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Splicing and spliceosome formation of the yeast *MAT***a***1* transcript require a minimum distance from the 5′ splice site to the internal branch acceptor site

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ABSTRACT

Small deletions of 6, 7, and 12 nucleotides introduced between the 5' splice site and the internal branch acceptor site of the first intron of the yeast *MAT***a***1* gene completely abolish accurate splicing *in vitro* in these constructs. Splicing only occurs at an alternative 5' splice site which was found in the first exon of the *MAT***a***1* gene and which is used both *in vivo* and *in vitro*. The splicing defect cannot be cured by expanding the distance from the branch point to the 3' splice site. If the alternative 5' splice site is deleted as well in these constructs, neither spliced products nor spliceosomes are formed. Our findings especially lead to the conclusion that a minimum distance between the 5' splice site and the internal branch acceptor site of the intron is required for the formation of splicing complexes and for accurate splicing.

INTRODUCTION

Haploid cells of the yeast *Saccharomyces cerevisiae* that belong to different mating types are able to mate and form diploids. The two mating types, **a** or α, are determined by the *MAT* locus on chromosome III which is occupied either by the **a** or α allele (1). From *MAT*α as well as from *MAT***a** two transcripts (α1, α2, and **a**1, **a**2) are made by bidirectional promoters (2, 3). According to the model of Strathern *et al.* (4) the *MAT*α gene products either activate (α1), or repress (α2) different sets of genes in haploid α cells. In contrast, the gene product of *MAT***a***1* does not seem to have any regulatory function in haploid **a** cells because the **a**-specific genes are constitutively expressed. Only in diploid **a**/α cells, where both the *MAT***a** and *MAT*α alleles are present, could a function be hypothetically assigned to **a**1: the products of the *MAT***a***1* gene and the *MAT*α*2* gene act together to turn off the **a**-specific, α-specific, and the haploid specific genes, and to repress the inhibition of meiosis- and sporulation-specific genes.

The *MAT***a***1* gene has also attracted interest because it is the only known gene in the yeast *Saccharomyces cerevisiae* that contains more than a single intron (5). The two introns found here contain the highly conserved sequences at the 5' splice site (GTATGT), the 3' splice site (PyAG), and the internal branch acceptor site (TACTAAC) which have been documented as stringent structural requirements for correct and efficient removal of introns from mRNA precursors in yeast (6-15). In contrast to all other described yeast introns the two introns are extremely small, the first one comprising 54 and the second one 52 nucleotides.

These natural "mini-introns" therefore lack most of the non-conserved sequences, which are generally found between the three highly conserved sequence elements and which may, at least in some cases, influence splicing efficiency (16) or even spliceosome assembly (17).
As the expression of the *MAT***a**1 gene is cell-type specific and restricted to haploid **a** cells and diploid **a**/α cells, we chose to investigate whether there are any detectable differences when a *MAT***a***1* primary transcript is subjected to splicing extracts prepared either from **a** cells or from α cells. Concentrating our analysis on the first intron, we furthermore wanted to investigate, whether this intron has already reached a critical minimum size for accurate and efficient splicing.

Since even small deletions introduced in the non-conserved region between the 5' splice site and the internal branch acceptor site completely abolished accurate splicing *in vitro*, we examined which step of the splicing process was affected. Spliceosome formation analysis showed that already the first step of the reaction, the formation of a splicing complex, was blocked. On the other hand, spliceosomes were still formed when alternative 5' splice sites were present in the first exon. Because cryptic 3' splice sites could not restore splicing complex formation, our findings eventually lead to the conclusion that the minimum size of (at least) this yeast intron is not merely defined by its overall length, but by the distances from the 5' splice site to the internal branch acceptor site and from this site to the 3' splice site.

MATERIALS AND METHODS

Materials

All restriction endonucleases, the Klenow fragment of *E. coli* polymerase I, and T4 DNA polymerase were purchased from Boehringer, Mannheim. T4 DNA ligase and the cap-analog $m^7G(5')ppp(5')G$ were obtained from New England Biolabs, and T4 polynucleotide kinase from NEN. FPLC-pure mung bean nuclease was purchased from P-L Biochemicals, and reverse transcriptase from Life Sciences. α-^{32}P-UTP (29.6 TBec/mmol), γ-^{32}P-ATP (185 TBec/mmol), and SP6 RNA polymerase were obtained from Amersham. The following oligodeoxynucleotides were synthesized with an automated DNA synthesizer from Applied Biosystems:
5'-CTTTAGTCAAATTACTTTCCA-3' (E2),
5'-GATCTCATACGTTTATTTATG-3' (E3),
5'-CTTTCCATTATAAAATTATAAAGA-3' (EE1),
5'-CTTTCCATTATAAATACAAATATCATC-3' (EEa),
5'-CGTTTATTTATGAACCAAACTC-3'(EE2),
5'-GTTGTCCTTCTGGATCCTCTTTGATATA-3' (MB),
5'-GTAAATTGATTCCCGGGCTATCCTTG-3' (MS),
5'-GTTAGTAAATTGATTCAATCCGATACCTTGAAATGAAAAC-3' (7C),
5'-CCATTATAAACTGAAAGGCTATGTATTGTTAGTAAATT-3' (Δ7A).

Bacterial and yeast strains

The *E. coli* K12 strain JM109 (*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1,* λ⁻, Δ*(lac-proAB), [F', traD36, proAB, lacI^q ZΔM15]*) was used as bacterial host for propagation of M13 phages and for cloning steps with pSP64, pUC13 and M13mp19 (RF) vectors. WK6 (*galE, Δ(lac-proAB), strA / F' lacI^q, ZΔM15, proA⁺B⁺*) and WK6mutS (*galE, Δ(lac-proAB), strA, mutS215::Tn10 / F' lacI^q, ZΔM15, proA⁺B⁺*) were used for oligodeoxynucleotide-directed *in vitro* mutagenesis by the gapped duplex DNA method. Transformation of *E. coli* cells was done as described by Hanahan *et al.* (18).

Yeast splicing extracts were prepared from EJ101 (MATα, *trp1, pro1-126, prb1-112, pep4-3, prc1-126*), IM1783 (*MAT*a, *trp1, leu2, ura3, his4, can1*) and IM1784 (*MAT*α, *trp1, leu2, ura3, his4, can1*). The gene replacement experiments were done in the yeast strain KN79 (*MAT*α, *ho, leu2, trp1*). JJ1C (*MAT*α, *arg1, thr1*) was used as mating tester strain.

Construction of SP6-*MAT***a***1* fusion plasmids and generation of deletion mutants (see Figure 1)

From pC54, a yeast shuttle vector containing the complete *MAT***a***1* locus on a HindIII fragment (19), a 1.1 kb XbaI-HindII fragment, containing the entire *MAT***a***1* coding region with 5' and 3' flanking sequences, was first cloned into the XbaI and SmaI sites of M13mp19 (20). A single BamHI site was created in this construct (M13-MATa1), in front of the first putative *MAT***a***1* transcription start site (21), by site directed *in vitro* mutagenesis according to Newman *et al.* (11) using the oligodeoxynucleotide MB. Single stranded DNA from this construct (M13-BMAT) was used for three further site directed *in vitro* mutageneses. First, a SmaI site was created in the first intron of the *MAT***a***1* gene (M13-BMAT-S) with the oligodeoxynucleotide MS. Second, the complete first intron was exactly deleted (M13-BMAT-ΔIVS), with the help of an exon 1 - exon 2 junction oligodeoxynucleotide (EE1). Third, a stretch of seven nucleotides in the first intron between the 5' splice site and the internal branch acceptor site was changed to its complementary sequence with the oligodeoxynucleotide 7C. Each mutation was confirmed by DNA sequence analysis according to Sanger *et al.* (22). From all the constructs (M13-BMAT, M13-BMAT-S, M13-BMAT–ΔIVS and M13-BMAT-7C) the BamHI-EcoRI fragment containing the complete *MAT***a***1* structural gene was inserted into the BamHI and EcoRI sites of the transcription vector pSP64 to create pBEMAT which contains the wildtype *MAT***a***1* coding sequence, pBEMAT-S which harbors a unique SmaI site in the first intron, pBEMAT-ΔIVS which is lacking the complete first intron, and pBEMAT-7C in which a stretch of seven nucleotides in front of the TACTAAC-box is changed to its complementary sequence.

