I–R system of hybrid dysgenesis in *Drosophila melanogaster*: analysis of the mitochondrial DNA in reactive strains exhibiting different potentials for *I* factor transposition

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In the I–R hybrid dysgenesis system, *Drosophila melanogaster* strains fall into two categories denoted inducer (I) and reactive (R). Among the reactive strains we can distinguish strains with weak, medium or strong reactivity levels. These levels are inherited in a complex way involving both chromosomal and nonchromosomal determinants, the nonchromosomal determinant being mainly maternally inherited. We were interested in determining the molecular basis of this maternal transmission. In this article we analyse the possible implication of the mitochondrial DNA in the determination of the reactivity levels. The mtDNA was analysed in lines with very different reactivity levels with the aim of correlating sequence differences with reactivity levels. The mtDNA was analysed by sequencing and restriction fragment length. No correlation was established between reactivity level and mtDNA sequence. This may favour the hypothesis that epigenetic changes would be responsible for the different reactivity levels and their transgenerational transmission.

Keywords: *Drosophila melanogaster*, maternal component, mtDNA sequence, reactive strains.

Introduction

I–R hybrid dysgenesis was described in the early 1970s (Picard & L'Héritier, 1971). In this system *Drosophila melanogaster* strains fall into two categories denoted inducer (I) and reactive (R). Inducer flies carry active copies of the *I* factor (a LINE-like retrotransposon) on their chromosome, whereas reactive flies do not bear any active *I* factor in their genome. The dysgenic traits are observed in F₁ females resulting from a dysgenic cross between reactive females and inducer males. In the oogenesis of these F₁ females (called SF females for 'Sterile Females'), the transposition of active *I* elements may occur at a high rate, leading to high levels of mutations, chromosomal rearrangements and nondisjunctions. These genetic abnormalities are associated with nonhatching of some eggs. The percentage of these nonhatching eggs in such a dysgenic cross may vary

within a wide range, depending on the reactive parental stock used in the cross (Bucheton *et al.*, 1976). According to the percentage of the nonhatching eggs, one can distinguish reactive strains with a weak, medium or strong reactivity level. All available data indicate that in a dysgenic cross, the reactivity level of the R mothers regulates the transposition rate of the *I* factor.

Significant characteristics of the reactivity levels are the following: (i) reactivity levels are determined by a cellular state present in the mature oocytes of the reactive females (Bucheton & Bregliano, 1982) which is inherited mainly maternally from one generation to the next, the chromosomes appearing as the major determinants only after several generations (Bucheton & Picard, 1978); (ii) nongenetic factors such as females ageing, inhibitors of nucleotide synthesis and gamma rays can modify the reactivity levels and these modifications are maternally heritable, cumulative over generations and always reversible when the treatment is stopped (Bucheton, 1979; Bregliano *et al.*, 1995; Laurençon & Bregliano, 1995); (iii) reactivity levels correlate with recombination and DNA repair during female meiosis, suggesting that

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reactivity may correspond to a more general biological function beyond the sole regulation of the *I* factor transposition (Laurençon *et al.*, 1997).

As mentioned above the reactivity levels are inherited in a complex way involving both chromosomal and nonchromosomal determinants, the nonchromosomal determinant being mainly maternally inherited. This was shown by using balanced chromosomes in order to replace, over three generations of crosses, the nuclear genome of a strong reactive strain by that of a weak one. Such lines were strongly reactive at first and underwent a progressive evolution toward a weak reactivity level. This evolution could take as long as 10 generations (Bucheton & Picard, 1978). A reciprocal scheme gave the same kind of results (i.e. a progressive evolution to strong reactivity level; J.C. Bregliano, unpublished results). We were interested in determining the molecular basis of the maternally inherited component of the reactivity levels. Mitochondria and symbiotes are good examples of cytoplasmic components which could interfere with the chromosomal determinants and modify the cell functioning. In the past, several assays aimed at relating the reactivity levels to the presence of symbiotes have failed (for review see Bregliano & Kidwell, 1983). The mitochondrial DNA (mtDNA) had also been analysed to test its possible involvement in the I-R system of dysgenesis. The study was performed by comparison between inducer and reactive strains; no difference was found between these two types of strain (Picard & Wolstenholme, 1980).

