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Nuclear control of the expression of the cytochrome *b* gene in yeast mitochondrial DNA

(apocytochrome b gene, Saccharomyces cerevisiae, CBP1 gene)

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SUMMARY

The mitochondrial gene encoding apocytochrome b is mosaic, containing two intervening sequences in the Saccharomyces cerevisiae strain D273-10B. Five nuclear genes and one mitochondrial gene have been identified that are necessary for the correct processing of the mitochondrial apocytochrome b premessenger RNA. The apocytochrome b mRNA from the wild-type strain has been characterized by Northern blot analyses, S1 mapping of the 5' and 3' ends and reverse transcription of the exon/intron boundaries. Apocytochrome b pre-messenger RNA processing has been investigated by Northern blot analyses of RNA from the wild-type strain, mitochondrial mit$^-$ and ρ^- mutant strains and nuclear respiratory deficient pet mutants. CBP1, a nuclear gene complementing one set of pet mutants defective in apocytochrome b mRNA processing, has been cloned and sequenced, revealing an open reading frame that could code for a basic protein of 76,000 daltons.

INTRODUCTION

Mitochondrial biogenesis is the coordinated process of assembling functional respiratory organelles from mitochondrial and nuclear gene products. In *Saccharomyces cerevisiae* two major enzyme complexes of the electron transfer chain, $CoQH_2$-cytochrome c reductase and cytochrome oxidase, as well as the energy-producing ATP synthetase are hybrid enzymes. These contain subunits encoded by the nuclear genome which are translated on cytoplasmic ribosomes and subunits encoded by mitochondrial DNA translated on mitochondrial ribosomes. How is the synthesis, transport and assembly of cytoplasmic subunits of these hybrid enzymes coordinated with that of mitochondrial components? Yeast undergoing derepression initially require a cytoplasmic translation product(s) to stimulate the synthesis of the mitochondrion-coded subunits (TZAGOLOFF 1971), suggesting nuclear control of mitochondrial biogenesis. Whether control is manifested at the level of transcription, mRNA processing or translation has not been clearly demonstrated. This paper will

focus on the gene structure of apocytochrome *b*, the only mitochondrion-coded subunit of $CoQH_2$-cytochrome c reductase, and on nuclear control of its expression at the level of pre-messenger RNA processing. The apocytochrome *b* gene is mosaic, containing two intervening sequences in the D273-10B strain and five in the KL14 strain (HAID et al. 1979; BORST & GRIVELL 1978; SLONIMSKI et al. 1978; NOBREGA & TZAGOLOFF 1980). Five nuclear genes necessary for the precise excision of the two introns in the apocytochrome *b* pre-messenger RNA have been identified by complementation analysis of induced mutations (DIECKMANN et al. 1982). In addition to these nuclear gene products, there is genetic evidence (DWHALE et al. 1981; DE LA SALLE et al. 1982) that a mitochondrial translation product encoded in the first intervening sequence of the apocytochrome *b* gene of D273-10B (equivalent to I_4 in KL14) is also involved in the processing of that intron. Mitochondrial biogenesis may be coordinated at the level of mRNA splicing.

DNA AND mRNA STRUCTURE

The apocytochrome *b* gene in *Saccharomyces cerevisiae* strain D273-10B has been sequenced (NOBREGA & TZAGOLOFF 1980) and the mRNA has been characterized by reverse transcription and S1 nuclease mapping (BONITZ et al. in press). The first of the two intervening sequences contains a long open reading frame in register with the first exon that could code for a basic protein of 44,000 MW. Two transcripts appear to be read from the apocytochrome gene, differing only in length of the

