Journal of Applied Phycology **10:** 581–588, 1999.

Characterization of the UDP-glucose pyrophosphorylase gene from the marine red alga *Gracilaria gracilis*

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Received 16 October 1998; revised 20 October 1998; accepted 21 October 1998

Key words: UDPglucose pyrophosphorylase, polysaccharide biosynthesis, red algae

Abstract

UDP-glucose pyrophosphorylase (UGPase) is a key enzyme in carbohydrate metabolism, particularly polysaccharide biosynthesis, in red algae. In this report, we characterize at the genomic and cDNA levels the putative UGPase gene of the agarophytic red alga *Gracilaria gracilis*. The gene is single-copy, devoid of introns, and produces two kinds of transcripts that differ in size by 332 basepairs. The large and small transcripts appear to utilize distinct polyadenylation signals. The putative protein has 495 amino acids, and is about 50% identical in sequence to its homologs in plants, animals and fungi. Sequencing of the genomic clone revealed that another gene, potentially encoding a DNA helicase and containing a 76 bp-intron near its 3′ end, occurs 376 bp downstream of the UGPase gene.

Introduction

UDP-glucose (UDPGlc) plays a key role in carbohydrate metabolism in red algae (Rhodophyceae). Its main biosynthetic function is as precursor of UDP-galactose (UDPGal), the D-galactosyl donor in the biosynthesis of galactans and of floridoside (α-D-galactopyranosyl-(1$\rightarrow$2)-glycerol) (Su & Hassid, 1962; Kremer & Kirst, 1981; Manley & Burns, 1991). Galactans, such as the commercially important agarans and carrageenans, are the most abundant component of the cell walls in most red algae (Craigie, 1990), whereas floridoside is a key photosynthetic product that functions as a short-term low-molecular-weight carbohydrate reserve (Kremer, 1978; Macler, 1986). There is some evidence that UDPGlc also serves as a minor glucosyl donor in the biosynthesis of floridean starch (Nagashima et al., 1971).

The key enzyme for the biosynthesis of UDP-Glc is UDP-glucose pyrophosphorylase (UGPase; EC 2.7.7.9), for which UTP and Glc1P are substrates. Plant, animal, and eubacterial UGPases are well characterized at the protein and gene levels (e.g., Turnquist & Hansen, 1973; Kleczkowski, 1994). The occurrence of this enzyme in red algae was first demonstrated in the early 1960s when UDPGlc and other metabolites of galactan biosynthesis were isolated from *Porphyra perforata* (Su & Hassid, 1962). More recently, Manley and Burns (1991) demonstrated UGPase activity in the red alga *Pterocladia capillacea*. However, characterization of red algal UGPases at the protein and gene levels has not yet been accomplished.

Herein we report the cloning of the nuclear gene encoding UGPase from the agarophytic marine red alga *Gracilaria gracilis* (Stackh.) Steentoft, Irvine & Farnham, and describe some features of its cDNA and genomic sequences.

Materials and methods

Construction and screening of genomic libraries and sequencing of clones

DNA was extracted from *G. gracilis* ('grass' strain) as described by Zhou & Ragan (1993), partially digested with *Sau*3AI, and ligated to the Lambda-DASH II vector (Stratagene, LaJolla, CA). The recombinant phage was packaged using the Gigapack III Gold Packaging Extract (Stratagene) and propagated in *Escherichia coli* XL1-Blue MRA (P2) strain (Stratagene).

A homologous UGPase probe was produced by a PCR-mediated approach. Degenerate primers were designed based on highly conserved regions of UGPases, as revealed by a multiple alignment of UGPase sequences obtained from the NCBI protein database. With genomic DNA from *G. gracilis* as template, PCR amplification with one pair of primers ('A', 5′-GGNGGN[TC]T[GT]GGNAC-[GT][AT]C[GT]ATGGG-3′, corresponding to the highly conserved amino acid sequence GGLGTSMG, and 'B', 5′-[GA]T[TC]NCC[GA]TG[GT]CCNGG-[GT]GG[GA]TACCA-3′, corresponding to the highly conserved amino acid sequence WYPPGHGD) yielded a product that was confirmed by subsequent sequencing to encode part of a potential UGPase gene. Using this PCR product as a probe, we screened the genomic library of *G. gracilis* we have prepared and the *G. gracilis* genomic library constructed by Zhou & Ragan (1994). Positive clones were purified using standard protocols, and subjected to automated sequencing on an ABI 373 'stretch' sequencer (Perkin Elmer – Applied Biosystems, Foster City, CA) using the manufacturer's protocols.

