

An hsp70 homolog is encoded on the plastid genome of the red alga, *Porphyra umbilicalis*

Michael Reith and Janet Munholland

Institute for Marine Biosciences, National Research Council of Canada, 1411 Oxford St., Halifax, Nova Scotia B3H 3Z1, Canada

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A PCR experiment using *Porphyra umbilicalis* DNA as the template and degenerate oligonucleotides representing conserved regions of hsp70 amino acid sequences generated a 1 kb product that hybridized exclusively to the plastid DNA of this red alga. DNA sequencing of two contiguous *EcoRI* plastid DNA clones revealed a 620 amino acid open reading frame with 71% identity to the *dnaK* gene of the cyanobacterium, *Synechocystis* 6803. Northern hybridization experiments detected a 2.3 kb transcript that is present in control (15°C) cultures and increases approximately 7-fold upon heat shock (75 minutes at 30°C).

dnaK; Heat shock; hsp70; Plastid genome; Red alga; *trnG*(GCC)

1. INTRODUCTION

The 70 kDa heat shock proteins (hsp70s) are a ubiquitous, highly conserved group of proteins originally detected because of their abundance following heat stress (see [1] for review). As might be expected for proteins induced by stress conditions, the hsp70s, in the presence of ATP, have been shown to help renature and to prevent aggregation of denatured proteins [2,3]. More recently, evidence has accumulated indicating a role for hsp70s in the normal translocation of proteins through membranes. Bacterial hsp70 proteins (encoded by the *dnaK* gene) facilitate the export of proteins through the plasma membrane [4]. In eukaryotic cells, hsp70 homologs reside in both the mitochondria and the endoplasmic reticulum, where they aid in the refolding and assembly into complexes of proteins transported into these organelles [5,6]. In addition to a number of other functions, cytoplasmic hsp70s are thought to maintain precursor proteins in an unfolded, transport-competent state [7,8]. Chloroplast localized hsp70 homologs have recently been reported that presumably carry out functions similar to their mitochondrial homologs [9,10]. In land plants, these chloroplast-localized hsp70s are presumably encoded in the nucleus since they have not been detected in the three chloroplast genomes that have been completely sequenced [11–13]. In this communication, we report the cloning and sequencing of a gene for an hsp70 protein from the red alga, *Porphyra umbilicalis*, that is encoded

on the plastid genome. The hsp70 product of this gene is presumably localized in the plastid.

2. MATERIALS AND METHODS

DNA was extracted [14] from *P. umbilicalis* conchocelis cultures grown in D-11 enriched seawater [15] or from thallus material collected at Avonport, N.S. Nuclear and plastid DNA fractions were separated by centrifugation on Hoescht 33258 - CsCl density gradients [16]. RNA was isolated from conchocelis cultures according to MacKay and Gallant [17] and poly(A)⁺ RNA was purified by passage over oligo(dT) cellulose [18]. Construction and screening of a plastid DNA *EcoRI* clone bank in λ ZAPII (Stratagene) was carried out according to standard methods [18]. In vivo excision of the insert and Bluescript plasmid from λ ZAPII clones was carried out according to the supplier's recommendations. PCR reactions contained 0.5 μ g *P. umbilicalis* total DNA and 100 pmol each oligonucleotide primer (Fig. 1A) in the standard 100 μ l reaction recommended by the supplier (Perkin Elmer Cetus). Cycle parameters were 5 min at 94°C followed by 33 cycles of 30 s at 42°C, 1 min at 72°C and 30 s at 94°C and a final cycle of 30 s at 42°C and 5 min at 72°C. Southern and Northern blotting were carried out as described previously [19]. Southern hybridization was done using the ECL Kit (Amersham) according to the manufacturer's recommended conditions. The probe for the Northern hybridization consisted of a region of the 3' end of the gene (bases 1576–1868, Fig. 3) cloned into pTZ19R. A labelled anti-sense RNA was transcribed from the T7 promoter using a RNA Transcription Kit (Stratagene) and was hybridized according to the supplied protocol. Transcript amounts were quantitated by densitometry of the autoradiogram on a Beckman DU-64 spectrophotometer. DNA sequencing was carried out on double-stranded plasmid templates using synthetic oligonucleotide primers and Sequenase (US Biochemicals).

