Human snRNP polypeptide D1 promotes pre-mRNA splicing in yeast and defines nonessential yeast Smd1p sequences

Brian C.Rymond*, Luis A.Rokeach^{1,*} and Sallie O.Hoch¹

T.H.Morgan School of Biological Sciences, University of Kentucky, Lexington, KY 40506-0225 and

¹The Agouron Institute, 505 Coast Boulevard South, La Jolla, CA 92037, USA

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ABSTRACT

Parallel investigations of yeast and metazoan pre-mRNA splicing have documented enormous complexity in the nucleic acid and protein components of the cellular splicing apparatus, the spliceosome. The degree to which yeast and metazoan spliceosomal proteins differ in composition and structure is currently unknown. In this report we demonstrate that the human small nuclear ribonucleoprotein (snRNP) polypeptide D1 complements the cell lethality, splicing deficiency, and snRNA instability phenotypes associated with a yeast *smd1* null allele. Mutational analysis of yeast *SMD1*, guided by a comparison of the predicted yeast and human proteins, reveals that a large, nonconserved portion of Smd1p is dispensable for biological activity. These observations firmly establish D1 as an essential component of the cellular splicing apparatus and suggest that yeast and metazoa are remarkably similar in the polypeptides guiding early snRNP assembly.

INTRODUCTION

The excision of intervening sequences (introns) from messenger RNA precursors is a ubiquitous feature of eukaryotic gene expression (reviewed in 1–2). Three intron sequences, the 5' splice site, the branchpoint region, and 3' splice site provide chemically reactive nucleotides to the two-step reaction pathway of nuclear pre-mRNA splicing. The enzyme catalyzing this RNA processing reaction is a large ribonucleoprotein termed the spliceosome. In both metazoa and yeast, spliceosome assembly occurs through the ordered association of the U1, U2, and U4/5/6 small nuclear ribonucleoprotein (snRNP) particles with the pre-mRNA substrate. Each snRNP is composed of one or more small nuclear RNAs (snRNAs), a set of common proteins (B, B', D1, D2, D3, E, F, G, as defined in metazoa) and a variable number of snRNP-specific or unique polypeptides. According to current models, the numerous trans-acting RNAs and proteins of the spliceosome function principally to organize the pre-mRNA

substrate into a splicing competent state. The active site of the spliceosome, though likely RNA-based, remains ill defined.

In both mammalian cells and *Xenopus* oocytes, snRNP assembly begins in the cytoplasm (reviewed in 3,4). An RNA-free, 6S complex (5,6) composed of core proteins D1, (D2 or D3), E, F, G initiates snRNP assembly by binding to the Sm site, a single-stranded region flanked by short hairpin structures, present on the U1, U2, U4, and U5 snRNAs. While the sequences mediating core protein association may vary somewhat between snRNA species, each contains the single-stranded RNA consensus element RA(U)₃GR (reviewed in 7). Early nuclease protection studies indicated that the Sm consensus element interacted directly with core proteins (8). This suggestion was reinforced by the recent demonstration that core protein G specifically crosslinks to the Sm site of isolated snRNP particles (9). The established snRNA-core protein complex increases snRNA stability, enhances the binding of other snRNP polypeptides, and is necessary for the subsequent trimethylation of the snRNA cap and import of the snRNP particle into the nucleus (3,4). Nuclear translocation requires a cytoplasmic transport factor distinct from that which mediates the import of SV40 T antigen-like nuclear localization signals (10). A trimethylguanosine (TMG) snRNA cap enhances nuclear import, although the degree of stimulation associated with cap hypermethylation differs between snRNP species (U1, U2 > U4,U5; see 10 and references within). Inside the nucleus, the role(s) of the common snRNP polypeptides in promoting spliceosome assembly and splicing are unknown. In vitro, mutations to the human U4 Sm site block core protein assembly during snRNP reconstitution and inhibit but do not abolish pre-mRNA splicing (11). A cautious explanation for this observation is that (at least) certain snRNP Sm-site/core protein interactions are dispensable after nuclear importation.

