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# A novel gene that is up-regulated during recovery from cold shock in Drosophila melanogaster

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#### Abstract

Gene expression during recovery at 25°C (rearing temperature) after cold shock (0°C) was studied in *Drosophila melanogaster* using a subtraction technique. A novel gene (*Frost*, abbreviated as *Fst*) was considerably up-regulated during recovery after cold shock. In addition, a prolongation of cold shock was more effective for induction. In contrast to cold shock, *Fst* gene did not respond to heat shock. This gene is apparently the same as the unidentified gene, CG9434. *Fst* has high internal repeats not only in nucleotide but also in amino acid sequences. In addition, FST protein has a proline-rich region. The deduced amino acid sequence revealed a modular structure; i.e., a signal peptide in the N-terminal region followed by a long hydrophilic region. Therefore, this protein is likely to be directed into ER and secreted into extracellular space.

#### 1. Introduction

Sudden exposure to low temperature causes severe damage to organisms and their tissues, and normally elicits the expression of stress proteins. However, if organisms are first exposed to low but non-freezing temperatures they undergo cold acclimation that increases their tolerance to subsequent low temperature exposure. A variety of biochemical and physiological changes occur during cold acclimation (Thomashow, 1990). In addition, changes in gene expression after cold treatment appear to be related to cold acclimation at least in plants (for example, Goodwin et al., 1996; Knight et al., 1996, 1997; Monroy et al., 1998).

In insects, it has been reported that heat-shock proteins (HSPs) play an important role in the acquisition of cold tolerance. Denlinger et al. (1992) observed in *Lymantria dispar* that chilled eggs are more tolerant to cold and express stress proteins more abundantly than unchilled ones. In addition, there are observations that cold shock elicits HSP expression and prior exposure to mildly elevated temperatures reduces cold-shock injury in *Drosophila* and *Sarcophaga* (reviewed by Denlinger et al., 1991). Moreover, it is well known in many cold-water marine fish and terrestrial arthropods that hemolymph or blood proteins such as anti-freeze proteins (AFPs or thermal hysteresis proteins; THPs) and their activator proteins contribute to cold tolerance (reviewed by Duman et al., 1991). On the other hand, it has been reported in plants that an elevation of cytosolic Ca<sup>2+</sup> is involved in environmental stresses (for example, Knight et al., 1996, 1997). In addition, Perotti et al. (1990) reported in human cells that cold shock resulted in endonuclease activation and apoptosis: the prolonged elevation of cytosolic free Ca<sup>2+</sup> induces apoptosis by stimulating Ca<sup>2+</sup>/Mg<sup>2+</sup>-dependent endonucleases and modulating Ca<sup>2+</sup>/ calmodulin dependent enzymatic activities (Knight et al., 1996, 1997). This link among Ca<sup>2+</sup>, cold stress and the expression of stress-inducible Ca<sup>2+</sup>-binding protein (DCA) was reported in *Drosophila melanogaster* (Goto, 2000).

However, there have been few reports of genes other than AFP, HSP and DCA in cold tolerance or acclimation in insects. Here I report the discovery of a gene that responds to cold shock in *D. melanogaster*.

#### 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 and heat shock

Prior to experiments, vials that contained food medium were cooled (0°C) or warmed (37°C). Flies maintained at 25°C were transferred to cooled or warmed vials and exposed to cold (0°C) for 2 or 8 h or heat (37°C) for 30 min. After cold shock, flies were allowed to recover at 25°C for 2 h. On the other hand, heat-shocked flies were used for experiments just after the exposure.

All flies entered a coma within 1 min after the onset of the heat or cold exposure, but they recovered from the coma within 10 min when they were returned to 25°C.

#### 2.3. RNA extraction and mRNA purification

RNA extraction and poly(A)+ RNA purification were performed using RNAzol B (TEL-TEST) and Dynabeads mRNA purification kit (DYNAL) according to the suppliers' instructions.

#### 2.4. Subtraction, differential screening and RACE

These techniques were performed using Clontech PCR-Select cDNA Subtraction kit, PCR-Select Differential Screening kit and Marathon cDNA Amplification kit (Clontech).

