An essential splicing factor, \textit{SLU7}, mediates 3' splice site choice in yeast

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Recently, we have reported the identification of several genes that exhibit genetic interactions with the U5 snRNA. Two of these genes, \textit{SLU4} and \textit{SLU7} (\textit{SLU}: synergistic lethal with U5 snRNA), encode products required for the second catalytic step of splicing. To analyze the specific roles of \textit{SLU4} and \textit{SLU7}, we have determined how mutants influence the relative usage of competing 3' splice sites. We find that mutations in \textit{SLU7} eliminate the normal 20-fold preference for 3' splice sites located >22 nucleotides downstream of the branchpoint. In contrast, mutations in \textit{SLU4} inhibit usage of all 3' splice sites, regardless of their location. This suggests that \textit{SLU7} is involved in the process of 3' splice site choice, whereas \textit{SLU4} fulfills a generic requirement for the second step. We show that \textit{SLU7} is an essential gene that contains a small motif with striking similarity to the cysteine-rich zinc knuckle of retroviral nucleocapsid proteins, which has been implicated in RNA binding. Mutational analysis of \textit{SLU7} indicates that this motif influences the efficiency, but not the sequence specificity, of 3' splice site selection. The identification of a component of the constitutive splicing machinery that can promote 3' splice site choice has potentially important implications for alternative splicing.

[Key Words: Pre-mRNA splicing, \textit{Saccharomyces cerevisiae}, \textit{SLU} mutants, U5 snRNP, alternative splicing]

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Nuclear pre-mRNA introns are defined by three conserved sequence elements. Two mark the 5' and 3' exon-intron boundaries while a third, the branchpoint sequence, is located just upstream of the 3' splice site. Unlike autocatalytic introns, these intron sequences are not sufficient to catalyze their own removal [Cech and Bass 1986]. Instead, numerous cofactors, including the small nuclear ribonucleoprotein (snRNP) particles, are essential for splicing. These factors bind to the pre-mRNA in a defined hierarchy that culminates in the assembly of a splicing-competent structure termed the spliceosome. Once this complex is assembled, intron removal proceeds by two consecutive catalytic steps. The first step entails a transesterification reaction between the 5' exon-intron junction and an adenosine residue within the branchpoint sequence that results in cleavage of the 5' exon from the intron. During the second step, cleavage occurs at the intron-3' exon border and the free 5' exon is concomitantly ligated to the 3' exon [Padgett et al. 1986; Guthrie 1991].

Given the complex matrix of interactions that occur between the splicing factors and the pre-mRNA, a key problem in understanding the splicing process has been to determine which of these factors are responsible for the primary recognition of the pre-mRNA. The U1 and U2 snRNPs are important determinants of 5' splice site and branchpoint recognition (respectively) that function by direct base-pairing between snRNA and pre-mRNA [for review, see Steitz et al. 1988]. Unambiguous identification of the factors that specify the 3' splice acceptor site has proven to be less straightforward. At least seven mammalian proteins (IBP, 70K, hnRNP C, hnRNP A1, hnRNP D, U2AF, PTB) have been reported to bind at the polypyrimidine tract preceding the AG at the 3' splice site [Chabot et al. 1985; Choi et al. 1986; Gerke and Steitz 1986; Tazi et al. 1986; Ruskin et al. 1988; Swan-son and Dreyfuss 1988; Garcia-Blanco et al. 1989; Buvoli et al. 1990]. The situation is complicated further by the fact that in addition to its role in specifying the 3' border of intron cleavage, the mammalian 3' splice site region functions to recruit the U2 snRNP into pre-spliceosomes before cleavage at the 5' splice site [Reed 1989; Smith et al. 1989; Zhuang and Weiner 1990]. A similar complica-tion may also pertain in the fission yeast \textit{Schizosaccharomyces pombe}, where the AG dinucleotide is clearly recognized by the U1 snRNP before the first step of splicing [Reich et al. 1992].

The situation in \textit{Saccharomyces cerevisiae} is potentially simpler because Rymond et al. [1987] have demonstrated that 3' splice site sequences are largely dispensable for spliceosome assembly and 5' splice site cleavage. This result argues that the 3' splice site sequences of \textit{S. cerevisiae} introns are likely to function solely in the second step of splicing. If so, then \textit{S. cerevisiae} provides an ideal system for the identification of trans-acting factors specifically required for 3' splice site selection.
To apply the power of a genetic approach to this problem, we have employed two recently described tools. The first is a set of splicing substrates in which two alternative 3' splice sites compete in cis. Experiments using these constructs suggested that the efficient utilization of 3' splice sites separated from their branchpoints by >22 nucleotides is likely to require the activity of a factor that binds 3' splice sites, with a preference for polyuridine [Patterson and Guthrie 1991]. The majority of yeast introns follow this configuration, which has been termed 3' Long (3'L; Parker and Patterson 1987). In contrast, yeast introns with a branchpoint to 3' splice site distance of 10–15 nucleotides [3' Short or 3'S; Parker and Patterson 1987] are not enriched for uridines; the short spacing in these introns may obviate the requirement for the putative uridine-binding factor [Patterson and Guthrie 1991].

