Size and position of intervening sequences are critical for the splicing efficiency of pre-mRNA in the yeast *Saccharomyces cerevisiae*

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ABSTRACT

The size of the 309 bp actin gene intron of the yeast *Saccharomyces cerevisiae* was enlarged by inserting DNA fragments of different lengths and sequence. Enlarging the intron above 551 bp, the largest known yeast intron, led to a decrease in splicing efficiency. The effect on transcript splicing was dependent on the length of the inserted fragments rather than their sequence. It was also observed that insertion of the actin gene intron into different regions of the normally unsplit yeast YP2 gene, significantly influenced the efficiency of splicing of the resulting transcripts. The splicing efficiency decreased with the increase of the distance between the mRNA cap site and the intervening sequence.

INTRODUCTION

Intervening sequences in eukaryotic pre-mRNAs considerably vary in number (from one to over 50) and size (from 48 bp to 32.4 kb) (1-3). They are found to disrupt the 5' untranslated, the protein-coding or, less frequently, the 3' untranslated regions (1,4-6). In contrast to higher eukaryotes where most protein-coding genes transcribed by RNA polymerase II contain introns, in the yeast *Saccharomyces cerevisiae* disrupted genes seem to be an exception rather than the rule. It is remarkable, however, that with the exception of the mating type gene MATa1, which contains two small introns of 54 and 52 bp at the beginning and end of the protein-coding region (7), all other known split genes in *S.cerevisiae* contain only one intron of rather uniform length (about 300 to 500 nucleotides) which is located either within or between the first few codons of the primary transcript or, less frequently, in the 5' untranslated region (8-12). Another striking feature of all *S.cerevisiae* introns is the occurrence of the highly conserved and essential sequence

elements 5'-GTATGT-3' at the 5' splice site and 5'-TACTAAC-3' within the 3' part of the intron preceding the PyAG utilized in the splicing reaction (13-15). Nevertheless, recent findings suggest that the basic mechanism of pre-mRNA splicing is common to all eukaryotes: in mammalian cells (16-18) as well as in yeast (19) the same splicing intermediate, the so-called lariat structure, is generated with the 5' terminal guanosine nucleotide of the intron forming a 2'-5' phosphodiester bond with an A-residue of the 3' intron region. In yeast this A-residue is part of the conserved TACTAAC-box.

Here we report on our observation that in the yeast *S.cerevisiae* in addition to the conserved intron sequences, the length of an intervening sequence and its location within the primary transcript are of critical importance for the efficiency of splicing.

MATERIALS AND METHODS

Materials

Restriction enzymes were purchased from Boehringer Mannheim, New England Biolabs or Bethesda Research Laboratories and used under conditions recommended by the suppliers. E.coli DNA polymerase, Klenow fragment and T4 DNA ligase were from Boehringer Mannheim, and T4 RNA ligase was from Bethesda Research Laboratories. (α - ^{32}P)-dNTPs (3000 Ci/mmol) were obtained from Amersham.

Bacterial and yeast strains

For all cloning experiments the E.coli strain RR1 (F^- pro leu thi lacy str^r r_k^- m_k^-) was used. Yeast transformations were performed with the haploid *S.cerevisiae* strain YNN27 (a trp1-289 ura3-52 gal2) or AH22 (a leu2-3 leu2-112 his4-519 can1).

Construction of hybrid genes

Plasmid pYA301 containing the entire yeast actin gene on a 3.4 kb BamHI-EcoRI fragment (20) was cut with BglII and HindIII, filled in with Klenow enzyme and religated, generating a 522 bp deletion in the coding region of the yeast actin gene. The 2.88 kb BamHI-EcoRI fragment from the resulting plasmid was filled in with Klenow enzyme, PstI linker were added, and inserted into the PstI site of the yeast-E.coli shuttle vector

pMP78-1 (21), giving rise to pMP78-1/D3.