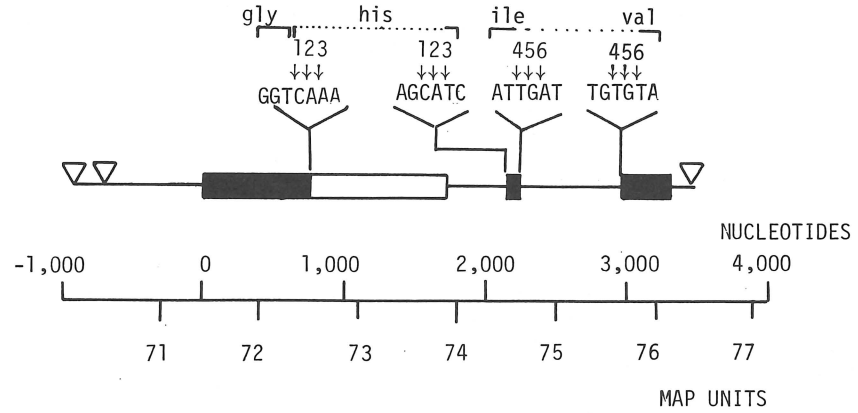

Figure 1. Structure of the apocytochrome *b* gene and mRNA. Black bars denote coding sequences. The open bar represents the open reading frame in the first intron that is in register with the first exon. Open triangles mark the two 5' ends and the unique 3' end of the RNA transcripts. Nucleotide sequences at the exon/intron boundaries are shown. Arrows denoting the three possible splice sites at each junction are paired by number to indicate joinings that preserve the amino acid sequence across the boundaries.

5' leader sequence. The longer transcript has a 5' terminus approximately -942 nucleotides upstream of the AUG start codon, whereas the shorter transcript starts only -723 nucleotides upstream (see Figure 1). The two transcripts are present in approximately equimolar amounts in mitochondrial RNA extracted from derepressed, glucose-grown, wild-type yeast. Both have the same 3' terminus some 110 nucleotides downstream of the TAA stop codon. The first intervening sequence (1401 nucleotides) occurs between nucleotides +766 and +2167 and the second (700) between +2199 and +2899. Interestingly, the correct splice junctions of both introns can be generated by one of three different splices involving successive nucleotides at the exon/intron boundaries (see Figure 1). This is possible because of the repetition of a two nucleotide sequence at the upstream and downstream boundaries in phase with the reading frame, e.g., the CA of the histidine codon occurs both after the exon 1 glycine codon and before the exon 2 proline codon. The histidine codon can be generated by splicing before the C, after the C or after the CA. The boundary nucleotides in intron 1 are dissimilar to those in intron 2, and neither intron displays the Chambon dinucleotide sequences (BREATHNACH & CHAMBON 1981).

Processing of the apocytochrome *b* precursor RNA was analyzed by Northern blot analysis (ALWINE et al. 1977) of wild-type mitochondrial RNA separated electrophoretically on agarose gels, transferred to DBM paper and hybridized to short ^{32}P-labeled DNA probes prepared from restriction fragments of the various exon and intron regions of the apocytochrome *b* gene. Hybridization of DNA probes from the first and third exons, E_1 and E_3, to mitochondrial RNA from the wild-type strain D273-10B yields four transcripts at 4.3, 3.6, 2.9 and 2.1 kb (see Figure 2). The composition of these transcripts is defined by hybridization analyses with intron probes. Only the 4.3 and 3.6 kb transcripts hybridize to an I_1 probe, whereas the 4.3 and 2.9 kb transcripts hybridize to an I_2 probe. Therefore, the largest transcript at 4.3 kb is presumed to be the full-length transcript containing both introns. I_2 (700 nucleotides) alone has been excised from the 3.6 kb transcript and I_1 (1401 nucleotides) alone is absent from the 2.9 kb intermediate. The 2.1 kb transcript hybridizes only to exon probes and to 5' and 3' extension probes. This transcript is ten to 100-fold more abundant than the three precursor species and is presumed to be the mature mRNA. The splicing of I_1 and I_2 appears not to be an ordered process since both the 3.6 kb and the 2.9 kb species are present in wild-type mitochondria. The 2.9 kb intermediate is five to ten-fold more abundant than the 3.6 kb transcript, suggesting that the rate of excision of I_1 from full-length precursor is much faster than the rate of I_2 removal.

