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Original research article

Putative DNA-dependent RNA polymerase in Mitochondrial Plasmid of *Paramecium caudatum* Stock GT704

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ABSTRACT

Mitochondria of *Paramecium caudatum* stock GT704 has a set of four kinds of linear plasmids with sizes of 8.2, 4.1, 2.8 and 1.4 kb. The plasmids of 8.2 and 2.8 kb exist as dimers consisting of 4.1- and 1.4-kb monomers, respectively. The plasmid 2.8 kb, designated as pGT704-2.8, contains an open reading frame encodes for putative DNA-dependent RNA polymerase (RNAP). This study reveals that this RNAP belongs to superfamily of DNA/RNA polymerase and family of T7/T3 single chain RNA polymerase and those of mitochondrial plasmid of fungi belonging to Basidiomycota and Ascomycota. It is suggested that RNAP of pGT704-2.8 can perform transcription without transcription factor as promoter recognition. Given that only two motifs were found, it could not be ascertained whether this RNAP has a full function independently or integrated with mtDNA in carrying out its function.

1. Introduction

Plasmids are extrachromosomal genetic elements found in various organisms. Two types of plasmid structures known so far, namely the double-stranded closed circular plasmid which commonly found in cells of prokaryotes and some eukaryotes, and single- and double-stranded linear plasmids found in fungi, yeast, and ciliates (Tallei *et al.* 2002; Hausner 2003).

Linear plasmid has terminal inverted repeat at both ends and covalently-bound terminal protein (TP) at 5′ end which is similar to some viral genome structure (Andrade *et al.* 2009). Two open reading frames (ORFs) of these plasmids encode for DNA and RNA polymerases which are required for their replication (Griffiths 1995; Oeser and Tudzynski 1989; Chan *et al.* 1991). RNA polymerase (RNAP) in the linear plasmids are DNA-directed RNAP (DNA-dependent RNAP) and abbreviated as RNAP, which serves to catalyze the synthesis of a DNA chain using DNA as a template. The RNAP characteristic is similar to those of the bacteriophage and yeast mitochondria. The 5′ end, besides being involved in replication, also has a role in the integration of the plasmid into mitochondrial genome (Griffiths 1995; Andrade *et al.* 2009; Andrade *et al.* 2013). Mitochondrial plasmid of *Moniliophthora perniciosa* can integrate fully into the mitochondrial genome (Andrade *et al.* 2009). The plasmid is likely to have a function in the aging process (senescence) (Chan *et al.* 1991; van Diepeningen *et al.* 2008).

Paramecium is the first example in which an mtplasmid was discovered among ciliates. *Paramecium caudatum* stock GT704 has type-II DNA with two sets of mtplasmids. The 8.2 and 2.8 kb DNAs are dimers of the 4.1 and 1.4 kb DNA, respectively (Tallei *et al.* 2002; Endoh *et al.* 1994). The plasmid 2.8 kb (designated as pGT704-2.8) has an ORF for RNAP. This study aimed at characterizing RNAP encoded by pGT704-2.8 and analyzing its relationship with RNAPs from other organisms to predict its function in mitochondria.

2. Material and Methods

2.1. Stock and culture

From previous research conducted by Tallei *et al.* (2002), *P. caudatum* stock GT704 used in this study contains plasmid type II. Plasmid type II always has four types of plasmid DNA with sizes of 8.2, 4.1, 2.8, and 1.4 kb. Plasmid DNA 8.2 and 2.8 kb are always in the form of dimer, each of monomer molecule of 4.1 and 1.4 kb, respectively. The cells of *P. caudatum* were grown at 25 °C in fresh lettuce juice medium inoculated with non-pathogenic strains of *Kleibsiella pneumoniae* 1 day before use.