This recombinant plasmid was used to insert DNA fragments of varying length derived from different genes into the single XhoI site of the actin gene intron after treatment of the DNAs with the Klenow fragment of DNA polymerase I. The hybrid genes Gl1⁻, Gl2⁻ and Gl3⁻ were generated by inserting either one, two or three copies of a 173 bp TaqI-BamHI fragment from the second exon of the rabbit β -globin gene (22), all in opposite orientation with respect to the direction of globin gene transcription (Fig.1). The insertion of a 473 bp HindIII fragment containing the 398 bp intron and 23 bp and 38 bp of the first and second exon of the yeast ribosomal protein gene rp51A (23), flanked by HindIII linker sequences, gave rise to the hybrid genes rp1⁺ and rp1⁻ (one fragment inserted in correct and opposite orientation, respectively) (Fig.1).

In another set of intron enlargements, a 1.11 kb BstEII fragment from the yeast β -tubulin coding region (24) was isolated from the recombinant plasmid pYA102 (25) and inserted into the XhoI site of the actin gene intron in the yeast-duck gene chimera YRp7/YDH1 described previously (20). The resulting hybrid genes Tub1⁺ and Tub3⁺ contained one or three copies of the BstEII fragment in correct orientation, Tub1⁻ and Tub2⁻ contained one or two fragments, respectively, in the opposite orientation with respect to the direction of tubulin gene transcription (Fig.3).

To construct the different plasmids containing the YP2 gene with the yeast actin intron inserted in different regions of the gene, a 2.2 kb BamHI fragment from the plasmid pYA102 (25), containing part of the β -tubulin gene and the entire YP2 gene, was inserted into the BamHI site of pUC82 (pUC8 (26) in which the PstI and the EcoRI site were eliminated by cutting, Klenow fill-in and religation) and pUC83 (pUC8 from which the HincII-HindIII region of the polylinker segment was deleted) to generate pUC82-YP2 and pUC83-YP2, respectively. These plasmids were used to insert into different restriction sites a 544 bp AluI fragment from the *S.cerevisiae* actin gene containing the intron and bordering exon sequences. From the resulting recombinant plasmids YP2I,II,III and IV (see Fig. 5) the 2.74 kb BamHI frag-

ments were then inserted into the BamHI site of the yeast-E.coli shuttle vector YRp7 (27).

All clones were identified by a miniscreen procedure (28) of transformed E.coli strain RR1.

Yeast transformation

Hybrid genes in the high-copy number plasmid pMP78-1, which contains the selectable marker LEU2 (21), were used to transform spheroblasts of the *S.cerevisiae* strain AH22 (29). Hybrid genes in the low-copy number plasmid YRp7, which contains the selectable marker TRP1 (27), were used to transform spheroblasts of the *S.cerevisiae* strain YNN27 (30). Methods for the preparation of spheroblasts with zymolase and the procedures for the transformation and selection of yeast have been described (29,31).

RNA isolation and analysis

Methods for the isolation of RNA and its analysis by Northern blotting have been described previously (13). Nitrocellulose filters were rehybridized according to Thomas (32).

RESULTS

Intron size and splicing efficiency

The *S.cerevisiae* actin gene intron has a length of 309 bp and is located within codon four (8,9). In previous studies we have shown that as long as the conserved 5' intron-exon boundary and the intron-contained TACTAAC-box are left intact, large parts of the intron around the single XhoI restriction site could be deleted without affecting the correct and efficient excision of the remaining intervening sequence (15,33). This suggests that a significant part of the intron plays a minor if any role in the recognition and excision of the intervening sequence. In studying the feature of excised introns, which for easier detection among total cellular RNA was modified to contain foreign sequences, we made the observation that the ratio of spliced versus unspliced transcripts varied considerably depending on the size of the inserted fragments.

