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Use of a synthetic DNA oligonucleotide to probe the precision of RNA splicing in a yeast mitochondrial petite mutant

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ABSTRACT

In some strains of Saccharomyces cerevisiae the mitochondrial gene coding for 21S rRNA is interrupted by an intron of 1143 bp. This intron contains a reading frame for 235 amino acids: Unassigned Reading Frame (URF). In order to check whether expression of this URF is required for proper splicing of precursors to 21S rRNA, the precision of RNA splicing was analysed in a petite mutant, where no mitochondrial protein synthesis is possible anymore. We have devised a new assay to monitor the precision of the splicing event. The method is of general application, provided that the sequence of the splice boundaries is known. In the case of the 21S rRNA it involves the synthesis of the DNA oligonucleotide d(CGATCCCTATTGTC) complementary to the 5' d(CGATCCCTAT) and 3' d(TGTC) borders flanking the intron in the 21S rRNA gene. The oligonucleotide is labelled with ^{32}P at the 5'-end,hybridised to RNA and subsequently subjected to digestion with S_1 nuclease. Resistance to digestion will only be observed if the correct splice-junction is made.

The petite mutant we have studied contains a 21S rRNA with the same migration behaviour as wildtype 21S rRNA. In RNA blotting experiments, using an intron specific hybridisation probe, the same intermediates in splicing are found both in wild type and petite mutant. Finally the synthetic oligonucleotide hybridises to petite 21S rRNA and its thermal dissociation behaviour is indistinguishable from a hybrid formed with wildtype 21S rRNA.

We conclude that expression of the URF, present in the intron of the 21S rRNA gene, is not required for processing and correct splicing of 21S ribosomal precursor RNA.

INTRODUCTION

In some strains of Saccharomyces cerevisiae the mitochondrial gene coding for 21S rRNA is interrupted by an intron of 1143 bp [1,2]. The DNA sequence of this intron contains an open reading frame [2], that can possibly code for a protein 235 amino acids long. Such reading frames called URF's (Unassigned Reading Frames), have also been detected in human mt DNA and in other regions of yeast mt DNA e.g. in the introns of the genes coding for subunit I of cytochrome oxidase and cytochrome b [3,4,5].

There is strong evidence that the URF present in the second

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intron of the gene for cytochrome b is involved in splicing of cytochrome b precursor RNA's: by splicing out the first intron, the DNA sequences coding for the N terminal part of cytochrome b are linked to those coding for the URF [6] and with antibody against cytochrome b a new fusion protein can be detected [7,8]. Mutants located in the URF sequence are disturbed in RNA maturation and it has been proposed that the "fusion" protein functions as an RNA maturase [6,9].

In wildtype yeast strains containing a split gene coding for the large ribosomal RNA, precursor RNA's still containing the intron and intermediates in splicing have been described [1]. Similar intermediates have also been observed in petite mutants retaining the gene for 21S rRNA, but since these intermediates were detected by electronmicroscopy [10], it is unknown if the splice-junction is made with the same precision in the petite mutant as in wildtype 21S rRNA. To study the precision of RNA splicing we have devised a novel assay to analyse splicing enzymes: a DNA oligonucleotide of sequences complementary to 10 nucleotides from the 5' exon flanking the intron and 4 nucleotides of the 3' exon flanking the intron was synthesised and labelled with ^{32}P at its 5' end. When precursor RNA is correctly spliced an RNA sequence complementary to the oligonucleotide is generated and this can be identified because it completely protects the labelled fragment from digestion with S_1 nuclease. With this test we show that splicing of the 21S rRNA precursor takes place in the petite mutant with precision even in the absence of proteins coded for by mt DNA and that expression of the reading frame present in the intron of this gene is not required for the maturation of 21S rRNA.