Compared with the metazoan system, very little is known about the early events of yeast snRNP assembly. The low abundance of yeast snRNP particles has hampered the identification of snRNP polypeptides by traditional biochemical techniques. Yeast genetic approaches have been very helpful for the isolation of

* To whom correspondence should be addressed

⁺ Present address: Department de Biochimie Faculte de Medecine, Universite de Montreal, C.P. 6128, Montreal, Quebec H3C 3J7, Canada

PRP (pre-mRNA processing) genes involved in the splicing process but have not yielded candidates coding for common snRNP polypeptides (1,2). Nevertheless, several lines of evidence suggest that analogous core snRNP structures exist in yeast. First, the yeast U1, U2, U4 and U5 spliceosomal snRNAs, while diverged greatly from their metazoan counterparts in primary structures, do retain the conserved single-stranded Sm site consensus (although the U1, U4, and U5 snRNAs lack the associated downstream hairpin) (7). The yeast Sm sequences are biologically functional. Injection of yeast snRNAs into *Xenopus* oocytes readily converts most spliceosomal snRNAs into an RNP form precipitable with antibodies specific for the core proteins (i.e., anti-Sm antibodies) (12). Second, anti-Sm antibodies will, albeit with low efficiency, precipitate native yeast snRNP particles (13,14,15). Third, certain Sm site point mutations are growth inhibitory or lethal in yeast (16). The Sm mutant phenotypes correlate with decreased snRNP stability and increased snRNA decay and appear to reflect the primary importance of the Sm site rather than a general sensitivity of spliceosomal snRNAs to nucleotide changes. This latter point is shown most dramatically by the fact that, in vivo, the 186 nucleotide human U2 snRNA can substitute for its 1175 nt yeast U2 counterpart (17). Finally, *SMD1* (18), identified as a genetic neighbor of *PRP38* (19), encodes a small basic polypeptide (Smd1p) that is similar in structure to human D1. Furthermore, cells metabolically depleted of Smd1p activity display characteristics consistent with the loss of a core polypeptide: a) pre-mRNA splicing is inhibited, b) the U1, U2, U4, and U5 snRNAs are selectively reduced in abundance and, c) the precipitability of residual spliceosomal snRNAs with anti-TMG cap antibodies is decreased (18).

In this communication, we address the question of *SMD1* function in snRNP biogenesis using a demanding genetic test, in vivo complementation of a yeast *smd1* null allele by a cDNA copy of human D1. The results of this test and a deletion analysis of *SMD1* strongly support the view that Smd1p is the yeast D1 core polypeptide and provide insight into the structural requirements for Smd1p activity.

MATERIALS AND METHODS

Plasmid and strain construction

The *smd1::URA3* null allele was created by substituting the yeast *URA3* gene (20) for *SMD1* codons 76–147. A 2kb *XbaI*–*PstI* subclone with *SalI* sites flanking the *SMD1* residues to be deleted (18) was cleaved with *SalI* and rendered blunt-ended with mung bean nuclease. *URA3* DNA, on a similarly blunted 1.2 kb *HindIII* fragment, was ligated into the remaining *SMD1* sequence. The resultant *smd1::URA3* allele was then released from the cloning vector by digestion with *XbaI* and *PstI* and used to replace one chromosomal copy of *SMD1* in diploid strain MGD407 (18) by homologous recombination. Putative disruptants were selected as Ura⁺ colonies on media lacking uracil and the correct insertion event was confirmed by southern blot analysis. Plasmid pHSMD1368 (21) was introduced by transformation into the heterozygous (i.e., *SMD1/smd1::URA3*) diploid strain. Selected transformants were sporulated and their asci dissected on galactose-based YEP medium (22). The haploid progeny were scored for the presence of the chromosomal *smd1::URA3* disruption and plasmid pHSMD1368 on synthetic media lacking uracil and leucine, respectively. All strains were scored for growth on galactose-based and glucose-based media.

