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vestigial suppressor genes and resistance to aminopterin in *Drosophila melanogaster*

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We have shown that the *vestigial* (*vg*) mutant of *D. melanogaster* has a perturbed nucleotide metabolism compared to various wild-type strains. The mutant is particularly spontaneously resistant to aminopterin. The resistance seems to correlate with an increase in dihydrofolate reductase (DHFR) activity and quantity. The DHRF is a target enzyme of aminopterin. Our results suggest that the vg^+ gene could be a regulatory gene acting on the DHFR gene. The wing mutant phenotype being due to a decrease in the thymidylate pool (dTMP) (Silber *et al.*, 1989).

In order to understand better the action of the mutant gene on nucleotide metabolism, we have induced suppressor genes of the mutant phenotype by mutagenesis with ethyl methanesulfonate (EMS) and bromouridine (BUR). The suppressor strains obtained display a phenotype intermediate between wild-type and *vg* phenotype. The action of three independent suppressor genes on eight parameters of nucleotide metabolism is reported here [three enzyme activities, resistance to aminopterin and to fluorodeoxyuridine (FUdR), auxotrophy test and the ability to use exogenous thymidine and uridine].

In comparison to the original *vg* strain, major changes for the parameters tested are observed. The most striking effects are obtained with the vg^{BUR27} strain, which is highly sensitive to aminopterin and to fluorodeoxyuridine and didplas the highest thymidine kinase (TK) and DHFR activities within the strains tested. The potential actions of suppressor genes on the *vg* mutant are discussed.

Keywords: aminopterin, DHFR, *D. melanogaster*, nucleotide metabolism, suppressor genes, TK and HGPRT activities.

Introduction

The *vestigial* mutant is principally characterized by wing reduction. We have shown that another phenotype, aminopterin resistance, can be associated with the mutation. *vg* mutants are spontaneously resistant to aminopterin, and inhibitor of the dihydrofolate reductase (DHFR) enzyme. This resistance seems to be due to an increase in the DHFR activity compared to the wild-type strain. Other parameters, like the wing phenotype, are affected by the inhibitor. Indeed heterozygotes vg/vg^+ and $defvg/vg^+$, heterozygotes for a deficiency of the *vg* gene, grown at a high concentration of aminopterin, produced phenocopies of the mutant. Of all the inhibitors of pyrimidine or purine metabolism tested, only fluorodeoxyuridine (FUdR) produced phenocopies of the mutant in heterozygous flies in a manner similar to aminopterin. The FUdR phosphorylated to FdUMP is a competitive inhibitor of the thymidylate synthetase enzyme which catalyses the dUMP to dTMP reaction. Aminopterin and FUdR could stop the thymidylate (dTMP) synthesis. We confirmed that the effect observed is specifically associated with the depletion of the dTMP pool because the addition of thymidine (TdR) to the medium with the inhibitor rescues the wing phenotype. Probably phosphorylated in dTMP by thymidine kinase (TK), the nucleotide saves the flies from the action of aminopterin. These results lead us to the hypothesis that the mutant phenotype is due to a depletion of dTMP (Silber *et al.*, 1989).

In order to understand better the effect of the mutation at the level of nucleotide metabolism, suppressor genes were induced by EMS and bromouridine (BUR), *vg* flies were screened on the basis of a change in wing phenotype. Several mutants were isolated and suppressor genes localized on the second or third autosome (Bazin & Silber, 1982). Three mutant strains (vg^{BUR27},

vg^{EMS4} vg^{EMS6}) were tested for eight parameters linked to nucleotide metabolism in order to see if there is a correlation between the wing size change and their nucleotide metabolism. We show that the mutagenesis has induced major changes in each strain. The results and hypotheses on the mode of action of the suppressor genes are discussed.