In the light of the recent data suggesting that differences in reactivity levels are correlated with differences in a basic biological process such as repair and meiotic recombination (Bregliano *et al.*, 1995), it appeared that looking for differences between inducer and reactive strains was not the appropriate way to address the question of the molecular basis of this maternal component. It appeared more relevant to analyse the mtDNA in isogenic strains with different levels of reactivity. In *Drosophila* some cases of heteroplasmy are known for which the relative proportion of the different mtDNA variant can change. A *Drosophila subobscura* strain possesses two types of mtDNA, the normal one and a variant deleted in the coding region. These two types of mtDNA are regularly transmitted over generations and their relative amounts appear to be tissue specific and vary during the course of development (Béziat *et al.*, 1993; Lecher *et al.*, 1994). In *D. mauritiana* as well as in *D. melanogaster* one case of heteroplasmy is known where two mtDNA length variants that differ in the A + T-rich region coexist. In both cases the proportion of the long mtDNA variant increases in offspring as the females age (Solignac *et al.*, 1987; Kann *et al.*, 1998).

As mentioned above ageing is one of the nongenetic factors which can modify the reactivity level of the females. The reactivity level of strong or medium reactive females decreases as the females get older. This age-dependent variation is maternally transmitted over several generations (Bucheton, 1979).

This similarity in the age-dependent evolution of the reactivity levels in our strains and of the proportion of different mtDNA variants in others induced us to work on the following assumption: if two mtDNA variants are present in the reactive strains, their relative amounts might be under the control of the chromosomal determinants but could also be modulated by nongenetic factors. If each of these variants is involved in some way in the determinism of the reactivity levels, these levels would depend on the relative amounts of each variant. On that hypothesis, we must assume that a line with a low level of reactivity will be enriched in one type of mtDNA variant while an isogenic line, with a high level of reactivity, will be enriched in the other variant; the comparison of the mtDNA between these two lines would be expressed as differences between these sequences.

In this paper we describe our results from research on a length or a sequence difference between mtDNA of *D. melanogaster* lines with the same genetic background and with very strong and weak reactivity levels.

Materials and methods

Drosophila stocks and culture conditions

The *estM* stock is a strongly reactive stock bearing the *ebony* mutation. With this stock we used the age-dependent variation of the reactivity level to obtain isogenic lines with very different levels of reactivity (Bucheton, 1979). Sublines bred with a short generation pattern, i.e. each generation being recovered from young mothers (1–3 days old), retain a strong reactivity level. In sublines bred with a long generation pattern, i.e. each generation being recovered from old mothers (30–45 days old), the reactivity level progressively decreases to reach a low value, which remains stable as long as the long generation pattern is maintained. These sublines are denoted SG (for Short Generation) and LGx (for Long Generation with x indicating the number of long generations). *Paris* is a reactive stock obtained in Paris in 1952. This stock was very weakly reactive for many years and then became polymorphic for reactivity levels. *P1m* and *PF9b* are lines, respectively, weak and strong, isolated from this stock nearly 15 years ago and maintained with short generation pattern. Culture conditions were as described in Bregliano *et al.* (1995).

Measure of the reactivity level

The reactivity levels were measured by the percentage of nonhatching eggs after a dysgenic cross between reactive females and inducer males (B2' or Canton S strains) as previously described (Bregliano *et al.*, 1995).

DNA analysis

Fifteen μg of total DNA was analysed by Southern blot (Southern, 1975) after digestion by *Hind*III, *Eco*RI or *Mbo*I. Probes were *Drosophila melanogaster* mtDNA fragments prepared from mtDNA sequences cloned in pGEM7 Zf or pGEM3 Zf (a gift from S. Alziari) and describe in Béziat *et al.* (1993). Probes were ^{32}P -labelled by random priming (Amersham Megaprime kit).

