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Construction of a yeast actin gene intron deletion mutant that is defective in splicing and leads to the accumulation of precursor RNA in transformed yeast cells

(BAL-31 deletions/RNA splicing/S1 nuclease mapping)

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ABSTRACT **The actin gene in yeast *Saccharomyces cerevisiae* is interrupted by a 309-base-pair intron within the protein-coding region. By using nuclease BAL-31, several intron deletion mutants were constructed to define sequences at the 5′ splice junction that are required for RNA splicing. Extensive parts of the intron can be removed without affecting correct splicing. One mutant gene from which the invariant thymidine residue in the second intron position was deleted led to the accumulation of large amounts of unspliced actin mRNA when introduced into yeast cells through a recombinant high-copy-number plasmid. No evidence for the usage of alternative splice sites was obtained.**

The occurrence of split protein-coding genes can be regarded as a general feature of the eukaryotic genome. Split genes have been found in many organisms from the unicellular yeast to man (see refs. 1 and 2 for review). The transcription products of genes interrupted by introns are colinear precursor molecules from which the intervening sequences are excised by a so-called splicing mechanism.

A comparison of the nucleotide sequences around splice junctions in more than 100 genes from very distantly related eukaryotes shows only limited structural similarities (see refs. 2 and 3 for review). All introns of protein-coding genes studied so far start with the dinucleotide 5′ G-T 3′ and end with the dinucleotide 5′ A-G 3′. Otherwise, only a few bases around the two highly conserved residues at the 5′ and 3′ splice sites are similar. The consensus sequences for both splice sites are six to eight nucleotides long (2–7).

There are indications from studies involving genes with large intron and exon deletions that sequences distant from the exon/intron boundaries do not affect correct splicing (8–10), but those sequences that are absolutely required have not been defined. In a recent study, it was shown that a point mutation in an adenovirus splice junction changing the T residue of the conserved G-T to a G residue prevented splicing of the RNA product (11).

The actin gene of the yeast *Saccharomyces cerevisiae* is interrupted within the coding region by a 309-base-pair intron (12, 13). The nucleotide sequences at the intron–exon junctions share the characteristics with the splice junctions in genes of higher eukaryotes—i.e., they follow the G-T/A-G rule. This finding led to the conclusion that the splicing mechanism might be identical in all eukaryotes (12). However, from studies in which foreign split genes were introduced into yeast, there are indications that transcripts originating from those genes are not correctly spliced (ref. 14; unpublished data).

In a search to delineate the minimal sequences required for a correct excision of intervening sequences from the primary transcripts in yeast, actin gene mutants were constructed with intron deletions of varying extent and tested in transformed yeast cells. Here, it is shown that significant parts of the intron can be removed without an impairment of splicing and that deleting the T residue of the dinucleotide G-T at the 5′ splice site results in the accumulation of unspliced actin precursor RNA in the transformed cells.

MATERIALS AND METHODS

Materials. [α-^{32}P]dNTPs and [γ-^{32}P]ATP (2,000–3,000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) were obtained from Amersham. Restriction endonucleases were from Bethesda Research Laboratories and Boehringer Mannheim. BAL-31 and S1 nucleases were purchased from Bethesda Research Laboratories.

Construction of Intron Deletion Mutants. The plasmid pYA208 carrying the yeast actin gene (12, 15) was linearized with the endonuclease *Xho* I, which cuts within the intron. Approximately 30 μg of DNA in 200 μl of digestion buffer (20 mM Tris·HCl, pH 8/0.6 M NaCl/12.5 mM $MgCl_2$/12.5 mM $CaCl_2$/1 mM EDTA) were digested with 2 units of BAL-31 nuclease. Aliquots were taken at 1-min intervals, and the extent of deletion was calculated from the length of appropriate restriction fragments. The BAL-31-digested plasmids were religated with T4 DNA ligase and cloned in *Escherichia coli* RR1. DNA sequence analysis of different deletion mutants was performed as described by Maxam and Gilbert (16) with restriction fragments 5′-end-labeled at the *Cla* I restriction site at position 269 of the intron. All experiments involving recombinant DNA conformed to the guidelines of the Bundesminister für Forschung und Technologie of the Federal Republic of Germany.

