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Structure, expression and regulation of a nuclear gene encoding a mitochondrial protein: the yeast L(+)-lactate cytochrome *c* oxidoreductase (cytochrome b_2)

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The yeast L(+)-lactate cytochrome *c* oxidoreductase or cytochrome b_2 is a component of the mitochondrial intermembrane space. The protein is encoded by the nuclear genome, synthesized as a larger precursor in the cytoplasmic compartment, and then proteolytically processed to its mature form during its import into the mitochondria. The structural gene for yeast cytochrome b_2 has been cloned. The complete nucleotide sequence of the gene with its 5′ and 3′ flanking regions was determined. The deduced primary structure of the cytochrome b_2 precursor reveals an unusually long amino terminal extension of 80 amino acids. A variety of potentially significant sequences were identified in the region flanking the structural portion of the gene. Transcript mapping with both S1 nuclease and primer extension methods reveals that the site of RNA synthesis is 56–66 bp downstream from a putative TATA box. By Northern blot analysis and gene disruption, it is shown that there is only a single copy of the cytochrome b_2 gene per haploid yeast nucleus. The cloned cytochrome b_2 gene was used to probe specific mRNA levels and demonstrate that cytochrome b_2 expression is transcriptionally repressed by glucose and induced by lactate. The inactivation of the chromosomal cytochrome b_2 gene by integrative transformation led to a deficiency in L(+)-lactate dehydrogenase activity and consequently to the inability to use L(+)-lactate as a sole source of carbon. This is the first reported mutation affecting the structural gene of cytochrome b_2.

Key words: cytochrome b_2 gene/nucleotide sequence/gene disruption/cytochrome b_2 gene expression/mitochondrial protein import

Introduction

Yeast L(+)-lactate cytochrome *c* oxidoreductase or cytochrome b_2 (EC 1.1.2.3) is a soluble protein of the intermembrane space of mitochondria which catalyses the transfer of electrons from L(+)-lactate to cytochrome *c* (Morton *et al.*, 1961; Hasegawa and Ogura, 1961; Labeyrie and Slonimski, 1964). This enzyme is a bifunctional tetrameric protein which carries two prosthetic groups; one heme and one flavin per subunit of 55 000 daltons (Jacq and Lederer, 1974).

Cytochrome b_2 biosynthesis is subject to three levels of regulation: (i) it is induced by oxygen during respiratory adaptation (Slonimski, 1953); (ii) it is repressed by glucose fermentation in aerobic conditions (Galzy and Slonimski, 1957); and (iii) it is specifically induced by lactate (Somlo, 1965). Five chromosomal genes CYP1–CYP5 involved in the regulation of the synthesis of cytochrome b_2 and iso1 and iso2 cytochromes c have been described by Clavilier *et al.* (1976). One of them, CYP1, is being studied by Verdière *et al.* (1985). Like most mitochondrial proteins, cytochrome b_2 is synthesized in the cytoplasm as a larger precursor before being directed to its specific submitochondrial destination, the intermembrane space (Gasser *et al.*, 1982).

I have isolated the complete cytochrome b_2 gene, and I report: (i) its entire nucleotide sequence. The deduced amino acid sequence gives new information on the primary structure of the mature protein, and identifies a long and complex pre-sequence of 80 residues. Several features of this pre-sequence and its role in directing cytochrome b_2 to its distinct mitochondrial compartment are discussed. The DNA sequences of the 5′ and 3′ flanking regions which reveal of number of interesting structural features are reported and discussed; (ii) transcript mapping experiments to identify the transcriptional start region; (iii) the analyis of the mRNA level under inducing and repressing conditions by using the cloned gene as a probe; (iv) the inactivation of the chromosomal gene by integrative transformation, thus creating the first known mutation affecting the expression of the cytochrome b_2 gene. The deficiency in L(+)-lactate cytochrome *c* oxidoreductase activity and the consequent inability to use L(+)-lactate as the sole source of carbon demonstrate that the cyto-

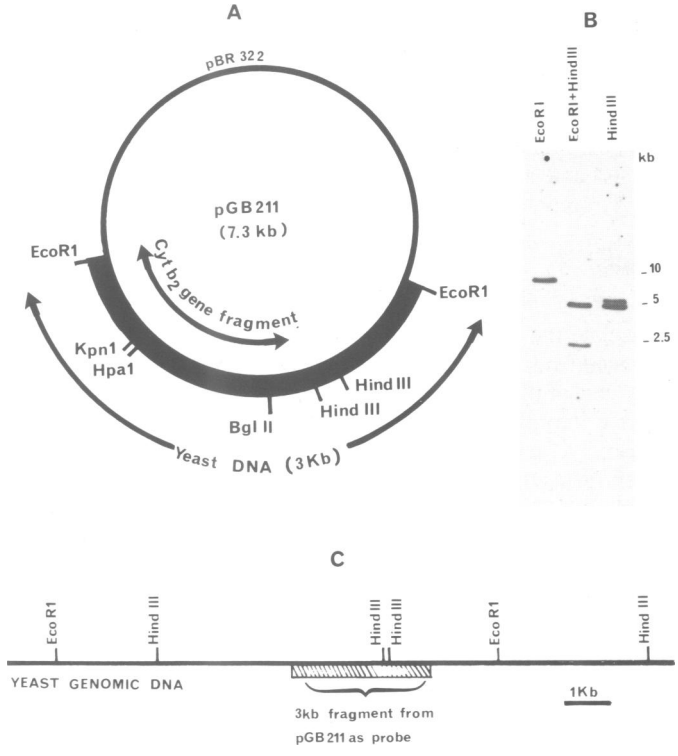

Fig. 1. (**A**) Schematic description of plasmid pGB211. (**B**) Southern blot analysis of nuclear DNA from D261. 5 μg of nuclear DNA were cleaved with the restriction enzymes indicated. The hybridization probe employed is the nick-translated *Eco*RI fragment from the recombinant plasmid pGB211. (**C**) Genomic environment of cytochrome b_2 gene.