3′ RACE

mRNA was extracted from laboratory-grown *G. gracilis* using the Invitrogen FastTrack 2.0 kit (Invitrogen, Carlsbad, CA). The 3′ end of the UGPase transcript was reverse-transcribed using the Pharmacia T-primed Ready-To-Go kit (Pharmacia Biotech, Uppsala), and PCR-amplified using primer f2b (positions 746–767, Figure 1) as the gene-specific primer, together with an anchor primer (5′-ATTCGCGGCCGCAGGAATT-3′). As two PCR products were found on the gel, these were separately reamplified by PCR using the same primers. The PCR products were desalted by centrifugation through Centricon-100 concentrators (Amicon, Beverly, MA) with 2 mL distilled water, and used directly for sequencing.

Southern analysis

Southern blotting experiments were carried out following standard protocols (Sambrook et al., 1989). The probe was synthesized and labeled with α-^{32}P-dCTP using the Random Primed DNA labeling kit (Boehringer Mannheim Canada, Laval, Qué.), using a portion of the cloned DNA fragment (positions –33 to 424 in Figure 1; amplified by PCR) as template.

Results and discussion

Cloning of the G. gracilis *UGPase gene*

Using a homologous probe to screen a *G. gracilis* genomic library, two clones were initially recovered, each of which was found to contain a UGPase-coding region truncated near the 3′-end; one clone (λ27) was sequenced. To obtain the sequence of the 3′-end and 3′-flanking region, another clone (λUGPb) was isolated from another *G. gracilis* genomic library (Zhou & Ragan, 1994). The two clones were 100% identical in sequence throughout the 339-bp region of overlap (data not shown); and as UGPase is furthermore encoded by a single gene in *G. gracilis* (see below), λUGPb almost certainly contains the same gene as λ27. The final sequence, reconstructed from clones λ27 and λUGPb (Figure 1), contains an ORF of 496 codons. Comparison of the deduced amino acid sequence with other UGPase sequences (Figure 2) shows significant sequence identity between the deduced protein and UGPases from *Solanum tuberosum* (50%), human (51%) and *Saccharomyces cerevisiae* (48%), suggesting strongly that the isolated gene encodes a UGPase. We designate this gene as *GgUGP*.

The 5′ flanking region has proven recalcitrant to sequencing. Also proving difficult was the sequencing of the 5′-end of the transcript by the 5′-RACE technique, using methodology that has yielded the 5′ ends of other *G. gracilis* genes (*GgGALT1* and *GgSBE1*; Lluisma & Ragan, 1998a).

Features of the gene

A number of ATG codons in-frame with the UGPase-encoding ORF are present just downstream of the only potential TATA box (Figure 1). In the absence of data on the location of the 5′ end of the transcript, it is not possible to provide a definitive identification of the start codon. The first ATG codon downstream of the