Materials and methods

Strains and media used

The wild-type strain Oregon (Or) came from Dr Anxolabéhère's laboratory (Paris, France), the *vestigial* mutant (*vg*), described in Lindsley & Grell (1968), came from Dr Gans's laboratory (Gif s/yvette France). The vg^{EMS4}, vg^{EMS6} and vg^{BUR27} revertants were induced in the *vg* strain by ethylmethanesulfonate (EMS) or bromouridine (BUR) treatments (Bazin & Silber, 1982). Strains were raised in bottles containing 50 g of an agar, cornmeal, yeast, nipagin and water medium at 25°C. Aminopterin was dissolved in 0.2 N NaOH and FUdR in water, which were added at the lowest possible temperature that allowed satisfactory mixing.

Effects of inhibitors

The concentrations of inhibitors used were 3 mg kg^{-1} aminopterin and 1 mg kg^{-1} FUdR. Fertilized embryos were placed on the different media tested (aminopterin, FUdR, Sang, Sang + TdR and Sang + UdR) and the number of adults emerging was determined. The resistance was estimated by the ratio: number of adults emerging/number of eggs tested compared to the control.

Following Falk & Nash (1974), auxotrophy was tested on Sang-defined medium (Sang, 1956) and thymidine (TdR) (2 mg kg^{-1}) or uridine (UdR) (2 mg kg^{-1}) were added to test the possibility that the flies used exogenous bases or nucleosides.

Enzyme assays

Flies were sonicated in 5×10^{-2} M Tris pH 7.4, then centrifuged for 30 min at 30,000 ***g***. The supernatant was stored at −20°C and used for the determination of enzyme activities. Each extract contained equal numbers of males and females. Each measurement was repeated three time and the same fly extract was used for the DHFR, HGPRT and TK assays. Protein concentration was determined according to Lowry *et al.* (1951). All chemicals were puchased from Sigma and tritiated products were from Amersham.

Hypoxanthine guanine phosphoribosyltransferase (HGPRT) activity was assayed according to Albertini & De Mars (1970). HGPRT is an enzyme of the purine salvage pathway which transforms hypoxanthine and guanine into inosine monophosphate (IMP) and guanidine monophosphate (GMP). The final reaction mixture was composed of 10 μl Tris pH 7.4, 5 μl 5×10^{-2} M $MgCl_2$, 5 μl 5×10^{-2} M phosphoribosyl pyrophosphate (PRPP), 5 μl (^{3}H)hypoxanthine (1.8 Ci $mmol^{-1}$) and 10 μl of adult fly extract. The mixture was incubated at 37°C. Aliquots of 5 μl were transferred at 5-min intervals onto cellulose-coated sheets. Bases, nucleotides and nucleosides were separated by ascending chromatography in 5 per cent Na_2HPO_4 and were visualized under ultraviolet light. The spots were then cut out, dried and scintillation counted.

The DHFR assay was as described by Alt *et al.* (1976). The final reaction mixture consisted of 0.1 mM (^{3}H) folic acid (10 mCi $mmol^{-1}$), 1 mM NADPH, 0.125 M potassium acetate pH 4.3, 1 mg ml^{-1} bovine serum albumin, in a total volume of 200 μl. After incubation at 37°C for 5 min, the reaction mixture was chilled on ice and 20 μl 0.027 M unlabelled folic acid were added as carrier. The folic acid was precipitated by the addition of 10 μl glacial acetic acid and 20 μl 0.17 M zinc sulphate. Ten minutes later, the mixture was precipitated and the radioactivity in 200 μl of the supernatant was counted. One unit of enzyme was defined as the amount necessary to reduce 1 nmol folic acid in 15 min. Thymidine kinase (TK) was assayed as described by Robert de Saint Vincent & Buttin (1973). The standard incubation mixture contained 0.1 mM (^{3}H) TdR (72 Ci $mmol^{-1}$), 3 mM ATP, 3 mM $MgCl_2$, 50 mM Tris–HCl buffer pH 8 and fly extract in a total volume of 25 μl. Incubation was carried out at 37°C for 10 min. Samples of the incubation mixture (5 μl) were transferred directly onto polyethyleneimine cellulose squares (1 × 1 cm). Unreacted substrate was removed from the reaction product by washing for 10 min with 1 mM ammonium formiate. The squares were dried, placed in scintillation vials and counted.

