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Heavy heat shock induced retrotransposon transposition in *Drosophila*

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Summary

The phenomenon of transposition induction by heavy heat shock (HHS) was studied. Males of a *Drosophila* isogenic line with a mutation in the major gene *radius incompletus* (*ri*) were treated by HHS (37 °C for 1 h followed by 4 °C for 1 h, with the cycle repeated three times) and crossed to untreated females of the same line. The males were crossed 5 d after heat shock, and also 9 d after HHS. Many transpositions were seen in the F1 larvae by *in situ* hybridization. The rate of induced transposition was at least 2 orders of magnitude greater than that of the control sample, and was estimated to be 0·11 events per transposable element copy per sperm. Two 'hot' subdivisions for transpositions, induced probably during the post-meiotic stage of spermiogenesis, were found: 43B and 97DE. Three-quarters of all transpositions were localized in these positions. In other sites the rates of induced transpositions were $(1{\cdot}3–3{\cdot}2) \times 10^{-2}$ events per occupied segment per sperm, 1 order of magnitude greater than those of the control.

1. Introduction

Transposable elements (TEs) constitute a substantial component of the *Drosophila* genome (Finnegan, 1992). According to experimental estimates (Harada *et al*., 1990; Nuzhdin & Mackay, 1995), the rates of spontaneous transpositions are approximately 10^{-5}–10^{-3} events per TE copy per sperm per generation. In recent years, direct and indirect evidence has accumulated in favour of the phenomenon of TE transposition being induced by external stress factors, of which temperature treatment and γ-irradiation are the most important. Strand & McDonald (1985) demonstrated that heat shock (HS) induces transcription (necessary for retrotransposition) of the *copia* retrotransposon. Junakovic *et al*. (1986) used Southern blotting with probes containing fragments of *copia*-like TEs to show that HS treatment of flies causes induction of transposition of the *297*, *412*, *B104*, *MDG-1* and *copia* TEs. Vasilyeva *et al*. (1988) and Zabanov *et al*. (1990) *used in situ* hybridization to reveal considerable changes in *412*, *MDG-1* and *copia* TE patterns in polytene chromosomes of heterogeneous 'temperature' lines, developed after a stepwise temperature treatment (29 °C ⇒ 18 °C). These results were confirmed by Southern blot hybridization (Vasilyeva *et al*., 1995). We succeeded in demonstrating, by means of *in situ* hybridization, induction of transposition of the *412* TE in several *Drosophila* isogenic lines after treatment by HS, heavy heat shock (HHS) and γ-irradiation (Ratner *et al*., 1992*a*, *b*; Anikeeva *et al*., 1994; Zabanov *et al*., 1995; Vasilyeva *et al*., 1997); HHS induction of B*104* TE transpositions has also been shown in one isogenic line (Zabanov *et al*., 1994).

However, other investigators (Arnault & Biémont, 1989; Arnault *et al*., 1991; Arnault & Dufournel, 1994; C. Biémont, personal communication) failed to demonstrate transposition induction for nine TEs by HHS in *Drosophila* inbred lines. Arnault & Dufournel (1994) questioned the reality of the phenomenon in their critical review. Therefore, we have conducted additional experiments on the induction of *412* transpositions by HHS in the isogenic line N 51, with

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a detailed description of the experimental procedure. These data, in comparison with our previously published data, corroborate the features of the phenomenon revealed earlier by us.

2. Materials and methods

(i) *Isogenic line N51*

Isogenic line N 51 of *Drosophila melanogaster* was obtained in 1991 by the standard method of isogenization from a heterogeneous line (*riC*) having the mutation *radius incompletus* (*ri*); line *riC* was described earlier (Vasilyeva *et al.*, 1988). The cross was performed with a balancer line, in which all three large chromosomes contained crossover suppressors and were marked by visible dominant mutations (Ashburner, 1989): M5 (X chromosome), Cy/Pm (second chromosome) and D/Sb (third chromosome).