Interestingly, only when third exon probes are used, a fifth, small RNA fragment 400-500 nucleotides in length hybridizes in an amount comparable to the hybridization of precursor species. S1 mapping and reverse transcriptase studies have shown (BONITZ et al. in press) that two transcripts differing in length by 50 nucleotides are contained in the band. Both fragments have the same 3' terminus as the mature transcript,

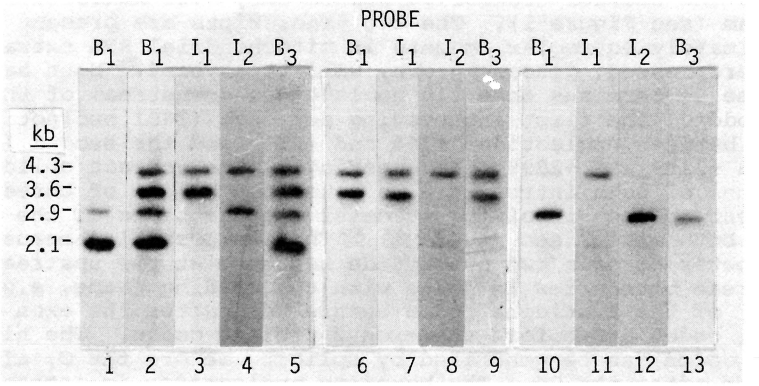

Figure 2. Northern hybridization analyses of mitochondrial RNA's. In lane 1, wild-type mitochondrial RNA. The 4.3 kb and 3.6 kb precursors are in low abundance in wild type and do not show up unless the film is severely overexposed. As marker strains we use RNA from mit$^-$ mutants with mutations in exon regions which tend to accumulate enough of each precursor to act as markers in normally exposed films. Lanes 2-5, strain *10-152*, an exon 1 mit$^-$ mutant. Lanes 6-9, *9-226* RNA, an I_1 mit$^-$ mutant. Lanes 10-13, *6-200* RNA, an I_2 mit$^-$ mutant. Probes: B_1 = *Hpa*II-*Rsa*I fragment of M4; I_1 = *Hae*III-*Rsa*I fragment of M4; I_2 = *Rsa*I-*Dde*I fragment of M4; E_3 = *Rsa*I-*Rsa*I fragment of M11. See NOBREGA & TZAGOLOFF 1980 for identification of ρ^- strains M4 and M11 and restriction map.

but the shorter fragment has a 5' terminus three to ten nucleotides upstream of the I_2/E_3 boundary. The longer of the two fragments has been identified as E_2E_3 by reverse transcription. These transcripts could be stable products of misprocessing of the I_2/E_3 and I_1/E_2 boundaries respectively. They could also be stable intermediates of intron processing. However, the corresponding half-molecules have not yet been detected in blots of wild-type mitochondrial RNA.

MITOCHONDRIAL PROCESSING: MITOCHONDRIAL MUTANTS

Two mit$^-$ mutations which abolish apocytochrome *b* production, *9-226* and *6-200*, have been mapped to I_1 and I_2 respectively. Northern blots of mitochondrial RNA extracted from these mutant strains were tested for hybridization with exon and intron probes from the apocytochrome *b* gene (see Figure 2). The blots revealed that *9-226* has the 4.3 kb precursor and the 3.6 kb intermediate but not the 2.9 intermediate or the 2.1 kb mature mRNA. Conversely, *6-200* has the 4.3 kb precursor and the 2.9 kb intermediate but not the 3.6 kb intermediate or the mature message. The result suggests that the excision of I_1 is independent of that of I_2 and vice versa.

