Research article

A SMALL SEQUENCE IN DOMAIN V OF THE MITOCHONDRIAL LARGE RIBOSOMAL RNA RESTORES *Drosophila melanogaster* POLE CELL DETERMINATION IN UV-IRRADIATED EMBRYOS

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Abstract: The mechanism by which the mitochondrial large rRNA is involved in the restoration of the pole cell-forming ability in *Drosophila* embryos is still unknown. We identified a 15-ribonucleotide sequence which is conserved from the protobacterium *Wolbachia* to the higher eukaryotes in domain V of the mitochondrial large rRNA. This short sequence is sufficient to restore pole cell determination in UV-irradiated *Drosophila* embryos. Here, we provide evidence that the conserved 15-base sequence is sufficient to restore luciferase activity *in vitro*. Moreover, we show that the internal GAGA sequence is involved in protein binding and that mutations in this tetranucleotide affect the sequence's ability to restore luciferase activity. The obtained results lead us to propose that mtlrRNA may be involved either in damaged protein reactivation or in protein biosynthesis during pole cell determination.

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Abbreviations used: mtlrRNA – mitochondrial large ribosome RNA; mtlsRNA – mitochondrial small ribosome RNA

INTRODUCTION

In many animals, localized cytoplasmic factors called determinants have been postulated to play an essential role in the commitment of early embryonic cell lineage [1]. The factors required for germ-line establishment are localized in a specific region of the egg cytoplasm, or germ plasm [2-3]. In *Drosophila*, the formation of the germ line progenitors, the pole cells, is induced by polar plasm localized in the posterior pole region of early embryos. The polar plasm contains polar granules, which act as a repository for the factors required for pole cell determination.

Despite extensive studies, researchers have only identified and characterized the functions of a few components of the polar granules [4-7]. Using *Drosophila melanogaster* as a model, it was observed that UV irradiation of the posterior pole of *Drosophila* embryos damaged the polar granules and impaired germ cell formation [8]. Interestingly, pole cell determination was restored when the posterior pole of irradiated embryos was microinjected with mitochondrial large ribosomal RNA (mtlrRNA), which is one of the identified components of the polar granules. Electron microscopy demonstrated that the mtlrRNA in the pole plasm is present outside the mitochondria and localized on the polar granules [9]. Several experiments have demonstrated that mtlrRNA which is transported out of the mitochondria and localized in the pole plasm is regulated by a mechanism under the control of different genes such as *osk*, *vas tud* and *gcl* [9-12]. Different hypotheses were proposed to explain how mtlrRNA restores pole cell determination after UV irradiation [7, 13]. Interestingly, during the stage from ovideposition to pole cell determination, small RNA (mtsrRNA) also localizes on the polar granules. This suggests that both small and large mitochondrial RNA form ribosomes on which the polar granule-stored mRNA encoding the pole cell-forming factors can be translated. However, the molecular basis of this phenomenon is still unknown.

In this study, we identified a 15-ribonucleotide sequence in domain V of the 16S mitochondrial rRNA. The sequence is highly conserved from the Rickettsiae to the Vertebrates. This small RNA sequence, similarly to the region's full-length mtlrRNA, is able to restore pole cells in UV-irradiated embryos. Our results suggest that the mtlrRNA in the polar granules could contribute to maintaining the active state of the proteins essential for pole cell determination.

MATERIALS AND METHODS

Microinjection

Five-day old *Drosophila* females were allowed to lay eggs for 2 days at 25ºC on apple medium. Following this 2-day long synchronization, the embryos were collected 30 min after egg laying (AEL). The embryos were dechorionated with 3% sodium hypochlorite, washed in distilled water and UV irradiated (280 nm, 345 J m^{-2} min^{-1}) in the posterior region. The irradiated embryos were covered with oil and microinjected in the posterior pole with 10 nl of 1 nM mtlRNAs or

with oligonucleotides depending on the type of experiment. To quantify the pole cell formation, the injected embryos were allowed to develop to blastoderm stage in oil at 25ºC, and then observed under a light microscope.

