Obtaining mutations of the gene *Trithorax-like* and measuring its spontaneous mutability in *Drosophila melanogaster*.

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In connection with the necessity of obtaining mutations of the gene *Trithorax-like* (*Trl*, 3L 70 F 1-2) for analysis of its functions, γ-irradiation was used for their induction, and a scheme of crosses has been developed to isolate them. The gene *Trl* is a modifier of the gene *Bithorax*, and its mutations are not visible in the usual sense of the word. However, they are lethal in homozygote or in compounds with each other. That is why mutations *Trl** that arose were detected by the absence of wild type flies in the test for allelism with the earlier known allele Trl^{R85} (= *Trl*85) of this gene (here and hereinbelow, cases of emergence of mutations of the gene *Trl* are designated as *Trl**). Because of the impossibility to observe mutations *Trl** in the 1st generation, the developed scheme includes two crosses. In them, four strains are used: irradiated + / +, marker *D* / *Sb*, and tester strains *Trl*85 / *D* and *Trl*85 / *Sb*. The scheme of crosses is presented in Figure 1.

Chromosomes 3 with dominant markers *D* (*Dichaete*) and *Sb* (*Stubble*) which are lethal in homozygotes carry rearrangements (inversions) that hinder crossing-over, due to which any homologous chromosome, when transmitted via genotypes *D* / + and *Sb* / +, conserves its allelic composition unchanged, which is necessary for prevention of the loss of the newly arisen mutations. P_i designates producers of generation F_i.

The first crossing $P_1 \rightarrow F_1$ may be called individualizing, because, when picking out of F_1 a single fly, we obtain thereby for the test for allelism one gamete from which this fly originated. The second crossing $P_2 \rightarrow F_2$ may be well called detecting, since it represents a test for allelism of the newly arisen mutation in the irradiated chromosome 3 with the laboratory allele *Trl*85, so that F_2 compounds formed die in the presence of a *Trl** mutation in the chromosome studied, which is detected by the absence of wild type flies. At the same time, this second crossing is multiplying, because the newly arisen mutation is multiplied in it, getting into several F_2 individuals, so that, by crossing appropriate flies we obtain a stable strain with the given mutation. The detecting cross has two versions which are separated by the vertical line in the Figure. If a fly *D* / + is taken from F_1 for gamete analysis, it is crossed with the strain *Trl*85 / *Sb*, as shown of the left side on the scheme. If a *Sb* / + fly is taken from F_1 for analysis, it is crossed with the strain *Trl*85 / *D* as shown on the right.

In the two versions, equal phenotypes emerge in F_2 which are indicated in the scheme under the genotypes, and mutations *Trl** are detected by the absence of flies *Trl*85 / + that have the wild phenotype (+). In this case, a control cross *D* × *Sb* or *Sb* × *D* (depending on what virgin females were found among F_2 flies) is made with flies from F_2 , which in fact replicates the test for allelism. Simultaneously with the control cross, a strain with a *Trl** mutation is set up as a *Sb* × *Sb* cross in the left version of the detective crossing or a *D* × *D* cross in its right version, but by no means vice versa, or else, instead of a strain with a new mutation *Trl**, we will obtain a strain with the laboratory allele *Trl*85. If the checking confirms the presence of a *Trl** mutation, then the strain is saved.

Before the individualizing cross *D* / *Sb* × + / +, either males or virgin females of wild type + / + were irradiated, *i.e.* the mutability was estimated as average for spermia and ova, irrespective of the gamete type. For the detecting cross, both males and virgin females *D* / + and *Sb* / + were taken from F_1, because in F_1 there were rather few flies: due to dominant lethals (DLM) induced with γ-rays at a