Four different deletions were introduced in the first intron of the *MAT***a***1* gene, between the 5' splice site and the internal branch acceptor site, in the pBEMAT-S construct using the following protocol: pBEMAT-S (2μg) was linearized with SmaI, phenol-extracted and precipitated with 2.5 volumes ethanol. The DNA was then incubated for 5 min at 37°C in 33 mM Tris-acetate (pH 7.9), 66 mM potassium acetate, 10 mM magnesium acetate, 0.05 mM

DTT, 0.01 mg/ml BSA, and 3 units of T4 DNA Polymerase, without deoxynucleotide triphosphates. The T4 DNA polymerase was inactivated by heating the reaction mixture to 65°C for 5 min. In order to cleave off the overhanging ends, the ethanol precipitated DNA was incubated in 30 mM sodium acetate, 20 mM $ZnCl_2$, and 5 % glycerol with 1 unit mung bean nuclease for 5 min at 37°C. The phenol extracted and ethanol precipitated DNA was then incubated in the presence of 50 µM dNTPs, 50 mM Tris-HCl (pH 7.2), 10 mM $MgSO_4$, 0.01 mM DTT, and 5 µg/ml BSA with 5 units Klenow enzyme, in order to produce higher yields of blunt ends. After religation with T4 DNA ligase the DNA was ethanol precipitated and digested with SmaI. The phenol-extracted and ethanol precipitated reaction products were used to transform JM109. DNA sequence analysis according to Chen and Seeburg (23) of the plasmids isolated from several transformants revealed deletions in both directions of the SmaI site in the first intron of the *MAT***a***1* gene. The sequences of the selected mutants pBEMAT-SΔ1, pBEMAT–SΔ6, pBEMAT–SΔ7, and pBEMAT–SΔ12 are partly presented in Figure 1 B. pRMAT, pRMAT-SΔ7, and pRMAT-ΔIVS were constructed by subcloning the RsaI fragments, which contain most of the *MAT***a***1* gene but lack 72 bp of the first exon including the two alternative 5' splice sites, of pBEMAT, pBEMAT-SΔ7, and pBEMAT-ΔIVS, into the SmaI site of pSP64. The construct pRMAT-SΔ7 was furthermore used as substrate for generating a length-compensatory mutation in the first intron. For that purpose, the 1kb BamHI/EcoRI insert of pRMAT-SΔ7 was ligated into the BamHI and EcoRI sites of the phasmidvector pMc5-8/R. In this construct (pMcMAT-SΔ7) seven nucleotides were inserted between the internal branch acceptor site and the 3' splice site of the first intron by the gapped duplex DNA method according to Kramer and Fritz (24) with the oligodeoxynucleotide Δ7A (pRMAT-SΔ7A).

Gene replacements

In the construct M13-MATa1 (see Fig. 1A) four different mutations were generated by oligodeoxnucleotide site directed mutagenesis *in vitro* according to Newman *et al.* (11): the deletion of intron 1 with the oligodeoxynucleotide EE1, the deletion of intron 2 with the oligodeoxynucleotide EE2, the deletion of both introns with the oligodeoxynucleotides EE1 and EE2, and finally the insertion of a unique SmaI site in the first intron with the oligonucleotide MS. A 4.2 kb HindIII fragment containing the complete *MAT***a***1* locus was subcloned from the plasmid pC54 (19) into the HindIII site of pUC13. This construct was completely digested with BglII and partially with XbaI, so that the *in vitro* mutagenized *MAT***a***1* sequences could be inserted as BglII/XbaI fragments. Yeast strain KN79 (*MATα, ho, leu2, trp1*) was cotransformed essentially as described by Rudolph *et al.* (25) with YEP13 and the isolated 4.1-4.2 kb HindIII fragments which carried in one case the unmutagenized *MAT***a** locus as a control and in the other cases the different intron deletions (Δ1, Δ2, Δ1/2), or the SmaI mutation. Leu$^+$ transformants were screened by mating with a *MATα* tester strain for replacement of the *MATα* sequences by the different *MAT***a** sequences. Correct integration of

the mutated *MAT***a** sequences at the *MAT* locus was verified by Southern blots of genomic DNA (data not shown).

Preparation of synthetic *MATa1* transcripts

RNA precursors were synthesized *in vitro*, essentially as described by Melton *et al.* (26), in 20 µl reactions containing 1 µg MboII-or BstNI-digested template DNA, 40 mM Tris-HCl (pH 7.5), 10 mM DTT, 2 mM spermidine, 6 mM $MgCl_2$, 10 µg/ml BSA, 0.5 mM of each nucleotide triphosphate, 1.5 mM m^7GpppG and 5 units SP6 RNA polymerase. Reactions were carried out at 40°C for 1 h, and the products were separated on 6% polyacrylamide / 8 M urea gels. Full length transcripts were eluted from excised gel pieces in 0.5 M ammonium acetate, 0.01 M magnesium acetate, 0.1 % SDS, and 0.1 mM EDTA for 3 hours, and precipitated with 2.5 volumes of ethanol. In order to obtain ^{32}P-uridine labeled transcripts 20 µCi of α-^{32}P-UTP were added to the reaction mixture.

In vitro splicing reactions

In vitro splicing reactions were performed essentially as described by Lin *et al.* (27) in a total volume of 30 µl at 30°C for 60 min containing around 20 ng (200 fMoles) of unlabeled precursor RNA. Reactions were stopped by the addition of 300 µl stop-buffer (100 mM NaCl, 4 mM EDTA, 67 mM sodium acetate, pH 5.0, 0.2 % SDS, 6 µg/ml *E. coli* tRNA) and 300 µl phenol. After phenol-extraction nucleic acids were precipitated with 2.5 volumes of ethanol, rinsed with 70 % ethanol and dried in vacuo.

Isolation of total yeast RNA

Isolation of total RNA from yeast cultures using hot phenol has been described previously (28).

Primer extension analyses

Approximately 50 µg total RNA or half of the phenol extracted and ethanol precipitated nucleic acids of the 30 µl *in vitro* splicing reactions were incubated in the presence of 1 ng ^{32}P-endlabeled oligodeoxynucleotide E2 or E3, 50 mM Tris-HCl (pH 8.3), 150 mM KCl, 0.5 mM EDTA, 1 mM DTT, 7 mM $MgCl_2$, 50 µM dATP, 50 µM dTTP, 50 µM dCTP (or dGTP), and 250 µM ddGTP (or ddCTP) for 2 min at 65°C in a total volume of 24 µl. The reaction mixtures were slowly tempered down to 42°C and 5 units reverse transcriptase were added. After 1 h incubation at 42°C, alkaline hydrolysis of the RNA (28) and ethanol precipitation, the primer extension products were analyzed along oligodeoxynucleotide size markers or DNA sequencing reactions on 12 % polyacrylamide / 8 M urea gels.

Glycerol gradient sedimentation analysis of splicing complex formation

Glycerol gradient sedimentation analysis of spliceosome formation was done essentially as described by Brody and Abelson (29). ^{32}P-labeled transcripts were incubated for 15 min at 30°C, the reaction mixtures (30 µl) were chilled on ice and layered on precooled (4°C) 15 % - 40 % glycerol gradients containing 20 mM HEPES (pH 7.0), 100 mM KCl, 2 mM $MgCl_2$, and 0.2 mM DTT. Centrifugations were carried out in a SW41 TI rotor

(Beckman) at 30,000 rpm for 16 h at 4°C. The gradients were fractionated from bottom to top in 400 µl aliquots and the radioactivity of each fraction was determined as Cerenkov cpm.

