Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 1496–1500, March 1983
Biochemistry

Yeast is unable to excise foreign intervening sequences from hybrid gene transcripts

(RNA splicing/S1 nuclease mapping/yeast transformation)

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Communicated by Robert Perry, December 1, 1982

ABSTRACT **To investigate whether transcripts from foreign split genes are correctly processed in yeast cells we have constructed two hybrid genes by inserting into the split yeast actin gene an intron-containing fragment from either the *Acanthamoeba* actin I gene or the duck α^D-globin gene. The hybrid genes were inserted into the autonomously replicating yeast plasmid YRp7, which was then used to transform yeast cells. It was found that the yeast but not the foreign intervening sequences were excised from the chimeric transcripts. This indicates that the recognition of intervening sequences or the splicing mechanism of RNA polymerase II transcripts is not universal.**

Introns in all eukaryotic genes transcribed by RNA polymerase II have two common structural features: their sequences begin with the dinucleotide 5′-G-T-3′ and end with the dinucleotide 5′-A-G-3′ (1). Besides these invariant nucleotides, there is limited structural similarity at and around the intron–exon junctions. Consensus sequences of about 10 nucleotides for the 5′ and 3′ splice sites have been derived from a comparison of more than 100 splice junctions (2, 3).

Because of the similarity of splice junction sequences in species as distant as yeast and man, it has been speculated that splicing may not be species specific. Experiments supporting this notion have shown that, for instance, mouse and rat genes inserted into simian virus 40 (SV40) vectors are transcribed in monkey cells and their transcripts are properly spliced (4, 5) and that transcripts of rabbit and chicken genes are correctly spliced in mouse cells (6, 7). Even a hybrid intervening sequence with the 5′ splice region from the mouse β-globin gene was properly excised from a chimeric transcript in monkey cells (8), although the splice junction sequences of the two donor genes do not exhibit obvious similarities, except for the conserved G-T and A-G dinucleotides. All these studies were undertaken with genes and cells from higher eukaryotes and it is likely that, at least in vertebrates, the splicing machinery for RNA polymerase II transcripts is interchangeable.

The actin gene of the unicellular eukaryote *Saccharomyces cerevisiae* contains an intron with 5′ and 3′ intron–exon junction sequences resembling the eukaryotic consensus sequences (9, 10). An indication that the RNA splicing mechanism in yeast might nevertheless differ from that in higher eukaryotes came from a study in which the mouse β-globin gene was introduced into yeast cells via a self-replicating recombinant plasmid (11). In this case the first β-globin intervening sequence was not removed, but this could also be related to the incorrect initiation and termination observed with this gene transcript.

We report here on the construction of two hybrid genes in which either an ameba actin or a duck globin gene fragment containing an intron has been inserted into the middle of the yeast actin gene. We show that in transformed yeast cells the splicing of the chimeric transcripts is selective in the sense that the yeast intervening sequences are excised but not the foreign ones.

MATERIALS AND METHODS

Enzymes and Reagents. Restriction endonucleases, T4 polynucleotide kinase, and S1 nuclease were purchased from Bethesda Research Laboratories; *Escherichia coli* DNA polymerase I and its Klenow fragment and phage T4 DNA ligase were from Boehringer Mannheim. [α-^{32}P]dNTPs and [γ-^{32}P]ATP (2,000–3,000 Ci/mmol; 1 Ci = 3.7 × 10^{10} Bq) were obtained from Amersham.