Yeast Transformation. The mutated yeast DNA from selected clones was excised with *Pst* I from the recombinant plasmid and inserted into the *Pst* I site of the hybrid vector pMP78-1 (17). DNA was cloned in *E. coli* RR1, selecting for chloramphenicol resistance. Recombinant plasmids carrying the mutated actin genes were used to transform the *S. cerevisiae* strain AH22 (a *leu2-3 leu 2-112 his4-519 can1*) (18). Transformation was performed by published procedures (18, 19) using zymolase for the preparation of sheroplasts. Transformants were grown on minimal plates without the addition of leucine.

DNA and RNA Analysis of Yeast Transformants. DNA was isolated from yeast transformants as described (20). To differentiate between the chromosomal actin gene and actin genes within the high-copy-number plasmid, the DNA was digested with a combination of the restriction enzymes *Cla* I and *Bam*HI. The resulting DNA fragments were transferred to nitrocellulose filters (21) and hybridized as described (15) to a nick-translated

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Abbreviation: kb, kilobase(s).

(22) 2.2-kilobase (kb) *Hin*dIII/*Pst* I DNA fragment containing the 5′ region of the actin gene up to codon 259 (12) and part of the "protein 2" gene, which is located 5′ to the actin gene (15). Polyadenylylated RNA was isolated as described (15), denatured by glyoxylation, and separated on a 1.5% agarose gel (23). Length markers were *E. coli* 16S RNA, 1,541 nucleotides (24); yeast 26S RNA, 3,360 nucleotides (25); yeast 17S RNA, 1,781 nucleotides (26); and the *Taq* I fragments of pBR322, 1,443 and 1,307 nucleotides (27). The nucleic acids were transferred to diazobenzyloxymethylated paper (28), and hybridization was performed with a nick-translated 2.2-kb *Hin*dIII/*Pst* I fragment (2×10^8 dpm/μg) mentioned above. Correctly spliced and unspliced RNA transcripts were analyzed by using the S1 mapping procedure (29, 30) as described (31).

RESULTS

The Functional Splice Region at the Donor Site Is Short. Fig. 1 shows the organization of the yeast actin gene and the extent of several intron deletions whose effect on the excision of the intervening sequence from the transcription product was examined in transformed yeast cells.

The conveniently located *Xho* I restriction site at positions 62–67 of the intron allowed the linearization of the recombinant plasmid pYA208 harboring the actin gene (12), the deletion of intron sequences with the nuclease BAL-31, and the religation of blunt-ended plasmids. DNA sequence analysis determined the extent of deletions in recloned plasmids that had preserved the *Hin*fI restriction site located within the two codons immediately preceding the intron start (see Fig. 2). The deletions resulting from BAL-31 digestion were much more extensive in the 3′ direction of the intron, obviously because of several T-rich segments that follow the *Xho* I restriction site. The wild-type actin gene and the mutated genes were integrated into the *Pst* I site of the hybrid plasmid pMP78-1 (17), which autonomously replicates in *E. coli* and yeast and is a recombinant of pBR325 (32) and a 3.3-kb *Hin*dIII fragment from pJDB219 (19) that carries the yeast *LEU2* gene and sequences of the yeast 2-μm plasmid. The plasmid pMP78-1 is a high-copy-number plasmid with 50 or more molecules per cell and allows the selection of colonies containing cells that after transformation change the phenotype from $Leu2^-$ to $Leu2^+$.

The surprising result was that, regardless of the orientation of the yeast actin gene in the recombinant plasmid, no yeast transformants could be recovered either with the wild-type actin gene or with the intron deletion mutants listed in Fig. 1, with the exception of the mutant gene YAIM48. In contrast, we obtained viable yeast transformants with the wild-type actin gene integrated into the low-copy-number plasmid YRp7 (20). Because the yeast genome contains only one actin gene, the expression of a large number of additional actin genes, introduced into cells through the vector pMP78-1, was obviously not compatible with the survival of such transformed cells. Most likely, the enormously increased synthesis of actin caused the inhibition of cell growth.

What led the introduction of pMP78-1–YAIM48 recombinants to the formation of viable transformants? Inspection of the mutant sequence shows (Fig. 2) that the deletion spans the region from nucleotide 2 to 164 of the intron. The T residue of the conserved dinucleotide G-T at the start of the intervening sequence was deleted. Therefore, it was concluded that the transcription product of this actin gene mutant was not correctly spliced and, hence, did not result in the synthesis of large amounts of actin. Indeed, only a tetrapeptide, Asp-Ser-Gly-Ala, could be formed from the unspliced RNA because of a translational stop codon in the reading frame.