Spliceosome analyses on native gels

Splicing reactions (30 µl) with ^{32}P-labeled transcripts were carried out as described above. After 0, 1, 2, 5, and 20 min 5 µl aliquotes were withdrawn and reactions were stopped by adding 2.5 µl ice cold stop solution containing 50 mg/ml Heparin, 5 mM DTT and 5 U/µl RNasin (17). The reactions were kept on ice until they were loaded with 2.5 µl loading buffer (50 % glycerol, 2.5 x TBE, 0.05% Bromophenol blue) on an agarose-polyacrylamide composite gel (0.5% agarose, 3% acrylamide/bisacrylamide with a cross-linking ratio of 160:1, 0.5 x TBE, 200 x 200 x 0.15 cm). Samples were fractionated by electrophoresis at 100 V and 4°C for 15 h and subsequently blotted onto DEAE paper (Whatman DE81).

RESULTS

Construction of SP6-*MATa1* fusion plasmids carrying different mutations in the first intron of the *MATa1* gene

The main goal of this study was to determine the minimum length requirements of and in the first intron of the *MATa1* gene for efficient splicing *in vitro*. To introduce specific changes into the nucleotide sequence of the *MATa1* gene, the entire gene plus 5' and 3' flanking sequences were first cloned as a 1.1 kb XbaI-HindII fragment into M13mp19 (Figure 1A). Via oligodeoxynucleotide directed mutagenesis a BamHI site was created 3 nucleotides upstream of the first putative *MATa1* transcription start site so that the entire gene without the *MATa1* promoter region could be cloned later as a BamHI-EcoRI fragment into an SP6 transcription vector. Two separate additional changes were made in this mutagenized M13-*MATa1* construct. First, a SmaI site was created in the nonconserved region of the first intron between the 5' splice site and the internal branch acceptor site. This SmaI site was used later to remove parts of the intron sequence in this region. Then, to have a transcript lacking exactly the complete first intron, the intron was precisely deleted using an exon 1 - exon 2 junction oligodeoxynucleotide.

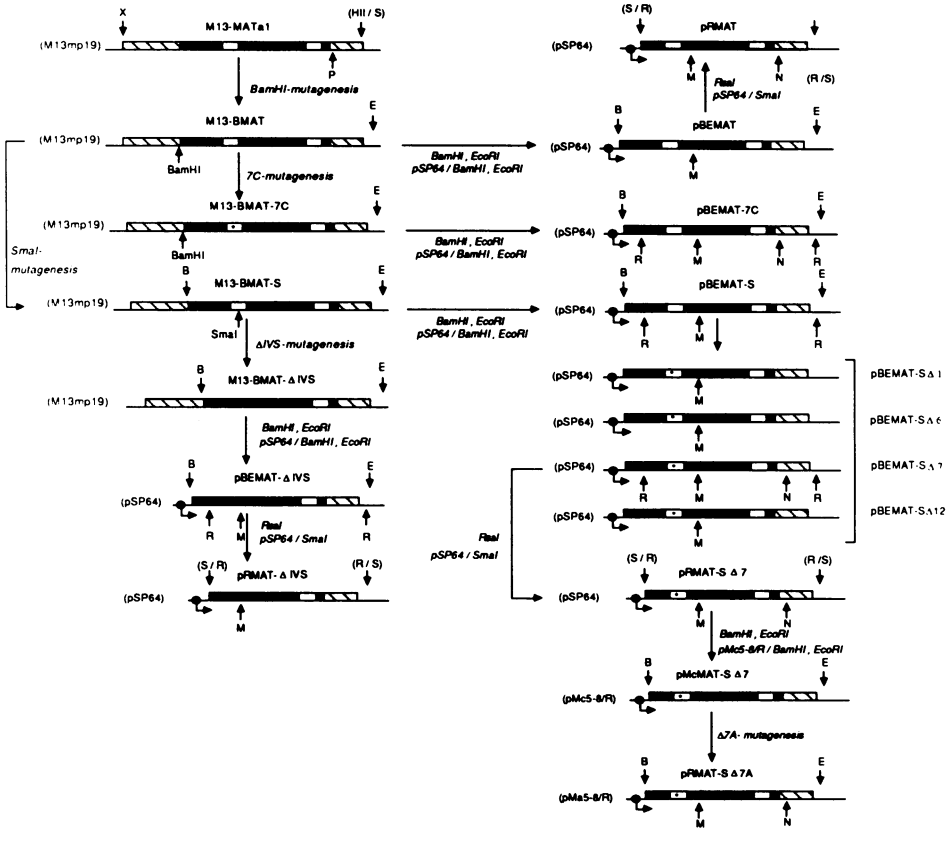
After cloning the mutagenized structural parts of the *MATa1* gene as BamHI-EcoRI fragments into the transcription vector pSP64, thus generating the plasmids pBEMAT, pBEMAT-S, and pBEMAT-ΔIVS, four deletion mutants were generated from pBEMAT-S in which the distance between the 5' splice site and the internal branch acceptor site was shortened (Figure 1B). These constructs cover the deletion of a single nucleotide at the SmaI site (pBEMAT-SΔ1), deletions of 6 and 7 nucleotides (pBEMAT-SΔ6 and pBEMAT-SΔ7) and a deletion of 12 nucleotides (pBEMAT-SΔ12) in which the first nucleotide of the the conserved TACTAAC sequence is already removed.

In vitro splicing of SP6-*MATa1* transcripts which have different mutations in the first intron

MATa1 transcripts comprising the first exon, the first intron and part of the second

exon were synthesized from MboII digested pBEMAT, pBEMAT-S, pBEMAT-SΔ1, pBEMAT-SΔ6, pBEMAT-SΔ7, and pBEMAT-SΔ12 (see Figure 1) and incubated in a yeast splicing extract. Splicing of the different transcripts was analyzed with a reverse transcription assay. Primer extensions were carried out in the presence of ddGTP with the 5' labeled oligodeoxynucleotide E2 as a primer which hybridizes to nucleotides 8-28 of the second exon, and the RNAs which had been subjected to the splicing extract as templates. As can be seen in Figure 1C, the primer extension product of an unspliced precursor has a length of 31 nucleotides, or 34 nucleotides, if reverse transcription stops at the second cytidine residue of the template RNA; a correctly spliced mRNA yields a 37 nucleotide-long cDNA.
When the splicing efficiencies of the transcripts from pBEMAT, which contains the wildtype intron sequence, and pBEMAT-S, in which a row of three thymidines had been changed to CGG to create a SmaI site, were compared, no obvious differences could be detected (Figure 2A, lanes WT and S). Conversely, the transcript derived from pBEMAT-SΔ1, in which one nucleotide had been deleted in the nonconserved region between the 5'splice site and the