Construction of Hybrid Genes. A 3.4-kilobase (kb) *Bam*HI/*Eco*RI fragment from the recombinant plasmid pYA208 (9, 12) harboring the yeast actin gene was inserted into the *Bam*HI and *Eco*RI sites of pBR322 to generate pYA301. The 2.4-kb *Hin*dIII fragment containing the *Acanthamoeba* actin I gene was isolated from clone pAA1 (13). This fragment and the plasmid pYA301 were cut with *Bgl* II and *Kpn* I and the mixture of fragments was ligated with T4 DNA ligase. The recombinant plasmid pYA301-1 with the *Bgl* II/*Kpn* I yeast fragment replaced by the intron-containing *Acanthamoeba Bgl* II/*Kpn* I fragment was isolated, cut with *Eco*RI and *Bam*HI, and ligated with the *Eco*RI/*Bam*HI-cut yeast vector YRp7 (14) in which one of the *Eco*RI sites had been previously deleted by partial *Eco*RI cutting and fill-in with the Klenow fragment of DNA polymerase I. To construct the yeast–duck gene chimera, a 4-kb *Eco*RI/*Bam*HI fragment from clone αDG-1 (15) containing part of the α^D-globin gene of the duck *Cairina moschata* (starting from the codon for amino acid 23, in which the *Eco*RI site is located) and the entire α^A-globin gene was subcloned in pBR322, cut with *Eco*RI, and filled in with the Klenow enzyme. After cutting with *Kpn* I (single restriction site located within the second intron of the α^D-globin gene), a 510-base-pair (bp) fragment flush-ended on one side and with sticky ends on the other side was generated. This fragment was used to replace the *Bgl* II/*Kpn* I fragment of the yeast actin gene in pYA208 after the *Bgl* II site had also been made flush by filling with the Klenow enzyme. The 3.3-kb *Eco*RI/*Bam*HI fragment of this recombinant plasmid was then inserted into the yeast vector YRp7 to obtain YRp7/YDH1. A chimeric gene containing a second yeast intron was constructed by inserting a 469-bp intron-containing fragment from the split *S. cerevisiae* ribosomal protein gene *rp 51* (16) into the yeast actin gene. The subcloned fragment (kindly provided by M. Rosbash) contained the 398-bp intron (17), 5′ and 3′ bordering sequences 23 bp and 38 bp long, and flanking *Hin*dIII linker sequences. This fragment was inserted into the *Hin*dIII

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Abbreviations: SV40, simian virus 40; kb, kilobase(s); bp, base pair(s).

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site located within codons 258/259 of the actin gene. The hybrid gene was subsequently introduced into YRp7 to generate YRp7/301-3. The plasmids YRp7/301-1, YRp7/YDH1, and YRp7/301-3 contain both an *Eco*RI/*Bam*HI fragment harboring the hybrid gene and a 1.4-kb *Eco*RI fragment with the yeast *TRP1* gene and a yeast replication origin, *ars1* (18). All clones were identified by a miniscreen procedure (19) of transformed *E. coli* strain RR1. Cloning experiments were performed under L2–B1 conditions, following the rules of the Bundesminister für Forschung und Technologie of the Federal Republic of Germany.

RNA Analysis of Yeast Transformants. The recombinant plasmids containing the hybrid genes were used to transform spheroplasts of the *S. cerevisiae trp1-289* strains SX1-2 (YNN34) or YNN27 (14, 20) according to published procedures (21, 22). Total cellular RNA, isolated from logarithmically growing cells (OD_{600} 1.5–2.0) as described (23), was denatured by glyoxylation (24), separated on a 1.5% agarose gel in 10 mM sodium phosphate buffer at pH 6.7, and transferred bidirectionally to diazotized paper (25). One paper was hybridized (12) with a nick-translated 1,008-bp *Xho* I/*Hin*dIII yeast DNA fragment spanning the actin gene region from nucleotide 62 of the intron to the codons 258/259 (9). To specifically identify hybrid RNAs, the second filter was hybridized either with a nick-translated 2.4-kb *Hin*dII fragment containing the *Acanthamoeba* actin I gene (13) or with the 510-bp *Eco*RI/*Kpn* I fragment of the duck α^D-globin gene (see above). S1 nuclease mapping (26, 27) was performed as described (13, 23). RNA·DNA hybrids were formed at 50°C for 4 hr and S1 nuclease digestions were performed at 30°C and the enzyme concentrations indicated in the figures.

