Some Cis- and Trans-Acting Mutants for Splicing Target Pre-mRNA to the Cytoplasm

Pierre Legrain* and Michael Rosbash
Department of Biology
Brandeis University
Waltham, Massachusetts 02254

Summary

We designed a strategy to identify splicing factors that act by preventing pre-mRNA transport into the cytoplasm. A yeast synthetic intron was inserted into a lacZ gene so that only the pre-mRNA could be translated to produce β-galactosidase activity. Deletion of either of the 5' splice junction sequence GUAUGU and the branchpoint sequence UACUAAC resulted in a dramatic increase in pre-mRNA translation, indicating its cytoplasmic localization. In ma6 and ma9 mutant strains assayed at the nonpermissive temperature, splicing inhibition occurred simultaneously with a large increase in pre-mRNA translation. Similarly, a point mutation in U1 snRNA decreased splicing efficiency and increased pre-mRNA translation. From these results, we conclude that early acting factors, probably including U1 snRNA, and the RNA6 and RNA9 gene products, interact in vivo with the 5' splice junction and the branchpoint sequence to commit the pre-mRNA to the splicing pathway, thereby preventing its transport to the cytoplasm.

Introduction

In eukaryotic cells, primary transcripts are processed in the nucleus prior to transport to the cytoplasm. For intron-containing transcripts, this process includes splicing, i.e., the removal of introns. Splicing events take place in large complexes called spliceosomes (Brody and Abelson, 1985; Grabowski et al., 1986; Fromdowoy and Keller, 1985). The spliceosome assembly pathway has been investigated almost exclusively in vitro; it is similar if not identical in yeast (Saccharomyces cerevisiae) and mammalian cells and includes the ordered binding of U2, U4/6, and Ul snRNPs to the pre-mRNA (Fikiel et al., 1986; Konarska and Sharp, 1986; Cheng and Abelson, 1987). More recently, several attempts have been made to characterize early steps in this pathway that may precede the binding of U2 snRNP (Legrain et al., 1988; Ruby and Abelson, 1988) from these and other in vitro experiments, several proteins or complexes have emerged as good candidates for early factors that bind to introns; these include hnRNP proteins (Choi et al., 1986), U1 snRNP (Bindereif and Green, 1987; Zillmann et al., 1987; Ruby and Abelson, 1988), and a U2 snRNP auxiliary factor, U2AF (Huskin et al., 1988). However, a direct in vivo approach is also needed to identify factors responsible for the early recognition of introns in primary transcripts.

In yeast, a class of thermosensitive (ts) mutant strains (ma mutants), originally identified based on the absence of ribosome accumulation at the nonpermissive temperature (Hartwell et al., 1970), have been shown to affect splicing in vivo (Rosbash et al., 1981; Fried et al., 1981; Teem and Rosbash, 1983). The analysis of whole cell extracts derived from several of these mutant strains indicated that splicing and spliceosome assembly are specifically inactivated by incubation at high temperature. This approach has led to the definition of various blockage points in the in vitro spliceosome assembly pathway (Lustig et al., 1986; Lin et al., 1987). However, to the best of our knowledge, no similar analysis has succeeded in establishing an in vivo assembly pathway. Since the splicing deficiency associated with most of these mutants is correlated with increased levels of pre-mRNAs (Rosbash et al., 1981; Fried et al., 1981; Jackson et al., 1988; Vijayaraghavan and Abelson, personal communication), it is likely that the unsplashed pre-mRNAs were not instantly degraded but accumulated, presumably in the nucleus. One can hypothesize that, for mutants in very early steps of the splicing pathway, the pre-mRNA may be directed to the pathway for non-intron-containing transcripts (i.e., transport to the cytoplasm). In contrast, for mutants in later steps, the pre-mRNA may be blocked within the spliceosome pathway (i.e., the presence of bound splicing factors may prevent pre-mRNA transport to the cytoplasm). However, a substantial accumulation of pre-mRNA may not be observed in the former situation since the half lives of cytoplasmic pre-mRNAs are unpredictable and may vary according to the specific characteristics of each intron (e.g., size, premature termination of translation; see Hoekema et al., 1987).