There is genetic evidence that the open reading frame encoded in intron 2 of the apocytochrome *b* gene in the KL14 strain (not present in D273-10B) is translated and is involved in the splicing of that intervening sequence (LAZOWSKA et al. 1980). Mutations in the first intron of the D273-10B apocytochrome *b* gene (equivalent to I_4 in KL14), which also contains an open reading frame in register with the preceding exon, block excision of that intervening sequence. Unlike the situation for I_2 in KL14, it was not clear whether the mutations in I_1 of D273-10B resulted in 1) a defective maturase or 2) disruption of RNA structure interfering with recognition by the processing enzyme(s). Mutations in introns without open reading frames (i.e., *6-200* in I_2) block splicing, suggesting that another approach should be used to investigate the necessity of translation of the D273-10B I_1 reading frame for I_1 excision.

ρ^- petites were generated from wild-type D273-10B and selected for retention of the apocytochrome *b* gene concomitant with loss of the genes necessary for mitochondrial translation, i.e., 15S and 21S rRNA genes and the clustered tRNA genes. Mitochondrial RNA of one such petite, DS8, was probed with ^{32}P-labeled DNA fragments from the apocytochrome *b* gene (see Figure 3). DS8 has the 4.3 kb precursor and the 3.6 kb intermediate but not the 2.9 kb intermediate or the mature mRNA. An aberrant 3.1 kb intermediate hybridizes to the I_1 and the I_2 probe; however, another intermediate at 2.4 kb hybridizes only to the I_1 probe. Intron 1 appears to have been incorrectly spliced and retains a portion that hybridizes to the I_1 probe. This result implies that the I_1 open reading frame encodes a protein necessary for the correct splicing of I_1. The I_1-encoded element is also necessary for the proper excision of I_4 in the *oxi3* gene transcript, which codes for subunit 1 of cytochrome oxidase. No mature mRNA for cytochrome oxidase subunit 1 is present in Northern blots of mitochondrial RNA of the *9-226* mutant. The possibility exists that an RNA transcript encoded in I_1 is necessary for both subunit 1 and apocytochrome *b* messenger RNA processing, but it seems likely that the I_1 reading frame is indeed translated on mitochondrial ribosomes and the protein is active in mitochondrial RNA splicing. The protein encoded in I_1 cannot be wholly responsible for excision of the first intervening sequence in the apocytochrome *b* gene. Nuclear mutations that block excision of intron 1 will be discussed in the following section.

MITOCHONDRIAL mRNA PROCESSING: NUCLEAR MUTANTS

A collection of nuclear respiratory deficient mutants (*pet* mutants), were screened for defective splicing of apocytochrome *b* mRNA. Five of the 130 complementation groups of Mendelian mutants unable to grow on the non-fermentable carbon source glycerol were found to be defective in the processing of the 4.3 kb precursor RNA, as revealed in hybridizations of Northern blots of total mitochondrial RNA with an exon probe (see Figure 4). Three of the complementation groups (Groups 26, 69 and 85) have the 4.3 kb precursor and the 3.6 kb intermediate but not the 2.9 kb intermediate or the mature transcript. These nuclear mutants are blocked in the excision of the first but not the

second intervening sequence, as was the case for the mitochondrial mutation *9-226*. One complementation group, Group 36, also has the 4.3 kb precursor transcript but has the 2.9 kb intermediate instead of the 3.6 kb transcript. It also lacks the mature RNA, and therefore has a phenotype similar to that of *6-200*. The fifth complementation group, Group 60, has a complicated phenotype with respect to the pattern of transcripts hybridizing to apocytochrome *b* exon and intron probes. The 4.3 kb transcript is apparent in blots of mitochondrial RNA in some of the Group 60 mutants (see Lane 13, *C286* of Figure 4), but the 3.6, 2.9 and 2.1 kb species are not detectable above the

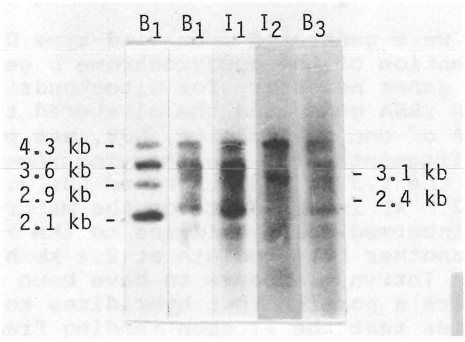

Figure 3. Northern hybridization analysis of a ρ^- mutant mitochondrial RNA. Lane 1, *10-152* RNA as marker. Lanes 2-5, DS8 RNA. Probes are the same listed for Figure 2.

