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Transcriptomic profiling of *Ichthyophthirius multifiliis* reveals polyadenylation of the large subunit ribosomal RNA

Jason W. Abernathy [a], De-Hai Xu [b], Ping Li [a], Phillip Klesius [b], Huseyin Kucuktas [a], Zhanjiang Liu [a,*]

[a] The Fish Molecular Genetics and Biotechnology Laboratory, Department of Fisheries and Allied Aquacultures and Program of Cell and Molecular Biosciences, Aquatic Genomics Unit, 203 Swingle Hall, Auburn University, AL 36849, USA
[b] Aquatic Animal Health Research Laboratory, Agricultural Research Service, United States Department of Agriculture, 990 Wire Road, Auburn, AL 36831, USA

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ABSTRACT

Polyadenylation of eukaryotic transcripts is usually restricted to mRNA, providing transcripts with stability from degradation by nucleases. Conversely, an RNA degradation pathway can be signaled through poly (A) tailing in prokaryotic, archeal, and organellar biology. Recently polyadenylated transcripts have also been discovered in rRNA in some eukaryotes including humans and yeast. Here we report the discovery of polyadenylated rRNAs in the ciliate teleost parasite *Ichthyophthirius multifiliis*, an important fish pathogen. Through large-scale analysis of ESTs, a large contig composed of the 28S rRNA with poly (A) tails was identified. Analysis using multiple sequence alignments revealed four potential polyadenylation sites including three internal regions and the 3′ end of the rRNA. Further analysis using a polyadenylation test, re-sequencing, and gene-specific PCR using primers flanking the presumed poly (A) sites confirmed the presence of polyadenylated rRNA in this parasite. The functions of polyadenylation of rRNA in this organism are largely unknown at present, but the presence of internal polyadenylation sites, along with the presence of truncated segments of the rRNA, may suggest a role of the polyadenylation in the degradation pathway, a function typical of prokaryotes, archaea, and organelles. These results are in congruence with reports of a similar phenomenon in humans and yeast.

1. Introduction

Three main classes of RNA exist in eukaryotes consisting of ribosomal RNA (rRNA), messenger RNA (mRNA), and transfer RNA (tRNA). In protein synthesis, rRNA and ribosomal proteins form the ribosomes where protein synthesis occurs; mRNAs encode the primary sequence of proteins for which the proper amino acids are transferred to the growing peptide chain by tRNA. In addition to these three major classes of RNAs, several other classes of RNAs have been reported including hybrid transfer-messenger RNA (tmRNA), small nuclear and small nucleolar RNA (snRNA and snoRNA), and micro-RNA (miRNA).

Important features of RNA are the modifications that occur during or immediately after transcription; in particular 5′ methylated reverse capping and 3′ polyadenylation. Polyadenylation is evident not only in the mRNA of all domains of eukaryotes but also in some organellar RNA: mitochondrial RNA (mtRNA) or chloroplast RNA (cpRNA). Typically, eukaryotic mRNA is polyadenylated on the mature transcript with implications for stability, signaling, and nuclear transport in compartmentalized cells, and can also serve as a binding domain (Beelman and Parker, 1995; de Moor and Richter, 2001; Edmonds, 2002; Mangus et al., 2003; Shatkin and Manley, 2000). In contrast, polyadenylation of mRNA in prokaryotes and archaea, as well as polyadenylation with certain mtRNA or cpRNA, signals RNA degradation (Dreyfus and Regnier, 2002, Kushner, 2004, O'Hara et al., 1995, Slomovic et al., 2005). In recent RNA examinations, exceptions to the stability versus decay rule are rising and coexisting mechanisms seem to occur (Kao and Read, 2005; LaCava et al., 2005; Slomovic et al., 2005; Vanacova et al., 2005; West et al., 2006; Wyers et al., 2005).

Polyadenylation is typically thought to be exclusive to translated RNA. Conversely, non-translated rRNAs have recently been shown to exhibit polyadenylation in some eukaryotes. Discoveries have been forthcoming of rRNA polyadenylation in humans (Slomovic et al., 2006), yeasts *Candida albicans* and *Saccharomyces cerevisiae* (Fleischmann and Liu, 2001; Fleischmann et al., 2004; Kuai et al., 2004), and the protozoan Kinetoplastid *Leishmania* (Decuypere et al., 2005). Here we report the polyadenylation of the large subunit rRNA in the ciliate protozoan *Ichthyophthirius multifiliis* (Ich).

