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# Homology between bacterial DNA and bovine mitochondrial DNA encoding cytochrome *c* oxidase subunit III

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A segment of mitochondrial DNA encoding the bovine cytochrome *c* oxidase subunit III gene was isolated and inserted into an *Escherichia coli* plasmid vector. A 556 base pair fragment of the insert DNA representing about 70% of the 3′-end of the subunit III gene was used to search for homology with bacterial DNA from strains that contain heme $aa_3$-type cytochrome *c* oxidases. *Bacillus subtilis, Thermus thermophilus,* and PS3 DNAs all showed strong hybridization to the probe, whereas *Paracoccus denitrificans* and *Rhodopseudomonas sphaeroides* DNAs showed only weak hybridization to the probe, even under low stringency conditions.

Cytochrome-*c* oxidase; DNA hybridization; DNA; DNA homology; (Bovine heart, Bacteria)

## 1. INTRODUCTION

Cytochrome *c* oxidase (EC 1.9.3.1) is an integral inner mitochondrial membrane protein that contains heme $aa_3$-type cytochromes [1]. Similar cytochrome *c* oxidases occur in the cytoplasmic membranes of several genera of bacteria [2]. The eukaryotic enzyme is complex, e.g., 9–12 protein subunits occur in the oxidase from beef heart [1], while the prokaryotic enzyme is much simpler in structure, having only 2–3 subunits [2].

Both the prokaryotic and eukaryotic enzymes mediate the transfer of electrons from ferrocytochrome *c* to molecular oxygen in the final catalytic reaction of the respiratory chain. The eukaryotic enzyme also translocates protons across the inner mitochondrial membrane in a vectorial manner [3]. Subunit III of the bovine heart enzyme has been implicated as a crucial component of proton translocation activity (review [4]) as shown by chemical modification experiments with dicyclohexyl carbodiimide (DCCD) [5] and by subunit III depletion experiments [6].

The proton translocating activity of the prokaryotic enzyme has not been as closely examined, but cytochrome *c* oxidases isolated from several bacterial species do mediate proton movement across membranes [7–9]. Our interest lies in identifying those protein domains involved in the vectorial proton translocation reaction of the prokaryotic enzyme, and in determining whether bacteria contain genetic information encoding a subunit that is structurally and/or functionally analogous to the bovine subunit III. Consequently, we have isolated the subunit III gene (CO III) from bovine mitochondrial DNA in order to search for homology between this DNA and bacterial genomes encoding heme $aa_3$-type cytochrome *c* oxidases. CO III DNA hybridized strongly to a

This work is dedicated to the memory of G. Egan

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number of DNA sequences from the bacterium PS3, *Thermus thermophilus*, and *Bacillus subtilis*, but hybridized only weakly to DNA from *Rhodopseudomonas sphaeroides* and *Paracoccus denitrificans*.

## 2. MATERIALS AND METHODS

### 2.1. *Isolation of bacterial and mitochondrial DNA*

Bacterial strains (see table 1) were grown in liquid medium with vigorous aeration under conditions described in [7–11]. DNA was isolated from the cells by the method in [12]. Mitochondria were prepared from beef heart [13] and the DNA was isolated according to [14]. Plasmid DNA that contained the CO III gene was isolated from *Escherichia coli* and purified by cesium chloride centrifugation [15].

### 2.2. *Preparation of Southern transfers and hybridization*

Restriction fragments of genomic bacterial DNA were transferred from agarose gels to nitrocellulose filters by the Southern technique as modified in [16]. Hybridization of the Southern transfers to radioactive probe DNA was carried out according to methods in [15]. The temperatures for hybridization and washing were determined by the equation:

$$T_m = 69.3 + 0.41(G + C)\% - 650/L \quad (1)$$

where $L = 556$, the number of nucleotides in the probe, and $(G + C)\% = 45$ [17].

### 2.3. *Plasmid construction and radioactive labeling*

Plasmid pMt4.8 was constructed by the insertion of a 4.8 kilobase pair (kbp) *Eco*RI fragment of mitochondrial DNA into *E. coli* plasmid pBR322. A 589 base pair (bp) *Pvu*II fragment that contained approx. 70% of the CO III gene (nucleotides 9198–9779 on the mitochondrial DNA map [17]) was isolated from pMt4.8 and inserted by blunt end ligation into the *Sma*I site in pUC13 [18] to yield pMt589 (fig.1A). The 556 bp *Hin*dIII-*Tth*111I restriction fragment of pMt589 (fig.1B) was labeled with [$\alpha$-$^{32}$P]dCTP by the replacement synthesis method of James and Leffak [19], which labels the DNA to high specific activity during complete and uniform resynthesis. The 556 bp probe DNA fragment was gel purified after labeling.