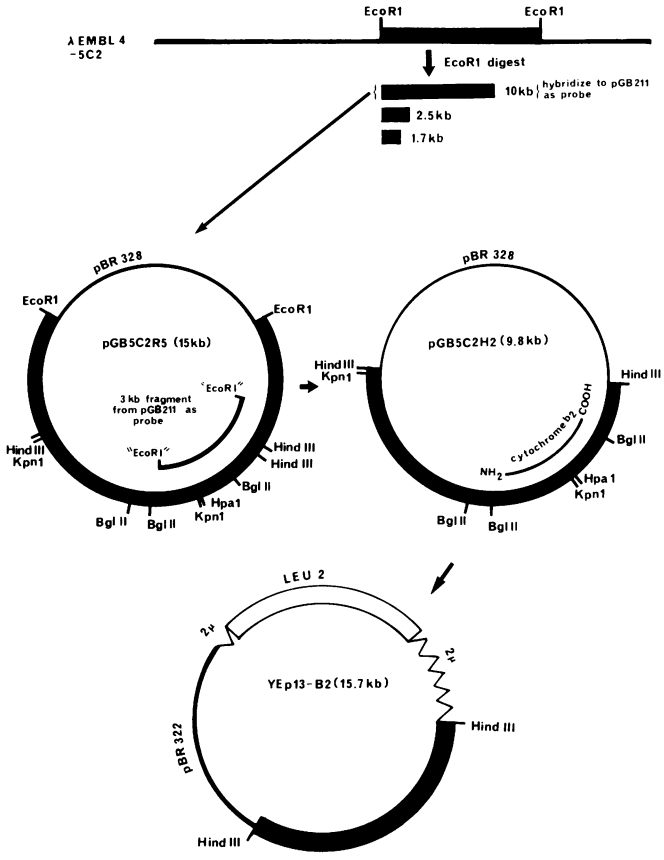

Fig. 2. Schematic description of λ EMBL4-5C2 recombinant carrying a 10-kb *Eco*RI yeast DNA fragment which contains the complete cytochrome b_2 gene. This 10-kb *Eco*RI fragment and a 5-kb *Hind*III sub-fragment were inserted into pBR328 to give the recombinant plasmids pGB5C2R5 and pGB5C2H2, respectively. The 5-kd *Hind*III fragment was inserted into the shuttle vector YEp13 to give the recombinant plasmid YEp13-B2.

chrome b_2 gene is present as a single copy and that a single protein is able to catalyse the oxidation of L(+)-lactate in *Saccharomyces cerevisiae*.

Results

Isolation of the gene encoding cytochrome b_2

To isolate the complete gene encoding yeast cytochrome b_2, I benefited from the characterization of a 3-kb DNA fragment carrying part of the gene (Guiard and Buhler, 1984). This fragment, inserted into pBR322 yielded the recombinant plasmid pGB211 (Figure 1A) which was used as a probe. Southern blot analysis of genomic DNA after digestion by *Eco*RI, *Hind*III and *Eco*RI + *Hind*III (Figure 1B) indicated that the gene encoding cytochrome b_2 exists as a single copy and is contained on a 10-kb *Eco*RI fragment (Figure 1C). This result is confirmed by the gene disruption experiment (cf. below).

A genomic library was constructed in the λ vector EMBL4 (see Materials and methods) and screened using nick-translated plasmid pGB211 as the probe. Starting with the recombinant phage EMBL4-5C2 (Figure 2), the 5-kb *Hind*III sub-fragment containing the entire cytochrome b_2 gene was subcloned into pBR328 (pGB5C2H2; Figure 2).

Nucleotide sequence analysis of the cytochrome b_2 gene

A restriction map of the 2.5-kb region containing the cytochrome b_2 structural gene and its 5′ and 3′ flanking regions is shown in Figure 3, together with the strategy used to determine its

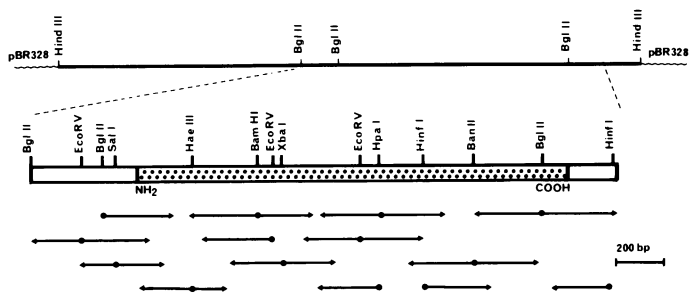

Fig. 3. Restriction map of the cytochrome b_2 region in plasmid pGB5C2H and strategy for sequencing the gene for cytochrome b_2 protein. The arrows indicate the length and the direction of sequence determined from 5′ end-labeled DNA fragments (closed circles). The position of the open reading frame is shown.

nucleotide sequence. The nucleotide sequence (2422 bp) thus obtained is shown in Figure 4. It reveals the presence of an open reading frame which could encode a protein of 591 amino acids. The deduced mol. wt. is 65 522. From the comparison with the published N-terminal amino acid sequence data (Guiard *et al.*, 1974, 1975), it is clear that the mature cytochrome b_2 starts at position 81. The translation initiation site for yeast cytochrome b_2 is probably the first methionine codon AUG, at nucleotide +1, downstream of the nonsense codon TAG at position −4. This means that the pre-sequence of the yeast cytochrome b_2 precursor contains 80 amino acids. The deduced mol. wt. of this pre-sequence (8953 daltons) agrees well with the earlier estimate of 10 000 daltons which was based on SDS-polyacrylamide gel electrophoretic analysis of immunoprecipitated precursor and mature yeast cytochrome b_2 (Gasser *et al.*, 1982). The present work establishes the complete sequence of the mature protein with the mol. wt. of 56 569 and 511 amino acid residues, and confirms the partial amino acid sequence presented previously (Guiard *et al.*, 1974; Ghrir *et al.*, 1984).