The design of the second genetic tool derived from observations tying U5 snRNP function to both 3' splice site binding and to the second catalytic step of splicing [Chabot et al. 1985; Gerke and Steitz 1986; Tazi et al. 1986; Patterson and Guthrie 1987; Winkelman et al. 1989; Frank et al. 1992]. We searched for mutations that exacerbated the growth defects of mutants carrying lesions in the conserved loop of the U5 small nuclear RNA (snRNA). By restricting our attention to alleles that are lethal in the presence of mutant U5 and conditionally lethal (either temperature or cold sensitive) in the presence of wild-type U5, we identified 13 complementation groups [Frank et al. 1992]. Two of these [SLU] synthetic lethals with U5 mutants, slu4-1 [an allele of PRP17; Vijayraghavan et al. 1989] and slu7-1, were shown to be specifically defective in the second catalytic step of splicing.

In this paper we present the first test of the hypothesis that SLU4 and SLU7 are 3' splice site recognition factors. We asked whether conditional mutations in these genes alter the relative use of competing 3' splice sites, reasoning that such a phenotype would be indicative of a defect in 3' splice site selection. We find that slu4-1 inhibits the splicing of both of the alternative sites. In contrast, the slu7-1 allele is defective in splicing 3'L introns but is fully functional in splicing 3'S introns. We propose that SLU7 functions in the selection of 3'L introns. Interestingly, sequence analysis of the essential SLU7 gene reveals a cysteine-rich zinc knuckle motif implicated previously in RNA binding by retroviral nucleocapsid proteins.

Results

3' splice site competition studies

To determine the effect of slu4-1 and slu7-1 mutations on 3' splice site choice, we introduced a set of 3' splice site competition reporter constructs [Fig. 1; described below] into wild-type and mutant cells. RNA was isolated from these cells, which were grown under semipermmissive conditions [30°C], and then analyzed by primer extension. This assay provides a measure of the steady-state levels of the alternatively spliced products [mature–proximal (MP) and mature–distal (MD)] as well as the splicing intermediates [precursor (P) and lariat–intermediate (L)]. To interpret these data we have modeled 3' splice site selection in terms of a simple competition between pairs of splice sites. According to this model [see Materials and methods], the ratio of MP RNA to MD RNA (MP/MD) is directly proportional to the ratio of the frequencies with which their respective 3' splice sites are selected. Although a change in the ratio of MP/MD is indicative of a change in the relative competitiveness of the two splice sites, it does not indicate whether one site is activated or the other is deactivated. However, the efficiency of selecting an individual 3' splice site is proportional to the ratio of its MD and MP level to the level of L (MD/L or MP/L). Thus, by measuring MD/L and MP/L, it is possible to determine whether a mutant is defective in using one or both competing 3' splice sites. Accordingly, our analysis of SLU4 and SLU7 is based on a consideration of these parameters.

We first examined the splicing of a construct (+0WT) in which a distal, 3'L splice site was placed in competition with a proximal 3'S splice site [Fig. 1; Patterson and Guthrie 1991]. This construct tests whether a mutant is defective in splicing either 3'S- or 3'L-type splice sites. Figure 2, A and B, shows the results of a primer extension analysis of +0WT RNAs isolated from slu4-1, slu7-1, and wild-type cells grown at a semipermissive temperature (30°C). [Fig. 2 also shows the analysis of a construct in which two 3'L splice sites were placed in competition; these results will be discussed below.] Measurements of MP/MD, MP/L and MD/L for each of the strains are summarized in Table 1A. In agreement with previous studies [Patterson and Guthrie 1991], we observe that in a wild-type strain, use of the distal site was strongly favored relative to the use of the proximal site (MD/MP = 22). A similar pattern of splicing was also observed in the slu4-1 strain [MD/MP = 18]. In contrast, when the +0WT construct was placed in the slu7-1 background we observed that use of the proximal site was approximately equivalent to use of distal site [MD/MP = 0.8].

The effect of slu7-1 on the ratio of MD to MP suggested that either the proximal 3' splice site had been activated or that the distal 3' splice site had been deactivated. That the latter case is more likely is indicated by the measurements of MP/L and MD/L, which reflect the efficiencies of using the individual sites. The observation that slu7-1 caused a 16-fold decrease in MD/L [Fig. 2A, B; Table 1A] clearly indicated that slu7-1 was defective in using the distal 3' splice site. In contrast, slu7-1 had little or no significant effect on MP/L, suggesting that use of the proximal site was unimpaired. In sum, these results indicated that the slu7-1 mutation inhibited the use of the distal, 3'L splice site of the +0WT construct but had no effect on the proximal 3'S site.

Unlike slu7-1, slu4-1 caused a decrease in both MP/L [threefold] and MD/L [fourfold]. The degree of inhibition of each site was approximately equal, as the ratio of MD/MP was not significantly changed in slu4-1, relative to
Figure 1. 3' Splice site reporter constructs. (A) Partial nucleotide sequences of the RNA substrates (+0WT and +AWT) used in this study. Each sequence begins at the branchpoint sequence UACUAAC (the branchpoint adenosine is in boldface type) and extends to the AG acceptor site of the distal 3' splice site. The competing 3' splice acceptor sites, which are in boldface type, are classified as being either 3'S or 3'L based on the distance between the branchpoint and the AG dinucleotide [for more details, see text and Parker and Patterson (1987)]. Details of the construction of the reporter plasmids are given in Patterson and Guthrie (1991) and C. Lesser and C. Guthrie (in prep.). (B) A diagram of the MP and MD products that arise from the use of the competing splice sites.

wild type. Hence, *slu*4-1 inhibited the splicing of both the proximal (3'S) and the distal (3'L) splice sites, indicating that this defect is independent of the spacing and uridine content of the 3' splice site.