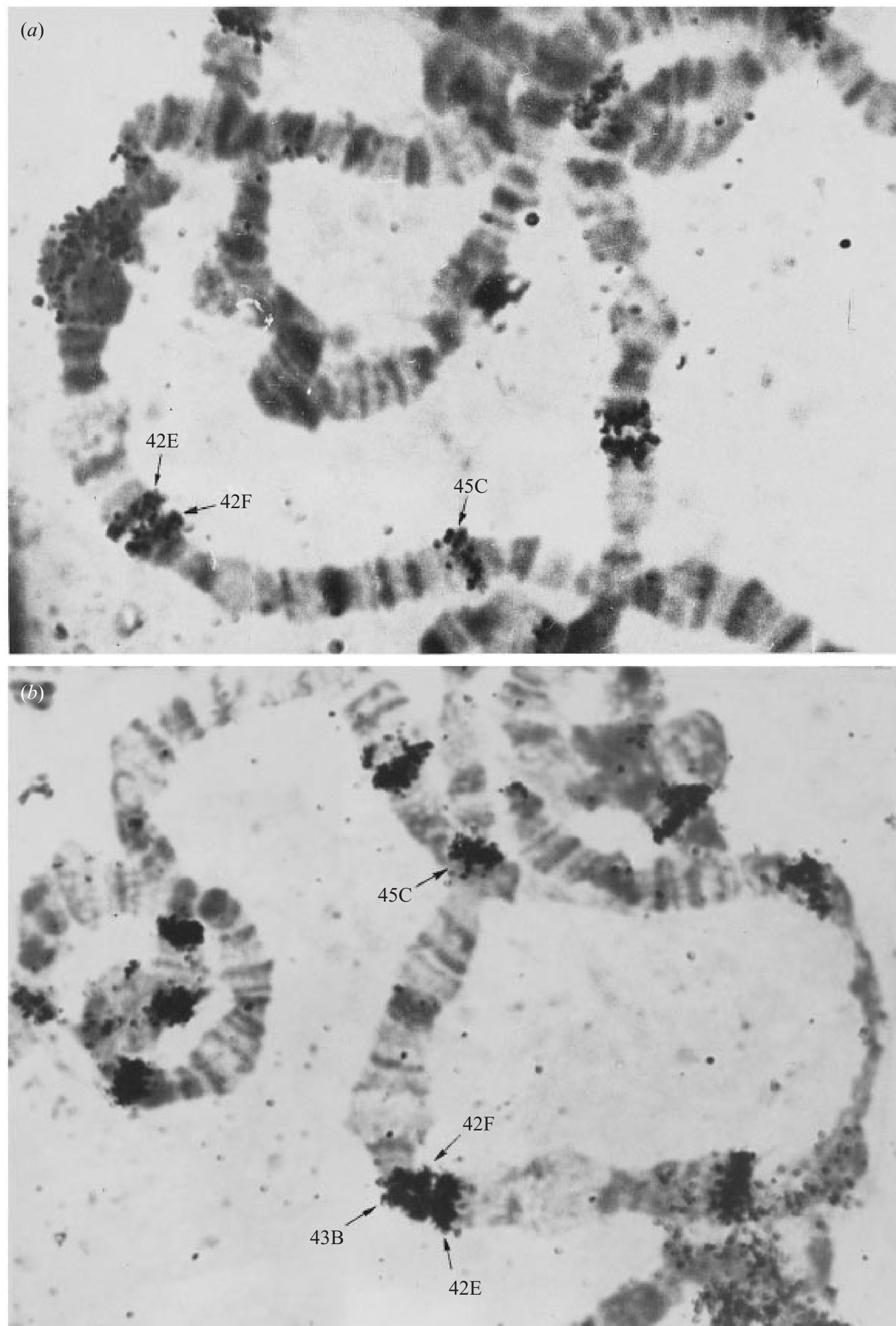

Fig. 1. Polytene chromosomes of *D. melanogaster* isogenic *ri* line N 51 after *in situ* hybridization with a probe containing a copy of the *412* transposable element (TE). Some segments include a localized label. (*a*) Control specimen: a copy of *412* TE is absent in segment 43B. (*b*) Experimental (HHS-2) specimen: a copy of *412* is included in segment of 43B. See text for details.