In a series of hybrid gene constructions we therefore varied the intron size as well as its sequence by inserting different DNA fragments into the same XhoI site of the actin gene intron. The different gene constructs, introduced in a yeast-E.coli

Fig.1 Construction of yeast actin gene intron enlargements in the XhoI site of pMP78-1/D3. The orientation of the inserted fragments relative to the direction of transcription of the gene they were derived from is indicated. The conserved TACTAAC sequence within the yeast actin and rp51A gene introns is indicated by an arrow. Solid blocks: exon. Open blocks: intron. Hatched blocks: untranslated region.

shuttle vector, were then used to transform yeast and total cellular RNA was examined by Northern blot analysis for the extent of transcript splicing. Disregarding possible differences in the stability of spliced and unspliced transcripts, the ratio of spliced to unspliced RNA (measured by counting the radioactivity in different bands detected on RNA blots) was used to get a rough calculation of the splicing efficiency. The presence of foreign sequences within intron and coding sequence enabled easy detection of transcripts on RNA blots.

As outlined in Fig.1, by blunt end insertion of different copies of a 173 bp TaqI-BamHI fragment from the second exon of the rabbit β -globin gene (22), the actin gene intron was enlarged from 309 bp to 482 bp (G11⁻), 655 bp (G12⁻) or 828 bp (G13⁻). Whereas the 482-nucleotide intervening sequence was very efficiently removed from the transcripts formed, a significant amount of unspliced precursor RNA was detected when the intron size was further increased (Fig.2A). As the (G+C) content of the inserted DNA fragment is rather high (52.6%) compared to that of the actin gene intron (32%) it could be argued that the base composition of the hybrid intron is the critical factor in changing the splicing efficiency. We therefore introduced into the actin gene intron a subcloned 473 bp HindIII fragment with a (G+C) content of 35.9% from the yeast ribosomal protein gene rp51A (23). By this manip-

Fig.2A Northern blot analysis of pMP78-1/D3/GI transcripts. Three micrograms of glyoxylated, total cellular RNA from transformed yeast was separated on a 1.5% agarose gel, transferred to a nitrocellulose filter, hybridized to a nick-translated 524 bp BglII-AvaII fragment from the protein-coding region of the yeast actin gene and rehybridized to a nick-translated 571 bp PstI-BamHI fragment from the rabbit β -globin gene. Numbers to the right indicate the nucleotide lengths of RNA and DNA fragments coelectrophoresed as marker. Open circles represent unspliced transcripts; closed squares represent spliced transcripts.

Fig.2B Northern blot analysis of pMP78-1/D3/rp transcripts. The nitrocellulose filter was hybridized to a nick-translated 524 bp BglII-AvaII fragment from the protein-coding region of the yeast actin gene and rehybridized to a nick-translated 473 bp HindIII fragment containing the yeast rp51A gene intron and short exon sequences.

Fig.3 Construction of yeast actin gene intron enlargements in the XhoI site of the yeast-duck hybrid gene YDH1 (20). The orientation of the inserted fragments with respect to the direction of the yeast tubulin gene is indicated. The conserved TACTAAC sequence within the yeast intron is indicated by an arrow. Solid blocks: exon. Open blocks: intron. Hatched blocks: untranslated region. Cross-hatched blocks: duck α^D -globin coding region.

ulation two types of intron were generated with the fragment being in the correct ($rp1^+$) or the opposite orientation ($rp1^-$) with respect to the direction of transcription of the $rp51A$ gene (Fig.1). Here again the transcripts from gene $rp1^-$, with an intervening sequence enlarged to 782 nucleotides, were less efficiently spliced than those containing introns with a length of less than 500 nucleotides (Fig.2A and 2B). The proportion of spliced to unspliced RNA (about 80%) was indeed comparable to that found with the genes $G12^-$ and $G13^-$.