DNA amplification and sequencing

The sequenced templates were obtained by PCR amplification of five overlapping fragments covering the mtDNA from position 37 to position 14 462 (positions refer to the numeration of the published sequence ID.MIDMMU375; (Lewis *et al.*, 1995)). Five pairs of primers were used, and their sequences are given below.

- 1-R: GTAAATCATGCAGTTTTCTG (37),
- 1-L: GATCCATTATGACCTGATGG (4267);
- 2-R: TCAGAAATCTGTGGGGCTAATC (3671),
- 2-L: TTGTTTATATGTGCTGGGGC (7209);
- 3-R: GAAGCAGCTGCATGATATTGAC (5444),
- 3-L: GTATGTGAAGGGGCCTTAGG (9633);
- 4-R: CAGCTTGTAACGTTCTGGC (9127),
- 4-L: AGATTGCGACCTCGATGTTG (12 943);
- 5-R: AGGACGAGGAATTTATTACGG (10 794);
- 5-L: ATTAATCCACGATGGACCTTAC (14 463).

The numbers refer to the positions of the 5' end of each primer on the previously published sequence (MIDMMU375) and letters to the direction of elongation with respect to that sequence. Fragments were amplified with a mix of Taq DNA and Pwo DNA polymerases (Expand™ High Fidelity PCR System, Boehringer Mannheim) according to the manufacturer's instructions. Sequences were subcontracted to the private company, Genome Express (SA).

Results

Comparison of restriction fragment lengths

The fragment length polymorphism was analysed on *Hind*III, *Eco*RI or *Mbo*I digestions of mtDNA from two couples of isogenic lines to study regions previously mentioned as polymorphic, for example the *Hind*III

fragment encompassing the A + T-rich region or some *Mbo*I fragments (Solignac *et al.*, 1983; Hale & Singh, 1991). The pair of isogenic lines used were the *estM* LG49-*estM* SG (reactivity levels, respectively, 10% and 100%) for which the difference in the reactivity levels is a consequence of ageing of the females and *P1m*-*PF9b* (reactivity levels, respectively, 8% and 100%) for which the difference in the reactivity level is constitutive (see Materials and methods). The overall length of the mtDNA is about 18.3 kb for the *Paris* strain and 18.8 kb for the *estM* one. This is due to the length of the *Hind*III fragment encompassing the A + T-rich region which measures 4.6 kb and 5.1 kb for the *Paris* strain and *estM* strain, respectively.

No difference in the length of the different restriction fragments was detected in relation to the reactivity level. With this technique, deletion or insertion less than 100 bp could not be detected.

Comparison of the sequence of the coding region

It has been shown, in some kinds of heteroplasmy, that the relative proportions of the different variants could be age-dependent (Solignac *et al.*, 1987; Kann *et al.*, 1998), so we preferred, in a first approach, to compare the nucleotide sequence in lines which have constitutive different levels of reactivity, i.e. *P1m* and *PF9b*, to avoid differences due to a possible age-dependent effect.

A sequence of 14 365 nucleotides, which corresponds to 96.4% of the coding sequence, has been determined, on both strands, for the two strains *P1m* (reactivity level 8%) and *PF9b* (reactivity level 100%). It starts at position 46 of the sequence previously published in the data bank (ID. MIDMMU375) and ends at position 14 417. Each base has been sequenced at least twice (usually three or four times) and we found no difference between the two mtDNA sequences coming from these two lines. In these technical conditions, we would have detected the presence of two DNA variants with at least one nucleotide change and whose proportions differ by at least 30% between the two strains. Below this value it becomes possible that the background noise hides the presence of the variants.