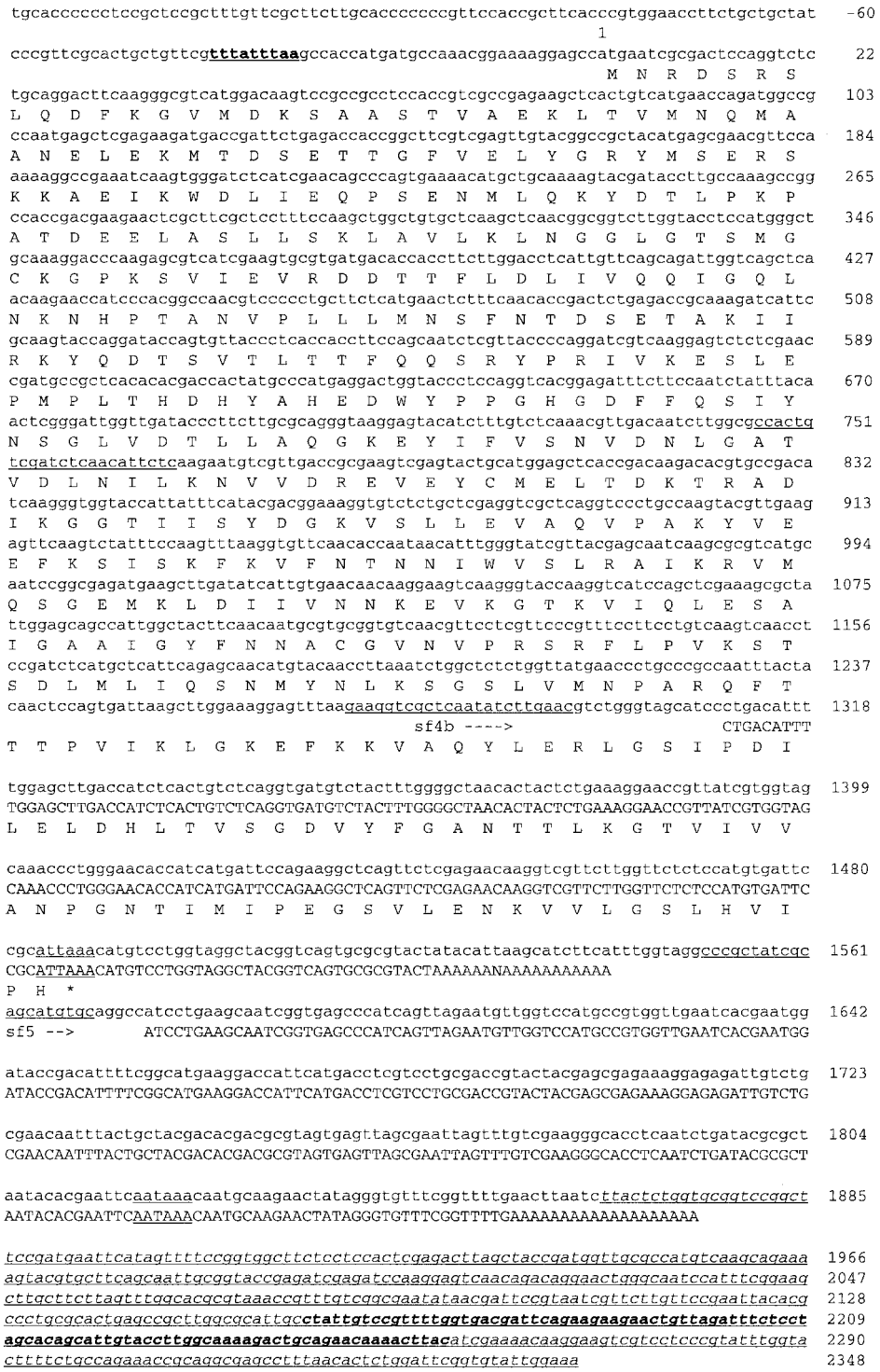

Figure 1. The nucleotide sequence of *GgUGP*. The numbering scheme assigns +1 to the first position of the putative start codon (marked with 1). The putative TATA box is shown in underlined boldface. The conceptual translation is shown below the coding region; * marks the stop codon. The 3′-RACE revealed two polyadenylation sites; the putative polyadenylation signal (ATTAAA) of the first site, which overlaps with the termination codon, and that of the second site (AATAAA), are underlined. The sequences of the 3′-ends of the cDNAs are aligned with the genomic sequence and shown in uppercase letters. Positions of the sequencing primers f4b and f5 are underlined, as is the position of primer f2b used in the RTPCR (positions 746–767). The portion of the sequence representing the 3′ end of a putative DNA helicase gene (encoded on the complementary strand) is italicized and underlined. Within the helicase gene region, the putative intron is shown in boldface.

