Nucleotide substitutions in a yeast mitochondria *cis*-acting mutant located in the last intron of the apocytochrome *b* gene

C.A. Bonjardim and F.G. Nóbrega*

Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, CP 20780, 01498 São Paulo SP, Brasil

Received 28 December 1983; revised version received 22 February 1984

The region of mitochondrial DNA corresponding to the intron mutant M6-200 in *Saccharomyces cerevisiae* D273-10B has been isolated, and the nucleotide sequence of a 519 bp *Rsa*I fragment has been determined. Three nucleotide substitutions were found at nucleotides +2650 (G⟶T), +2668 (G⟶A) and +2798 (A⟶G), all within the genetically defined location in the gene. Particular significance can be attributed to the first two changes (+2650 and +2668), that can be genetically isolated from the third substitution and, in addition, alter conserved sequence features detected in a study [(1982) Biochimie 64, 867–881] of fungal mitochondrial introns.

Mitochondrial intron mutant *DNA sequence* *Apocytochrome* b *gene* Saccharomyces cerevisiae

1. INTRODUCTION

The gene for mitochondrial apocytochrome *b* in *Saccharomyces cerevisiae* has a mosaic structure [1] and some of its introns contain open reading frames and harbor mutants that are *trans*-recessive and code for a maturase [2,3] that is necessary for the correct splicing of the message. The other class of introns usually contain no open reading frames and do not depend upon mitochondrial protein synthesis for its excision. Mutations that map in these introns are *cis*-dominant and are probably unable to provide the splicing enzymes with the correct RNA substrate [3,4]. To understand more fully the molecular basis of the *cis*-acting mutations we determined the nucleotide substitutions in M6-200/A1 [5] a COB region mit$^-$ that maps inside the last intron of the 'short' apocytochrome *b* gene [6] by sequencing the genetically defined region cloned and amplified in PB 347, a derived ϱ^- mutant. A preliminary communication of this work has been presented [7].

* To whom correspondence should be addressed

2. MATERIALS AND METHODS

The PB347 clone used here was isolated from the mit$^-$ M6-200/A1 (α, auxotrophic, ϱ^+, ω^+, mit$^-$) [8] by mutagenesis with ethidium bromide [9]. This ϱ^- was genetically characterized by crosses with testers from the cytochrome *b* region [10], *oxi3* gene (a M10-150/4D) [11], tRNA^{Asp} gene (a M7-37/70 C) [12] and a COB region ϱ^-, derived from an isomitochondrial yeast strain that is able to restore respiratory competence in all cytochrome *b* mit$^-$ mutants utilized [10]. Genetic procedures used are described in [13].

Yeast mitochondria were purified from protoplasts as in [14]. The mitochondria were lysed in buffered 2% sarkosyl, phenol extracted and the DNA purified on CsCl–ethidium bromide gradients [15].

Restriction endonucleases were obtained from Bethesda Research Laboratories, MD. *Rsa*I was prepared as in [16]. DNA restriction fragments were purified from 5% polyacrylamide gels, treated with bacterial alkaline phosphatase (Worthington Biochemicals, NY) and labeled at the

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

5'-ends with bacteriophage T4 polynucleotide kinase (PL Biochemicals, WI) and [γ-^{32}P]ATP (>3000 Ci/mmol). The labeled ATP was prepared under a FINEP contract at Dr J.C.C. Maia's laboratory (IQUSP – São Paulo) as in [17] using carrier-free $^{32}P_i$ obtained from CNEN-São Paulo.

The procedures for manipulation and labeling of the DNA fragments, strand separation, elution of bands, chemical derivatization reactions and thin gel high-voltage sequencing follow the detailed protocols in [18].