In the experiment described above, the cells were grown continuously at a semipermissive temperature (30°C) so that the levels of the various RNA species would be at a steady state. We then asked whether the change in RNA levels in the *slu*7-1 mutant could be correlated with its temperature-sensitive growth and splicing phenotypes [Frank et al. 1992]. We did not test the cold-sensitive defect of *slu*7-1. To do so, we analyzed +0WT RNA that was isolated from cells shifted to a nonpermissive temperature (37°C) for either 4 or 8 hr (for details, see Materials and methods). The latter time point corresponds to the time after a shift to 37°C when growth of a *slu*7-1 culture is effectively blocked. A primer extension analysis of RNAs isolated from wild-type and *slu*7-1 cells is shown in Figure 2C, and the data are summarized in Table 1B. We observed that the ratio of MD/MP for *slu*7-1 fell from 0.77 to 0.26 upon heat shifting for 8 hr, whereas the wild-type strain exhibited only a slight change in MD/MP (from 22 to 20). Thus, the defect exhibited by the *slu*7-1 cells in splicing the distal site of +0WT was more pronounced upon shifting to a nonpermissive temperature. More significantly, this loss of distal splicing in *slu*7-1 was accompanied by an increase in the absolute level of MP RNA in *slu*7-1, relative to wild type. We interpret this to mean that at a nonpermissive temperature, proximal splicing was still competitive: *slu*7-1 was not defective in splicing the proximal site, even under conditions in which it could not support viability. The results of the 4-hr shift were similar to those of the 8-hr shift, indicating that we had observed the full extent of the 37°C *slu*7-1 phenotype.

The analysis of +0WT splicing in *slu*7-1 strongly suggested that this mutation causes a defect in the utilization of 3'L splice sites. To test this hypothesis further, we examined the effect of *slu*7-1 on the pattern of splicing a construct, +AWT, which places two 3'L sites in competition. This construct was made by inserting 14 cytosine and adenosine residues between the branchpoint and the proximal splice site, hence placing the proximal splice site 24 nucleotides downstream of the branchpoint. On the basis of the results with +0WT, we predicted that *slu*7-1 would use both splice sites inefficiently.

An example of the primer extension analysis of RNA produced by the +AWT construct in *slu*4-1, *slu*7-1, and wild-type cells is shown in Figure 2, A and B, and the data obtained from this experiment are summarized in Table 1A. The analysis revealed that the *slu*7-1 mutation inhibited splicing significantly at both sites, relative to wild type, because both MP/L and MD/L were reduced (11- and 12-fold respectively). As a result, the overall decrease in mature mRNA production was accompanied
by an increased level of lariat-intermediate in slu7-1. Furthermore, the observation that the ratio of MD/MP in +AWT was unaffected by slu7-1 argues that the magnitude of the splicing defect was similar at both sites. These findings support the hypothesis that slu7-1 is defective in splicing all 3' splice sites, regardless of their relative distances from the branchpoint. Similarly, the defect of slu7-1 was not sensitive to the uridine content upstream of the splice sites, because the uridine-rich distal site was affected to the same extent as the uridine-poor proximal site.

The pattern exhibited by slu4-1 in splicing the +AWT construct was similar to its pattern of splicing the +0WT construct. In particular, slu4-1 caused a significant reduction in both MP/L and MD/L [12- and 20-fold, respectively], indicating that use of both the +AWT proximal and distal splice sites was inefficient relative to wild type; this result is clearly evidenced by the accumulation of lariat-intermediate in slu4-1. slu4-1 thus caused a general inhibition in the use of all 3' splice sites tested. Unlike the situation with slu7-1, we found that slu4-1 caused a slight [approximately twofold] decrease in the ratio of MD/MP. Thus, although both the proximal and the distal sites were used inefficiently in slu4-1, the defect was somewhat more severe in the case of distal splicing, suggesting that SLU4 might function in "measuring" the branchpoint to AG spacing.

Cloning the SLU7 gene

The unique phenotype that the slu7-1 strain exhibited in splicing the 3' splice site competition constructs led us to clone and sequence the wild-type SLU7 gene. SLU7 was cloned by complementation of the slu7-1 cold-sensitive phenotype with a yeast genomic DNA plasmid library (Guthrie and Fink 1991, chapter 14). Cold-resistant transformants were then streaked at 37°C to test for complementation of the slu7-1 heat-sensitive phenotype. Two independent, temperature-resistant transformants were isolated, which carried identical complementing plasmids (pYS7-1). The complementing activity of pYS7-1 was localized to a 2.5-kb fragment (plasmid pYS7-7). The DNA sequence of both strands of this fragment was subsequently determined (Fig. 3). This fragment contains one large open reading frame (ORF1), which is predicted to encode a 382-amino-acid protein of ~44 kD.

To determine whether ORF1 was identical to the SLU7 gene, we replaced over three-quarters of ORF1 with the TRP1 gene and used this construct to disrupt a SLU7/slu7-1 heterozygous diploid [for details, see Materials and methods]. Disruption of ORF1 produced two classes of diploids. The first class consisted of diploids that were wild type with respect to growth at 37°C and gave rise, upon sporulation, to two wild-type Trp−
Table 1. Quantitation of primer extension data

