Expression of *Drosophila* homologue of senescence marker protein-30 during cold acclimation

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Abstract

Gene expression during cold acclimation at a moderately low temperature (15°C) was studied in *Drosophila melanogaster* using a subtraction technique. A gene homologous to senescence marker protein-30 (SMP30), which has a Ca<sup>2+</sup>-binding function, was up-regulated at the transcription level after acclimation to 15°C. This gene (henceforth referred to as *Dca*) was also expressed at a higher level in individuals reared at 15°C from the egg stage than in those reared at 25°C. Moreover, *DCA* mRNA increased at the senescent stage in *Drosophila*, although SMP30 is reported to decrease at senescent stages in mammals. In situ hybridization to polytene chromosomes revealed that the *Dca* gene was located at 88D on chromosome 3R. The 5' flanking region of this gene had AP-1 (a transcription factor of SMP30) binding sites, stress response element and some other transcription factor binding sites. The function of *DCA* was discussed in relation to the possible regulation of cytosolic Ca<sup>2+</sup> concentration. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Drosophila*; Cold acclimation; *Dca*; Senescence marker protein-30; Senescence

1. Introduction

In general, ectothermal organisms, from bacteria to higher plants or vertebrates, become more cold tolerant when maintained at low temperature. In bacteria, plants or fish, this change in cold tolerance is suggested to be associated with changes in fatty acid composition of phospholipids; percentages of saturated fatty acids in phospholipids decrease in individuals reared at low temperature, and this change results in the maintenance of membrane functions at cold (Hochachka and Somero, 1984; Hazel and Williams, 1990). However, Ohtsu et al. (1998, 1999) found that *Drosophila* species exhibit an inverse response; individuals acclimated to low temperature decreased the degree of unsaturation of phospholipids. Thus, the relation between the qualitative changes of phospholipids and cold tolerance is still unclear in *Drosophila*. On the other hand, it is well known in insects that cryoprotectants such as glycerol, sorbitol or trehalose are often accumulated when insects are maintained at low temperatures (reviewed by Storey and Storey, 1991). In addition, novel proteins called ‘cold acclimation proteins’ have been recently reported in some bacteria and plants which were acclimated to low temperature (Neven et al., 1993; Berger et al., 1997; Monroy et al., 1998). Moreover, in many cold-water marine fishes and terrestrial arthropods, antifreeze protein and ice-nucleating proteins were reported (reviewed by Lee, 1991). However, there have been no reports of such proteins in *Drosophila*. Here, I report an attempt to find genes that are specifically expressed during acclimation at moderate cold in *Drosophila melanogaster* using subtraction technique.

2. Materials and methods

2.1. Flies

*D. melanogaster* Meigen (Canton S strain) was maintained under laboratory conditions (continuous light at 25°C) on cornmeal–malt medium, and used for experiments 7 days after eclosion.
2.2. Cold acclimation

Flies maintained at 25°C were transferred to vials (containing food medium) that were cooled to 15°C prior to experiment, and then maintained at 15°C (continuous light) for 1 or 2 days. Half lethal temperature (temperature that kills half the population when exposed for 24 h) was 3.0°C for individuals reared at 25°C to the 7-day adult stage, and 1.0°C for those acclimated to 15°C for 1 day. When flies were reared at 15°C from the egg stage to the 14-day adult stage, half lethal temperature was −3.4°C.

2.3. RNA extraction and mRNA purification

RNA was extracted according to Goto et al. (1998). mRNA was purified from total RNA using BioMag mRNA purification kit (PerSeptive Biosystems) according to the supplier’s instruction.

2.4. Subtraction and differential screening

2.4.1. cDNA synthesis

ds CDNAs were synthesized from 4 μg of poly(A)+ RNA with oligo (dT) according to Sambrook et al. (1989) and digested with Rsal.