In order to use pre-mRNA translation as an assay for pre-mRNA transport from nucleus to cytoplasm, we designed a synthetic intron with an open reading frame and inserted it into the open reading frame of a β-galactosidase gene. The pre-mRNA was poorly spliced and accumulated in vivo. Surprisingly, a small proportion of the pre-mRNA was translated. Deletion of either one of the two important yeast sequences for spliceosome assembly (the 5' splice junction or the branchpoint sequence) was sufficient to allow the pre-mRNA to resemble a non-intron-containing transcript in that most of the RNA was transported to the cytoplasm and translated. The results support the hypothesis that it is indeed the interaction of intron sequences with trans-acting splicing factors that prevents pre-mRNA transport and translation.

This construct was then introduced into several ma mutant strains to check whether the translation of the pre-mRNA was increased at the nonpermissive temperature, i.e., when splicing was inhibited. Two mutants (ma6 and ma9) strongly exhibited such a phenotype. Similarly, when that construct was introduced into a U1 snRNA mutant strain (Seraphin et al., 1988), splicing efficiency was...
Figure 1. Structure of the PLint Plasmid

The sequence of the artificially synthesized BamHI fragment is depicted with the predicted amino acid sequence and the numbering, starting at the β-galactosidase initiation codon, printed below (see Experimental Procedures for cloning strategy). Restriction sites are overlined with the position of the cut indicated. The intron sequence is boxed with consensus sequences shaded. The main features of the pLGSD5 (Guarente et al., 1982) and PLint plasmids are indicated.

decreased and pre-mRNA translation significantly increased, suggesting that U1 snRNP could be one of the players in the process of intron recognition and pre-mRNA commitment to the splicing pathway.

Results

A Small Synthetic Intron with an In-Frame Coding Sequence Is Spliced In Vivo

We constructed a short, synthetic version of the RP51A intron with an open reading frame (Figure 1). The intron (63 nucleotides) is flanked by short exons, and the sequence contains multiple restriction sites that allow for easy modification of the reading frame and the generation of multiple, small deletions (see Experimental Procedures for the cloning strategy). The DNA was cloned in the plasmid pLGSD5, which encodes a hybrid CYCl-IacZ gene (Figure 1; Guarente et al., 1982). This new plasmid (PLint) also codes for β-galactosidase.

A wild-type strain (DB745) was transformed with PLint and, as a control, pLGSD5. RNAs were analyzed by primer extension after induction of the gene by the addition of galactose (Figure 2). In the absence of galactose, no β-galactosidase-specific RNA was detected in either transformant (Figure 2, lanes 1 and 5). After addition of galactose, specific RNAs accumulated during the first 6 hr. In pLGSD5-transformed cells, multiple bands (68–109 nucleotides long) were detected (Figure 2, lanes 2–4), corresponding to the multiple starts of transcription described for the CYCl gene (Faye et al., 1981; Teem and Rosbash, 1983). In PLint-transformed cells (Figure 2, lanes 6–8), two sets of bands were detected: high molecular weight bands (152–193 nucleotides) and smaller bands (89–130 nucleotides). These groups of bands corresponded to the primary transcripts with multiple starts of transcription and to their spliced products, respectively. The proportion of spliced RNA was about 10%–20%. From this result, we conclude that the PLint intron-containing...
pre-mRNA is spliced in vivo, although with a relatively poor efficiency.

From the PLint construct (in which both the pre-mRNA and the mRNA contain the β-galactosidase sequence in-frame with the CYC7 AUG initiation codon), we derived two variants in which the β-galactosidase coding sequence is in-frame with the CYC7 AUG for only the pre-mRNA or only the spliced mRNA (these constructs, and cells transformed with them, are hereafter referred to as Nde<sup>A</sup><sub>C</sub> and Acc<sup>A</sup>, respectively; see Experimental Procedures and Figure 3A). In addition, a third variant was constructed with the β-galactosidase coding sequence out of frame for both the mRNA and the pre-mRNA lacZ (Sty<sup>A</sup>; see Figure 3A). DB745 cells were transformed with these constructs, and β-galactosidase activity was assayed at different times after the addition of galactose (Figure 3B). Sty<sup>A</sup> did not display any activity, as expected for an entirely out-of-frame construct. Acc<sup>A</sup> exhibited almost as much activity as PLint, i.e., about 10% of the activity found in pLGSD5 transformed cells. Surprisingly, Nde<sup>A</sup><sub>C</sub> gave rise to significant β-galactosidase activity, well above background and about 3% of that derived from pLGSD5; this could not be attributed to the mRNA which is out-of-frame and similar to that encoded by Sty<sup>A</sup>. Therefore, we conclude that some intron-containing pre-mRNA molecules can exit the nucleus and be translated. Incidentally, the PLint construct, which has both the pre-mRNA and the mRNA-encoded β-galactosidase sequences in-frame, gave rise to an activity approximately equal to the sum of the Acc<sup>A</sup> and Nde<sup>A</sup><sub>C</sub> activities (Figure 3B). Western blot experiments verified that the amounts of β-galactosidase-containing fusion proteins were accurately reflected by the β-galactosidase activity measurements (Figure 3C): anti-β-galactosidase antibodies detected a large amount of protein in pLGSD5 transformed cells (lanes 1–4), much less in Acc<sup>A</sup> (lanes 5–8), and even lower amounts in Nde<sup>A</sup><sub>C</sub> (lanes 9–12).