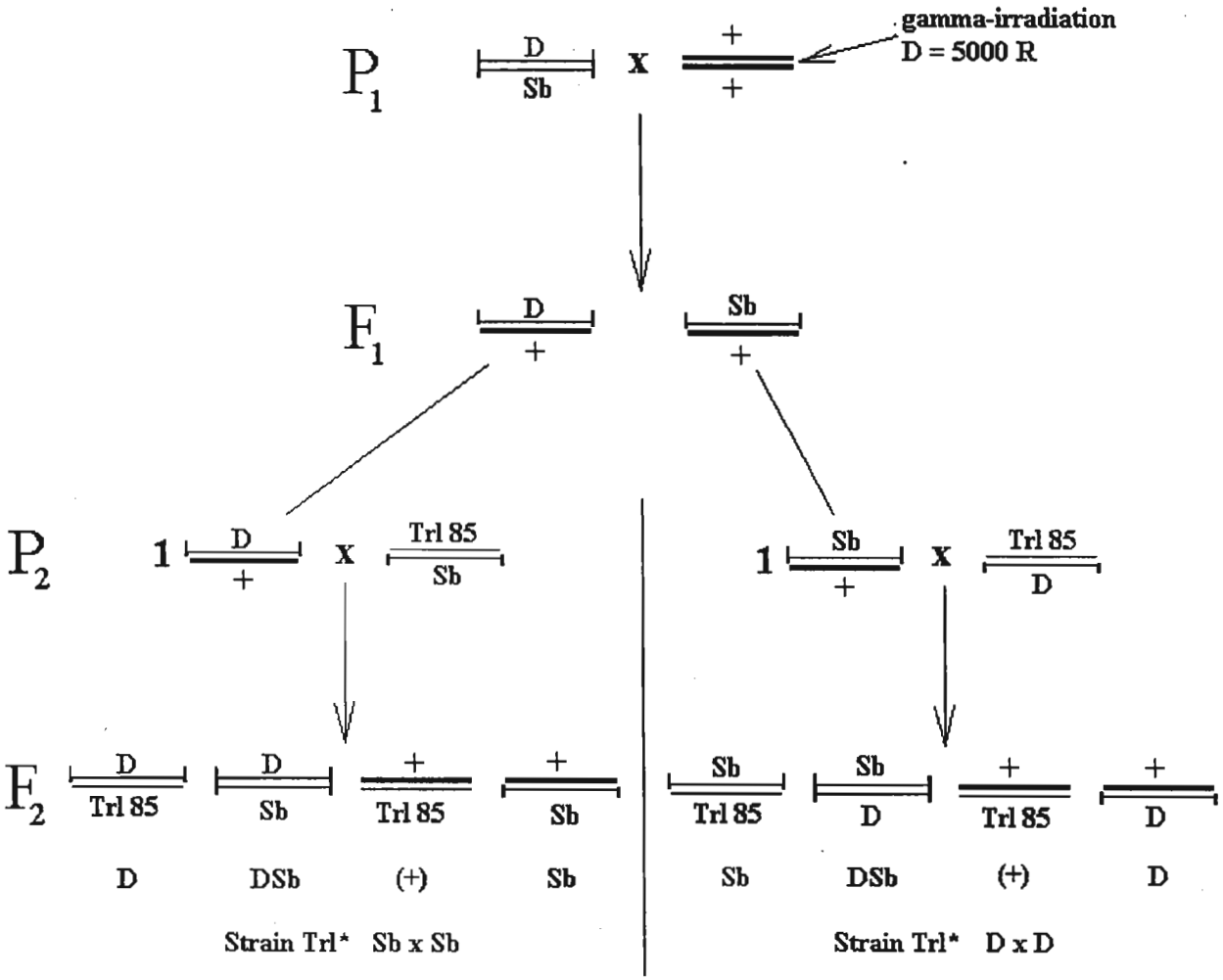

Figure 1. The scheme of crosses for detection of mutations of the gene *Trl*. Stabilized marker chromosomes *D* and *Sb* are marked with transverse dashes at the ends; irradiated wild-type chromosomes are marked with a fat line. The number 1 before the F_2 parent's genotype means that only one fly of the given genotype is used in the cross, *i.e.* that in each of such crosses only one gamete or irradiated P_1 parent is studied. The presence of mutation *Trl** in irradiated chromosome is established by the absence of wild-type flies (+) in F_2. For other explanations, see the text.

