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

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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 *Eco*RI 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 orginally 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 aggregatation 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.

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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 *Eco*RI clone bank in λZAPII (Stratagene) was carried out according to standard methods [18]. In vivo excision of the insert and Bluescript plasmid from λZAPII clones was carried out according to the supplier's recommendations. PCR reactions contained 0.5 μg *P. umbilicalis* total DNA and 100 pmol each oligonucleotide primer (Fig. 1A) in the standard 100 μl 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

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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 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 λZAPII. 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.

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	T					

B.

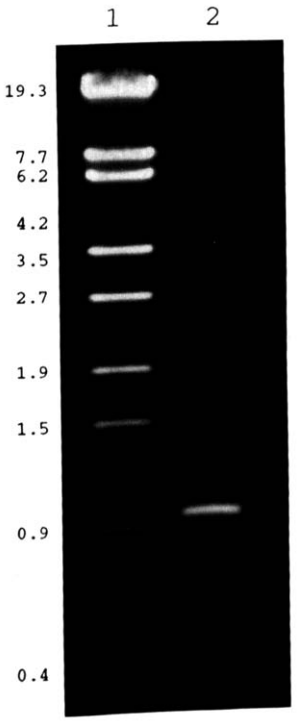

Fig. 1. (A) Oligonucleotide primers used in the PCR experiment. Numbers at the ends of the amino acid sequence refer to the *E. coli dnaK* amino acid sequence. (B) Results of the PCR experiment following electrophoresis on a 1% agarose gel (lane 2). Size marker (lane 1) is λ DNA digested with *Sty*I.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

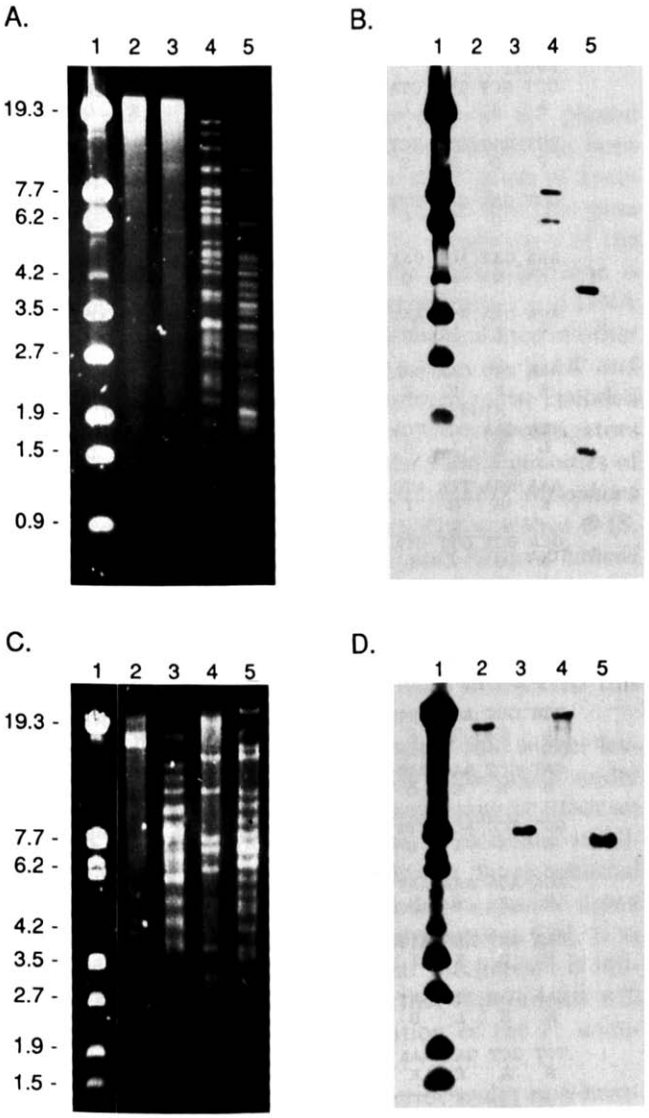

Fig. 2. Southern hybridization of the PCR-generated hsp70 probe to *P. umbilicalis* DNA. (A) Ethidium bromide-stained 0.7% agarose gel with 3 μg of nuclear (lanes 2 and 3) or 1 μg of plastid (lanes 4 and 5) DNA digested with *Eco*RI (lanes 2 and 4) or *Hin*dIII (lanes 3 and 5). (B) A Southern blot of the gel in (A) hybridized to the PCR-generated hsp70 probe. (C) Ethidium bromide stained 0.7% agarose gel of plastid DNA (1 μg) 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 *Sty*I. Fragment sizes are indicated in kilobases.