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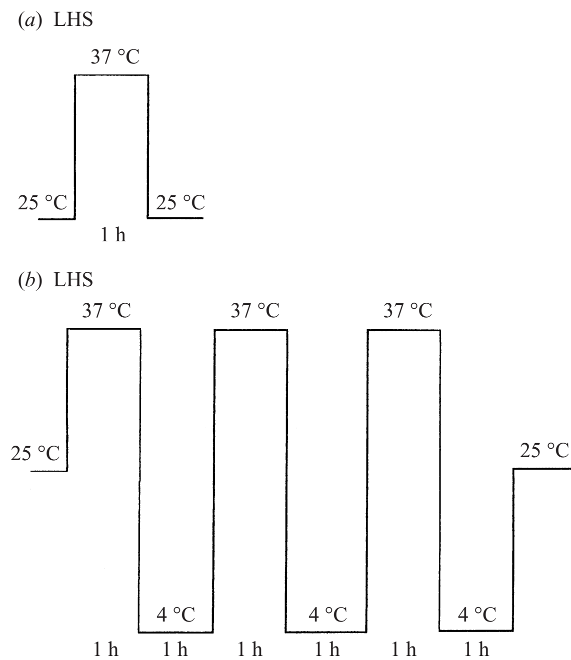

Fig. 2. The procedures for temperature treatments: (*a*) light (usual) heat shock (LHS); (*b*) heavy heat shock (HHS) – combined heat-cold shock.

Line N 51 has been used many times by us (Goryachkovskaya & Vasilyeva, 1991; Anikeeva *et al.*, 1994; Ratner & Vasilyeva, 1996). In a control sample of 100 males of this line, the proportion that were sterile was 2%. Two control samples were used for the determination of the *412* TE localization pattern: 17 larvae (1991) and 10 larvae (1997). Based on the *in situ* hybridization of a probe containing a copy of *412* TE (see below) with polytene chromosomes, it was shown that the pattern contains 31 sites (segments) of this TE localization on Bridges' cytological map. The patterns of the two samples were the same and stable, no polymorphic site of *412* being found. The absence of *412* TEs in other segments was certain in all larvae of both samples. Furthermore, a very detailed search for TE polymorphisms in the 43B and 97DE segments was negative (Fig. 1*a*); segments were identified precisely, and mistakes were ruled out. These data act as a control for 'hot' sites (segments) of *412* induced transpositions (see Table 1).

(ii) *Heavy heat shock*

Heavy heat shock (HHS) is a stressful treatment: 86 males 2–4 d of age after emergence were placed in an incubator at 37 °C for 1 h, and then transferred to another incubator at 4 °C. The cycle was repeated three times (Fig. 2). Thus this is a combined heat-cold shock for 6 h; this treatment is, however, short in comparison with the length of the stages of spermatogenesis. Death was not observed, though the proportion of sterility among treated males (20%) was greater than in the control sample (2%).

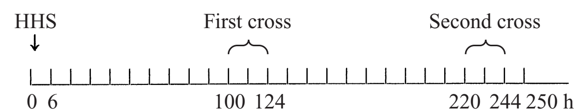

Fig. 3. Scheme of the experiment. The first cross of treated males with untreated virgin females of the same isogenic line N 51 was done 100 h after HHS (i.e. on the fifth day) for 24 h; the second cross of the same treated males with new untreated virgin females of the same line N 51 was done 220 h after HHS (i.e. on the ninth day) for a day.