Results

We postulated that the wing phenotype in the *vestigial* mutant results from a decrease in the dTMP pool while its aminopterin resistance seems to correlate with an increase in DHFR activity. We analysed three independent, partial, revertant strains of the *vestigial* mutation, induced by EMS or BUR mutagenesis. The tests were done shortly after mutagenesis and the strains were isogenic so we postulate that the differences observed between the *vg*, vg^{BUR27}, vg^{EMS4} and vg^{EMS6}

mutations are solely the results of the action of mutagenesis.

vg^{BUR27}, vg^{EMS4} and vg^{EMS6} phenotypes are more or less similar (see Fig. 1), i.e. an intermediate phenotype between *vg* and wild-type. We would expect that suppressor strains would display intermediate results for the parameters tested as well (if there were a close relation between these parameters and the wing phenotype).

The results we observed with the *vg* strain (Table 1) are in agreement with previous experiments. The *vg* mutant is resistant to aminopterin, sensitive at high concentration to FUdR (Silber *et al.*, 1989), displays a TK activity lower than the wild-type strain, a spontaneous HGPRT activity (Silber & Becker, 1981) and a higher DHFR activity than the wild-type strain (Silber *et al.*, 1989).

We did not find any correlation for the revertant strains between the wing phenotype and variation of the nucleotide metabolism parameters. We observed that vg^{BUR27} and vg^{EMS6} survive particularly well on Sang medium, which constitutes an auxotrophic test, this being correlated with a weak utilization of TdR and UdR nucleotides. By contrast, vg^{EMS4} flies do not survive well on Sang medium but are well able to utilize the nucleotides tested.

The other prominent results are the high sensitivity of the vg^{BUR27} strain to FUdR (even compared to *vg*) and to aminopterin (even compared to Or) and we observed a significant increase in TK and DHFR