A



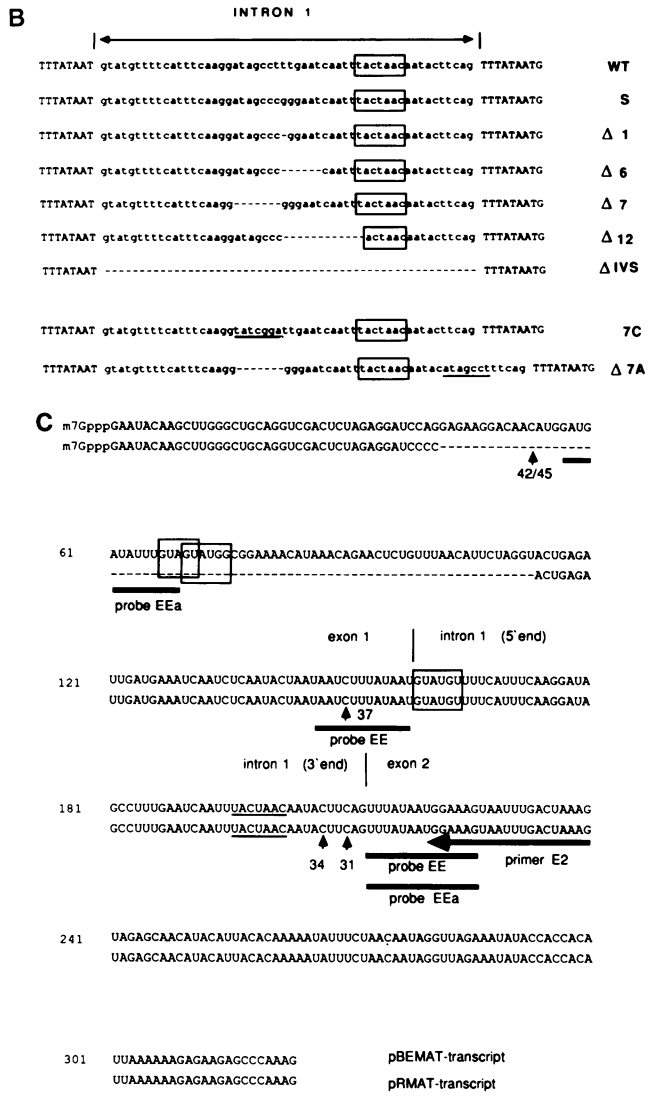


FIG. 1. (A) Outline of strategy for producing mutations in the *MATa1* ene and for generating SP6-*MATa1* fusions.
M13-MATa1 is M13mp19 containing a 1.1 kb XbaI-HindII fragment which includes the *MATa1* gene plus flanking regions inserted in the XbaI and SmaI sites of the polylinker. In this construct a BamHI site was created by oligodeoxynucleotide directed mutagenesis just in front of the first transcription start site of the *MATa1* gene (M13-BMAT). M13-BMAT single stranded DNA served as template for changing 7 nucleotides (M13-BMAT-7C) or introducing a SmaI site (M13-BMAT-S) between the 5' splice site and the internal branch acceptor site of the first intron of the *MATa1* gene. M13-BMAT-ΔIVS was obtained from this construct by exactly deleting the entire first intron. Subcloning of the BamHI-EcoRI fragments from M13-BMAT, M13-BMAT-7C, M13-BMAT-S, and M13-BMAT-ΔIVS which contain the

entire *MAT***a***1* structural gene into pSP64 lead to pBEMAT, pBEMAT-7C, pBEMAT-S, and pBEMAT-ΔIVS, respectively. pBEMAT-S served as substrate for introducing small deletions in the first intron (pBEMAT-SΔ1, pBEMAT-SΔ6, pBEMAT-SΔ7, and pBEMAT-SΔ12). Subcloning of the RsaI fragments, including most of the *MAT***a***1* structural gene but lacking 72 bp of the first exon, from pBEMAT-SΔ7, pBEMAT, and pBEMAT-ΔIVS into the SmaI site of pSP64 lead to pRMAT-SΔ7, pRMAT, and pRMAT-ΔIVS. A BamHI-EcoRI fragment of pRMAT-SΔ7 was ligated into pMc58/R (pMcMAT-SΔ7). In this construct, 7 nucleotides between the internal branch acceptor site and the 3' splice site of the first intron were inserted to give pRMAT-SΔ7A. Thin lines represent vector sequences, solid bars coding exon sequences, hatched bars nontranslated leader and trailer exon sequences, and open bars intron sequences. Asterisks indicate the deletions introduced in the first intron. SP6 transcription start sites are indicated by rectangular arrows. B (BamHI), E (EcoRI), HII (HindII), M (MboII), N (BstNI), P (BglII), R (RsaI), S (SmaI), X (XbaI).
(B) Nucleotide sequence of the first intron and the adjacent parts of the surrounding exons of the *MAT***a***1* gene.
Below the wildtype sequence (WT), the SmaI mutation (S) and five different deletions of 1 (Δ1), 6 (Δ6), 7 (Δ7), and 12 (Δ12) nucleotides in the first intron and of the entire first intron (ΔIVS) are shown. 7C is a construct in which a stretch of 7 nucleotides in front of the internal branch point was replaced by the complementary sequence. In Δ7A, seven nucleotides are deleted between the 5'splice site and the internal branch acceptor site and 7 nucleotides are inserted between the branch point and the 3'splice site. Boxed areas indicate the conserved heptanucleotide sequences around the internal branch acceptor site.
(C) Nucleotide sequence of the two SP6 transcripts generated from MboII digested pBEMAT and pRMAT DNA.
Boxed areas indicate the 5' splice site at the 5' end of the first intron and the alternative 5' splice sites in the first exon. Bars represent the 3' halves and 5' halves of the two exon-exon junction oligodeoxynucleotides EE1 and EEa. The long horizontal arrow stands for primer E2 which was used for reverse transcription. Small vertical arrows indicate the primer extension endpoints when reverse transcription was carried out with primer E2 in the presence of ddGTP. Unspliced precursor RNA yields reverse transcripts of 31 and 34 nucleotides length. In the case of a correctly spliced mRNA, the reverse transcription leads to a 37 nucleotide-long transcript. If the alternative 5' splice sites at positions 67 and 70 are used, reverse transcription gives rise to cDNA transcripts of 42 and 45 nucleotides length, respectively. The first exon of the pRMAT transcript is 72 nucleotides shorter (indicated by a dashed line), and does therefore not contain the two alternative 5' splice sites.

internal branch acceptor site, exhibited a distinctly reduced splicing efficiency (Figure 2A, lane Δ1). To measure this reduced splicing efficiency more accurately, a longer pre-mRNA which also contained the second intron of the *MAT***a***1* gene was synthesized, spliced *in vitro* and the splicing products were analyzed by reverse transcription with oligodeoxynucleotide E2 in the presence of ddCTP (Figure 3). This analysis allowed us to compare on a quantitative level the amounts of the cDNA products deriving from spliced RNA (61 nucleotides) and from unspliced RNA, in which termination of reverse transcription occurred at the second guanosine residue (54 nucleotides). Scanning of the autoradiogramms revealed that the splicing efficiency of the first intron of the Δ1 transcript was only around 40 % of the splicing efficiency of the first intron of the wildtype transcript. In all of the examined constructs the splicing efficiency of the second intron, which was measured by primer extension in the presence of ddGTP with the oligodeoxynucleotide E3 which primes at the boundary of intron 2 and exon 3, was unchanged.