Although a negative criterion, the failure of mutated actin genes integrated in the high-copy-number plasmid to produce viable yeast transformants was used successfully as a screening procedure for the identification of actin genes generating correctly processed mRNA.

Accumulation of Precursor RNA in Cells Transformed with the Splice-Defective Mutant Gene. RNA from transformed yeast cells was enriched for poly(A)-containing molecules, denatured, and blotted onto diazobenzyloxymethylated paper after gel electrophoretic separation. Ethidium bromide staining showed an RNA species about 1,530–1,550 nucleotides long, which was found in large amounts in cells transformed with the recombinant plasmid pMP78-1–YAIM48 (regardless of the orientation of the actin gene; Fig. 3*A*, tracks 3 and 4) but not in cells transformed with the vector pMP78-1 alone (Fig. 3*A*, track 2). According to the length estimation on agarose gels, this RNA was roughly 100–150 nucleotides longer than the mature actin mRNA.

Fig. 3*B* shows that this RNA hybridized strongly to an actin gene probe containing exon and intron sequences (tracks 3 and 4), and it is evident that, compared to the spliced actin mRNA (track 2), the intensity of the radioactive band was related to the large amount of precursor RNA seen on the stained gel.

The amount of precursor RNA clearly was related also to the high copy number of the recombinant plasmid within the cells. Fig. 4 shows a Southern blot of restriction endonuclease-digested total cellular DNA from yeast strain AH22 used for transformation (track 3) and of the same cells transformed with the recombinant plasmid carrying the mutated actin gene in the two orientations (tracks 1 and 2).

In Fig. 3*B* there are, besides the strongest hybridizing actin precursor (band a), two other radioactive bands (b and c) cor-

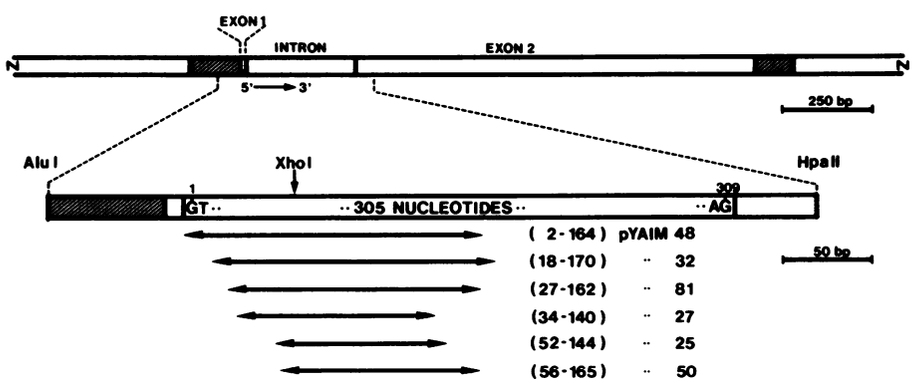


FIG. 1. Organization of the *S. cerevisiae* actin gene (▨, 5′ and 3′ untranslated regions) and the extent of intron deletions tested in yeast transformation. Figures in parentheses are intron nucleotides deleted. bp, Base pair.

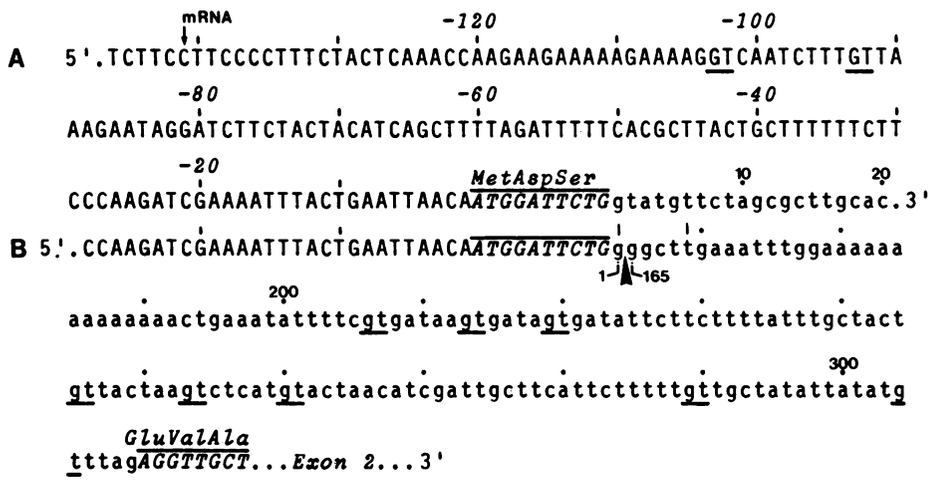


FIG. 2. Nucleotide sequence of the wild-type actin gene (*A*) and the mutant gene YAIM48 (*B*). Note that the sequence upstream from the intron nucleotide 1 is identical in both genes. Intron nucleotides 21–164 are not shown. The mRNA start site and the fusion point of intron nucleotides 1 and 165 in the deletion mutant are indicated by an arrow and an arrowhead, respectively.