(iii) *Scheme of the experiment* (Fig. 3)

The treated males were individually placed for 24 h with 2–5 untreated virgin females of the same isogenic line N 51 100 h (on the fifth day) after HHS; later the same males were placed for 24 h with new females 220 h (9 d) after HHS. Squash preparations of the polytene salivary chromosomes were made from the larvae of the next generation (F1) for subsequent *in situ* hybridization.

(iv) In situ *hybridization*

In situ hybridization of a probe containing *412* DNA with polytene chromosomes was done using a standard technique (Finnegan *et al.*, 1978; Ashburner, 1989), using a [^{3}H]DNA-labelled pOR708 probe which contains a full-length copy of *412* TE cloned into the pAT153 vector (Shepherd & Finnegan, 1984) (the clone was received from K. O'Hare). The standard exposure time of the preparations, which were covered with photoemulsion of M type (NIICHIMPHOTOPROJECT, Moscow) was 15–30 d. The intensively labelled bands on the *Drosophila* polytene chromosomes were localized on Bridges' cytological map (Fig. 1). Although the polytene chromosomes are diploid, in the F1 derived from crossing of the treated males with untreated females all the new transpositions are detected, being present in haploid sperm of treated males.

(v) *Statistical analysis*

The statistical significance of the differences between transposition rates per site was tested by Fisher's method with transposition of the frequencies using the formula (Snedecor, 1956)

$$\phi = 2\arcsin\sqrt{p}.$$

Then the Fisher's criterion is

$$F_\phi = (\phi_1 - \phi_2)^2 \frac{k_1 k_2}{k_1 + k_2},$$

where k_1 and k_2 are the sizes of the samples being compared. In our case (see Tables 2 and 3) $k =$ (number of larvae) × (number of sites), because we

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estimated the transposition rate per site. In the tables a double asterisk corresponds to $P < 0{\cdot}01$, and a triple asterisk to $P < 0{\cdot}001$. The cases with $P > 0{\cdot}05$ were insignificant.

3. Results

Examples of labelled chromosome preparations from the control crosses (*a*) and after HHS (*b*) are shown in Fig. 1. The change in *412* TE pattern after HHS is evident: the label in the 43B segment (and also in 97DE out of screen) is absent in the control sample (Fig. 1*a*) and present in the experimental (Fig. 1*b*). The pattern of *412*, revealed in the control samples, was completely stable (see above).

The main results for *412* transposition after HHS are given in Table 1. The larvae were investigated after the first and second crosses of treated males with untreated females of the isogenic line N 51. A total of 193 transpositions in 18 segments were revealed after the first cross in a sample of 85 larvae. The majority of transpositions ($138/193 = 0{\cdot}715$) were found in two 'hot' sites (43B and 97DE); the other 55 were located in different sites. Thirteen transpositions were revealed after second cross in a sample of 49 larvae, seven of these were located in the same 'hot' segments (43B and 97DE) as in the first cross, their proportion being $7/13 = 0{\cdot}538$.

Table 1. *Induction of TE* 412 *transpositions by heavy heat shock after the first and the second crosses based on the data of* in situ *hybridization in F1 larvae*

Segments of Bridges' map	Larvae of F1 after first cross (5th day) (85 larvae)	Larvae of F1 after second cross (9th day) (49 larvae)
3C	1	—
4B	1	—
22B	1	—
28A	4	—
34B	4	2
43B	59	4
56E	4	2
60B	17	—
63A	1	—
67A	1	—
75C	2	—
83D	1	—
86D	6	—
87B	2	1
87F	2	—
94D	1	—
97DE	79	3
98E	6	1
18 segments	193 (55)	13 (6)

Only the new locations of transpositions are shown; the figures in parentheses are the number of transpositions minus those at 'hot' sites (43B and 97DE). Induction is significant in both cases ($P < 0{\cdot}001$ in the first cross, $P < 0{\cdot}01$ in the second cross).