Henceforth, the cDNA derived from flies acclimated to 15°C for 1 day and reference cDNA derived from control flies reared at 25°C to the 7-day adult stage were referred to as ‘tester’ and ‘driver’ respectively. Digested cDNAs were phenol-extracted, ethanol-precipitated and dissolved in 5.5 μl of water.

2.4.2. Adaptor ligation and subtractive hybridization

The following procedure was mainly according to Gurskaya et al. (1996). One μl of tester and driver were diluted with 5 μl of water. Then, 2 μl of tester or driver solution was mixed with 2 μM Adaptor 1 or 2 μM Adaptor 2R (Table 1) in 10-μl reactions. After ligation at 16°C for 18 h with 350 U of T4 DNA ligase (TaKaRa), the products were added with 1 μl of 0.5 M EDTA, heated at 70°C for 5 min and stored at −20°C.

For the first round of the subtractive hybridization, 1.5 μl of Rsal I-digested driver was mixed with 1.5 μl of Adaptor 1-ligated tester or Adaptor 2R-ligated tester: the former and latter mixtures were referred to as ‘forward A’ and ‘forward B’, respectively. In addition, 1.5 μl of Rsal I-digested tester was mixed with 1.5 μl of Adaptor 1-ligated driver or Adaptor 2R-ligated driver: the former and latter mixtures were referred to as ‘reverse A’ and ‘reverse B’, respectively. These mixtures were added with 1 μl of hybridization buffer (200 mM HEPES–HCl, pH 7.5/2 M Na/0.08 mM EDTA). After mineral oil was overlaid on the solutions, DNA was denatured at 98°C for 1.5 min and annealed at 68°C for 12 h.

For the second round of the subtractive hybridization, 1 μl of Rsal I-digested driver or Rsal I-digester tester was mixed with 1 μl of hybridization buffer and 2 μl of water. These solutions were overlaid with mineral oil and heated at 98°C for 1.5 min. Forward A and reverse A mixtures were mixed with forward B and reverse B, respectively. Then, forward mixture was added with 1 μl of freshly denatured driver, and reverse mixture was added with 1 μl of denatured tester. After incubation at 68°C for 24 h, samples were mixed with 200 μl of dilution buffer (20 mM HEPES–HCl, pH 8.3/50 mM NaCl/0.2 mM EDTA, pH 8.0) and stored at −20°C.

2.4.3. Selective PCR amplification

Primary PCR used 1μl of the sample and final concentration of 0.2 mM of dNTP, 0.4 μM of PCR primer 1 (Table 1) 1×PCR reaction buffer as formulated by Clontech and 1×Advantage cDNA polymerase mix (Clontech) in total volume of 25 μl. After incubation at 75°C for 5 min and heating at 94°C for 25 s, 35 cycles of PCR (10 s at 94°C; 30 s at 66°C; 1.5 min at 72°C) were conducted.

Secondary reaction used the same components for the primary reaction, except that the primers were Nested PCR primer 1 and 2R (Table 1) and the target was 1 μl of 10-fold-diluted primary PCR products. PCR conditions were 15 cycles of 10 s at 94°C, 30 s at 68°C, and 1.5 min at 72°C. Henceforth, PCR products derived from the forward mixture and from the reverse one in subtractive hybridization were referred to as ‘forward subtracted cDNAs’ and ‘reverse subtracted cDNAs’, respectively.

2.4.4. Differential screening

Forward subtracted cDNAs were ligated into plasmid using pGEM-T Vector (Promega) according to the supplier’s instruction.

cDNA inserts from colonies were amplified with colony direct PCR. The reaction used 0.5 U of AmpliTaq (Perkin Elmer) and final concentration of 1×PCR buffer II as formulated by Perkin Elmer, 0.5 μM of Nested PCR primer 1 and 2R (Table 1), 0.2 mM of dNTP and 1.5 mM of MgCl2 in a total volume of 20 μl. Amplification was performed with 28 cycles of 30 s at 93°C, 30 s at 68°C, and 30 s at 72°C.

