Volume 13 Number 12 1985 Nucleic Acids Research

Streptomycin-resistance of *Euglena gracilis* chloroplasts: identification of a point mutation in the 16S rRNA gene in an invariant position

Paul-Etienne Montandon, Paul Nicolas[1], Peter Schürmann and Erhard Stutz*

Laboratoire de Biochimie végétale, Université de Neuchâtel, Chantemerle 18, CH-2000 Neuchâtel, Switzerland, and [1]Département de Biologie générale et appliquée, Université de Lyon I, 43 Bvd du 11 Novembre 1918, F-69622 Villeurbanne Cédex, France

Received 15 April 1985; Revised and Accepted 23 May 1985

ABSTRACT

We sequenced the chloroplast 16S rRNA gene of two Euglena gracilis mutants which contain streptomycin-resistant chloroplasts (Sm^r 139.12/4 and Sm^r 139.20/2). These mutants are known to contain a single intact rrn operon per circular chloroplast genome. Nucleotide sequence comparison between a 16S rRNA gene of wild type Euglena gracilis, strain Z, with streptomycin-sensitive chloroplasts, and the 16S rRNA gene of both Sm^r-strains reveals a single base change (C to T) at position 876. This position is equivalent to the invariant position 912 of the E. coli 16S rRNA gene. The analogous position is also conserved in all chloroplast small subunit RNA genes from lower and higher plants sequenced so far. Light dependent protein synthesis with purified chloroplasts from streptomycin-resistant cells is not inhibited by streptomycin. Based on the results reported here we postulate linkage between the observed point mutation on the 16S rRNA gene and streptomycin-resistance of chloroplast 70S ribosomes.

INTRODUCTION

In lower and higher plants mutants with antibiotic-resistant chloroplasts are known for some time. In tobacco (1) and Chlamydomonas (2), e.g., such antibiotic-resistance is often uniparentally inherited, i.e., such mutations most likely are located on the chloroplast genome. For the unicellular alga Euglena gracilis several mutants with antibiotic-resistant chloroplasts have been described (3). Since, however, Euglena lacks a sexual reproduction it is impossible, by classical genetic means, to unequivocally trace the mutation. We may, however, assume that antibiotic-resistance of Euglena chloroplasts can also be due to altered chloroplast genes.
Streptomycin binds to the 30S subunit of bacterial ribosomes at a specific site (4). Using the bifunctional cross-linking reagent phenyldiglyoxal it was possible to link streptomycin specifically to the ribosomal proteins S1, S5, S11 and S13 (but not S12) and to 16S rRNA (but not 23S rRNA) (5).

This latter observation is in agreement with earlier data (6) which showed that E. coli 16S rRNA provides binding sites for streptomycin. Streptomycin-resistant mutants of E. coli, described so far always have an altered protein S12 (7), but no mutants with an altered 16S rRNA have been described. Such mutations being recessive would be difficult to retrieve, since E. coli, like other eubacteria, contains multiple rrn operons. The use of recombinant DNA techniques along with classical genetics made it possible to search for rRNA mutants. It was, e.g., recently reported (8) that a single base change on the 16S RNA confers spectinomycin-resistance in E. coli. Lemieux et al. (9) studied chloroplast DNA recombinations in interspecies hybrids of Chlamydomonas. They showed linkage between a uniparental locus for streptomycin-resistance and a chloroplast DNA restriction fragment coding for 16S rRNA. Several antibiotic-resistance mutations have been located on mitochondrial rRNA genes (e.g. 10,11). It is noteworthy that mitochondria contain a single set of rRNA genes per circular genome.

Nicolas et al. (12) found one class of UV-induced streptomycin-resistant Euglena mutants which have a specific deletion in the rDNA region of the chloroplast genome, which lost two rrn operons keeping one per circular unit. The authors suggest that streptomycin-resistance is somehow related to this deletion event which leaves a single altered 16S rRNA gene.

We have previously analysed on a nucleotide level the chloroplast 16S rRNA gene of rrn operon A of Euglena gracilis, Z-strain (13). We have also mapped and sequenced the single chloroplast gene for the ribosomal protein S12 (14). This allowed us to perform a comparative sequencing study of each of these chloroplast genes which both may be involved in conferring streptomycin-resistance. We show in this report that two streptomycin-resistant mutants contain a 16S rRNA gene which differs at one position from the wild type gene, however we could not find an altered S12 protein gene.