3. RESULTS

3.1. *Characterization of PB 347*

This ϱ^- was selected among 104 clones derived from M6-200/A1 after treatment with ethidium bromide. These clones were cytoplasmic petites that spanned the mitochondrial apocytochrome *b* (COB) region in *S. cerevisiae* D273-10B as shown by their failure to be restored to respiratory competence after crossing to a COB region ϱ^-, an *oxi*3 mit$^-$ tester (aM10-150/4D) and a tRNA^{Asp} syn$^-$ tester (aM7-37/70C). Nevertheless these clones gave colonies able to grow in non-fermentable substrates after crossing to all mit$^-$ COB testers except M6-200 indicating that they carried this mitochondrial mutation. Twenty clones were picked at random, re-purified and tested for stability of marker retention. Mitochondrial DNA was purified from 9 of the most stable ϱ^- (>90%) and

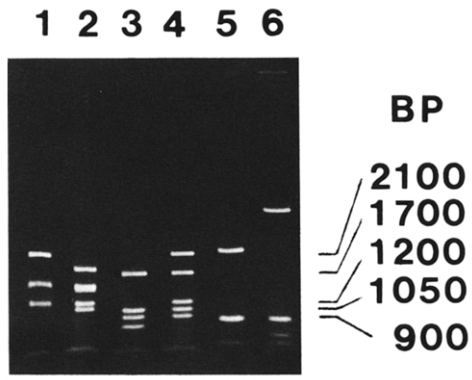

Fig.1. Restriction digests of PB347 (lanes 1,3,5) and DS400/A12 (lanes 2,4,6) mitochondrial DNAs electrophoresed on a 0.9% agarose gel [10]. (1,2) *Hin*fI, (3,4) *Mbo*I, (5,6) *Rsa*I. The size of DS400/A12 *Mbo*I fragments (lane 4) is indicated in base pairs [6].

the complexity and size of the retained DNA segment were analysed by restriction endonuclease cleavage and agarose gel electrophoresis. The chosen clone (PB 347) has a mitochondrial genome consisting of a circular DNA with a repeat unit length of 5.1 kbp and in fig.1 its restriction fragments are compared with the well characterized DS400/A12 ϱ^- [10]. The genetic and physical mapping of PB 347 located the unique sequence retained in this clone between 69.4 and 76.7 map units as shown in fig.2.

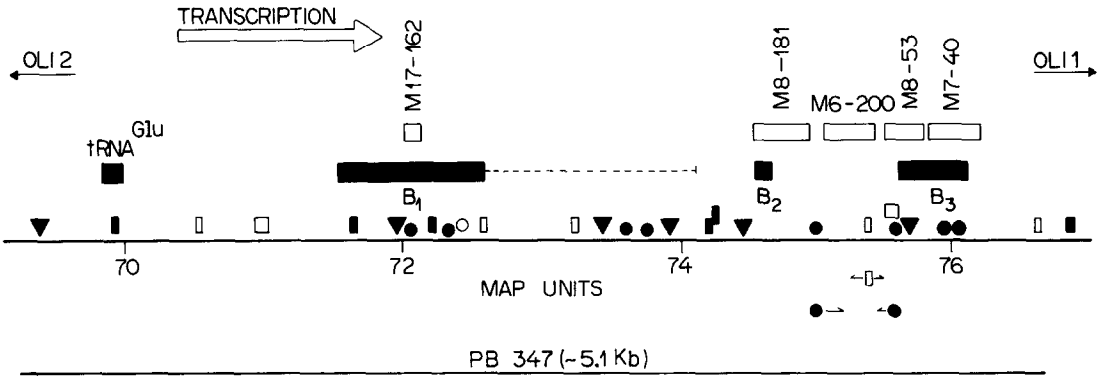

Fig.2. Restriction map and location of PB347 mitochondrial DNA relative to the COB region genetic markers. B1, B2 and B3: exon regions of the apocytochrome *b* gene. The approximate limits of the mit$^-$ mutations are indicated by the open boxes [6]. The arrows show the direction and approximate distances sequenced from the restriction sites indicated. The interrupted line indicates the open reading frame in the first intron. Restriction sites: *Mbo*I (▼), *Eco*RI (○), *Hae*III (□), *Hin*cII (□), *Hin*fI (■), *Rsa*I (●).