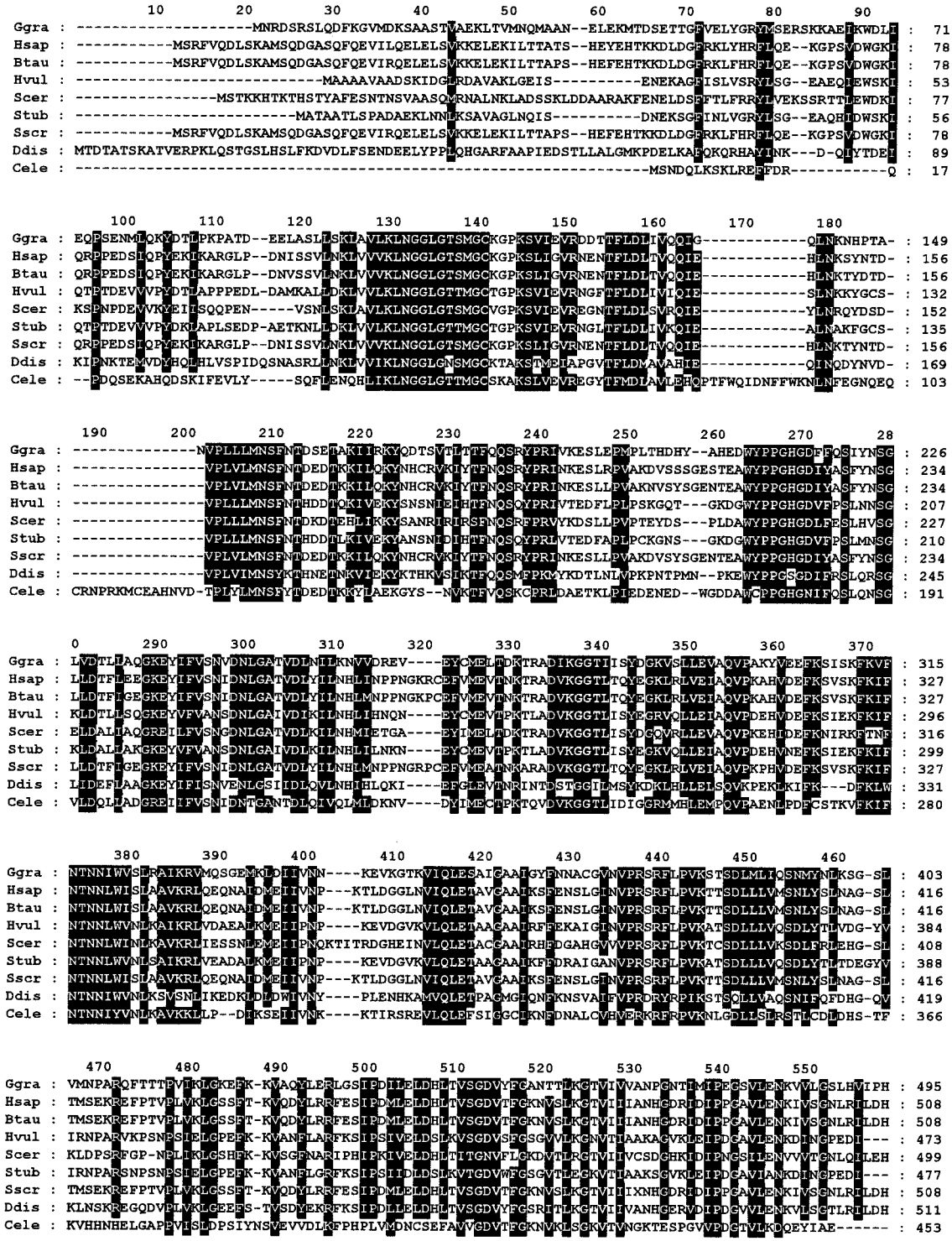

Figure 2. Alignment of UDP-glucose pyrophosphorylase (UGPase) sequences. The sequences were obtained from the NCBI database and aligned, together with the UGPase sequence from *G. gracilis*, using CLUSTAL W (Thompson et al. 1994) under its default parameters: pairwise alignments = slow (accurate), gap opening penalty = 10, gap extension penalty = 0.10 or 0.05, and the BLOSUM series (Henikoff & Henikoff, 1992) for scoring. Residues shown in white on a black background are conserved in at least 90% of the sequences. Ggra, *Gracilaria gracilis* UGPase, this paper (NCBI accession number AF100788); Hsap, *Homo sapiens* UGPase (731050); Btau, *Bos taurus* UGPase (731049); Hvul, *Hordeum vulgare* UGPase (2117937); Scer, *Saccharomyces cerevisiae* UGPase (UGP1, 1585157); Stub, *Solanum tuberosum* UGPase (322794); Sscr, *Sus scrofa* UGPase (1752677); Ddis, *Dictyostelium discoideum* UGPase (136738); Cele, *Caenorhabditis elegans* UGPase (1326259).