background. In general, probes from all parts of the gene hybridize to a background smear of many sizes of transcripts between 3.6 and 1.5 kb. Two anomolous transcripts of 2.6 and 1.9 kb appear when I_1 or E_3 probes are hybridized to blots of mitochondrial RNA from Group 60 mutants. Since neither transcript hybridizes to an E_1 probe, the 2.6 kb ($I_1E_2I_2E_3$) and 1.9 kb ($I_1E_2E_3$) fragments are products of misprocessing or are uncommonly abundant, true intermediates of the first intron excision reaction(s).

ISOLATION AND SEQUENCING OF NUCLEAR GENES

The wild-type nuclear genes complementing the Group 36, 60 and 69 mutations have been cloned (DIECKMANN et al. 1982b), and the DNA nucleotide sequence of two of the genes, CBP1 (Group 60) and CBP2 (Group 36), has been determined (DIECKMANN & TZAGOLOFF, in preparation; McGRAW & TZAGOLOFF, in preparation). A CV13 (YEp13) (BROACH et al. 1979) plasmid bank with 5-20 kb inserts of wild-type DNA representing the entire genome from *Saccharomyces cerevisiae* strain W87 was used as transforming DNA for selection of the wild-type genes. CV13 is a *Saccharo-*

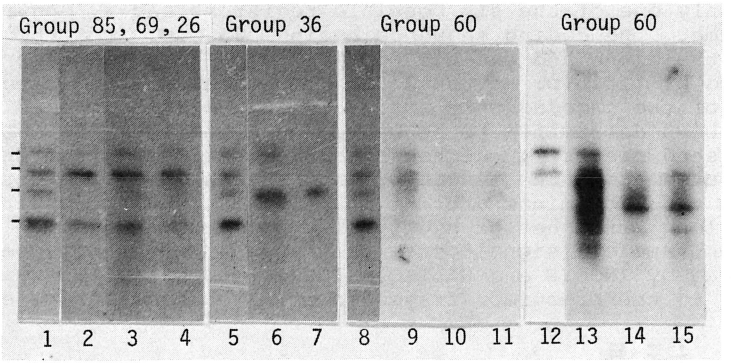

Figure 4. Northern blot analyses of nuclear *pet* mutant mitochondrial RNA. Marker RNA: Lane 1, *17-162*; lanes 5 and 8, *516-45*; lane 12, *10-152*. Like *10-152*, *17-162* is also an exon 1 mit$^-$ mutant. *516-45* is an aspartyl tRNA syn$^-$ mutant that also accumulates the precursors. Probes used for lanes 1-11: *Hinf*I/*Hinf*I fragment of ρ^- N24; lanes 12-15: *Taq*I/*Mbo*I fragment of M4. Lane 2--*N493*; lane 3-*C110*; lane 4--*C31*; 6--*C237*; 7--*C89*; 9--*C286*; 10--*E3*; 11--*N5-26*; 13--*C286*; 14--*E3*; 15--*N5-26*. See NOBREGA & TAZGOLOFF 1980 for identification of ρ^- strain N24.

myces cerevisiae/E. coli hybrid vector containing 1) pBR322, which provides an origin of replication and selection by ampicillin resistance and/or tetracycline resistance in *E. coli*; 2) an *Eco*RI fragment of the 2μ yeast plasmid, which provides an origin of replication in yeast; and 3) a *Pst*I fragment of yeast nuclear DNA encoding the *LEU2* gene, which provides selection in *leu2* strains of yeast. The plasmid bank was constructed by partially digesting total yeast DNA with *Sau*3A and ligating the resulting fragments into the *Bam*HI site of pBR322, thereby inactivating the *tet*^{r} gene (NASMYTH & REED 1980). A double leucine auxotrophy, *leu2-3leu112*, was introduced into mutants representative of each group. Yeast spheroplasts transformed with the plasmid carrying the wild-type gene were selected by plating on glycerol medium lacking leucine. Double selection for growth on glycerol and growth without leucine guards against selecting revertants of the nuclear *pet* mutations. Small DNA preparations made from yeast transformants were used to transform *E. coli* strain RR1 to ampicillin resistance.