responding to a length of about 1,250 and 880 nucleotides, respectively. The origin of the larger of the two bands is unclear, but it could simply be a defined degradation product. This RNA is too short for a processed actin gene transcript that had used an alternative 5′ splice site upstream or downstream from the mutated splice region. We have noted, however, that the length of band b would fit an RNA starting at the 3′ splice site. The additional hybridizing band c corresponds to the mRNA for "protein 2," whose gene is located 5′ to the actin gene (15, 31) and which was partly represented in the hybridization probe used.

To further characterize the accumulated actin precursor RNA with respect to the intervening sequence it contained, an S1 nuclease protection experiment was performed. A 5′-end-labeled 456-nucleotide *Hin*fI fragment of the wild-type gene, spanning the region between codons 2 and 3 of exon I and between codons 51 and 52 of exon II was hybridized to total cellular RNA derived from pMP78-1–YAIM48-transformed yeast cells. The results of a sequence determination on a 6% polyacrylamide gel (Fig. 5) established that the bulk of the protected radioactive DNA fragments ended around nucleotide 165 of the intron—i.e., within the region where the wild-type and the YAIM48 sequences deviate because of the deletion of nucleotides 2–164 (see also Fig. 2). In addition, two significantly weaker radioactive fragments, 121 and 122 nucleotides long and representing the 3′ intron–exon junction, were observed. When RNA from cells transformed with the vector alone was used in S1 mapping, only the latter two fragments were observed (data not shown). The intensity of label in these two sets of prominent protected fragments reflects quite well the amounts of spliced actin mRNA, which most likely originated entirely from the single chromosomal gene, and that of the unspliced precursor RNA generated from the mutated actin genes within the recombinant plasmids. There was no indication for the usage of alternative splice sites, although several GT sequences, underlined in Fig. 2, are present within the remaining intron and the 5′ untranslated region.

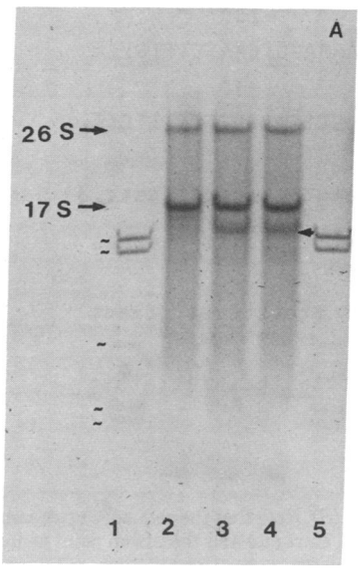
DISCUSSION

The advantage to the study in split yeast genes of the nucleotide sequences required for a correct splicing is the possibility of examining the biological consequences of site-directed mutations after reintroduction of the mutated genes into the homologous cells. Although the nucleotide sequences at the intron–exon borders of the yeast actin gene (12, 13) resemble those found in higher eukaryotes (2–7), there are indications of differences in the splicing mechanism for RNA polymerase II transcripts in yeast and other eukaryotes (ref. 14; unpublished data). Therefore, the elucidation of the minimal sequences necessary for the excision of intervening sequences from yeast gene transcripts and their comparison with sequences required in genes of other eukaryotes should prove to be informative.

The search for splice-defective actin gene mutants was facilitated by the finding that the wild-type actin gene brought into cells with a high-copy-number recombinant plasmid gave no viable transformants. Because in contrast, the actin gene introduced into cells through the low-copy-number plasmid YRp7 led to the formation of transformants, it was concluded that a gene dosage effect, the overproduction of actin, was responsible for the inhibition of cell growth. A similar observation has been made recently with yeast cells transformed with a yeast phosphatase gene (*pho-5*). Growth inhibition was observed in cells transformed with a high-copy-number plasmid after the expression of the phosphatase genes was induced (A. Hinnen, personal communication).