In this experiment, the cDNAs synthesized from flies reared at 25°C as adults for 7 days and from 7 days old flies that were cold shocked at 0°C for 8 h and then returned to 25°C for 2 h were used for the subtraction. In addition, cDNA derived from the forward subtraction (non-shocked subtracted from cold shocked) and cDNA derived from the reverse subtraction (cold shocked subtracted from nonshocked) were referred to as cold-shock-enriched and control-enriched cDNAs, respectively.

#### 2.5. Northern hybridization

Poly(A)+ RNAs were electrophoresed on denaturing gels and transferred to nylon membranes according to Sambrook et al. (1989). The probe was labeled using a DIG DNA Labeling kit (Boehringer Mannheim) according to the supplier's instruction. Hybridization and detection were performed using a DIG DNA Luminescent Detection kit (Boehringer Mannheim). *D. melanogaster rp49* gene probe was used as a control. All experiments were conducted in triplicate and representative blots are shown.

#### 2.6. 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 kits (PE Applied Biosystems).

#### 3. Results and discussion

#### 3.1. Selective PCR amplification and differential screening

After the selective PCR amplification, cold-shock-enriched cDNAs were subcloned into pGEM-T vector (Promega). For differential screening, 94 fragments were amplified with PCR and blotted onto two membranes. Among them, five clones showed stronger signals when detected using cold-shock-enriched cDNAs as probes than when detected using control-enriched cDNAs as probes (data not shown).

#### 3.2. Northern hybridization analysis

Northern hybridization was performed using the above five fragments as probes. It appeared that a gene was expressed at low level in naive flies but was clearly upregulated in cold-shocked flies (Fig. 1A). On the other hand, the remaining four genes showed only slight differences between cold-shocked and control flies (data not shown). Using this positive fragment as a probe for Northern hybridization, two bands of approx. 1.2 and 1.0 kb in length were observed (Fig. 1B). In addition, the signals were considerably stronger in flies given a 2-h cold shock than in the controls, and the signals were stronger in flies cold shocked for 2 h (Fig. 1A).

In insects, HSP70 has been extensively studied in relation to cold shock, but it was induced by heat shock as well as cold shock (reviewed by Denlinger et al., 1991). In yeast, TIP1 gene has been investigated in the relation to cold shock, but the gene was also induced not only by cold but also by heat (reviewed by Thieringer et al., 1998). Therefore, I investigated whether this positive gene could be induced by heat. Northern hybridization revealed that this gene did not respond to heat shock (Fig. 1C).

Henceforth, this positive gene is referred to as *Frost*, abbreviated as *Fst*.

#### 3.3. RACE

After sequencing of the *Fst* fragment, Sub2-R3 and Sub2-F6 primers (Table 1) were designed and used for 5'- and 3'-RACE reactions, respectively. Single and double band(s) was(were) amplified in 5'- and 3'-RACE reactions, respectively. These products were subcloned and sequenced using primers listed in Table 1. For 5'-RACE products, ten independent clones were sequenced to obtain the full sequence at the 5' end of the mRNA. Both long and short 3'-RACE products had poly(A) tails at the 3<sup>'</sup> ends. In addition, they had identical sequences except that the long product had a long 3<sup>'</sup> UTR.

Fig. 2 shows full sequence of the long *FST* cDNA (the combination of 5'-RACE and long 3'-RACE products) with a length of 1,167 bp and an ORF of 834 bp that encodes 278 amino acids (the nucleotide sequence is available from DDBJ/GenBank/EMBL under accession number AB043874). The short cDNA (the combination of 5'-RACE and short 3'-RACE products; 981 bp) had a poly(A) tail from position 959 in the long cDNA (DDBJ/ GenBank/EMBL accession number is AB059240). The lengths of the long and short cDNAs correspond to the bands observed in Northern hybridization. Because the annealing site of Sub2-R3 primer was not in the short cDNA, 5'-RACE reaction using this primer could not amplify the short mRNA (see Fig. 2 and Table 1). The sequence, GTCTAGG, around the terminator codon agrees with the consensus sequence (Kozak, 1986). In addition, this gene had a rapid RNA degradation signal at the 3' end (Fig. 2; Sakai et al., 1989; Yost et al., 1990; Gillis and Malter, 1991). Moreover, it is characteristic that this gene had high internal repeats, especially at the 3' half of the ORF (analyzed data not shown).