In the first cross, offspring of 19 treated males were observed, 3–5 larvae from each. Transpositions were found in all these groups, most showing only the pairs in the 'hot' positions, 43B and 97DE. In the second cross, descendants of 12 treated males were observed, 4–5 larvae from each. Transpositions were seen in 7 of these 12 groups, some of which were into the same 'hot' sites (7 of 13).

Table 2 shows the results of statistical estimation of the difference in transpositions between two 'hot' sites (43B and 97DE) and the others (usual) in both the first and second crosses. The numbers of total (I), 'hot' (II) and usual (III) induced transpositions per larvae in the first cross are much greater than in the second. But the difference in the proportions of 'hot' transpositions among the total in the first and second crosses is insignificant. This means that phenomenon of 'hot' sites is real in both the first and second crosses after HHS induction, but the level of induction in first cross is 1 order of magnitude greater than in the second cross.

Estimation of the rates of spontaneous and induced transpositions was done by the relation (Shakhmuradov *et al.*, 1989; Ratner *et al.*, 1992*a*, *b*)

$$\Delta n = \lambda \frac{n(m-n)}{m},$$

where Δn is the average number of transpositions seen per sperm per generation, n is the number of occupied sites in the isogenic line prior to the transpositions, m is the number of specific target positions for TE insertions per genome, $(m-n)$ is the number of unoccupied target positions for TE insertions, and λ is the transposition rate measured by the number of events per TE copy in the isogenic line per generation. This formula assumes that the probability of a visible transposition is proportional to the number of initial TE copies per haploid genome and to the proportion of unoccupied target positions of insertion. It thus assumes that all the events are independent and random. In our case (isogenic line N 51) $n = 31$ (see above); m is estimated indirectly as a sum total of positions were *412* has ever been found in all our *ri* lines and populations (control line, 'selection' and 'temperature' lines, isogenic lines, etc.) (Ratner *et al.*, 1992*a*, *b*), $m = 86$. We accept that this figure is a lower estimate of the number of possible positions. $\Delta n = 193/85 = 2{\cdot}27$ events per generation (Table 1).

To estimate the rate of spontaneous transpositions in isogenic line N 51 we use the same method, $\Delta n < 1/27 = 0{\cdot}037$ events per haploid genome per generation, because no transposition was found in the respective control sample (27 larvae). The upper limit of the 95% confidence interval for a Poisson dis-

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Table 2. *Statistical significance of 'hot' and usual induced transpositions in the first and second crosses after heavy heat shock*

	First cross	Second cross	Significance of difference
(I) Total transpositions	85 larvae from 19 treated males with 193 transpositions in 18 sites	49 larvae from 12 treated males with 13 transpositions in 6 sites	
	193/85 = 2·27 induced transpositions per larva	13/49 = 0·265 induced transpositions per larva	$P < 0{\cdot}001$
(II) 'Hot' transpositions	2 'hot' sites, 138 induced transpositions	2 'hot' sites, 7 induced transpositions	
	138/193 = 0·715 of 'hot' transpositions among total	7/13 = 0·538 of 'hot' transpositions among total	$P > 0{\cdot}05$
	138/85 = 1·623 induced 'hot' transpositions per larva	7/49 = 0·143 induced 'hot' transpositions per larva	$P < 0{\cdot}001$
(III) usual transpositions	16 usual sites, 55 transpositions	4 usual sites, 6 transpositions	
	55/85 = 0·647 induced usual transpositions per larva	6/49 = 0·143 induced usual transpositions per larva	$P < 0{\cdot}001$

Table 3. *Summary table of the estimates of transposition rates in control samples and after heat shock*

