DNA sequence analysis of diuron-resistant mutations in the mitochondrial cytochrome *b* gene of *Saccharomyces cerevisiae*

J.-P. di Rago, X. Perea[+] and A.-M. Colson

University of Louvain, Laboratoire de Genetique Microbienne, Place Croix du Sud, 4, 1348 Louvain-la-Neuve, Belgium and *[+]Centre de Genetique Moleculaire du CNRS, Gif-sur-Yvette, France*

Received 10 September 1986

Diuron (3-[3,4-dichlorophenyl]-1,1-dimethylurea), an inhibitor of mitochondrial respiration, blocks the yeast respiratory chain between cytochrome *b* and c_1. Diuron-resistant mutants of *Saccharomyces cerevisiae* have been selected and several mutations localized to the mitochondrial cytochrome *b* gene. The present paper identifies specific DNA base changes within the cytochrome *b* gene conferring diuron-resistance. DNA sequence analysis was done utilizing primer extension of crude mitochondrial RNA preparations in the presence of reverse transcriptase. Five independent diuron-resistant mutations have been sequenced.

(Saccharomyces cerevisiae) *Diuron resistance* *Mitochondrial cytochrome* b *split gene* *DNA sequence analysis* *Primer extension* *Mutational alteration*

1. INTRODUCTION

Diuron (3-[3,4-dichlorophenyl]-1,1-dimethylurea) is an inhibitor of the yeast respiratory chain which blocks the electron flow between cytochromes *b* and c_1 [1]. Several diuron-resistant mutants of *Saccharomyces cerevisiae* have been selected on glycerol in the presence of inhibitory concentrations of diuron [2]. The mutations were found to be of mitochondrial heredity and were located at two loci of the apocytochrome *b* gene. The *diu1* locus was found to belong to exon 4 and the *diu2* locus has been attributed to exon 1 of the long cytochrome *b* gene [3,4]. The cytochrome *b* gene carries other drug-resistant loci (fig.1). In addition to *diu2*, exon 1 carries three loci *ana1*, *muc1* and *myx1*; exon 4 carries *ana2* and *fun1*; exon 5 carries *muc3* and exon 6 carries *muc2* and *myx2* (fig.1). The wild type sequence of the yeast cytochrome *b* gene is known [8] and a hydropathy plot of the deduced amino acid sequence has been used to construct a protein folding model [9]. The identification of specific DNA sequence changes within the cytochrome *b* gene codons of drug-resistant mutants has recently become practical. Thus, identified nucleotide changes responsible for resistant phenotypes can now be localized precisely within their respective exons and to a particular segment of the cytochrome *b* hydropathy plot. We describe, here, the determination of the exact DNA base changes within the cytochrome *b* gene of five independently isolated diuron-resistant mutants. The mutational alterations will be discussed in relation to their deduced amino acid change, to their particular position in the current protein folding model of the cytochrome *b* protein and to the conserved or unconserved nature of the implicated amino acid.

This paper will be reported in part in the 4th EBEC Reports

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

2. MATERIALS AND METHODS

2.1. *Strains*

KL14-4A, a *his1 trp2 oli1-1 cap1-321 par1-452*; IL125-2B, *his1 leu*; D273-10B/A21, *met oli1-625 ery3-624 par1-626*; KL14-4A/*diu2-726*; KL14-4A/*diu2-728*; KL14-4A/*diu2-766*; IL125-2B/*diu1-721*; D273-10B/A21/*diu1-724*. All diuron-resistant mutations but one, *diu2-766* (from Colson, unpublished), have been mapped in [3].

2.2. *Sequence analyses*

The experimental procedure was as described in [10]. Primers of about 20 nucleotides were synthesized. The choice of the primers (P1–P5) depended on the position of the mutations in the gene (fig.1). The location of each primer has to be downstream from the mutated sites. Since diuron-resistant mutations are mapped at two loci (*diu1* and *diu2*) and belong to the first and the fourth exon of the cytochrome *b* split gene, several primers were needed. For exon 1, three primers (P1–P3) were used whereas two primers (P4 and P5) were utilised for exon 4.