RESULTS

The yeast actin gene, which is interrupted by a 309-bp intron within the codon for amino acid four (9, 10), was manipulated such that another intervening sequence either from yeast or from a different eukaryote was inserted downstream from its own intron, thereby generating chimeric genes with two introns and normal yeast transcription initiation and termination sequences. These hybrid genes were inserted into the extrachromosomally replicating low copy number plasmid YRp7 (14) and used to transform spheroplasts of auxotrophic yeast mutants (*trp1*). Transformants were selected on tryptophan-deficient agar plates and then grown under selective pressure in minimal medium without tryptophan. RNA was isolated from logarithmically growing cells, denatured and separated on agarose gels, transferred bidirectionally to diazotized paper, and identified by using specific hybridization probes.

As can be seen in Fig. 1 (lane 1), two RNA species, about 1,550 and 1,430 nucleotides in length, were detected in cells transformed with the yeast–*Acanthamoeba* hybrid gene when a yeast actin hybridization probe was used. With the same probe, only the 1,430-nucleotide-long RNA was found in untransformed cells (lane 2). This RNA species represents the mature yeast actin mRNA transcribed from the chromosomally located actin gene (23). With the *Acanthamoeba* actin hybridization probe, which under the stringent hybridization conditions used gave a signal only with the homologous sequences, only the larger of the two RNA species was labeled (lanes 3 and 4). This result strongly suggested that the 1,550-nucleotide RNA was a partially processed transcript originating from the actin gene chimera, because it contained yeast and *Acanthamoeba* actin sequences and was larger than the mature yeast actin mRNA by about 120 nucleotides, consistent with the length of the *Acanthamoeba* intervening sequence (129 nucleotides).

This possibility was tested by S1 nuclease mapping of the junctions of the intervening and coding sequences. As illustrated in Fig. 2, a 1,322-bp *Bst*NI/*Bam*HI fragment of the hybrid gene, 5′-end-labeled at its *Bst*NI site, was hybridized to total RNA from transformed cells and digested with S1 nuclease. The *Bst*NI restriction site resides in the *Acanthamoeba* gene (codon 137). Because there are numerous base differences between the yeast and the *Acanthamoeba* coding sequence upstream from this site, the 5′-end-labeled fragment could be used to specifically identify hybrid transcripts. The DNA fragments protected from S1 nuclease were separated on a 6% polyacrylamide sequencing gel in parallel with marker fragments of known length. As shown schematically in Fig. 2, a S1-protected 96-base fragment was expected if the *Acanthamoeba* intervening sequence was excised correctly and a 530-base fragment should have been protected if the yeast but not the *Acanthamoeba* intervening sequence was excised from the hybrid transcript. A precursor RNA containing both intervening sequences should protect a fragment 989 nucleotides in length because the 5′ end of the yeast actin mRNA lies 141 nucleotides upstream from the ATG initiation codon (23). The autoradiogram (Fig. 2) clearly shows that the major S1-nuclease-resistant DNA fragment was about 530 nucleotides long and that a 96-base fragment was not observed, even after longer film exposure. This result established that the yeast intervening sequence was properly excised from the hybrid transcript, whereas the *Acanthamoeba* intervening sequence was not.

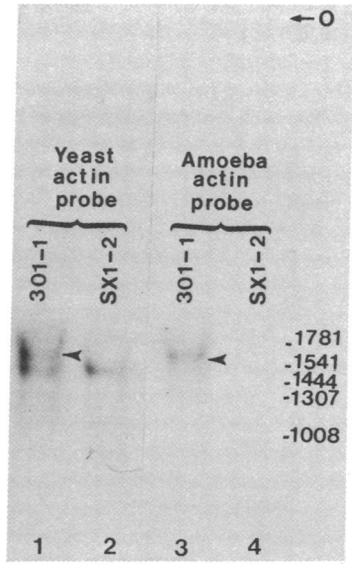

FIG. 1. Analysis of the yeast–*Acanthamoeba* hybrid actin gene transcripts. Approximately 10 μg of denatured total cellular RNA from the untransformed yeast strain SX1-2 (YNN34) (slots 2 and 4) and from the same yeast transformed with the hybrid gene YA301-1 were separated on a 1.5% agarose gel and transferred to diazotized paper. Separate filters were hybridized to either a yeast (lanes 1 and 2) or an *Acanthamoeba* actin gene probe (lanes 3 and 4). The position and length in nucleotides of RNA and DNA markers are indicated; from top to bottom: yeast 17S rRNA (28), *E. coli* 16S rRNA (29), two *Taq* I fragments of pBR322 (30), and a *Xho* I/*Hin*dIII fragment from the yeast actin gene (9). Arrows point to the hybrid transcripts.

