Vol. 189, No. 1, 1992
November 30, 1992

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 445-449

INCREASED MITOCHONDRIAL ACTIVITIES IN PIGMENTED (MELANIZED) FISH CELLS AND NUCLEOTIDE SEQUENCE OF MITOCHONDRIAL LARGE rRNA

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Received October 17, 1992

Summary: Using the subtracted probe approach, we had previously isolated eight cDNAs whose corresponding RNAs are more abundant in pigmented (melanized) than in closely related unpigmented goldfish cell lines. We report here that two of these are of mitochondrial origin, suggesting that pigmentation is accompanied by higher content (activity) of mitochondria. We also present the complete nucleotide sequence of the full length cDNA for the large mitochondrial rRNA, showing the presence of a polyA tail, two polyadenylation signals and a long open reading frame potentially encoding for a polypeptide of 166 amino acids and with no known protein homologue. It is however unknown whether such a polypeptide is actually produced.

The content of mitochondria of different tissues varies with their energy demand, e.g. the abundance of mitochondria in cardiac muscle. During an investigation on melanogenesis, utilizing a number of related pigmented and unpigmented cell lines derived from a dedifferentiated goldfish erythrophore tumor cell line (1-3), we cloned eight cDNAs whose corresponding RNAs are more abundant in pigmented than in unpigmented cells. We report here that two of these RNAs are of mitochondrial origin, corresponding to cytochrome oxidase subunit III and to mitochondrial large rRNA. These results suggest a correspondence of pigmentation with enhanced mitochondrial activity, presumably to meet additional energy demand incurred for the formation of melanosomes. We also present the complete sequence of the latter cDNA which contains a polyA tail, two polyadenylation signals, and a long open reading frame, the significance of which is unclear.

Materials and Methods:

All reagents, including those used for cloning and sequencing, are from common commercial sources and are listed in ref. 4 and 5. The cell lines used, unpigmented cell lines P15D and P4-1-JD and pigmented cell lines P15DI and P11, were cultured according to Chou et al (2,3). Total RNAs from both pigmented and unpigmented cells were extracted with guanidinium thiocyanate (6). PolyA$^+$ RNA was isolated by the batch affinity chromatography method (7). PolyA$^+$ RNA was also purified by using QuickPrep mRNA purification kit for the construction of cDNA library in plasmid. Details of these procedures are described in ref. 4 and 5. The P11 cDNA library in lambda Gem4 (1X10^6 pfu) was screened with a subtracted probe (8) from pigmented cell (P11) vs. unpigmented cell (P4-1-JD).

0006-291X/92 $4.00

Dot and Northern blotting of polyA+ RNA from the four cell lines was performed according to Sambrook et al (7). Eight cDNA clones were isolated whose corresponding RNAs were more abundant in pigmented than in unpigmented cells, ranging from two to five fold. Sequencing by the dideoxynucleotide chain termination method showed that one of them (designated as fmel-3) has an open reading frame coding for a peptide homologous to the C terminal of cytochrome oxidase subunit III (see results) while another cDNA of 0.7 kb contains an open reading frame coding for a peptide with no known protein homologue. The latter cDNA was used to isolate the full length cDNA, designated as fmel-2, as follows. A cDNA library (from the pigmented cell line P15DI) in plasmid (pSPORT1) was constructed by use of the SuperScript plasmid system according to manufacture's instruction. $1X10^5$ colonies were then screened by the 0.7 kb fragment. An 1.6 kb cDNA was obtained and shown to contain the same sequence in the 0.7 kb fragment. This cDNA was then completely sequenced.

To determine the origin of fmel-2 cDNA, two primers (5'-AGCTCAGACAGATAG-3', corresponding to nucleotides 538 to 552 and 5'-GGCCAATGCTCAAGCACG-3', corresponding to nucleotides 1526 to 1543) were used to amplify DNA from isolated mitochondrial DNA and nuclear genomic DNA by forty cycles of PCR amplification. The resulting material was analyzed by gel electrophoresis.

Results:

Sequence of full length cDNA fmel-2. Sequencing of the 0.7 kb fragment of fmel-2 showed that it contained a long open reading frame in its 5' end, followed by a 3' terminal with two polyadenylation sequences and a polyA tail. The deduced amino acid sequence from this open reading frame showed no homology to any known protein. We therefore proceeded to clone and sequence the full length fmel-2. Fig. 1 shows the nucleotide sequence with a long open