Analysis of the sequence of the *Paris* lines

The sequence we found for the *Paris* lines (ID. AJ400907) differs from that already published (ID. MIDMMU375) by 69 differences. Among them is the 6 bp-insertion (positions 7131–7136 in MIDMMU375) already mentioned by Rand *et al.* (1994) which could correspond to a cloning artefact in the sequence of Garesse (Garesse, 1988). Two differences correspond

Table 1 Sequence comparison between the previously published sequence (MIDMMU375) and that of the *Paris* strain (AJ400907) of *Drosophila melanogaster*

Genes	Bases (MIDMMU375)	Bases (<i>Paris</i>)	Position (<i>Paris</i>)	Amino- acid	Position in codon
<i>ND2</i>	C	T	630	L	3
	G	A	769	S	3
	C	T	694	L	1
	A	G	750	G	3
	T	A	797	Y(L)	
	A	T	798	Y(L)	
	A	C	1027	L(I)	1
<i>CO I</i>	G	A	1633	M	3
	A	T	1738	L	3
	A	G	1795	G	3
	A	G	1816	P	3
	T	C	1820	L	1
	G	A	1888	G	3
	T	C	2119	Y	3
	C	T	2146	H	3
	C	T	2620	P	3
	C	T	2821	I	3
<i>CO II</i>	G	A	2923	P	3
	T	C	3476	P	3
	T	C	3542	A	3
<i>ATPase 6</i>	T	C	4055	S(L)	2
	A	C	4206	G	3
	A	G	4386	T	3
	T	C	4558	P(S)	1
	A	G	4579	V(M)	1
<i>CO III</i>	C	T	4721	F	3
	A	G	5171	Q	3
	C	T	5306	F	3
<i>ND3</i>	A	G	5902	M	3
Noncoding	A	T	5926		
Noncoding	T	A	5928		
<i>tRNA Glu</i>	C	T	6264		
<i>ND5</i>	C	T	6959	I(M)	3
	G	A	7133	H	3
	G	A	7823	F	3
	G	A	7841	F	3
<i>ND4</i>	A	G	8897	H(Y)	1
	C	A	8898	V	3
	A	G	8958	Y	3
	T	A	8997	S	3
	A	G	9140	L	1
<i>ND4L</i>	G	A	9736	L	1
<i>ND6</i>	C	T	9943	Y	3
	C	T	10178	L	1
	C	T	10342	P	3
<i>cyt b</i>	T	G	10878	G(V)	2
	T	A	10888	V	3
	A	C	10905	S(T)	

Table 1 (Continued)

Genes	Bases (MIDMMU375)	Bases (<i>Paris</i>)	Position (<i>Paris</i>)	Amino- acid	Position in codon
	C	A	10906	S(T)	
	C	T	11218	N	
	C	A	11232	N(T)	2
	T	C	11513	L	1
<i>ND1</i>	T	A	11846	F(I)	1
	G	C	11869	G(P)	
	G	C	11870	G(P)	
	C	A	11940	F(L)	3
	T	G	12013	T(N)	2
	C	T	12044	M(V)	1
	T	C	12579	L	3
Large rRNA	A	T	13241		
	T	A	13242		

Bases (MIDMMU375) refer to the bases in that sequence.

Bases (*Paris*) refer to the bases in that strain.

Position (*Paris*) refers to the number of the base in the *Paris* sequence.

Amino-acid refers to the one encoded by the *Paris* sequence. The amino-acid encoded by the MIDMMU375 sequence is indicated in parentheses.

Position in codon refers to the position of the modified base in the codon.

The entries in italics in column 3 refer to nucleotides in which there are possible errors either in the *Paris* sequence or in the previously published sequence, so they were not taken in account as amino-acid substitutions.

to a one-base deletion in the *Paris* strain (position 13 214 and 13 288 in MIDMMU375). The 61 remaining differences probably reflect strain-dependent variations such as have been observed in other *D. melanogaster* mtDNA sequence comparisons (de Bruijn, 1983; Kaneko *et al.*, 1993; Ballard & Kreitman, 1994; Rand *et al.*, 1994; Lewis *et al.*, 1995). These differences are summarized in Table 1. Genes for rRNA or tRNA are modified three times and two differences are in a noncoding region. Of the 56 remaining differences 44 are synonymous and 12 are

nonsynonymous. If we consider the overall pattern of the amino-acid substitutions, some regions appeared to be more subject to substitutions than others, i.e. the region of the ATPase 6 subunit gene and of the ND1 subunit gene. Kaneko *et al.* (1993) have studied the polymorphism in the ATPase 6 subunit gene; we found the amino-acid substitutions mentioned by these authors (positions 4055, 4558, 4579, see Table 1). This suggests that these amino-acid polymorphisms are widespread in *D. melanogaster*. In this region we found two other polymorphisms which are silent mutations