putative TATA box is in the right context as a translation initiation site, but is probably too proximate (6 bp). Another ATG codon, 30 bp downstream of the putative TATA box (Figure 1), is also in the right context for translation initiation; its 5′-flanking sequence (GCC**ATG**, the start codon in bold) conforms to the canonical sequence, RCY**ATG**, at translation initiation sites in red algal genes so far characterized (Zhou & Ragan, 1996). We thus provisionally designate this codon as the translation start site of the UGPase gene.

The amino acid sequence deduced from the genomic sequence of *GgUGP* shows significant sequence similarity with other UGPase sequences throughout its entire length (Figure 2); no region in the deduced sequence appears to be an 'insertion' relative to the other sequences, providing *a priori* evidence that *GgUGP* is devoid of introns. Other intron-free nuclear genes have been reported in red algae, e.g., genes for triose phosphate isomerase in *G. gracilis* (Zhou & Ragan, 1995c) and for GapC in *Chondrus crispus* (Liaud et al., 1993). In contrast, the UGPase gene from potato is interrupted by 19 introns (Borovkov et al., 1997), while that of *Dictyostelium discoideum* is interrupted by three introns (Ragheb & Dottin, 1987).

Reverse transcription and PCR amplification of the 3′ end of *GgUGP* transcripts were carried out to determine the location of polyA sites and polyA signals. Two PCR products differing in size were obtained, and sequence was obtained from both using primer sf4b; the sequence of the 3′ end of the smaller amplicon is shown in Figure 1. The longer transcript was identical throughout the region of overlap; the sequence of its 3′ end was obtained using another primer, sf5 (Figure 1). Alignment of the sequences with the genomic sequence (Figure 1) confirms that there are two types of UGPase transcripts in *G. gracilis*, differing in size by 332 bp due to alternative polyadenylation sites. Each site apparently has its own polyA signal, AUUAAA for the shorter transcript, found 33 bp upstream of the polyA site; and AAUAAA for the larger one, 32 bp upstream of the other polyA site. The polyA signal for the shorter transcript overlaps with the termination codon, a situation observed with another *G. gracilis* gene, polyubiquitin (Zhou & Ragan, 1995b). Interestingly, the polyubiquitin gene also has an alternative polyA site with its own polyA signal, and, just like the UGPase transcript, the polyA signal for the longer polyubiquitin transcript is also AAUAAA. The presence of UAAA signal in both polyadenylation sites in *GgUGP* and in the polyubiquitin gene probably indicates the importance of this highly conserved polyadenylation signal in *G. gracilis*. The UAAA motif has been observed in all gene trancripts so far characterized from *G. gracilis* (see Zhou & Ragan, 1996). The relevance of alternative polyadenylation sites in the *GgUGP* transcript to the physiology of *G. gracilis* remains to be evaluated.

Features of the deduced protein

GgUGP as delineated above potentially encodes a 495-amino acid protein that shares a high level of sequence identity (around 50%) with UGPases from other eukaryotes (Figure 2). Affinity-labelling studies of potato (Kazuta et al., 1991) and bovine (Konishi et al., 1993) UGPases have identified conserved lysine residues (K336, K402, K444, K486, K488, numbered according to the alignment in Figure 2) that are at, or close to, the substrate-binding site. Three of these (K336, K402, K444) have been confirmed by site-directed mutagenesis to be important for enzyme activity (Katsube et al., 1991); K444 is involved in catalysis of pyrophosphorylation *per se*, while K336 and K402 are believed to be involved in binding pyrophosphate or α-D-glucose-1-phosphate. These residues are highly conserved among UGPases, including GgUGP. There are, however, highly conserved residues for which mutation does not adversely affect enzyme activity, at least in the human liver UGPase (Chang et al., 1996); examples of such mutations include C140S, H311R, W263S, R437H, R471Q, and R495H. One of these sites, the highly conserved H311, is substituted in GgUGP with N.