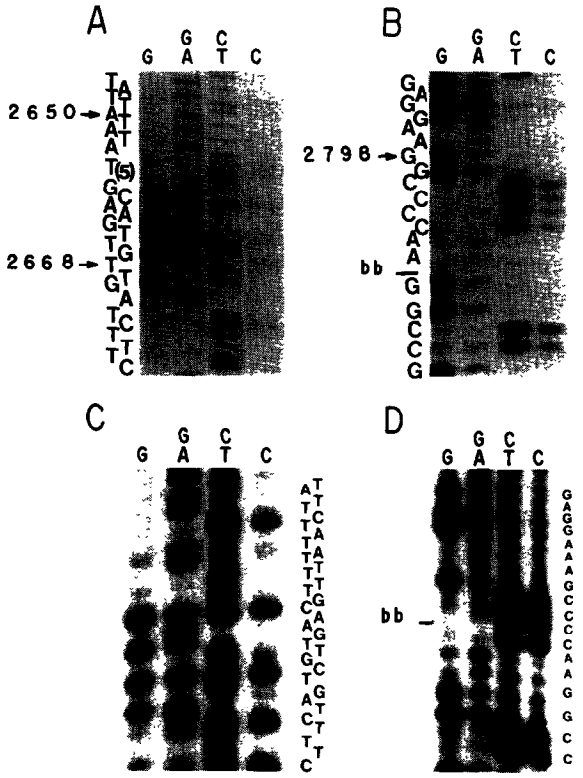

Fig.3. Nucleotide substitutions in the M6-200 intron mutant. The figure shows autoradiographs of the 5′-end-labeled DNA fragments subjected to chemical degradation as in [18]. The products have been resolved on 10% polyacrylamide gels, the location of the substitutions being indicated as in [6]; bb, location of the bromophenol blue marker dye. Sequence of the mutant 519 bp *Rsa*I fragment labeled at the *Hae*III site: (A) Upstream sequence. The mutational alterations shown (C⟶A and C⟶T) correspond to a G⟶T transversion and a G⟶A transition in the non-transcribed strand. (B) Downstream sequence. The mutation is an A⟶G transition in the non-transcribed strand. Corresponding sequences of the wild-type mitochondrial DNA: (C) Upstream sequence from the labeled *Hpa*II site. The mitochondrial DNA was obtained from the ϱ^- DS400/A12 [6]. (D) Downstream sequence from the labeled *Hae*III site. The mitochondrial DNA was purified from the ϱ^- DS400/M4 [6].

3.2. *DNA sequencing of the mutant*

Previous work established the entire nucleotide sequence of 7.6 kbp of wild-type mitochondrial DNA retained in the petite clone DS400/A12 derived from *S. cerevisiae* D273-10B. This unique sequence contains a glutamyl tRNA gene and the 3 exons of the apocytochrome *b* gene (fig.2). It allowed a more precise mapping of the COB mutants [6,10,19], M6-200 being located in the last intron between positions +2539 and +2800 [6]. Analysis of restriction digests with the purpose of purifying the DNA fragments that spanned the above region failed to reveal the *Rsa*I site that could be predicted by the published sequence [6] starting at nucleotide +2769. This prompted a revision of the sequence and it has been determined that there is indeed no *Rsa*I site and the unique *Hae*III site nearby is flanked by two *Hpa*II sites [20]. To sequence the region where the M6-200 mutation was previously [6] mapped the 519 bp *Rsa*I fragment (nucleotide +2446 to +2964) from a digest of PB 347 mitochondrial DNA was electrophoretically purified in a 5% polyacrylamide gel. The eluted DNA was digested with *Hae*III and the two resulting fragments (332 bp and 187 bp) were individually isolated, labeled and sequenced.

