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Gene Sequence and Expression of an Analog of Proliferating Cell Nuclear Antigen (PCNA) in the Alga *Tetraselmis chui* and Detection of the Encoded Protein with Anti-Rat PCNA Monoclonal Antibody

LI-CHUAN CHENG,[1]† SHENG-PING L. HWANG,[2] AND JENG CHANG[1]*

Institute of Marine Biology, Taiwan Ocean University, Keelung 202-24,[1] and Institute of Zoology, Academia Sinica, Nankang, Taipei 115-29,[2] Taiwan, Republic of China

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To identify a phytoplankton cell cycle marker detected by a monoclonal antibody against mammalian proliferating cell nuclear antigen (PCNA) (S. Lin, J. Chang, and E. J. Carpenter, J. Phycol. 30:449–456, 1994), a PCNA gene fragment was isolated by reverse transcription-PCR from the marine unicellular alga *Tetraselmis chui* Butcher (*Prasinophyceae*). The gene fragment was 616 bp in length and contained an open reading frame of 205 amino acids. The deduced amino acid sequence showed 80 and 88% similarity to human and rice PCNA, respectively. Southern hybridization indicated that the isolated gene fragment was part of the *T. chui* genome, with up to three copies in each haploid nucleus. Northern hybridization was used to detect a PCNA mRNA with a size of 1.2 kb from an exponentially growing algal culture. The *T. chui* gene fragment has been cloned into an expression vector, and a fusion protein was subsequently generated. Anti-rat PCNA simultaneously recognized the PCNA fusion protein and a single 33-kDa band in *T. chui* total protein extract. Our results indicate that *T. chui* PCNA is highly similar to its mammalian counterpart and that anti-rat PCNA is a good tool for detecting phytoplankton PCNA in general.

Species-specific growth rate in situ is an important parameter in the study of phytoplankton ecology. Cell cycle analysis, which monitors how cells travel through successive phases of the cell division cycle and eventually enter mitosis, has been proven to be a reliable way to estimate growth rate (4, 25, 32). This method does not require any form of incubation at the time of sampling, and errors caused by loss due to grazing and other processes appear to be small (7, 8). Until recently, cellular DNA content was the method commonly used to identify a cell's position in the cell cycle. However, since the quantitative determination of DNA content in individual cells requires both expensive equipment and a lengthy processing time, more convenient cell cycle markers to further improve this method are in demand (17, 20).

Proliferating cell nuclear antigen (PCNA) is the auxiliary protein of eukaryotic DNA polymerase δ (for reviews, see references 6, 15, and 35). During DNA replication, three identical PCNA monomers form a ring-shaped "sliding clamp" that holds a DNA molecule at the center. This structure stabilizes the binding of polymerase δ to the DNA template so that long stretches of new DNA can be synthesized without interruption. PCNA is also involved in the regulation of the cell cycle. When errors occur during DNA replication, a special protein, $p21^{WAF1}$, is generated to bind PCNA as well as several other key proteins (33). As a result, the progress of the cell cycle is effectively blocked.

PCNA is a highly conserved protein in both amino acid sequence and biochemical function. The molecular mass of PCNA from various organisms ranges from 30 to 36 kDa. PCNA proteins with similar amino acid sequences have been found in mammals (27), higher plants (22, 30, 31), insects (26), and yeast (3). Cloned PCNA from rice has been shown to stimulate the activity of human DNA polymerase δ (24). Conversely, calf thymus PCNA is able to increase the product length of wheat DNA polymerases B and CII (16). Furthermore, human $p21^{WAF1}$ can form a stable complex with pea PCNA, indicating that the binding site for the regulatory protein on PCNA is well preserved in different organisms (2).