3. RESULTS

In order to generate a homologous hsp70 probe for *Porphyra umbilicalis*, a PCR experiment was performed using degenerate oligonucleotides representing two highly conserved regions of the hsp70 amino acid sequence (Fig. 1A). The primary product of the PCR

Correspondence address: M. Reith, Institute for Marine Biosciences, National Research Council of Canada, 1411 Oxford St., Halifax, Nova Scotia B3H 3Z1, Canada. Fax: (1) (902) 426 9413.

reaction was a DNA fragment of the expected size, approximately 1 kb (Fig. 1B). Direct DNA sequencing of the PCR product confirmed that it contained the expected segment of the *hsp70* gene (unpublished). The PCR product was purified by electrophoresis through low-melting-temperature agarose and hybridized to Southern blots of *P. umbilicalis* nuclear and plastid DNA. Unexpectedly, the probe hybridized exclusively to specific plastid DNA fragments (Fig. 2B). Further

A.

5' OLIGO

AMINO ACID	142	V	P	A	Y	F	N	D	148
OLIGO SEQUENCE	5'	GTA	CCA	GCA	TAT	TTT	AAT	GA	3'
		C	C	C	C	C	C		
		G	G	G					
		T	T	T					

3' OLIGO

AMINO ACID	471	Q	I	E	V	T	F	D	477
PREDICTED SEQUENCE	5'	CAA	ATA	GAA	GTA	ACA	TTT	GA	3'
		G	C	G	C	C	C		
			T		G	G			
					T	T			

OLIGO SEQUENCE (REVERSE COMPLEMENT)	5'	TC	AAA	AGT	AAC	CTC	AAT	CTG	3'
			G	C	C	T	G	T	
				G	G				
				T	T				

B.

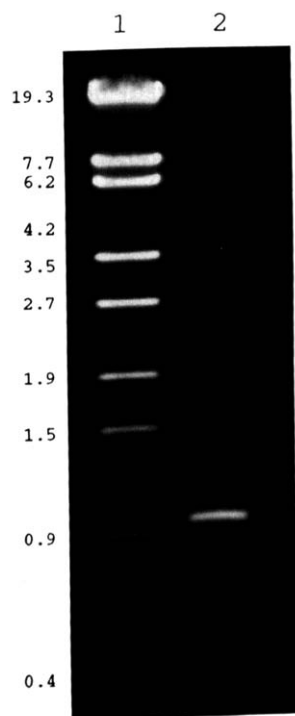
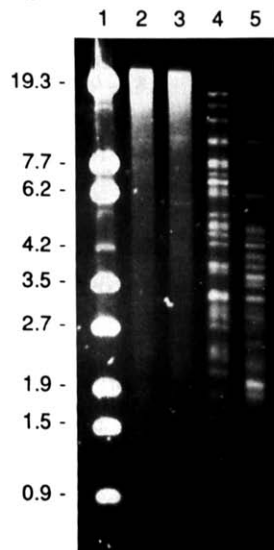


Fig. 1. (A) Oligonucleotide primers used in the PCR experiment. Numbers at the ends of the amino acid sequence refer to the *E. coli* *dnaK* amino acid sequence. (B) Results of the PCR experiment following electrophoresis on a 1% agarose gel (lane 2). Size marker (lane 1) is λ DNA digested with *Sst*I. Fragment sizes are indicated in kilobases.

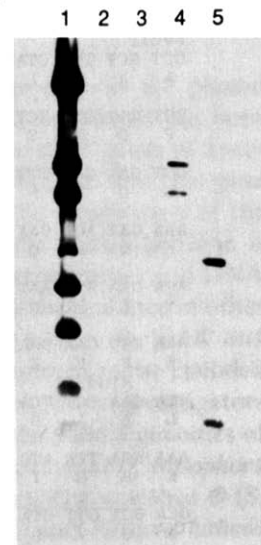
experiments (Fig. 2D) confirmed the hybridization of the probe to plastid DNA fragments and demonstrated the single copy nature of the *hsp70*-like gene.