RNA from yeast cells transformed with the yeast actin/duck α^D-globin gene chimera exhibited, on RNA blots, two species about 1,420 and 1,290 nucleotides in length when hybridized to a nick-translated yeast actin gene fragment (Fig. 3, lane 4). The untransformed cells contained only the larger of the two, the mature actin mRNA (lane 3). In the transformed cells the 1,290-nucleotide-long RNA was the only RNA detected with a duck globin gene probe (lane 2), identifying this RNA as the hybrid transcript. Because the globin gene fragment used to replace the *Bgl* II/*Kpn* I yeast actin gene fragment in the hybrid was about 150 bp shorter, the difference of roughly 130 nucleotides

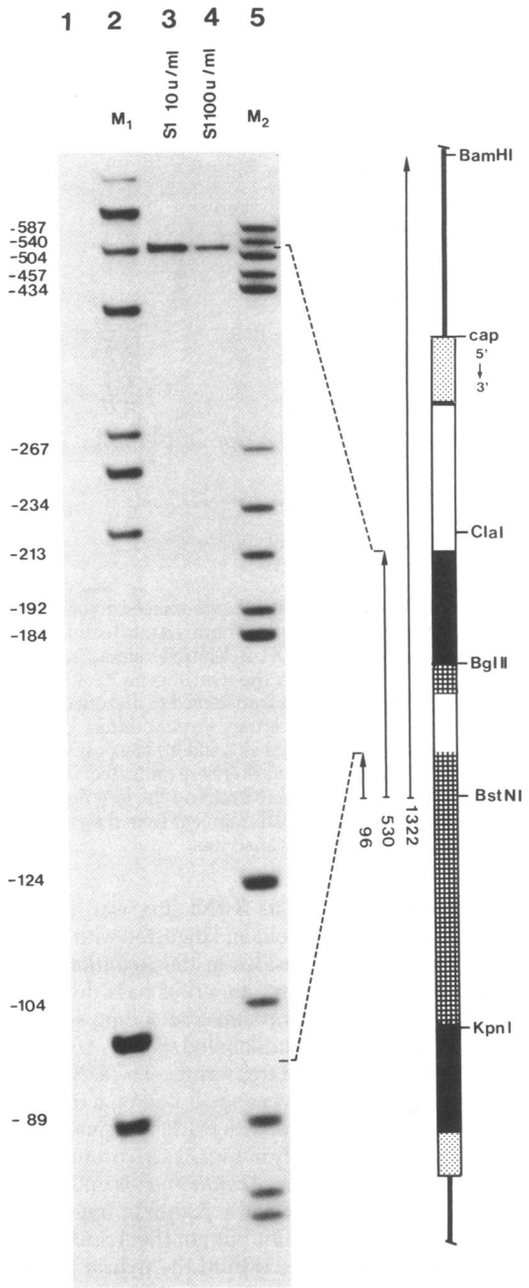

FIG. 2. S1 nuclease mapping of the 3′ splice site of the yeast *Acanthamoeba* hybrid transcript. Approximately 100 μg of total RNA from transformed cells was hybridized to a 5′-end-labeled *Bst*NI/*Bam*HI hybrid gene fragment and subjected to S1 nuclease digestion at the enzyme concentrations indicated. As a control, a hybridization mixture of 100 μg of *E. coli* tRNA and the same labeled DNA fragment was digested with S1 nuclease at 100 units (u)/ml. Protected fragments were separated on a 6% polyacrylamide/8.3 M urea sequencing gel (31). 5′-End-labeled *Alu* I (M_1, lane 2) and *Hae* III (M_2, lane 5) fragments of pBR322 served as length markers. The lengths in nucleotides of the *Hae* III fragments are indicated on the left margin of the autoradiogram. The structure of the gene chimera and possible S1-nuclease-resistant fragments are shown on the right. Solid bar, yeast coding region; stippled bar, yeast untranslated regions; cross-hatched bar, *Acanthamoeba* coding region; empty bars, intervening sequences.