The large fragment gels were readable up to 180 nucleotides (both strands) and for the small fragment reading was sharp up to 160 nucleotides providing ample overlap. Three nucleotide substitutions were found at nucleotides +2650 (G⟶T), +2668 (G⟶A) and +2798 (A⟶G) as shown in fig.3. The substitutions at +2650 and +2668 (fig.3A) were also confirmed by sequencing the non-transcribed strand labeled at the *Rsa*I site (not shown).

4. DISCUSSION

The mit$^-$ mutant M6-200 is located in the last intron of the short apocytochrome *b* gene [6]. It was induced with manganese and no revertants could be obtained [11]. M6-200 lacks the characteristic apocytochrome *b* polypeptide on SDS-polyacrylamide gels of mitochondrial translation products although a shorter polypeptide (~22 kDa) was detected [3,5]. The splicing defect present in M6-200 specifically blocks the removal of the last intron although it does not prevent the splicing of the first intron [4]. A phenotypic similar defect is caused by a nuclear mutation (cbp 2), and the corresponding gene was recently cloned by complementation [21]. The multiple substitutions found in M6-200 are consistent with the mutant stability and have been found by others [2] in some manga-

nese-induced mit$^-$. It is unlikely that any of the mutations found was introduced by the ethidium bromide used to induce the ϱ^- clone of this mutant. Previous extensive sequencing of primary and secondary petite clones from the COB region did not reveal the concomitant appearance of point mutations [6]. The nucleotide substitutions found (fig.4) fall well inside the region covered by the complementing ϱ^- DS400/N31 [6]. One of them (A⟶G at +2798) can be abolished by recombination with ϱ^- DS400/M11 that spans part of the second intron and the last exon (+2675 to +6125) of the apocytochrome *b* gene [6]. Nevertheless DS400/M11 does not restore respiratory competence in M6-200 [6] pointing to a significant role for the other two substitutions in causing the mutant phenotype. It is worth mentioning that this mutation (+2798) modifies a 14 base sequence that has an inversely homologous counterpart located 98 nucleotides from the second exon (B2), introducing a mismatch in a possible secondary structure of the apocytochrome *b* transcript that could be important as a recognition site for the splicing enzymes as suggested in [6]. The next upstream substitution (G⟶A, +2668) affects the G residue in the consensus sequence TCANNGACTA found in [22] to be conserved among introns of fungi. This region is homologous to the R sequence element described in [23] and, as suggested by these authors, able to pair with the complementary S sequences located further downstream in mitochondrial introns (fig.4B). The last substitution found (G⟶T, +2650) would abolish the G-C base pair that closes a conserved secondary structure stem common to the apocytochrome *b* intron and the large 21 S rRNA intron of ω^+ strains [22] (fig.4A). The importance of the G residue at the consensus sequence is suggested by its change to a T in mutants that map at the fourth intron of the long COB gene (box 9-M281) [24] and at the