The Group 60 mutant *N5-26* (*a, ade, leu2-3leu2-112*) was transformed with the plasmid bank, and two different clones were selected that complemented the *N5-26* mutation. pG60/T10 was the clone containing the larger insert of nuclear DNA, 6.7 kb in length (see Figure 5). As determined by restriction mapping, the smaller clone was wholly contained within the 6.7 kb sequence but lacked 800 b.p. on one end of the insert. A

2.4 kb subclone of pG60/T10 was isolated and sequenced by the method of Maxam and Gilbert (1977).

Only one of the six possible registers had a long reading frame. The coding sequence begins 385 b.p. from the 5' end of the clone and extends for 1962 b.p. to a TGA stop codon, followed by 110 b.p. at the 3' end. The calculated molecular weight of the encoded polypeptide is 76,000 daltons, and the pI has been determined to be between 9.6 and 10.1 (BEYCHOK, unpublished results). Although charged residues are evenly distributed along the length of the protein, both the N-terminus and the C-terminus are enriched for basic residues. There are no long stretches of hydrophobic residues indicative of transmembrane or "signal" sequences anywhere in the sequence (reviewed by INOUYE and HALEGOUA 1980). The basic N-terminus is similar to the precursor fragments of mitochondrial and chloro-

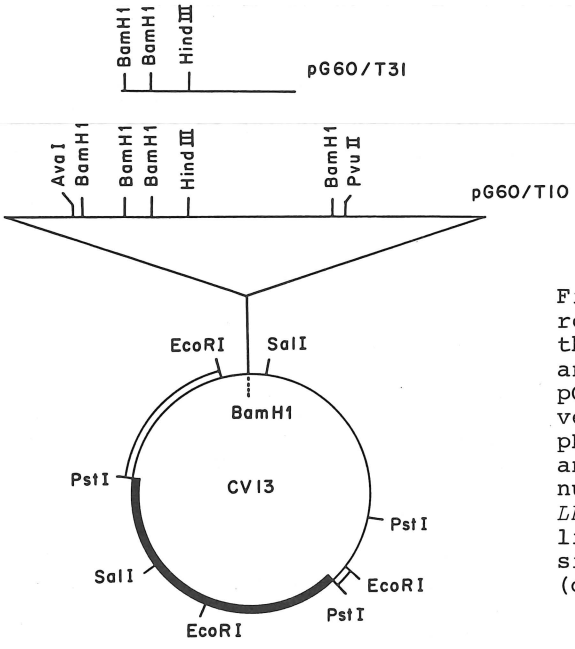

Figure 5. Partial restriction map of the pG60/T10 plasmid and its subclone pG60/T31. The CV13 vector consists of pBR322 (thin line) and a fragment of yeast nuclear DNA with the *LEU2* gene (solid arc) ligated to the *Pst*I site of yeast 2 μ DNA (open arc).

plast polypeptides that are transported from the cytoplasm concomitant with cleavage of the presequence (SCHMIDT et al. 1979; CORUZZI et al., submitted for publication; CORUZZI, personal communication; SEBALD, personal communication). In the 5' upstream portion of the clone, two "Hogness box" sequences are present (GOLDBERG 1979). These may be important in initiation of transcription of this gene, as they are for other nuclear genes in eukaryotes. This gene has been named CBP1 for *cytochrome b processing 1*. (See Figures 6 and 7.)