The localization of the *hsp70* gene to the plastid genome was further established by screening a plastid DNA library of *Eco*RI fragments in the vector λ ZAPII. Southern hybridization experiments had indicated that the *hsp70* probe hybridized to two *Eco*RI fragments (Fig. 2B, lane 4), and two types of *Eco*RI clones, containing fragments of either 6.4 or 7.8 kb, were recovered from the library. DNA sequencing of the ends of these clones revealed the presence of different regions of an

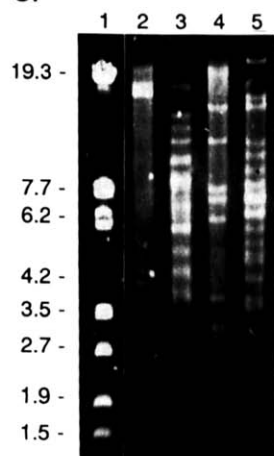
A.



B.



C.



D.

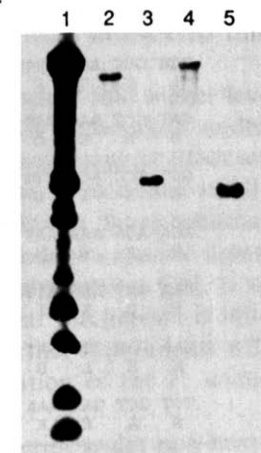


Fig. 2. Southern hybridization of the PCR-generated *hsp70* probe to *P. umbilicalis* DNA. (A) Ethidium bromide-stained 0.7% agarose gel with 3 μ g of nuclear (lanes 2 and 3) or 1 μ g of plastid (lanes 4 and 5) DNA digested with *Eco*RI (lanes 2 and 4) or *Hind*III (lanes 3 and 5). (B) A Southern blot of the gel in (A) hybridized to the PCR-generated *hsp70* probe. (C) Ethidium bromide stained 0.7% agarose gel of plastid DNA (1 μ g) digested with *Kpn*I (lane 2), *Pst*I (lane 3), *Sal*I (lane 4) or *Sst*I (lane 5). (D) Southern blot of the gel in (C) hybridized to the *hsp70* probe. Lane 1 in A-D is λ DNA digested with *Sst*I. Fragment sizes are indicated in kilobases.