PCR products were mixed with the same volume of 0.6 N NaOH. One μl of these mixtures were blotted on to two nylon membranes.

Forward and reverse subtracted cDNAs were digested with Rsal I, EcoT52 I and Sma I, and applied to QIAquick PCR purification kit (QIAGEN) in order to remove the Adaptor 1 and 2R. DNA was labeled with Digoxigenin-11dUTP using DIG DNA labeling kit (Boehringer Mannheim).

Each membrane was hybridized with probes derived from forward and reverse subtracted cDNAs, respectively. Hybridization and detection was performed using
Table 1
Sequences of primers and adaptors

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>DF-1</td>
<td>5'-CTC CAG CAG GAT TTT ACC GGT GCT T-3'</td>
</tr>
<tr>
<td>DF-2</td>
<td>5'-GAC CTC CAA AAG CCA CCG AGG TGA T-3'</td>
</tr>
<tr>
<td>DF-3</td>
<td>5'-AAC AGG GTG CGA GTG ACC TT-3'</td>
</tr>
<tr>
<td>DF-5</td>
<td>5'-TGA AGA TAT CGC CAC TGT CGG-3'</td>
</tr>
<tr>
<td>DF-6</td>
<td>5'-CGA GTT CCA CGT AGT ACA GAC TCT G-3'</td>
</tr>
<tr>
<td>DF-7</td>
<td>5'-CCT CAC ACC TAC ACA TAG CA-3'</td>
</tr>
<tr>
<td>DF-8</td>
<td>5'-GCA ACA CTC GAA TAA GGC A-3'</td>
</tr>
<tr>
<td>DR-1</td>
<td>5'-AGG TGC AGC CGG ATC TGA AGG AAA A-3'</td>
</tr>
<tr>
<td>DR-2</td>
<td>5'-TCA TCG TCC AGT GGG ATG GAG TCT C-3'</td>
</tr>
<tr>
<td>DR-3</td>
<td>5'-CTA CAA TCA GAG CAC CGG CG-3'</td>
</tr>
<tr>
<td>DR-5</td>
<td>5'-CCA CTA ACT ACG ACC GGG TT-3'</td>
</tr>
<tr>
<td>DR-X</td>
<td>5'-AAG GAG TCG GGA CAG GGG CAC AGT T-3'</td>
</tr>
<tr>
<td>DR-X2</td>
<td>5'-TTG CTC GCG CTC TCA AGA-3'</td>
</tr>
<tr>
<td>DR-X3</td>
<td>5'-GTG TGT GAG ATT GCG AAT GG-3'</td>
</tr>
<tr>
<td>M13-M4</td>
<td>5'-GTT TTC CCA GTC ACG AC-3'</td>
</tr>
<tr>
<td>M13-RV</td>
<td>5'-CAG GAA ACA GCT ATG AC-3'</td>
</tr>
<tr>
<td>Nested PCR primer 1</td>
<td>5'-TCG AGC GGC CGC CCG GGC AGG T-3'</td>
</tr>
<tr>
<td>Nested PCR primer 2R</td>
<td>5'-AGC GTG GTC GCG GCC GAG GT-3'</td>
</tr>
<tr>
<td>PCR primer 1</td>
<td>5'-CTA ATG CTA CAC ATG ATG CCA-3'</td>
</tr>
<tr>
<td>Adaptor primer 1</td>
<td>5'-CCA TCC TAA TAC GAC TCA TTA TAG GCC-3'</td>
</tr>
<tr>
<td>Adaptor 1</td>
<td>5'-CTA ATA CTA CTC ACT ATA GGG ACC TCT A-5'</td>
</tr>
<tr>
<td>Adaptor 2R</td>
<td>5'-CTA ATG CTA CTC ACT ACA TGG ACC TCT A-5'</td>
</tr>
<tr>
<td>RACE adaptor</td>
<td>5'-CTA ATG CTA CTC ACT ATA GGG ACC TCT A-5'</td>
</tr>
</tbody>
</table>

DIG luminescent detection kit (Boehringer Mannheim) according to the supplier’s instruction.