3. RESULTS AND DISCUSSION

Several mutational alterations have been detected (table 1). In exon 1, mutation *diu2-766* induces a transversion from A to T which will change

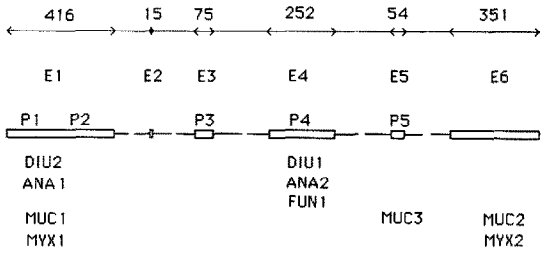

Fig.1. Drug-resistant loci of the cytochrome *b* split gene. The exons are numbered from E1 to E6. The length of each exon is given in base pairs. The primers utilized for the sequencing experiments are numbered from P1 to P5. The position of the primers is given on the basis of their first and last bp numbers on the cytochrome *b* split gene. P1, 189–208; P2, 279–298; P3, 2716–2734; P4, 4554–4573; P5, 6118–6140. DIU, diuron-resistance [3]; ANA, antimycin-resistance [5]; FUN, funiculosin-resistance [5]; MUC, mucidin-resistance [3,6]; MYX, myxothiazol-resistance [7].

Table 1

Mutational alterations in the cytochrome *b* gene of diuron-resistant mutants

Mutation	Exon	Codon change	Amino acid	
			Change	Number
diu2-766	E1	AUU UUU	Ile Phe	17
diu2-726	E1	AAU AAA	Asn Lys	31
diu2-728	E1	AAU AAA	Asn Lys	31
diu1-721	E4	UUU UCU	Phe Ser	225
diu1-724	E4	UUU UCU	Phe Ser	225

The mutations have been mapped in the cytochrome *b* split gene. The position of the diuron-resistant loci and the exons limits are given in fig.1. The position of the deduced amino acid change is presented in fig.2

codon AUU into UUU resulting in the replacement of amino acid no.17 isoleucine by a phenylalanine. In the same exon, two independent mutations *diu2-726* and *diu2-728* carry the same transversion from T to A which changes codon AAU into AAA leading to the replacement of amino acid no.31 asparagine by lysine. In exon 4, two other independent mutations *diu1-721* and *diu1-724* were found to carry a transition from T to C altering codon UUU into UCU, thus changing amino acid no.225 from phenylalanine to serine.

Two observations favor the fact that the detected alterations are indeed those conferring diuron-resistance: first, the existence of independent mutants which carry the same nucleotide change; second, the absence of polymorphisms among the tested strains and the published sequence of the cytochrome *b* gene.

The position of each mutated amino acid has been indicated on a protein folding model of cytochrome *b* adapted from [9] (fig.2). On this basis, all mutations appeared to be located in hydrophilic segments of the protein and were located on either side of the membrane. The 32 kDa protein of chloroplast photosystem II is known to bind diuron and mutations resulting in single amino acid changes within this protein have also been shown to confer diuron-resistance [11]. In contrast to the position of the altered amino acids of the yeast cytochrome *b* diuron-resistant mutants, the changed amino acids of the 32 kDa protein seem to be located in a lipophilic segment

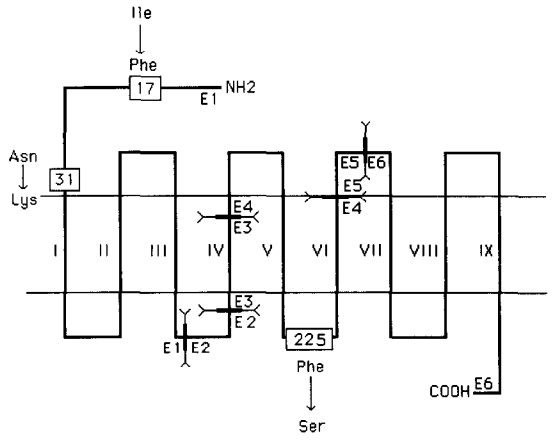

Fig.2. Altered amino acids in the cytochrome *b* polypeptide of diuron-resistant mutants. Mutation *diu2-766* was found to alter amino acid no.17. Mutations *diu2-726* and *diu2-728* were found to affect the amino acid no.31. Mutations *diu1-721* and *diu1-724* were found to alter amino acid no.225. The exons are numbered E1–E6. The exon limits are indicated by ⟩—⟨.

