Mitochondrion 23 (2015) 25–31

Contents lists available at ScienceDirect

Mitochondrion

journal homepage: www.elsevier.com/locate/mito

MitoMatters

Transcription and processing of mitochondrial RNA in the human pathogen *Acanthamoeba castellanii*

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ARTICLE INFO

Article history:
Received 12 January 2015
Received in revised form 18 May 2015
Accepted 22 May 2015
Available online 28 May 2015

Keywords:
Acanthamoeba castellanii
Mitochondrial genome
Mitochondrial transcription
Polycistronic transcripts
Transcript processing
tRNA punctuation

ABSTRACT

The size, structure, gene content and organisation of mitochondrial genomes can be highly diverse especially amongst the protists. We investigated the transcription and processing of the mitochondrial genome of the opportunistic pathogen *Acanthamoeba castellanii* and here we present a detailed transcription map of the 41.6 kb genome that encodes 33 proteins, 16 tRNAs and 2 rRNAs. Northern hybridisation studies identified six major polycistronic transcripts, most of which are co-transcriptionally processed into smaller mono-, di- and tricistronic RNAs. The maturation of the polycistronic transcripts is likely to involve endonucleolytic cleavage where tRNAs serve as processing signals. Reverse transcription polymerase chain reactions across the intervening regions between the six major polycistronic transcripts suggest that these transcripts were once part of an even larger transcript. Our findings indicate that the mitochondrial genome of *A. castellanii* is transcribed from only one or two promoters, very similar to the mode of transcription in the mitochondria of its close relative *Dictyostelium discoideum*, where transcription is known to occur from only a single transcription initiation site. Transcription initiation from a minimal number of promoters despite a large genome size may be an emerging trend in the mitochondria of protists.

1. Introduction

Mitochondria are thought to have originated from an endosymbiotic event between a primitive eukaryotic cell and an α-proteobacterial ancestor (Margulis, 1970; Gray et al., 1999; Mercer et al., 2011). During the course of its evolution, the bacterial endosymbiont underwent many changes where most of its genes were lost or transferred to the nucleus of the eukaryotic host cell (Adams et al., 2002; Adams and Palmer, 2003; Barbrook et al., 2010). Although the mitochondria retained some of the original genetic information, their genomes can be extraordinarily diverse in their size, structure, gene content and gene organisation, depending on the state of their evolution and the organism in which they are found (Gray et al., 1998).

The smallest sequenced mitochondrial genome belongs to the human malaria parasite, *Plasmodium falciparum*, at less than 6 kb in size; it encodes only three protein and two ribosomal RNAs (rRNAs) (Wilson and Williamson, 1997). In contrast, the largest mitochondrial genomes sequenced belong to plants, ranging in size from 200 kb to over 2000 kb (Palmer et al., 2000; Bevan and Lang, 2004). These genomes can be far more complex than their metazoan and protozoan counterparts, due to the presence of large non-coding regions arising from genetic recombination and gene duplications (Newton, 1988; Gray, 1989; Binder et al., 1996; Holec et al., 2006). However, a common set of genes seems to be present in most mitochondrial genomes sequenced to date; the set includes genes encoding the large and small subunit rRNA, some transfer RNAs (tRNAs) and several proteins required for oxidative phosphorylation (Andersson and Kurland, 1999; Adams and Palmer, 2003; Gray, 2012).

The mechanism of gene expression can also be highly variable amongst mitochondrial genomes of different organisms with transcription occurring either from a single control region as found in the human mitochondrial genome, or from multiple promoters, which has been shown to be the case in plants, fungi and some protozoa (Jaehning, 1993; Tracy and Stern, 1995; Taanman, 1999; Asin-Cayuela and Gustafsson, 2007; Gagliardi and Binder, 2007). In many cases, mitochondrial genes located close together are often found to be co-transcribed into polycistronic transcripts, a mechanism that is hypothesised to assist in the regulation of gene expression (Wissinger et al., 1988; Binder et al., 1996; Takvorian et al., 1997; Barbrook et al., 2010). Maturation of these polycistronic transcripts may occur through either co- or post-transcriptional processing, mediated via tRNA cleavage and other processing mechanisms, to produce RNA species containing individual coding sequences (Ojala et al., 1980, 1981; Costanzo and Fox, 1990). The smaller RNAs may be subjected to additional post-transcriptional modifications, such as RNA editing, which occurs in the mitochondria of a variety of species to produce fully functioning RNA transcripts (Lonergan and Gray, 1993; Scott, 1995; Simpson and Thiemann, 1995; Barth et al., 1999; Simpson, 2003).