Considering the basic function of PCNA, it is not surprising that PCNA is preferentially synthesized shortly before and during the S phase when genomic DNA is replicated (5). This pattern of expression makes PCNA a good indicator of actively dividing cells, and the detection of PCNA has become useful in the treatment of cancer (29). By the same token, PCNA should be a good indicator for actively growing phytoplankton in the ocean. It has been shown that a monoclonal antibody made against rat PCNA (anti-rat PCNA) is able to recognize protein bands with a molecular mass of about 36 kDa in four phytoplankton species belonging to distantly related taxonomic groups and that the PCNA-like band disappears if the antibody has been preincubated with purified calf thymus or *Drosophila* PCNA (17). With the same antibody, immunocytochemical staining indicates that the PCNA-like protein is localized in the nucleus of *Dunaliella tertiolecta* cells, and the percentage of cells with positive staining correlates well with the S-phase fraction in a partially synchronized population (19). Field tests of this new cell cycle marker were conducted during two cruises to the North Atlantic Ocean and Caribbean Sea (18). The nucleus of an ecologically important diatom, *Ethmodiscus rex*, was successfully labeled by the anti-PCNA antibody, and estimates of in situ growth rate were obtained.

Despite these results, some basic questions about the PCNA-like protein in phytoplankton remain unanswered. In some phytoplankton tested, more than one protein was labeled by the antibody, and the amount of the labeled protein did not

* Corresponding author. Phone: 886 2 462-2192, ext. 5308. Fax: 886 2 463-3152. E-mail: b0176@ntou66.ntou.edu.tw.

† Present address: Institute of Zoology, Academia Sinica, Nankang, Taipei 115-29, Taiwan, R.O.C.

change with the age of the culture (17). On the other hand, when *Ethmodiscus* cells were stained, both nucleus and chloroplasts were labeled by anti-PCNA (18). As an organelle of prokaryotic origin, chloroplasts should have neither DNA polymerase δ nor PCNA. These observations thus raised the questions of whether phytoplankton possesses the PCNA protein and whether the anti-rat PCNA simply cross-reacted with an unrelated phytoplankton protein which may or may not oscillate with the course of the cell cycle.

To investigate the true identity of the PCNA-like phytoplankton protein recognized by anti-rat PCNA, we isolated a gene fragment from a unicellular alga, *Tetraselmis chui*. Based on sequence similarity, we showed that *T. chui* does contain a PCNA gene homologous to its counterpart in other eukaryotic organisms. Furthermore, we showed that the anti-rat PCNA recognized the His-tagged fusion protein that was generated from *Escherichia coli* transformed by a recombinant plasmid containing the algal PCNA gene fragment.

MATERIALS AND METHODS

Algal culture and growth conditions. The unialgal culture of the marine microalga *Tetraselmis chui* Butcher (*Prasinophyceae*) clone TA was provided by H.-M. Su of the Tunkang Marine Laboratory, Pintung, Taiwan. The culture was grown in f/2-enriched sea water medium at 20°C (13), and continuous illumination was provided at 160 microeinsteins m^{-2} s^{-1}.

Total RNA isolation. Exponentially growing algal cells were used for total RNA isolation by the guanidinium thiocyanate-phenol-chloroform extraction method (9). Briefly, cells were harvested by centrifugation, followed by sonication in a buffer containing 4 M guanidinium thiocyanate to release cell contents. Next, the crude cell extract was subjected to phenol-chloroform extraction in order to remove proteins. The extracted RNA was then precipitated with isopropanol, redissolved in Tris-EDTA buffer, and stored at −70°C.

Reverse transcription (RT)-PCR. Three degenerate primers, named PC5A, PC5B, and PC3C, were designed according to conserved regions of PCNA revealed by amino acid sequence alignment. PCNA sequences of various species were obtained from GenBank. Primer sequences were 5′-(C/T)TI CA (A/G) GCI ATG GA (C/T) (A/T) (C/G) I (A/T) (C/G) I CA (C/T) GT-3′ for PC5A, 5′-GA (A/G) GGI TT (C/T) GA (A/G) CA (C/T) TA (C/T) (A/C) GI TG (C/T) GA-3′ for PC5B, and 5′-C (C/T) TC (A/G) TC (C/T) TC (C/T) TC IAT (C/T) TT IGG IGC-3′ for PC3C (I, inosine). The expected product sizes were 640 and 616 bp for primer pairs PC5A/PC3C and PC5B/PC3C, respectively. First-strand cDNA synthesis was conducted at 42°C for 1 h in a solution containing 10 μg of *T. chui* total RNA (denatured at 65°C for 10 min, then chilled on ice), 2.5 U of reverse transcriptase per μl, 2.5 μM oligo(dT), 2.5 μM random hexamer, 5 mM $MgCl_2$, and 10 mM deoxynucleoside triphosphate (final volume, 20 μl). Subsequently, the PCR mixture, containing 2.5 U of Replitherm DNA polymerase (Epicentre Technologies, Madison, Wis.), 1× Replitherm reaction buffer, 1 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphate, and 100 pmol of primers, was added to the denatured first-strand cDNA mixture (final volume, 100 μl). PCR was carried out on a Biometra DNA thermocycler at the following settings: 94°C for 1 min, 52°C for 1 min, 72°C for 1 min for 35 cycles, followed by 72°C for 10 min for 1 cycle. The PCR mixture was then stored at 4°C. The amplified products (10 μl) were examined by 1.5% 0.5× Tris-borate agarose gel electrophoresis.

