Characterization of two *Drosophila melanogaster* cytochrome c genes and their transcripts

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ABSTRACT

Analysis of total *Drosophila melanogaster* DNA by genomic blot hybridization indicates that two cytochrome c-like sequences exist in the *Drosophila* genome. These two sequences, DC3 and DC4, have been isolated from a Charon 4A-D. *melanogaster* genomic library. DC3 and DC4 are located within a 4 kb region of DNA, at position 36A 10-11, on the left arm of chromosome 2. The nucleotide sequence of these two clones has been determined. Both DC3 and DC4 can encode functional cytochrome c proteins. The polypeptide sequences predicted by these two genes, however, differ at 32 amino acid residues. DC4 is expressed at varying, but relatively high levels throughout *Drosophila* development. In contrast, DC3 is expressed at constant, but relatively low levels throughout development.

INTRODUCTION

Cytochrome c is a small, heme-containing protein found in the mitochondria of all eucaryotic cells. It is one of a series of proteins involved in the electron transport chain, and is therefore essential for aerobic respiration. Due to structural and functional constraints, the cytochrome c proteins of widely divergent organisms exhibit considerable amino acid homology¹. Assuming this homology to be retained at the DNA level, it was considered likely that a cytochrome c gene from one organism could be used as a specific hybridization probe to isolate the cytochrome c genes of other widely divergent organisms. Utilizing this interspecies homology, cytochrome c genes from six species have now been isolated.

In this communication, we describe the isolation and characterization of two *Drosophila melanogaster* cytochrome c genes. These two genes are linked and can encode two functional, but structurally different, cytochrome c proteins. The two genes are differentially expressed and appear to be independently regulated.

MATERIALS AND METHODS

The *Drosophila melanogaster* genomic library² was constructed by randomly shearing genomic DNA from the *D. melanogaster* strain Canton-S. Fragments approximately 20 kb long were isolated after centrifugation through a sucrose gradient. *EcoRI* linkers were ligated onto these fragments, which were then ligated onto the purified *EcoRI* arms of the lambda phage Charon 4A³. From the expected size of the *Drosophila* DNA inserts (20 kb) and the approximate size of the *Drosophila* genome (1.5×10^5 kb), it was estimated that screening approximately 35,000 phage would ensure, with 99% probability, that the entire *Drosophila* genome was represented⁴. The hybridization probe was the 0.95 kb *Bam*HI-*EcoRI* fragment of pMC1⁵, which contains the entire coding region of the mouse 'somatic' cytochrome c gene. Hybridization and washing conditions were the same as those previously described⁶. DNA sequencing was performed by the dideoxynucleotide chain-termination procedure of Sanger *et al*⁷. Genomic DNA and polyadenylated RNA were obtained from Thomas Fuerst (Cornell University) and J. Tso (Cornell University).

RESULTS

Isolation of Two *Drosophila melanogaster* Cytochrome c Genes

A Charon 4A-*Drosophila melanogaster* genomic library was screened, using the mouse cytochrome c gene, MC1, as a specific hybridization probe. Approximately 200,000 plaques were analyzed and seven recombinant clones isolated. Restriction enzyme and hybridization analysis revealed that all seven clones contain overlapping fragments of the same region of *Drosophila* DNA. Further analysis was therefore restricted to a single recombinant

Fig. 1. Partial restriction enzyme map and DNA sequencing strategy of two *D. melanogaster* cytochrome c genes. The direction of transcription of DC3 and DC4 is from right to left. Arrows indicate the nucleotide sequencing strategy used.

Fig. 2. Nucleotide sequences of two *D. melanogaster* cytochrome c genes. The nucleotide sequences of DC4 and DC3 are aligned for maximum homology. The amino acid sequence predicted by DC4 is displayed on the line above the nucleotide sequences and the amino acid sequence predicted by DC3 is presented on the line below the nucleotide sequences. The numbering system corresponds to DC4. Nucleotides are numbered on the line below the nucleotide sequences and amino acid residues are numbered on the line above the nucleotide sequences. Nucleotides -660 to -501 are not shown for DC3. Putative control sequences are overlined in DC4 and underlined in DC3.

[illegible]

phage, Ch4A-DC1. This clone contains two hybridizing regions approximately 3.5 kb apart (Fig. 1). Both hybridizing regions were subcloned into pUC13⁸, and designated pDC3 and pDC4. The nucleotide sequence of DC3 and DC4 was determined (Fig. 2). Both genes appear to encode functional, but structurally different, cytochrome c proteins.