between the measured length of the mature actin and the chimeric mRNA suggested that the 140-nucleotide duck globin intervening sequence was not excised from the hybrid transcript. The S1 nuclease mapping experiment illustrated in Fig. 4 clearly proved this assumption. A 1,470-bp *Hin*cII/*Bam*HI DNA fragment of the hybrid gene with the 5′-end-labeled *Hin*cII site residing within codon 93 of the second globin gene exon was hybridized to total cellular RNA isolated from the yeast transformant and the hybrids were subjected to S1 nuclease digestion. On excision of the globin intervening sequence a 196-nucleotide S1-nuclease-protected fragment should have been generated. The occurrence of a 600-nucleotide protected fragment would signify the correct removal of the yeast intervening sequence from the hybrid transcript. As shown in Fig. 4, only the large nuclease-resistant fragment was observed. By electrophoresis on a gel with higher resolution in the region of the large-sized DNA fragments it was shown that the 600-nucleotide fragment exactly maps the 3′ splice site of the yeast actin intron (data not shown).

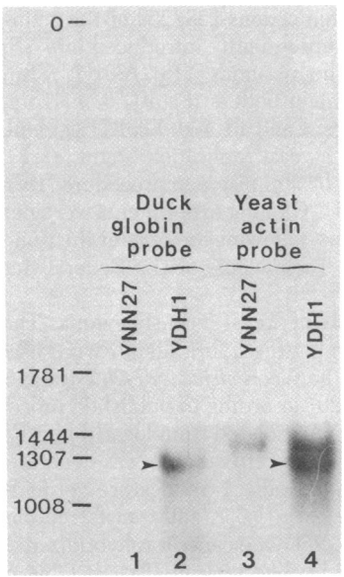

FIG. 3. Analysis of yeast–duck hybrid gene transcripts. RNA from the untransformed yeast strain YNN27 (lanes 1 and 3) and from the same yeast transformed with the hybrid gene YDH1 (lanes 2 and 4) was analyzed as described in the legend to Fig. 1, using as hybridization probe a nick-translated duck α^D-globin gene fragment (lanes 1 and 2) or a yeast actin gene fragment (lanes 3 and 4). The positions of length markers are indicated on the left margin.

To exclude the possibility that only one splicing event is allowable during the processing of the actin mRNA precursor, a hybrid gene with a second intron derived from a yeast ribosomal protein gene (*rp 51*) was tested (Fig. 5). Because the mature actin mRNA and the correctly spliced RNA derived from the hybrid gene are different in length, it could be concluded from a RNA blot analysis (data not shown) that both introns were removed from the hybrid transcript.

The 5′ splice junction of the *rp 51* intron–exon border (17) was mapped with S1 nuclease by using an 813-bp *Bgl* II/*Bst*NI hybrid gene fragment 3′-end-labeled at its *Bgl* II site located within codons 191/192 of the actin gene. In the case of correct splicing a 204-bp fragment should be protected from S1 nuclease digestion in addition to a 234-bp fragment resulting from hybrids between the labeled fragment and the mature actin mRNA, which are complementary only up to the point of insertion of the foreign DNA fragment at the *Hin*dIII site of the actin gene. The experimental result presented in Fig. 5 clearly shows that the *rp 51* intervening sequence was correctly removed from the chimeric transcript.