A cDNA transcript of 37 nucleotides, which would have been the indication for a

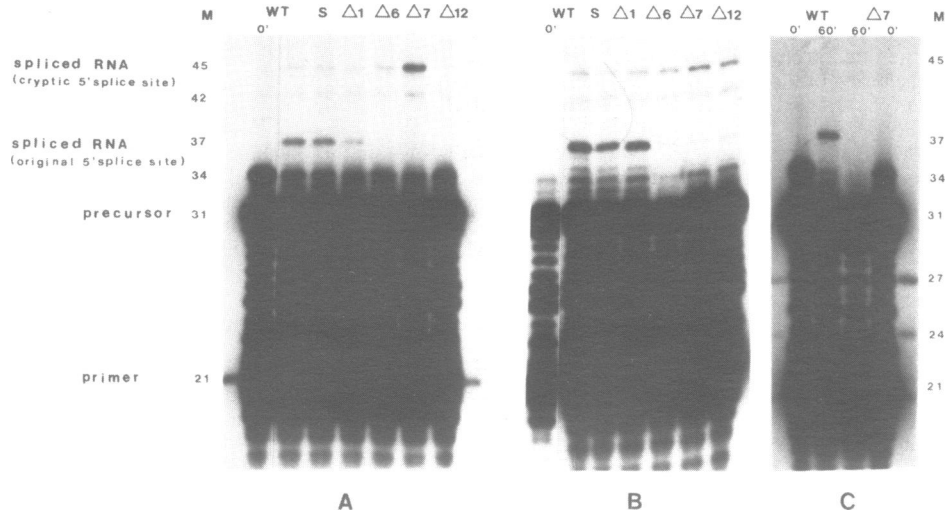


FIG. 2. Primer extension analysis of *in vitro* spliced RNA.
SP6 transcripts of MboII-digested template DNAs were incubated for 60 min (if not indicated otherwise) at 30°C in yeast splicing extracts prepared from cells of mating type α (A and C) and mating type **a** (B). RNA was isolated from the reaction mixture and reverse transcription was carried out in the presence of ddGTP with the 5' ^{32}P-labeled *MATa1* exon 2 specific oligodeoxynucleotide E2 as primer. The cDNA transcripts were separated on 12 % polyacrylamide / 8 M urea gels. (A) and (B): WT (pBEMAT), S (pBEMAT-S), Δ1 (pBEMAT-SΔ1), Δ6 (pBEMAT-SΔ6), Δ7 (pBEMAT-SΔ7), Δ12 (pBEMAT-SΔ12). (C): WT (pRMAT), Δ7 (pRMAT-SΔ7). Primer extension products of 31 and 34 nucleotides correspond to unspliced precursor RNA, of 37 nucleotides to correctly spliced RNA, of 45 nucleotides to alternatively spliced RNA (see Figure 1C).

correct splicing reaction, could not be detected, when the transcripts of pBEMAT-SΔ6, pBEMAT-SΔ7, and pBEMAT-SΔ12 were used as substrate in the *in vitro* splicing reaction (Figure 2A, lanes Δ6, Δ7, and Δ12). This result strongly suggests that in these transcripts the distance from the 5' splice site to the internal branch acceptor site had been shortened too far and therefore a complete and accurate splicing reaction could no longer take place. Correct splicing could be restored by inserting into the Δ7 transcript a stretch of seven different, i. e., complementary nucleotides at the same position (Figure 4, lane 3). This result indicates that the absence of splicing of at least the Δ7 transcript was not caused by the loss of a specific sequence context required for splicing. Conversely, splicing could not be restored by inserting between the internal branch point and the 3' splice site the previously deleted seven nucleotides (Figure 4, lane 6). Especially this direct comparison of two introns with the same overall length, but different distances between the conserved sequence elements (Figure 4, lanes 4 and 6), demonstrates that a minimum distance between the 5' splice site and the internal branch point is required for accurate splicing.

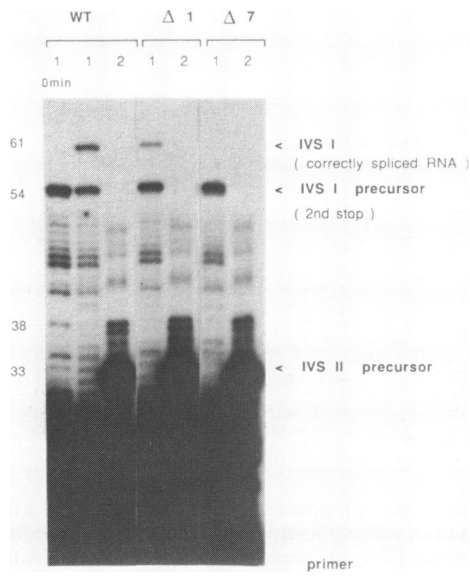


FIG. 3. The deletion of a single nucleotide in the first intron leads to a reduced splicing efficiency.
Transcripts of BstNI digested pBEMAT (WT), pBEMAT-SΔ1 (Δ1), and pBEMAT-SΔ7 (Δ7) which contain the entire *MAT***a***1* coding region including both introns were incubated in a yeast α-cell splicing extract for 60 min. The RNAs were extracted from the reaction mixtures and divided into two parts. Primer extension analyses were done either with the 5' ^{32}P-labeled exon II specific primer E2 in the presence of ddCTP (1) or with the 5' ^{32}P-labeled exon III specific primer E3 in the presence of ddGTP (2). (1), the 61 nucleotide-long cDNA derives from an RNA in which the first intron was correctly excised; the 54 nucleotide-long cDNA transcript derives from an RNA in which the first intron was not excised and reverse transcription stopped at the second guanosine residue. (2), the 33 nucleotide-long cDNA transcript derives from an RNA in which the second intron was not excised; a cDNA transcript of 38 nucleotides length would correspond to an RNA with a correctly excised second intron.

Surprisingly, an additional cDNA transcript of 45 nucleotides length was present in all lanes of Figure 2A, aside from the 0 min control experiment. A slightly increased amount of this 45 nucleotides long transcript was found in lane Δ7. A possible explanation for this reverse transcript was that it derived from alternatively spliced RNA. Indeed, two alternative 5' splice sites, GUAGUA and GUAUGG, located in the first exon 92 and 89 nucleotides, respectively, upstream of the normal 5' splice site (see Figure 1C) could be identified. As shown in Figure 1C, the utilization of these two 5' splice sites would lead to reverse transcripts 42 and 45 nucleotides long. The 42 nucleotide-long cDNA which can also be detected in all lanes of Figure 2A, even in the 0 min control, can possibly also derive from reverse transcription termination at a more distant cytidine residue of the RNA precursor, as did the 39 nucleotide-long transcript which can be observed only in the 0 min control. To test the hypothesis that

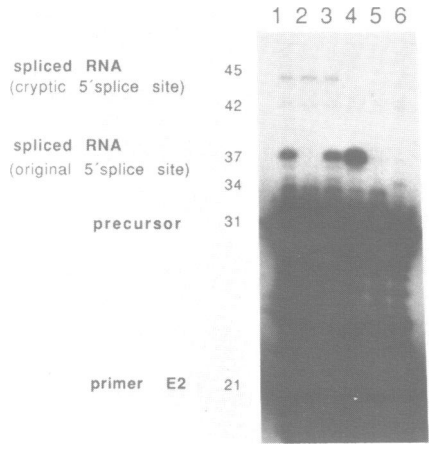


FIG. 4. Effects of different length-compensatory mutations introduced into the first intron. Primer extension analysis of *in vitro* spliced *MATa1* transcripts was done with the 5' ^{32}P-labeled exon II specific primer E2 in the presence of ddGTP. A cDNA transcript of 31 nucleotides corresponds to unspliced precursor, a 37 nucleotide-long cDNA results from a correctly spliced transcript, the 45 and possibly also the 42 nucleotide-long reverse transcripts result from alternatively spliced transcripts. Transcripts were made from BstNI digested pBEMAT (1), pBEMAT-SΔ7 (2), pBEMAT-7C (3), pRMAT (4), pRMAT-SΔ7 (5), and pRMAT-SΔ7A (6).

alternative splicing had indeed occurred, a Northern blot of *in vitro* spliced transcripts from pBEMAT-SΔ7 was hybridized with an oligodeoxy-nucleotide (EEa) complementary to the alternative exon-exon junction 89 nucleotides upstream of the 5' splice site of the first intron, and a band of the correct size was obtained (data not shown). To provide further proof, SP6-*MATa1* fusions with a shortened first exon (pRMAT and pRMAT-SΔ7, Figure 1A and C) were constructed which no longer contain the alternative 5' splice site(s). When the pRMAT-SΔ7 transcript was incubated in the splicing extract and the reaction products were analyzed by primer extension analysis, the 45 nucleotide band was no longer present, even after prolonged exposure (Figure 2C, lane Δ7). This result again suggests that the 45 nucleotide cDNA product in Figure 2A (lane Δ7) came from an alternatively spliced transcript through the use of one of the alternative 5' splice sites in the first exon. Data which are presented below and which were obtained through the analysis of total RNA isolated from different *MATa1* mutant strains corroborate this conclusion.