## 3. RESULTS AND DISCUSSION

### 3.1. *Hybridization studies with bacterial DNAs*

The probe for the hybridization experiments was a segment of bovine heart mitochondrial CO II DNA comprising approx. 70% of the 3′-end of the gene, including the region that encodes the DCCD-binding site (fig.1A,B). We initially examined hybridization of the CO III DNA probe to *Hin*dIII-digested bacterial DNA samples (fig.2A) under high stringency conditions (hybridization and washing at 65°C), and saw essentially no binding to the probe. At this temperature, based on a $T_m$ value of 68.3°C calculated from eqn 1, and on the observation that the $T_m$ of a DNA duplex decreases by approx. 1°C for every 1% increase in mismatched base pairs [15], the bacterial and probe DNAs would have to be about 95% homologous to observe hybridization; this stringency level may be too high considering the amount of evolutionary divergence likely to have occurred between the genes.

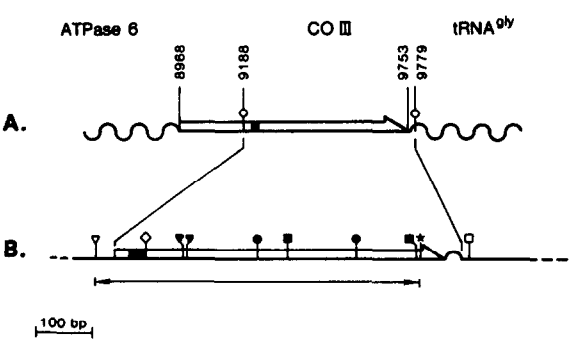


Fig.1. Restriction fragment map of bovine mitochondrial subunit III DNA and plasmid pMt589 DNA. The subunit III gene (CO III, nucleotides 8968–9753 in the bovine mitochondrial DNA) is transcribed in the direction indicated by the open arrow; the black rectangle is the region encoding the DCCD binding site (A). The open circles (○) signify *Pvu*II restriction targets. Line B is the insert of the 3′-end of the CO III gene in the plasmid pMt589. Additional symbols for restriction targets are: *Hin*dIII (▽), *Rsa*I (◇), *Fnu*4HI (▼), *Ava*II (●), *Acc*I (■), *Tth*111I (★), and *Eco*RI (□). The bottom line indicates the DNA fragment used as probe in hybridization experiments.