MATERIALS AND METHODS

The following yeast strains were used: S. cerevisiae KL14-4A, S. cerevisiae JSI-3D, S. carlsbergensis and the petite strains F41 and 23 both derived from S. cerevisiae JSI-3D and described before [11], S. cerevisiae LH 25F2, a petite lacking the 21S rRNA gene [12] and the ρ^0 petite S. cerevisiae S288C/II. Culture conditions and the preparation of mt DNA, mt RNA and mt ribosomes have been described [13,14,15]. The oligonucleotide d(CGATCCCTATTGTC) was synthesised, purified and analysed by a methodology described

before [16]. For an intron-specific probe a HinfI DNA fragment was isolated from petite 23 DNA; this fragment almost exclusively consists of intron sequences. Exon sequences were competed against by carrying out the hybridisation in the presence of S. carlsbergensis mt RNA, a strain with a continuous 21S rRNA gene.

DNA fragments were 5' labelled according to Seeburg et al. [17] or internally labelled by nick translation [18]. mt RNA was electrophoresed in a 1.25% agarose gel (90 mM Tris-borate pH 8.0,2.5 mM EDTA) and transferred to DBM paper according to the procedure of Alwine et al. [19] using sodium phosphate pH 5.5 [20]. Hybridisation conditions were as described [12]. S_1 nuclease analysis was carried out according to the procedure of Berk and Sharp [21] with the modifications described [12]. The products were analysed on 20% polyacrylamide gels as described by Maxam and Gilbert [22].

The melting behaviour of the hybrid between DNA fragment and 21S rRNA was analysed as described [23] with the only exeption that rRNA was fixed on DBM paper instead of nitrocellulose [24].

RESULTS

The petite mutant F41 is a large petite (20 kb), retaining the split gene for 21S rRNA with flanking 5' and 3' regions [11]. We have analysed mt RNA preparations of wildtype Saccharomyces strains and the mt RNA isolated from petite F41 by agarose gel electrophoresis (fig. 1). The position for 21S rRNA is indicated. In the RNA of petite F41 a 21S rRNA species is found that comigrates with the 21S rRNA of the other strains (fig. 1) and hybridises with labelled DNA fragments containing 21S rRNA gene sequences (experiments not shown). The RNA's were transferred from agarose and coupled to DBM paper and hybridised with a ^{32}P labelled DNA fragment representing intron sequences (see Materials and Methods). The specificity of hybridisation is illustrated in fig. 1, lane d', since no hybridisation is found with mt RNA isolated from S.carlsbergensis, a strain with an uninterrupted gene for 21S rRNA. With the other two wildtype strains, containing an intron in the large rRNA gene, precursor RNA's are found (fig. 1 lanes a' and b') as described earlier [1]. The same precursors, although in different concentrations, are found in the petite mu-

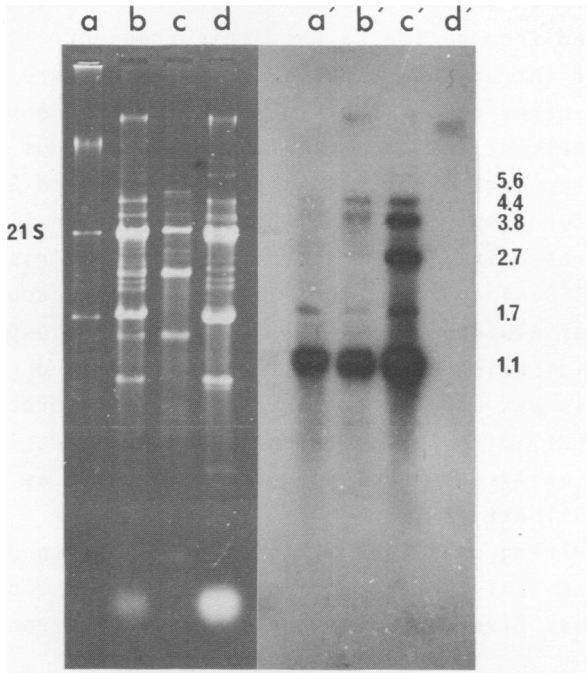

Fig. 1. Detection of 21S ribosomal precursor RNA's in wildtype strains and a petite mutant. mt RNA preparations (2 µg) were electrophoresed on a 1.25 % horizontal agarose gel (see Methods) and RNA bands visualised by staining with ethidium. RNA's were subsequently transferred to DBM paper and hybridised with a ^{32}P-labelled intron-specific DNA fragment (see Methods). RNA precursors still containing intron sequences are shown in lanes a', b', c' and d' and their approximate length is indicated in Kb. a: S. cerevisiae Kl 14-4A, b: S. cerevisiae JS1-3D, c: petite mutant F41, d: S. carlsbergensis.

tant (fig. 1 lane c'). These experiments suggest that in the petite mutant, despite the absence of mitochondrially synthesised proteins, normal processing of RNA takes place leading to mature 21S rRNA.