GCA ATA TAT AAT TAT ATC TAG TTT AGC AAG CAT GAC AAT ACA GCT AAC TAT ATA ATA AAT TTA TTT GTA AAA ATT	-13
AAA <u>GAG GTG</u> TTC ATG GGT AAA GTT GTT GGA ATT GAC TTA GGA ACA ACT AAT TCT GTA ATT GCT GTT ATG GAA GGA	63
G K P T V I P N A E G F R T A S V V A Y T K S G	21
GGG AAA CCT ACT GTA ATA CCA AAT GCA GAG GGT TTT AGA ACT ACC GCT TCT GTT GTT GCT TAT ACT AAA AGT GGA	138
G K P T V I P N A E G F R T A S V V A Y T K S G	46
GAT AAA TTA GTT GGG CAA ATT GCT AGG CAA GCC GTT ATT AAT CCT GAG AAT ACT TTT TAT TCT GTA AAA AGA TTT	213
D K L V G Q I A R Q A V I N P E N T F Y S V K R F	71
ATA GGA AGA AAA CAA AAT GAA ATT TCT CAA GAA ATT AGA CAA ACC TCA TAT AAT GTA AAA ACT AGT GGC TCA AGT	288
I G R K Q N E I S Q E I R Q T S Y N V K T S G S S	96
ATA AAA ATT GAA TGT CCT GCA TTA AAT AAA GAT TTT GCG CCT GAA GAA ATT TCT GCT CAA GTA TTA AGA AAA CTT	363
I K I E C P A L N K D F A P E E I S A Q V L R K L	121
GTA GAA GAT GCC AGT ACA TAT TTA GGT GAA ACG GTT ACA CAG GCT GTT ATT ACA GTG CCA GCT TAT TTT AAC GAT	438
V E D A S T Y L G E T V T G I T V P A Y T F N D	146
TCT CAA AGA CAG GCA ACA AAG GAT GCA GGT AAA ATC GCA GGC TTA GAT GTA CTT AGA ATT ATT AAT GAA CCT ACA	513
S Q R Q A T K D A G K I A G L D V L R I I N E P T	171
PvuII GCT GCT TCG CTA TCA TAT GGG TTA GAT AAA CAA AAT AAT GAA ACT ATT CTT GTT TTT GAT CTT GGA GGA GGC ACA	588
A A S L S Y G L D K Q N N E T I L V F D L G G G T	196
TTT GAT GTA TCT ATC TTA GAA GTT GGA GAT GGG GTT TTT GAA GTT CTG TCT ACA TCT GGA GAT ACA CAT TTA GGT	663
F D V S I L E V G D G V F E V L S T S G D T H L G	221
GGT GAT GAC TTT GAC CAG CAA ATT GTT GAA TGG TTA ATC AAA GAT TTT AAA CAA AGT GAA GGA ATT GAC CTC GGT	738
G D D I V E W L I K Q S E G I D L G	246
AAA GAT AGA CAA GCA CTT CAA AGA TTA ACA GAA GCC TCT GAA AAG GCA AAA ATT GAA CTA TCA AAC TTG ACT CAG	813
K D R Q A L Q R L T E A S E K A K I E L S N L T Q	271
ACA GAA ATT AAT TTA CCA TTT ATT ACA GCA ACG CAA GAT GGA CCA AAA CAC TTA GAA AAA ACT GTG ACT AGA GCA	888
T E I N L P F I T A T Q D G P K H L E K T V T R A	296
AAA TTT GAA GAA CTC TGC TCT AGA CTA ATA GAT AAA TGT AGC ATC CCT GTT AAT AAT GCC TTA AAA GAT GCA AAA	963
K F E E L C S R L I D K C S I P V N N A L K D A K	321
HindIII CTA GAA GCT TCA AGT ATT GAT GAA GTT GTC TTA GTT GGT GGA TCT ACA AGA ATT CCA GCT ATA CAA CAA ATG GTT	1038
L E A S S I D E V V L V G G S T R I P A I Q M V	346
AAA AGA TTA ATC GGA AAA GAT CCT AAT CAA AGT GTA AAT CCT GAT GAA GTT GTT GCT ATC GGA GCT GCT GTT CAA	1113
K R L I G K D P N Q S V N P D E V V A I G A A V Q	371
GCA GGT GTT CTA GCA GGC GAA GTT AAA GAT ATT TTA CTA TTA GAT GTT ACT CCA TTA TCT TTA GGC GTT GAA ACT	1088
A G V L A G E V K D I L L L D V T P L S L G V E T	396
CTT GGT GGT GTT ATG ACA AAA ATT ATT CCA AGA AAT ACT ACT ATT CCT ACT AAA AAA TCT GAA GTA TTC TCT ACA	1263
L G G V M T K I I P R N T T I P T K K S E V F S T	421
PvuII GCT GTA GAT AAT CAG CCT AAC GTA GAA ATT CAA GTG CTT CAA GGA GAA AGA GAG CTT ACT AAA GAT AAT AAG AGC	1338
A V D N Q I V E I Q V L Q G E R E L T K D N K S	446
CTA GGC ACA TTT CGT TTA GAT GGT ATT ATG CCT GCA CCA AGA GGT GTA CCT CAA ATT GAG GTT ACT TTT GAT ATT	1413
L G T F R L D G I M P A P R G V P Q I E V T F D I	471
GAT GCT AAT GGT ATT TTA TCT GTG AAA GCA AAA GAA AAG GCA ACT GGG AAG GAG CAA TCT ATT ACT ATA TCT GGC	1488
D A N G I L S V K A K E K A T G K E Q S I T I S G	496
GCT TCC ACA TTG CCT AAA GAT GAT GTA GAA AGA ATG GTA AAA GAA GCC GAA GAA AAC TTT GAC GTA GAT CAA AAA	1563
A S T L P K D D V E R M V K E A E E N F D V D Q K	521
AGA AGA AAA AAT ATT GAC ATA AGA AAT CAG GCA GAA TCA CTG TGC TAC CAG TCT GAA AAA CAA GTC AAA GAG TTT	1638
R R K N I D I R N Q A E S L C Y Q S E K Q V K E F	546
GAA GAT AAG ATT GAT GAA GAA CTA AAA AAT AGA ATA ACA AAC TTA ATT AGC GAG CTG CGA TCT AAT TTA GAG AAA	1713
E D K I D E E L K N R I T N L I S E L R S N L E K	571
GAA GAG CTG GAT AGT ATT GAA GCT AAT TCC GAA AAA TTA CAG AAT GCA TTA ATG GAA ATT GGG AAA AAT GCT ACT	1788
E E L D S I E A N S E K L Q N A L M E I G K N A T	596
TCT GCT GAA AAA GAT ACC CAA AAT GCA TCA AAT GAT GAC ACG GTT ATT GAT ACA GAC TTT TCT GAA GCT AAG TAA	1863
S A E K D T Q N A S N D D T V I D T D F S E A K	620
AAA ATA A <u>AG CGG GTA AGC GAT TGA ACG CGC GAC ATC AAC CTT GGC AAG GTT GCG CTC TAC CAC TGA GCT ATA CCC</u>	1938
GC A TTG AAG TTA TTA TTA CAA ATA TTT TGA TAT TTG TCA ATC TAC TAA TTG GCG TGT AAT TAA AAT AAC AAA AAT	2013
ATA GAC TTA TCT ATC TCA GTT AAA AAA GTA CTT ATA TTC TTT TAT AAG TAC TTT TTT TGA TGA AAA AAT TAA AGA	2088