The *smd1Δ20* and *smd1Δ27* alleles were constructed by site-directed mutagenesis (23) using mutagenic oligonucleotides AACAGTAATGGTATTAGAGGCAATACCATA and AGTAATGGTATTGCGTTGCAATACATAAAC. The mutant alleles were confirmed by DNA sequence analysis of the entire *SMD1* gene. The *SMD1* deletion derivatives were subcloned into the *TRP1*-containing YCplac22 plasmid for yeast transformation into wild-type strain MGD353 46D *SMD1* (18), or strains bearing the *smd1::URA3* or *smd1::LEU2* (18) alleles. In each case where a chromosomal *smd1* null mutation was assayed, a second plasmid with a galactose inducible yeast (*GAL1::SMD1*) or human (*GAL1::HD1*) allele was also present. The complementing activities of *smd1Δ20* and *smd1Δ27* alleles were assayed at 30°C in the null background by transcriptionally repressing the *GAL1*-promoter fusion derivatives in YEP media containing 2% glucose.

RNA methods

RNA was extracted from whole cell lysates (24) of cultures grown continuously at 30°C on YEP galactose (22) or from cultures shifted to glucose-based media for 10 hours. All cultures were diluted sufficiently at the time of transfer to obtain a measured cell density at the time of harvest of less than 0.6 (O.D.₆₀₀). Samples of 10–20 μg of total RNA were resolved on 1% agarose/formaldehyde gels (for splicing analysis) or denaturing 7M urea, 5% polyacrylamide gels (for snRNA analysis) and subsequent northern transfers probed with random-primed probes complementary to *RP51A* intron/exon sequences or the U1, U2, U4, U5, and U6 snRNA as described (19).

RESULTS

In order to assay the ability of human D1 to function in yeast we first created a null allele of its putative cognate yeast gene, *SMD1*. The *URA3* gene was inserted into the yeast-specific region between conserved segments I and II (figure 1) as described previously in the construction of an analogous null allele, *smd1::LEU2* (18). All *SMD1* coding sequences 3' to the point of insertion (i.e., codons 76–146) were deleted. A diploid strain heterozygous at the *SMD1* locus for this *smd1::URA3* disruption was then transformed with plasmid pHSMD1368 (21) which carries the human D1 gene fused to the nutritionally regulated *GAL1* promoter (*GAL1::HD1*). Three independent diploid transformants were sporulated and their meiotic products dissected to obtain haploid strains expressing only the human *GAL1::HD1* fusion gene. As summarized in figure 2, the genotypes and growth characteristics of the meiotic progeny demonstrated that: i. the *smd1::URA3* disruption mutation is lethal, ii. pHSMD1368, encoding human D1, complements the *smd1::URA3* lesion, and iii. complementation depends on transcriptional activation of *GAL1::HD1*. Since we have no measure of the relative Smd1p and D1 protein abundance in yeast, it is uncertain whether the unit activity of the human protein is equivalent to its yeast counterpart. Nevertheless, based on these observations we conclude that under the conditions employed the human D1 polypeptide effectively substitutes for yeast Smd1p.

SnRNP proteins D1, (and perhaps D2 or D3), E, F, and G assemble into a cytoplasmic RNA-free core prior to recruitment by the U1, U2, U4, and U5 snRNAs and incorporation into the spliceosome (25). Given the number of D1 proteins involved and the overall complexity of the spliceosome (26), multiple

Figure 1. Comparison of the human and yeast Smd1p proteins. The dark boxes represent regions of high local sequence similarity. Smd1p residues 1–28 (segment I) and 88–109 (segment II) share 75% and 77% sequence identity, respectively, with human D1. Small case letters identify the residues (amino acids 61–87) unique to yeast Smd1p. The carboxyl-terminal 36 amino acids are composed of 33% (yeast) and 44% (human) lysine and arginine residues.