TE Isogenic line (n), Experiment	No. of larvae in a sample	No. of new TE positions per a sample	Δn per sperm per generation	λ, transposition rate per sperm per TE copy
(1) *412*				
Line N 49(26)				
Control	167	< 1	< 0·018	$< 6{\cdot}9 \times 10^{-4}$
LHS	49	30	0·61	$3{\cdot}4 \times 10^{-2}$***
HHS	312	408	1·31	$7{\cdot}2 \times 10^{-2}$***
(2) *412*				
Line N 51(31)				
Control	27	< 1	< 0·11	$< 3{\cdot}5 \times 10^{-3}$
HHS-1	54	38	0·75	$3{\cdot}8 \times 10^{-2}$***
(3) *412*				
Line N 51(31)				
Control	27	< 1	< 0·11	$< 3{\cdot}5 \times 10^{-3}$
HHS-2:				
First cross	85	193	2·27	$1{\cdot}1 \times 10^{-1}$***
First cross without 'hot' sites	85	55	0·647	$3{\cdot}2 \times 10^{-2}$***
Second cross	49	13	0·265	$1{\cdot}3 \times 10^{-2}$**
(4) *412*				
Line N 66(29)				
Control	14	< 1	< 0·19	$< 6{\cdot}5 \times 10^{-3}$
HHS	57	29	0·50	$2{\cdot}6 \times 10^{-2}$**
(5) *B104* (*roo*)				
Line N 49(70)				
Control	27	< 1	< 0·11	$< 1{\cdot}4 \times 10^{-3}$
HHS	165	292	1·77	$8{\cdot}0 \times 10^{-2}$***

Ratner *et al.* (1992*a*, *b*), Anikeeva *et al.* (1994), Zabanov *et al.* (1994), Vasilyeva *et al.* (1997), this paper.
TE, transposable element; LHS, light (common) heat shock; HHS, heavy heat shock (alternating heat-cold shock); HHS-1, previous experiment (Anikeeva *et al.*, 1994); HHS-2, present experiment. In all cases (apart from HHS-2, second cross) the males were crossed on the fifth day after heat shock. In all cases experimental estimates are significant in comparison with control ones.

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tribution corresponds to $\Delta n = 0{\cdot}11$ transpositions per sperm. This value was used as the estimate of the rate of spontaneous transpositions per sperm. This leads to an estimate of transposition rate in the control, $\lambda_0 = 3{\cdot}5 \times 10^{-3}$ transpositions per sperm per TE copy. The real value could be smaller. For example, in the data of Harada *et al.* (1990), other laboratory *Drosophila* lines have a rate of spontaneous transposition of $5{\cdot}7 \times 10^{-5}$ per sperm per TE copy.

The main results of the estimation of transposition rates are shown in Table 3 (experiment 3, HHS-2). First, transposition induction is evident in all experiments after HHS treatment: the rates are increased by 1–2 orders of magnitude in comparison with estimates for spontaneous transpositions. Second, almost three-quarters of all induced transpositions in the first cross were concentrated in two 'hot' sites (43B and 97DE). If these events are excluded from the first cross, the rate of transposition becomes $\lambda = 3{\cdot}2 \times 10^{-2}$, i.e. it is the same order of magnitude as that after the second cross, $\lambda = 1{\cdot}3 \times 10^{-2}$. Note that both these estimates are 1 order of magnitude greater than the rate of spontaneous transpositions.

Hence, it can be suggested that there are two levels of transposition induction: (1) a 'basic' level (1 order of magnitude) with transpositions that appeared in the progeny of second cross, and (2) a level of superinduction (2 orders of magnitude) with transpositions that appeared only in descendants of the first cross after HHS. Two 'hot' sites (43B and 97DE) make the main contribution to the superinduction of transpositions.

4. Discussion

We have confirmed the phenomenon that *412* TE transpositions were induced by HHS: Table 3 shows comparative estimates of the rates of HS-induced transpositions in all our experiments (Ratner *et al.*, 1992*a*, *b*; Anikeeva *et al.*, 1994; Zabanov *et al.*, 1994; present paper). In all cases, a cross of treated males with untreated females was done on the fifth day after HS. The data are also shown for usual (light) heat shock (LHS) (Ratner *et al.*, 1992*b*).