MATERIALS AND METHODS

Euglena gracilis strains

We use the wild type Euglena gracilis, Z-strain. Streptomycin-resistant mutants (Sm^r) were obtained by UV-irradiation (3). Different classes of streptomycin-resistant strains were obtained and are described elsewhere (12). The mutants Sm^r 139.20/2 and Sm^r 139.12/4 characteristically have a

single rrn operon per chloroplast genome. They were selected from a single mutated cell and are able to grow photoautotrophically in the presence of 500 ug/ml of streptomycin. This is a tenfold concentration of streptomycin required to induce 50% bleaching of the wild type strain.

Growth conditions

Euglena gracilis, Z-strain, is routinely grown in a modified Hutner's medium with vitamin B12 at 50 ng/l (15). The same medium was used to grow the strain Sm^r 139.12/4. We also used the Euglena broth (Difco) for strain 139.20/2.

Cloning and sequencing of chloroplast DNA

Chloroplasts from the wild type strain and strain Sm^r 139.12/4 were isolated according to (16) and the DNA was purified according to (17). Chloroplasts of strain Sm^r 139.20/2 were isolated according to (18).

The 16S rRNA genes of the chloroplast genome of the wild type (13) and the two Sm^r mutants (12) have been mapped. We inserted the DNA fragment BamHI-D containing the rrn A of the wild type strain into pBR322 and we inserted the fragment EcoRI-P of the single rrn operon of Sm^r 139.20/2 and Sm^r 139.12/4 into pBR322. The clones were screened following the procedures of (19). Recombinant DNA was isolated and purified as described (13). Selected DNA fragments were isolated according to Wienand et al. (20).

DNA fragments were blunt ended with E. coli DNA polymerase (Klenow fragment), inserted into the Hinc II site of M13 mp9 DNA (21) and sequenced according to (22). T4 gene 32 protein was used in addition in the experiment shown in Fig. 2 (C. Kraik, UCSF, private communication).

Protein synthesis in organello

In all cases (wild type, Sm^r-mutant) the cells were grown in a modified Hutner's medium (15) for 4 days in the light. Chloroplasts were isolated and purified as described (16). Chlorophyll content was determined according to Arnon (23). 100 µl aliquots of chloroplasts at a concentration of 1 mg chlorophyll per ml were preincubated in the light at 20°C in the suspension medium (0.33 M sorbitol, 50 mM Tricine-KOH, pH 8.5) containing the inhibitor at the indicated concentration. After 30 minutes 20 µl aliquots were removed from the preincubation mixture and diluted to 180 µl of fresh suspension medium free of inhibitor, but supplemented with a mixture of aminoacids (minus methionin) at 50 µM each and 9.3 µCi of ^{35}S-Met ($>$1000 Ci per mmole). Protein synthesis proceeded in saturating red light at 20°C for 1 h.

5 µl aliquots were removed at intervals as specified in the legend to Fig. 1. TCA precipitable counts (^{35}S-Met) were determined according to (24).

RESULTS

Chloroplast protein synthesis of Euglena gracilis is known to be inhibited by streptomycin (25). We isolated intact chloroplasts from the wild type strain and the streptomycin-resistant mutants (Sm^r 139.20/2) and measured the incorporation rate of ^{35}S-Met in a light dependent polymerisation assay. The results shown in Fig. 1 can be summarized as follows : 1. ^{35}S-Met incorporation into total TCA precipitable counts is strictly light dependent. Virtually no incorporation was measured in the dark (not shown), i.e., the observed protein synthesis occurs within and not outside of the chloroplast and is therefore not due to contaminating streptomycin-resistant 80S cytosolic