DISCUSSION

The hypothesis that there may be one enzymatic mechanism for splicing RNA polymerase II transcripts common to all eukaryot-

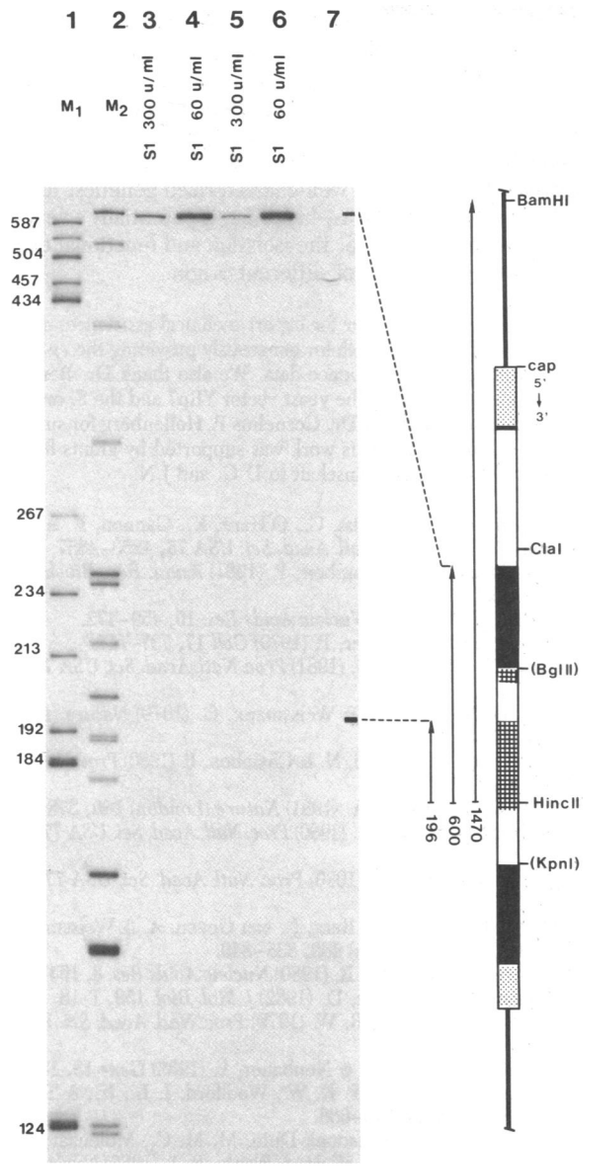

FIG. 4. S1 nuclease mapping of the 3′ splice site of the yeast–duck hybrid gene transcript. RNA from two yeast transformants (lanes 3 to 6) or *E. coli* tRNA (lane 7) was hybridized to a *Hin*cII/*Bam*HI hybrid gene fragment 5′-end-labeled at its *Hin*cII site. Hybrids were digested with S1 nuclease at enzyme concentrations indicated; the hybridization mixture containing the *E. coli* tRNA was digested with nuclease at 60 units (u)/ml. In lanes 1 and 2 end-labeled *Hae* III and *Hpa* II fragments of pBR322 were electrophoresed on the sequencing gel. The lengths in nucleotides of the *Hae* III marker fragments are indicated on the left. On the right, the structure of the gene chimera and possible nuclease-resistant fragments are shown. Yeast and foreign DNA sequences are illustrated as in Fig. 2.