| Strain     | RNA          | MD/MP | MP/L   | MD/L   |
|------------|--------------|-------|--------|--------|
| WT         | +OWT-TRP     | 21.9 ± 1.9 | 7.0 ± 0.8 | 153.9 ± 22.2 |
| slu7-1     | +OWT-TRP     | 0.8 ± 0.0  | 12.1 ± 3.6 | 9.3 ± 2.7   |
| slu4-1     | +OWT-TRP     | 18.3 ± 1.4 | 2.1 ± 0.2  | 38.2 ± 4.5  |
| WT         | +AWT-TRP     | 2.3 ± 0.1  | 2.9 ± 0.5  | 6.8 ± 1.3   |
| slu7-1     | +AWT-TRP     | 2.4 ± 0.0  | 0.3 ± 0.0  | 0.6 ± 0.1   |
| slu4-1     | +AWT-TRP     | 1.4 ± 0.0  | 0.2 ± 0.0  | 0.3 ± 0.1   |
| WT         | +OWT-LEU     | 5.5 ± 0.2  | 34.2 ± 4.5 | 181.2 ± 16.6|
| slu7-1     | +OWT-LEU     | 0.2 ± 0.0  | 41.1 ± 2.6 | 7.2 ± 0.4   |
| slu7-7–CCSS| +OWT-LEU     | 3.1 ± 0.0  | 18.4 ± 1.5 | 56.1 ± 4.3  |

(MD/MP) The ratio of mature-distal to mature-proximal RNA; (MP/L) the ratio of mature-proximal to lariat intermediate RNA; (MD/L) the ratio of mature-distal to lariat intermediate RNA. The data, which were obtained by PhosphorImaging, are expressed as the mean ± S.E.M. of three independent samples. [A] The analysis of +OWT and +AWT RNA in wild type (WT), slu4-1, slu7-1, and slu7–CCSS. The identity of the +OWT construct used is designated either +OWT-TRP or +OWT-LEU; these constructs differ in the reading frames of the MD and MP mRNAs (for details, see Materials and methods). [B] The analysis of +OWT RNA after shifting wild-type and slu7-1 cultures to 37°C for the designated time.

spores and two inviable spores. In the second class, the diploids were temperature sensitive and produced two temperature-sensitive Trp– spores and two inviable spores. In no case did a temperature-sensitive diploid give rise to a wild-type spore. We infer from these results that the temperature-sensitive diploids were generated by disruption of the wild-type SLU7 gene through integration of the ORF1–TRP1 construct, which left only the slu7-1 gene intact. The wild-type diploids, on the other hand, resulted from disruption of the slu7-1 allele. We conclude that the integrated ORF1 gene disruption is tightly linked to the slu7-1 allele and, therefore, that the complementing plasmid pYS7-7 does encode SLU7. Furthermore, because all diploids tested produced only two viable Trp+ progeny upon sporulation, we conclude that SLU7 is essential for the viability of S. cerevisiae.

An examination of the predicted SLU7 protein sequence revealed a region that bears striking similarity to a cysteine-rich zinc knuckle motif of retroviral nucleocapsid proteins [Table 2; Berg 1986; South and Summers 1990]. The hallmark of this motif is the consensus sequence CX2CX3HX4C, which coordinates zinc in a manner reminiscent of classical zinc finger proteins [Schiff et al. 1988; Roberts et al. 1989; Green and Berg 1990; Summers et al. 1990; Fitzgerald and Coleman 1991]. Note that the relative spacing of cysteine and histidine residues is strictly specified in the case of nucleocapsid proteins, unlike the case of zinc finger proteins. In addition to this consensus sequence, the SLU7 motif shares several other features with its retroviral counterparts, including (1) highly conserved glycine residues at positions 5 and 8, (2) charged residues at positions 6 and 13, (3) a polar residue at position 3, and (4) several lysine or arginine residues directly following the zinc knuckle (Berg 1986; Katz and Jentoft 1989; South and Summers 1990). We thus wanted to address the intriguing possibility that the putative zinc knuckle of SLU7 might bind RNA in some capacity during splicing. To test this idea, we mutated two of the conserved cysteine residues within the zinc knuckle, C122 and C125, to serine (the double mutant is referred to as slu7–CCSS). We first tested whether slu7–CCSS could support viability in the absence of the wild-type SLU7 gene product. To this end, a LEU2 plasmid carrying slu7–CCSS (pYS7CCSS–LEU) was transformed into a strain, yS7GK, in which the chromosomal SLU7 gene was disrupted by TRPI; wild-type SLU7 activity was provided by pYS7-7, a URA3 plasmid. To assay mutant activity,
Figure 3. Nucleotide and deduced amino acid sequence of the SLU7 gene. The sequence of the smallest slu7-1 complementing fragment is shown along with the amino acid sequence of the largest ORF. The sequence of the zinc knuckle, amino acids 122–135, is indicated by overlining.
Table 2. **Alignment of zinc knuckles**

|   | 1 | 5 | 10 | 14 |
|---|---|---|----|----|
| SLU7 | C RN C GE A G H K E KD C M E KP R K M Q K L V P |
| RSV-a | C YT C G S P G H Y Q A Q C P K R K S G N S R E R |
| RSV-b | C Q L C N G M G H N A K Q C R K R D N G Q G Q R P G |
| MoMuLV | C A Y C K E K G H W A K D C P K R P G P R G P R P |
| MMTV-a | C F S C G K T G H I R K D C K E E G S K R A P P G |
| MMTV-b | C P R C K K G Y H W K S E C K S K F D K D G N P L P |
| HIV-1-a | C F N C G K E G H I A R N C K A P R K K G |
| HIV-1-b | C W K C G R E G H Q M K D C T E R Q A N F L G K I W |
| Ty3-2 | C F Y C K K E G H R L N E C R A R K A S S N R S |
| Copia | C H H C G R E G H I K K D C F H Y K R I L N N K N K |
| CNBP | C F K C G R S G H W A R E C P T G G G G R G M R S |
|   | C Y R C G E S G H L A K D C D L Q E D A |
|   | C Y N C G R G G H I A K D C K E P K R E R E Q |
|   | C Y N C G K P G H L A R D C D H A D E Q K |
|   | C Y S C G E F G H I Q K D C T K V K |
|   | C Y R C G E T G H V A I N C S K T S E V N |
|   | C Y R C G E S G H L A R E C T I E A T A |