RNA levels were analyzed in these cells by primer extension. Results obtained at different times after galactose addition are shown in Figure 3D. Spliced products were detected from both the Acc<sup>A</sup> and Nde<sup>A</sup><sub>C</sub> groups. The level of spliced RNA from Acc<sup>A</sup> was approximately 10%–20% of the pre-mRNA level (Figure 3D, lanes 5–8). This RNA profile was similar to that observed with PLint-transformed cells (Figure 2) and correlated well with the β-galactosidase activity from Acc<sup>A</sup> compared with that from pLGSD5. Nde<sup>A</sup><sub>C</sub> contained noticeably less spliced RNA (Figure 2, lanes 9–12). This result can be explained by a decreased cytoplasmic half-life of an out-of-frame mRNA as compared with in-frame mRNAs (Losson and Iaconato, 1979; Hoekema et al., 1987). The level of pre-mRNA found for Nde<sup>A</sup><sub>C</sub> was similar to that for Acc<sup>A</sup> and did not correlate with the β-galactosidase activity found for Nde<sup>A</sup><sub>C</sub> (Figure 3B). We conclude that in Nde<sup>A</sup><sub>C</sub> (and in PLint-transformed cells as well), most of the pre-mRNA was unavailable for translation, presumably because it was blocked in the nucleus. However, a small proportion (about 3%) of this pre-mRNA escaped the block and was translated. The only difference between Nde<sup>A</sup><sub>C</sub> and pLGSD5 transcripts is the presence of an intron. We presume that the inability of 95% of the pre-mRNA to be translated is due to an interaction of nuclear factors with some intron sequences. This is further supported by the deletion experiment described below.

**Deletion of Either One of the Consensus Splicing Signals Allows a Pre-mRNA to Be Translated Efficiently**

To investigate the critical sequences that prevent a pre-mRNA from being translated, we derived several deletion mutants from the PLint construct (Figure 4A). AA, AC, and AD are deletions of the 5' splice site, the TACTAAC box and the 3' splice site of the intron, respectively; AB is an internal deletion lying between the 5' splice site and the TACTAAC box (see Green, 1986, for a review of intron consensus sequences). The β-galactosidase coding sequence in all of these constructs is in-frame with the CYC7 AUG, as in Nde<sup>A</sup><sub>C</sub>. DB745 cells were transformed with these constructs, and a summary of the results obtained for β-galactosidase activity is given in Figure 4A. AA and AC expressed high activities, within a factor of two of that observed in pLGSD5-transformed cells and much higher than that observed in Nde<sup>A</sub><sub>C</sub>. On the contrary, both AB and AD gave rise to low activities, although higher than that detected in Nde<sup>A</sub><sub>C</sub>. By Western blot analysis, we verified that the amounts of these various β-galactosidase fusion proteins correlate with the detected enzymatic activities (data not shown). As analyzed by primer extension, pre-mRNA levels from AD and AD were indistinguishable as those found from the other constructs tested (Figure 4B; compare with Figures 2 and 3D). No detectable AB spliced RNA was observed (Figure 4B, lanes 1–4), as expected for an intron whose size falls below the minimum size required for splicing (Wieringa et al., 1984; Thompson-Jager and Domdey, 1987; Köhler and Domdey, 1988). Primer extension analyses from AD (Figure 4B, lanes 5–8) showed an additional strong band, corresponding to the lariat intermediate as deduced from the expected size of an extension product up to the branch point (Piklinsky et al., 1983); this indicates that, in vivo, at least some of the AD primary transcripts undergo spliceosome assembly and the first step of splicing, i.e., 5' splice site cleavage and lariat formation. This result is similar to those obtained in vivo, in yeast, for point mutations in the 3' splice site (Vijayraghavan et al., 1986; Fouser and Friesen, 1987) and in vitro with splicing substrates deleted or mutated in their 3' splice site (Reed and Maniatis, 1985; Rymond and Rosbash, 1985, 1986). The deletion mutant results show that the presence of both key consensus splicing signals (i.e., the 5' splice site and the TACTAAC box) is necessary for an efficient discrimination between yeast transcripts that either contain or lack an intron. The retention of 95% of the PLint pre-mRNA in the nucleus is directly related to the presence of both splicing consensus sequences. The increased activities observed in AB and AD constructs as compared with Nde<sup>A</sub><sub>C</sub> suggest that: an optimal recognition of an intron requires a minimal distance of the 5' splice site and the TACTAAC box; and the 3' splice site may contribute to the recognition process of a yeast...
introns, although weakly as compared with the 5′ splice site and the TACTAAC box. These experiments also confirm that the recognition process of the intron can be assayed by an analysis of Nde°Acc° pre-mRNA translation.