To investigate whether the splice-junction in F41 rRNA is the same as in wildtype 21S rRNA, we have synthesised a DNA oligonucleotide complementary to 21S rRNA containing 10 nucleotides from the 5' exon border flanking the intron and 4 nucleotides from the 3' exon border flanking the intron (fig. 2). This synthetic DNA fragment was labelled with ^{32}P at its 5' end and hybridised with various RNA preparations. Hybrids were treated with S_1 nuclease and the fate of the DNA fragment was analysed by polyacrylamide

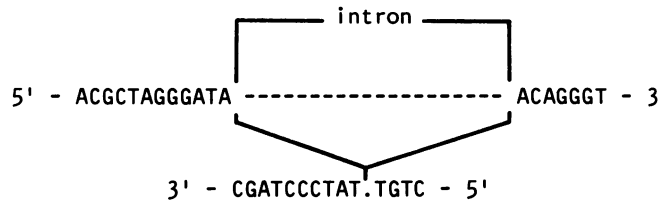

Fig. 2. Nucleotide sequence at 5' and 3' exon borders flanking the intron in the gene for 21S rRNA (anti-sense strand) and the complementary nucleotide sequence of the synthetic DNA fragment.

gel electrophoresis and autoradiography of the gel (fig. 3). In fig 3 lane a the input fragment is shown. This is completely digested with S_1 nuclease (lane b) also after hybridisation with yeast RNA preparations lacking 21S rRNA, isolated from a ρ^0 petite (with no mt RNA at all) or from a ρ^- petite lacking the gene for 21S rRNA (lanes c and d). The DNA fragment is fully protected against S_1 nuclease when hybridised to wildtype and petite F41 21S rRNA (lanes e and f).

Although it has been reported that S_1 nuclease can detect a single nucleotide mismatch [25] this is not always the case [1] and probably dependent on the nucleotidesequence surrounding the mismatch. A single mismatch in a hybrid with a 14 nucleotide long DNA fragment has a destabilising effect resulting in a difference of 10^oC in Tm with the perfect hybrid as observed in a model study with øX 174 DNA [23]. We therefore immobilised wildtype and F41 petite rRNA on DBM paper and constructed hybrids with the ^{32}P-labelled fragment. Fig. 4 shows the thermal dissociation of the fragment from the immobilised 21S rRNA. No difference in thermal melting between mutant and wildtype RNA can be observed.

We conclude that the splice-junction in petite F41 rRNA remaining after excision of the intron from the precursor RNA, is identical in nucleotide sequence to the one found in wildtype 21S rRNA.

DISCUSSION

An unexpected discovery of determining the nucleotide sequence of the genomes of human [3] and yeast mt DNA [4,5,6] is the finding of long reading frames whose function is as yet unknown (Un-