Table 2 Polymorphic sites in the ATPase 6 subunit gene

Strains	Nucleotide position					
	4055	4206	4386	4553	4558	4579
<i>mel-1</i>	L (TTA)	G (GGA)	T (ACA)	N (AAT)	S (TCT)	M (ATA)
<i>mel-2</i>	S (TCA)	G (GGT)	T (ACA)	N (AAT)	P (CCT)	V (GTA)
<i>SP-1</i>	S (TCA)	G (GGT)	T (ACA)	K (AAA)	S (TCT)	M (ATA)
<i>IR-17</i>	S (TCA)	G (GGT)	T (ACA)	N (AAT)	P (CCT)	V (GTA)
<i>Paris</i>	S (TCA)	G (GGC)	T (ACG)	N (AAT)	P (CCT)	V (GTA)
Position	4055	4206	4386	4553	4558	4579
Codon number	12	62	122	177	180	187

mel-1 and *mel-2* correspond to the sequences published by de Bruijn (1983); *SP-1* and *IR-17* correspond to sequences published by Kaneko *et al.* (1993).

Position: refers to the nucleotide position in the sequence of the *Paris* strains.

Codon number: refers to that of the previously published sequence MIDMMU375.

(positions 4206 and 4386). Position 4206 (a glycine residue) appeared to be highly polymorphic, as three different glycine codons were found in the different strains analysed so far (see Table 2 and Kaneko *et al.*, 1993).

Discussion

We have compared the mtDNA between lines of *Drosophila melanogaster* which exhibit strong and low reactivity levels, with the aim of looking for differences in the sequence which could be correlated with these reactivity levels. The aim of this study is based on the assumption that differences in the mitochondrial sequence between these two strains could involve the presence of two variants (heteroplasmy) in each strain, the relative amounts of which might be correlated with the reactivity level.

In *Drosophila melanogaster* the mtDNA is about 18 kb to 19 kb long and composed of an A + T-rich region (4 kb to 5 kb long) which contains the replication origin and a coding region encoding for 13 polypeptides all implicated in oxidative phosphorylation, coding for 22 tRNA and two rRNA. We analyse the mtDNA by restriction fragment length and DNA sequencing. The analysis of the restriction fragment length allowed us to compare the *Hind*III fragment encompassing the A + T-rich region and the sequencing approach allowed us to compare more precisely the coding region. About 96% of the coding region was sequenced in two lines of the *Paris* stock.

Within the limits of these techniques we can assume that no heteroplasmy is linked to the modulation of the reactivity levels. This result strengthens the hypothesis that this modulation is due to epigenetic changes.

Within the past years our group has shown that the reactivity levels are closely linked to a DNA repair-recombination system which has been called VAMOS for VARIability MODulation System. As for the reactivity levels, the changes induced on the VAMOS are transmitted by females only and may be slowly accumulated over generations (Laurençon & Bregliano, 1995). Polycomb group and trithorax group gene products are responsible for the maintenance of repressed and active expression patterns of many regulatory genes. Recent data show that epigenetically determined active or repressed states can be transmitted to the next generation through the female germ-line, suggesting that a protein-based cellular memory mechanism can be propagated through meiosis (Cavalli & Paro, 1998). Recently Jensen *et al.* (1999) have shown that the transposition of incoming *I* factor could be silenced by the prior transgenesis of a fragment of this factor. This repression of the *I* factor activity develops in a generation-dependent manner, via female germ-line

transmission. This autoregulation presents the characteristic features of homology-dependent gene silencing or cosuppression (Jensen *et al.*, 1999). Cosuppression is mediated by a cellular system whose molecular basis is unknown so far. The epigenetic system responsible for transgenerational transmission might imply only nuclear chromatin but we cannot exclude the possibility that it could lie in a complex regulatory process involving biochemical functions of mitochondria.

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