Mapping the cytochrome b_2 transcript

The open reading frame described above delineated the coding region of the pre-cytochrome b_2. To determine precisely the initiation position of the transcripts, the 5′ end of the cytochrome b_2 gene was mapped by measuring the RNA-dependent protection of radiolabeled DNA probes from S1 nuclease digestion (Figure 5A and B). To confirm these data, primer extension experiments were carried out as described in Figure 5C. These agree with results obtained from S1 mapping. The two procedures suggest slight heterogeneity of the 5′ terminus of cytochrome b_2 transcripts and place the location of the transcriptional start between positions −34 and −44 upstream of the ATG.

One-step disruption of the cytochrome b_2 gene

To eliminate the possibility of more than one gene coding for the cytochrome b_2 protein, and to analyse the phenotype of a strain without cytochrome b_2 activity, we have used the one-step disruption method described by Rothstein (1983). The strategy used is described in Figure 6. The mutant DBY-U4 thus obtained had become URA^+ and did not contain any pBR328 DNA. When genomic DNA was analysed by Southern blotting using the 1.8 kb *Bgl*II fragment from pGB5C2H2 as a probe, the 5-kb *Hind*III band of DBY746 was found to be replaced by a larger band of 6.2 kb corresponding to the size of the *Hind*III fragment of plasmid pGB5C2H2-U carrying the cytochrome b_2-URA3 region (Figure 7A). A control experiment performed with the 1.2-kb URA3 *Hind*III fragment as a probe confirmed this result. Therefore in the mutant DBY-U_4 the native cytochrome b_2 region

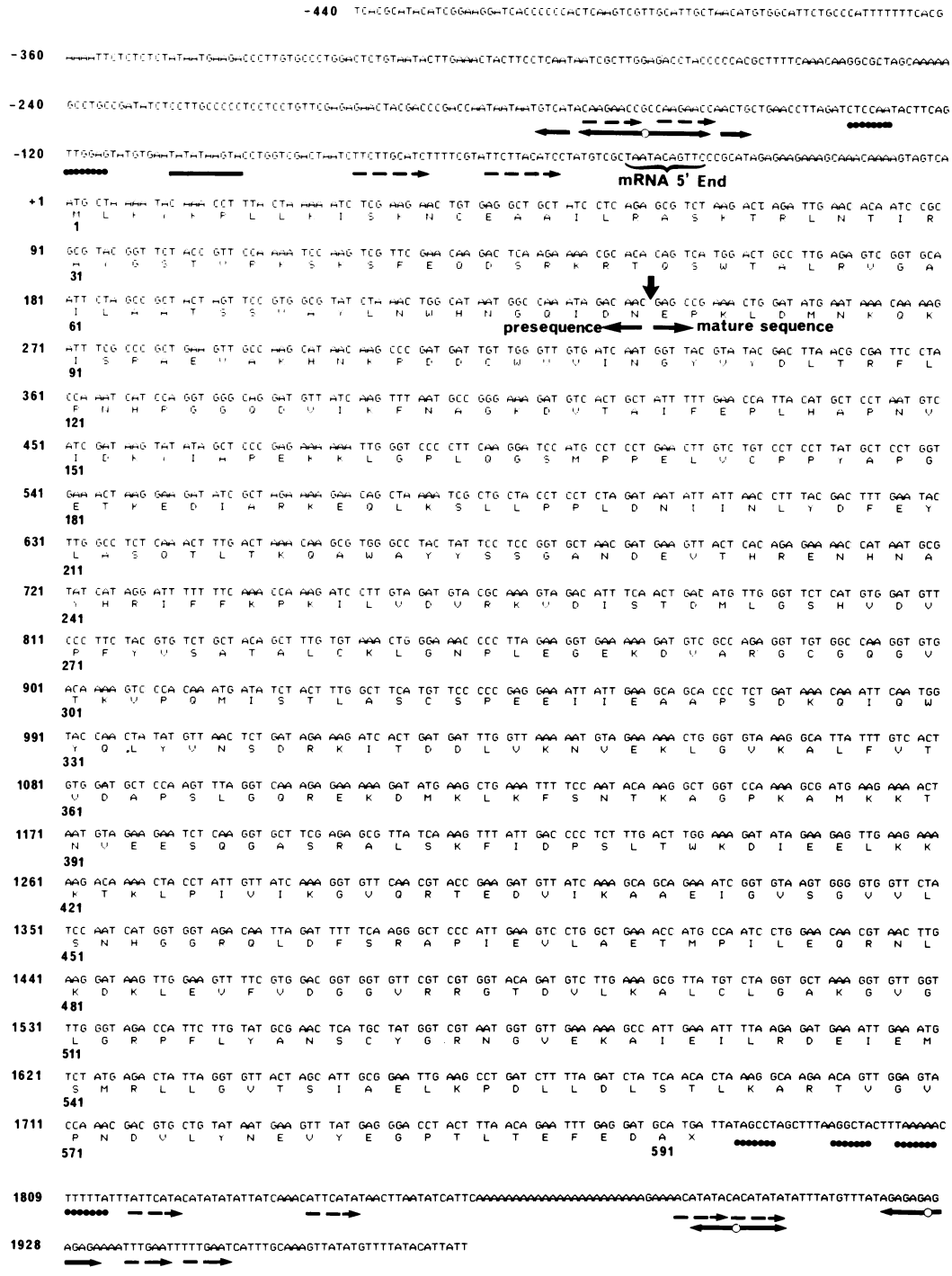

Fig. 4. Nucleotide sequence of the complete gene for cytochrome b_2 protein with its flanking regions. The 2422 bp sequence with the deduced amino acid sequence encoded by the 1773 bp open reading frame is shown. The putative 'TATA' box is indicated by underlining with a solid line. Inverted repeats are underlined with filled circles, direct repeat sequences with dotted arrows. Palindromic sequences are indicated by arrows joined at the point of symmetry. A brace indicates the extent of 5′ terminal heterogeneity of the mRNA species. All numbers are relative to the ATG at position +1.