D1-mediated protein and RNA contacts are likely. Even though human D1 differs from Smd1p in size and sequence it functions well as the sole D1 source in yeast and does not display dominant-negative effects when co-expressed with the native yeast polypeptide. The robust growth of pHSM1368-transformed *smd1::URA3* cells and their sensitivity to the transcriptional repression of the *GAL1::HD1* is clearly evident in a plate assay of galactose-grown and glucose-grown cultures (figure 3). At 30°C, *smd1::URA3* mutants expressing *GAL1*-driven human D1 gene grow with a generation time only slightly longer (approximately 1.25-fold) than wild-type strains expressing *SMD1*. In contrast, after 10–12 hours on glucose-based media cell division ceases (data not shown).

Splicing efficiency is indistinguishable whether yeast express human D1 or Smd1p. The pre-mRNA/mRNA ratios of the intron-containing *RP51A*, *ACT1* and *CYH2* transcripts do not change as a function of the D1 source (figure 4A and data not shown). As previously reported (18), metabolic depletion of Smd1p by transcriptional repression of a *GAL1::SMD1* allele inhibits splicing; pre-mRNA levels rise and mRNA levels fall from intron-containing genes (lanes 2 and 6). Repression of *GAL1::HD1* likewise inhibits splicing in the *smd1::URA3* null strain (lanes 4 and 8). Since splicing inhibition is noted 4–5 hours after transcriptional repression of either *GAL1::SMD1* or *GAL1::HD1*, a large pool of functional D1 protein is probably not present in these cells (18 and data not shown).

The U1, U2, U4, and U5 spliceosomal snRNAs are stable in strains that express either *GAL1::SMD1* or *GAL1::HD1* (figure 4B). *GAL1::HD1* expression also restores the efficient precipitation of these spliceosomal snRNAs by anti-trimethylguanosine antibodies (B. Rymond, unpublished observations), a characteristic lost in the null allele background (18). In contrast, repression of either *GAL1*-fusion gene causes a drop in the TMG-capped spliceosomal snRNAs in cells where this is the only D1 (or Smd1p) source (figure 4B). U6 snRNA, which lacks the Sm site of core protein association, remains unchanged. The drop in the TMG-capped snRNA levels is concomitant with the rise in pre-mRNA and plateaus near the time cell division ceases (data not shown). The most likely explanation for the snRNA decrease is that these RNAs are less stable in the absence of Smd1p/D1, a state brought about by repression of the *GAL1* fusion genes and dilution of existing protein pools through cell division. While it is formally possible that *smd1::URA3* generates a truncated peptide with partial biological activity, this activity is not sufficient to support cell growth, facilitate pre-mRNA splicing, or maintain normal levels of TMG-capped snRNAs. *GAL1::HD1* expression complements each of these functions.

Figure 2. Genetic characterization of human D1 function in yeast. Diploid yeast heterozygous for a chromosomal *smd1::URA3* disruption and transformed with the galactose inducible *GAL1::HD1* fusion gene on plasmid pHSM1368 (21) were sporulated and the meiotic progeny scored for growth on galactose (GAL) and glucose (GLU) based media. + indicates growth, - no growth; the *LEU2* and *URA3* genes complement auxotrophic mutations in the host strain and confer growth competence on media lacking leucine and uracil, respectively.

The yeast Smd1p polypeptide is longer than human D1 by 27 amino acids (see figure 1.) Since *GAL1::HD1* complements the yeast *smd1::URA3* null allele, all of the peptide elements necessary for yeast Smd1p function must be represented in human D1. By extension of this argument, sequences unique to the yeast protein may be dispensable for human D1 activity. To test this hypothesis two new alleles, *smd1Δ20* and *smd1Δ27*, were created. The peptides produced from these genes lack the overlapping yeast-specific sequences MASLYLTGGQQPTAS-DNIAS and AMASLYLTGGQQPTASDNIASLQYINI, respectively (see figure 1). Each was assayed for the ability to complement *smd1::LEU2*. The nutritionally regulated *GAL1::SMD1* gene provided wild-type Smd1p in the test strains when grown on galactose medium. The constitutively expressed deletion derivatives were the sole source of Smd1p on glucose medium (see Materials and Methods). Surprisingly, the two deletion constructs presented strikingly different results in the plate assay, *smd1Δ20* completely complemented the *smd1::LEU2* null allele while *smd1Δ27*, lacking the entire yeast-specific segment, was unable to support even modest growth (see figure 2). Northern analysis of RNA isolated from these strains confirmed that *smd1Δ27* failed to support pre-mRNA splicing