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1234567890 1234567890 1234567890 1234567890 1234567890
ATTAAATCAT TCTTTTACCT GAGTATGGGC GACAGAAAAG GTTCCACAAA      50
GCGATAGAAA TAGTACCGCA ACGCAAAGCT GAAAGAGAAA TGAAATAACC     100
CATATAAGCA ATAAAAAGCA AAGATTAAAC CTTGTACCTT TTGCATCATG     150
ATTTAGCCAG TACACCCAAG CAAAGAGACC TTTAGTTTGA AACCCCGAAG     200
CCAGGTGAGC TACCCCGAGA CAGCCTATTG AGGGCCAACC CGTCTCTGTG     250
GCAAAAGAGT GGGAAGAGCT CCGGGTAGAG TGACAGACCT ACCGAACCTG     300
GTGATAGCTG GTTGCCTAAG AAATGGATAG AAGTTCAGCC TCGTACTCCC     350
CAAATCAAAT AAACATTAAT AAGACAACAA GAGAAACATA CGAGAGTTAG     400
TTAAAGGGGG TACAGCCCTT TGACAAAGGA TACAACCTTT CTAGGAGGAT     450
AAAGATCATA ATACATAAAA CATACTGTTC TAGTGGGCCT AAAAGCAGGC     500
CACCTAGATA GAAAGCGTTA AGCTCAGACA GATAGAAGTT TATTATCCTG     550
ATAATATATC TTACTCCCCT AAATACTATT AGGCCAACCC ATGCCCACAT     600
GGAAGAGATT ATGCTAAAAT GAGTAACAAG AAGCCCCGCC CTTCTCCAAG     650
CACAAGTGTA AGCCAAATCG GACAAGCCAT TGGCAACTAA CGAACTCAAC     700
CAAAGAGAGC AATGTGGTAT CACAAAAAAC CTAGAAAAAC CCACAACCTA     750
AATATCGTTA CCCCCACGCT GGAGTGCAAC AAAGCAAAGA CTAAAAGAAA     800
AGGAAGGAAC TCGGCAAACA CAAGCCTCGC CTGTTTACCA AAAACATCGC     850
CTCCTGCAAC ACAACCAAGT ATAGGAGGTC CAGCCTGCCC AGTGACTACA     900
AGTTCAACGG CCGCGGTATT TTGACCGTGC AAAGGTAGCG CAATCACTTG     950
TCTTTTAATT AGAGACCTGT ATGAATGGCT AAACGAGGGC TTAACTGTCT    1000
CCCCTTTCCA GTCAGTGAAA TTGATCTACC CGTGCAGAAG CGGGTATAAT    1050
AATACAAGAC GAGAAGACCC TTTGGAGCTT AAGGTACAAA ACTCAACCAC    1100
GTTAAGCAAC TCAATAAAAA GTGAAAACTT TGTGGAACAT GAGATTTTAC    1150
CTTCGGTTGG GGCGACCACG GAGGAAAAAA AAGCCTCCAG GTGGAACGGG    1200
AAAATTTCCT AAAACTAAGA GAGACATCTC TAAGCCACAG AACATCTGAC    1250
CAAATATGAT CCGGCTAATA CAGCCGATCA ACGAACCAAG TTACCCTAGG    1300
GATAACAGCG CAATCCTCTC CCAGAGTCCA TATCGACGAG GGGGTTTACG    1350
ACCTCGATGT TGGATCAGGA CATCCTAATG GTCAGCCGCT ATTAAGGGTT    1400
CGTCTGTTCA ACGATTAAAG TCCTACGTGA TCTGAGTTCA GACCGGAGCA    1450
ATCCAGGTCA GTTTCTATCT GTAACGCTCT TTTCCTGTGC AAAGGATCGG    1500
AAAAGAGGGG CCAATGCTCA AGCACGCCCC ACCCCTAATT TATGAAAACA    1550
AATAAATAAA ATAAAGGGAG GCCAAAACCC CAGCTGGCCA AAATAAGGAC    1600
ATACTGGAAA AAAAAAAAA AAAAA                                1626
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Fig. 1. Nucleotide sequence of fmel-2, the full length cDNA for the large mitochondrial rRNA. The polyA tail and the two polyadenylation signals are underlined. There is an open reading frame beginning with nucleotide 712 and ending with nucleotide 1209.