The ciliate protozoan *I. multifiliis* is a devastating freshwater teleost parasite. The parasite is widespread; it affects many freshwater fish species around the world. It causes great loss to both the aquaculture and ornamental fish industries. Ich is responsible for the disease

* Corresponding author. Aquatic Genomics Unit, Department of Fisheries and Allied Aquacultures, 203 Swingle Hall, Auburn University, AL 36849, USA. Tel.: +1 334 844 4054; fax: +1 334 844 4694.
E-mail address: zliu@acesag.auburn.edu (Z. Liu).

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ichthyophthiriosis or 'white spot' disease with characteristic white spot cysts forming under the fish gill or epidermis. Also, typical of unicellular members of the phylum Ciliophora, Ich exhibits nuclear dimorphism. It possesses the characteristic macronucleus and micronucleus. Ich has been shown to be closely related phylogenetically to species of *Tetrahymena* (Abernathy et al., 2007; Eisen et al., 2006; Wright and Lynn, 1995) such as *T. thermophila*, a free-living non-parasitic ciliate protozoan. From a comparative view to *Tetrahymena* (Eisen et al., 2006; Prescott, 1994), Ich may also undergo both germ line and somatic cell divisions, generally where the micronucleus contains the germ line DNA that can be replenished through meiotic conjugation, and the macronuclear DNA that is transcriptionally active to support the cell. There has been evidence for asexual reproduction in Ich for many years, with possible evidence that some sexual regeneration may occur [for a review see Matthews, 2005]. One such piece of evidence arises from the major difficulties encountered in maintaining viable isolates of Ich in the laboratory setting. Multiple passages of an Ich isolate on a fish host lead to a significant decline in infectivity, relating to senescence (Matthews, 2005; Xu and Klesius, 2004). It is speculated that this type of induced senescence could be due to a lack of recombination of the germ line. Therefore, undertaking a study of senescence-related genes would be an important step in better understanding Ich development, reproduction, and parasitic nature.

A set of highly abundant transcripts containing poly (A) tails at the 3′ end was initially identified during EST analysis (Abernathy et al., 2007). BLASTX similarity comparisons of these transcripts yielded hypothetical proteins related to senescence proteins in antisense orientation, with low to moderate similarity ($e^{-6} \geq$ E-value $\geq e^{-78}$ for various segmented alignments). We continued to study these transcripts to test for senescence-related expression, and we learned that the antisense transcripts did not exist using Northern blot analysis, but its related sense strand transcripts were highly expressed in all three stages and all ages of Ich tested. Such results led to more extensive BLAST searches using both BLASTX and BLASTN. BLASTN searches revealed that these transcripts were highly similar (E-value = 0) to ribosomal RNAs, in particular to portions of the large subunit ribosomal RNA in many organisms, including closely related *Tetrahymena* species, or to precursor-rRNA in *T. thermophila* with alignment to the large subunit. However, the putative identity as rRNA is in contradiction of the fact that the transcripts were found to be polyadenylated. Therefore, we set out to further characterize these transcripts, obtain more of their sequences, and test for polyadenylation. Here we present the 28S rRNA sequence from the ciliate protozoan *I. multifiliis*, and provide evidence for polyadenylation of the large subunit rRNA.

2. Materials and methods

2.1. Cells, RNA isolation, and analysis

A single Ich isolate was obtained from a local pet shop with an outbreak of ichthyophthiriosis. Ich was cultured on fish as previously described (Abernathy et al., 2007). Samples were washed in phosphate-buffered saline (PBS; pH 7.2) and flash-frozen in liquid nitrogen. Cells were stored in a −80 °C freezer until usage. Total RNA was isolated from each of the three Ich life-stages using the RNeasy Plus Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The kit uses a spin column to eliminate DNA contamination. RNA quality was assessed by using denaturing agarose gel electrophoresis containing formaldehyde (Liu, 2007a).