2.5. Northern hybridization

Poly(A)+ RNAs were electrophoresed in denaturing gel and transferred to nylon membrane according to Sambrook et al. (1989). Hybridization and detection were made as above. D. melanogaster ras2 gene probe, pUC8-HB-1.2 kb, was used as a control (Bishop and Corces, 1988; Juni et al., 1996).

2.6. RACE

To sequence the full length of cDNA, RACE (Rapid Amplification of cDNA Ends) was performed according to Siebert et al. (1995). ds cDNA was ligated to RACE adaptor (Table 1). The ligation product was diluted to 250-fold with water. Primary reaction for nested PCR used 1 μl of the sample and a final concentration of 0.2 mM of dNTP, 0.2 μM of Adaptor primer, 0.2 μM of DF-2 primer for 5'-RACE but DR-2 primer was used instead of DF-2 for 3'-RACE (Table 1) and 1× Advantage cDNA polymerase mix (Clontech). Amplification was performed for 30 s denaturing at 94°C, 5 cycles of 5 s at 94°C and 2 min at 72°C, 5 cycles of 5 s at 94°C and 2 min at 72°C, and 25 cycles of 5 s at 94°C and 2 min at 68°C.

Secondary reaction used the same components for the primary reaction, except that the primers for 5'- and 3'-RACE were DF-1 and DR-1 (Table 1), respectively, and the targets were 1 μl of 250-fold-diluted primary products. PCR conditions were as above, except that the final step was 20 cycles.

2.7. DNA extraction

Genome of D. melanogaster was extracted with QIAGEN genomic-tip (QIAGEN) according to supplier’s instruction.

2.8. Amplification of 5' flanking region

Genome was digested with Dra I, Sca I, Eco RV, Pvu II and Stu I. Digested products were ligated to RACE adaptor and diluted to 10-fold with water.

Primary reaction for nested PCR used the same components that were used in RACE, except that the primer was DF-2 (Table 1) and the targets were 1 μl of the adaptor-ligated genomes. Amplification was performed with 30 s denaturing at 94°C, 5 cycles of 5 s at 94°C and 3 min at 72°C, 5 cycles of 5 s at 94°C and 3 min at 70°C, and 25 cycles of 5 s at 94°C and 3 min at 68°C.

Secondary reaction used the same components that were used in the primary reaction, except that the target was 1 μl of 100-fold-diluted primary products and the primer was DF-6 (Table 1). PCR conditions were the
same as for the primary reaction, except that the final step was 15 cycles. The amplified fragments were ligated into pGEM-T Vector (Promega).

2.9. Sequencing

Plasmids were purified using QIAPrep Spin Miniprep kit (QIAGEN). The sequence was obtained from a 373A DNA sequencer (PE Applied Biosystems) using Dye Primer and Dye Terminator Cycle Sequencing FS Ready Reaction kit (PE Applied Biosystems).

2.10. In situ hybridization

In situ hybridization to salivary gland chromosomes was performed using DIG-labeled probe according to the method of Langer-Safer et al. (1982) and Engels et al. (1986).

3. Results

3.1. Selective PCR amplification and differential screening

After the selective PCR amplification, forward subtracted cDNAs showed six major and some minor bands in agarose–gel electrophoresis. For differential screening, 72 colonies were amplified and blotted onto the membranes. Among them, 12 clones showed stronger signals when detected using forward subtracted cDNAs as probes than when detected using reverse subtracted cDNAs as probes (data not shown). According to their length and restriction sites, these 12 clones were subdivided into six groups and these groups coincided with the major six bands observed in the electrophoresis of forward subtracted cDNAs, respectively (data not shown).