The molecular weight of the deduced polypeptide was 29.3 kDa and the estimated pl was 3.73. This amino acid sequence was rich in E (glutaminic acid; 16.5%), T (threonine; 12.5%), G (glycine; 11.5%) and S (serine; 10.8%), especially in E (28.3%), T (20.7%) and S (16.7%) in the C-terminal half of the protein (residues from 134 to 278; Fig.2).

#### 3.4. Homology analyses

The homology analyses revealed that the *FST* cDNA shares little identity to known sequences except to the *D. melanogaster* unidentified gene, CG9434 (DDBJ/GenBank/ EMBL accession number is AC020051; DAD accession number is AE003683) (Adams et al., 2000), located at position 85E2 on chromosome 3R. The identities are 98.9% for the nucleotides, and 98.6% similarity and 99.3% positive for the protein. Therefore, *Fst* is apparently the same as the CG9434 gene. However, there is a gap in the alignment analysis between the *FST* cDNA and genomic sequence of the CG9434 gene; i.e., a gap of 24 nucleotides between position 828 and 829 in the *FST* cDNA, suggesting that the genome DNA copy contains a 24 nt intron (Fig. 2). In addition to the reports from the *Drosophila* EST project that this gene was expressed at embryonic and larval-early pupal stages (Berkeley Drosophila Genome Project, personal communication), the present study revealed that this gene also was expressed at a low level in adult flies. There are no reports on the mutant alleles of this gene.

#### 3.5. Deduced FST protein

The amino acid sequence also has high internal repeats at the C-terminal half (analyzed data not shown). In fact, FST protein contains nine PEEST (Pro-Glu-Glu-Ser-Thr) sequences (Fig. 2). In addition, this protein has some PEST regions that are thought to mediate rapid degradation of proteins (Rogers et al., 1986).

There is a proline-rich region at the middle of the FST protein. Although this motif is found in small proline-rich proteins (SPRs) cloned from mammals (Kartasova and van de Putte, 1988), its function is still unknown. SPR proteins are components of the cornified cell envelope (CE) of stratified squamous epithelia. In addition, *spr* genes are expressed in response to UV damage, chemical treatment, and in hyperproliferative or malignant disease (reviewed by Tesfaigzi and Calson, 1999). However, FST protein showed little similarity to SPRs (data not shown).

The hydropathy profile (Kyte and Doolittle, 1982) suggests that this protein has a modular structure; i.e., a short hydrophobic region in the N-terminus and the following hydrophilic region (Fig. 3). The 18 amino acids at the N-terminus have the characteristic features of a signal peptide in both length and amino acid composition (Nielsen et al., 1997). Signal peptides direct proteins into the ER and the protein is thus likely to enter the secretory pathway. On the other hand, particular amino acid sequences at the C-terminus cause proteins to be retained in the ER (Horton and Nakai, 1997), but such sequences are absent in FST. In addition, PSORTII (Horton and Nakai, 1997) also predicts that the protein would be secreted into extracellular space.

#### 4. Conclusion

- 1. A novel gene (*Frost*, abbreviated as *Fst*) was up-regulated during recovery from cold shock in *D. melanogaster*. A prolongation of cold shock was more effective for induction. In contrast to cold shock, this gene did not respond to heat shock.
- 2. The complete nucleotide sequence of *Fst* gene revealed an ORF of 834 bp that encodes 278 amino acids. The putative amino acid sequence is rich in glutaminic acids. In addition, the protein has high internal repeats at the C-terminal half and a proline-rich region at the middle. Moreover, FST protein has a signal peptide at the N-terminal end, suggesting that it would be secreted into extracellular space.
- 3. Fst gene is apparently the same as the D. melanogaster unidentified gene, CG9434. The gene has at least one intron.