Fig. 3. Nucleotide sequence and deduced amino acid sequence of *P. umbilicalis* *dnaK*. Selected restriction sites are shown above the sequence. A potential ribosome binding site is underlined. The *tmG*(GCC) gene encoded on the complementary strand is boxed. The stem-loop structure is indicated by the horizontal arrows. This sequence will appear in the EMBL, Genbank and DDBJ Nucleotide Sequence Databases under the accession number X62240.

hsp70-like gene on each one. The connection between the two *Eco*RI clones was confirmed by sequencing of the PCR product through the *Eco*RI site. DNA sequencing of the clones was continued to determine the entire coding region of this gene (Fig. 3). The sequence revealed an open reading frame of 1860 bp, encoding a 620 amino acid protein with a predicted mol. wt. of 67.6 kDa. The encoded protein shows a very high degree of identity to the *Synechocystis* 6803 *dnaK* protein (71%) [20]. Proteins encoded by the *dnaK* genes from *Escherichia coli* [21], *Bacillus subtilis* [22] and *Caulobacter crescentus* [23] and the yeast hsp70 gene, *SSC1*, which encodes a mitochondrial hsp70 [24], are 52–56% identical to the protein encoded by the *P. umbilicalis* plastid gene. This protein is also 43–48% identical to hsp70 proteins of eukaryotes ranging from *Trypanosoma brucei* to maize and human. The region of homology between the *P. umbilicalis* *dnaK* gene product and other hsp70 proteins extends throughout most of the amino acid sequence except for approximately 100 amino acids at the carboxy end of the protein. Homology between other hsp70 proteins also breaks down in this region, suggesting that it is not evolutionarily well-conserved.

Immediately upstream of the methionine initiation codon is a probable ribosome binding site with the sequence GAGG. Eight bases downstream of the TAA stop codon and encoded on the opposite strand is a gene for a glycine tRNA (*trnG*(GCC)). While the complementary strand of *trnG* might provide sufficient secondary structure for transcription termination, a more typical transcription termination structure begins 172 bp after the stop codon (bases 2035–2071, Fig. 3). This stem-loop structure consists of a 15-base inverted repeat with a loop of 6 bases and ends in a run of Ts.