Some Mutations in Splicing Trans-Acting Factors Affect the Recognition Process of an Intron
Thermosensitive ma mutant strains (ma2–ma11; Hartwell et al., 1970) are known to be affected in splicing activity when the cells are shifted to the nonpermissive temperature (Rosbash et al., 1981). Indeed, when some of these strains (ma2, 6, 8, 9, 11) were transformed with the Acc° plasmid (for which only the mRNA codes for enzyme activity), β-galactosidase was undetectable when the cells were shifted to 35°C before galactose induction (data not shown). These mutants were also transformed with the Nde°Acc° and pLGSD5 plasmids. Detailed analyses were performed with an ma6 mutant strain, and

---

Figure 3. Structure and Expression of Variants from PLint Construct
(A) Characteristics of the different predicted amino acid sequences derived from the pre-mRNA or the mRNA synthesized by the different plasmids. Also shown is their ability to code for β-galactosidase.

(B) β-galactosidase activity assayed in DE745 cells transformed with various plasmids. Aliquots of cells were taken at different times after the addition of galactose. Background levels are below 0.5 U and the Sty° construct never exhibited activities above background.
results for \( Nde^{o}Acc^{o} \) activity, expressed as a percentage of pLGSD5 activity, are summarized in Figure 5A. At 26°C (open histograms), \( Nde^{o}Acc^{o} \) activity was higher in the \( ma6 \) mutant strain than in DB745 strain (wild-type cells). At 35°C (shaded histograms), this activity increased dramatically in \( ma6 \) cells while it decreased in DB745 cells. Two series of control experiments were performed. First, cells that grew at 37°C, i.e., revertants for the thermosensitivity phenotype, were isolated, and two clones were analyzed for \( Nde^{o}Acc^{o} \) expression (rev2 and rev4 in Figure 5A). Both of them exhibited a fairly high \( \beta \)-galactosidase activity at 37°C but failed to manifest increased activity at 35°C. Second, diploid cells were obtained by crossing \( ma6 \) mutant cells and DB745 wild-type cells. After sporulation, tetrads were dissected and cells derived from ten spores were transformed and analyzed for \( Nde^{o}Acc^{o} \) expression. They exhibit different activities at 26°C (open histograms) which do not correlate with thermosensitivity (growth phenotypes are depicted in Figure 5A). However, assays at 35°C divided the cells into two groups. Those that dramatically increased their \( Nde^{o}Acc^{o} \) activity as compared with 26°C were all thermosensitive, whereas those that showed similar or diminished activity at 35°C were thermoresistant. From these two series of experiments (i.e., revertant cells and spore analysis), we conclude that the \( ma6 \) mutation gives rise to a substantial increase in pre-mRNA translation at the nonpermissive temperature. However, differences in genetic backgrounds influence the translation of \( Nde^{o}Acc^{o} \) pre-mRNA at the permissive temperature.

Similar analyses were performed with other \( ma \) mutants and, as a control, five non-splicing-related thermosensitive mutants (Figure 5B). In addition to \( ma6 \), \( ma9 \) also displayed a very high expression of \( Nde^{o}Acc^{o} \) when cells were assayed at the nonpermissive temperature. Two other mutants, \( ma2 \) and \( ma8 \), showed a less marked but significantly increased activity at 35°C as compared with 26°C. \( ma11 \) mutant cells are not significantly affected by shift in temperature. We were unable to analyze other \( ma \) mutants due to the weak galactose induction observed in these strains (\( ma3 \), \( ma4 \), and \( ma6 \)). All five non splicing related thermosensitive mutants (\( sec1 \), \( sec5 \), \( sec9 \), \( sec10 \), and \( cdc9 \)) exhibited the same or lower \( Nde^{o}Acc^{o} \) expression at 35°C as at 26°C (Figure 5B). We conclude that \( ma6 \) and \( ma9 \) mutants express, in addition to the previously described splicing defect, a new phenotype, i.e., they allow a substantial fraction of pre-mRNA translation despite the presence of an intron.