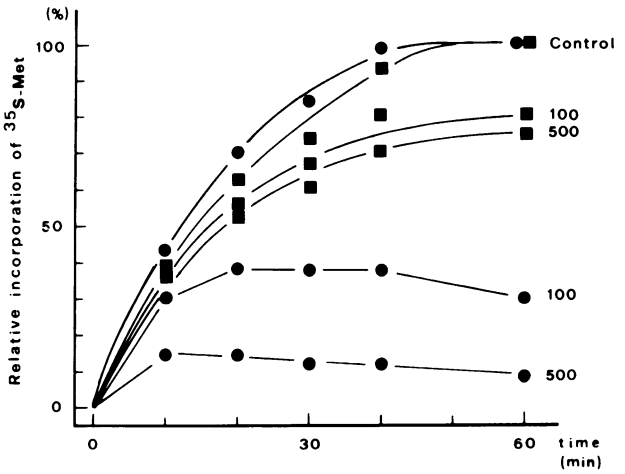

Figure 1. Light dependent polypeptide synthesis of purified chloroplasts from the Euglena gracilis, Z-strain, and the mutant Sm^r 139.20/2. ^{35}S-Met incorporation into TCA precipitable counts (24) are plotted against time of incubation. The values are expressed in % of the controls. Wilde type 100% : $8.05 \cdot 10^6$ counts per min per mg chlorophyll · 60 min; Sm^r 139.20/2 100% : $11.88 \cdot 10^6$ counts per min per mg chlorophyll · 60 min; no incorporation of ^{35}S-Met occurs in the dark. In the presence of lincomycin (1 ug per ml) ^{35}S-Met incorporation stays at background level with both kinds of chloroplasts (not shown). Aliquots were taken after 10, 20, 30, 40 and 60 minutes. The streptomycin-sulfate concentrations in the incubation mixture are indicated in the graph as 100 and 500 for 100 ug and 500 ug per ml. -●- wilde type; -■- Sm^r 139.20/2.

ribosomes. 2. After one hour of incubation protein synthesis of Sm^r 139.20/2 chloroplasts is reduced for about 20 and 25% in the presence of 100 and 500 ug/ml streptomycin, respectively, while the corresponding values are 70 and 92% for the wilde type chloroplasts. 3. The five fold increase of inhibitor has little additional effect on the resistant chloroplasts, but a more pronounced effect on the wilde type chloroplasts. 4. The shape of the inhibition curves (wilde type) shows that the relative inhibitory effect increases with time, due to the adverse effect of the induced misreading. 5. The antibiotic lincomycin (26) was used in parallel experiments and inhibited at 1 µg/ml ^{35}S-Met incorporation in both kinds of chloroplasts to about 95% (not shown). In conclusion we can say that the observed chloroplast streptomycin-resistance must be due (solely ?) to an altered proteosynthetic apparatus, affecting most likely the ribosomes.

Streptomycin-resistance may involve both mutations of genes for ribosomal proteins and/or mutations on 16S rRNA. Among the ribosomal proteins the small-subunit protein S12 is a likely candidate. We therefore cloned and sequenced the single chloroplast gene for S12 of the Sm^r 139.20/2 mutant and compared it with the already known sequence of the wild type strain. We could not find a difference, in particular we did not see a mutation at position equivalent to Lys 42 and/or Lys 87 of the E. coli S12 gene (7). The results are not shown.

We sequenced the chloroplast 16S rRNA gene of Sm^r 139.20/2 and compared it with the 16S rRNA wild type (Z-strain) gene. We located a single altered nucleotide at position 876. Numbering of positions is according to (13). We show in Fig. 2 the relevant part of the sequencing gel and include the corresponding sequence of the wilde type gene (WT) and the second mutant Sm^r 139.12/4 . We note that in both streptomycin-resistant mutants the identical position is altered (C to T). The control sequence shown was obtained from the wilde type strain which was used as starting culture for the UV-mutation experiments (12). The displayed sequence matches exactly the previously published sequence, i.e., we can exclude a possible laboratory strain specific micro-heterogeneity. We have also sequenced the coding strand. The result is the same (data not shown). Position C 876 of the Euglena chloroplast genome is equivalent to position C 912 of E. coli 16S rRNA gene. This is one of five bacterial invariant nucleotides (27) and is conserved also in all chloroplast 16S rRNA genes

Table 1. Postulated streptomycin-resistance site of the chloroplast 16S rRNA gene of Euglena gracilis and comparison with the analogous sequences of other small subunit rRNA genes.

Organism		1					2						2a								912 1a •												Ref.
Ec	e	5' - G	G	A	G	T	A	C	G	G	C	C	G	C	A	A	G	G	T	T	A	A	A	A	C	T	C	A	A	A	T	G	A - 3'	28,29
Pv	e	-																															-	30
An	e	-								C	A								T		G	G									G		-	31
Hv	a	-	A							T									A			G				T					G		-	R 32
Eg	c	-								C	T	T				C	A	A	G			G									G		-	13
EgR	c	-								C	T	T				C	A	A	G			G				T					G		-	R -
Cr	c	-					T			C	T							A	G			G									G		-	33
Zm	c	-								T	T							A	A			G									G		-	34
Nt	c	-								T	T							A	A			G									G		-	35
Sc	m	- A								T	T	A						T	A	A		G									A	C	-	36
Asn	m	-					A		T	A	T	G						C	A		A	T				G					C		-	? 37
Zm	m	-									T							A	C	C		G									G		-	38
Mm	m	-	A		C				T	A	G		C	A	T			C													G		-	39
Hs	m	- A	C		C					A	G		C	A	C			C													G		-	40
Sc	n	-					T				T				A					C		G				T					G		-	R 41
Zm	n	-					T				T									C		G				T					G		-	R 42
Xl	n	-					T				T	T						A		C		G				T					G		-	R 43
Rn	n	-					T				T	T						A		C		G				T					G		-	R 44