Cons.  

|   | C X X C X X X X H X X X X C X X X . . . |

The zinc knuckle motif of **SLU7** (amino acids 122–135) is shown in alignment with other representative zinc knuckle motifs. The consensus sequence, CXX2CX2HX2C, is indicated by Cons. The examples shown are as follows: Rous sarcoma virus (RSV; Schwartz et al. 1983); Moloney murine leukemia virus (MoMuLV; Shinnick et al. 1981); Mouse mammary tumor virus (MMTV; Jacks et al. 1987); human immunodeficiency virus (HIV; Wain-Hobson et al. 1985); yeast Ty element 3-2 (Ty3-2; Hansen et al. 1988); Copy element (Copia; Mount and Rubin 1985); and cellular nucleic acid binding protein (CNBP; Rajavashisth et al. 1989).

Leu + transformants were streaked onto 5-fluoro-orotic acid (5-FOA), which selects against cells carrying the **URA3** gene and, hence, the wild-type SLU7 plasmid (Guthrie and Fink 1991). Under these conditions, cells could live only if **slu7**-CCSS provided some degree of SLU7 activity. We observed that **slu7**-CCSS supported yeast cell growth comparable to wild-type SLU7 under all conditions tested (data not shown). Therefore, disruption of the SLU7 zinc knuckle does not appreciably affect the growth characteristics of yeast.

As a more sensitive test of SLU7 activity, we again turned to the 3' splice competition construct +0WT and asked whether **slu7**-CCSS might affect the pattern of competitive splicing. Results of the primer extension analysis of +0WT RNA isolated from SLU7, **slu7**-1, and **slu7**-CCSS cells are shown in Figure 4 and summarized in Table 1A. By this assay, the activity of **slu7**-CCSS is measurably different from the activities of both SLU7 and **slu7**-1. That is, **slu7**-CCSS caused the levels of both MP and MD to fall relative to L, approximately twofold and threefold respectively. Unlike **slu7**-1, therefore, **slu7**-CCSS is moderately defective in splicing both 3'S and 3'L splice sites. Finally, the decrease in the ratio of MP/MD [from 5.5 in the case of wild-type to 3.1] observed for **slu7**-CCSS indicated that the distal site was slightly more sensitive to this mutation than was the proximal site. The phenotype of the **slu7**-CCSS allele is significant because it links SLU7 activity to 3'S splicing, that is, wild-type SLU7 activity is required for the efficient splicing of these splice sites, as well as for 3'L sites.

**Discussion**

The SLU4 and SLU7 genes were identified on the basis of their genetic interaction with the U5 snRNA [Frank et al. 1992] and are both required for the second catalytic step of splicing ([Vijayaraghavan et al. 1989; Frank et al. 1992] note that SLU4 is allelic to **PRP17**). In this paper we have begun to address the specific mechanisms by which they act and whether their roles can be linked to the function of the U5 snRNP. In the simplest view, one can imagine several modes of action for a factor required for step two: (1) selection of a specific 3' splice site among several potential sites [e.g., 3'L vs. 3'S]; (2) identification of the 3' splice site as a fixed landmark [e.g., the first AG 3' to the branchpoint]; or (3) promotion of the cleavage/ligation reaction. Because the latter two roles are presumably generic requirements for all introns, we sought to distinguish the first mechanism by employing a construct in which two potential 3' splice sites compete in cis. An alteration in the ratio with which these sites are used should be indicative of mutation in a factor that mediates splice site choice.

**slu7**-1 affects 3'L but not 3'S splicing

Our experimental evidence strongly suggests that SLU7 is a factor involved in the selection of 3'L-type splice sites, which are located >22 nucleotides downstream of the branchpoint and are normally preceded by a uridine-rich sequence [Parker and Patterson 1987]. The **slu7**-1 mutant was defective in splicing the +0WT distal site and both the distal and proximal sites of +AWT, but it spliced the +0WT proximal site normally. As discussed above, both of the +AWT splice sites are 3'L-type splice sites, as is the +0WT distal site. The +0WT proximal site, on the other hand, is located just 10 nucleotides

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Figure 4. Effects of zinc knuckle mutations on +0WT splicing. The effect of slu7-CCSS, slu7-1, and wild-type on splicing +0WT RNAs was measured by primer extensions. The products of splicing at the proximal or distal 3' splice sites are designated MP or MD, respectively [for details, see Fig. 1B]. L, an internal control (the U1 snRNA) and the products of the endogenous CUP1 gene are also indicated. [A] The analysis of +0WT RNA extracted from cells grown at a semipermissive temperature (30°C)j [B] a longer exposure of the gel shown in A, which highlights the levels of L. Note that the +0WT construct examined in this experiment differs from the +0WT construct examined in Fig. 2 with respect to the reading frames of the competing messages. Consequently, the MD/MP ratios are not directly comparable between the two experiments.

downstream of the branchpoint and is therefore similar to 3'S splice sites. The phenotype of the slu7-1 mutation can thus be explained by a decrease in the ability of the spliceosome to productively utilize splice sites located the typical distance (>22 nucleotides) from the branchpoint. Because the slu7-1 allele had little effect on the splicing of a 3'S site, use of this type of site probably bypasses the SLU7 activity that this mutation disrupts.

