated versions were overexpressed (overexpression was confirmed by Western blot-
Figure 4.—Point mutations in motif IV. (A) The wild-type sequence of motif VI is indicated (the numbers specify the position of the respective residue in the wild-type sequence). The lethal substitutions are listed above the sequence; viable mutants are listed below. The 1 and 2 indicate the growth phenotypes of the 5-FOA survivors (scored according to the criteria in Figure 3). (B) Temperature-sensitive growth of PRP16 alleles compared with wild-type PRP16 (wt).

The lethal substitutions are listed above the sequence; viable mutants are listed below. The + and − indicate the growth phenotypes of the 5-FOA survivors (scored according to the criteria in Figure 3). (B) Temperature-sensitive growth of PRP16 alleles compared with wild-type PRP16 (wt).

(Continued)

d) suggesting that the deletion mutants were not capable of binding to the spliceosome (data not shown). Each of the GAL-PRP16 missense and truncation alleles described above (on 2 μ LEU2 plasmids) was tested for growth on galactose-containing 5-FOA medium. None of these high-copy alleles supported cell growth (not shown). Hence, the lethal phenotype was not reversed by increased gene dosage and enhanced transcription.

DISCUSSION

We have presented a mutational analysis of the GxxGxGKT, DExH, SAT, and QRxGRxGRxxPG motifs in the splicing factor Prp16, an RNA-dependent NTPase. Our results demonstrate that individual residues within the targeted motifs are important for Prp16 function. Motif I corresponds to Walker motif A, which binds the β and γ phosphates of the nucleotide in the crystal structures of several NTPases (Pai et al. 1990; Story and Steitz 1992; Subramanya et al. 1996; Korolev et al. 1997). In Prp16, residues Gly-378, Lys-379, and Thr-380 in this motif could not be substituted by alanine. However, K379R grew (albeit at reduced efficiency), arguing that a basic side chain is essential for ATP binding. Biochemical studies of NPH-II and HCV NS3 protein showed that replacement of the equivalent lysine residues by alanine dramatically reduced NTPase and...
Mutational Analysis of Prp16

Mutational Analysis of Prp16

In their discussion of the crystal structure of the HCV NS3 RNA helicase domain, Yao et al. (1997) suggest that motif III (S/TAT) is part of the “switch region” responsible for the transmission of conformation changes triggered by NTP hydrolysis. In mammalian eIF-4A, replacement of SAT by AAA yielded a protein that could hydrolyze ATP but was defective in RNA unwinding (Pause and Sonenberg 1992). Single alanine substitutions of either threonine residue in the TAT motif of NPH-II inactivate the RNA helicase function without abolishing ATPase activity (C. Gross and S. Shuman, personal communication). In Prp16, Ser-505 and Thr-507 in motif III can be replaced by alanine. It will be interesting to see if the correlation between the essentiality of these specific conserved residues and RNA helicase activity persists in other DExH box members.

The role of motif VI (QRxGRxGRxxPG) in NTP hydrolysis and RNA unwinding has been established (Gross and Shuman 1996; Pause et al. 1993). Gln-685, Arg-686, Gly-688, Arg-689, and Arg-692 mutations in Prp16 abolish its function in vivo. Alanine substitutions of the corresponding residues in NPH-II caused severe defects in RNA unwinding and ATP hydrolysis (Gross and Shuman 1996). Gly-691 and Gly-696 are not essential for Prp16 function but are important for the catalytic activities of NPH-II (Gross and Shuman 1996). Mutations in this motif in eIF-4A abolish cross-linking of eIF-4A to RNA, prompting the speculation that the defect in enzymatic activity is caused by the inability to bind RNA (Pause et al. 1993). A 53-amino-acid segment of the plum pox potyvirus RNA helicase that includes motif VI was found to be sufficient for RNA binding, as determined by Northwestern assays (FernaÂndez et al. 1995). In contrast, vaccinia NPH-II can bind RNA with high affinity in spite of mutations in motif VI that abolish ATPase activity (Gross and Shuman 1996).

We show that lethal mutations of Prp16 within motifs I, II, and VI cause a dominant negative growth defect when overexpressed in a wild-type PRP16 cell. We infer that the mutant versions retain partial function and interfere with the wild-type Prp16 protein. In particular, we suggest that the mutants can bind to the spliceosome but are impaired for ATP hydrolysis. This suggestion is based on the studies of Prp16-1 protein, demonstrating that the mutant protein with reduced ATPase activity competes with wild-type protein for spliceosome binding (Schwer and Guthrie 1992b). Preliminary studies of the mutant Prp16-K379A in vitro support this hypothesis; recombinant Prp16-K379A protein is inactive for splicing and it inhibits the splicing reaction when provided in excess over wild-type Prp16 protein (our unpublished results). In a genetic screen for dominant negative mutants of Prp2, Plumpton et al. (1994) isolated Prp2dn in which the serine in motif III (SAT) was replaced by leucine in Prp2. The corresponding mutation,
when introduced by site-directed mutagenesis in HRH1, the human homolog of Prp22, caused a dominant negative splicing defect and negatively affected export of mRNA in mammalian cells (Ohno and Shimura 1996). Our analysis demonstrates that Ser-505 and Thr-507 are not essential for Prp16 function, as they could be substituted by alanine. However, the S505L mutant of Prp16 is lethal and causes a dominant negative growth defect upon overexpression (Figure 5 and data not shown). Thus, although the serine residue in the SAT motif of Prp16 is not essential per se, changing it to the bulkier leucine results in a nonfunctional protein.

This study shows that conserved amino acid residues important for NTPase activity of the DExH-box proteins NPH-II and HCV NS3 protein are essential for Prp16 function in vivo. Because lethal mutations within the conserved motifs are dominant when overexpressed, we assume that these motifs are important for the catalysis of NTP hydrolysis rather than for binding of the Prp16 protein to the spliceosome. Our finding that overexpression of nonviable mutants that were truncated from the C and N termini does not interfere with wild-type Prp16 suggests that the segments flanking the NTase domain are required for spliceosome binding.

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