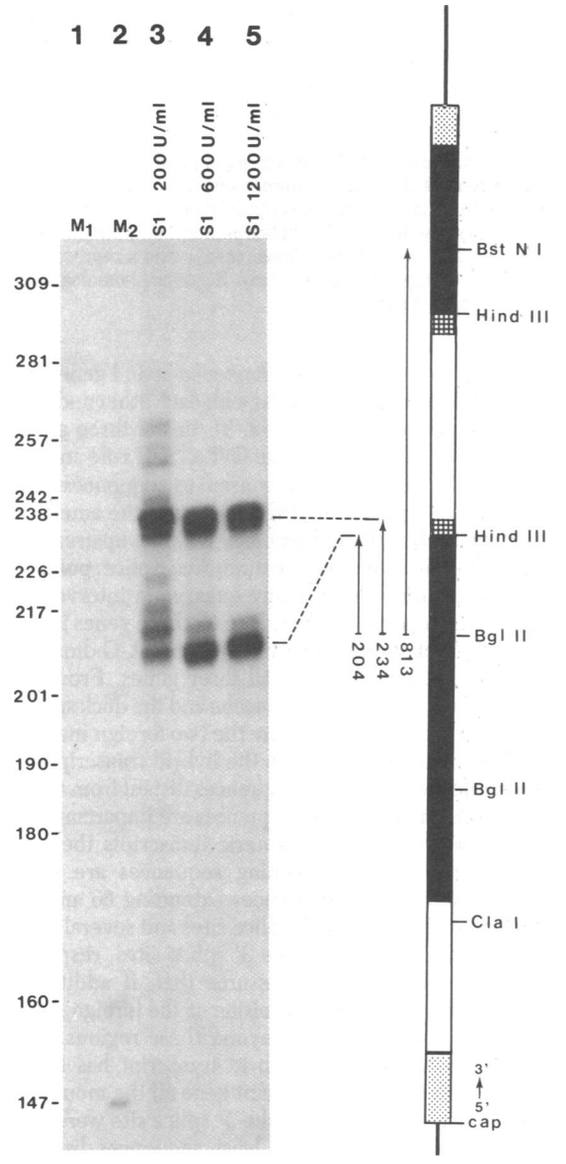

FIG. 5. S1 nuclease mapping of the 5′ splice site of the yeast *rp 51* intervening sequence in the yeast–yeast hybrid transcript. The S1 nuclease concentrations (U, units) used to digest the hybrids formed between the 3′-end-labeled *Bgl* II/*Bst*NI hybrid gene fragment and total cellular RNA from a yeast transformant are indicated (lanes 3–5). The length in nucleotides of *Alu* I (M_1) and *Hpa* II (M_2) marker fragments derived from plasmid pBR322 are shown on the left. On the right, the structure of the hybrid gene and the nuclease-resistant fragments are indicated. Yeast *rp 51* gene sequences are illustrated as the foreign sequences in Fig. 2. Note that in comparison with Figs. 2 and 4 the 5′→3′ direction of the gene is changed.

ic cells is supported by the high sequence conservation of the exon–intron junctions and by the observation that transcripts of many vertebrate genes are correctly spliced in nonhomologous mammalian cells (4–8).

The observation that yeast cells failed to splice an incomplete rabbit β-globin gene transcript (11) could be taken to indicate that the splicing mechanisms in yeast and rabbit differ, but it could also be argued that a complete transcript is a prerequisite for RNA splicing and that the prematurely terminated globin RNA was therefore not a substrate for the yeast splicing enzyme(s). In addition, the possibility was discussed (11) that the splicing enzyme(s) and the incomplete globin transcript were in different compartments of the transformed yeast cells.

In this report we clearly demonstrate the inability of the yeast cell to excise an *Acanthamoeba* and a duck intervening sequence from hybrid transcripts that are properly initiated and terminated and from which an adjacent yeast intervening sequence is correctly removed. In contrast, a second yeast intervening sequence is properly excised from a chimeric transcript, arguing against the possibility that only one intervening sequence can be removed from one transcript. This must mean that the yeast splicing machinery is unable to recognize essential structures necessary for the excision of the *Acanthamoeba* and the duck intervening sequences. This is clear evidence against the universal nature of the splicing mechanism.