Cloning and *in vitro* transcription

Wolbachia pipientis 23S rDNA was cloned from *Wolbachia* and isolated from *Drosophila hydei* embryos following the protocol of Braig *et al.* [14]. The pellet of *Wolbachia* cells was suspended in a lysis buffer (10 mM Tris-HCl, pH 7.5; 100 mM NaCl; 10 mM Na$_2$EDTA; 1% SDS) for DNA extraction. The 23S rDNA gene was amplified by PCR using the oligonucleotides (A) 5'-TAT GGGCCC**AAATAAATTATTAAGAGCACTTG**-3' and (B) 5'-TATGGG CCC**ATTAGTACCAGTTAGCTTCACAT**-3'. 16S mitochondrial rDNA was extracted from *Anopheles gambiae* and *Drosophila melanogaster* as follows. Fifteen mosquitoes were homogenized in an isolation medium (0.25 M sucrose; 10 mM Tris-HCl, pH 7.4; 1 mM EGTA; 1% bovine serum albumin), and the suspension was centrifuged 3 times for 5 min at 300 *g*. The supernatant was centrifuged again for 10 min at 3,000 *g*, and the pellet was dissolved in the isolation medium without bovine serum albumin, then centrifuged for 5 min at 7000 *g*. The new pellet (mitochondria) was dissolved in a lysis buffer (10 mM Tris-HCl, pH 7.5; 100 mM NaCl; 10 mM Na$_2$EDTA; 1% SDS) for DNA extraction. 16S mitochondrial rDNAs were PCR amplified using the following oligonucleotides pairs:

A. gambiae: (A) 5'-TATGGGCCC**AAATTATTTTAATAATTTTCATTTA**-3' and (B) 5'-TATGGGCCC**TAAATTGATAATAATTATTTATTAA**-3'; and
D. melanogaster: (A) 5'-TATGGGCCC**GTTAGTTTTTATTTATTAATTTTT**-3' and (B) 5'-TATGGGCCC**TTTATATTAATAATATTCTTATAA**-3'.

At the extremities of each oligonucleotide, an *Apa*I restriction site was introduced for successive cloning of the amplified DNAs into the *Apa*I site of the *pCRII-TOPO* vector (Invitrogen). The constructs were linearized with *Xba*I, and *in vitro* transcribed using a MEGAscript^{TM} High Yield transcription kit (Ambion).

Synthetic oligonucleotides

The RNA and DNA oligonucleotides used were synthesized by M.W.G. Biotech (Tab. 1). Dro-GAGA (5'-UAUUUUG**GAGA**GUUCAUA-3') contains the conserved domain of 16S mitochondrial rRNA of *Drosophila melanogaster*. The GAGA tetranucleotide is indicated in bold characters. Dro-1-mut (5'-UAUUUUG **GA**C**A**GUUCAUA-3') is the same sequence with a single base mutation (underlined). Anoph-GAGA (5'-AUUUUUUA**GAGA**GUUCAUAU-3') contains the conserved domain of 16S mitochondrial rRNA of *Anopheles gambiae.* Anoph-2-mut (5'-AUUUUUUA**G**UU**A**GUUCAUAU-3') has two base mutations (underlined). Wol-GAGA (5'UGGGGCUG**GAGA**AGGUCCCA-3') contains the conserved domain of23S rRNA of *Wolbachia pipientis*. Wol-1-mut (5'-UGGGGCUG**GA**C**A**AGGUCCCA-3') and Wol-all-mut (5'UGGGGCU C**CCCC**AGGUCCCA-3') have mutations in the underlined bases. Wol-DNA

(5'-TGGGGCTG**GAGA**AGGTCCCA-3') is a DNA oligonucleotide containing the same sequence as Wol-GAGA.

Tab. 1. The oligoribonucleotide sequences used for the *in vivo* and *in vitro* assays.