The polypeptide sequence predicted by DC4 is very similar, but not identical, to a published *Drosophila* cytochrome c amino acid sequence⁹. The nucleotide sequence predicts that residues 50, 60 and 61 are aspartate, asparagine and glutamate, respectively. The published amino acid sequence shows that these three residues are asparagine, glutamate and aspartate. All three differences could easily be due to errors involved in amino acid sequence analysis. Therefore, the polypeptide sequence predicted by the nucleic acid data is probably correct.

DC4 encodes a protein 107 amino acids long. DC3 encodes a protein 104 amino acids long. When aligned for maximum homology, the two proteins differ at 32 amino acid residues. DC4 encodes a protein which is very homologous with other cytochrome c proteins. In contrast, DC3 encodes a cytochrome c protein which is relatively unique. In Fig. 3, the predicted polypeptide sequences of DC3 and DC4 are compared to a composite amino acid sequence of 30 cytochrome c proteins¹⁰. Thirteen of the amino acid residues predicted by DC3 are not found in the composite sequence. Twelve of these differences, however, occur at variable or hypervariable positions, and most result in substitutions of a conservative nature. The only substitution that occurs at an invariant position is at residue 70, where an aspartate is predicted by DC3 and an asparagine is usually seen. Although residue 70 is evolutionarily invariant, it does not appear to be an absolutely essential residue for cytochrome c function. Therefore, since none of the amino acid substitutions predicted by DC3 involve

Fig. 3. Comparison of the predicted amino acid sequences of DC3 and DC4, and a composite sequence of 30 cytochrome c proteins. The polypeptide sequences deduced from the nucleotide sequences of DC3 and DC4 are presented. A composite polypeptide sequence of 30 different cytochrome c proteins is presented on the line(s) above the DC3 and DC4 sequences. The predominant residue at each amino acid position of the 30 different cytochrome c sequences is presented on the bottom line of the composite sequence. Underlined amino acids indicate those residues encoded by DC3 which differ from the composite sequence. (-) indicates positions at which gaps have been introduced into the composite sequence to maximize homology. Gaps have also been introduced into the DC3 and DC4 sequences to maximize homology.

Fig. 4. Hybridization analysis of *D. melanogaster* genomic DNA. Each lane contains five μ g of genomic DNA from adult Canton-S *D. melanogaster* flies. Samples were digested with *Eco*RI (E), *Hind*III (H) and/or *Sst*I (S), resolved by gel electrophoresis, transferred to nitrocellulose and hybridized at 37°C to the 32 P-labeled *Eco*RI-*Sst*I fragment of pDC3 (coding region of DC3) and the 32 P-labeled *Hind*III-*Hind*III fragment of pDC4 (coding region of DC4). Size standards are the *Eco*RI-*Hind*III digestion fragments of λ phage DNA.

essential residues, the cytochrome c protein encoded by DC3 is probably functional.

Complexity of Cytochrome c-Like Sequences in the *Drosophila melanogaster* Genome

In order to investigate the complexity of cytochrome c-like sequences in the *Drosophila melanogaster* genome, total DNA was isolated from adult Canton-S flies, cut with various restriction endonucleases, fractionated by electrophoresis, transferred to nitrocellulose and hybridized to a mixed probe containing the coding regions of both DC3 and DC4 (Fig. 4). Two hybridizing fragments are observed in each lane, indicating that no other

Fig. 5. Hybridization analysis of polyadenylated RNA from four stages of *D. melanogaster* development. Each lane contains six μ g of polyadenylated RNA from first instar (1), third instar (3) or pupa (P), or three μ g of polyadenylated RNA from adult (A). Samples were resolved by gel electrophoresis, transferred to nitrocellulose and hybridized at 23°C to, (A) the ³²P-labeled PstI-PstI fragment of pDC4 (3'-noncoding region of DC4), or (B) the ³²P-labeled SstI-HindIII fragment of pDC3 (3'-noncoding region of DC3). Size standards are the PstI, EcoRI and EcoRI-HindIII digestion fragments of pDC3. The X-ray film in Fig. 5B was exposed approximately seven times longer than the film in Fig. 5A.

cytochrome c-like sequences exist in the *Drosophila* genome. The size of these two hybridizing fragments correspond exactly with the size of the restriction enzyme fragments expected from digestion of DC3 and DC4 (Fig. 1). In situ hybridization analysis has determined that DC3 and DC4 are located on the left arm of chromosome 2, at position 36A 10-11 (R. MacIntyre, personal communication).