The relative use of 3'L splice sites is influenced by two considerations: [1] optimal branchpoint to 3' splice site spacing, and [2] uridine tract strength [Patterson and Guthrie 1991]. In principle, disruption of the factors responsible for either of these preferences could inhibit 3'L splicing. Our data do not support the hypothesis that the slu7-1 mutation is defective in polyuridine recognition, because a 3'L splice site with little or no uridine enrichment (the proximal site of +AWT) was affected to the same extent as uridine-rich sites. In other words, slu7-1 caused little, if any, change in uridine specificity relative to the overall effect on 3'L splicing. In a preliminary set of experiments, we have found that slu7-1 responds to increases in uridine strength almost to the same degree as does wild type [data not shown]. These results suggest that the slu7-1 mutation does not disrupt a polyuridine-binding domain, but they do not rule out the possibility that other domains of SLU7 might function to bind RNA

Zinc knuckle mutations confer a modest phenotype

Intriguingly, the SLU7 gene encodes a 14-amino-acid motif that is suspected to function in RNA binding: the cysteine-rich zinc knuckle motif of retroviral nucleocapsid proteins [Berg 1986; Katz and Jentoft 1989; South and Summers 1990]. Several in vitro studies have demonstrated that these nucleocapsid proteins can bind RNA nonspecifically [Karpel et al. 1987; Jentoft et al. 1988; Roberts et al. 1989]. Furthermore, mutations in the zinc knuckle, most notably in the conserved cysteine residues, result in the production of viral particles that are devoid of viral genomic RNA but, instead, package host RNA. These findings have led to the proposal that this
motif specifically binds retroviral genomic RNA during retroviral particle assembly (Gorelick et al. 1988, 1990, Meric and Goff 1989; Aldovini and Young 1990, Dupraz et al. 1990). In contrast to the retroviral studies, we found that mutating two of the conserved cysteine residues of the SLU7 zinc knuckle motif (C122 and C125; slu7-CCSS) produced no discernible growth phenotype in cells homozygous for this mutation. slu7-CCSS did, however, cause a moderate, but reproducible, decrease in the splicing of both 3'S and 3'L introns. Thus, an intact zinc knuckle promotes efficient splicing per se, but it is not preferentially required for 3'L introns and therefore does not specifically bind to the uridine-enriched tracts of 3'L splice sites. If this motif does function in RNA recognition (which has yet to be rigorously shown for any protein listed in Table 2), another candidate substrate is the U5 snRNA, because slu7-1 was isolated in a genetic screen for factors that interact with the conserved loop of this RNA. We are currently testing this possibility.

The lack of strong growth or splicing phenotypes conferred by slu7-CCSS was surprising, given the strong similarity to retrovirial zinc knuckles, which are essential for viral RNA packaging. Our results clearly limit the possible roles of the SLU7 zinc knuckle in RNA binding and/or splicing. It is possible that the SLU7 zinc knuckle functions as an RNA-binding domain but that its activity is not rate limiting under the growth conditions tested.

slu4-1 is more typical of step-2 mutants.

In contrast to slu7-1, slu4-1 is defective in splicing both 3'S and 3'L splice sites. This phenotype indicates that SLU4 is required for a more general process that occurs during the splicing of all 3' splice sites (e.g., recognition of AG dinucleotides). A preliminary examination of other second-step splicing mutants revealed that their behavior in the competition assay was more similar to that of wild-type or slu4-1 than to that of slu7-1 [data not shown]. To date, we have tested mutations in all published second-step factors [prp18-1 (Vijayraghavan et al. 1989), prp16-2 (Schwer and Guthrie 1991), slu4-1 and slu7-1], as well as a variety of other mutations that are thought to interact with SLU7 or the U5 snRNP [prp8-1 (Lossky et al. 1987), prp4-1 (Bordonn6 et al. 1990), slu1–slu12 (Frank et al. 1992), and several U5 snRNA point mutations]. Our results to date indicate that slu7-1 confers a unique phenotype. The phenotypic differences between slu7-1 and slu7-CCSS, however, underscore the need for multiple alleles in any such analysis.

A model of SLU7 function

The slu7-1 mutation eliminates the normal 20-fold preference of 3'L over 3'S sites, this reduction can be totally accounted for by inhibition of 3'L sites, rather than activation of 3'S sites. Because neither of the SLU7 mutations that we have tested are affected by the uridine content of 3'L sites, SLU7 probably does not recognize these introns by a sequence-specific mechanism. Rather, the role of SLU7 is likely to be dependent on a generic feature of this class of introns, presumably the distance (>22 nucleotides) between the branchpoint and the AG dinucleotide. We have proposed previously that the recognition of distantly spaced 3' splice sites is likely to require the participation of a factor (PRPX) that binds to 3' splice sites with an affinity that is proportional to the uridine content adjacent to the AG dinucleotide (Parker and Patterson 1987; Patterson and Guthrie 1991). According to this view, the normal hierarchy of 3' splice sites ([U-rich 3'L > U-deficient 3'L > 3'S]) would be determined by the poor affinity of PRPX for splice sites that are low in uridine content or sterically constrained (or both). A common feature of all 3'L sites would thus be their recognition by PRPX.