Ec, Escherichia coli; Pv, Proteus vulgaris; An, Anacystis nidulans; Hv, Halobacterium volcanii; Eg, Euglena gracilis, wilde type; EgR, Euglena gracilis, Sm^r 139.20/2; Cr, Chlamydomonas reinhardii; Zm, Zea mays; Nt, Nicotiana tabacum; Sc, Saccharomyces cerevisiae; Asn, Aspergillus nidulans; Mm, Mus musculus; Hs, Homo sapiens; Xl, Xenopus laevis; Rn, Rattus norvegicus; e, eubacterium; a, archaebacterium; c, chloroplast; m, mitochondrium; n, nucleus. The top line in the table gives 32 positions of the E. coli 16S rRNA gene around the invariant position 912. The analogous sequences of the other organisms are exactly aligned. Blanks mark sequence identity. In the second last column we mark by R if the corresponding ribosomes are streptomycin-resistant. The headings 1,2,1a,2a identify the base-paired regions as shown in Fig. 3.

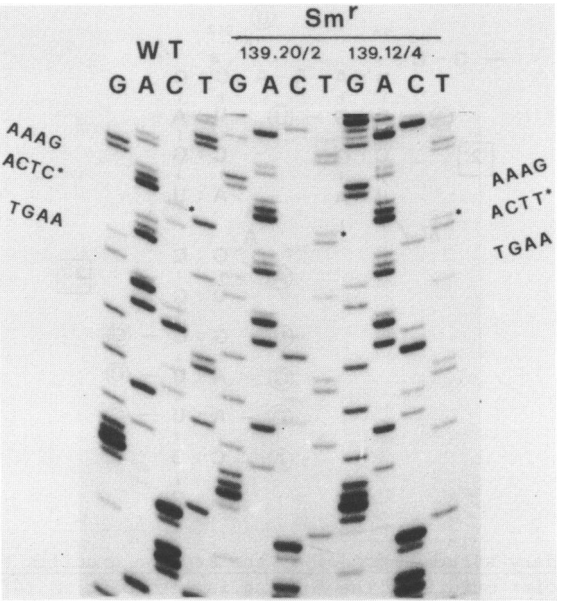

Figure 2. Radiograph of sequencing gel. We show the sequence (RNA-like DNA strand) of a segment of the restriction fragment HpaI-TaqI [positions 827 to 925 (13)] which carries the point mutation marked with an asterisk. WT, wilde type.

published so far. In Table 1, we list the relevant sequences of the small subunit rRNA genes of prokaryotes, cell organelles and eukaryotes. We indicate, to the best of our knowledge, whether the corresponding ribosomes are resistant to streptomycin. From this sequence listing it becomes apparent that streptomycin-resistance positively correlates with the presence of a T (U) at that position in the small subunit rRNA gene which is equivalent to the bacterial invariant position 912. All organellar small subunit rRNA genes analysed so far contain a C, with the single exception of Aspergillus nidulans which has a G at this position. As far as we know, it is not established whether Aspergillus mitochondrial 16S rRNA interacts with streptomycin. It is clear, of course, that other structural components of the small subunit are also involved in streptomycin binding and its interferences with the translation step.

Several research groups (45,46,47) have proposed elaborate secondary structure models for the small subunit rRNA of prokaryotes and eukaryotes.

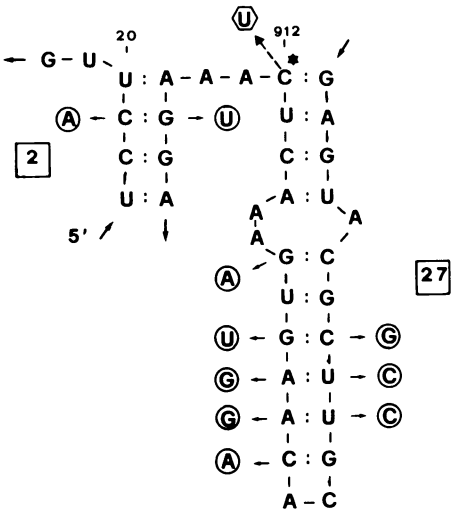

Figure 3. Secondary structure model of the Euglena gracilis 16S rRNA region containing the point mutation. The folding is according to (47). The Euglena 16S rRNA sequence is displayed and compared with the E. coli counterpart, differing positions are circled. The altered position (C → U) is marked by an asterisk. In line with the model of (49) and to facilitate orientation we mark the E. coli positions 20 and 912 (equivalent to 876 of E. gracilis) and mark stem No 2 and the helix region No 27.