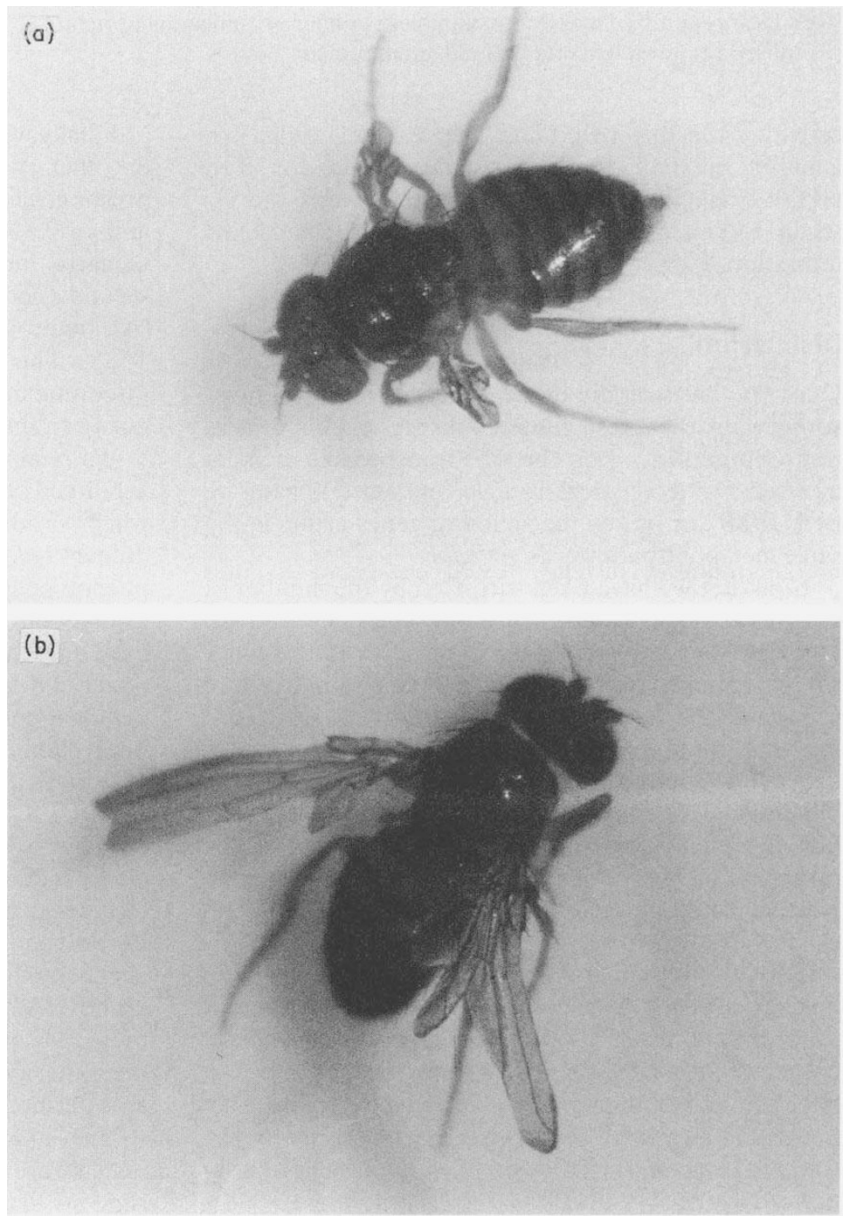

Fig. 1 Phenotype of the *vestigial* (a) and the revertant strains (b).

Table 1 Resistance of the strains *vg*, vg^{EMS6}, vg^{EMS4}, vg^{BUR27} and Or, to FUdR (1 mg kg^{-1}) and to aminopterin (3 mg kg^{-1}), the auxotrophic requirements of these strains were tested on Sang medium, Sang + TdR (2 mg kg^{-1}) and Sang + UdR (2 mg kg^{-1}) and is expressed as a measure relative to the control response. Levels of enzyme activities TK, HGPRT and DHFR

	Control		FUdR		Aminopterin		Sang		S + TdR		S + UdR		Enzyme activities		
Strains	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	Eggs	Adults	TK*	HGPRT*	DHFR†
vg	1712	1355	200	14	279	159	1300	93	1495	158	800	207	9	30	4.3
%/T	100		8.9		72.0		9.0		13.5		32.7				
vg^{EMS6}	520	360	114	4	590	138	400	124	600	267	700	344	8.9	33	3.1
%/T	100		5.0		33.0		44.8		64.3		70.9				
vg^{EMS4}	800	672	200	51	325	60	400	19	400	124	700	357	20	10	3.6
%/T	100		30.3		22.0		5.6		36.9		60.7				
vg^{BUR27}	700	566	200	2	330	10	500	146	500	185	600	221	40	8	5.9
%/T	100		1.2		3.7		36.1		45.8		45.6				
Or	2092	1681	200	91	459	127	2415	323	1420	290	589	162	17	0	2.0
%/T	100		56.6		34.4		16.6		25.4		34.2				

*Results are expressed in (10^{-3}) nanomoles per hour per milligram protein.
†Results are expressed in units per milligram protein.

activities for this revertant. The vg^{EMS4} results, for aminopterin and FudR resistances, and for TK, HGPRT and DHFR activities, tend towards the Or strain; the main differences residing in TdR and UdR utilization.

Discussion

Our hypothesis on the mode of action of the suppressor genes is that these genes could act: (i) either on *vg* transcription, or (ii) on the 412 transposable element inserted in the classical *vestigial* mutant (Williams & Bell, 1988), or (iii) by the action of genes acting in the same metabolic pathway as *vestigial*.