Of the actin gene intron deletion mutants tested, the only one defective in splicing had a deletion that included the T residue of the invariant G-T dinucleotide at the 5′ splice site. The deletion coming second closest to the 5′ splice site (mutant YAIM32) included intron nucleotides 18–170. The inability of this mutant gene to form viable yeast transformants led to the conclusion that the RNA transcript was most likely spliced correctly. In four of the mutants, the intron sequences fused to the deletion end point at the 5′ side—i.e., next to intron nucleotides 1 (in YAIM48), 17 (in YAIM32), 26 (in YAIM81), and 55 (in YAIM50)—were, with a difference of only a few residues, identical. Taking this into account, our results show that (*i*) significant parts of the intron can be deleted without affecting correct splicing, (*ii*) the maximum length of the splice signal sequence downstream from the 5′ intron/exon junction lies within the region of nucleotides 1 and 17 of the intron, and (*iii*) the T residue of the conserved dinucleotide G-T is most likely an essential part of the splicing signal.

The 5′ splice region of the yeast actin gene has the structure 5′ C-T-G-G-T-A-T-G-T 3′ which is rather similar to the 5′ donor consensus sequence 5′$\frac{A}{C}$-A-G-G-T-$\frac{A}{G}$-A-G-T 3′. In the splice-defective intron deletion mutant YAIM48, this region was



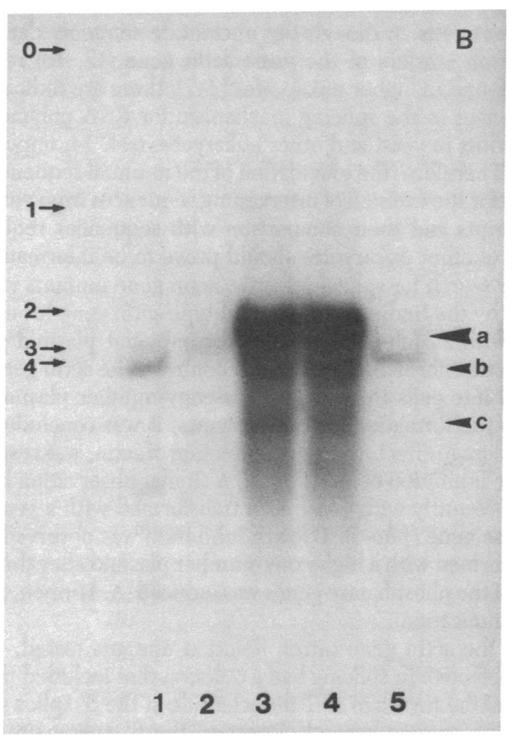


FIG. 3. RNA stained with ethidium bromide on 1.5% agarose gel (*A*) and hybridized to a nick-translated actin gene probe (*B*). Total RNA from cells transformed with either the hybrid vector pMP78-1 (track 2) or with the recombinant plasmid pMP78-1–YAIM48 carrying the actin gene in two orientations (tracks 3 and 4) was passed once over an oligo(dT)-cellulose column. *Taq* I restriction fragments of pBR322 (tracks 1 and 5) served as additional length markers. (*A*) Arrow on the right points to the unspliced precursor RNA. (*B*) Unspliced actin mRNA (band a) and 1,250- and 880-nucleotide RNAs (bands b and c; see text). Arrows 1, 2, 3, and 4 indicate the positions of yeast 26S and 17S rRNA and the 1,443- and 1,307-nucleotide *Taq* I fragments, respectively.

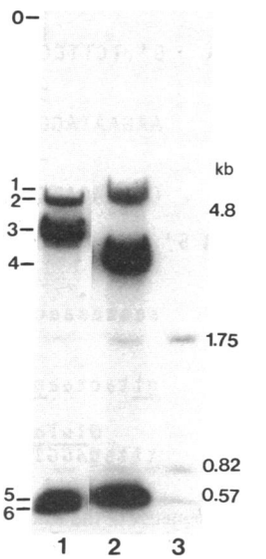


FIG. 4. Southern blot of *Cla* I/*Bam*HI-digested DNA from untransformed cells (track 3) and from cells transformed with the recombinant plasmid carrying the mutated actin genes in both orientations (tracks 1 and 2). Hybridization was performed with a nick-translated actin DNA-containing fragment. The lengths of the hybridizing chromosomal DNA fragments are indicated. Only parts of the 4.8-kb and the 1.75-kb fragments are contained in the cloned DNA. The 0.57-kb *Cla* I fragment spans the region between intron nucleotide 269 and codons 178/179. The 0.82-kb *Cla* I/*Bam*HI fragment contains part of the intron; in the deletion mutant, this fragment is shortened to 0.65 kb (band 5). Depending on the orientation in the recombinant plasmid, fragments containing actin DNA and vector sequences are either 5.4 kb and 3.84 kb (bands 2 and 3, track 1) or 6.24 kb and 3 kb (bands 1 and 4, track 2).