As shown in Figure 5, SLU7 could serve to "recruit" the PRPX–3' splice site complex to the assembled spliceosome. The slu7-1 phenotype would result from the inability of mutant SLU7 to interact productively with PRPX. Hence, slu7-1 would splice all 3'L sites inefficiently, without influencing the relative competition between such sites (which is determined by the affinity of PRPX for the splice site RNAs). The use of 3'S sites, on the other hand, would be unaffected by slu7-1, because

Figure 5. Model of SLU7 function. This model shows an assembled spliceosome immediately before the selection of a 3' splice site (in this case, a 3'L site). The 3' splice site is bound by a putative 3' splice site-binding factor, PRPX. Splice site selection occurs when SLU7 binds to the PRPX-3' splice site complex, thus recruiting the site into the spliceosome. The phenotype of the slu7-1 mutant is hypothesized to arise from a block in the recruitment process, as indicated by a broken line.
recognition of such sites does not normally require the SLU7–PRFX interaction. The modest inhibition of 3′ splicing caused by slu7–CCSS indicates that SLU7 might also play some role [albeit minor] in recognizing features common to all 3′ splice sites, for instance, the AG dinucleotide.

SLU7 is depicted [Fig. 5] as an intrinsic component of the spliceosome, rather than as a specialized factor associating only with 3′L complexes, for several reasons. First, the phenotype of the putative zinc knuckle mutant slu7–CCSS argues that perturbation of SLU7 function can also [modestly] affect the use of 3′S sites. Second, the slu7-1 mutant exhibits genetic interactions with U5 as well as with prp16 and prp18 mutants [Frank et al. 1992]. In that U5 [Patterson and Guthrie 1987] and PRP16 [Couto et al. 1987] are known to be required for splicing of 3′S as well as 3′L introns, it is likely that these are all constitutive components of the spliceosomal machinery. Moreover, in light of recent provocative evidence for an interaction between the U5 snRNA and the exon 2 sequences of CYH2 [Newman and Norman 1992], it is tempting to speculate that the SLU7–PRFX complex and the U5 snRNA might function in concert to select 3′ splice sites. Biochemical experiments are in progress to directly test these predictions. Finally, preliminary experiments in which splicing extracts have been immunodepleted of SLU7 protein indicate that this protein is required for the second, but not the first, step of splicing [data not shown], as would be predicted for a factor that functions solely in 3′ splice site selection. This result suggests that the second-step defect exhibited by the slu7-1 allele does not arise from a neomorphic [novel gain-of-function] change in the SLU7 gene, rather, the mutant phenotype is an effect of disrupting an essential function of SLU7.

Implications

The development of a sensitive in vivo assay for 3′ splice site competition revealed that alterations in spacing and sequence can profoundly alter 3′ splice site choice in S. cerevisiae [Patterson and Guthrie 1991]. Using these constructs as reporters, we have now identified a transacting factor [SLU7] that functions in the selection of 3′ splice sites. Because SLU7 is likely to be a component of the constitutive splicing machinery, this work lends credence to the emerging belief from mammalian systems that even tissue-specific splice site choice can be determined primarily by ubiquitous splicing factors [Ge and Manley 1990; Krainer et al. 1990]. It is an intriguing possibility that SLU7 analogs will be found to influence splice site selection in higher eukaryotes, where alternative splicing abounds.

Materials and methods

Genetic manipulations

All yeast genetic techniques, including transformations, diploid selection, sporulation, dissection, and the plasmid shuffle were as described in Guthrie and Fink [1991].

RNA analysis

In most cases, cells were grown at 30°C on the appropriate selective media to an OD_{600} of 0.8–1.0 then harvested. For the heat shifts, cells were first grown in media without tryptophan at 30°C to an OD_{600} of 1.0. Aliquots of the cultures were diluted (such that the final OD_{600} would be 1.0) into media without tryptophan that had been prewarmed at 37°C and then grown for either 4 or 8 hr before being harvested. RNA was extracted by the method of Wise et al. [Guthrie and Fink 1991, chapter 28] except that the guanadium buffer was replaced by the following buffer: 50 mM Tris [pH 7.4], 100 mM NaCl, 10 mM EDTA, and 1% SDS.

Primer extensions were performed as follows: Thirteen micrograms of RNA was mixed with 1 ng of 32P-labeled oligonucleotide in a volume of 6.4 μl and added to 1.6 μl of annealing buffer (250 mM Tris-Cl at pH 8.3, 300 mM NaCl, 50 mM DTT). After heating for 3 min at 68°C, this mixture was frozen in dry ice/ethanol and allowed to thaw on ice. To this mixture was added 11.3 μl of reverse transcriptase buffer [18 mM Tris-HCl at pH 8.3, 21 mM NaCl, 3.6 mM DTT, 11 mM Mg(OAc)$_2$, 0.72 mM in each dNTP] and 14 units of AMV reverse transcriptase [Life Sciences, Inc]. The reaction mixtures were incubated at 37°C for 3 min and then at 42°C for 20 min. Reactions were stopped by adding 10 μl of formamide dyes and heating at 90°C for 3 min. Products were resolved on 6% polyacrylamide/7 M urea gels, which were subsequently dried for 1 hr. Primer extension products were quantitated by PhosphorImaging (Molecular Dynamics). All numerical results are based on an analysis of three independent transformants. t-Tests were performed for all sets of triplicates to assess the statistical significance of the data [Dowdy and Wearden 1983]. In discussing the results, the term significant was used to signify P > 95% for a given test.