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http://dx.doi.org/10.1016/j.mito.2015.05.003
1567-7249/

While a great deal is known about gene expression in organisms with small mitochondrial genomes, such as animals, less is known about mitochondrial gene expression in organisms with larger mitochondrial genomes such as plants and protists. In 2009, Le et al. demonstrated that the large (55.5 kb) mitochondrial genome of the protozoan *Dictyostelium discoideum* is transcribed from only a single transcription initiation site. The resulting large polycistronic transcript is co-transcriptionally processed into eight secondary transcripts, which are further processed into mature mono-, di- and tricistronic RNA molecules (Barth et al., 2001; Le et al., 2009).

In recent years' *Acanthamoeba castellanii* a free living protist, closely related to *D. discoideum* has gained interest from various scientific communities. This is due to the versatility of this organism, as it not only plays a role in the environment, regulating microbial communities (Rønn et al., 2002; Rosenberg et al., 2009), it can act as a Trojan horse for microbial pathogens (Greub and Raoult, 2004; Hilbi et al., 2007) and can cause serious human diseases such as cutaneous lesions, amoebic keratitis and a rare, granulomatous amoebic encephalitis (Marciano-Cabral and Cabral, 2003; Khan, 2007; Visvesvara et al., 2007). As part of a comprehensive effort to explore the mitochondrial genetics in protists, the mitochondrial genome of *A. castellanii* has been fully sequenced (Burger et al., 1995).

A. castellanii contains a circular mitochondrial genome that is 41.6 kb in size, carrying genes encoding large and small subunit rRNAs, 16 tRNAs, and 33 proteins involved in the electron transport chain, oxidative phosphorylation and ribosomal structure (Burger et al., 1995). All genes that are encoded on the same strand, are densely packed with relatively short intergenic regions, and in some cases overlap by up to 38 bp (Burger et al., 1995). It is of interest to note that the *A. castellanii* mitochondrial genome contains a single continuous ORF for the genes encoding cytochrome oxidase subunits 1 and 2 (*cox1* and *2*) and has all but two of its ribosomal proteins present in a single cluster (Lonergan and Gray, 1996). The mitochondrial genome of *A. castellanii* shares these features with that of *D. discoideum* (Burger et al., 1995, 2003; Gray et al., 2004).

This report presents a detailed transcription map of the mitochondrial genome in *A. castellanii*. In Northern hybridisation studies, six polycistronic transcripts were detected, most of which appear to be processed further into smaller transcripts. Reverse Transcription PCR (RT-PCR) confirmed the polycistronic nature of these transcripts and indicated that the polycistronic transcripts derived from an even larger primary transcript. In most cases, processing of the RNA molecules seems to occur through the excision of tRNAs, which act as processing signals. The existence of a large primary transcript that is co-transcriptionally processed into mature RNAs indicates that the mitochondrial genome of *A. castellanii* requires the presence of only one or two promoters for efficient gene expression. This mode of transcription is therefore very similar to the mitochondrial genome of its close relative *D. discoideum*.

2. Materials and methods

2.1. Strain and culture conditions

A. castellanii strain Neff (ATCC® 30010) was grown axenically in PYG medium (2% (w/v) Proteose Peptone, 0.1% (w/v) Yeast Extract, 1% (v/v) 0.4 M $MgSO_4 \cdot 7H_2O$, 0.8% (v/v) 0.05 M $CaCl_2$, 3.4% (v/v) 0.1 M Sodium Citrate, 1% (v/v) 0.005 M $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (ferrous ammonium sulfate), 1% (v/v) 0.25 M Na_2HPO_4 and 1% (v/v) 0.25 M KH_2PO_4) at 28 °C until the culture reached the required cell density.