Cloning and DNA sequence analysis. RT-PCR products from *T. chui* were purified with low-melting-point agarose gel and then ligated into a pGEM-T vector (Promega, Madison, Wis.). Subsequent transformation and plasmid DNA isolation were performed by standard methods (28). The sequencing of the cloned segment was done on an ABI Prism 377A DNA sequencer (Perkin-Elmer, Branchburg, N.J.). The nucleic acid and deduced amino acid sequences were analyzed with Lasergene software (DNASTAR Inc., Madison, Wis.) as well as the BESTFIT program from the Wisconsin sequence analysis package of the Genetics Computer Group (12).

Fusion protein generation. A 616-bp *Sph*I-*Pst*I restriction fragment containing the PCNA gene segment was inserted into a *Sph*I-*Pst*I-digested pQE-31 vector (Qiagen). The recombinant pQE31 vector was verified by DNA sequencing. To generate fusion protein, an overnight culture of *E. coli* strain SG 13009 [pREP4] transformed with pQE31 containing the PCNA gene fragment was diluted 1:10 in fresh Luria-Bertani medium containing kanamycin (25 μg ml^{-1}) and ampicillin (100 μg ml^{-1}) and incubated at 37°C for 2 h. Subsequently, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 2 mM and incubated at 37°C for another 3 h. *E. coli* cells were then harvested by centrifugation and dissolved in 1× sample buffer. After being boiled at 100°C for 5 min, samples were centrifuged and the supernatants were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% polyacrylamide).

Preparation of DIG-labeled DNA and RNA probes. In vitro transcription was conducted in a mixture of *Nco*I-digested PCNA gene fragment containing pGEM-T vector (1 μg), RNase inhibitor (20 U), digoxigenin (DIG)-11–UTP (Boehringer Mannheim) containing nucleoside triphosphate labeling mixture, 1× buffer, and SP6 RNA polymerase (40 U) at 37°C for 2 h. DIG-11–dUTP-labeled DNA probes were generated by PCR with the restriction fragment containing a PCNA gene segment as the template and 1.3 mM (each) dATP, dCTP, and dGTP; 0.63 mM dTTP; 0.13 mM DIG-11–dUTP; 2.5 U of Replitherm DNA polymerase (Epicentre Technologies); and 100 pmol of primers. PCR conditions were as follows: 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min for 35 cycles and 72°C for 10 min for 1 cycle.

Southern blot analysis. Total DNA was isolated from *T. chui* cells by the phenol-chloroform extraction method, and excess polysaccharide was removed by the addition of cetyltrimethylammonium bromide (Sigma) (11, 28). Southern blotting was performed according to standard procedures (28). Twenty-five micrograms of genomic DNA from *T. chui* was digested with restriction endonucleases *Eco*RI, *Pst*I, *Hin*dIII, and *Xba*I. The enzyme concentration used was 8 U μg of DNA^{-1}, and digestion was conducted at 37°C for 6 h. The digested DNA was separated on a 0.8% 0.5× Tris-borate agarose gel, followed by transfer to nylon membranes. The membranes were prehybridized for 2 h in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50% formamide–2% blocking solution–0.1% sodium lauryl sarcosine–0.02% SDS at 42°C. Hybridization was conducted in prehybridization buffer containing 25 ng of DIG-labeled DNA probes ml^{-1} at 42°C overnight. Subsequently, the membranes were washed with 2× SSC–0.1% SDS for 5 min twice at room temperature, followed by another two washes with 0.5× SSC and 0.1% SDS for 15 min each at 55°C. The locations of the nucleic acid fragments that contained the PCNA gene were determined by AMPPD chemiluminescence (Boehringer Mannheim) following the manufacturer's protocol.