3.2. Northern hybridization analysis

Northern hybridization was performed using the above six fragments as probes. It appeared that a gene was clearly up-regulated in flies acclimated to 15°C for 1 day (Fig. 1), while the remaining five showed only slight differences between acclimated and control flies (data not shown). In Northern hybridization using this positive fragment as a probe, two bands of approx. 1.6 and 1.1 kb in length, were observed, but the signal of the 1.6 kb band was very weak or sometimes undetectable. The major signal became weaker in flies acclimated to 15°C for 2 days than in those acclimated for 1 day, but still stronger than in control flies (Fig. 1). Henceforth, this positive gene was referred to as Dca (Drosophila cold acclimation gene).

3.3. In situ hybridization analysis

In order to confirm the location of Dca gene, in situ hybridization to salivary gland chromosomes was performed. The probe hybridized at 88D on chromosome 3R (Fig. 2).

3.4. RACE

The Dca fragment was sequenced using M13-M4, M13-RV, Nested PCR primer 1 and 2R. After the sequencing, DF-1, DF-2, DR-1 and DR-2 primers were designed and used for 5'- and 3'-RACE reactions (Table 1). Single and double bands were amplified in 5'- and 3'-RACE reactions, respectively. These PCR products were subcloned and sequenced using DF-1, DF-5, DR-1, DR-3, M13-M4 and M13-RV primers (Table 1). For 5'-RACE products, 10 independent clones were sequenced in order to obtain the full sequence at the 5' end of mRNA. Sequences of both long and short 3'-RACE products were identical except that long product had two inserted sequences (Fig. 3). The long cDNA (combination of 5'-RACE and long 3'-RACE products)
has a shorter open reading frame (ORF) than the short cDNA (combination of a 5'-RACE and short 3'-RACE products) because the insertion had a terminator codon. In addition, both ends of the insertion followed Chambon's rule (Fig. 3); the 5' end of the intron has GT and the 3' end of the intron has AG (Breathnach and Chambon, 1981). Therefore, it is considered that the longer cDNA is hnRNA, and therefore one of the insertions is, at least, an intron (Fig. 3). Because the DF-1 primer extended over two exons, 5'-RACE using this primer did not amplify hnRNA. Thus, translatable mRNA would be the short cDNA (combination of 5'-RACE and short 3'-RACE products) with a length of 1022 bp and ORF of 909 bp, which encodes 303 amino acids (Fig. 4; the nucleotide sequence was available from DDBJ/GenBank/EMBL under accession number AB029490). The length of this short cDNA corresponds to the major band observed in Northern hybridization. The molecular weight of the deduced polypeptide was 33.3 kDa and the estimated $pI$ was 6.21. In the 3' untranslated region, two RNA instability motifs (ATTTA) were observed (Fig. 4). Other consensus motifs were searched by PROSITE (Bairoch et al., 1997) and SignalP (Nielsen et al., 1997) database; there are several motifs concerning post-translational modification, such as casein kinase II phosphorylation, protein kinase C phosphorylation, $N$-myristoylation and $N$-glycosylation sites. There is no signal peptide in the deduced amino acid. The analysis by PSORT II (Horton and Nakai, 1997) predicted that the protein is mainly located in the cytosol.

The computer assisted homology analyses revealed that the Dca shared similarity with the senescence marker protein-30 (SMP30) which has been cloned in rat, mouse and human in nucleic acid (52.1–55.3%) and amino acid sequences (33.4–34.8%; Fig. 5). Therefore, it is concluded that the gene, Dca, is the Drosophila homologue of SMP30.