2.2. Purification of mitochondria

The cell suspension was filtered with gauze to remove dirt, centrifuged, and washed twice in a Dryl solution (Tsukii *et al.* 1994). Mitochondrial purification was performed following the method

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of Goddard and Cummings (1975) with slight modification. The centrifuged cells were resuspended in two times volume of ice cold mannitol solution (0.44 M mannitol, 1 mM KCl, 1 mM Tris-HCl pH 7.5, and 0.25% bovine serum albumin). Cells of *P. caudatum* were crushed using a homogenizer until almost all the cells ruptured. Homogenized cells were resuspended in ice cold mannitol and centrifuged at 600 *g* for 6 minutes at 0 °C. Supernatant was centrifuged at 5,000 *g* for 6 minutes. Mitochondrial pellets were resuspended in 1 mL of mannitol solution containing 30 U of DNAse I (Takara Shuzo Co., Japan) and 5 mM $MgCl_2$. Mitochondrial suspension was incubated on ice for 25 minutes and added with mannitol solution containing 10 mM EDTA, followed by centrifugation at 5,000 *g* for 6 minutes. Pellets were used for pure mitochondrial fraction.

2.3. Extraction of mitochondrial DNA and plasmids

Mitochondria were lysed in 0.1 M Tris-HCl (pH 8.0), 50 mM EDTA, 1% (w/v) sodium dodecyl sulfate and 0.1 mg/mL proteinase K for 50 °C for 3 hours. Lysate was extracted twice with phenol (same volume) and treated with proteinase K (Tsukii *et al.* 1994).

2.4. DNA sequencing

Nucleotide sequencing of pGT-704-2.8 kb was conducted at Genetics Laboratory, University of Kanazawa, Japan.

3. Results

Mtplasmids 8.2 and 2.8 kb are dimers of monomers 4.1 and 1.4 kb, respectively. Sequence analyzed was dimer 2.8 kb (pGT704-2.8) (GenBank accession: FJ222255). Nucleotide Basic Local Alignment Search Tool result shows that pGT704-2.8 encodes for putative DNA-directed RNAP. This RNAP has sequence identity with RNAP encoded by several mtplasmids found in fungi, namely pHC of *Hebeloma circinans* (75%), pLP of *Pleurotus ostreatus* (74%), pFv of *Flammulina velutipes* (70%), pEM of *Agaricus bitorquis* (59%), pAL2-1 of *Podospora olymera* (55%), *kal* of *Gelasinospora sp.* (54%), kalilo of *Neurospora intermedia* (54%), and pCLK of *Claviceps purpurea* (53%).

Nucleotide sequence of pGT704-2.8 was translated using special code for mitochondria. The following is the amino acid sequence of putative DNA-directed RNAP:

MNHRDKYQEYENIYDYDEKSS**INSLEA**NNLHKIDALLVKHVLEVLD
VIT**IHDCF**GTRIKDVAKLIDVVNIYYQRYTSNDNYSIFILX

The sequences are in conserved domain of RNAP (Figure 1). The figure shows that RNAP of pGT704-2.8 is a part of single chain RNAP family and resembles RNAP of T-even phages group (T3/T7). Basic Local Alignment Search Tool protein search reveals that this putative protein has identity with RNAP of other mtplasmids found in *Hebeloma circinans* (41%), *Pleurotus ostreatus* (40%), *Agaricus bitorquis* (39%), *Flammulina velutipes* (39%), *Claviceps purpurea* (32%), *Gelasinospora* sp. (30%), *Neurospora intermedia* (29%), *Podospora anserina* (30%), and RNAP of Phage T3 (53%).