Northern blot analysis. Total RNA (20 μg) was fractionated on 1.2% agarose gels containing 1.2 M formaldehyde and EDTA-borate buffer (14). Gels were transferred to nylon membranes for 3.5 h by the downward alkaline transfer method (10). The membranes were prehybridized for 2 h at 65°C in the same prehybridization buffer used for Southern blot analysis. Hybridization was carried out in prehybridization buffer containing 50 ng of DIG-labeled anti-sense RNA probe ml^{-1} at 65°C overnight. After hybridization, membranes were washed twice with 2× SSC–0.1% SDS for 5 min at room temperature, followed by another two washes with 0.5× SSC–0.1% SDS at 65°C for 15 min each. Finally, CDP-STAR chemiluminescent detection was conducted following protocols from the manufacturer (Boehringer Mannheim).

Western blot analysis. *T. chui* cells were harvested by centrifugation at 4,000 rpm (Jouan GR 422) for 10 min, followed by sonication in 1× sample buffer. After being boiled at 100°C for 5 min, samples were centrifuged, and the supernatants were separated by SDS–12.5% PAGE at 100 V for 1 h. The gels were electrophoretically transferred to nitrocellulose membranes in 25 mM Tris (pH 8.8), 192 mM glycine, and 20% methanol. The resultant blots were blocked in 5% nonfat milk in PBST (1× PBS and 0.1% Tween-20) for 1 h at room temperature and then incubated with a monoclonal anti-rat PCNA antibody (clone PC-10; Oncogene Research) (34) diluted to 1:250 for 1.5 h at room temperature. After several washes in 5% nonfat milk in PBST, the blots were incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G secondary antibody (Bethyl Lab., Inc., Montgomery, Tex.) diluted 1:10,000 for 1 h at room temperature. After several washes in PBST and a 15-min wash in PBST containing 0.5 M NaCl, the immune complexes were revealed by enhanced chemiluminescence (Amersham) following the protocol from the manufacturer.

Nucleotide sequence accession number. The nucleic acid sequence of the cloned PCNA gene fragment has been submitted to GenBank under accession number AF012212.

RESULTS AND DISCUSSION

With RT-PCR, both PC5A/PC3C and PC5B/PC3C primer pairs generated a single product with a size around 600 bp (Fig. 1). In accordance with the primer design, the product from primer pair PC5A/PC3C was slightly larger. Subsequently, the shorter product was cloned, and two positive colonies were used to determine the nucleic acid sequence (616 bp in length). An open reading frame of 205 amino acids was deduced from the nucleic acid sequence, and the gap-free alignment showed a high degree of similarity between the deduced amino acid sequence of the *T. chui* fragment and PCNA sequences found in other organisms (Fig. 2). The similarity was especially high between *T. chui* and rice, reaching 88%, while the lowest similarity was between *T. chui* and yeast at 67% (Table 1). Although the isolated gene fragment was about 20% shorter than the full-length PCNA coding region, the similarity clearly indicates that it is the PCNA gene in *T. chui* (Fig. 2).

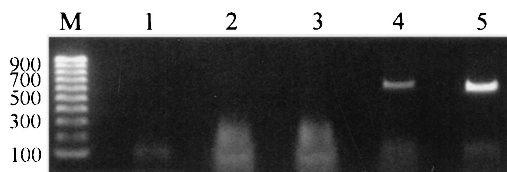

FIG. 1. Amplification of PCNA gene fragments from *T. chui*. Lane 1, negative control with RNA replaced by distilled water during RT-PCR; lanes 2 and 3, negative control without the addition of reverse transcriptase when the primer pair PC5A/PC3C or PC5B/PC3C was in use, respectively; lane 4, amplified RT-PCR product with the primer pair PC5A/PC3C; lane 5, amplified RT-PCR product with the primer pair PC5B/PC3C; lane M, DNA molecular size marker in base pairs. The original image was captured with a Kodak DCS 420 digital camera via MGDS program, and annotation was added with Aldus Photostyler software.