Since the expression of the *MATa1* gene depends on the cell type, we also chose to test whether the splicing efficiency of the *MATa1* transcript is different in splicing extracts prepared from different cell types (a and α). These extracts had not revealed any differences when a transcript of the intron-containing yeast actin gene was used as substrate of the splicing

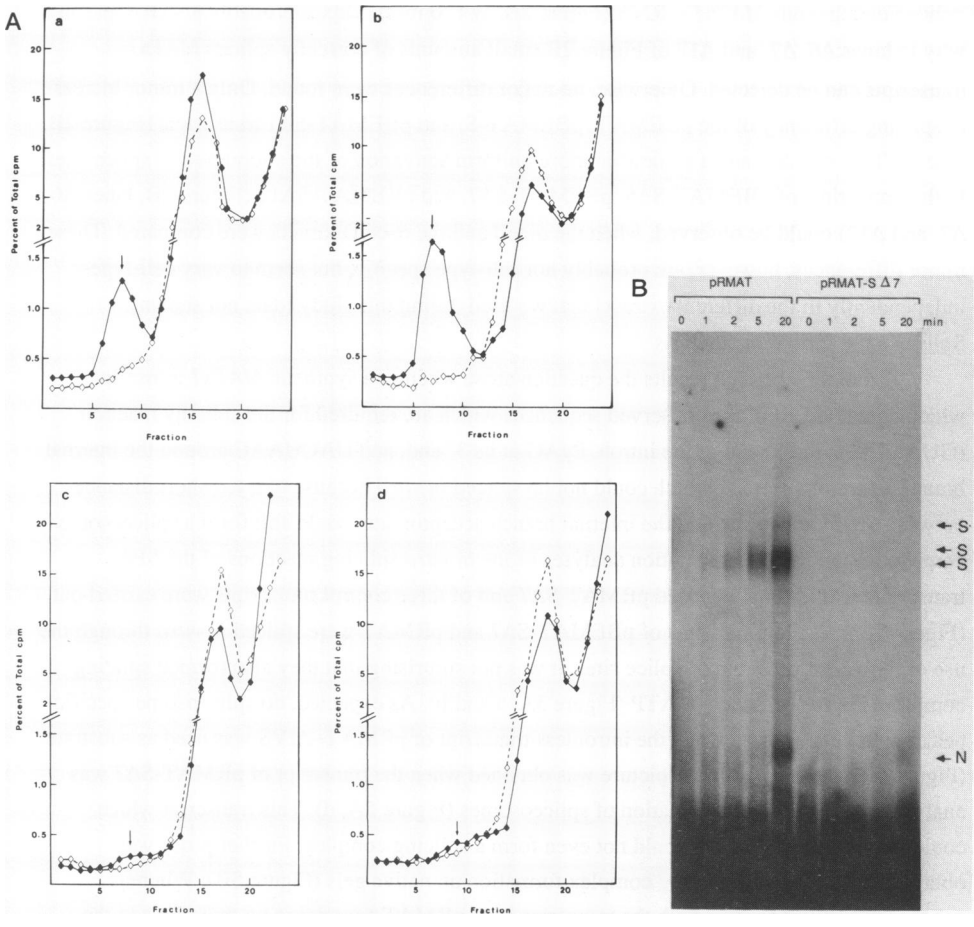


FIG. 5. Effects of intron deletions on spliceosome formation.
(A) Glycerol Gradient Sedimentation Analysis.
^{32}P-labeled SP6-transcripts of MboII digested pRMAT (a), pBEMAT-SΔ7 (b), pRMAT–ΔIVS (c), and pRMAT-SΔ7 (d) were incubated for 15 min at 30°C with (♦) and without (◊) ATP. Then the reaction mixtures (30 µl) were sedimented through 15 % - 40 % glycerol gradients. The gradients were fractionated, the radioactivity of each fraction was determined as Cerenkov counts, and the percentage of radioactivity per fraction was calculated. The spliceosome specific peak is indicated with an arrow.
(B) Analysis on native gels.
^{32}P-labeled SP6-transcripts of MboII digested pRMAT and pBEMAT-SΔ7 were incubated in a yeast splicing extract. Aliquots were withdrawn at 0, 1, 2, 5, and 20 min, and the reactions were stopped by adding a heparin-containing buffer. The samples were fractionated on a native composite gel. S, splicing complexes; N, nonspecific pre-mRNA complexes.

reaction (data not shown). The primer extension analyses of *in vitro* spliced *MAT***a***1* transcripts after incubation in an α-cell extract and in an **a**-cell extract are presented in Figures 2A and 2B, respectively. First we realized that the **a**-cell extract contains small amounts of correctly

spliced endogenous *MAT***a***1* mRNA (Figure 2B, WT, 0 min). This is probably also the reason why in lanes Δ6, Δ7, and Δ12 of Figure 2B small amounts of correctly spliced *MAT***a***1*-transcripts can be detected. Otherwise, no major differences were found. Only a minor increase in splicing efficiency of the pBEMAT, pBEMAT-S, and pBEMAT-SΔ1 transcripts (Figure 2B, lanes WT, S, and Δ1) and a somewhat more uniform activation of the alternative 5' splice sites in the transcripts of pBEMAT-SΔ6, pBEMAT-SΔ7, and pBEMAT-SΔ12 (Figure 2B, lanes Δ6, Δ7, and Δ12) could be observed, when the **a**-cell and the α-cell extracts were compared. These minor differences, however, are probably not cell-type specific, but seem to vary cell-type-independently in the different extracts that we used during this study (data not shown).

Spliceosome formation analysis

From the obtained results the question arose whether a synthetic *MAT***a***1* transcript which contained all of the conserved sequences which are required for the splicing reaction (GUAUGU at the 5' end of the intron, PyAG at its 3' end, and UACUAAC around the internal branch acceptor site), but which could not be spliced *in vitro* because of a too short distance between the 5' splice site and the internal branch acceptor site, could still form a spliceosome complex. Therefore sedimentation analyses of the *in vitro* splicing reactions of the SP6-transcripts from MboII digested pRMAT-SΔ7 and of three control transcripts were carried out (Figure 5A). As the transcripts of pBEMAT-SΔ7 and pRMAT were spliced *in vitro* through the use of, however, different 5' splice sites, it was not surprising that they also formed splicing complexes in the presence of ATP (Figure 5A, a and b). As expected, no spliceosome specific peak could be identified when the intronless transcript of pRMAT–ΔIVS was used as substrate (Figure 5A, c). A very similar picture was obtained when the transcript of pRMAT-SΔ7 was analyzed for the possible formation of spliceosomes (Figure 5A, d). This transcript which could not be spliced *in vitro* could not even form a splicing complex. Further proof was obtained by comparing splicing complex formation on native gels (Figure 5B). Whereas spliceosomes were formed with the transcript from pRMAT containing no mutations in the first intron, no such complex was formed with the Δ7-transcript derived from pRMAT-SΔ7. These results indicate that in addition to the known splice signals a minimum distance between the 5' splice site and the internal branch acceptor site is needed for the assembly of any kind of the described splicing complexes (17, 30, 31).