Strains

The following yeast strains were employed in this study: TR3, MATα ura3 lys2 his3 ade2 trp1, yDFA7, MATα slu7-1 ura3 lys2 his3 ade2 trp1 trp1 trp2 snr7::LEU2 pBWURA–SNR7; yDFA7C, MATα slu7-1 ura3 lys2 his3 ade2 trp1 leu2; yDFA4, MATα slu4-1 ura3 lys2 his3 ade2 trp1 leu2 snr7::LEU2 pBWURA–SNR7, SNR7D, MATα ura3 lys2 his3 ade2 trp1 leu2 snr7::LEU2 pBWURA–SNR7, and yDFA7–GK1, MATα ura3 lys2 his3 ade2 trp1 leu2 slu7::LEU2 pYS7-7.

Plasmids

The plasmids employed in this study are listed below. pSE358, pSE360, and pUN100 were the gift of Steve Elledge [Baylor College of Medicine, Houston, TX]. The yeast genomic DNA library, in YCP50, was a gift of Mark Rose [Princeton University, Princeton, NJ]. Bluescript II SK(+) was purchased from Stratagene.

pG1 + 0WT::TRP, pG1 + 0WT::LEU, and pG1 + AWT::TRP were constructed by J. Umen [University of California at San Francisco]. These constructs were made by replacing the actin intron sequence of the pG1–ACT1–CUP1 fusion [C. Lesser and C. Guthrie, in prep.], with the appropriate 3′ splice site sequences [the origin of the competition constructs is described in Patterson and Guthrie [1991]]. The parental vector is a 2μ plasmid, and transcription of the ACT1–CUP1 fusion utilizes the GPD1 promoter. The + 0WT constructs in the pG1 + 0WT::TRP and pG1 + 0WT::LEU plasmids differ with respect to the reading frames encoded by the competing messages [in + 0WT::TRP, the MD is in-frame, whereas in + 0WT::LEU, the MP is...
in-frame). Because the relative stabilities of the mRNAs differ according to their reading frame, the MD/MP ratios are not directly comparable between these constructs.

pYS7-7 is the parental slu7-1 complementing clone obtained from the Rose library (6.5-kb insert), pYS7-7 is the 2.5-kb Clal–EcoRI fragment of pYS7-1 insert cloned into YCP50. pBS7-CR is the Clal–EcoRI fragment of pYS7-1 in Bluescript. pBS7-GK is pBS7-CR with a TRP1 replacement of pBS7-1. The sequence of pBS7-1 is the parental plasmid shuffle at 25°C and 30°C. Strains cured of the SLU7-URA plasmid pYS7-7 were streaked onto YEP at 18°C, 25°C, 30°C, and 37°C to test for conditional defects.

For the competition assays, pYS7CCSS–URA and pYS7–URA were transformed into a derivative of strain yDF7-GK in which SLU7 was provided by pYS7–LEU. Transformants were cured of pYS7–LEU and retransformed with the plasmid pG1 + 0WT–LEU.

**A model of 3′ splice site selection**

To model the process of 3′ splice site selection, we extended the hypothesis of Pikielny and Rosbash (1985) to include both steps of the splicing pathway and a branch in the pathway that represents alternative splicing at the 3′ splice site:

\[
\begin{align*}
\text{p} & \xrightarrow{k_T} \text{l} \xrightarrow{k_L} \text{MD} \xrightarrow{k_{MD}} \text{MP} \xrightarrow{k_{MP}} \text{MP}_{\text{degradation}} \\
& \quad \xrightarrow{k_{DMP}} \text{MP}_{\text{degradation}} \\
& \quad \xrightarrow{k_{DMp}} \text{L} = k_{DL} + k_{MD} + k_{MP} + k_{Dp} + k_{L} \\
& \quad \xrightarrow{k_{MD}} \text{MD}_{\text{degradation}} \\
& \quad \xrightarrow{k_{DM}} \text{MD}_{\text{degradation}} \\
& \end{align*}
\]

proximal, \text{MD} = \text{mature-distal, } k_T = \text{rate of transcription, } k_L = \text{rate of lariat formation, } k_{MP}, k_{MD} = \text{rates of splicing at proximal } (\text{MP}) \text{ and distal } (\text{MD}) \text{ splice sites, } k_{DP}, k_{DL}, k_{DMP}, k_{DMD} = \text{degradation rates of the various products and intermediates. At steady state } (dp/dt = 0, \text{dL}/dt = 0, \text{dMP}/dt = 0, \text{dMD}/dt = 0):

\[
\begin{align*}
\text{MP/MD} & = \frac{k_{MP} k_{DMD}}{k_{MD} k_{DMP}} \\
\text{MP/L} & = \frac{k_{MP}}{k_{DMP} K_{DL} + k_{MD} + k_{MP} + k_{Dp} + k_{L}} \\
\text{MD} & = \frac{k_{MD} k_{L}}{k_{DMD}} \\
\text{MP} & = \frac{k_{MP} L}{k_{DMP}}
\end{align*}
\]

MP/MD is therefore proportional to the ratio of the individual rates of MP and MD production (\text{MP} and \text{MD}), MP/L and MD/L provide a measure of these individual rate constants. MP/MD, MP/L and MD/L are also influenced by message stability (\text{K}_{DMP} and \text{K}_{DMD}). The slu4-1 and slu7-1 mutations must, however, influence rates other than the mature mRNA degradation rates, because one effect of these mutations is to increase the absolute level of L, a change that cannot be attributed to altered message stability.

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Note added in proof
The nucleotide sequence data reported in this paper have been submitted to the EMBL, GenBank, and DDBJ nucleotide sequence data bases.

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