Figure 3. Plate assay for *GAL1::HD1*, *GAL1::SMD1*, and *SMD1*-deletion derivative activity. Haploid strains bearing only wild-type *SMD1* (*SMD1*), a wild-type and human D1 copy (*SMD1*, *GAL1::HD1*), a *URA3*-disruptant and a human D1 copy (*smd1::URA3*, *GAL1::HD1*), and a *LEU2* disruptant transformed with both *GAL1::SMD1* and a *SMD1* deletion derivative (*smd1::LEU2*, *GAL1::SMD1*, *smd1*Δ20; *smd1::LEU2*, *GAL1::SMD1*, *smd1*Δ27) were assayed at 30°C for growth on galactose-based and glucose-based medium.

(data not shown). Thus, much but not all of the yeast-specific domain is dispensable for Smd1p function.

DISCUSSION

To date, only 4 splicing-associated proteins have been identified with likely counterparts in both yeast and mammals: the U5 snRNP PRP8 protein (27), the U1 snRNP A (28) and 70K (29,30) proteins and the common (or shared) D1 snRNP protein (18). We have demonstrated that human core snRNP polypeptide D1 can substitute for yeast Smd1p to promote pre-mRNA splicing. Collateral phenotypes associated with the loss of Smd1p activity (e.g., decrease in intracellular snRNA abundance, changes in anti-TMG precipitability of snRNAs) are also relieved when either human D1 or a Smd1p deletion derivative designed to mimic the D1 structure is expressed as the sole source of this essential polypeptide. By establishing the functional equivalence of Smd1p and human D1, this study provides the first direct evidence that individual 6S core polypeptides are necessary for pre-mRNA splicing in vivo; analogous mechanisms of yeast and human snRNP biogenesis are anticipated.

The yeast and human D1 polypeptides differ most obviously in the fact that the yeast polypeptide contains an additional 27 amino acids. In this regard, the null phenotype of *smd1*Δ27 was surprising as it was designed to express a protein with a clean deletion of the 'yeast-specific' peptide sequence. The structure of this polypeptide differs from that produced by the functional derivative *smd1*Δ20 allele by only 7—apparently non-conserved—amino acids. While this change appears minor, it should be remembered that the choice of deletion end-points in *smd1*Δ27 was largely arbitrary, guided solely by a computer-selected (31,32) best-fit match of primary sequence similarity. The computer algorithms cluster regions of similarity, the 'extra' sequences of Smd1p may actually be scattered throughout the polypeptide. Weakly conserved (or nonconserved) sequences necessary for proper protein folding, stability or function apparently exist in the region covered by the *smd1*Δ27 deletion.

Figure 4. Northern analysis of *GAL1::HD1*-transformed strains. RNA was extracted from strains bearing the endogenous yeast *SMD1* allele or disruptant derivatives (see figure legend 3) containing the *GAL1*-driven *SMD1* or human D1 genes during growth on galactose (lanes 1–4) or 10 hours after shift to *GAL1*-repressing, glucose-based medium (lanes 5–8). A) Hybridization with intron plus exon sequences of the yeast *RP51A* gene. The positions of the *RP51A* precursor (pre-mRNA) and correctly spliced messenger RNA (mRNA) are indicated. B) Hybridization of RNA samples described in panel A with probes specific for the yeast U1, U2, U4, U5, and U6 snRNA genes. U5 snRNA is present as a long (U5L) and short (U5S) form differing in the length at the 3' ends (7).

Regardless, the fact that almost 75% of the unique yeast segment can be removed without perceptibly impeding Smd1p function argues strongly that Smd1p and human D1 operate similarly to support snRNP assembly. The precise function(s) of Smd1p/D1, whether a simple shield to protect the snRNA, a snRNP assembly or targeting agent, or an essential component of the spliceosome remain to be established (but see below).