dose of 5000 R, zygotes' survival did not exceed 35-38%. The method of calculation of the zygotes' survival at a preset dose of irradiation was developed by us in our previous work (Ivanov, 1998a). Therein, the dependence of the mean number $\tilde{A}$ of DLM in the genome on the γ-irradiation dose D in Roentgens

$$\tilde{A}(D) = 0.0205 + 0.000204\,D$$

was used, whose free term is equal to the average number A of spontaneous DLM in a complete genome (autosomes + X chromosome). For the dose D = 5000 R, we obtain a value $\tilde{A}$ (5000) = 1.0380 from which the zygotes' survival is calculated. When P_1 females are irradiated, an F_1 female's zygote receives an irradiated genome from her mother and an unirradiated one from her father, *i.e.* on the average $\tilde{A}$ + A DLM, while an F_1 male's zygote receives an irradiated genome from his mother and an unirradiated incomplete genome from his father. That is, on the average $\tilde{A}$ + (1 –

s) A DLM, where s is the proportion of genes of X chromosome in the complete genome. Then, at the primary sex ratio of 1:1, the mean number of DLM received by an average, "intersexual" zygote is equal to the arithmetic mean of these quantities, *i.e.* $\tilde{A} + (1 - s / 2) A$. The probability of this zygote not receiving a single DLM which is equal to the probability of survival, if one neglects other mortality factors, is exp $\{- [\tilde{A} + (1 - s / 2) A]\}$, which at $\tilde{A} = 1.0380$, $A = 0.0205$ and $s = 0.19$ is 0.348 $\approx$ 35%. If, however, P_1 males are irradiated, then an F_1 female's zygote receives $A + \tilde{A}$ DLM, and an F_1 male's zygote formed by an unirradiated maternal genome and an irradiated incomplete paternal genome receives $A + (1 - s) \tilde{A}$ DLM. Then the "average" zygote receives on the average $A + (1 - s / 2) \tilde{A}$ DLM, whence the probability of its survival (*i.e.* of the absence of any DLM in it) is exp$\{- [A + (1 - s / 2) \tilde{A}]\}$, which at our values gives 0.383 $\approx$ 38%.