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Table 1

Nucleotide sequence of primers

Oligonucleotides	Sequence (5 -3 )	Position of 5' base <sup>a</sup>
Sub2-F1	CCGACTCTGAGGATGATAGCG GTTC	603
Sub2-F2	TACCAGTCAGGCACCAGAGGA AATC	475
Sub2-F5	GCTCCGGTGACGATGAAA	129
Sub2-F6	GTCAGGCACCAGAGGAAATCA CCACCG	480
Sub2-R1	ACACGATGTGGGTTCATGAGG CTTC	1068
Sub2-R3	TGGGTTCATGAGGCTTCAATC CTAACCC	1060
Sub2-R4	AATGGCGAGTGCGTGACATAG TAACACG	1091
Sub2-R5	TCCTCAGAACCGCTATCA	633
Sub2-R6	TCCGGAGCCTGAGTGGTATCC TCAGAAC	651

<sup>a</sup> Positions correspond to cDNA in Fig. 2.



Fig. 1. Northern hybridization analyses using *Fst* and *rp49* probes. (A) Lane 1: control flies (reared at  $25^{\circ}$ C to the 7th day of the adult stage); lane 2: those allowed to recover at  $25^{\circ}$ C for 2 h after exposure to  $0^{\circ}$ C for 2 h; lane 3: those allowed to recover at  $25^{\circ}$ C for 2 h after exposure to  $0^{\circ}$ C for 8 h. (B) The details of the signals detected by *Fst* probe in lane 1 of A (arrow heads indicate bands). (C) Lane 1: control flies (reared at  $25^{\circ}$ C to the 7th day of the adult stage); lane 2: those exposed to heat at  $37^{\circ}$ C for 30 min; lane 3: those allowed to recover at  $25^{\circ}$ C for 2 h after exposure to  $0^{\circ}$ C for 8 h. Positions and sizes of the marker are indicated at the right. Five micrograms of poly(A)+ RNAs were applied to each lane.