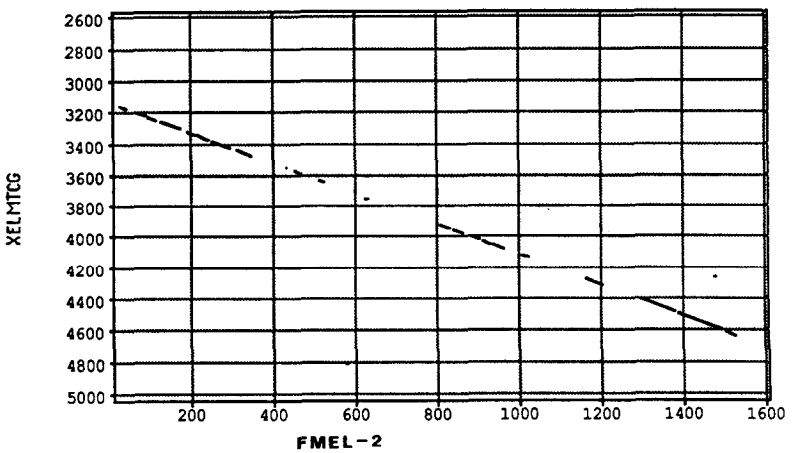

Fig. 2. **Matrix plot of fmel-2 and the large mitochondrial rRNA of *Xenopus*.** These two sequences are clearly homologous. However, it should be pointed out the 3' end of fmel-2 (after the polyadenylation signals) does not show homology to the *Xenopus* rRNA.

reading frame (nucleotides 712-1,209, capable of coding for 166 amino acids), provided that the codon TGA is read as coding for tryptophane in the fish mitochondria as in other mitochondria. When a computer search showed no known homologous protein, we searched for nucleotide homology and found that it is clearly homologous to several large mitochcondrial rRNA, particularly that of *Xenopus*. (Fig.2)

The RNAs corresponding to cDNAs fmel-2 is more abundant in pigmented than in unpigmented cell lines. After the isolation of full length fmel-2, it was used for Northern blots of RNA from two pairs of pigmented/unpigmented cell lines. (Fig. 3) The corresponding RNA is approximately 5 times more abundant in P11 than in P4-1JD, the pigmented and unpigmented cell lines originally used for cDNA isolation. In the pair of reversible pigmentation cell lines P15D and P15DI, the corresponding RNA is 50% more abundant in the pigmented cells than in the unpigmented cells. Although this difference is slight, it should be mentioned that the unpigmented P15D cells grows much more slowly (generation time more than three times longer) than the pigmented P15DI cells and thus should have much reduced energy demand for growth.

Mitochondrial origin of RNA corresponding to fmel-2. Because a portion of the 3' end of fmel-2 (about 100 nucleotides) is different from other mitochondrial rRNA and because, in one incidence, an apparent mitochondrial ribosomal RNA gene was found to be incorporated into the nucleus and resulted in a transcript with the 3' terminal derived from nuclear sequence (9), we examined the origin of fmel-2 RNA by PCR amplification with two appropriate primers and DNA from either the nucleus or the mitochondrion. The results (Fig.4) show clearly that the anticipated polynucleotide was obtained only with mitochondrial DNA, thus establishing the mitochondrial origin of this RNA.

fmel-3 contains partial sequence for fish cytochrome oxidase polypeptide III. Partial sequencing of fmel-3 showed that 28 amino acids deduced from fmel-3 DNA sequence is

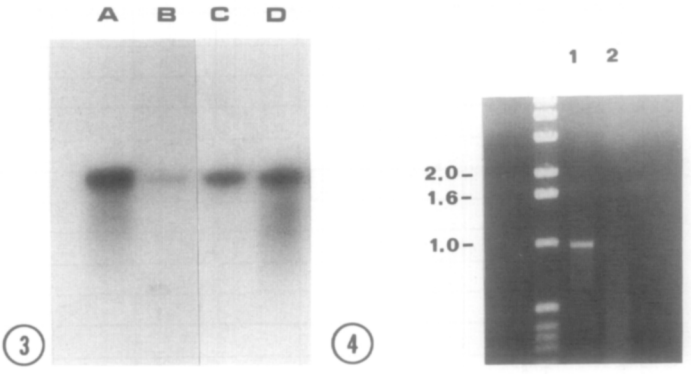

Fig. 3. The RNA corresponding to fmel-2 is more abundant in pigmented than in unpigmented cells. Same amounts of total RNA from two pairs of pigmented/unpigmented cells were subjected to Northern blot. In the case of the cell lines used for the the cloning of fmel-2, the corresponding RNA is approximately 5x more abundant in the pigmented P11 cells (lane A) than in the unpigmented P4-1JD cells (lane B). In the case of the cell lines capable of undergoing reversible differentiation/dedifferentiation, this RNA is 50% more abundant in the pigmented P15DI cells (lane D) than in the unpigmented P15D cells (lane C). It should,however, be mentioned that the latter cells, grown in the absence of fish serum, had a generation time that was at least 3x longer than the pigmented counterpart.

Fig. 4. The fmel-2 RNA is of mitochondrial origin. PCR was performed with nuclear and mitochondrial DNA and the two primers listed under Materials and Methods. It might be pointed out that one of the primers is directed to a sequence in the middle of fmel-2 (homologous to the large mitochondrial rRNA of *Xenopus*) while the second primer is directed to the 3' end of fmel-2 where there is no homology with the *Xenopus* rRNA. Lane 1: PCR with mitochondrial DNA. Lane 2: PCR with nuclear DNA. The sizes of three of the markers are indicated on the left. The results clearly show that the fmel-2 RNA is of mitochondrial origin.

identical to the C terminal of cytochrome oxidase subunit III from carp and nearly identical (one amino acid difference) to that of *Xenopus levis*. It also shares strong homology with cytochrome oxidase subunit III from other species (Fig. 5). It is thus clear that fmel-3 codes for the C-terminal of the fish cytochrome oxidase subunit III.