Because U1 snRNP has been implicated in early steps of the spliceosome assembly pathway (Bindereif and
Figure 4. Deletion Mutant Analysis

(A) Shown is the amino acid sequence at the beginning of the β-galactosidase fusion protein. DNA sequence corresponding to PLint insertion is boxed with the small exonic sequences shaded. The intron is indicated by its consensus sequences, and deleted portions are shown as dark grey boxes with the number of removed nucleotides printed below. On the right, β-galactosidase activities detected 3 hr after the addition of galactose are given, normalized to 1000 U for pLGSD5. The numbers are mean values of three independent experiments that gave similar results (±15%). Similar relative values were observed at 1 hr or 2 hr after galactose induction.

(B) Primer extension analyses for deletion mutants. RNA from cells transformed with ΔA (lanes 1–4) or ΔD (lanes 5–8) were analyzed in a similar manner as in Figures 2 and 3D. Pictures are from the same autoradiogram and the intensities of bands are directly comparable. P: pre-mRNA; LI: lariat intermediate band (see text).

Figure 5. Pre-mRNA Translation in ma Mutant Cells

Histograms depict the β-galactosidase activities of Nde’rev transformed strains as a percentage of that of pLGSD5 in the same strains, assayed 3 hr after galactose induction. Similar histograms were obtained in several independent experiments. Open boxes, cells assayed at 26°C; shaded boxes, cells assayed at 37°C.

(A) Results are given for the ma6 mutant, DB745, two revertant strains originated from the ma6 mutant (rev2 and rev4) and spores derived from a diploid strain ma6 x DB745 (tetrad 2A, 2B, 2D; tetrad 4A, 4C, 4D; tetrad 5A, 5B, 5C, 5D). The ability to grow at 37°C (i.e., nonpermissive temperature) is indicated below each pair of histograms.

(B) Results are given for different ma mutants (ma2, ma6, ma8, ma9, and ma11) as well as for five other thermosensitive mutants (cdc9, sec1, sec5, sec9, and sec10). None of these strains grow at 37°C.
Splicing and Pre-mRNA Transport into the Cytoplasm

Green, 1987; Zillmann et al., 1987; Ruby and Abelson, 1988; Seraphin et al., 1988), we checked whether Nde\(^{6}\)Acc\(^{6}\) expression was specifically increased in strains that carry U1 snRNA mutants (Seraphin et al., 1988). Of the three mutants tested (U1-3C, U1-5A, and U1-4U, named by the position and the character of the mutation; Seraphin et al., 1988; B. Seraphin, personal communication), the first two showed no specific phenotype, either for the expression of Acc\(^{6}\) (where only the mRNA encodes \(\beta\)-galactosidase) or Nde\(^{6}\)Acc\(^{6}\) (where only the pre-mRNA encodes \(\beta\)-galactosidase) compared with the wild-type U1-bearing strain (U1-wt; data not shown). On the contrary, U1-4U cells showed a strong splicing defect as assayed by Acc\(^{6}\) \(\beta\)-galactosidase activity and a reproducible 2-fold increase in the translation of Nde\(^{6}\)Acc\(^{6}\) pre-mRNA (Figure 6; note the logarithmic scale). This increase is significant, we believe, because: the various U1 strains were precisely isogenic (see Table 1); the U1-4U mutant strain is viable (in contrast to the ma mutant strains assayed at the nonpermissive temperature); and there was no previous molecular phenotype associated with the U1-4U mutation as assayed on several intron-containing transcripts.