changed to 5′ C-T-G-G-G-G-C-T-T 3′, leaving identical only the first nucleotide (G) and the sixth residue (T) downstream from the putative splice point. Two of the changes produced might be of special importance, the replacement by a purine of the otherwise conserved T residue in intron position 2 and the exchange of the G residue by a pyrimidine in the fifth intron position.

That the T residue in the second intron position is required for proper splicing was recently shown in a study in which the T was replaced by a G in an adenovirus 5′ splice junction (11). Another adenovirus mutant defective in splicing has been described by Solnick (33). It was found that two adjoined G residues, five and six nucleotide downstream from the 5′ splice point, were changed to A-T. The nucleotide in position 5 of the introns is nearly exclusively a purine and most often a G residue (2, 3). This is also true for the yeast actin gene intron; therefore, the G → A change in the adenovirus mutant and the G → T change in our actin gene mutant in position 5 might contribute to the splicing defect.

A deletion of a pentanucleotide starting with the invariant T residue at the 5′ splice junction has been found in the α2-globin gene of an individual with α-thalassaemia (34). Mutations within the GGT 5′ splice region have also been observed in the β-globin gene of patients with β-thalassaemia (35, 36), but in no case has it been demonstrated that these gene alterations caused a defect in RNA splicing.

Two other features of the splice-defective yeast mutant merit comment. First, the unspliced RNA was found in large amounts within the transformed cells. Because it is difficult to cleanly separate nuclear and polysomal fractions from disrupted yeast cells, we have not attempted to investigate where in the cell the precursor RNA is accumulated. Second, alternative splice sites are most likely not at all or very inefficiently used so that they were not detected. In simian virus 40-β-globin recombinants in which the 5′ splice junction of the second globin gene intron

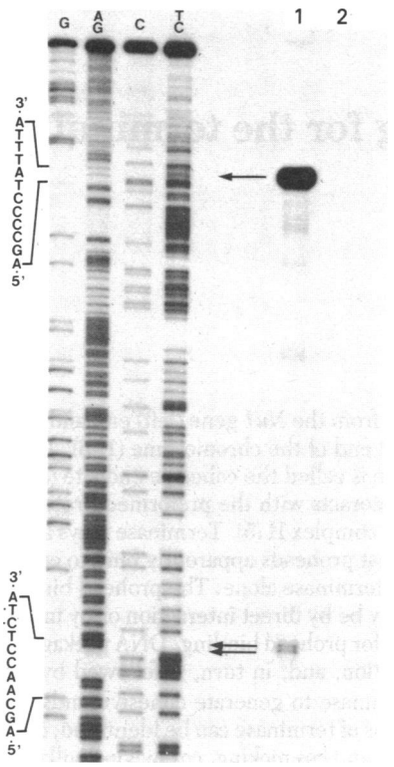


FIG. 5. DNA fragments protected from S1 nuclease digestion in hybrids between total RNA from pMP78-1–YAIM48-transformed cells (track 1) or *E. coli* tRNA (track 2) and a 5'-end-labeled *Hin*fI fragment from the wild-type gene spanning the entire intron. Chemical cleavage fragments of the same DNA fragment were coelectrophoresed. Labeled fragments corresponding to the 3' intron–exon border and the region of sequence divergence (cluster of four C residues) between the wild-type and the mutant gene are indicated by arrows.

was deleted, new splice sites located upstream and downstream from the deleted one were used (37). In the yeast actin gene, a potential 5' splice site with the conserved central GGT region lies 103 nucleotides upstream from the ATG initiation codon (Fig. 2). Because the mature actin mRNA, including the poly(A)tail, is between 1,400 and 1,430 nucleotides long, a RNA of about 1,300 to 1,350 nucleotides was expected if this splice region would have been used. Although we have not excluded the possibility that the polyadenylylated 1,250-nucleotide-long RNA that was detected on RNA blots in addition to the unspliced actin mRNA was a product resulting from alternative splicing, the length of this RNA makes such an assumption unlikely.

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