In order to replace yeast Smd1p in vivo, the human D1 polypeptide must interact favorable with (or at least not interfere with the functions of) other snRNP polypeptides. So, while a 6S core polypeptide complex similar to that which mediates metazoan snRNP assembly (5,6) has yet to be identified in yeast, our results predict that such a structure is likely to exist. If one assumes that human D1 and Smd1p serve similar functions in their respective organisms, then the metazoan snRNP assembly model becomes a useful framework to begin our evaluation of

Smd1p function. Early studies on snRNP assembly revealed that the Sm consensus, (RA(U)₃GR), was both necessary and sufficient to promote core protein binding to artificial RNA substrates (33). More recently, Jaroloski and Mattaj (34) have shown that segments of natural snRNAs, including sequence-independent single-stranded structures removed from the Sm consensus, are important determinants of early snRNP assembly. Their data support the view that the sequence context of the Sm site is an important parameter in promoting the stable binding of the core protein unit. A 'favorable' context might, for example, improve snRNP assembly by balancing a relatively weak Sm site/protein interaction with enhanced core protein binding elsewhere on the snRNA. The presence of multiple determinants for Sm core protein interaction offers an explanation for the observation in yeast that many mutations to the U5 Sm consensus are benign, with little or no apparent effect on snRNP function (16).

Which of the 6S core polypeptides bind snRNA to nucleate snRNP assembly? While the geometry of core protein/snRNA is far from clear, enough experimental detail is available to speculate about this question. Polypeptide G is a likely candidate to make sequence-specific snRNA contacts since this polypeptide is uniquely crosslinked to the Sm site of snRNP particles (9). Curiously, in studies where filter-bound core proteins are simply incubated with radiolabeled RNA, D1 was the only core protein with detectable nucleic acid binding properties (35,36). Indeed, D1 appears to bind RNA very tightly, with little or no sequence specificity. The D1 carboxyl terminus containing 9 (G-R) repeats (21) may promote this non-specific RNA binding through multiple electrostatic interactions with snRNA phosphates. The retention of many basic residues (but poor primary structure conservation) in the Smd1p carboxyl terminus reinforces the notion that point charge attraction between lysine and arginine side chains and RNA phosphates may be an important functional feature of this end of the molecule. While many models are possible, the data are consistent with polypeptide G serving to promote snRNP assembly through nucleotide-specific contacts at the Sm site and D1, via contact with G (or another core polypeptide), serving to buttress this interaction through stable, largely non-specific contacts elsewhere on the snRNA. The newly identified sequence-independent RNA elements required for U1 and U5 snRNP assembly (34) are promising targets of D1 interaction. Ostensibly, peptide sequences mediating protein-protein contacts between D1 and other conserved snRNP peptides will be constrained by the steric requirements for productive association. Segments I and II of Smd1p/D1 (see figure 1) offer well-conserved structures worth probing for such activities.

Yeast and metazoan splicing differ somewhat in precise pre-mRNA substrate requirements, snRNA primary structures, and in the order of addition (or necessity of) certain splicing factors (1,2). For example, proteins that mediate alternative splice site selection and differential splice site activation in metazoa (1,37) are unlikely to be present in yeast where the majority of the interrupted genes contain a single, constitutively spliced intron. Notwithstanding such distinctions, the similarities of spliceosome assembly profiles and splicing reaction pathways argue strongly for conservation of the basic eukaryotic splicing apparatus. This point has been strengthened by the functional replacement of yeast Smd1p by human D1 and like success in experiments involving U2 snRNA (17). The success of these experiments encourages the use of yeast genetics to study the fundamental characteristics of eukaryotic snRNP assembly and function. In addition, these

data suggest that it may be practical to use human cDNAs to complement *prp* splicing mutations and thereby employ yeast as a biological trap to harvest conserved components of the human splicing apparatus.

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