was replaced by the plasmid-borne cytochrome b_2::URA3 region which must have integrated by homologous recombination. The plasmid-borne gene is interrupted at a position corresponding to amino acid 335, and the inserted 1.2-kb fragment carrying the URA3 gene has stop codons in all three reading frames. The mutant DBY-U4 should be able to make a short NH_2-terminal fragment of the cytochrome b_2 protein. However, the mutant lacks any protein recognized by different antibodies raised against the authentic cytochrome b_2 protein (Figure 7B-1), even when specifically directed against the heme binding domain at the N terminus of cytochrome b_2. We suppose, therefore, that the new protein is either not synthesized or too unstable. The mutant DBY-U4 is unable to use L(+)-lactate as its sole source of carbon (Figure 7C) and does not contain any L(+)-lactate dehydrogenase activity (DBY-746: 21.7 units, DBY-U4: <0.1 unit).

The transformation of the mutant DBY-U4 with the plasmid YEp13-B2 (Figure 2) results in the presence of immunodetectable cytochrome b_2 in the cells (Figure 7B-4). The same experiment performed with the wild-type strain increases the level of the protein 2- or 3-fold (Figure 7B-3). This means that the 5-kb

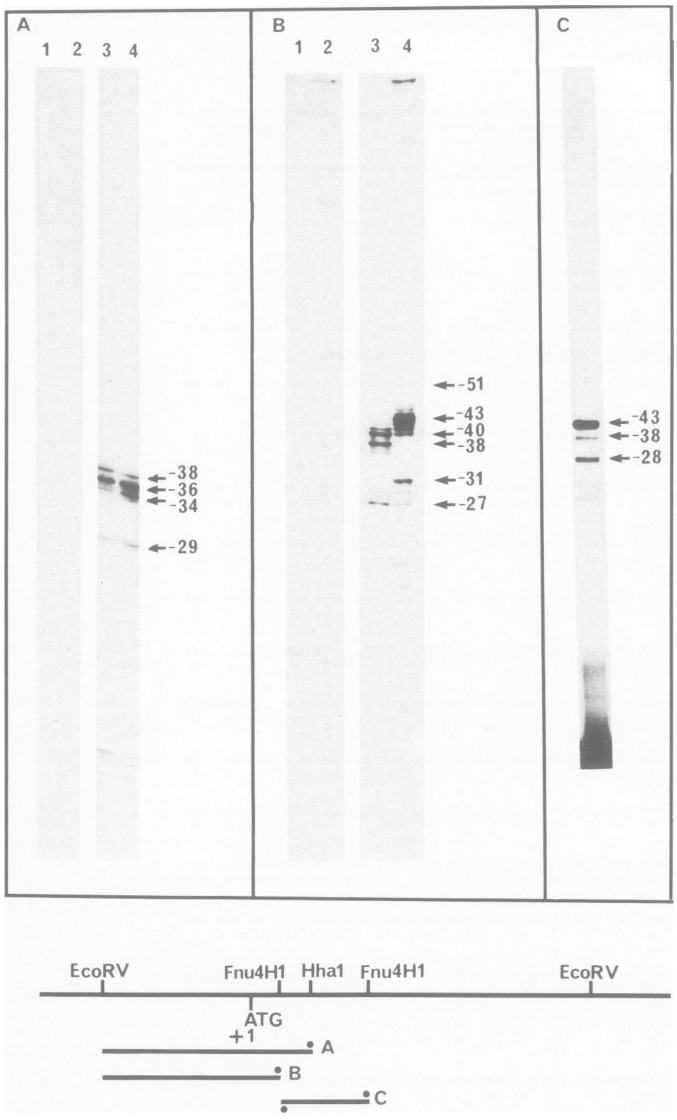

Fig. 5. Mapping of the 5′ ends of the cytochrome b_2 transcripts. S1 nuclease mapping of the 5′ end with: (**A**) The single strand 5′ end-labeled 321 bp *Hha-Eco*RV. (**B**) The single strand 5′ end-labeled 277 bp *Fnu*4h1-*Eco*RV. **Line 1**: no RNA, 150 U S1 nuclease; **line 2**: no RNA, 20 U S1 nuclease; **line 3**: RNA from lactate-grown yeast culture, 150 U S1 nuclease; **line 4**: RNA from lactate-grown yeast culture, 20 U S1 nuclease. (**C**) Primer extension mapping of the double-stranded 5′ end-labeled 141 bp *Fnu*4H1. Schematic representation of DNA fragments used as probes for S1 mapping and primer extension mapping. Closed circles give the position of the 5′ end labeled.