TABLE 1. Sequence identity and similarity between the cloned *T. chui* gene fragment and PCNA genes from other eukaryotic organisms according to the Genetics Computer Group BESTFIT program

Organism	Nucleic acid sequence identity (%)	Amino acid sequence (%)	
		Identity	Similarity
Human	69	64	80
Xenopus	65	61	80
Drosophila	60	62	82
Oryza sativa (rice)	72	76	88
Saccharomyces cerevisiae	68	39	67

The Southern blot analysis revealed one to three hybridization bands in each of the four distinctive restriction enzyme digestions (Fig. 3a), indicating that the PCNA gene is an integral part of the *T. chui* genome. Since the four restriction endonucleases used do not possess any cutting site within the DNA probe, the three-band pattern after *Eco*RI, *Hin*dIII, and *Xba*I digestion suggests that the *T. chui* genome may contain three copies of the PCNA gene (Fig. 3a). An alternative explanation is that the *T. chui* genome contains only a single copy of the PCNA gene, as suggested by *Pst*I digestion (Fig. 3a), and that the existence of extra hybridization bands in the other three enzyme digestions is a result of additional cutting sites located in the intron regions of the PCNA gene (23).

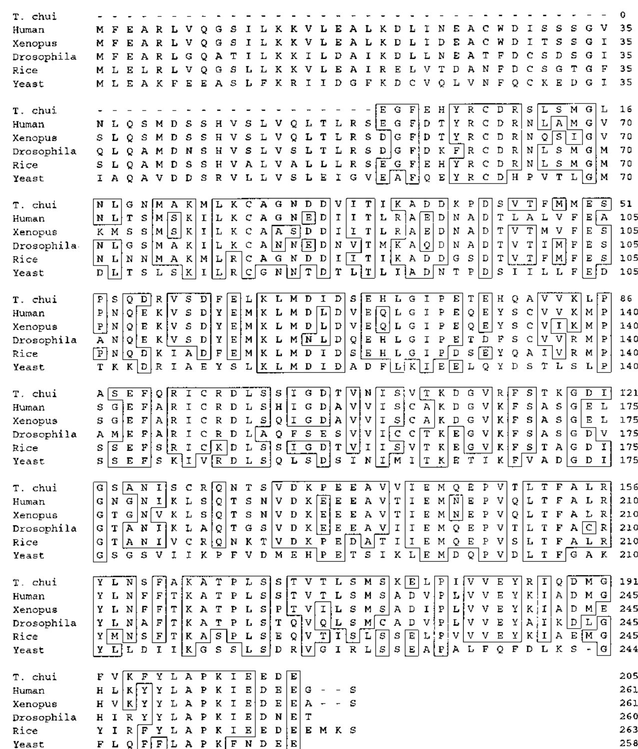

FIG. 2. Amino acid sequence alignment for PCNA from *T. chui* (partial sequence), human, *Xenopus*, *Drosophila*, rice (*Oryza sativa*), and yeast (*Saccharomyces cerevisiae*). Conserved amino acid residues found in four or more sequences are boxed. Sequences from organisms other than *T. chui* were obtained from GenBank, and amino acid numbering started at methionine 1. For *T. chui*, the first amino acid deduced from the RT-PCR product was labeled amino acid 1.

The 1.4-kb hybridization band revealed by Northern blot analysis indicated that the *T. chui* PCNA gene is actively transcribed in the exponential growth period (Fig. 3b). In contrast, the mRNA of rice PCNA is 1.2 kb long (30), and two species of PCNA mRNAs exist in rat with sizes of 1.1 and 0.98 kb (23).

Upon the addition of IPTG, production of a 32-kDa His-tagged fusion protein was increased in *E. coli* transformed with the pQE-31 vector containing the *T. chui* PCNA gene fragment (Fig. 4a, lane 2). When the anti-rat PCNA antibody was used to perform immunodetection, the fusion protein was heavily labeled on the Western blot (Fig. 4b, lane 2). Interestingly, under the uninduced condition the same antibody detected a single major band at the 32-kDa position. This result indicates that the polypeptide encoded by the *T. chui* gene fragment is still synthesized by *E. coli* in the absence of IPTG, although at a much lower level (Fig. 4b, lane 1). Based upon mobility, the PCNA fusion protein had a molecular mass of 32 kDa, which is substantially higher than the 23-kDa molecular mass calculated from the amino acid sequence (Fig. 2). A similar anomaly in mobility has been reported for both PCNA and PCNA fusion proteins from other organisms, such as rat (23, 34).