In Fig. 6, the sequences surrounding the 5′ and 3′ splice sites

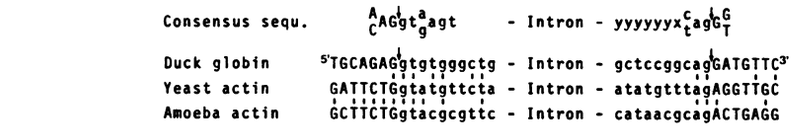

FIG. 6. Comparison of the intron–exon junction sequences of the yeast actin gene (9, 10), the *Acanthamoeba* actin I gene (13), and the duck α^D-globin gene, first intron (unpublished data) with the eukaryotic consensus sequences (2). The splice points are indicated by arrows. Intron nucleosides are shown in lower case; y and x represent pyrimidines and any nucleoside, respectively. Sequence homologies are indicated by vertical lines.

of the yeast actin gene, the *Acanthamoeba* actin I gene, and the duck α^D-globin gene are compared with each other and with the eukaryotic consensus sequences (2, 3). In the three genes the junction sequences conform to the G-T...A-G rule and exhibit homology similar to that of the consensus sequences. The sequences at the 5′ splice site of the yeast and the ameba genes show a particularly striking homology, 5 bases upstream and 3 bases downstream from the presumptive splice point being identical. In addition, as in many eukaryotic intervening sequences, the fifth intron nucleotide in all three genes is a G residue. At the 3′ splice site, however, only the A-G dinucleotide at the intron end is common to all three genes. From the sequences surrounding the *Acanthamoeba* and the duck splice sites it is not immediately apparent why the two foreign intervening sequences were not excised from the hybrid transcripts.

It is possible, however, that sequences distant from the splice sites of the foreign intervening sequences are important for their recognition and use. In the chimeric transcripts the *Acanthamoeba* and the duck intervening sequences are still surrounded by their natural sequences extending 66 and 25 nucleotides upstream from the 5′ splice sites and several hundred nucleotides downstream from the 3′ splice sites, respectively. Therefore, one would have to assume that, if additional sequences were required for the excision of the foreign intervening sequences, they would lie beyond these regions. Correct splicing of a SV40–β-globin hybrid transcript has been observed, although in the recombinant gene all the mouse globin sequences 18 bp upstream from the 5′ splice site were deleted (4), indicating that, in this gene at least, sequences distant from the 5′ splice site are not important for correct splicing.

It is also possible that some structural features of the *Acanthamoeba* and the duck intervening sequences themselves preclude their accessibility for the yeast splicing enzyme(s), although there is no precedent for this in vertebrate cells. It is conceivable, however, that for the excision of intervening sequences in the yeast cell yeast-specific sequences located within or outside the intron are required for the recognition by the splicing enzyme(s). Our preliminary data show that this might indeed be the case. In this context it is interesting to note that extensive parts of the yeast actin gene intron can be deleted without an effect on splicing (32), and this is also true for SV40 deletion mutants (33, 34).

There is another explanation for the failure to excise the foreign intervening sequences in yeast, but we feel it to be rather unlikely. The *Acanthamoeba* actin I gene and the duck α^D-globin gene could be functionless genes that are not transcribed or whose transcripts could not be spliced, even in the homologous organisms. In addition to the fact that the sequences surrounding the splice junctions of the two foreign genes conform to the eukaryotic consensus sequences, nucleotide sequence data on the *Acanthamoeba* actin I gene (13) and the duck α^D-globin gene (unpublished results) give no indication for a pseudogene nature of these two genes.

In summary, the results presented in this paper make it highly likely that the splicing mechanism in yeast differs in some important aspect(s) from that in a simple eukaryote, *Acanthamoeba*, and in a vertebrate, the duck. Whether the splicing of RNA polymerase II transcripts in yeast differs from that in other eukaryotes, for instance in plant cells, as well, is not certain at present. This question is of considerable interest as the yeast *S. cerevisiae*, because of its well-characterized genetics, its short generation time, and its easy handling, is potentially a very useful eukaryotic organism for the isolation and functional expression of eukaryotic genes of different origin.

We thank Angela Fiebiger for expert technical assistance and Drs. Pikielny and Michael Rosbash for generously providing the *rp 51* subclone and unpublished sequence data. We also thank Dr. Ronald W. Davis for kindly providing the yeast vector YRp7 and the *S. cerevisiae* strain SX1-2, and we thank Dr. Cornelius P. Hollenberg for supplying the yeast strain YNN27. This work was supported by grants from the Deutsche Forschungsgemeinschaft to D.G. and J.N.

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