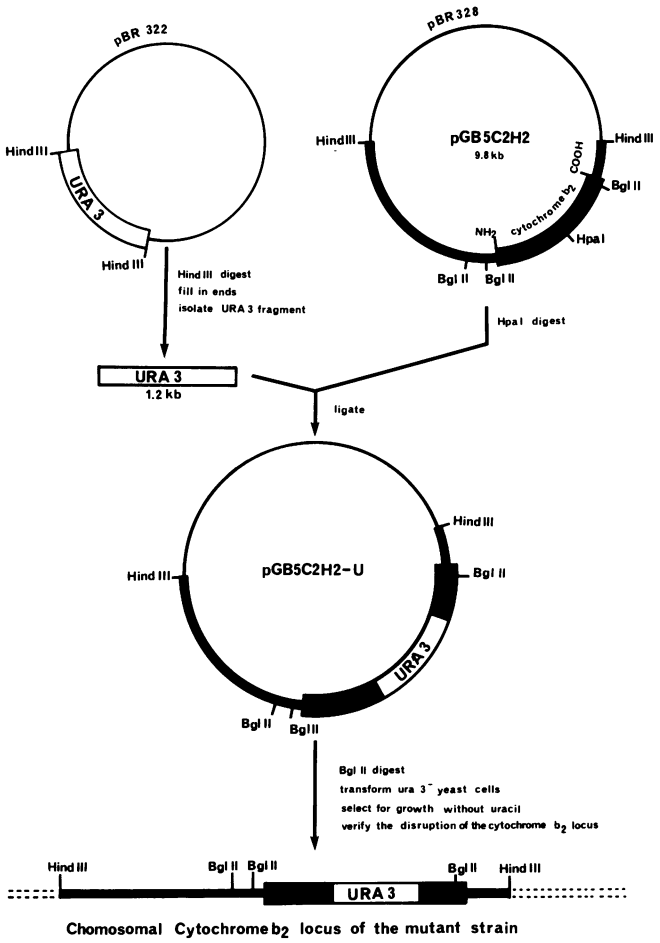

Fig. 6. Construction of an insertion mutation in the nuclear gene for the cytochrome b_2 protein.

*Hind*III fragment contains the signals necessary for cytochrome b_2 expression.

Cytochrome b_2 transcription

The effects of different carbon sources on cytochrome b_2 steady-state mRNA level were investigated. Total mRNA prepared from strain D261 grown on glucose, ethanol or lactate was subjected to Northern blot analysis (Figure 8). A single band of 1.9 kb was seen when total mRNA from ethanol- or lactate-grown cells was probed with the 0.8-kb *Eco*RV fragment. The intensity of this band is 2.5-fold reduced in ethanol as compared with lactate. In glucose-repressed cells no specific mRNA was detected.

Discussion

The sequence of *Saccharomyces cerevisiae* cytochrome b_2 gene contains some interesting structural features which are presented in Figure 4. A region with homology to the consensus sequence 5′ $TATA_T^AAT_T^A$ 3′ (Breathnach and Chambon, 1981) believed to be involved in promoter recognition by eucaryotic RNA polymerase II was found between positions −106 and −97 with the following sequence TATATAA(GTA) and lay 50−60 bp upstream from the region of transcription initiation. This sequence also contains the nonanucleotide motif ATATAAGTA founded immediately upstream of several mitochondrial genes and also in putative mitochondrial origins of replication (Osinga *et al.*, 1984). *In vitro* experiments clearly illustrate that mitochondrial initiation of transcription occurs in this box (Osinga *et al.*, 1984). An identical sequence has been described preceding the transcription initiation site of the yeast histone H4 gene (Smith and Andresson, 1983). The significance of such homology is unclear and it could be purely coincidental. S1 nuclease protection and reverse transcription experiments have shown that transcription initiation sites for cytochrome b_2 gene occur in a region located 56−64 nucleotides downstream from the TATATAA sequence. This distance seems to be characteristic of yeast genes and can vary from 40 to 70 nucleotides (Bajwa *et al.*, 1984).

The cytochrome b_2 transcript level is strongly related to growth conditions. Like many nuclear genes encoding mitochondrial components (Szekely and Montgomery, 1984; Federoff *et al.*, 1983; Lustig *et al.*, 1982; St John and Davis, 1981; Zitomer *et al.*, 1979) glucose fermentation represses the expression of the cytochrome b_2 gene at the level of transcription.