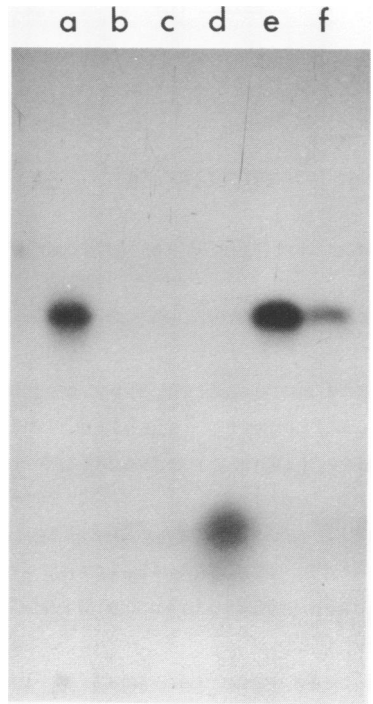

Fig. 3. Precision of splicing in a petite mutant assayed with the synthetic DNA fragment. The ^{32}p-labelled fragment was hybridised with different mt RNA preparations (5 µg) and subsequently treated with S1 nuclease (100 µ/ml), with the exeption of lane a. After alcohol precipitation pellets were dissolved and analysed on a 20% polyacrylamide gel. The autoradiogram of the gel is shown. a: input ^{32}P-labelled DNA fragment without S_1 nuclease. b: ^{32}P-labelled DNA fragment after treatment with S_1 nuclease. c: idem plus ρ^o RNA. d: idem plus mt RNA from a petite lacking the 21S rRNA gene. e: idem plus wildtype 21S rRNA, isolated from the large ribosomal subunit. f: idem plus total mt RNA from petite F41.

assigned Reading Frames: URF's), with the exeption of the URF in the second intron present in the gene coding for cytochrome b. There, evidence indicates that this URF codes for part of a protein involved in the maturation of cytochrome b precursor RNA's [6]. We have therefore tried to assess if the URF present in the intron interrupting the gene for 21S rRNA [2] has a similar function in processing of 21S precursor RNA's.

We have selected a mitochondrial petite mutant F41, retaining the gene for 21S rRNA with flanking regions in which no mitochon-

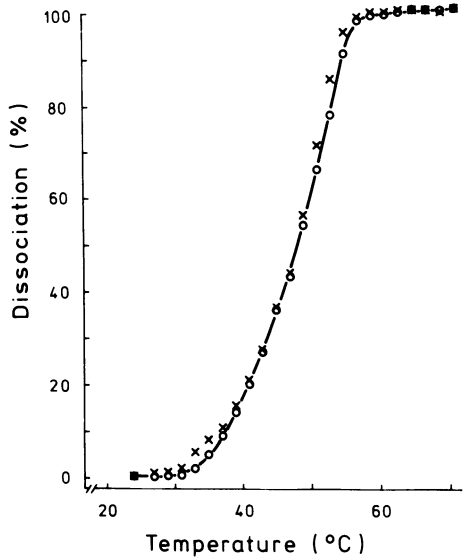

Fig. 4. Thermal dissociation of hybrids constructed with wildtype and petite 21S RNA. RNA was fixed to DBM paper and hybridised with the ^{32}P-labelled DNA fragment. Radioactivity lost from the filter after temperature increases of 2^{o} C was counted and cumulatively plotted (for details see Methods).
o: wildtype 21S rRNA, x: petite 21S RNA.

drial protein synthesis is possible anymore. The same precursor rRNA's and intermediates of processing are present in this mutant as are found in wildtype strains. Also the precision of splicing out the intron does not differ from the wildtype. We have synthesised a 14 nucleotide length DNA oligonucleotide consisting of 10 nucleotides from the 5' exon flanking the intron and 4 nucleotides of the 3' exon flanking the intron. This DNA fragment when hybridised to petite or wildtype 21S rRNA, is in both cases fully resistant against single strand specific S_1 nuclease and no difference in thermal dissociation can be found. In a study with a 14 nucleotide length DNA fragment hybridised to wildtype øX 174 DNA or DNA from an amber mutant, a difference of 10^{o}C was found between the two hybrids, indicating that a single mismatch in a hybrid of short length has a drastic destabilising effect. We conclude therefore that petite mutant and wildtype 21S rRNA have the same splice-junction and that expression of the URF in the intron of the 21S rRNA gene is not required for processing or splicing of 21S pre-

cursor RNA, although a minor role e.q. in the efficiency of RNA maturation cannot be excluded.

Our experiments support the feasibility of using a short synthetic DNA fragment, complementary to a splice-junction, as a specific probe to assay enzymes involved in RNA splicing since even a short fragment of only 14 nucleotides length survives the S_1 nuclease treatment with utmost specificity only when complementary sequences are provided. We have performed the hybridisation under several conditions of stringency with the same specific results (experiments not shown). Apparently all mispaired combinations of oligonucleotide and RNA are eliminated during subsequent treatment with S_1 nuclease.