The monoclonal anti-rat PCNA detected a single band from *T. chui* total protein extract (Fig. 4b, lane 3). Since the same monoclonal antibody recognized the *T. chui* PCNA fusion protein (Fig. 4b, lane 2), the single band detected in *T. chui* protein extract must be the PCNA of this alga. Based on the mobility of the labeled band, *T. chui* PCNA should have a molecular mass of 33 kDa. The size of *T. chui* PCNA is the same as that of the PCNA-like protein in *D. tertiolecta* but is

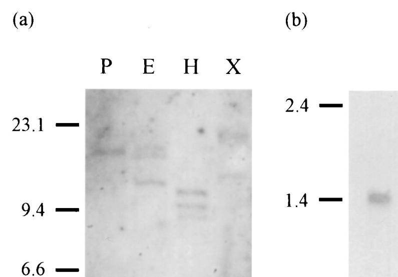

FIG. 3. Southern and Northern blot analyses of the PCNA gene and its mRNA in *T. chui*. (a) Southern blot analysis. *T. chui* genomic DNA digested individually by restriction endonucleases *Pst*I (P), *Eco*RI (E), *Hin*dIII (H), and *Xba*I (X) was separated on an agarose gel and transferred to a nylon membrane. The digested genomic DNA was then hybridized with a DIG-labeled PCNA DNA probe. (b) Northern blot analysis. Twenty micrograms of *T. chui* total RNA was fractionated and transferred to a nylon membrane. The blot was hybridized with a DIG-labeled antisense PCNA RNA probe. The positions and sizes (in base pairs) of molecular size markers are labeled on the left of each panel.

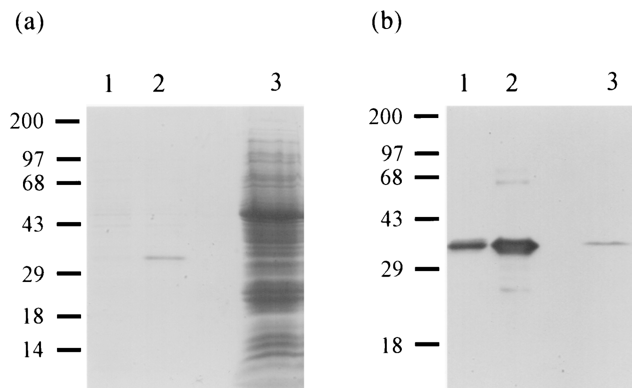

FIG. 4. The production of *T. chui* PCNA fusion protein and immunodetection by the anti-rat PCNA antibody. (a) Crude extract of *E. coli* containing the recombinant PCNA gene fragment was separated on an SDS-PAGE gel and stained with Coomassie blue. The *E. coli* extract under untreated and IPTG-treated conditions is shown in lanes 1 and 2, respectively. Total protein extracted from a *T. chui* culture is shown in lane 3. (b) Proteins in an SDS-PAGE gel run simultaneously with that in panel a were transferred to a nitrocellulose membrane and detected with the anti-rat PCNA antibody. Lane assignment is the same as that in panel a.

somewhat smaller than those of other phytoplankton tested (17).

In a marine diatom, *Skeletonema costatum*, anti-rat PCNA recognized an additional 19-kDa protein band that did not change in intensity with the age of the culture (17). Apparently, in some phytoplankton species, anti-rat PCNA may cross-react with proteins of an unknown nature. Whether the cross-reacting proteins will cause false positives in immunocytochemical staining requires further study.

Our results provide strong support for the conclusion that phytoplankton proteins detected by anti-rat PCNA are truly algal PCNA (17). Therefore, we now have a firmer foundation upon which to base the use of the anti-rat PCNA antibody as a tool to detect actively dividing phytoplankton cells in a natural environment (18). By enumerating PCNA-positive cells in water samples, the in situ growth rates of several phytoplankton species can be estimated simultaneously by a slightly modified cell cycle analysis equation (21). In addition, in case the commercially available antibody fails to detect PCNA in some species, as has occurred in a slime mold (1), the same procedure can be repeated to generate a PCNA fusion protein specifically for those species and to use it to generate a workable antiserum.

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