2.2. Sequence analysis

The Ich ESTs used for assembly of the initial contiguous sequences (contigs) are from GenBank dbEST accession numbers EG957858–EG966289. The ESTs were generated by sequencing a unidirectional cDNA library enriched for full-length cDNAs (Abernathy et al., 2007; Liu, 2007a,b). All ESTs were sequenced from the 5′ end. The sequences were assembled into contigs using ContigExpress in the Vector NTI version 10.3.0 software (Invitrogen, Carlsbad, CA, USA) with an overlap length cutoff set at 40 bp and sequence identity cutoff of the overlap set at 90%. All other settings were at the default values. The contig used for this study was the largest contig in the assembly containing 764 ESTs. The contig was visually inspected for polyadenylated regions. During the course of this study, EST resources grew significantly in the dbEST database of the GenBank. Using the latest release, version 031408 (25,084 ESTs), we were able to assemble the contig to include the mature 3′ end of the rRNA molecule as compared with the top 4 megablast hits.

Sequence identity was determined using BLASTN at the National Center for Biotechnology Information (NCBI). Multiple sequence alignments were conducted using ClustalW with the 2677 bp Ich consensus sequence from the contig (herein 28S rRNA), along with the top homologous sequences generated from BLAST searches.

2.3. Northern blot

Two separate Northern blot analyses (Sambrook et al., 1989) were performed on the three life-stages of Ich. Briefly, total RNA was isolated using the RNeasy Plus kit according to the manufacturer's provided protocol. Total RNA (3 μg each of the three Ich life-stages) was separated by electrophoreses on a 1% agarose gel containing formaldehyde. The gel was UV visualized, then rinsed twice for 10 min in DEPC-treated water to destain and remove excess formaldehyde. The gel was dipped in 20× SSC buffer. A downward capillary transfer was performed for 4 h using positively charged nylon membranes (Millipore, Bedford, MA) and 20× SSC buffer. After transfer, the membranes were dipped in 20× SSC buffer, and RNA was fixed to the membrane by UV-crosslink using the UV Stratalinker 2400 (Stratagene, La Jolla, CA, USA) using the auto-crosslink setting. The membranes were pre-hybridized in 5 mL ULTRAhyb-Oligo buffer (Ambion, Austin, TX, USA) for 1.5 h at 42 °C. Labeled probes were added and hybridized overnight at 42 °C in a hybridization oven. The membranes were washed twice for 30 min each in a wash buffer (2× SSC, 0.5% SDS) and exposed to X-ray film for 4 h at room temperature. As our initial BLASTX analysis indicated the contig under consideration was similar to senescence proteins, but in an antisense orientation, two probes were used to determine the presence of sense-strand transcript (using an antisense probe: 5′-GACCAGAGGCTGCTAACCTTGGAGACCTGATGCGGTTATG-3′) and antisense transcript (using a sense probe: 5′-CATAACCGCATCAGGTCTCCAAGGTTAGCAGCCTCTGGTC-3′). The probes were end-labeled using ATP [γ-^{32}P] (MP Biomedicals, Solon, OH, USA).

2.4. Testing for polyadenylation at the full 3′ end

One-hundred nanograms of total RNA from each of the three Ich life-stages was pooled and reverse transcribed using an oligo (dT) adapter primer 5′-GGTGAGCCCGCGTCACGG$(T)_{12}$-3' as designed elsewhere (Decuypere et al., 2005) using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's protocol. A polyadenylation test was conducted as previously described (Salles

Table 1
PCR primer sequences and product sizes assuming there are no internal poly (A/T) tracts as predicted from the consensus sequences.

	Upper primer	Lower primer	Predicted PCR product sizes	Observed PCR product sizes
First poly (A) site	GGTCTCCAAGGTTAGCAGC	AGGCCGAAGCCACTCTAC	200 bp	200 bp
Second poly (A) site	ACATGCCTGCGCATAAG	GTTGAATTGCGTCACTTTGA	200 bp	200 bp
Third poly (A) site	TGCCGTGAAGCTACCATC	GACTCTTTCGTCTTCAGCC	150 bp	150 bp