2.2. Isolation of A. castellanii genomic DNA

Large scale isolation of *A. castellanii* genomic DNA (gDNA) was performed as described by Noegel et al. (1985). *A. castellanii* cells were harvested via centrifugation at 2000 rpm for 10 min at 4 °C and lysed with nuclear lysis buffer (0.01 M NaCl, 0.01 M Mg acetate, 0.03 M Hepes, 10% (w/v) Sucrose and 2% (v/v) NP-40). The nuclei were collected through centrifugation at 6000 rpm for 10 min at 4 °C and treated with hot (65 °C) EDTA/Sarkosyl (0.2 M EDTA and 2% (w/v) N-Lauroylsarcosine sodium salt) to lyse the nuclei and release the gDNA. The gDNA was separated on a CsCl density gradient, collected, ethanol precipitated and stored in TE (0.01 M Tris–HCl; pH 8.0, 0.001 M EDTA) buffer at 4 °C.

2.3. Creation of digoxigenin (DIG) labelled DNA probes

Random-primed labelling with DIG-High Prime was performed using the procedure described by the manufacturer (Roche). 1 μg of gene specific PCR product was heat denatured at 100 °C for 10 min and then rapidly cooled. Subsequently DIG-High Prime labelling mixture was added, the reaction was mixed thoroughly and then incubated at 37 °C for 1–20 h. The reaction was terminated with the addition of 2 μl 0.2 M EDTA. The labelled probes were concentrated through ethanol precipitation and resuspended in TE buffer.

2.4. RNA preparation and analysis

2.4.1. RNA extraction

Total *A. castellanii* RNA was isolated using TRIzol® reagent according to the supplier's instructions (Life Technologies). The cells were grown to a density of 5×10^6 cells ml^{-1}, pelleted and lysed with 1 ml of TRIzol® reagent. To allow separation of the RNA from protein, 0.2 ml of chloroform was added and the mixture was centrifuged at maximum speed in a bench top centrifuge (Eppendorf centrifuge 54151). The aqueous phase was collected and the RNA recovered by isopropanol precipitation and resuspended in sdDEPC-treated H_2O.

2.4.2. Northern hybridisation

Samples containing approximately 5 μg μl^{-1} of total RNA were separated on a 1–1.5% (w/v) agarose gel prepared with DEPC TAE (Tris-acetate) buffer, at 100 V for 2 h and then transferred onto a nylon membrane (GE Healthcare). The membrane was subsequently probed with gene-specific DIG-labelled DNA probes, and a fluorescent substrate for alkaline phosphatase-based detection, Enhanced Chemifluorescence (ECF) (GE Healthcare Life Sciences), was used for detection. The membrane was left in the dark at room temperature for up to 1 h. Fluorescence was detected by scanning the membrane using the Amersham Storm 860 imaging system (GE Health Bio-Sciences).

2.4.3. Reverse transcriptase polymerase chain reaction

Prior to RT-PCR, the total RNA samples were incubated with DNase I (DNA-free kit, Ambion) following the manufacturer's instructions to remove any contaminating DNA. The DNase-treated total RNA and appropriate gene specific 3′ primers (Table 2) were heat denatured at 65 °C for 5 min to remove secondary structures. The RNA template was reverse transcribed using ThermoScript™ Reverse Transcriptase (Invitrogen) at 55 °C for 1 h and the enzyme was denatured at 85 °C for 5 min. The resulting cDNA was amplified via PCR with gene-specific 5′ and 3′ primers (Table 2) for 30 cycles at 94 °C for 1 min, 55 °C for 1–1.5 min and 72 °C for 2 min. PCR reactions using gDNA or DNase-treated RNA as template served as positive or negative controls, respectively.

3. Results

3.1. Acanthamoeba mitochondrial genes are co-transcribed into large polycistronic transcripts

The *A. castellanii* mitochondrial genome is 41.6 kb in size and, like most protozoan mitochondrial genomes, carries a core set of 33 genes encoding proteins for oxidative phosphorylation and energy