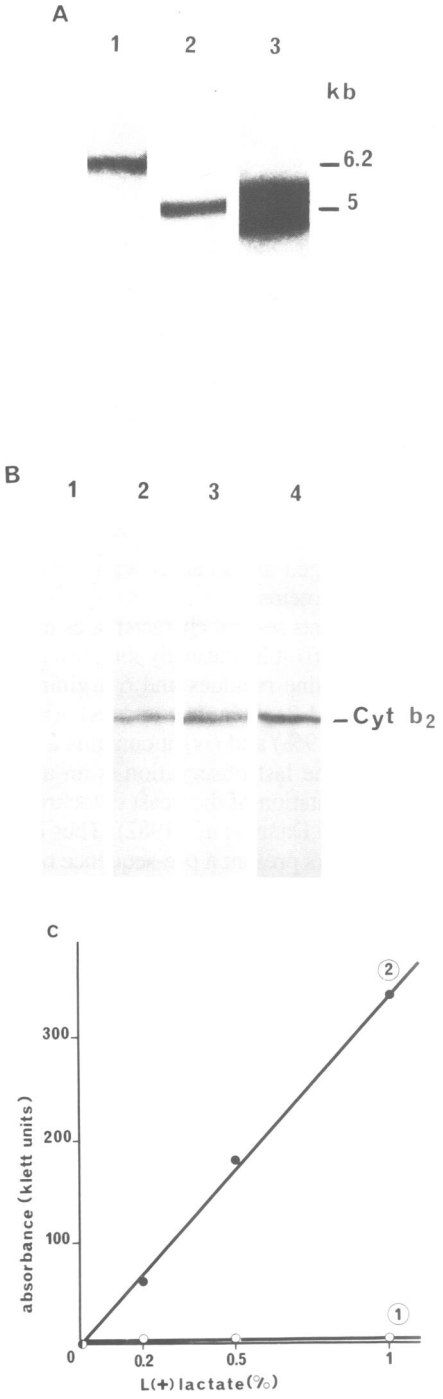

Fig. 7. The interrupted cytochrome b_2 gene has replaced the wild-type gene in the yeast nucleus. **1**, URA3 transformant: DBY-U4; **2**, wild type: DBY-746; **3**, wild type transformed by YEp13-B2; **4**, DBY-U4 transformed by YEp13-B2. (**A**) DNA was cut with *Hind*III and analysed by Southern blotting using the nick-translated 2-kb *Eco*RI-*Hind*III fragment from plasmid pGB211. (**B**) Protein was extracted from the cells and analyzed by immunoblotting with polyclonal antibody against cytochrome b_2. (**C**) Yeast strains were grown in exhausted medium on L(+)-lactate. The strains were each grown on three different concentrations of L(+)-lactate until the stationary state was reached. Maximum cellular growth (expressed as maximum absorbance in Klett units) of the cell suspension is linearly related to substrate concentration.

In this case the modulation of cytochrome b_2 expression extends over a range >200-fold and is a convenient model system for studying glucose repression. Thirdly, cytochrome b_2 mRNA synthesis is de-repressed in aerobic yeast cultures growing exponentially on ethanol or lactate. The amount of cytochrome b_2 mRNA in lactate growth conditions is increased 2.5-fold as compared with ethanol. This observation shows that lactate induction first observed by Somlo (1965) also occurs at the transcriptional level.

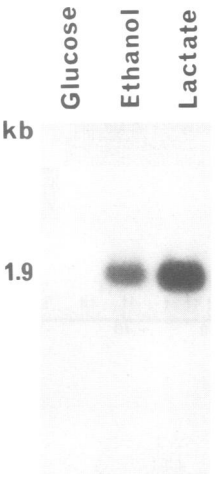

Fig. 8. Northern analysis of cytochrome b_2 gene. RNA was isolated from D261 strains and hybridization was carried out with nick-translated 0.8 kb *Eco*RV-*Eco*RV fragment from plasmid pGB5C2H2. Each lane contained 20 μg of total RNA from the strains grown on 2% lactate, 10% glucose or 1% ethanol.

The regulation of the cytochrome b_2 gene shares extensive similarities with those of the CYC1 gene, encoding the iso1 cytochrome *c* protein (Clavilier *et al.*, 1976; Clavilier, unpublished data). Recently Guarente *et al.* (1984) have proposed that the catabolic repression of CYC1 expression is caused by decreasing intracellular heme levels and can be mediated by two activation sites (UAS1 and UAS2) upstream from the transcription initiation region. These sites were shown to bear properties similar to the enhancer sequences of higher eucaryotes by their ability to act at various distances from the transcription initiation site and in both orientations (Guarente and Hoar, 1984). A sequence upstream of the cytochrome b_2 putative 'TATA box' reveals two homologous sequences TTCTTGGCGGTT (−155 and −166) and TTATTGGTCGGGT (−181 to −193) which are on the transcribed strand and present strong similarities to UAS1 and UAS2, but are in the opposite orientation. From this observation we would predict a transcriptional induction of the cytochrome b_2 gene by heme, as for the CYC1 gene. The biological role of these two sequences in the regulation of the cytochrome b_2 gene merits investigation.

Using the gene disruption technique the first yeast mutant which lacks cytochrome b_2 has been constructed (Figure 7). This mutant is devoid of L(+)-lactate dehydrogenase activity and cannot grow on exhausted medium supplied with L(+)-lactate (Figure 7C). These observations underline the physiological importance of cytochrome b_2 enzymatic activity and confirm the hypothesis presented by Labeyrie and Slonimski (1964) that only this protein is able to catalyse the oxidation of L(+)-lactate in yeast.

The deduced sequence of mature cytochrome b_2 confirms the partial primary structure presented previously (Guiard *et al.*, 1974; Ghrir *et al.*, 1984).

The most interesting feature of pre-cytochrome b_2 is clearly its deduced amino-terminal transient pre-sequence of 80 residues. Like the majority of mitochondrial proteins, cytochrome b_2 is initially synthesized in the cytoplasm as precursor with higher