An alternative 5' splice site in the first exon is also used *in vivo*

Since the alternative 5' splice site located in the first exon of the *MAT***a***1* gene was used only to a very low extent in the *in vitro* splicing reaction, one could have speculated that this result was only an artifact of the *in vitro* system. Therefore we tested whether this alternative 5' splice site is also used *in vivo*. For this purpose total RNA was isolated from 6 different yeast strains which had been constructed before. These strains were isogeneous except from the sequences at the *MAT* locus which either contained the wildtype *MAT*α sequence or different *MAT***a** sequences. The *MAT***a** sequences differed in so far as the *MAT***a***1* gene was present either in its wildtype form or in mutagenized configurations in which either intron 1 or

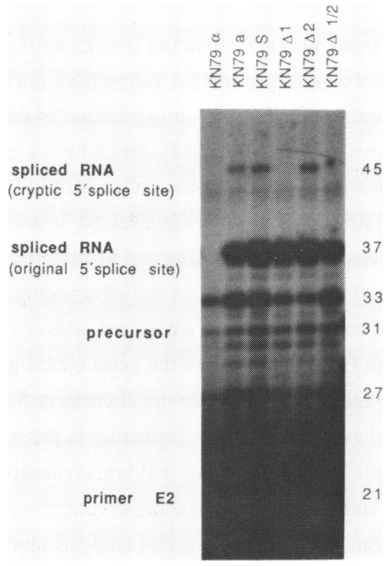


FIG. 6. Primer extension analysis of total RNA isolated from different chromosomal *MAT***a***1* mutants.
50 µg total RNA isolated from 6 different yeast strains were reverse transcribed in the presence of ddGTP using the 5' ^{32}P-labeled *MAT***a***1* exon 2 specific oligodeoxynucleotide E2 as primer. Reverse transcripts of 37 and 45 nucleotides length correspond to differently spliced *MAT***a***1* transcripts in which either the 5'splice site of the first intron (37) or the alternative 5' splice site in the first exon 89 nucleotides upstream (45) were used. A 31 nucleotide-long cDNA would be generated from primary transcripts where the first intron was not excised. The 33 nucleotide-long cDNA transcript which is present in all lanes derives from crosshybridization of the primer with 27S rRNA.

intron 2 or both introns were deleted, or in which intron 1 contained the previously described SmaI mutation. 50 µg of total RNA from each strain was reverse transcribed in the presence ot ddGTP with the 5' ^{32}P-labeled oligodeoxynucleotide E2, which hybridizes to the 5' proximal portion of the second exon (see Figure 1C), as a primer. In Figure 6 the results of the reverse transcription analyses are shown. RNA from α cells was used as a negative control and as expected no *MAT***a***1* specific reverse transcripts could be detected. RNA from all **a** strains yielded high amounts of reverse transcripts derived from correctly spliced *MAT***a***1* mRNA. Only those RNAs which derived from strains containing the first intron of the *MAT***a***1* gene gave rise to a reverse cDNA transcript which corresponds exactly to an RNA generated through the use of at least one of the alternative 5' splice sites in the first exon (89 nucleotides upstream of the 5' splice site in the first intron) and the 3' splice site of the first intron. Although this alternatively spliced *MAT***a***1* mRNA species is present in only very small amounts, this result demonstrates that the use of this alternative 5' splice site is obviously not an artifact of the *in vitro* splicing system and that alternative splicing occurs in yeast *in vivo*, too.

DISCUSSION

The main conclusion from our data is that the formation of yeast spliceosomes and the subsequent accurate excision of a yeast pre-mRNA intron does not only require the presence of the conserved sequence elements of the intron, but in addition to that also a critical minimum distance between these elements. Several lines of evidence have especially contributed to this conclusion. (i) If the overall length of the first intron of the *MATa1* gene is reduced from 54 to 48 nucleotides, the intron can no longer be correctly excised; instead alternative 5' splice sites located in the first exon are used. (ii) Since splicing can be restored by reinserting at the same position the identical number of nucleotides with a different sequence, the observed splicing defects can not be contributed to the deletion of a required accessory sequence element, like those described by Newman (17) for the intron of the yeast *CYH2* gene, but just to the reduced distance. (iii) If in addition to reducing intron size the alternative 5' splice sites are deleted, no splicing is observed at all, and even spliceosome formation is prevented. (iv) The reduction of the distance between the 5' splice site and the internal branch acceptor site cannot be compensated by the possible activation of one or more cryptic 3' splice sites located further downstream in the second exon. The primer extension analysis used here with an oligodeoxynucleotide that starts reverse transcription 6 nucleotides downstream of the first intron would not have detected alternative splicing patterns caused by cryptic 3' splice site activation. The absolute lack of spliceosome formation for the transcript of pRMAT-SΔ7, however, proves that a possible extension of the size of the intron into the downstream direction was not able to substitute for the deletions introduced in the nonconserved region between the 5' splice site and the internal branch acceptor site, and that splicing of our deletion mutant transcripts could not be restored by the use of cryptic 3' splice sites. A more direct proof was obtained by inserting between the branch point and the 3' splice site the same heptanucleotides sequence that had been previously deleted between the 5' splice site and the internal branch point. This intron, although having the same overall length as the wildtype intron, but different distances between the three conserved sequence elements, could not be excised. The obvious lack of spliceosome formation for the transcript of pRMAT-SΔ7 finally also excludes that the absence of spliced products could be due to an accumulation of the intron-exon 2 lariat and that the splicing reaction was blocked after the formation of this reaction intermediate.

Other results obtained in our laboratory from studies of the splicing process *in vivo* also strongly justify this conclusion. A yeast actin mini-intron with an overall length of 73 nucleotides could be excised from the primary transcript if the distance between the 5' splice site and the internal branch acceptor site was 44 nucleotides, but not if this distance was reduced to 30 nucleotides (16).

For introns of the yeast *Saccharomyces cerevisiae* this specific distance requirement might be a common rule. In other species, however, this distance requirement seems to be

different. In the fission yeast *Schizosaccharomyces pombe*, introns even smaller than the *MAT***a***1* introns of *Saccharomyces cerevisiae* have been found in the *NDA2*, *NDA3*, and *YPT2* genes (32-34), and also the distance between the 5' splice site and the internal branch acceptor site can be smaller, e. g. 28 and 29 nucleotides in the first and the third intron, respectively, of the *NDA3* gene or 31 nucleotides in the first intron of the *YPT2* gene (35). One of the smallest known introns which can be spliced in a mammalian system is the intron of the SV40 small t antigen. The distance from the 5' splice site to the internal branch acceptor site in this intron is 49 nucleotides (36). Interestingly, Fu and Manley (37) observed that splicing of the SV40 early pre-mRNA to produce large T antigen, thereby excising a 346 nucleotide-long intron from the pre-mRNA, is at a competitive advantage relative to the production of small t antigen mRNA. However, expansion of the small t antigen intron from 66 to 77 nucleotides resulted in a substantial increase in the amount of small t mRNA generated relative to large T mRNA. In agreement with the data presented here, Fu and Manley suggested that their results may imply that the minimum length requirement for splicing is not based on the distance between the 5' and 3' splice sites but rather on the distance between the 5' splice site and the internal branch acceptor site.

Different intron length requirements in *Saccharomyces cerevisiae* and other species could also reflect differences in factors, e.g. snRNPs, that are involved in splicing. Ares (38) and Kretzner *et al.* (39) have already shown that the *S. cerevisiae* U2 and U1 snRNA analogs (snR20 and snR19) are significantly larger than their *S. pombe* or mammalian counterparts. Ordered pathways of snRNP binding to the precursor have been proposed recently for mammalian pre-mRNA splicing (40) and for *S. cerevisiae* pre-mRNA splicing (27, 32, 33, 39, 41). According to these models the U1-snRNP binds first to the 5' splice site of the precursor followed by the interaction of the U2-snRNP with the internal branch point. The apparent failure of pRMAT-SΔ7 to form any kind of splicing complex could very well reflect that the ordered assembly of the complex is prevented here by the shortened distance between the two conserved sequence elements. The shortened distance might, for example, sterically hinder the interactions of the U1- and/or U2-snRNPs with the mRNA-precursor. The formation of spliceosomes with other transcripts in which the same sequence in the intron was deleted (pBEMAT-SΔ7) or partly replaced (pBEMAT-S, data not shown), but alternative 5' splice sites were present, furthermore demonstrates that it was not the loss of a specific sequence within the intron that caused this defect.