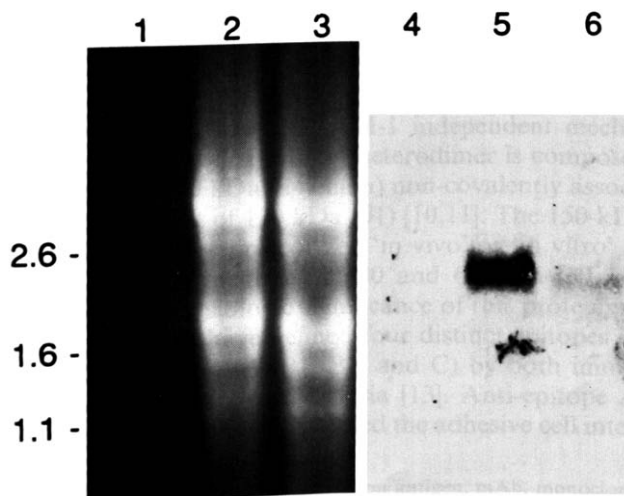


Fig. 4. Northern analysis of the *dnaK* transcript. (Lanes 1–3) Ethidium bromide-stained agarose gel. (Lanes 4–6) Northern hybridization results using the *P. umbilicalis* *dnaK* probe. Lanes 1,4, poly(A)⁺ RNA (1 μ g) from a control culture; lanes 2,5, total RNA (10 μ g) from a heat-shocked culture; Lanes 3,6, total RNA (10 μ g) from a control culture. Size markers (in kb) are derived from restriction fragments of the 6.2 kb *Eco*RI clone.

Northern blot analysis using a probe from the 3' end of the gene was carried out with total RNA from control (15°C) and heat shocked (75 min at 30°C) cultures as well as poly(A)⁺ RNA from a control culture. A single transcript of approximately 2.3 kb was detected (Fig. 4) in both total RNA lanes, while no transcripts were seen in the poly(A)⁺ RNA lane. The absence of hybridization to the poly(A)⁺ RNA lane demonstrates that the transcript detected is organellar rather than nuclear in origin. Approximately 7-fold higher levels of the transcript were detected in the RNA from the heat-shocked culture, suggesting that the plastid-encoded hsp70-like gene responds to heat stress.

4. DISCUSSION

The presence of an hsp70-like gene in the plastid genome of the red alga *Porphyra umbilicalis* has been detected. Given its similarity to the *dnaK* genes of *Synechocystis* 6803 and bacteria, we propose that this gene be given the *dnaK* designation. The localization of the *dnaK* gene of *P. umbilicalis* to the plastid genome is supported by both hybridization experiments and DNA sequencing data that have revealed the presence of other nearby genes, such as *trnG*(GCC) (Fig. 3), *psbK* and *petG* (unpublished), which are expected to be encoded on the plastid genome. In addition, other investigators have detected *dnaK* homologs in the plastid genomes of other rhodophyte and chromophyte algae: *Cyanophora paradoxa* (D. Bryant, pers. comm.), *Cryptomonas* Φ (S. Douglas and P. Liu, pers. comm.) and *Pavlova lutherii* [25]. These findings suggest that non-green-plastid-types have retained the *dnaK* gene from the original endosymbiont in their plastid genomes, while the land plants and probably green algae have transferred this gene to the nucleus [11–13].

It is interesting that approximately 7-fold higher levels of *P. umbilicalis* *dnaK* mRNA accumulate under conditions of heat-shock. These results suggest either an increase in the rate of transcription or increased stability of the mRNA under these conditions. Since bacterial heat-shock genes require the presence of a specific sigma factor, σ^{32} , for high levels of transcription [26], it is tempting to speculate that a similar mechanism is utilized in *P. umbilicalis* plastids. Further experiments will be required to explore the regulation of the *P. umbilicalis* *dnaK* gene.

The presence of the *dnaK* transcript under non-heat-shock conditions indicates a role for this protein in the normal function of the plastid. Given the high degree of similarity of this protein to its prokaryotic and mitochondrial counterparts, several different functions are likely. One probable role is in the translocation and folding of proteins transported into the plastid from the cytoplasm as has been found for yeast mitochondrial *SSC1p* [5]. In *E. coli*, the *dnaK* protein has been shown to facilitate the export of proteins [4], a process that is

mirrored in plastids in the transport of proteins to the thylakoid lumen [27]. Thus, a plastid-localized *dnaK* protein might also maintain proteins destined for the lumen in a translocation-competent state. The *E. coli dnaK* protein has also been implicated in the replication of both phage and host DNA [28] and a similar role could be envisioned for the plastid version. Finally, the ability of all hsp70 proteins to refold denatured proteins is also a likely function of the plastid *dnaK* protein, considering the heat-inducible nature of the gene.

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