Table 1. Yeast Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>DB745</td>
<td>MATA</td>
</tr>
<tr>
<td>DJY366 (ma2)</td>
<td>MATA</td>
</tr>
<tr>
<td>SpB6.66 (ma8)</td>
<td>MATA</td>
</tr>
<tr>
<td>SpW3.31 (ma8)</td>
<td>MATA</td>
</tr>
<tr>
<td>JmB646 (ma9)</td>
<td>MATA</td>
</tr>
<tr>
<td>A6355 (ma11)</td>
<td>MATA</td>
</tr>
<tr>
<td>NY5029 (sec1)</td>
<td>MATA</td>
</tr>
<tr>
<td>NY5049 (sec5)</td>
<td>MATA</td>
</tr>
<tr>
<td>NY5078 (sec28)</td>
<td>MATA</td>
</tr>
<tr>
<td>NY5045 (sec12)</td>
<td>MATA</td>
</tr>
<tr>
<td>R1775 (cdc9)</td>
<td>MATA</td>
</tr>
</tbody>
</table>

U1-wt | MATA |

U1-4U | MATA |

Strains were obtained from *J. Beggs, *J. Woolford, *P. Novick, or *J. Haber; *construction of these strains is described in Seraphin et al. (1988).
(Seraphin et al., 1988). Moreover, the two other U1 snRNA mutants gave results identical to those obtained with U1- wt (data not shown), emphasizing the particular phenotype of U1-4U. Thus, these results implicate U1 snRNP in the discrimination between transcripts with or without an intron at an early stage in spliceosome assembly.

**Discussion**

Our strategy for identifying cis- and trans-acting elements that interfere with pre-mRNA transport from the nucleus to the cytoplasm was to design a gene with a synthetic intron in-frame with the coding sequence. The pre-mRNA has all the characteristics of a spliceable nuclear substrate, but it also shares with mRNAs those normal features that allow translation. This artificial intron resulted in a low in vivo splicing efficiency; unspliced RNA was predominantly found, whereas the poorest natural yeast splicing substrate of which we are aware, MATa1 pre-mRNA, has less than 20% unspliced RNA (Miller, 1984).

The poor efficiency of PLint pre-mRNA splicing is probably in part due to the small size of the PLint intron (similar to the MATa1 intron size), as well as to the deletion of some as yet unidentified RP51A intronic sequences known to contribute to a high splicing efficiency (Pikielny and Rosbash, 1985; similar contributions of nonconsensus sequences have been described for the AGC1 and CYH2 genes [Newman, 1987]).

It is interesting that a small proportion of the artificial pre-mRNA was translated (Figure 3B), suggesting that pre-mRNA can escape the splicing pathway and exit the nucleus. We do not know if pre-mRNAs with more efficiently spliced introns are less efficiently translated. The translation of this particular pre-mRNA, however, is not a consequence of the large accumulation of pre-mRNA, because a similar amount of pre-mRNA translation is detected shortly (15 min) after galactose addition when pre-mRNA levels are far below those detected several hours later (see Figure 3B; also, data not shown). Moreover, the proportion of the pre-mRNA that is translated seems to be more or less constant during pre-mRNA accumulation, suggesting that this phenomenon is related to poor intron recognition by splicing factors rather than to titration of limiting factors by excess pre-mRNA.

Export of intron-containing RNA to the cytoplasm is seen in a particular form of alternative splicing, i.e., that in which an intron is retained in the mature mRNA (Breitbart et al., 1987). For example, human retroviral RNAs can be spliced at different sites or processed without splicing to code for regulatory and structural proteins, and to provide virion RNA (Varmus, 1988). Also, alternative splicing in the y fibrinogen gene (Crabtree and Kant, 1982) and in the Drosophila P element (Laski et al., 1986) are examples in which the retained intron modifies the 3' end of the coding sequence of the protein product.

The inability of most of the Nde\textsuperscript{Acc}\textsuperscript{c} pre-mRNA to be translated, presumably due to its retention in the nucleus, is directly related to the presence in the intron of both key consensus sequences; deletion of either the 5' splice site or the TACTAAC box is sufficient to direct most, if not all, of the pre-mRNA toward the translational apparatus (Figure 4). Therefore, no factor that binds solely to one of these sequences is able to trap a detectable amount of pre-mRNA in the nucleus. This result confirms previous reports from in vitro experiments that indicate that early splicing factors bind synergistically to these two consensus sequences; competition experiments have shown that the presence of both sequences is required for the formation of the U2 snRNP-independent commitment complex identified in the splicing pathway in vitro (Legrain et al., 1988); independently, it has been shown that U1 snRNP binding to the pre-mRNA is affected by mutations in the branchpoint consensus sequence (Ruby and Abelson, 1988). The fact that deletion of the 3' splice site sequence only modestly affects the level of translated pre-mRNA confirms that the 3' splice site is not a key sequence for intron recognition in yeast (Rymond and Rosbash, 1985). However, for an inefficiently spliced intron, e.g., PLint, the influence of the 3' splice site becomes detectable.