The reduced splicing efficiency of the transcript in which only one nucleotide was deleted in the first intron (pBEMAT-SΔ1) might also offer an explanation for the lower splicing efficiency observed *in vivo* for the second intron of the *MAT***a***1* gene (5), because in this intron the distance from the 5' splice site to the internal branch acceptor site is one nucleotide shorter than in the first intron. Since, on the other hand, the position of an intron in the primary transcript can directly influence splicing efficiency (42), it is not possible to

decide, whether the reduced splicing efficiency observed for the second intron of the *MATa1* gene results from the smaller distance between the 5' splice site and the internal branch point, or from its 3' terminal position, or from both.

Another observation we made during this study was the rather low *in vitro* splicing efficiency (1 - 2 %) of the pBEMAT transcripts which contain a 159 nucleotide-long first exon. Shortening the first exon to 85 nucleotides, however, increased the efficiency to a level comparable to the *in vitro* splicing efficiency of other intron containing yeast transcripts like the yeast actin gene (see Figure 4). There are several possible explanations for this phenomenon. (i) The size of the first exon might directly influence the splicing efficiency. (ii) A spliced mRNA with a shorter exon 1 might have a lower turnover rate, at least under the *in vitro* splicing conditions used here. (iii) The presence of the two alternative 5' splice sites might negatively influence the splicing efficiency directly; if these sites are deleted splicing efficiency increases. Since we have not yet introduced point mutations at the alternative 5' splice sites, we are presently not able to discriminate between the different possibilities.
For our analyses we have used extracts which had been prepared from cells of mating type **a** and α. Our results show that the *MATa1* transcript which is expressed *in vivo* only in haploid **a**-cells and diploid **a**/α-cells, but not in haploid α-cells, can also be accurately processed in a splicing extract prepared from haploid cells of mating type α. The minor differences revealed in the different extracts, however, are not cell-type specific, but vary in the four different extract preparations (two from **a**-cells and two from α-cells) used during this study.
This is the first time that alternative splicing has been demonstrated for a yeast gene *in vitro* as well as *in vivo*. Presently, we do not yet know whether the alternatively spliced product is also translated *in vivo*. If so, an essential biological function of the corresponding translation product which would be a nonapeptide is very unlikely, since the exact deletion of the first intron in the *MATa1* gene did not lead to any phenotypical difference during vegetative growth, mating, sporulation, or germination.

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REFERENCES

1. Mortimer, R.K. and Hawthorne, D.C. (1969) In Rose, A.H. and Harrison, J.S. (eds.), The Yeasts, Vol. 1. Academic Press, New York, pp. 385 - 460.
2. Tatchell, K., Nasmyth, K.A., Hall, D., Astell, C. and Smith, M. (1981) Cell **27**, 25 - 35.
3. Nasmyth, K.A., Tatchell, K., Hall, B.D., Astell, C. and Smith, M. (1981) Cold Spring Harbor Symp. Quant. Biol. **45**, 961 - 981.
4. Strathern, J., Hicks, J. and Herskowitz, I. (1981) J. Mol. Biol. **147**, 357 - 372.
5. Miller, A.M. (1984) EMBO J. **3**, 1061 - 1065.
6. Gallwitz, D. (1982) Proc. Natl. Acad. Sci. USA **77**, 2546 - 2550.
7. Langford, C.J. and Gallwitz, D. (1983) Cell **33**, 519 - 527.
8. Pikielny, C.W., Teem, J.L. and Rosbash, M. (1983) Cell **34**, 395 - 403.
9. Langford, C.J., Klinz, F.-J., Donath, C. and Gallwitz, D. (1984) Cell **36**, 645 - 653.
10. Teem, J., Abovich, N., Käufer, N., Schwindinger, W., Warner, J., Levy, A., Woolford, J., Leer, R., Van Raamsdonk-Duin, M., Mager, W., Planta, R., Schultz, L., Friesen, J., Fried, H. and Rosbash, M. (1984) Nucleic Acids Res. **12**, 8295 - 8312.
11. Newman, A.J., Lin, R.-J., Cheng, S.-C. and Abelson, J. (1985) Cell **42**, 335 - 344.
12. Parker, R. and Guthrie, C. (1985) Cell **41**, 107 - 118.
13. Cellini, A., Parker, R., McMahon, J., Guthrie, C. and Rossi, J. (1986) Mol. Cell. Biol. **6**, 1571 - 1578
14. Fouser, L.A. and Friesen, J.D. (1986) Cell **45**, 81 - 93.
15. Vijayraghavan, U., Parker, R., Tamm, J., Iimura, Y., Rossi, J., Abelson, J. and Guthrie, C. (1986) EMBO J. **5**, 1683 - 1695.
16. Thompson-Jäger, S. and Domdey, H. (1987) Mol. Cel. Biol. **7**, 4010 - 4016.
17. Newman, A. (1987) EMBO J. **6**, 3833 - 3839.
18. Hanahan, D. (1983) J. Mol. Biol. **166**, 557 - 580.
19. Astell, C. R., Ahlstrom-Jonasson, L., Smith, M., Tatchell, K., Nasmyth, B. and Hall, B. D. (1981) Cell **27**, 15 - 23.
20. Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene **33**, 103 - 119.
21. Nasmyth, K.A., Tatchell, K., Hall, B.D., Astell, C. and Smith, M. (1981) Nature **289**, 244 - 250.
22. Sanger, F., Nicklen, S. and Coulson A.R. (1977) Proc. Natl. Acad. Sci. USA **74**, 5463 - 5467.
23. Chen, E.Y. and Seeburg, P.H. (1985) DNA **4**, 165 - 170.
24. Kramer, W. and Fritz, H.-J. (1987) Methods Enzymol. **154** ,350-367.
25. Rudolph, H., Koenig-Rauseo, I. and Hinnen, A. (1985) Gene **36**, 87 - 95.
26. Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984) Nucleic Acids Res. **12**, 7035 - 7056.
27. Lin, R.-J., Newman, A.J., Cheng, S.-C. and Abelson, J. (1985) J. Biol. Chem. **260**, 14780 - 14792.
28. Domdey, H., Apostol, B., Lin, R.-J., Newman, A., Brody, E. and Abelson, J. (1984) Cell **39**, 611 - 621.
29. Brody, E. and Abelson, J. (1985) Science **228**, 963 - 967.
30. Pikielny, C.W., Rymond, B.C. and Rosbash, M. (1986) Nature **324**, 341 - 345.
31. Cheng, S.-C. and Abelson, J. (1987) Genes and Development **1**, 1014 - 1027.
32. Toda, T., Adachi, Y., Hiroaka, Y. and Yanagida, M. (1984) Cell **37**, 233 - 242.
33. Hiroaka, Y., Toda, T. and Yanagida, M. (1984) Cell **39**, 349 - 358.
34. Lehmeier, T. (1986) Diploma thesis, University of Marburg, FRG.
35. Gallwitz, D., Halfter, H. and Mertins, P. (1987) In Kinghorn J.R. (ed.), Gene Structure in Eucaryotic Microbes. SGM Special Publications 22, IRL Press, Oxford, pp. 27 - 40.
36. Noble, J.C.S., Pan, Z.-Q., Prives, C. and Manley J.L. (1987) Cell **50**, 227 - 236.
37. Fu, X-Y. and Manley, J.L. (1987) Mol. Cell. Biol. **7**, 738 - 748
38. Ares, M., Jr. (1986) Cell **47**, 49 - 59.
39. Kretzner, L., Rymond, B.C. and Rosbash, M. (1987) Cell **50**, 593 - 602.
40. Bindereif, A. and Green, M.R. (1987) EMBO J. **6**, 2415 - 2424.
41. Parker, R., Siliciano, P.G. and Guthrie, C. (1987) Cell **49**, 229 - 239.
42. Klinz, F. and Gallwitz, D. (1985) Nucleic Acids Res. **13**, 3791 - 3804.