In Fig. 3 we show, based on the model of Maly and Brimacombe (47), that part of the chloroplast 16S rRNA gene where the mutation was observed in the two streptomycin-resistant Euglena mutants. The base change C to U (E. coli position 912) is located at the end of the imperfect stem and loop structure No 27 and three bases apart from the long range interaction No 2 which links the central part with the 5' terminal part. The C* to U base change in the mutated 16S rRNA converts the last base pair (C - G) into a U - G pair. This slightly destabilizes this stem structure. For convenience we show base differences between the chloroplast and E. coli sequences. We note in the lower part of stem No 27 two compensating base changes, certainly a strong argument for the existence and importance of this hair-pin structure.

DISCUSSION

We profited from the observation that a certain class of streptomycin-resistant Euglena mutants contains a chloroplast genome with a single intact rrn operon. This single rrn operon per circular genome must be

the result of an inter- or intramolecular unequal crossing event between the tandemly arranged rrn operons A and C as discussed in detail elsewhere (12). Obviously, the chloroplast rRNAs in this class of mutants exclusively stems from this single operon and resistance-conferring mutations on the rRNA gene become rapidly apparent during the sorting out procedure.

We have previously sequenced the chloroplast 16S rRNA gene of the rrn operon A of wild type Euglena gracilis (13). We repeated this analysis with that wild type strain used by Nicolas et al. (12) in the UV-induced mutation experiments. We found a perfect sequence match between the two independently grown Z-strains within the crucial region and we therefore consider that the observed mutation in the two streptomycin-resistant strains is not due to an accidental sequence micro-heterogeneity.

The results presented here do not unequivocally prove a linkage between the point mutation on the 16S rRNA gene and the observed streptomycin-resistance of the chloroplasts. However the following arguments are in strong favor of such a linkage : 1) Two independently isolated streptomycin-resistant mutants of the same class show the same base change at the identical position. 2) The point mutation occurs at a eubacterial and chloroplastic invariant gene position. 3) The chloroplast gene for the S12 protein of Sm^r mutant 139.20/2 is unchanged. Furthermore it is interesting to note that the small subunit rRNAs of streptomycin-resistant 80S type ribosomes contain a U at the analogous position like the archaebacterial 16S rRNA. Archaebacteria are resistant towards streptomycin (48).

Antibiotic-resistance conferring point mutations on rRNA genes have been observed in several instances (49). It was, e.g., shown that spectinomycin-resistance in E. coli results from a mutation at position 1192 of the 16S rRNA gene where a C is replaced by a T (8). Paromomycin-resistance of yeast mitochondrial ribosomes is linked to a point mutation in the small subunit rRNA gene where a C is replaced by a G (10). It is noteworthy that this mutation also occurs at the end of a variable and imperfect helix, somewhat similar to the situation reported here. Lemieux et al. (9) observed that a nonmendelian mutation for streptomycin-resistance of the unicellular alga Chlamydomonas can be linked to a chloroplast DNA restriction fragment coding for 16S rRNA. This is the only report so far, where a relationship between streptomycin-resistance of chloroplasts and a possible mutation on the 16S rRNA was suggested.

Streptomycin interfers with several steps of the translation process but its precise interaction with the ribosome is not yet understood despite extensive studies (e.g. 5, 50). It is accepted now that streptomycin somehow binds to the small subunit region facing the large subunit. It binds to the 16S rRNA and several proteins but interestingly not to S12 which controls the interaction of streptomycin with the ribosome (7). Our observation of streptomycin-resistant ribosomes with an unaltered S12 protein gene is not in contradiction to this classical results obtained with E. coli, rather it shows that the 16S rRNA is directly involved in the read-out process, a concept strongly favored more recently (references in 49). It will be necessary now to test the postulated linkage between the observed point mutation and streptomycin-resistance, e.g., by appropriate in vitro polypeptide synthesis using reconstituted ribosomes and by bacterial transformation experiments using recombinant DNA techniques (e.g. 51).

ACKNOWLEDGEMENTS

We are very grateful to C. Aegerter and L. Salvi for technical assistance, C. Bachmann for secretarial help and R. Sahli (ISREC, Lausanne) for the kind gift of T4 gene 32 protein. This work received support from Fonds National Suisse de la Recherche Scientifique.

*To whom correspondence should be addressed

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