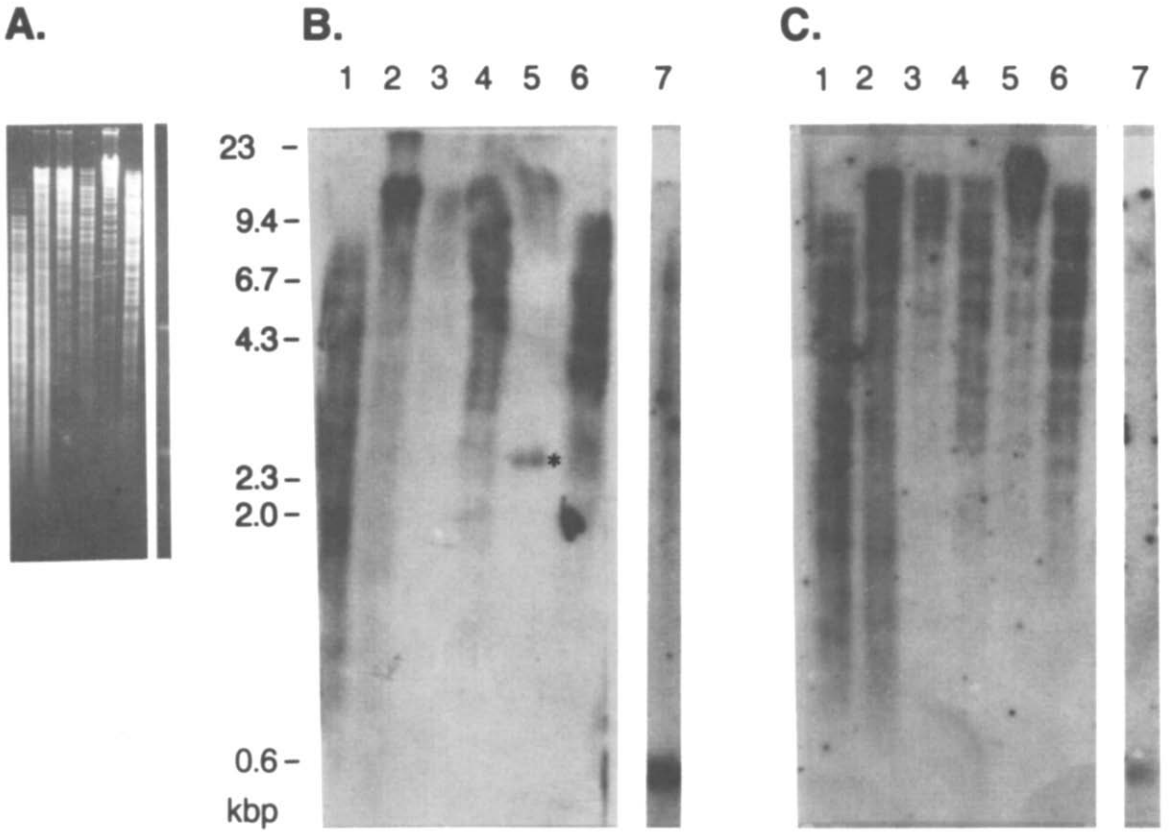


Fig.2. Hybridization of the CO III DNA to bacterial DNA. Panel A shows an ethidium bromide-agarose gel of *Hin*dIII-digested bacterial DNA, approx. 4 $\mu$g of DNA per lane. Bacterial DNA in each lane is: (1) *B. subtilis*; (2) *E. coli*; (3) *P. denitrificans*; (4) PS3; (5) *R. sphaeroides*; (6) *T. thermophilus*. Lane (7) contains pMt589 DNA digested with *Hin*dIII and *Eco*RI to release the 0.6 kb insert (lower band) from the 2.7 kb pUC13 DNA (upper band). Panel B is an autoradiogram of a Southern transfer of the bacterial DNAs hybridized to the *Hin*dIII-*Tth*111I fragment of pMt589 at 45°C and washed in 6 × standard saline citrate (SSC) at the same temperature. Numbers at left are the sizes (in kbp) of the molecular standard, *Hin*dIII-digested bacteriophage λ-DNA. Asterisk indicates artifactual binding of DNA probe to filter. This binding did not occur in any other experiments. Panel C as in B, but the hybridization and washing temperatures were both 25°C.

We next attempted to decrease the stringency conditions by lowering the temperature at which the hybridizations and washings were performed. At 50°C, where approx. 80% homology is required for hybridization, a few fragments hybridized weakly to the probe in lanes that contained *B. subtilis, T. thermophilus* and PS3 DNA (not shown). At 45°C, where the DNAs must be about 75% homologous to observe strong binding, a large number of bacterial DNA fragments that hybridized to the probe were seen (fig.2B). The abundance of fragments annealing to the probe indicates that there may be 'families' of homologous DNA, possibly of variable size and sequence, which are related to the subunit III probe: bacterial subunit III DNA may contribute to one or more of these families. The sizes of some of the more intensely hybridizing *Hin*dIII fragments are as follows: *B. subtilis*, 5.9 and 4.1 kbp; *T. thermophilus*, 9.4 and 3.6 kbp; PS3, 8.6 kbp. None of the other DNAs showed strong homology to the probe under these conditions, indicating that they either do not have DNA sequences that encode a subunit III-like protein, or the genetic information is rearranged so drastically that homology cannot be detected. The hybridization of PS3 and *B. sub-*

Table 1

The structure and function of bacterial cytochrome *c* oxidases

| Bacterial strains | No. of subunits in oxidase | Molecular mass of subunits (kDa) | $H^+$ pumping[a] | Reference |
|---|---|---|---|---|
| *Paracoccus denitrificans* | 2 | 45, 28 | +* | [7,22] |
| *Thermus thermophilus* HB8 | 2 | 55, 33 | + | [8] |
| PS3 | 3 | 56, 38, 22 | +** | [9,20] |
| *Bacillus subtilis* W23 | 3 | 57, 37, 21 | − | [10] |
| *Rhodopseudomonas sphaeroides* | 3 | 45, 37, 35 | − | [11] |

[a] (+) Proton pump activity; (−) no proton pumping activity observed; (*) DCCD-insensitive proton pumping activity; (**) DCCD-sensitive proton pumping activity

*tilis* DNAs with the probe suggests that a protein domain homologous to mitochondrial subunit III may exist in these bacteria. Both bacterial enzymes contain three subunits: in the case of PS3, its enzyme acts as a proton pump, and this activity is inhibited by DCCD (table 1) [20].