When ma6 and ma9 mutant cells, transformed with the Nde\textsuperscript{Acc}\textsuperscript{c} plasmid (which encodes the in-frame pre-mRNA), are shifted to the nonpermissive temperature, this pre-mRNA is very efficiently translated at a level comparable to that obtained with a non-intron-containing transcript (Figure 5B). Based on this quantitative difference observed between ma6 and ma9 mutants and other ma mutants, we suggest that RNA6 and RNA9 gene products are involved in the early steps of the spliceosome assembly pathway, i.e., those that lead to stable complex formation. Splicing deficiency in these mutants is, therefore, a consequence of an intron recognition defect, which leads to transport of the pre-mRNA to the translational apparatus. This interpretation is reinforced by the finding that Nde\textsuperscript{Acc}\textsuperscript{c} translation is observed rapidly after galactose induction (data not shown). Among ma mutations, ma2 is the only one described that affects a late step in the in vitro assembly pathway (Cheng and Abelson, 1987). In this mutant strain, the somewhat increased level of pre-mRNA translation at the nonpermissive temperature (compared with the permissive temperature) can be explained by at least two nonexclusive hypotheses. First, the late effect on in vitro spliceosome assembly referred to above may be accompanied by an early effect on the in vivo assembly pathway. Second, mutations in late factors could lead to pre-mRNA transport by blocking completion of the spliceosome pathway, resulting in the titration of early factors.

The experiments shown in Figure 6 suggest that U1 snRNP is involved at a similar, early point in spliceosome assembly. Although many experiments show that U1 snRNP is necessary for splicing (reviewed in Maniatis and Reed, 1987), its precise role is still undefined. However, both the definition of a U2-independent commitment step for in vitro splicing and the finding that U1 snRNP binds to the pre-mRNA early in in vitro spliceosome assembly (Legrain et al., 1988; Ruby and Abelson, 1988; Seraphin et al., 1988) prompted us to assay the expression of Nde\textsuperscript{Acc}\textsuperscript{c} in U1 snRNA mutant strains (Seraphin et al., 1988). When Nde\textsuperscript{Acc}\textsuperscript{c} was introduced into the U1-4U
Splicing and Pre-mRNA Transport into the Cytoplasm

transcripts. These three factors, and perhaps others, contribute to the formation of a stable committed complex which will then undergo further steps in spliceosome assembly and splicing. Interference with one of these factors (as regulatory factors might do) would shift the equilibrium toward another pathway, i.e., transport of the pre-mRNA to the cytoplasm (Figure 7). For example, human retroviral posttranscriptional regulation might act to apopitically interfere with commitment to the splicing pathway, leading to pre-mRNA cytoplasmic transport. In this scheme, the processing of non-intron-containing mRNA is a default process. Clearly, the strategy developed here can now be extended to a screen for new mutants affecting pre-mRNA transport to the cytoplasm and to the analysis of some specific regulated pathways. This type of analysis is not limited to yeast. Transport of pre-mRNA into the cytoplasm of mammalian cells could be assayed in a similar way. This should be a powerful strategy for elucidating some mechanisms involved in alternative splicing and nuclear RNA processing.

**Experimental Procedures**

**Strains and Plasmids**

Yeast strains are listed in Table 1. Nontransformed cells were grown in rich medium containing 2% glucose at 26°C. Cells transformed with pLGSD5 and PLint derivatives were grown in selective medium without uracil, with 2% lactate, 2% glycerol, and 0.05% glucose at 26°C. Yeast transformations (I0 et al., 1983), crosses, sporulation, and tetrad dissections were performed according to standard procedures. Galactose induction was achieved by adding 25% of a 20% galactose solution to cultures grown to between 0.5 and 1 OD600. Temperature shift experiments were made by a 2-fold dilution of a culture into fresh medium at 26°C or 35°C. Galactose was added 15 min later. pLGSD5 was kindly given by L. Guarente (Guarente et al., 1982). pTZ19Rmid (US Biochemical) was used for the initial cloning of PLint insert and for the generation of the different variants (see below).