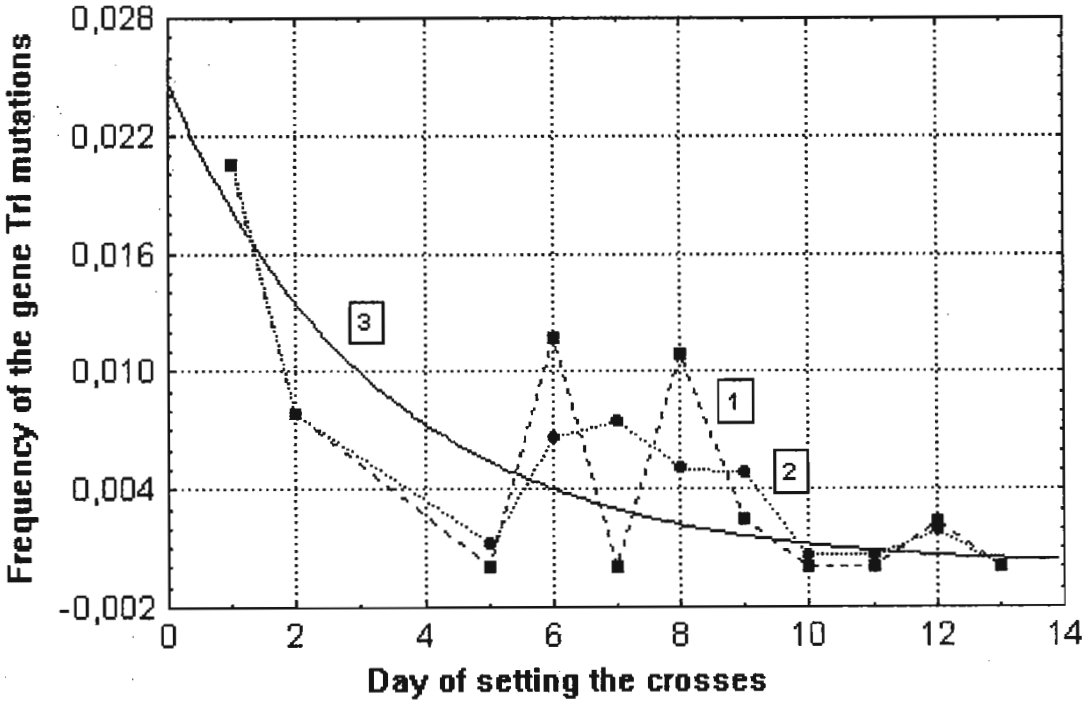

Figure 2. Dependence of the frequency of the gene *Trl* mutations on the day of setting the detecting crosses. 1 – empirical dependence; 2 – dependence levelled out by the method of moving average; 3 – exponential approximation $y(x) = 0.0247 \exp(-0.303 x)$.

For compensation of F_1 zygotes' mortality from DLM, we placed no less than six + females or about 10 *D / Sb* females of P_1 in each tube, and there were more than 50 tubes with $P_1 \rightarrow F_1$ crosses. Therefore there were surely more than 2500 flies available for detecting crosses.

While the development continued in crosses $P_1 \rightarrow F_1$, we picked out virgin females and males of tester strains for subsequent detecting crosses. The whole experiment was carried out at room temperature, without using any thermostat.

When isolating the newly arisen mutations *Trl**, the frequency of their occurrence was estimated. For an accurate recording of the number of gametes studied, the number of 1) performed, 2) failed, and 3) successful detecting crosses $P_2 \rightarrow F_2$ was counted. The latter is the number of gametes studied which can be checked by the difference between the two former numbers.

The performance of detecting crosses lasted, for technical reasons, 13 days out of which no crosses took place on the 3rd and 4th days because of lack of tubes. However, thanks to this it was

found that the frequency of mutations *Trl** in samples of crosses performed on different days clearly decreased from the beginning to the end of the period, as is shown in Table 1. This decrease of *Trl** mutation frequency in samples during the period of crosses $P_2 \rightarrow F_2$ is significant, which can be seen from the ratio of the coefficient $b = -0.303 \pm 0.0846$ of exponential approximation of the dynamics to its standard error: $t = b / s_b = -3.58$ at $f = 9$ degrees of freedom. This means that the hypothesis of $b = 0$ is rejected as implausible at the confidence level $\alpha = 1$ %.

This phenomenon finds the simplest explanation in a hypothesis that heterozygous carriers of mutations *Trl** have a heightened mortality and in time die out among the flies picked out for the detecting crosses. It is just for this reason that exponent was chosen for the time course of their frequency.

Table 1. Time course of the frequency of detected *Trl** mutations in samples of detecting crosses, and its approximation by means of the function $y(x) = 0.0247 \exp(-0.303x)$.

Day x_i	Data of crossing	Number of crosses $P_2 \rightarrow F_2$	failures	gametes examined	mutations detected	Frequency of mutations *Trl** x 10^3 observed	levelled y_i	Expected according to the function $y(xi) = 0.0247 \exp(-0.303 x_i)$
1.	13.01	217	22	195	4	20.5	20.5	18.24
2.	14.01	139	12	127	1	7.87	7.87	13.48
3.	15.01	-	-	-	-	-	-	9.96
4.	16.01	-	-	-	-	-	-	7.36
5.	17.01	166	12	154	0	0	1.26	5.44
6.	18.01	284	28	256	3	11.7	6.68	4.02
7.	19.01	126	12	114	0	0	7.56	2.97
8.	20.01	202	19	183	2	10.9	5.14	2.19
9.	21.01	445	33	412	1	2.43	4.93	1.62
10.	22.01	220	14	206	0	0	0.618	1.20
11.	23.01	125	4	121	0	0	0.604	0.89
12.	24.01	441	19	422	1	2.37	1.97	0.65
13.	25.01	193	22	171	0	0	0.101	0.48
	Total	2558	197	2361	12	-	-	-

Levelling out the empirical relation was performed at two stages: 1) by the method of moving average, using 5-term non-distorting Hildebrand formulae (Pollard, 1977), a leveled-out relation in the segment [5; 13] was obtained, 2) by the least squares method, the exponential function $y(x) = 0.0247 \exp(-0.303x)$ was found as an approximation of the relation throughout the whole time segment [0; 13]. The partial levelling by the former method was needed in order to get rid of 0 frequencies which cannot be logarithmized when building the auxiliary linear regression $\ln[y(x_i)] = \ln a + bx_i$. The empirical and the two levelling relations are represented graphically in Figure 2.