Analysis of *Drosophila melanogaster* Cytochrome c mRNA

Polyadenylated RNA was isolated from four stages of *Drosophila* development; first instar, third instar, pupa and adult. This mRNA was fractionated on an agarose-formaldehyde gel, transferred to a nitrocellulose filter and hybridized to the 3'-noncoding region of DC4

(Fig. 5A), or the 3'-noncoding region of DC3 (Fig. 5B).

The DC4 fragment hybridizes to a single mRNA species. This transcript is approximately 900 nucleotides long and is present at relatively high levels throughout *Drosophila* development. DC4 does not appear to be expressed at constant levels in the four stages of development examined. In fact, when adjusted for varying amounts of sample loaded, the ratio of expression of DC4 is approximately 6:1:2.4:6, for the first instar, third instar, pupa and adult, respectively.

The DC3 fragment also hybridizes to a single mRNA species. This transcript is approximately 2100 nucleotides long and is present at low levels throughout *Drosophila* development. Unlike DC4, the relative expression of DC3 does not appear to vary appreciably at different stages of development.

In general, DC4 is expressed at much higher levels than DC3. In fact, densitometry analysis indicates that in the third instar, where expression of DC4 is lowest, expression of DC4 is approximately 25 times greater than expression of DC3.

RNA was also isolated from the three segments of adult *Drosophila* flies; head, thorax and abdomen. RNA from these three segments was fractionated on an agarose-formaldehyde gel, transferred to nitrocellulose and hybridized to the 3'-noncoding region of DC3, or the 3'-noncoding region of DC4.

The DC3 transcript is present at low, but constant levels in each of the three segments (results not shown). Expression of DC4, however, is greatest in the thorax (Fig. 6). In fact, densitometry analysis indicates that the ratio of expression of DC4 is approximately 1.3:4:1 for the head, thorax and abdomen, respectively.

The thorax contains large amounts of adult flight muscles. These cells have a very high rate of respiration and contain numerous mitochondria. Therefore, it is not surprising that the DC4 cytochrome c gene is expressed at higher levels in the thorax than in the head or abdomen. In conclusion, it appears that expression of DC4 is developmentally regulated and tissue specific. In contrast, expression of DC3 appears to be constitutive, and unaffected by developmental variations.

Nucleotide Sequence Analysis of DC3 and DC4

The sequence, TATA, is often found approximately 30 bp upstream from the start of mRNA transcription¹¹. This sequence is found at position -103 in DC4 and at position -84 in DC3. The hexanucleotide sequence, AATAAA, is

Fig. 6. Hybridization analysis of total RNA from three segments of adult *D. melanogaster* flies. Each lane contains ten μ g of total RNA from head (H), thorax (T) or abdomen (A). Samples were resolved by gel electrophoresis, transferred to nitrocellulose and hybridized at 23°C to the 32 P-labeled PstI-PstI fragment of pDC4 (3'-noncoding region of DC4). Size standards are the same as Fig. 5.

commonly found approximately 20 bp upstream from the site of poly(A) addition¹². This sequence is found at position +514 in DC4 and at position +613 in DC3.

Assuming that transcription of DC4 starts approximately 30 nucleotides downstream from the putative promoter sequence (position -103) and polyadenylation occurs approximately 20 nucleotides downstream from the putative polyadenylation signal sequence (position +514) and by allowing an additional 300 nucleotides for the poly(A) tail, the resultant DC4 mRNA molecule is predicted to be approximately 910 nucleotides long. Since the observed DC4 transcript is approximately 900 nucleotides long (Fig. 5A), the size of the estimated and observed DC4 mRNA molecules agree.