Name	Sequences
Dro-GAGA	5'-UAUUUUGGAGAGUUCAUA-3'
Dro-1-mut	5'-UAUUUUGGA<u>C</u>AGUUCAUA-3'
Anoph-GAGA	5'-AUUUUUUAGAGAGUUCAUAU-3'
Anoph-2-mut	5'-AUUUUUUAG<u>UU</u>AGUUCAUAU-3'
Wol-GAGA	5'-UGGGGCUGGAGAAGGUCCCA-3'
Wol-1-mut	5'-UGGGGCUGGA<u>C</u>AAGGUCCCA-3'
Wol-all-mut	5'-UGGGGCU<u>CCCCC</u>AGGUCCCA-3'
Wol-DNA	5'-TGGGGCTGGAGAAGGTCCCA-3'

Denaturation and reactivation of firefly luciferase

100 nM firefly luciferase (Sigma) was denatured for 5 min by UV irradiation (280 nm, 345 J m^{-2} min^{-1}) or for 15 min by 1 μM H$_2$O$_2$ at the same protein concentration. The reaction was blocked by adding catalase (2.5 U/sample). The oligoribonucleotide concentration was equimolar to the luciferase concentration (100 nM). The protein activity was determined via incubation at 25ºC in the presence or absence of oligoribonucleotide with a Triathler Multilabel Tester (Hidex) luminometer using the Promega luciferase assay, as per the manufacturer's indications. The protein was diluted 1:10,000 to enter the linear range of light detection of the instrument. Protein reactivation was calculated as the percentage of the restored activity.

Electrophoretic mobility-shift assay (EMSA)

The eletrophoretic mobility-shift assay was performed using labelled wild-type (Wol-GAGA) or mutated (Wol-all-mut) oligoribonucleotides as probes, and native or denatured luciferase. The oligoribonucleotides were labelled at their 5' ends with T4 polynucleotide kinase (Roche), using [γ-^{32}P] ATP [15] purified with a Sephadex G-50 column (Probe Quant, Amersham). The radioactivity was quantified using a β-counter. Firefly luciferase (Sigma) at two different concentrations (32 μM and 10 μM) was denatured by UV-irradiation (280 nm, 345 J m^{-2} min^{-1}) for 5 min.

Native and irradiated luciferase (10 μM or 5 μM final concentrations) were incubated at 0ºC for 10 min with 2 μl of poly (dI-dC; 0.5 μg/μl), 1 μl of RNase inhibitor (50 U/μl), and 2 μl of 5X binding buffer (250 mM Tris-HCl, pH 7.9; 1 M NaCl; 42% glycerol). 2 μl of probes (30000 cpms; 1 μM) were then added and incubated for 10 min at 31ºC, and then for 10 min at 0ºC. The protein binding to the oligoribonucleotides was assessed by electrophoresis in non-denaturing polyacrylamide gel (6%), in 1X TBE, at 20 V/cm, for 2 h. After electrophoresis, the gel was dried and exposed overnight to X-ray film.

RESULTS

A 15-base sequence contained in domain V of mtlrRNA is capable of restoring pole cell determination

Mitochondrial large rRNA (16S rRNA) is capable of restoring *Drosophila* embryos that have an inability to form pole cells due to UV irradiation [10-11]. Crystallographic studies indicated that the mitochondrial 16S rRNA contains six structural domains constituted by a network of hairpins, bulges and loops [16-17]. Specific functions have been attributed to some of these domains. For example, domain V exhibits peptidyltransferase activity [18-19] and the sarcin-ricin loop is involved in the elongation process during protein synthesis [20-21]. This data indicates that distinct regions of this molecule have specific functions. Based on the assumption that evolutionarily conserved sequences/structures share similar functions, we tested the ability of the mitochondrial large rRNA from *Drosophila* and *Anopheles* and the cytoplasmic large rRNA from the *Drosophila* endosymbiont protobacterium *Wolbachia* to restore pole cell determination in UV-irradiated embryos. We included *Wolbachia* in this comparative analysis because this protobacterium emerged 2.5×10^9 years ago and is considered the common ancestor of mitochondria [22-23]. As shown in Tab. 2, all three mitochondrial large RNAs are capable of restoring pole cell formation when injected into the posterior pole of UV-irradiated *Drosophila* embryos. The percentage of embryos able to form pole cells after mtlrRNA injection is significantly higher than that of control embryos.

Tab. 2. The pole cell-inducing activity of the mitochondrial large ribosomal RNA.

Injected material	No. of embryos treated	Embryos developing to blastoderm stage		
		Total	With pole cells (%)	Significance[a]
H_2O (control)[b]	320	259	19 (7.3)	
Wol23S rRNA	333	223	96 (43)	p < 0.01
Dro16s rRNA	279	195	88 (45)	p < 0.01
Anoph16S rRNA	320	259	88 (40)	p < 0.01

[a]Probability was calculated vs the value of the control using the chi-square test. [b]Control embryos were UV irradiated and microinjected with distilled H_2O.