UGPase is single-copy in G. gracilis

Southern analysis was performed to determine copy number in the *G. gracilis* genome. The final wash was of moderate stringency (0.5X SSC/0.1% SDS, 65°C, 30 min, performed twice); at this stringency, multiple copies of other *G. gracilis* genes (*GgGALT1* and *GgSBE1*, Lluisma & Ragan, 1998b) were clearly detected. However, in the case of UGPase, only one band was found per lane (data not shown), indicating that the UGPase gene is single-copy in *G. gracilis*. A second band (< 1 kb) observed after restriction with *Xho*I can be explained by the presence of a *Xho*I restriction site (CTCGAG, positions 112–117 in Figure 1) within the region spanned by the probe. UGPase from potato (Borovkov et al., 1996) has also been shown to be single-copy, although allelic isoforms have been described from this plant (Sowokinos et al., 1997).

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G. gracilis      ......FQYTESRVLKARLRFLAEKYQIREDDFLVFDVSFVLQSFAKVQCCARRNLTVLLL
S. pombe         ...624YQYTESRVLKARLEFLRDTYQIREADFLTFD----------------------
M. musculus      ...625YVYTQSRILKARLEYLRDQFQIRENDFLTFD----------------------
D. discoideum    ...621---TESKVLRARLEFLRDRYQIRENEFLTFD----------------------

G. gracilis      NRHQNGQ*AMRQAAQCAGRVIRNKNDYGIVIFADKRFTRAKLRSKLPKWIAQFLSVDSLDL
S. pombe         --------AMRHAAQCLGRVLRGKDDHGIMVLADKRYGRSDKRTKLPKWIQQYITEGATNL
M. musculus      --------AMRHAAQCVGRAIRGKTDYGLMVFADKRFARADKRGKLPRWIQEHLTDSNLNL
D. discoideum    --------AMRTASQCVGRVIRGKSDYGIMIFADKRYNRLDKRNKLPQWILQFCQPQHLNL

G. gracilis      DLGTAIAEARTFLLDMAQPSVAKSR--VEEKPPENYEFIGSRTAPE*--------------
S. pombe         STDMSLALAKKFLRTMAQPFTASDQEGISWWSLDDLLIHQKKALKSAAIEQSKHEDEMDID
M. musculus      TVDEGVQVAKYFLRQMAQPFHREDQLGLSLLSLEQLQSEETLQRIEQIAQQL*--------
D. discoideum    STDMAISLSKTFLREMGQPFSREEQLGKSLWSLEHVEKQSTSKPPQQQNSAINSTITTSTT


G. gracilis      ---------------
S. pombe         VVET*----------
M. musculus      ---------------
D. discoideum    TTTTTSTISETHLT*
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Figure 3a. Alignment of the peptide fragment deduced from the portion of a putative DNA helicase gene found downstream of *GgUGP* (italicized portion in Figure 1) with the C-terminal portions of DNA helicase homologs from *Mus musculus* (NCBI accession number 2114484), *Dictyostelium discoideum* (RepD, 2058510), and *Schizosaccharomyces pombe* (RAD15, 5022); numbers at the start of sequences indicate positions in the original sequence of the first residue shown here. The 32-position insertion in the *Graciliaria gracilis* conceptually translated helicase sequence corresponds to a putative intron (Figure 3B and text) and as such would not actually occur in the protein. '-' indicates gaps inserted to achieve alignment; * indicates a position encoded by a stop codon. Alignment was produced using CLUSTAL W (Thompson et al. 1994).

An intron-containing gene occurs just downstream from GgUGP

We sequenced the region downstream from *GgUGP* and used the sequence to search the NCBI database (using BLASTX). The conceptual translation product of the sequence in this region has significant similarity with C-terminal portions of DNA helicases (Figure 3A), strongly suggesting that this region (italicized in Figure 1) represents the 3′ portion of a potential DNA helicase gene (encoded on the complementary strand relative to *GgUGP*). Its stop codon is 376 bp downstream of the stop codon of *GgUGP*. The proximity of these two genes is yet another indication that close spacing of genes in the *G. gracilis* genome may not be uncommon. The proximity of genes encoding polyubiquitin and mitochondrial aconitase (Zhou & Ragan, 1995a), and of genes encoding GALT and a putative peptidyl-tRNA hydrolase (Lluisma & Ragan, 1998b), has already been observed.