Discussion: Several years ago, we cloned a number of melanized cell lines after treatment of a dedifferentiated goldfish tumor (erythro-/xantho-phoroma) cell line (2,3). One of these, designated as P15, has the unusual ability to undergo reversible

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                        10        20
                        12345678901234567890123456789
 Consensus
fmel-3                  ALTILLGLYFTALQAMEYYEAPFTIADG
carp                    ALTILLGLYFTALQAMEYYEAPFTIADG
xenopus levis           TLTILLGLYFTALQAMEYYEAPFTIADG
human                   LITILLGLYFTLLQASEYFESPFTISDG
mouse                   LITIMLGLYFTILQASEYFETSFSISDG
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Fig. 5. fmel-3 encodes the C terminal 28 amino acids of cytochrome oxidase subunit III. The C terminal amino acid sequences of several vertebrate cytochrome oxidase subunit III (from SWISS-PROT protein sequence data bank) are aligned with the putative polypeptide coded by fmel-3. It is seen that the latter sequence is identical to that of carp, differs from that of *Xenopus* by only one amino acid, and is highly homologous to the other sequences.

dedifferentiation/redifferentiation by the omission/addition of fish serum in the culture medium. As these cells appear to be well suited to study the formation of the organelle melanosome, which is a major cellular component in the darkly pigmented cells, we undertook to identify those gene transcripts which are either unique or more abundant in the pigmented cells. For practical reasons, we used another pigmented cell line P11 and an unpigmented cell line P4 -1-JD (derived from culturing the pigmented cell line P4-1-J in the absence of fish serum) to prepare a subtracted probe. In what was an exploratory experiment, 8 cDNAs were obtained whose corresponding RNA is more abundant in P11 than in P4-1-JD. The results presented here show that two of these cDNAs are involved in mitochondrial function, a phenomenon that we tentatively attribute to reflect the increase of energy demand for the formation of melanosomes. As indicated in the Introduction, it is well known that different tissues have different abundance of mitochondria, in relation to the energy demand of the tissues. However, how this is regulated is unknown. It appears that the system described here may offer an opportunity to study the regulation of the cellular content of mitochondria.

We have also presented here the complete nucleotide sequence of goldfish large mitochondrial rRNA, which shares pronounced homology with the large mitochondrial rRNA of other organisms, particularly that of *Xenopus*. The sequence shows a puzzling feature, namely, the presence of all the elements of a gene coding for a protein, including a polyA tail, two polyadenylation signals, and a long open reading frame. Computer search failed to detect any protein with significant homology to the putative protein coded by this long open reading frame and we have at present no knowledge whether this is actually read and translated into a protein. In this context, we would like to mention that microinjection of Drosophila mitochondrial rRNA has been reported to restoration the pole-cell-forming ability of u.v.-irradiated *Drosophila* embryos (10). Whether this is a chance artifact or due to translation of information in this RNA is also currently unknown.

References:

(1) Matsumoto, J.; Ishikawa, T.; Prince Masahito; Takayama, S.; Taylor, J.D. and Tchen, T.T. **1984** *Differentiation* 27:36-45.
(2) Chou, S.; Taylor, J.D. and Tchen, T.T. **1989** *In vitro Cell. & Develop. Biol.* 25:813-820.
(3) Chou, S.; Yang, C.; Kimler, V.A.; Taylor, J.D. and Tchen, T.T. **1989** *Cell Differentiation and Development* 28:105-118.
(4) Peng, G. **1992** Dissertation. Wayne State University.
(5) Peng, G.; Taylor, J. D. and Tchen, T.T. **1992** Submitted.
(6) MacDonald,,R.J; Swift, G.H.; Przbyla, A.E. and Chirgwin, J.M. **1987** *Methods in Enzymology* 152:219-227.
(7) Sargent, T.D. **1987** *Methods in Enzymology* 152:423-432.
(8) Sambrook, J., Fritsch, E.F. and Maniatis, M. 1989 *Molecular cloning (2nd edition) Cold Spring Harbor Laboratory Press.*
(9) Nomiyama, H.; Fukuda, M.; Wakasugi, S.; Tsuzuki, T. and Shimada, K. **1985** *Nucleic Acids Res.* 13:1.
(10) Kobayashi, S. and Okada, M. **1989** *Development* 107:733-742.