Note that we have been surprised by the number of suppressor genes induced by EMS (nine independent) or BUR (four independent) mutagenesis. In this work we present only the results of the strains with the more pronounced pattern for the parameters tested. We verified that at the molecular level the suppressed strains display the same restriction pattern at the *vg* locus as our original *vg* strain (results not shown). So it seems that the phenotypic differences are due only to the presence of the suppressor genes. We also tested whether these suppressor genes could be suppressors of the 412 element as, for example, is $su(s^2)$. To do this we used the $vermillon^k$ (v^k) mutant, which is known to be caused by a 412 insertion. The induced suppressor genes do not act on the v^k phenotype. It seems likely, however, that 412 suppressor genes do not have an effect on all the mutations that are due to the insertion of the 412 element. This is the case with the $su(s^2)$ 412 suppressor gene which has no phenotypic action on *vg* derived from a 412 insertion (results not shown).

Finally we tested whether these suppressor genes act on other *vg* mutants. This was difficult to test as suppresor genes have no phenotype of their own. vg^{np}, vg^{al}, and vg^{83b27} strains were tested. This last strain is particularly interesting because it probably defines a second complementation group at the *vg* locus (Alexandrov & Alexandrova, 1987; Williams & Bell, 1988). The suppressor genes have no effect on these three mutants. Their action appears to be specific to our *vg* strain.

We retain the hypothesis that the suppressor genes act directly or indirectly in the same metabolic pathway which produces the *vg* phenotype. Our experiments (Silber *et al.*, 1989) lead us to the hypothesis that the *vg* phenotype is due to a depletion of the dTMP pool, the primary effect of the gene being located at the level of the DHFR enzyme. The isolation and the sequencing of the 3.8 kb cDNA of the *vg* locus (J. A. Williams, personal communication) show that there is no structural homology with known DHFR eucaryotic sequences. It appears that the effects that we report at the DHFR level in the *vg* strain are probably indirect but conclusive. If, as we think, the mutant phenotype is due to a depletion of the dTMP pool in the wing, every change in the balance of this pool will change the phenotype. We hypothesize that this pool may be changed by a difference in the transcription of the 3.8 kb mRNA or by mutations of genes which might alter the dTMP pool. In the first case, we might expect that the pattern of the parameters we tested would display an intermediate position between the Or and the *vg* strains. This is not the case with the strains for which results are presented here. Indeed, the evidence suggests that the pattern observed for the three strains

supports the second hypothesis. The existence of a closed inter-relation between the eight parameters tested made the interpretation of our results rather difficult. We had previously studied the nucleotide metabolism variability in different species (Silber *et al.*, 1985) and in different wild-type strains of *D. melanogaster* (Le Menn *et al.*, 1987a and b). These results indicated that with the exception of the *vg* mutant, aminopterin resistance is not correlated with DHRF activity but mainly with the use of salvage pathways and particularly TdR.

The result of the vg^{BUR27} strain seems to be the more interesting. There is a complete change compared to the original *vg* strain. The mutation is almost lethal on FUdR and aminopterin and, paradoxically, presents high TK and DHFR activities correlated with high viability on Sang medium. At this stage, it is impossible to know which enzymatic activity is responsible for this change of pattern in vg^{BUR27}. With respect to the two strains induced by EMS mutagenesis, vg^{EMS6} is closest to *vg* concerning resistance to the inhibitors and the enzymatic activities, tested, and vg^{EMS4} tends towards the wild-type strain. The main differences compared to *vg* and Or are the viability on Sang medium and the use of the exogenous nucleotides. One hypothesis is that vg^{EMS6} may have increased the dTMP pool compared to *vg* due to a better use of the endogenous pathway and vg^{EMS4} due to a better use of pyrimidine nucleosides.