In contrast, the size of the observed and estimated DC3 mRNA molecules do not agree. Assuming that transcription of DC3 starts approximately 30

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Amino acid	Codon sequence	<u><i>Drosophila melanogaster</i></u>		<u><i>Saccharomyces cerevisiae</i></u>		Chicken CC9	Mouse MC1	Rat RC4
		DC4	DC3	CTC1	CTC7			
Phe	UUU	0	1	2	3	3	1	2
Phe	UUC	4	2	2	1	1	3	2
Leu	UUA	0	0	1	2	1	0	0
Leu	UUG	0	2	5	3	1	1	1
Leu	CUU	0	2	1	0	1	1	1
Leu	CUC	0	2	0	0	1	1	1
Leu	CUA	0	0	1	0	0	1	0
Leu	CUG	8	0	0	0	2	2	3
Ile	AUU	0	2	2	3	3	2	2
Ile	AUC	5	0	2	1	3	3	3
Ile	AUA	0	2	0	1	1	1	1
Met	AUG	1	1	2	3	2	2	2
Val	GUU	4	1	1	1	1	2	2
Val	GUC	0	1	0	1	1	0	0
Val	GUA	0	0	0	1	1	0	0
Val	GUG	1	5	2	0	0	1	1
Ser	UCU	0	1	2	2	2	1	1
Ser	UCC	0	0	0	1	1	0	0
Ser	UCA	0	1	1	1	1	0	0
Ser	UCG	1	0	1	0	0	0	0
Pro	CCU	1	0	1	3	0	1	1
Pro	CCC	4	1	0	0	1	1	1
Pro	CCA	0	1	3	2	2	1	1
Pro	CCG	0	1	0	0	0	0	0
Thr	ACU	0	0	3	1	4	3	3
Thr	ACC	6	3	3	1	0	2	2
Thr	ACA	0	3	2	3	3	3	3
Thr	ACG	1	0	0	4	1	0	0
Ala	GCU	2	1	3	2	1	5	5
Ala	GCC	6	4	4	2	2	2	2
Ala	GCA	0	3	0	3	1	1	1
Ala	GCG	3	1	0	1	1	0	0
Tyr	UAU	0	1	3	3	2	2	2
Tyr	UAC	4	4	2	2	2	2	2
Stop	UAA	1	0	1	0	1	1	1
Stop	UAG	0	1	0	1	0	0	0
His	CAU	1	0	2	3	2	1	2
His	CAC	2	2	2	0	1	2	1
Gln	CAA	0	0	2	0	1	0	1
Gln	CAG	3	2	0	3	2	3	2
Asn	AAU	1	3	2	1	2	3	2
Asn	AAC	4	2	5	6	2	2	3
Lys	AAA	0	3	6	8	5	5	6
Lys	AAG	15	14	10	9	13	13	12
Asp	GAU	2	3	1	4	4	3	3
Asp	GAC	2	2	3	1	1	1	1
Glu	GAA	0	1	5	3	2	3	5
Glu	GAG	6	5	2	3	5	4	2
Cys	UGU	0	1	2	2	1	1	1
Cys	UGC	2	2	1	0	1	1	1
Stop	UGA	0	0	0	0	0	0	0
Trp	UGG	1	1	1	1	1	1	1
Arg	CGU	0	0	0	0	0	0	0
Arg	CGC	3	1	0	0	1	0	0
Arg	CGA	0	0	0	0	0	0	0
Arg	CGG	0	1	0	0	0	1	1
Ser	AGU	0	0	0	2	0	0	0
Ser	AGC	0	0	0	0	0	0	0
Arg	AGA	0	0	3	2	1	0	0
Arg	AGG	0	0	0	1	0	1	1
Gly	GGU	5	4	8	8	3	2	3
Gly	GGC	5	7	2	1	4	4	3
Gly	GGA	4	2	0	2	6	7	7
Gly	GGG	0	2	2	1	0	1	1

nucleotides downstream from the putative promoter sequence (position -84) and polyadenylation occurs approximately 20 nucleotides downstream from the putative polyadenylation signal sequence (position +613) and by allowing an additional 300 nucleotides for a poly(A) tail, the resultant DC3 mRNA molecule is predicted to be approximately 1000 nucleotides long. The actual DC3 transcript is approximately 2100 nucleotides long. Since there is a large discrepancy between the observed and estimated DC3 mRNA molecules, it is possible that the DC3 transcript is not initiated or polyadenylated at the putative promoter and polyadenylation sequences discussed above. Preliminary S1 nuclease analysis, however, indicates that transcription of DC3 begins approximately 20 bp downstream from the putative promoter sequence at position -84 (results not shown).

DISCUSSION

Drosophila melanogaster contains two functional cytochrome c genes, DC3 and DC4. These two genes are located 3.5 kb apart and have obviously arisen from an ancient duplication event. DC3 and DC4 can encode two separate cytochrome c proteins, which differ at 32 amino acid residues. The Unit Evolutionary Period (UEP) of cytochrome c is approximately 20 million years (MYs)¹³. This means that it takes approximately 20 MYs for two identical cytochrome c proteins to diverge by 1%. Assuming this calculation to be correct, DC3 and DC4 diverged approximately 600 MYs ago. Since insects are thought to have diverged from vertebrates approximately 600 MYs ago¹⁴, other insects may also contain two cytochrome c genes.