**Cloning Strategies for PLint and Derivatives**

The PLint sequence is derived from the short version (A2) of the RP51A intron (Pikielny et al., 1983). The basic strategy was to make a short intron of about 60 nucleotides, keeping as much as possible...
of the sequence near the consensus sequences, and adding
convenient restriction sites. Two oligonucleotides were synthesized:
U1235, 48 nucleotides long (5'-AATCGATACGACTTTTGAAATCAAAGAGTTGAATGTAATGCTCCGATCGACTTTGGAATGATAGTAATGTTGGTATCGAGAAGTTAG-3') and U1236, 63 nucleotides long:
(5'-AATCCGATACGACTTTTGAAATCAAAGAGTTGAATGTAATGCTCCGATCGACTTTGGAATGATAGTAATGTTGGTATCGAGAAGTTAG-3'). The 3' ends of U1235 and U1236 are complementary to each other. Their 5' ends can be ligated into a HindIII site and an EcoRI site, respectively. Two strategies have been followed with success. The first strategy was to anneal the two oligonucleotides, then to convert the structure into a double-stranded DNA with the Klenow fragment of E. coli DNA polymerase I, to cut that DNA with BamHI, and finally, to clone the fragment into pZ19R cut at the BamHI site. The second strategy was to ligate the two kinased oligonucleotides to pZ19R cut with EcoRI and HindIII (100-fold excess for each oligonucleotide); the DNA was then incubated with Klenow fragment, digested with Sall (to cut at the unique site in PLint: see Figure 1), gel purified, and religated.

The Acc2 and Sy6 constructs were obtained by linearizing the plasmid described above (PLint sequence in a pZ19R backbone) with Acc or Sty, respectively, and filling in with Klenow fragment. The NdeI/Acc2 construct was derived from the Acc2 construct by cutting at the NcoI site, trining in, and inserting a BamHI linker (New England Biolabs, #1003) to obtain the appropriate reading frame.

Deletion mutants IA, 1B, 1C, and 1D were deletions of Sty1-HindIII, HpaI-EcoRI, EcoRV-Stul, and Stul-NdeI fragments, respectively (see Figure 1 and 4A), followed by filling in when necessary to allow blunt-end ligations. Constructions were sequenced, both in the pZ19R backbone and in the pLGSOD backbone, with end-labeled primer DT320 (see below) and the Sequenase kit (US Biochemical) according to the manufacturer's recommendations.

**Primer Extension**

RNA was prepared according to Pikielny and Rosbash (1985). Primer extensions were performed as described in Teem and Rosbash (1985). The primer, DT320 (5'-CACTAGCAGACGCGG-3) is complementary to positions 27-42, numbered from the initiation codon for the $\beta$-galactosidase coding sequence in pLGSOD. Products were analyzed on an 8% polyacrylamide-urea gel.

**$\beta$-Galactosidase Assays**

Aliquots, 0.5 ml or 1 ml, were removed from cultures at different times after the addition of galactose. Cells were spun and then resuspended in 0.5 ml of 100 mM Na phosphate (pH 7.2) containing 10 mM KCl, 1 mM MgSO4, and 50 mM $\beta$-mercaptoethanol. Chloroform, 2.5 pl, was added, and cells were vortexed for 20 sec. One hundred microliters of sterile water was first incubated with rabbit anti-$\beta$-galactosidase (Cappel, #0631, diluted 1:1000) and then with 125I-labeled protein A (Amersham). Western Blot Experiments

Cells were collected and broken with glass beads by vortexing twice for 20 sec; then the supernatant was mixed with loading buffer for SDS-PAGE (Laemmli, 1970). Seven-and-a-half percent acrylamide gels were run and electroblotted onto nitrocellulose. The membrane was then incubated with rabbit anti-$\beta$-galactosidase (Cappel, #0631, diluted 1:1000) and then with 125I-labeled protein A (Amersham).

**Acknowledgments**

We would like to thank J. Beggs, L. Guarente, J. Haber, P. Novick, and J. L. Woolford for providing us with various biological materials and J. Beggs, J. Morran, and B. Seraphin for communicating unpublished results. We thank many members of the Rosbash laboratory, especially B. Seraphin, for helpful discussions. H. V. Colot, L. Kretzner, and B. Seraphin for critical reading of the manuscript, and T. Tishman for expert secretarial assistance.

P. L. is a fellow of the Fogarty International Center. This work was supported by grant FOS TO03928 to P. L. and grant GM23549 to M. R. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 18, 1988; revised March 2, 1989.

**References**


Seraphin, B., Kretzner, L., and Rosbash, M. (1986). A U1 snRNP pre-mRNA base pairing interaction is required early in yeast spliceosome assembly but does not uniquely define the 5' cleavage site. EMBO J. 5, 2533-2538.


ribonucleoproteins are required early during spliceosome assembly.