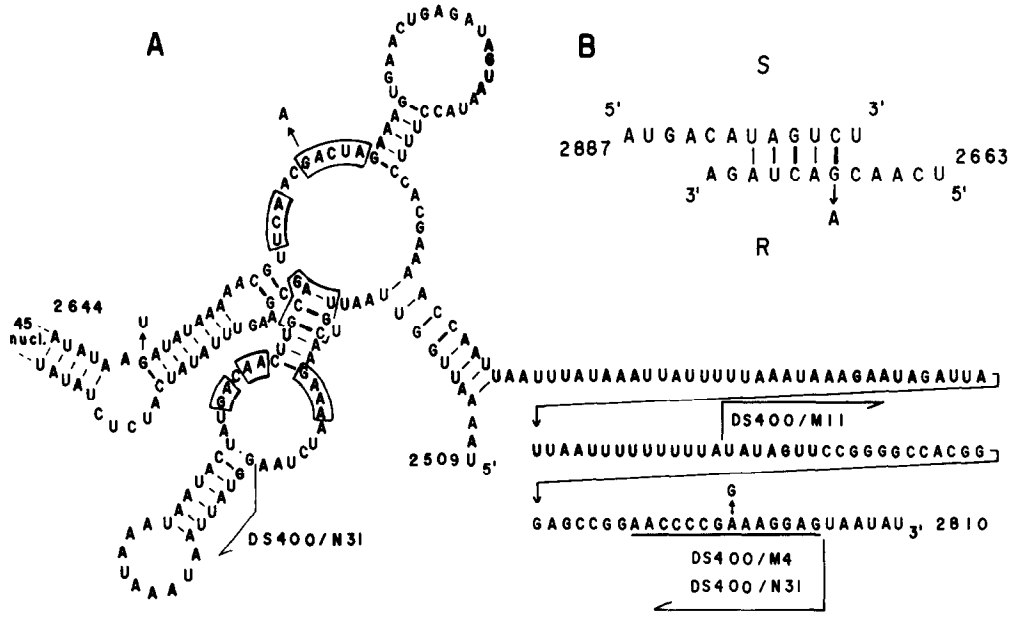

Fig.4. Schematic representation of the nucleotide sequence and secondary structure features of the transcript in the last intron of the 'short' COB gene indicating the changes found in M6-200. (A) Part of the secondary structure of the RNA transcribed from the last COB intron as proposed in [22] with the substitutions found in M6-200. Boxed stretches are common to most or all introns of group I [22]. The limits of the mitochondrial genomes of some ϱ^- mutants relevant to this work (DS400/M11, DS400/M4 and DS400/N31) have been marked. The continuous line indicates the 14 nucleotide sequence that has an inverted repeat counterpart at the beginning of this intron. The intron spans 738 nucleotides from position +2225 to +2962 [6,20]. (B) Possible pairing between sequence elements homologous to the R and S regions described in [23] and transcribed from the last COB intron. The arrow indicates the situation in M6-200. Nucleotide numbering follows [6,20].

homologous fourth intron of the *oxi3* mitochondrial gene (G192) [25]. It is changed to an A in mutant V353 also at the box 9 locus in the long apocytochrome *b* gene [26]. These 3 mutants are single base substitutions and revert with a frequency of 10^{-7} being located at the open reading frame in the corresponding introns. Authors in [27] suggested that the consensus sequence is possibly involved in the splicing of the many introns where it was found: namely, mitochondrial genes of fungi [10,24,25,28–30] or plants [31] and also in nuclear rRNA introns present in *T. thermophila* [32,33] and *P. polycephalum* [34]. The role of the individual mutations in M6-200 remains a question open to debate. The genetic data allow the exclusion of one mutation (+2798) alone as functionally nonessential to the maintenance of the changed phenotype in the presence of the other two (+2650 and +2668) substitutions. The mutation at +2668, being located at a crucial G residue in the consensus sequence [22–27], is probably sufficient to block intron processing by itself. The mutated sites mapped in M6-200, a *cis*-acting mutant that does not contain an open reading frame, bring additional evidence to support the concept that sequence determinants located in introns are important in transcript processing.

ACKNOWLEDGEMENTS

We thank Dr Alexander Tzagoloff for the yeast strains used. F.F. Dowbor and J. Lino provided expert technical assistance. This work was supported by grants from FAPESP, CNPq and FINEP. C.A.B. is recipient of a CNPq graduate studentship.