Cytochrome c genes from six species have now been isolated and sequenced; the CYC1¹⁵ and CYC7¹⁶ genes of the yeast Saccharomyces cerevisiae, the PoCYC gene of the fission yeast Schizosaccharomyces pombe¹⁷, the CC9(CC10) alleles of chicken⁶, the RC4 gene of rat¹⁸, the MC1 gene of mouse⁵, and the DC3 and DC4 genes of Drosophila melanogaster.

D. melanogaster contains two cytochrome c-like sequences per haploid genome. Chicken⁶ and S. cerevisiae¹⁶ contain one and two cytochrome c-like sequences, respectively. In contrast, mammals contain approximately 20-30 cytochrome c-like sequences¹⁹, most of which appear to be nonfunctional pseudogenes^{5,20}.

Fig. 7. Codon usage of seven cytochrome c genes. The codon usage of the DC3 and DC4 genes of D. melanogaster, the CYC1 and CYC7 genes of S. cerevisiae, the CC9 gene of chicken, the MC1 gene of mouse and the RC4 gene of rat are presented.

Chicken and S. pombe contain one functional cytochrome c gene per haploid genome. D. melanogaster and S. cerevisiae appear to contain two functional cytochrome c genes per haploid genome. The two Drosophila genes, DC3 and DC4, are differentially expressed. Expression of DC4 is relatively high, and is stage and tissue specific. In contrast, expression of DC3 is relatively low and is not stage or tissue specific. The two yeast genes, CYC1 and CYC7, are also differentially expressed²¹. In fact, CYC1 contributes approximately 90-95% of the total cytochrome c of the yeast cell²². Expression of CYC1 appears to be regulated by the level of intracellular heme²³. In contrast, CYC7 does not appear to be regulated by the level of intracellular heme²⁴. Therefore, both D. melanogaster and S. cerevisiae contain two differentially expressed cytochrome c genes; one gene which is expressed constitutively at low levels, and another gene which is expressed at high levels under tight regulatory control.

The two Drosophila cytochrome c genes are more homologous to CYC1 than to CYC7. Therefore, it appears that both DC3 and DC4 evolved from an ancestral CYC1 gene, rather than one gene evolving from CYC1 and the other gene evolving from CYC7. This observation is not surprising considering that the two Drosophila genes diverged approximately 300 MYs after insects and yeast diverged¹⁴.

DC3 and DC4 do not appear to contain intervening sequences. The S. cerevisiae and S. pombe cytochrome c genes also do not contain intervening sequences. In contrast, the mouse (MC1) and rat (RC4) cytochrome c genes each contain two intervening sequences, one in the 5'-noncoding region (position -12), and one in the coding region. These introns are located at identical positions and appear to be of comparable lengths. The chicken (CC9) cytochrome c gene contains an intervening sequence which interrupts the coding region at the same position as the coding region intron of MC1 and RC4. The chicken gene, however, does not appear to contain a 5'-noncoding region intron. It therefore appears that intervening sequences are not a constant feature of the cytochrome c gene throughout evolution.

A comparison of the codon usage of seven cytochrome c genes is presented in Fig. 7. In general, the codon usage of these genes is relatively random. However, the codon usage of DC4 is considerably biased. For example, each of the lysine (15), leucine (8), glutamate (6), isoleucine (5), phenylalanine (4), tyrosine (4), glutamine (3), and arginine (3) residues utilize the same codon sequence. Compared to DC4,

the codon usage of DC3 is relatively random. In addition, in a few instances the two genes utilize two different classes of codon sequences. For example, DC4 contains five isoleucine residues and utilizes the AUC codon five times. DC3, on the other hand, contains four isoleucine residues and utilizes each of the AUU and AUA codons twice, and does not use the AUC codon at all. Similarly, DC4 contains eight leucine residues and utilizes the CUG codon eight times. In contrast, DC3 contains six leucine residues and utilizes each of the UUG, CUU and CUC codons twice, but does not use the CUG codon. DC3 and DC4 therefore encode proteins having the same function, in the same organism, yet exhibit totally different patterns of codon usage.

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