The construction $rp1^+$ (Fig.1) represents an intron within an intron. Interestingly, the proportion of spliced to unspliced RNA (excision of all intron sequences) was even lower than with construction $rp1^-$ (Fig.2B). However, as can be seen in Fig.2B, several intermediate bands appear on the RNA blot probed with a fragment from the actin protein-coding region and these might correspond to RNAs with sequences spliced out from the 5' splice site of one intron to the 3' splice site of the other.

The relation between intron size and splicing efficiency was further explored by inserting into the actin gene intron several copies of a 1.11 kb BstEII fragment with a (G+C) content of 45.6% from the protein-coding region of the yeast β -tubulin gene (24) (Fig.3). These constructions were done with the yeast-duck hybrid gene YDH1 described previously (20), facilitating the specific detection of cellular transcripts with a duck globin

Fig.4 Northern blot analysis of the YDH1/Tub transcripts. Three micrograms of glyoxylated, total cellular RNA from transformed yeast was separated on a 1.0% agarose gel, transferred to a nitrocellulose filter, hybridized to a nick-translated 429 bp PstI-KpnI fragment from the duck α^D -globin gene (34) and rehybridized to a nick-translated 1.11 kb BstEII fragment from the yeast tubulin gene.

DNA probe. Regardless of the orientation of the fragment inserted, enlargement of the intron to 1.42 kb (Tub1⁺ and Tub1⁻) resulted in a dramatic decrease of the splicing efficiency to about 10% (Fig.4). Further increase in intron size, 2.53 kb in Tub2⁻ and 3.64 kb in Tub3⁺ (Fig.3), led to an almost complete inhibition of transcript splicing (Fig.4).

As can also be seen in Fig.4, the orientation of the fragments inserted heavily influenced the amounts of detectable transcripts. This result might be explained by either a sequence dependent reduction of the transcription rate, premature termination or reduced stability of the primary transcripts. That indeed comparable amounts of total cellular RNA from the different yeast transformants were loaded onto the gel was shown by longer exposure of the filter hybridized to the β -tubulin probe which detected comparable quantities of the 1600-nucleotide

Fig.5 Construction of actin gene intron-containing YP2 genes. The insertion site of the intron-containing fragment is indicated by an open arrow with the number representing the distance (in nucleotides) from the major YP2 cap site. Solid blocks: exon. Open blocks: intron. Hatched blocks: untranslated region.

β -tubulin mRNA from the chromosomal gene. These bands are not seen on the autoradiogram presented in Fig.4 since the transcripts derived from the plasmid are much more abundant.

Intron position and splicing efficiency

In previous studies we had shown that an artificial intron could be generated by placing the conserved TACTAAC-box with flanking sequences further downstream into the coding region of the yeast actin gene (13). Although not very efficient, splicing occurred between the normal 5' splice site and an alternative 3' splice site downstream from the TACTAAC sequence.

In order to test whether introns can be removed from transcripts formed of normally unsplit genes, we placed the actin gene intron into different parts of the ras-related YP2 gene, the 5' neighbor of the actin gene on yeast chromosome VI (35). As outlined in Fig.5 a 544 bp AluI fragment with 73 bp of the first exon, the 309 bp intron and 162 bp of the second exon was inserted into either the EcoRI, PstI or HincII restriction site, all residing in the protein-coding region but 132, 274 and 656 nucleotides downstream from the major transcription initiation site, respectively. The intron was also inserted into the BssHII site of the 3' untranslated region located 713 bp downstream from the major cap site. On the RNA blot shown in Fig.6 three different transcripts are detected with a YP2-specific hybridization probe: a faint band of 830 nucleotides representing the YP2 mRNA from the chromosomal gene (35) and two other bands of about 1100 and 1400 nucleotides with the expected length for the spliced and unspliced plasmid-derived transcripts. Calculation