REFERENCES

[1] Borst, P. and Grivell, L.A. (1980) Nature 289, 439–440.
[2] Lazowska, J., Jacq, C. and Slonimski, P.P. (1981) Cell 22, 333–348.
[3] Lamb, M.R., Anziano, P.Q., Glaus, K.R., Hauson, D.K., Klapper, H.J., Perlman, P.S. and Mahler, H.R. (1983) J. Biol. Chem. 258, 1991–1999.
[4] Bonitz, S.G., Homison, G., Thalenfeld, B.E., Tzagoloff, A. and Nóbrega, F.G. (1982) J. Biol. Chem. 257, 6268–6274.
[5] Slonimski, P.P. and Tzagoloff, A. (1976) Eur. J. Biochem. 61, 27–41.
[6] Nóbrega, F.G. and Tzagoloff, A. (1980) J. Biol. Chem. 255, 9828–9837.
[7] Bonjardim, C.A. and Nóbrega, F.G. (1983) Arq. Biol. Tecnol. 26, 162.
[8] Tzagoloff, A., Foury, F. and Akai, A. (1976) Mol. Gen. Genet. 149, 33–42.
[9] Macino, G. and Tzagoloff, A. (1979) Proc. Natl. Acad. Sci. USA 76, 131–135.
[10] Nóbrega, F.G. and Tzagoloff, A. (1980) J. Biol. Chem. 255, 9821–9827.
[11] Tzagoloff, A., Akai, A., Needleman, R.B. and Zulch, G. (1975) J. Biol. Chem. 250, 8236–8242.
[12] Trembath, M.K., Macino, G. and Tzagoloff, A. (1977) Mol. Gen. Genet. 158, 35–45.
[13] Coruzzi, G., Trembath, M.K. and Tzagoloff, A. (1979) Methods Enzymol. 50, 95–106.
[14] Faye, G., Kujawa, C. and Fukuhara, H. (1974) J. Mol. Biol. 88, 185–203.
[15] Sanders, J.P.M., Borst, P. and Weijers, P.J. (1975) Mol. Gen. Genet. 143, 53–64.
[16] Lynn, S.P., Cohen, L.K., Kaplan, S. and Gardner, J.F. (1980) J. Bacteriol. 142, 380–383.
[17] Walseth, T.F. and Johnson, R.A. (1979) Biochim. Biophys. Acta 562, 11–31.
[18] Maxam, A.M. and Gilbert, W. (1980) Methods Enzymol. 65, I, 499–560.
[19] Nóbrega, F.G. and Tzagoloff, A. (1980) FEBS Lett. 113, 52–54.
[20] Bonjardim, C.A. and Nóbrega, F.G. (1983) submitted.
[21] McGraw, P. and Tzagoloff, A. (1983) J. Biol. Chem. 258, 9459–9468.
[22] Michel, F., Jacquier, A. and Dujon, B. (1982) Biochimie 64, 867–881.
[23] Wayne Davies, R., Waring, R.B., Ray, J.A., Brown, T.A. and Scazzochio, C. (1982) Nature 300, 719–724.
[24] Weiss-Brummer, B., Rödel, G., Schweyen, R.J. and Kaudewitz, F. (1982) Cell 29, 527–536.
[25] Netter, P., Jacq, C., Carignani, G. and Slonimski, P.P. (1982) Cell 28, 733–738.
[26] De La Salle, H., Jacq, C. and Slonimski, P.P. (1982) Cell 28, 721–732.
[27] Burke, J.M. and RajBhandary, V.L. (1982) Cell 31, 509–520.
[28] Dujon, B. (1980) Cell 20, 185–197.
[29] Weiss-Brummer, B., Holl, J., Schweyen, R.J., Rödel, G. and Kaudewitz, F. (1983) Cell 33, 195–202.

[30] Waring, R.B., Wayne Davies, R., Lee, S., Grisi, E., Berks, M.M. and Scazzochio, C. (1981) Cell 27, 4–11.
[31] Fox, T.D. and Leaver, C.J. (1981) Cell 26, 315–323.
[32] Kan, N.C. and Gall, J. (1982) Nucleic Acids Res. 10, 2809–2822.
[33] Cech, T.R., Kyle Tanner, N., Tinoco, I., Weir, B.R., Zuker, M. and Perlman, P.S. (1983) Proc. Natl. Acad. Sci. USA 80, 3903–3907.
[34] Nomiyama, H., Sasaki, Y. and Takagi, Y. (1981) Proc. Natl. Acad. Sci. USA 78, 1376–1380.