It is noteworthy that among irradiated gametes that had gone through genotypes *D* / + and *Sb* / + of F_1, the mutation *Trl** frequencies were 10/1107 and 2/1254, respectively. These frequencies were significantly different at $\alpha = 1\%$. It occurs that chromosome *D* favors detection of mutation *Trl**, whereas chromosome *Sb*, on the contrary, decreases that chances of its detection. If one rules out the selective coupling of gametes, it is reasonable to hypothesize that *Trl** mutations survive better in *D* / *Trl** than in *Sb* / *Trl** compounds. Taking into account the increased mortality of *Trl** mutations in the latter, one has again to think that there are more occurring than detected *Trl** mutations. This is in good accordance with the hypothesis that F_1 flies carrying *Trl** have a heightened mortality, so that their frequency decreases in time. Probably, the decrease of frequency of *Sb* / *Trl** flies due to death occurs still more rapidly and includes the preimaginal stages.

It is clear that the estimate of the frequency of occurrence of *Trl** mutations must not be their total frequency in the gametes examined where it is decreased due to the death of a part of *Trl**

carrier, but be their frequency before the detecting crosses which can be estimated by the zero ordinate $y(0) = 0.0247$ of the constructing exponent. Therefore, the frequency of occurrence of mutations of gene *Trl* at a dose of γ-irradiation of 5000 R is

$$u\,(5000) = (24.7 \pm 17.7) \times 10^{-3}\,.$$

Therein, its error was calculated from the dispersion $s^2_a = 3.15 \times 10^{-4}$ obtained by means of regression analysis for the coefficient a of the approximating function $y(x) = a \exp(bx)$.

For estimation of the frequency of spontaneous mutations of the gene *Trl*, one needs to know by how many times its mutability increases as compared to the spontaneous one at the irradiation dose of 5000 R. According to our data presented in two papers (Ivanov, 1998a, b), dependence of the mean number ã of genic mutations in X chromosome on the dose D of γ-rays in Roentgens is a linear function $\tilde{a}\,(D) = a + kD$, where $a = (23.5 \pm 3.2) \times 10^{-4}$ is the mean number of spontaneous recessive lethals and visible mutations in the X chromosome, and $k = (22.1 \pm 2.8) \times 10^{-6}$ is a coefficient of proportionality or the increment of the number of such mutations per Roentgen. That is why the multiplicity of increase of the number of mutations in the X chromosome at a dose of D Roentgens as compared to the number of spontaneous ones is $\kappa(D) = \tilde{a}(D)/a = 1 + (k/a)D$, which, after substitution of numbers, gives a linear function of the dose: $\kappa(D) = 1 + 0.00940\,D$. Hence, $\kappa(5000) = 48.0 \pm 8.7$. Transferring this multiplicity from the whole chromosome that carries many genes to a single gene, let us assume that the multiplicity of increase of mutation frequency of gene *Trl* is the same. Then the sought frequency of its spontaneous mutations will be $u = u\,(5000)\,/\kappa(5000)$, which, given our numbers, gives a rather high mutability:

$$u = (5.15 \pm 3.80) \times 10^{-4}.$$

This result is unexpected. For a guaranteed obtaining *Trl** mutations we assumed that the rate of their spontaneous occurrence was not high and equal to $u = 10^{-5}$. The multiplicity of increase of the number of mutations being $\kappa\,(5000) = 48$, we expected that the gene's mutability would increase to $u\,(5000) = 5 \times 10^{-4}$. Then, to obtain at least one *Trl** mutation with a 95% guarantee, one had to examine about 6000 gametes. However, even a small sample of $n = 2361$ gave more than ten *Trl** mutations. This leaves no doubts as to the mutability of $u = 10^{-5}$ being an underestimation.