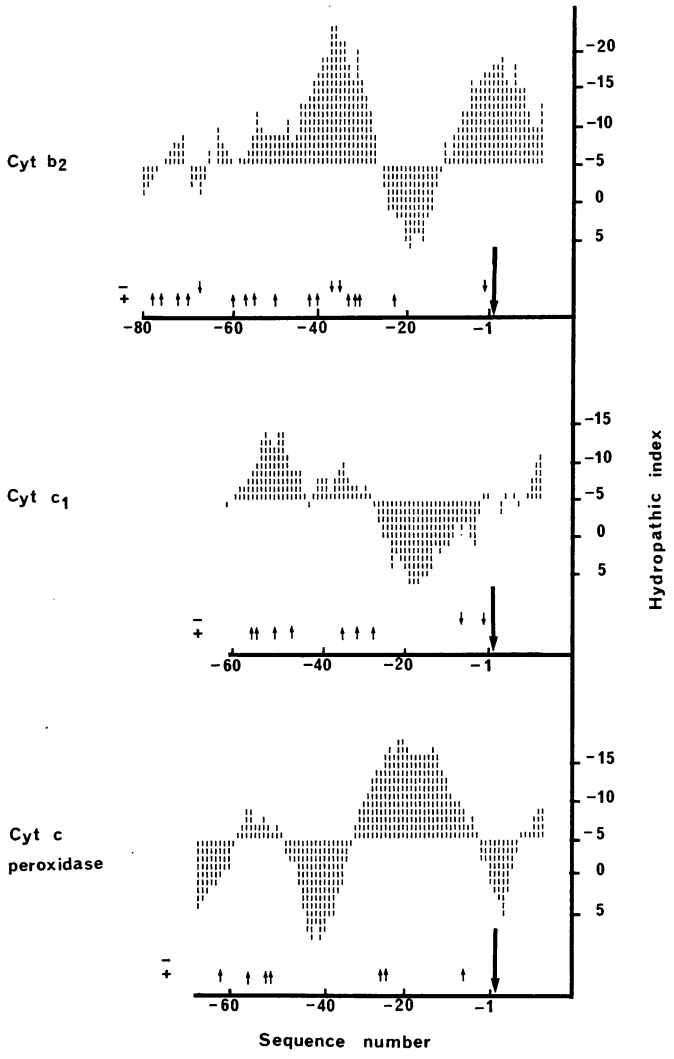

Fig. 9. Distribution of charged and lipophilic amino acid residues within the sequences of cytochrome b_2, cytochrome c_1 (Sadler *et al.*, 1984) and cytochrome *c* peroxidase (Kaput *et al.*, 1982). The hydropatic index is calculated according to Kyte and Doolittle (1982). The position of basic residues (positive charge) and acidic residues (negative charge) is indicated by vertical arrows. The larger arrow indicates the border between the transient pre-sequence and the mature sequence. Amino acid residues are numbered with respect to the second proteolytic cleavage site.

mol. wt. than its native form, and subsequently imported into mitochondria (Gasser *et al.*, 1982). It has been shown that the transient pre-sequences of mitochondria precursor polypeptides are essential for their translocation into mitochondria (Riezman *et al.*, 1983) and in a few cases carry information relating to the intramitochondrial destination of the corresponding mature polypeptide (Douglas *et al.*, 1984; Hurt *et al.*, 1984). What is the nature of this information carried by the cytochrome b_2 pre-sequence? A first step towards an answer is the structural analysis of the pre-sequence. Here the deduced primary structure of cytochrome b_2 pre-sequence is presented, with a comparison with those of cytochrome c_1 and cytochrome *c* peroxidase, two intermembrane-space hemoproteins of mitochondria (Sadler *et al.*, 1984; Kaput *et al.*, 1982). Indeed, the translocation pathway of these three molecules presents several common features (Gasser *et al.*, 1982; Daum *et al.*, 1982; Ohashi *et al.*, 1982) and it is interesting to analyze how the similarities between these two molecules and cytochrome b_2 can be extended to the level of their pre-sequence structures. Figure 9 shows the distribution

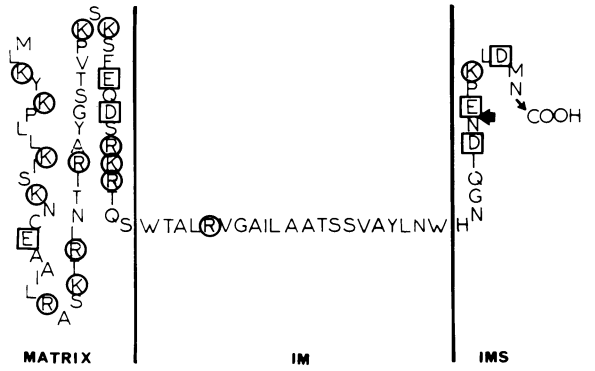

Fig. 10. Presumed orientation of the yeast cytochrome b_2 precursor across the mitochondrial inner membrane. Basic and acidic amino acids are indicated by circles and squares respectively. IM for inner membrane, IMS for intermembrane space. The large arrow indicates the second cleavage site.

of charged and uncharged amino acids within the pre-sequence of these three hemoproteins.

Cytochrome b_2 presents several characteristics in common with the two other proteins: (i) it is unusually long (80 residues), (ii) it is strongly basic (8 lysine residues and 6 arginine residues for 2 aspartic residues and 2 glutamic residues), (iii) it is rich in threonine and serine (19%) and (iv) it contains a long stretch of non-polar residues. The last observation is an agreement with a transmembrane orientation of the yeast cytochrome b_2 precursor as first suggested by Daum *et al.* (1982). Thus it is remarkable that the three precursors present a pre-sequence of similar structure even though their amino acid sequences are not obviously homologous. Such observations suggest that the three polypeptides could reach their correct intra-mitochondrial location by using a similar import mechanism, and are in agreement with the two-step import model presented by Reid *et al.* (1982) and Gasser *et al.* (1982) based on *in vitro* studies, as well as pulse-chase experiments with intact yeast cells. It proposes that the cytochrome b_2 precursor polypeptide initially translocates across mitochondrial membranes so that its 52 amino-terminal residues face the mitochondrial matrix. Non-polar residues 53 – 73 would then act as a transmembrane segment within the inner membrane interrupting the process of translocation into the matrix, and the rest of the sequence (residues 74 – 591) protrudes into the intermembrane space (Figure 10). A matrix protease removes part of the amino terminus exposed into the matrix; the exact cleavage site is unknown. A second proteolytic step which takes place on the outer surface of the inner membrane, cleaves the membrane-bound intermediate between the residues Gln80 and Asp81, and released mature cytochrome b_2 into the membrane space.

Materials and methods

Strains and plasmids

The strains used were: the *E. coli* K-12 derived HB101, JM103, C600, Q358, BHB2688, BHB2690 (Maniatis *et al.*, 1982) and the following *E. coli* vectors: pBR322 (Bolivar *et al.*, 1977), pBR328 (Soberon *et al.*, 1980). Plasmids were propagated in *E. coli* strain HB101 grown in Luria Broth supplemented with ampicillin (50 μg/ml, Maniatis *et al.*, 1982). The *Saccharomyces cerevisiae* strains used were D261 (Clavilier *et al.*, 1976), DBY.746 alpha delta *his, leu2* (3-112), delta *ura* (3-52), *trp* (1-25), *cyh*R and the yeast *E. coli* shuttle vector, YEp13 (Broach *et al.*, 1979). *S. cerevisiae* strains were grown on media containing 1% yeast extract, 2% bacto-peptone and either 10% glucose or 2% lactate (pH 4.6). To eliminate the respirable energy sources contained in the natural medium, it as first exhausted by growing the yeast strain for 96 h in the absence of any added substrate; the cells were removed by centrifugation followed by filtration through a 0.45 μ millipore filter. The desired energy source was added after sterilisation of the medium.