The rRNA large subunits from distantly related organisms maintain the capacity of restoring pole cell determination. This observation led us to the hypothesis that these molecules might share evolutionarily conserved sequences. The alignment of rRNAs from *Drosophila melanogaster*, *Anopheles gambiae* and *Wolbachia pipientis* shows that the three sequences have only 15 nucleotides in common (Fig. 1), and they belong to one of the hairpins of domain V that also contains the peptidyl-transferase site [24].

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W. pipientis 23S rRNA (143-187)            5'-ATAACAGGCTGATGGTGTTCGAGAGTTCATATCGACGACACCGT-3'

D. melanogaster 16S mtrRNA (12941-12985)   5'-TAACAGCGTAATTTTTTTTGGAGAGTTCATATCGATAAAAAAGA-3'

A. gambiae 16S mtrRNA (12857-12901)        5'-ATAACAGCGTAATTTTTTTAGAGAGTTCATATCGATAAAAAAGA-3'

D. yakuba 16S mtrRNA (12857-12901)         5'-ATAACAGCGTAATTTTTTTTGGAGAGTTCATATCGATAAAAAAGA-3'
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Fig. 1. The alignment (NCBI Blast) of *Wolbachia pipientis* 23S rRNA vs *Drosophila* and *Anopheles* mitochondrial genomes. The conserved 15-nucleotide sequences are in bold.

Three different oligoribonucleotides containing the conserved domain and short flanking region specific for *Drosophila, Anopheles* and *Wolbachia* (Dro-GAGA, AnophGAGA and Wol-GAGA; listed in Tab. 1) were able to restore the germ cell determination (Tab. 3) once injected into the posterior pole of UV-irradiated *Drosophila* embryos. The percentage of embryos able to form pole cells after oligoribonucleotide injection was significantly higher with respect to the control embryos, and comparable with the values reported in Tab. 2. The pole cell formation after microinjection of the Wol-GAGA oligonucleotide is shown in Fig. 2. These results suggest that the evolutionarily conserved small hairpin of mtlrRNA domain V is sufficient to partially restore pole cell determination.

Tab. 3. The pole cell-inducing activity of the oligonucletides containing the GAGA sequence after UV irradiation.

Injected material	No. of embryos treated	Embryos developing to blastoderm stage		
		Total	With pole cells (%)	Significance[a]
H_2O (control)[b]	333	239	12 (5)	
Wol-GAGA	308	222	89 (40)	$p < 0.01$
Dro-GAGA	310	213	87 (41)	$p < 0.01$
Anoph-GAGA	284	185	78 (42)	$p < 0.01$

[a]Probability was calculated vs the value of the control using the chi-square test. [b]Control embryos were UV irradiated and microinjected with distilled H_2O.
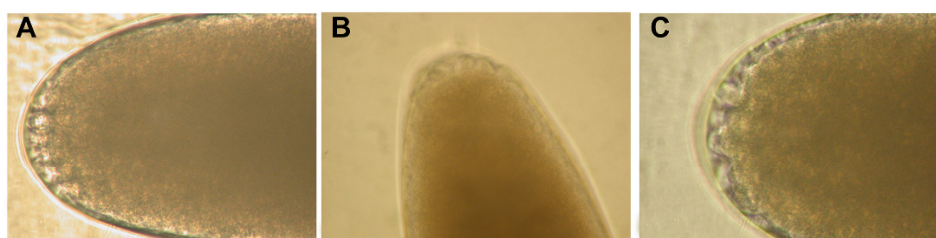

Fig. 2. Restored embryos after UV irradiation. A – control embryo. B – UV-irradiated embryo injected with Wol23S rRNA. C – UV-irradiated embryo injected with Wol-GAGA oligonucleotide.