1																A	AAC	AAT	AGC	GAA	13
14	ATG	AAG	GTC	СТА	ACT	CTT	ATC	стс	TTC	АСТ	GCC	TTG	GTG	GCA	GTG	GCT	тст	GGC	CGC	ccc	73
1	М	K	V	L	т	L	I	L	F	Т	Α	L	v	Α	V	Α	S	G	R	Ρ	20
71	ል አጥሪ	260	ഭദന	тсс	ጥጥሮ	CCA	ልልጥ	ጥጥጥ	ദദന	AGC	AAC	ጥርር	GCC	'acc	ACC	·ጥጥር	CCT	T DDT	n GGC	TCC	133
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134 41	GGTG	JAC	GAT	GAA	T	V		GAA		AGG	TGG W	AA'I' N	AGC	CAA 0	AA'I N	CAG	TGG W	AA'I' N	P	AA'I' N	193
	<u> </u>	2			-		<u>×</u>		<u>×</u>					2		26			-		
194	GGC	AAC	GGT	CAG	GGT	CAG	TGG	GAT	GGC	GAA	AAT	GGT	CAC	GGA	CAA	TCI	CAC	GGA	AAC	AAT	253
01	G	IN	G	Q	G	Q	W	U	G	Ľ	Ν	9	Q	G	Q	5	п	G	N	<u>IN</u>	80
254	CAA	GGC	СТС	GGC	CAA	GGA	AAC	GGA	CAC	GGA	AAC	AAC	CAI	CAT	GGT	CAT	CAT	GGC	AAC	ААТ	313
81	Q	G	L	G	Q	G	N	G	H	G	N	N	н	H	G	Н	Н	G	N	N	100
314	CAT	GGT	САТ	GGT	ААТ	GGT	CAA	GGC	CAC	GGT	GGA	CAG	CGC	сст	CCI	CCA	CCA	CCA	CCA	ACG	373
101	н	G	Н	G	N	G	Q	G	н	G	G	Q	R	P	Ρ	Ρ	Ρ	Ρ	P	т	120
374	GAT	ርጥር	ССТ	GAG	TTG	ACC	ACC	GAG	Сат	GAT	GTG	GTA	TCC	ACC	ACG	GAT	יGידיד	ACC	AGT	CCC	433
121	D	L	P	E	L	T	T	E	D	D	V	V	S	T	T	D	v	T	S	Р	140
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434 141	GCG	GAG E	GAG E	ACC T	ACT T	CTG L	GCA A	P	GAG	GTT V	P	GAA E	GAF E	STC1	'ACC T	AG'I: S	CAG 0	GCA A	CCA P	GAG	493 160
111		-	-	-	-	-		-	-	•	-					2	*		-	-	200
494	GAA	ATC	ACC	ACC	GGC	TCT	GAA	GAA	GGC	AGC	GGT	TCC	TCO	GAG	GAC	ACC	ACC	ACC	CTG	GCC	553
101	Е	T	т	т	G	5	E	E	G	5	G	5	5	E	D	т	т	т	Г	А	190
554	ССТ	GAA	GTT	CCG	GAG	GAA	TCC	ACC	АСТ	CAG	GCA	CCG	GAG	GAA	AGC	CACC	CACC	GAC	TCT	GAG	613
181	Р	Ε	v	<u>P</u>	E	E	S	<u>T</u>	т	Q	Α	<u>P</u>	E	E	S	<u>T</u>	т	D	S	Е	200
614	GAT	GAT	AGC	GGT	TCT	GAG	GAT	ACC	АСТ	CAG	GCT	CCG	GAA	AGAA	ACC	ACC	ACC	GAG	GAG	ССТ	673
201	D	D	s	G	s	Е	D	т	т	Q	Α	Ρ	Е	Ε	т	т	т	Е	Е	<u>P</u>	220
674	CAA	222	ጥሮሮ	<b>ACC</b>	ACC	GAG	CCT	ccc	GAA	GDD	TCC	'acc	'ac(	CAC	GCD		GDD	GAA	тCC	ACC	733
221	E	E	s	<u>_T</u>	S	E	A	<u>P</u>	E	E	S	<u></u>	S	E	A	<u>P</u>	E	E	S	<u>T</u>	240
		~ ~ ~													-						700
734	ACA T	GAG E	GAG E	CCC P	GAA E	GAA E	TCG S	ACA T	ACC T	GAG E	GCG A	C'I''1 T.	'G'I'A'. V	AGAA E	STCC	ACA: T	AG'I S	GAG E	IGCA A	P	260
211	-	-	-	-					-	-		_	•	-	-	-	-	_		=	
794	GAA	GAA	TCG	ACG	ACC	GAG	GCT	222	GAA	GAA	TCC			CGAI	IDT.	TCF	GCG	GTC	TAG	GAA	853
201	E	E	5	T	т	Е	А	<u>P</u>	E	E	_5_	_1	Т	D	5	5	А	v	~		270
854	GTT	AGT	TTC	ATT	GTT	ACC	AAG	GTI	TCT	GGI	GCA	\TTT	TC:	<b>FCA</b>	TAP	ATCO	TTT	AAA	ATT	TGG	913
914	CGT	TTC	CGA	GTA	ACA	AGG	TTA	TTA	AAC	CAAA	GCG	TTA	TAT	AAAC	GAA		ATGO	TGC	AAA	TAT	973
974	ТАА	АСТ	GTT	ATA	GCI	AAA	TTA	TTA	ACA	ATGI	TCT	CCC	CAG	AAGA	AGCO	GTA <i>I</i>	AAAC	CAI	TTC	TAG	1033
1034	GGT	TAG	GAT	TGA	AGC	стс	ATG	AAC	CCF	ACAI	CGI	GTI	TAC	FATO	<b>JTC</b>	ACGO	CACI	CGC	CAT	TTA	1093
1094	GTT	тта	TAG	СТС	CAA	CAA	TCI	GAA	CAA	\TT <b>F</b>		CAA	ACCO	GAAA	AAA	GAA	AAA		AAA	AAA	1153
1154	AAA	ААА	AAA	AAA	AA																1167

Fig. 2. Nucleotide (upper row) and deduced amino acid (lower row) sequences of the long cDNA. The termination codon (TAG) is indicated by asterisk. Arrow indicates the putative cleavage site of the signal peptide. The RNA instability signal is shown by double underline. The moderate and weak PEST sequences are indicated by closed and open boxes, respectively. A proline-rich region is indicated by ellipse. Several PEEST sequence repeats are indicated by underlines. Vertical line indicates a putative splicing site. Arrowhead indicates the beginning of poly(A) tail in the short cDNA. The nucleotide sequence is available from DDBJ/GenBank/EMBL under accession number AB043874.



Fig. 3. Hydropathy plot of the predicted FST protein (Kyte and Doolittle, 1982).