More recently, the gene defined by the Group 36 mutants

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5'-GATCGCTTGTTGTCGTGCGAACTACTGTTACGTGTGCGTTGTAAAGGCGTTGGAGTGG

TCTATGTGCGATGCCTGTGGATCCTCTGGGAGACTGACCGCCTCACCAGTGTACTAAAGGG

CCCCGTTCCCTCGCTTGTATCTCTATATATGTGTATACTATATTCATATTCATGTTGTCAA

AATCGGTCACGTGGTAAAGGGTCTGGTTTTTTTTTTTTCACTTTCTGCTCAAGAAAAAAAA

GAAACAATAAAGAAGTGGTATAATATCATCATGGAACGCTGTACGGGACGATGTGGGAAGC

GACGTCTGAAATCCAGTAATAATAAACAGTGAGCACTTTAGGATAGCAGTAGTTTGCATTT

TGCACGTTTCCCTTTCCATGCA . . . 1962 b.p. coding sequences . . . TGA ACGGCCAAACG

CACATTTACGATATAAATAACAAAGCAAATAATAACAAAACAAATTTCTGTAAATATATAC

TAAATCCAAGCATTGCATCCTAATTCTTTTCTTTTTTTGATC
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Figure 6. Sequence of the 5' upstream and 3' downstream regions of the CBP1 gene. "Hogness boxes" are underlined (------).

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MFLPRLVRYRTERFIKMVPTMTLRRINHSSRDPIQKQVLALIKANANLNDNDKLKIRKYW
SDMADYKSLRKQENSLLESSILHEVKIEDFISFINRTKTSSMTTRGIYRRECLYQCKKNLD
LVNQVVSQVSSVRHQKPLTTQLDTMRWCVDDAIGTGDIVMAADLFLLYYRLFTDDKKLDE
QYAKKIISVLAYPNPLHDHVHLVKYLQLNSLFESITGGGIKLTRFQLETLSNKALGLSNE
APQLCKAILNKLMNINYSLTNDLKLRDDQVLLAYKSIDENYRRGNVASVYSIWNKIKEHY
VSISAHDSRIIYKVFKICTHNRAYRSICSEMFWQLTPEYYCNNPLILPAIIDFITKQDSL
TMAKELMQNINRYTLPENHHIVWLNKRCLSSLLRMHLKFNDSNGVDRVLKQITTNFRALS
QENYQAIIIHLFKTQNLDHIAKAVKLLDTIPPGQAMLAYGSIINEVVDWKLASKVKFTDN
LMALVNDLLTKAHDFDPDHRNSLWNVVSALYIKKLCHYKKRDGKFVANAKKDIDLAKLLY
INAAKRSKTYWTKSNCNPFIASSPCDVKLKVNNQNRFTILRNIALSALQIGRTDIFLWAC
AELYQNGMTIEELKLDWNFILKHQIRNSEFKTNKEIIQDIKKHGVSAVKRYLR
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Figure 7. Amino acid sequence derived from the CBP1 gene. "*" below residues denotes position of the two basic amino acid residues lysine (K) and arginine (R). "^" denotes position of the acidic residues, glutamic acid (D) and aspartic acid (E).

has been sequenced. This gene also codes for a large, basic polypeptide (MW approximately 74,000) (MCGRAW & TZAGOLOFF, in preparation).

In summary, six genes have been implicated in the proc-

essing of the apocytochrome *b* mRNA. Three nuclear genes (represented by Groups 26, 69 and 85) are certainly involved in the excision of the first intervening sequence. CBP1 (Group 60) is most probably also involved in the proper excision of I_1 as is the mitochondrial gene in the intron itself. It may be of interest to note that the I_1 encoded protein, like the CBP1 and CBP2 polypeptides, is also a very basic protein. The sixth gene, CBP2 (Group 36), is the only one identified as yet with excision of I_2. Whether or not the various polypeptides involved in the excision of I_1 are acting in concert as an enzyme complex or in a stepwise pathway remains to be determined.

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