The GAGA tetranucleotide is essential for pole cell determination

To further investigate the mechanism underlying the ability of the small hairpin of domain V to restore determination, we established an *in vitro* assay based on the ability of the wild-type oligoribonucleotide to restore the enzymatic activity of firefly luciferase. After UV irradatiation, 30-70% of the luciferase activity was restored when wild-type oligoribonucleotides (Wol-GAGA) were added (Fig. 3). Similar results were obtained using H_2O_2 as a denaturant agent (Fig. 4).
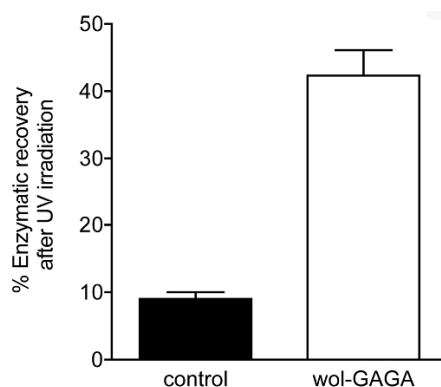

Fig. 3. The effects of the wild-type oligoribonucleotide on the enzymatic activity of UV-irradiated luciferase. The percentage recovery of enzymatic activity is shown after 5 min of incubation with the Wol-GAGA oligonucleotide.
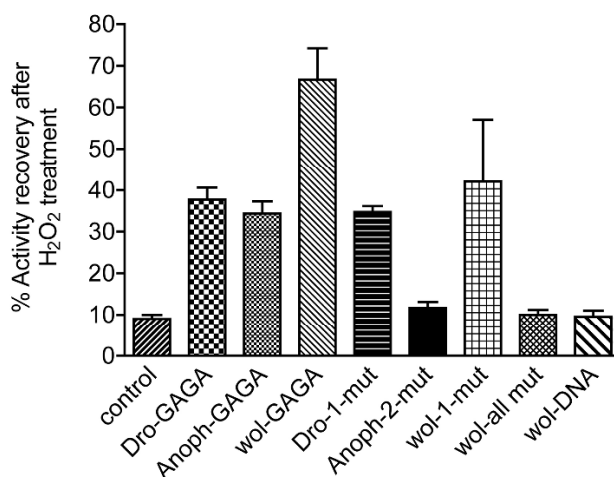

Fig. 4. The effects of wild-type and mutated oligoribonucleotides on luciferase activity after H_2O_2 treatment. The percentage recovery of enzymatic activity is shown after 5 min of incubation with synthetic oligoribonucleotides before the activity assay.

By analysing the domain V sequences of mtlrRNA from several species, we found that inside the 25-nucleotide sequence, there is a conserved tetranucleotide, namely GAGA, which is present in all animals (from Rotifera to

Vertebrates) that display pole cell production. To gain insight into the role of the GAGA tetranucleotide present in the mtlrRNA-conserved region of *Drosophila*, *Anopheles* and *Wolbachia*, we introduced mutations in this sequence (Tab. 1) and then tested its effects both *in vivo* and *in vitro*. Only the GAGA sequence was able to restore luciferase activity. Indeed, when mutated to CCCC (Wol-all-mut) or GUUA (Anoph-2-mut; Fig. 4), we observed a complete loss of the ability to restore enzymatic activity *in vitro*, and no restoration of pole cell production in UV-irradiated embryos. However, a single base mutation in the GAGA tetranucleotide did not affect oligoribonucleotide function. (Fig. 4).
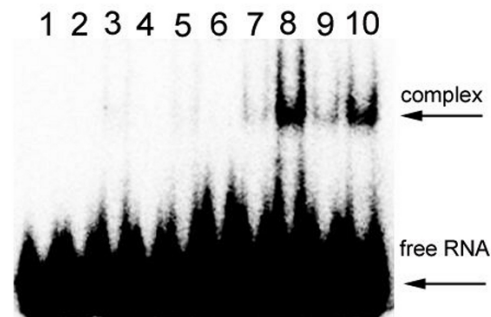

Fig. 5. A mobility shift assay of the binding of firefly luciferase to radioactive oligo Wol-GAGA (lanes 6-10) and oligo Wol-all-mut (lanes 1-5). Wol-GAGA and Wol-all-mut were present at a concentration of 1 μM. Lane 1: Wol-all-mut; lanes 2 and 3: respectively 5 and 10 μM Wol-all-mut incubated with denaturated luciferase; lanes 4 and 5: respectively 5 and 10 μM Wol-all-mut incubated with native luciferase; lane 6: Wol-GAGA; lane 7 and 8: respectively 5 and 10 μM Wol-GAGA incubated with denaturated luciferase; lanes 9 and 10: respectively 5 and 10 μM Wol-GAGA incubated with native luciferase.