Fig.6 Northern blot analysis of transcripts from actin gene intron-containing YP2 genes. Three micrograms of glyoxylated, total cellular RNA from transformed yeast was separated on a 1.5% agarose gel, transferred to a nitrocellulose filter and hybridized to a nick-translated subcloned 1.08 kb BamHI fragment containing the whole YP2 gene (a 1.08 kb BglII-BamHI fragment from pYA102 (25) was provided with BamHI linkers and inserted into the BamHI site of pUC8).

of the splicing efficiency of the RNAs transcribed from the modified YP2 genes showed that it decreased with the increase of the distance between the RNA cap site and the intron. Whereas the splicing efficiency was about 95% and 90% for the constructions I and II, respectively, it was about 70% for the construction III and only 60% for the RNAs with the intervening sequence located in the 3' untranslated region (Fig.6).

DISCUSSION

The experimental results described indicate that besides the conserved intron sequences previously identified to be required for yeast pre-mRNA splicing (13-15), the length and the location of intervening sequences are also critical for the efficiency with which primary transcripts are spliced in this unicellular organism. This is perhaps not surprising as a compilation of all 20 or so known split genes in *S.cerevisiae* shows that introns are in general between 300 and 500 nucleotides long and are located exclusively within the extreme 5' region of the RNA

(8-12). The only exception is the MATa1 gene with two small introns one of which disrupting the 3' part of the protein-coding region (7). Interestingly, this second intron, which contains a 'Homeo box' related sequence, is rather inefficiently spliced out of the MATa1 transcripts (7).

This finding compares with our observation that the efficiency of splicing decreases with the distance of an intervening sequence from the mRNA cap site. Since we have noticed a similar dependence of intron location and splicing efficiency in other constructions we tend to believe that this phenomenon could point to a mechanism by which a mRNA molecule is scanned for intervening sequences to be excised, starting at the cap site and proceeding in 5'→3' direction. The farther a hypothetical protein or ribonucleoprotein recognizing the cap site would have to travel along a RNA molecule the more likely would be the possibility of its dissociation from the RNA.

It is worth noting in this context that in mammalian in vitro splicing systems capped pre-mRNAs are much more efficiently spliced than uncapped transcripts (36,37). This finding has been interpreted to mean that a RNA-protein complex required for splicing is formed at the pre-mRNA cap structure (37).

Of course, another explanation for our observation would be that the secondary or tertiary structure of a RNA molecule could favour or render difficult the recognition and excision of an intervening sequence depending on its sequence environment.

Both explanations given above could also hold to rationalize our findings that stepwise enlargements of an intron lead to an increasing interference with the splicing of pre-mRNA. The decrease in splicing seems to be due to the size of the inserted fragments rather than to their sequence and only after the intron length was extended over 551 nucleotides, the largest S.cerevisiae intron known (10), was a significant inhibition of RNA splicing observed.

The amounts of detectable transcripts were heavily dependent on the orientation of the fragments from the β -tubulin gene coding region inserted into the yeast actin gene intron. Reduced levels of mature mRNA were also observed in two other cases, the avian leukosis virus env mRNA and the maize Adh1 mRNA, following

the insertion of DNA fragments into an intron of the corresponding genes (38,39).

Although a scanning model of pre-mRNA splicing in higher eukaryotic cells, originally proposed by Sharp (40), has been challenged (41), such a mechanism might nevertheless operate in the yeast *S.cerevisiae*. During its evolutionary history this ascomycete seems to have specialized on a splicing mechanism not only requiring highly conserved intron sequences but perhaps also involving a 5'→3' pre-mRNA scanning. With regard to the stringent requirement of the intron-contained TACTAAC-box we would like to stress that this is a feature unique to the yeast *S.cerevisiae*. Even other ascomycetes, like *Schizosaccharomyces pombe* and *Neurospora crassa*, have the less conserved sequence 5'-CTAPuC-3' (42-45) whose functioning in the splicing reaction has not been established yet. The two organisms further differ from *S.cerevisiae* in often containing several small introns in one nuclear gene scattered over the entire coding region.

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