Enzyme assay

Cytochrome b_2 assays were performed as described by Somlo (1965). Cells were grown in 15 ml of 2% lactate medium to an OD_{600} = 1, spun down, resuspended in 1 ml 0.6 mannitol, 10 mM Tris-HCl (pH 7.5), 1 mM phenylmethylsulfonyl fluoride and 0.5 ml glass beads (0.45 mm). The cells were broken by agitation on a vortex. The glass beads were removed from the extract used directly for determination of cytochrome b_2 activity. Activity was measured by monitoring the decrease of absorbance of ferricyanide at 420 nm; 1 unit is defined as one nmol of ferricyanide reduced per min and per OD of the culture.

DNA manipulation

The restriction endonuclease, ligase, alkaline phosphatase, DNA polymerase I, DNase I and T4 polynucleotide kinase were obtained from Biolabs and Boehringer, and used in accordance with suppliers' recommendations. DNA sequencing was performed according to Maxam and Gilbert (1980). 5′ end restriction fragments were labeled with T4 polynucleotide kinase. DNA fragments labeled at a single end were generated from fragments labeled at both ends by cutting with a second restriction enzyme or by strand separation.

Transformation. E. coli was transformed according to the technique of Mandel and Higa (1970). Yeast transformation was carried out by the LiCl procedure of Ito *et al.* (1983).

Analytical procedures

Proteins were separated electrophoretically on SDS-polyacrylamide gels (Laemmli, 1970) and then electrophoretically transferred to nitrocellulose sheets (Towbin *et al.*, 1979). Immunological detection was carried out as described (Guiard and Buhler, 1984).

Preparation and screening of the yeast genomic library

The vector, λ EMBL4 (Frischauf *et al.*, 1983) was used to clone *Eco*RI fragments obtained from a partial digestion of yeast strain D261 genomic DNA. The double cleavage of the vector with *Eco*RI and *Bam*HI digests the middle fragment, this procedure reduces the level of parental vectors produced in the ligation. Genomic fragments of an average length of 20 kb were selected by a sizing step on sucrose gradients. For ligation, the partially *Eco*RI-digested DNA was mixed with a molar excess of vector cut with *Eco*RI and *Bam*HI and ligated. Ligation mixtures were then directly packaged as described by Maniatis *et al.* (1982). The library was screened by a standard plaque hybridization protocol (Maniatis *et al.*, 1982). The recombinant plasmid pGB211 (Guiard and Buhler, 1984), was nick-translated and used as a probe.

DNA preparation

Phages were prepared according to Yamamoto *et al.* (1970) and purified by two cycles of CsCl equilibrium centrifugation. Phage DNA was prepared by extracting phage suspensions twice with saturated phenol/chloroform/isoamylalcohol (25:24:1 by vol.) followed by extraction with diethyl ether and precipitation with ethanol. Plasmids were prepared by an alkaline lysis protocol (Birnboim and Doly, 1979) followed by two cycles of CsCl-ethidium bromide equilibrium density centrifugation. *S. cerevisiae* genomic DNA was isolated by the method of Davis *et al.* (1980).

RNA preparation

Total RNA from *S. cerevisiae* was prepared by the method of Maccechini *et al.* (1979).

Electrophoresis of DNA, transfer to nitrocellulose sheets and hybridization

Agarose gel electrophoresis of DNA was carried out in TBE Buffer (Maniatis *et al.*, 1982). The DNA fragments were transferred from the gels to nitrocellulose filters by the method of Southern (1975). Hybridization was carried out at 42°C in 50% formamide, 5 × SSC, 5 × Denhardt's solution, 20 mM $NaPO_4$ at pH 6.5, 100 μg/ml yeast tRNA with probes nick-translated by the method of Rigby *et al.* (1977).

Electrophoresis of RNA, transfer to nitrocellulose sheets and hybridization

Total RNA was subjected to agarose gel electrophoresis in 50 mM boric acid, 5 mM borate, 10 mM sodium sulfate, 5 mM methylmercuric hydroxide (Bailey and Davidson, 1976). Transferred to nitrocellulose filters, RNA was then hybridized with nick-translated DNA probes as described by Thomas (1980).

Transcript mapping

S1 nuclease mapping was carried out according to the method of Berk and Sharp (1977) as modified by Weaver and Weissman (1979). The 5′ end-labeled 277 bp *Eco*RV-*Fnu*4H1 fragment and 321 bp *Eco*RV-*Hha*I fragment were strand separated and mixed with 100 μg of total mRNA. Aliquots were then treated with various concentrations of S1 nuclease for 40 min at 37°C. Samples were analysed on 8% sequencing gels along with a sequence ladder.

The primer extension experiment was carried out as described by Maniatis *et al.* (1982). The double-stranded 5′ end-labeled 141 bp *Fnu*4H1 fragment was hybridized with 100 μg of total mRNA at 52°C for 3 h. The hybrids were resuspended in a buffer containing 100 mM Tris (pH 8.0), 10 mM $MgCl_2$, 140 mM KCl; 28 mM β-mercaptoethanol and 1 mM of each deoxynucleotide triphosphate in the presence of 40 U of reverse transcriptase and 30 U of RNasin. Incubation was carried out at 42°C for 2 h. The sample was subjected to electrophoresis on an 8% sequencing gel with a sequence ladder.

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