Moreover, the *Wolbachia* DNA oligonucleotide (corresponding to the RNA sequence) was not able to restore luciferase activity *in vitro* (Fig. 4). These results indicate that only the RNA-GAGA sequence is relevant for the specific function.

To further investigate the function of the GAGA sequence, we tested whether this tetranucleotide mediates the interaction between the oligoribonucleotide and proteins. For this, we analysed the wild-type *Wolbachia* oligoribonucleotide and the mutant version Wol-all-mut for their ability to bind native or unfolded luciferase in the electro-mobility shift assay (EMSA). As shown in Fig. 5, only the wild-type oligoribonucleotide was able to bind increasing amounts of either folded or unfolded luciferase. These results indicate that the wild-type GAGA tetranucleotide directly interacts with proteins, and suggest that the restoration of protein functionality in the embryos could be mediated by this interaction.

DISCUSSION

UV irradiation of the posterior pole of *Drosophila* embryos prevents germ cell formation. Earlier studies [10-11] demonstrated that microinjection of the entire mitochondrial large rRNA repairs this defect. The authors have postulated that UV irradiation directly damages the mtlrRNA and restoration occurs by replacing it. More recently, it was proposed that mtlrRNA forms mitochondrial ribosomes able to produce proteins required for pole cell determination. In this study, we hypothesized an alternative mechanism based on the notion that UV light may damage proteins, inducing changes that affect their specific activity [25-26].

We identified a short RNA sequence belonging to domain V of the 16S mitochondrial rRNA. This sequence is highly conserved from Rickettsiae to Veretebrates. We demonstrated that this domain is the minimal requirement to restore *Drosophila* pole cell determination when injected into UV-irradiated early embryos.

In addition, we provided evidence that this 15-base sequence restores the activity of luciferase *in vitro* after denaturation. This ability is affected by the introduction of mutations within the conserved GAGA sequence. Interestingly, mutants unable to restore luciferase activity *in vitro* were also unable to restore the determination of pole cells *in vivo*, while oligoribonucleotides capable of restoring luciferase activity *in vitro* were also able to restore the formation of pole cells *in vivo*. This suggests that the two processes are regulated by the same molecular mechanisms.

We also demonstrated that this sequence directly binds luciferase *in vitro* after denaturation and restores luciferase enzymatic activity. The degree of conservation among different organisms may be related to the necessity of establishing interaction with many different proteins. Any change might thus restrict the number of possible interactions.

It is worth noting the chemical specificity of this functional domain, because a DNA sequence corresponding to the wild-type RNA oligonucleotide lacks this specific function. This probably depends on the unique property of RNA to fold up into complex tertiary structures. This small RNA sequence can interact with inactivated proteins contributing to the recovery of the native activity. Another hypothesis is that mtlrRNA could contribute to restoring ribosomal protein activity after UV irradiation, allowing the translation of protein involved in pole cell determination.

It has been well established that proteins are the major targets of photo-oxidation, and that loss of protein activity may occur [25-26]. The ability of mtlrRNA to reactivate proteins *in vitro* was extensively demonstrated [24, 27-30]. In *E. coli* and *B. subtilis*, it was also shown that the protein folding activity is confined to domain V of 23S rRNA [23, 26]. Furthermore, both 12 and 16S mitochondrial rRNA can promote refolding of denatured proteins from prokaryotic and eukaryotic origins [28].

The small hairpin belonging to domain V of mtlrRNA is able to restore luciferase activity *in vitro* and pole cell determination *in vivo*. These results suggest that it could operate in cellular processes involving the repair of inactivated proteins.

It is conceivable that the presence of the domain we described contrasts the denaturing effects and protects the protein function. Since polar granules of *D. immigrans* can functionally substitute those of *D. melanogaster* [31-32], the conserved oligoribonucleotide appears to be a conserved element essential for pole cell determination in Diptera (*Drosophila* and *Anopheles*).

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