of the protein. It should be pointed out however, that two of the three altered nucleotides in the 32 kDa protein were found to be located closer to either one or other side of the membrane. This situation is similar to that found with diuron-resistant mutations in yeast cytochrome *b*. These observations might suggest that diuron could act at two specific sites at either side of the membrane.

Diuron-resistance results when amino acids with more hydrophilic side chains are exchanged for the amino acids at positions 31 and 225, polar to positively charged and non-polar to polar, respectively. Steric effects may lead to diuron-resistance at positions 17 and 225 since resulting mutations change an aromatic phenylalanine into a non-aromatic amino acid serine residue (position 225) and an isoleucine into phenylalanine (position 17).

The mutated amino acid asparagine no.31 is conserved in cytochrome *b* isolated from a variety of species but is not conserved in chloroplast cytochrome b_6 which is known to be insensitive to diuron. The 17 kDa (subunit 4) protein of the chloroplast b_6f complex is homologous to the second half of the mitochondrial cytochrome *b* polypeptide [9]. The mutated amino acid phenylalanine no.225 is found at the appropriate position within the 17 kDa protein and is also conserved in cytochrome *b* isolated from *Aspergillus nidulans*. This amino acid is not conserved in mammalian (bovine, human, mouse) cytochrome *b* but it is replaced by an aromatic tyrosine residue in these species. The amino acid isoleucine no.17 is conserved in *A. nidulans* and in the cytochrome b_6 of the spinach chloroplast. Surprisingly, it is already changed into a phenylalanine in mammalian cytochrome *b* which apparently are naturally resistant to diuron. Finally, in yeast this mutated amino acid of exon 1 is in the third position of a short amino acid sequence Ser-Tyr-Ile-Ile. This sequence also surrounds the mutated amino acid no.225 in exon 4: Ser-Tyr-Phe-Ile. This sequence is found at two positions in yeast (17 and 225) and around amino acid no.17 in both yeast and *A. nidulans*.

ACKNOWLEDGEMENTS

A.-M.C. is a Research Associate to the Fonds National de la Recherche Scientifique, Belgium and J.P.d.R. has an IRSIA fellowship, Belgium.

REFERENCES

[1] Convent, B. and Briquet, M. (1978) Eur. J. Biochem. 82, 473–481.
[2] Colson, A.-M., Luu The Van, Convent, B., Briquet, M. and Goffeau, A. (1977) Eur. J. Biochem. 74, 521–526.
[3] Colson, A.-M. and Slonimski, P.P. (1979) Mol. Gen. Genet. 167, 287–298.
[4] Lazowska, J., Jacq, C. and Slonimski, P.P. (1980) Cell 22, 333–348.
[5] Colson, A.-M., Michaelis, G., Pratje, E. and Slonimski, P.P. (1979) Mol. Gen. Genet. 167, 287–298.
[6] Takacsova, G., Subik, J. and Kotylak, Z. (1981) Mol. Gen. Genet. 179, 141–146.
[7] Thierbach, G. and Michaelis, G. (1982) Mol. Gen. Genet. 186, 501–506.
[8] Nobrega, F.G. and Tzagoloff, A. (1980) J. Biol. Chem. 255, 9828–9837.
[9] Widger, W.R., Cramer, W.A., Herrman, R.G. and Trebst, A. (1984) Proc. Natl. Acad. Sci. USA 81, 674–678.
[10] Perea, X. and Jacq, C. (1985) EMBO J. 4, 3281–3288.
[11] Erickson, J., Rahire, M., Rochaix, J.-D. and Mets, L. (1985) Science 228, 204–207.