In the list of genes whose mutability has been measured, the latter seldom exceeded 10^{-4}. According to Dubinin's review (1976), the mutability in *D. melanogaster* varies from 3×10^{-5} to 1.5×10^{-4}. Therefore, our result of $u = 5 \times 10^{-4}$ for the gene *Trl* is extraordinary. At the same time, the observation that *Trl** mutants die out rapidly from the sample still before the detecting crosses (or at the crosses, which may point to a certain loss of cultures) lets us think that mutations of other genes may have the same property, due to which, if it was not taken into account, their mutability may have been underestimated. For example, if, when considering the rate of occurrence of some visible mutation, a part of mutations in F_1 may be eliminated at early stages of development due to a certain degree of their dominant lethality, their frequency among imagines will turn out to be lower than that of their occurrence. However, the idea that mutability of many genes is really underestimated is disproved by a simple reasoning. Assuming that our estimate of the gene *Trl* mutability is equal to the average mutability of a single gene, one can estimate the number of genes in X chromosome by means of dividing the total mutability in it by the average mutability of a gene, i.e. at a value of $23.5 \times 10^{-4} / 5 \times 10^{-4} \approx 5$. The absurdity of this result is obvious. There is no doubt that our estimate of the gene *Trl* mutability exceeds by far the average mutability of genes which must therefore be assumed to be very low. Thus, our estimate of the frequency of induced mutations of the *white* gene, when at a dose of 4500 R, we obtained 18 mutations per 55,259 gametes, permits estimating its spontaneous mutability at $u = (7.7 \pm 2.1) \times 10^{-6}$ which does not differ statistically from the estimate of $u = (2.9 \pm 2.0) \times 10^{-5}$ given by Dubinin.

That is why one may conclude that the gene *Trl* has its peculiarities (in particular, a heightened level of dominance of lethal effect in heterozygotes) which still require confirmation and explanation. Besides, the question arises whether this is not a case of instability in the locus. We were speaking at some length and were somewhat prolix in order to make the present work accessible to unbiased criticism and to let others find possible errors which we probably are not aware of.

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Seasonal fluctuations of *D. cestri* and *D. incompta,* two species of the *flavopilosa* group.

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The species of *flavopilosa* group (subgenus *Drosophila*) utilize flowers as exclusive source for feeding and breeding. This group was established by Wheeler *et al.* (1962) to include *D. flavopilosa* and thirteen new species from the neotropical region. Three other species *D. cestri, D. cordeiroi,* and *D. mariaehelenae* were added to the list (Brncic, 1978; Vilela, 1984). They are small to medium size flies and are mostly pale yellow in color. This body coloration is cryptic with respect to the flowers of *Cestrum* (Solanaceae), which in South America constitute the exclusive sites where the adults were seen fluttering, feeding, and ovipositing, and where larvae were seen growing and pupating.

Brncic (1983) has pointed that among flower breeding drosophilids there are cases in which only one species uses a specific flower source. For example in *Drosophila flavopilosa* and some *Zygothrica* species from Colombia, only a single preadult develops inside the flowers of their respective plant-host *Cestrum parqui* and *Salvia rubescens.* In some other species, as *Zaphriothrica dispar* that use large *Datura* flowers, hundreds of individuals have been found inside of a single flower. Brncic (1983) also reported that there are cases that more that one species could live in the

Figure 1 (next page). Seasonal fluctuations in drosophilids/flowers ratio (an estimative of occupation of the oviposition sites). A) Jardim Botânico location. The amount of collected flowers was 44229 in which 7353 drosophilids and 213 wasp parasites emerged. Obs: *Cestrum* flowers were collected weekly but the data were grouped by month. B) Estação Experimental de Silvicultura location. The collected flowers were 27660 and 5048 drosophilids emerged.

Figure 2 (following page). Seasonal fluctuation in *Drosophila cestri* and *D. incompta* frequencies. A) Jardim Botânico; B) Estação experimental de Silvicultura.