### 3.5. Sequencing of the 5' flanking region of Dca gene

To identify potential cis-regulator elements of Dca, the 5' flanking region of Dca gene was amplified by PCR. All PCR products showed a single band in electrophoresis, indicating that the Dca gene is present as a single copy in the genome of D. melanogaster. PCR products derived from Dra I- and Sca I-digested genomes show approx. 0.6 and 1.5 kb in length, respectively. These fragments were cloned and sequenced with the primer walking method using DF-6, DF-7, DF-8, DF-9, DF-10, DR-5, DR-X, DR-X2, DR-X3, M13-M4, M13-RV primers (Table 1). Fig. 6 shows the sequence of the 5' flanking region of Dca (the nucleotide sequence was available from DDBJ/GenBank/EMBL under accession number AB029490). The presence of an intron at the 5' flanking of the initiator codon was suggested. The exon–intron junction also followed Chambon's rule (Breathnach and Chambon, 1981). Since the ATG initiator codon is located at the beginning of the second exon, the first exon represents a 5' untranslated sequence. In the proximal promoter region, both the TATA sequence and the CAAT box were located. AP-1 (a transcription factor of SMP30) binding site, STRE (stress response element) and some other putative transcription factor binding sites were detected by TFSE-ARCH (Heinemeyer et al., 1998) (Fig. 6).
3.6. Accumulation of DCA mRNA in flies reared to a senescent stage and at low temperature

To investigate whether Dca changes expression according to aging or not, Northern hybridization was performed. It was observed that this gene was up-regulated in senescent (49–53 days after eclosion) flies in comparison to young (7 days after eclosion) ones. In addition, the expression level was higher in flies reared at 15°C from the egg stage to the 14-day adult stage than in control flies (reared at 25°C to the 7-day adult stage) (Fig. 7).

4. Discussion

A gene encoding a Drosophila homologue of the senescence marker protein-30 (Deca) was up-regulated after acclimation to 15°C. The Deca gene is present as a single copy in the genome, located at position 88D on chromosome 3R, and has some introns. From the deduced amino acid sequence, DCA protein is estimated to have a molecular weight of 33.3 kDa, pI of 6.2, and to be present in the cytosol.

The mammalian gene, SMP30, has also been reported to be preferentially expressed in the cytosol of hepatocytes and renal tubular epithelia (Fujita et al., 1992a,b).
The expression of this gene is maintained at a high level throughout the tissue maturing process (Fujita et al., 1996), but decreases during senescent stages in both sexes (Fujita and Maruyama, 1991; Fujita et al., 1996), but decreases during senescent stages in both sexes (Fujita et al., 1996). It is reported that DNA binding activity of AP-1 transcription factors decreases in old individuals maintained at low temperature in Drosophila (Fujita et al., 1998). It is therefore possible that DCA plays a role in maintaining cytosolic Ca\(^{2+}\) levels at low temperatures, and enhances cold tolerance in individuals maintained at low temperature in Drosophila.

In contrast to mammalian SMP30, expression of the Dca gene did not decrease but increased at the senescent stage in Drosophila. In senescent Drosophila, an increase in ATP-dependent Ca\(^{2+}\) uptake has been reported (Shi et al., 1994), suggesting a link between the up-regulation of Dca and Ca\(^{2+}\) uptake.

The transcriptional regulation of SMP30 in rat is assumed to be mainly dependent on AP-1 transcription factor which binds to the sequence located at the 5′ flanking region of the SMP30 gene (Murata and Yamaguchi 1993a,b, 1994; Fujita et al., 1998). It is therefore possible that DCA plays a role in maintaining cytosolic Ca\(^{2+}\) level.

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References

Berger, F., Normand, P., Potier, P., 1997. capA, a cspA-like gene that encodes a cold acclimation protein in a psychrotrophic bacterium...
Fig. 6. Nucleotide sequence of 5' flanking region, first exon, first intron and second exon of Dca. Exon sequences are shown in black boxes. The translation initiator codon is located in the second exon (indicated by arrow head). Nucleotides located at the 5' and 3' end of intron (indicated by bold) follow Chambon’s rule. Predicted TATA sequence and CAAT box sequences are indicated by rectangles. Other putative AP-1, STRE, HFH-2 and NIT2 binding sites, and GATA box are also indicated by rectangles. Arrows represent primers and their directions. The nucleotide sequence is available from DDBJ/GenBank/EMBL under accession number AB029491.