A potential 96-bp, phase-0 intron was also found in the DNA helicase gene region. As indicated by the alignment (Figure 3A), the otherwise strong sequence similarity is interrupted by the presence in the conceptually translated *G. gracilis* sequence of an apparent insertion, 32 amino acids in length including an in-frame termination, that does not occur in DNA helicase proteins from *Schizosaccharomyces pombe, Dictyostelium discoideum*, or mouse. Inspection of the nucleotide sequence (Figure 3B) reveals that this apparent amino acid insertion in fact corresponds to a putative intron whose sequences at the potential 5′ (AT:GTAAGT, where the colon indicates the presumptive exon-intron junction) and 3′ (TAG:G) splice sites and at the potential branch site (CTAAC) conform perfectly to the canonical sequences for spliceosomal introns in red algal nuclear genes (Zhou & Ragan, 1996). Notably, this intron is positioned close to the 3′ end of the gene; all other red algal genes characterized to date are devoid of introns at the 3′ region.

Conclusion

From the red alga *G. gracilis* we have cloned a single-copy nuclear gene that putatively encodes UGPase, a key enzyme of galactan biosynthesis. The characterization of this gene paves the way for more intensive studies of the biochemistry and molecular biology of UGPase and agar biosynthesis in this alga. The gene produces two transcripts that differ in length due to the presence of alternative polyadenylation sites; the possibility that this constitutes a mechanism for regulating

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tttccaatacaccgaatccagagtgttaaaggctcgcctgcggtttctggcagaaaagtaccaaatacgggaggacga
  F  Q  Y  T  E  S  R  V  L  K  A  R  L  R  F  L  A  E  K  Y  Q  I  R  E  D  D

cttccttgttttcgatgtaagttttgttctgcagtcttttgccaaggtacaatgctgtgctaggagaaatctaacagt
  F  L  V  F  D

tcttcttctgaatcgtcaccaaaacggacaatagcaatgcgccaagcggctcagtgcgcagggcgtgtaattcggaa
                                  A  M  R  Q  A  A  Q  C  A  G  R  V  I  R  N

caagaacgattacggaatcgttatattcgccgacaaacggtttacgcgtgccaaactaagaagcaagcttccgaaatg
  K  N  D  Y  G  I  V  I  F  A  D  K  R  F  T  R  A  K  L  R  S  K  L  P  K  W

gattgcccagttcctgtctgttgactccttggatctcgatctcggtaccgcaattgctgaagcacgtacttttctgct
  I  A  Q  F  L  S  V  D  S  L  D  L  D  L  G  T  A  I  A  E  A  R  T  F  L  L

tgacatggcgcaaccatcggtagctaagtctcgagtggaggagaagccaccggaaaactatgaattcatcggaagccg
  D  M  A  Q  P  S  V  A  K  S  R  V  E  E  K  P  P  E  N  Y  E  F  I  G  S  R

gaccgcaccagagtaa
  T  A  P  E  *
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Figure 3b. The nucleotide sequence of the 3′-portion of a putative gene encoding a DNA helicase homolog in *G. gracilis*; this sequence is complementary to the italicized portion in Figure 1 (positions 2348–1865). The conceptual translation is shown below the nucleotide sequence. The portion likely to be an intron is italicized; the dinucleotides conserved at the 5′ and 3′ ends (GT and AG, respectively) and at the branch site (AC) in red algal spliceosomal introns (Zhou & Ragan, 1996) are in bold and underlined.

gene expression remains to be investigated. The observation that another gene, potentially encoding a DNA helicase, is located just downstream of *GgUGP* adds to accumulating evidence that occurrence of closely spaced genes may not be unusual in the *G. gracilis* genome.

Acknowledgements

We thank the Marine Biology group of the NRC Institute for Marine Biosciences, especially Ms Colleen Murphy, for excellent technical assistance. AOL thanks the International Development Research Center of Canada for the PhD fellowship, and the University of the Philippines – Marine Science Institute for study leave. MAR thanks the Canadian Institute for Advanced Research, Program in Evolutionary Biology for fellowship support. Issued as NRCC no. 42277.

The DNA sequence reported herein has been deposited in GenBank under the accession number AF1007880.

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