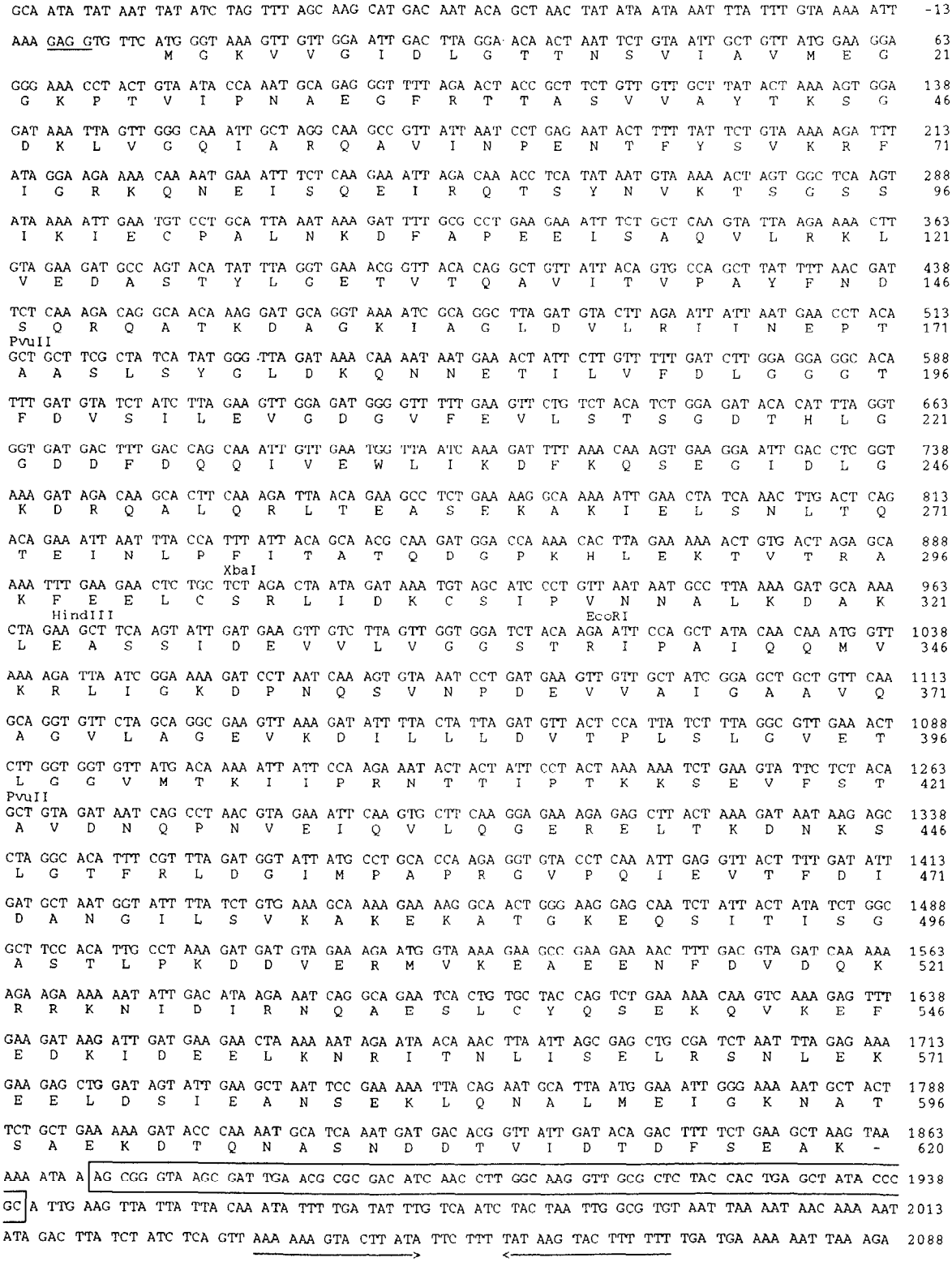

Fig. 3. Nucleotide sequence and deduced amino acid sequence of *P. umbilicalis dnaK*. Selected restriction sites are shown above the sequence. A potential ribosome binding site is underlined. The *trnG*(GCC) gene encoded on the complementary strand is boxed. The stem-loop structure is indicated by the 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.

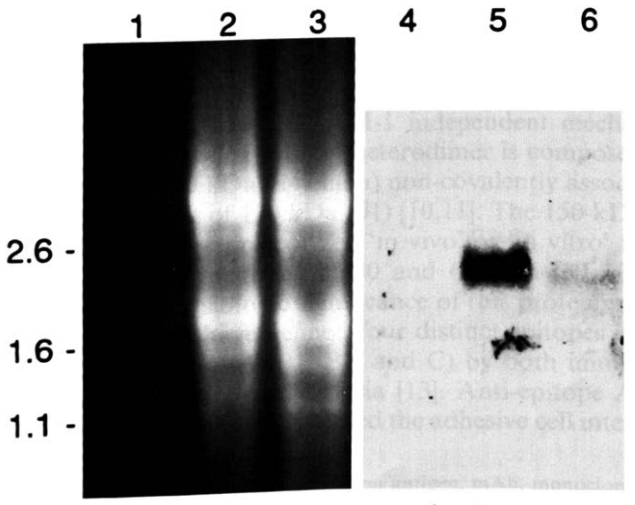

Fig. 4. Northern analysis of the *dnaK* transcript. (Lanes 1–3) Ethidium bromide-stained agarose gel. (Lanes 4–6) Northern hybridization results using the *P. umbilicalis dnaK* probe. Lanes 1,4, poly(A)$^+$ RNA (1 μg) from a control culture; lanes 2,5, total RNA (10 μg) from a heat-shocked culture; Lanes 3,6, total RNA (10 μg) 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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