In all cases, the rates of induced transpositions were 1–2 orders of magnitude greater than the maximum estimates of the rates in control samples, this difference being statistically significant. The rates were $(1{\cdot}3–11{\cdot}0) \times 10^{-2}$ events per initial occupied site of isogenic line per generation. Such results were demonstrated for the *412* TE in isogenic lines NN 49, 52 and 66 after HHS, for the *412* TE in line N 49 after LHS, and also for the *B104* TE in line N 49 after HHS.

The existence of 'hotspots' for induced transpositions is also common, being clear and significant in the larger samples. In the present experiment (HHS-2) the hotspots are 43B and 97DE for *412* TE; these were also shown to be hotspots in an earlier HHS experiment (HHS-1) (Anikeeva *et al.*, 1994). In the isogenic line N 49 the hotspots were 43B, 49CD and 66A for the *412* TE (Ratner *et al.*, 1992*a*, *b*); and in the isogenic line N 49, segments 17C, 34D, 44CD, 58DE, 65E and 86D were hotspots for *B104* (Zabanov *et al.*, 1994). Some hotspots for *412* are the same in different lines and experiments (e.g. 43B).

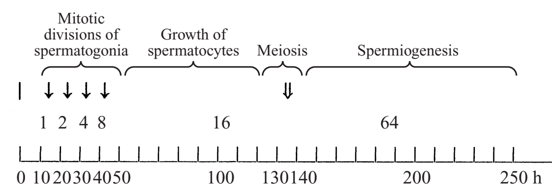

Fig. 4. Conventional chronology of spermatogenesis stages: formation of 64 mature sperm from one primary gonial cell (Lindsley & Tokuyasu, 1980; Haekstein, 1991). Vertical arrows indicate the times of spermatogonial mitoses; double arrow marks two meiotic divisions. See text for details.

A comparison of the results of the first and second crosses after HHS (Tables 1 and Table 3, experiment 3, HHS-2) indicates the timing of the transposition events. Fig. 4 shows the chronology of the stages of spermatogenesis at 25 °C, i.e. formation of 64 mature sperm from one initial gonial cell (Lindsley & Tokuyasu, 1980): the interval of 10–50 h after the division of the stem cell corresponds to the four mitotic divisions of spermatogonia; the next interval of 50–120 h corresponds to growth of the 16 spermatocytes; 120–140 h is when the two meiotic divisions occur; 140–250 h is the time of maturation of 64 spermatids. The total cycle of spermatogenesis from the gonial cell to the formation of 64 mature sperm is completed within 250 h.

The experiment is shown in Fig. 3. The first cross of the treated males with untreated females was done 100–124 h after HHS, i.e. on the fifth day; the second cross was done 220–244 h after HHS, i.e. on the ninth day. The same males were used, and thus all the sperm produced were evacuated after the first cross, and only the sperm matured more than 124 h (the fifth day) after HHS were used in the second cross. Therefore, the pool of gametes of the first cross contains sperm matured between 0 and 124 h and HHS, while the pool of gametes of the second cross contains sperm matured between 124 and 244 h after HHS.

Thus, the sperm used in the first cross were treated by HHS within 120–244 h after the appearance of their gonial cells (see Fig. 4), i.e. within the stages of meiosis and spermiogenesis. The sperm of the second cross were treated within 6–120 h after appearance of their gonial cells (see Fig. 4), i.e. within the stages of mitotic divisions of spermatogonia and growth of spermatocytes. Therefore, we conclude that super-

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induction of transpositions (predominantly to 'hot' target sites) occurred within meiosis and spermiogenesis, and the 'basic' level of induction occurred after HHS treatment during the mitotic divisions of spermatogonia and growth of spermatocytes.