The sequence of the 3.8 kb cDNA does not show extensive homology with known sequences (J. A. Williams, personal communication) and there is still no clear evidence that it is this 3.8 kb cDNA which is directly responsible for the mutant phenotype. The *vg* product could be a regulatory protein that could form a dimer [Rabinow & Birchler studying the interaction between *vg* and *Notch* suggest this possibility (1990)] and modulate another product which would be responsible for the wing phenotype. Only when we know precisely by which steps the *vg* product modulates aminopterin resistance (and DHFR activity) will we have a clear indication concerning the nature of these suppressor genes.

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References

ALBERTINI, R. AND DE MARS, R. 1970. Diploid azaguanine-resistant mutants of cultured human fibroblasts. *Science*, **169**, 482–485.

ALEXANDROV, I. D. AND ALEXANDROVA, M. V. 1987. A new *nw* allele and interallelic complementation at the *vg* locus of *Drosophila melanogaster*. *Drosophila Inf. Ser.*, **66**, 11–12.

ALT, F. W., KELLEMS, R. E. AND SCHIMKE, R. T. 1976. Synthesis and degradation of folate reductase in sensitive and methotrexate resistant lines of S-180 cells. *J. Biol. Chem.*, **251**, 3063–3074.

BAZIN, C. AND SILBER, J. 1982. Genetic studies of phenotypic revertants of the *vestigial* mutant in *Drosophila melanogaster*, induced by bromouridine and ethyl methanesulfonate. *Mut. Res.*, **105**, 65–72.

FALK, D. R. AND NASH, D. 1974. Sex linked auxotrophic and putative auxotrophic mutants of *Drosophila melanogaster*. *Genetics*, **76**, 755–766.

LE MENN A., BAZIN, C. AND SILBER, J. 1987a. Nucleotide metabolism variability in *Drosophila melanogaster*. *Mol. Gen. Genet.*, **208**, 408–411.

LE MENN, A., SILBER, J. AND BAZIN, C. 1987b. Hypoxanthine guanine phosphoribosyltransferase (HGPRT) activity and resistance to aminopterin in the wild-type and in *vestigial* mutant of *Drosophila melanogaster* during development. *Biol. Cell*, **59**, 15–20.

LINDSLEY, D. L. AND GRELL, E. M. 1968. Genetic variations of *Drosophila melanogaster*. *Carnegie Institute Washington Publications*, 627.

LOWRY, O. H., ROSEBROUGHT, N. J., FARR, A. L. AND RANDALL, R. J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265.

ROBERT DE SAINT VINCENT AND BUTTIN, G. 1973. Studies on 1β-D-arabinofuranosyl-cytosine-resistant mutant of Chinese-hamster fibroblasts. A mitochondrial deoxycytidine kinase devoid of activity on arabino-cytosine. *Eur. J. Biochem.*, **37**, 481–488.

RABINOW, L. AND BIRCHER, J. 1990. Interactions of *vestigial* and scabrous with the Notch locus of *Drosophila melanogaster*. *Genetics*, **125**, 41–50.

SANG, J. H. 1956. The quantitative nutritional requirement of *Drosophila melanogaster*. *Exp. Biol.*, **33**, 45–72.

SILBER, J. AND BECKER, J. L. 1981. Hypoxanthine guanine phosphoribosyltransferase (HGPRT) in the *vestigial* mutant of *Drosophila melanogaster*. Effect of inhibitors of the purine pathway. *Genetica*, **55**, 217–220.

SILBER, J. A., COSTE, C., BAZIN, C. AND LE MENN, A. 1985. Dihydrofolate reductase activity and resistance to aminopterin in various species of *Drosophila*. *Mol. Gen. Genet.*, **200**, 92–95.

SILBER, J., BAZIN, C. AND LE MENN, A. 1989. *Vestigial* mutants of *Drosophila* live better in the presence of aminopterin. Increased level of dihydrofolate reductase enzyme in the mutant. *Mol. Gen. Genet.*, **218**, 475–480.

WILLIAMS, J. A. AND BELL, J. B. 1988. Molecular organization of the *vestigial* region in *Drosophila melanogaster*. *EMBO J.*, **7**, 1355–1363.