Figure 2 shows the alignment of conserved domain of several fungal RNAPs which has high identity with RNAP found in pGT704-2.8. Figure 3 shows motifs resulted using Multiple EM for Motif Elicitation (MEME) software developed by Bailey and Elkan (1994). Based on this MEME motif, RNAP of pGT704-2.8 does not have solid motif on block X so it cannot be produced by the program, while the block XI can be generated. Figure 4 shows phylogenetic tree reconstructed using Geneious version 8.0.4. RNAP of pGT704-2.8 is in monophyletic group (clade 1) with RNAP of mtplasmids of *Hebeloma circinans*, *Pleurotus ostreatus*, and *Agaricus bitorquis*, all of them belong to division Basidiomycota which have two linear plasmids in their mitochondria. *Flammulina velutipes* also belongs to division Basidiomycota although it is in the same monophyletic group (clade 2) together with *Claviceps purpurea*, *Podospora anserina*, *Gelasinospora* sp., and *Neurospora intermedia* which belong to division Ascomycota that have one linear plasmid in their mitochondria.

4. Discussion

Linear mtplasmids have often been found in fungi and plants. The first discovery of this kind of plasmid in animal kingdom was in *P. caudatum* (protozoa: ciliates) (Tallei *et al.* 2002). Furthermore, the linear mtplasmid was found in *Oxytricha trifallax* (protozoa: ciliates) by Swart *et al.* (2012). This plasmid has a size of approximately 5 kb and has 251 bp sequence with identity 82% with its mitochondrial genome (mtDNA), so it is suggested that this mtplasmid had been integrated in the mtDNA.

Circular mtplasmid usually has one ORF encodes for one DNA polymerase or one reverse transcriptase, while linear mtplasmid generally has two ORFs encode for putative DNA polymerase and RNAP, in which their amino acid motifs have homology to viral polymerases (Griffiths 1995). In linear plasmids, DNA polymerase and RNAP each is encoded by one single plasmid, or encoded by different plasmid if there are more than one plasmids in mitochondria (Cermakian *et al.* 1997).

According to Griffiths (1995), the characteristics of the linear plasmid among others, have the ORF that encodes a viral type of DNA polymerase and RNAP similar to RNAP of bacteriophage or

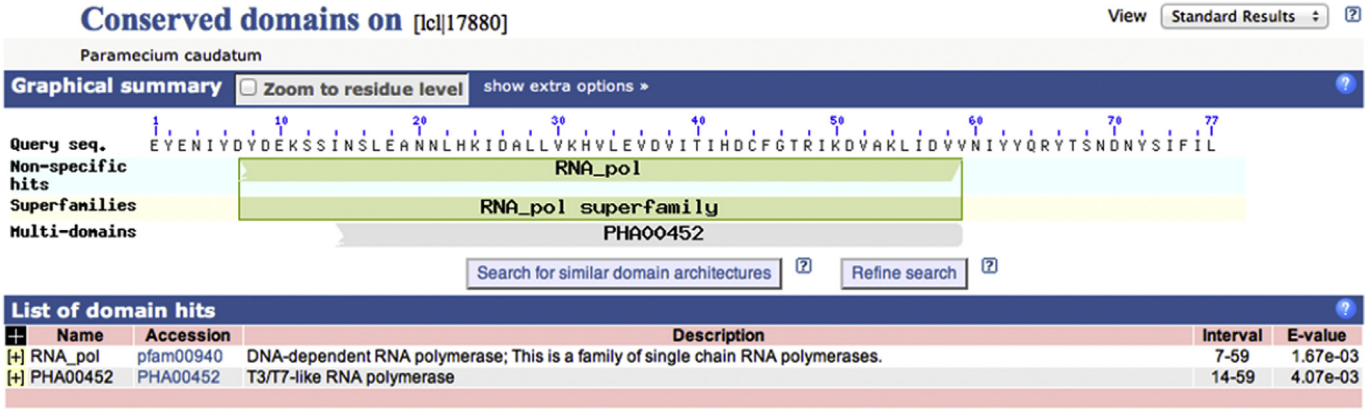

Figure 1. Conserved domain of RNA polymerase (RNAP) of pGT704-2.8 based on conserved domain search developed by Marchler-Bauer *et al.* (2011) showing that this RNAP belongs to DNA-dependent RNAP, a family of single chain RNAP and resembles T3/T7-like RNAP.