Because the DNAs of *P. denitrificans, R. sphaeroides* and *E. coli* did not show appreciable hybridization at 45°C, the stringency conditions were lowered further, hybridizing and washing at 25°C, to detect even very limited homology between the bacterial and CO III DNAs (fig.2C). At this temperature, hybridization is predicted to occur even if the two DNAs are only 50–60% homologous. Strong hybridization occurred again in the lanes containing *B. subtilis*, PS3 and *T. thermophilus* DNA, and the families of hybridizing sequences were more evident. Under these conditions, faint hybridization occurred in the lanes with *P. denitrificans, R. sphaeroides* and even *E. coli* DNA, which does not encode a heme $aa_3$-type cytochrome *c* oxidase [21]. This indicates that these bacteria may contain some DNA sequences similar to the CO III gene, but that these sequences have not been highly conserved. The hybridization observed did not result from some nonspecific binding of the probe DNA to bacterial DNA since it did not bind to *E. coli lacZ* DNA carried on the 2.7 kb pUC13 [18] segment of the pMt589 plasmid, but hybridized only to itself (fig.2A–C, lanes 7). Although *R. sphaeroides* has a three subunit oxidase (table 1), it does not pump protons [10], thus the bacterial DNA may not encode a subunit III-like protein. The very weak hybridization of the probe to *P. denitrificans* DNA under low stringency conditions is significant in view of the recent identification of a subunit III gene in *P. denitrificans* with approx. 30% nucleotide sequence homology to the subunit III probe [23]. This value is much less than the 50–60% homology level needed to produce strong hybridization to the subunit III probe in our experiments. The relatively strong hybridization observed with several other genomic DNAs reflects the presence of sequences with substantially more homology to the CO III DNA in these bacteria than in *P. denitrificans*. In the case of *T. thermophilus*, which contains a two subunit enzyme capable of mediating proton translocation, the bacterial DNA sequences show much stronger hybridization, hence a closer relationship, to the mitochondrial DNA. In light of the discovery of a possible subunit III gene in *P. denitrificans*, a subunit III gene may also be found in *T. thermophilus*.

### 3.2. *Detailed examination of PS3 DNA*

Since the PS3 bacterium showed high levels of homology with the subunit III probe, and because of the similarities between the bacterial and mitochondrial oxidases discussed above, we have investigated the DNA of PS3 further. Southern

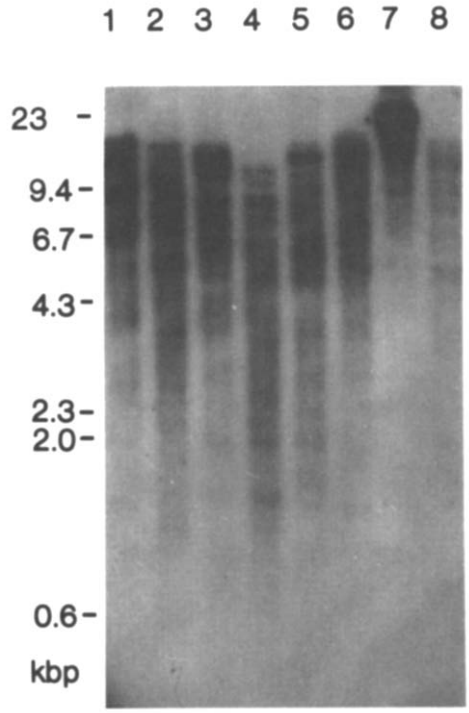


Fig.3. Hybridization of the CO III DNA to PS3 DNA. Experimental methods were as described in fig.2: hybridization and washing were carried out at 25°C. Restriction enzymes used to digest the PS3 DNA were as follows: (lane 1) *Bam*HI; (2) *HindIII; (3) Pst*I; (4) *Pvu*II; (5) *Sal*I; (6) *Sma*I; (7) *Xba*I; (8) *Xho*I.

transfers of PS3 DNA digested with a battery of restriction enzymes were hybridized with the subunit III probe under low stringency conditions (fig.3). As expected, the probe hybridized to a number of fragments in each lane, but hybridized intensely to only 1–3 fragments per lane. In addition, the probe hybridized to different sized restriction fragments in each of the lanes, indicating that the regions of bacterial homology lay on distinct restriction fragments. Clearly, hybridization to the CO III probe reveals only structural homology between regions of the PS3 genome and the bovine subunit III gene, not necessarily functional homology. This is apparent from the hybridization of the subunit III probe to sequences from *E. coli*, which does not encode a heme $aa_3$-type cytochrome *c* oxidase [21]. Nevertheless, in view of the weak hybridization to *P. denitrificans* DNA, which contains a distantly related subunit III gene, the strong signal from several discrete PS3 DNA restriction fragments is quite provocative. Consequently, isolation and characterization of several of these restriction fragments from a genomic PS3 library are currently in progress.

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