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Abbreviations: rRNA, ribosomal RNA; (k) bp, (kilo) base pair(s); DBM, diazo-benzyloxymethyl-paper.

REFERENCES

1. Bos, J.L., Osinga, K.A., Van der Horst, G., Hecht, N.B., Tabak, H.F., Van Ommen, G.J.B. and Borst, P., (1980) Cell 20, 207-214.
2. Dujon, B. (1980) Cell 20, 185-197.
3. Anderson, S., Bankier, A.T., Barrell, B.G., De Bruijn, M.H.L., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J.H., Staden, R. and Young, I.G., (1981) Nature 290, 457-465.
4. Bonitz, S. and Tzagoloff, A., (1980) J. Biol. Chem. 255, 9075-9081.
5. Bonitz, S., Coruzzi, G., Thalenfeld, B.E. and Tzagoloff, A. (1980) J. Biol. Chem. 255, 11927-11941.
6. Lazowska, J., Jacq, C. and Slonimski, P.P. (1980) Cell 22, 333-348.
7. Kreike, J., Bechman, H., Van Hemert, F.J., Schweyen, R.J., Boer, P.H., Kaudewitz, F. and Groot, G.S.P. (1979) Eur. J. Biochem. 101, 607-617.
8. Solioz, M. and Schatz, G., (1979) J. Biol. Chem. 254, 9331-9334.
9. Borst,P. and Grivell, L.A. (1981) Nature 289, 439-440.
10. Merten, S., Synenki, R.M., Locker, J., Christianson, T. and Rabinowitz, M. (1980) Proc. Natl. Acad. Sc. 77, 1417-1421.
11. Heyting, Ch. and Menke, H.H. Molec. gen. Genet. (1979) 168, 279-291
12. Tabak, H.F., Hecht, N.B., Menke, H.H. and Hollenberg, C.P. (1979) Curr. Gen. 1, 33-43.
13. Sanders, J.P.M., Heyting, Ch. Verbeet, M.Ph., Meijlink, F.C.P.W. and Borst, P. (1977) Mol. Gen. Gen. 157, 239-261.

14. Moorman,A.F.M., Grivell, L.A., Lamie, F. and Smits, H.L. (1978) Biochim. Biophys. Acta 518, 351-365.
15. Grivell, L.A., Reijnders, L. and Borst, P., (1971) Biochim. Biophys. Acta, 247, 91-103.
16. De Rooy, J.F.M., Wille-Hazeleger, G., Van Deursen, P.H., Serdijn, J. and Van Boom, J.H. (1979) Recl. Trav. Chim. (Pays Bas) 98, 537-548.
17. Seeburg, P.H., Shine, J., Martial, J.A., Ullrich, A. Baxter, J.D. and Goodman, H.M. (1977) Cell 12, 157-165.
18. Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg., P. (1977) J. Mol. Biol. 113, 237-251.
19. Alwine,J.C., Kemp, D.J. and Stark, G.R., (1977) Proc.Natl.Acad.Sc. 74, 5350-5354.
20. Van Ommen, G.J.B., Boer, P.H., Groot, G.S.P., De Haan, M., Roosendaal, E. and Grivell, L.A. (1980) Cell 20, 173-183.
21. Berk, A.J. and Sharp, P.A. (1977) Cell 12, 731-732.
22. Maxam,A.M. and Gilbert, W., (1977) Proc. Natl. Acad. Sc. 74, 560-564.
23. Wallace, R.B., Shaffer, J., Murphy, R.F., Bonner, J., Hirose, T. and Itakura, K. (1979) Nucl.Acid Res. 6, 3543-3557.
24. Hoeymakers, J.H.J., Borst, P., Van den Burg, J., Weissmann, C. and Cross, G.A.M. (1980) Gene 8, 391-417.
25. Shenk, T.E., Rhodes, C. Rigby, P.W.J. and Berg. P. (1975) Proc. Natl. Acad. Sc. U.S. 72, 989-993.