Transcription of full-length RNA TE copies by the host RNA polymerase II must be possible at any stage of spermatogenesis where *412* (and other TE) transpositions were discovered. This process is necessary for further translation of transposition enzymes, reverse transcription and insertion of TE DNA copies into new target sites (Arkhipova *et al*., 1995). Early autoradiographic studies led to the prevailing viewpoint that mRNA synthesis is largely completed before the meiotic stage of spermatogenesis (see reviews by Lindsley and Tokuyasu, 1980; Lifschitz, 1987; and Hackstein, 1991), when any translation is using pre-existing mRNA.

Many *Drosophila* genes indeed show this (Kuhn *et al*., 1988; Michiels *et al*., 1993; Porter *et al*., 1994; Yang *et al*., 1995; etc.). However, some undergo active transcription and expression throughout spermatogenesis (Hackstein, 1991; Bendena *et al*., 1991; etc.). Furthermore, the retrotransposon *micropia* of *D. hydei* has been shown to transcribe a full-length RNA and some other 'sense' and 'antisense' (regulatory?) RNAs in spermatogenesis (Lankenau *et al*., 1994). These were shown at the stage of early spermatocytes, but some persisted up to the stage of spermatid elongation. Therefore, there seems to be no general restriction of retrotransposon transcription during spermatogenesis.

It is also necessary to postulate that some molecular systems of response to temperature treatments were functioning at all the stages of spermatogenesis where *412* (etc.) transposition induction was observed. These may be the systems of response to heat shock (HS) and cold shock (CS) which enhance the transcription activity of subordinate genes. Indeed, some data have been accumulated in favour of transcription and transposition being induced by light HS in *Drosophila* (Strand & McDonald, 1985; Junakovic *et al*., 1986; Ratner *et al*., 1992*b*; Georgiev, 1991). An influence of low temperature on transcription activation of yeast *Ty* elements has been found (Paquin & Williamson, 1984). Bendena *et al*. (1991) showed that transcripts of *hsr-omega* locus, forming one of the largest HS-induced puffs in *Drosophila*, were absent at the primary spermatocyte stage, but appeared at the late spermatid stage. Correspondingly, primary spermatocytes do not respond by enhancing synthesis of mRNA fractions of the main HS genes (*hsr-omega* and *hsp70*) to HS treatment; in contrast, spermatids express a considerable response to HS by elevation of transcription of these two genes.

A role for the molecular system of response to HS in the temperature induction of transpositions seems probable (Vasilyeva *et al*., 1997). The discovery of many different motifs of HS-system functional sites within *412* (and other TE) DNA sequences argues in favour of this view (Ratner & Amikishiev, 1996). As to our case, it means that the genomic system of response to HS is associated with induction of TE transposition only at the stages of spermatogonia and post-meiotic spermiogenesis.

Third, the existence of 'hot' sites of HHS-induced transpositions (Ratner *et al*., 1992*a*, *b*; Anikeeva *et al*., 1994; Zabanov *et al*., 1994; Vasilyeva *et al*., 1997; this paper) can be explained by possible unpacking of specific sites accessible to insertion of TE *412*, etc., just at the stages of spermatogonia and spermiogenesis. This accessibility of target sites may be connected with active function of some genes (located in their neighbourhood) just in the cells of the germ line at the HS-sensitive stages of spermatogenesis.

Some indirect evidence argues in favour of this. $P \times M$ hybrid dysgenesis in *Drosophila melanogaster*, which is induced by a dysgenic cross and consequent mass transposition of *P*-elements, comprises a set of traits affecting the male germ line and meiosis (Engels, 1989). The genes controlling these traits are supposed to act at some stages of spermatogenesis. Castrillon *et al*. (1993) analysed 83 recessive autosomal male-sterile *Drosophila* mutations induced by insertions of single copies of the *P*-element. They clustered into 63 complementation groups, 58 loci of which were necessary for spermatogenesis: 12 affected spermatogonial proliferation, spermatocyte growth and meiosis, and 46 others were responsible for spermatid differentiation and maturation. However, it is clear that insertions to accessible unpacked loci do not necessarily produce lethals, sterile and defective mutations, as we found in our case.

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