GENETIC STUDIES OF GERMINAL MOSAICISM IN *DROSOPHILA MELANOGASTER* USING THE MUTABLE w^c GENE

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THE process of gonad differentiation and germ cell renewal in higher metazoans is a useful piece of information. Its usefulness extends from medical counselling where it can be used to determine the recurrence risks for gonadal mosaics (HARTL 1970) to evolutionary biology in which it enters into the equations for describing the probability of survival of a newly mutant gene (FISHER 1930). Two general experimental approaches have been used to elucidate this process, a cytological approach and a purely genetic approach (HANNAH-ALAVA 1965). In order to be effective the cytological approach requires that some morphological difference exist between the germ cells and the associated apical or synergistic cells. This is often the case, and as a consequence a substantial amount of cytological observation is available throughout the animal kingdom from arthropods to vertebrates. On the other hand, the cytological studies are of limited value because the identification of a germ cell depends on its morphology, not its function, and the possibility cannot be excluded that some of the "germ cells" may never undergo gametogenesis.

The genetic approach to gonadogenesis and germ cell renewal has as its main method brood-pattern studies among the progeny of individuals who are gonadal mosaics for point mutations or chromosome aberrations. This procedure depends for the most part on the induction of new mutations and hence conclusions based on it must include reservations about the possibility of differential viability between mutant and nonmutant cells.

Ideally one would study spontaneous mutations, but these occur too rarely to be of much use (ALEXANDER 1954). The optimum mutation rate for brood-pattern studies is one which is sufficiently high that almost every individual studied will have had at least one new mutation occur during his lifetime, but sufficiently low that very few individuals will have had more than one mutation occur. One genetic element which has this property is the recently discovered sex-linked mutable gene white-crimson (w^c) in *Drosophila melanogaster* (GREEN 1967, 1969a, 1969b). This paper reports the results of brood-pattern studies on

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the offspring of 16 gonadal mosaic w^c males. The results lead to the conclusion that Drosophila males have approximately 14–20 functional germ cells.

MATERIALS AND METHODS

Single w^c sn^3 males (w^c = white-crimson eye color, allele of white, X:1.5; sn^3 = singed bristles, X:21.0) were transferred without etherization to successive harems each consisting of 6 y^2 w^- *spl* sn^3 females (y^2 = yellow body, X:0.0; w^- = a viable deficiency of white; *spl* = split bristles, rough eyes, X:3.0). The resulting female progeny were scored for mutations of w^c. The mutations of w^c are most often to w^+, w, or w^i (ivory) and less often to w^{dc} (dark crimson) or w^{di} (dark ivory). No attempt was made to distinguish between mutations to w and w^i which are approximately equal in frequency (GREEN 1967). Dominant Notch (N = notched wings, X:3.0) deficiencies were also scored; these are also products of the mutator gene.

Each male was presented with nine consecutive harems. Each male was allowed to remain with the first harem for 48 hr, with the next four for 24 hr each, and with the last four for 48 hr each. The males were 0–2 hr old at the beginning of the experiment and about 14 days old at the end. We have obtained a good sampling of brood patterns of young and middle-aged males but not of elderly males because the experiment was terminated about 2 weeks before the males would ordinarily have become sterile.

Conventional Drosophila culture methods were used throughout the experiment.

RESULTS AND DISCUSSION

Among a total of 32 fertile males, 18 had at least one mutant offspring. Of these, 2 were almost sterile, one having but 1 offspring and one having 2. The analysis will be confined to the remaining 16 males which had apparently normal fertility. These 16 males gave rise to 45,624 females of which 1838 were mutant. If each of these mutations represented an independent mutation of w^c, then the mutation rate would be about 0.04. Obviously, not all of these are independent, so the value 0.04 is a gross overestimate. Yet it is useful in that one can calculate from it that one incurs an error of less than 0.0016 by neglecting the possibility of two identical and independent mutations in the same male. This is still a highly conservative estimate because 14/32 males were not gonadal mosaics. No counts were made of the progeny of these males, but the males did appear to be as fertile as the gonadal mosaic males. Correcting the upper limit of the mutation rate on this basis changes the above value of 0.04 into 0.02. The maximal probability of two identical and independent mutations in a single male then becomes 0.0004.

The 16 males manifested 24 mosaic patches, a mosaic patch being defined as a cluster of phenotypically identical mutants from a single male. Ten males had a single patch, four had two patches, and two had three mosaic patches. The multiply mosaic males probably arose by independent mutations of w^c rather than by a replicating instability because in the latter case one would expect a correlation between the size of the mosaic patches (as defined by the total fraction of mutant progeny) in a single individual. The observed correlation coefficient is 0.046 ($df = 4$) which does not differ significantly from zero.

The mosaic patches fall into two categories, those in which all of the mutants appear among the progeny of a single harem, and those in which the mutants are found in the offspring of every, or almost every, harem. Table 1 summarizes

TABLE 1

Distribution of mutant offspring arising in the progeny of a single harem

Male number	Mutation	Cluster size (number of affected females)	Harem in which cluster appeared	Total female progeny from male
1	w	10	1	3331
2	w	3	1	2957
2	w^+	10	2	2957
4	w^+	10	1	1805
8	w	33	1	1952
13	w^cN	8	5	2981
15	w^+	15	1	2948
19	w^+	2	1	3232
21	w^+	2	1	3147
25	w	6	1	3223
26	w^+	10	1	3275
26	w	6	1	3275
26	w^{dc}	10	5	3275
30	w	1	2	3181
31	w	2	1	2956
32	w	3	1	2845
32	w^cN	1	5	2854
32	w^{di}	1	7	2845

the 18 mosaic patches in the first category. These are most easily interpreted as arising from a mutation of w^c within a single cyst. Two kinds of spermatogonia are recognized in *Drosophila melanogaster*, indefinitive and definitive (in the terminology of HANNAH-ALAVA 1965). The maturation of the definitive spermatogonia has been well described (COOPER 1950; KAUFMANN and GAY 1963). A single cell becomes surrounded by a septal (cyst) membrane; it then undergoes 4 synchronous mitotic divisions, and the resulting 16 encysted cells undergo meiosis and spermiogenesis giving rise to a bundle of 64 mature spermatozoa. The bundle breaks apart, the sperms wriggle through the testicular duct into the seminal vesicle and ejaculatory duct, and from there they are eventually ejaculated (LEFEVRE and JONSSON 1962). This whole process has been timed autoradiographically (KAPLAN and SISKEN 1960; CHANDLEY and BATEMAN 1962; OLIVIERI and OLIVIERI 1965). The interval between the time of uptake of tritiated thymidine in spermatocytes to the time of insemination is about 10 days. Of this period, 4 days are spent in spermatocyte maturation, 5 days in spermiogenesis, and one day in processes related to insemination.

At successive stages in the maturation of a cyst there are 1, 2, 4, 8, 16, 32, and 64 nuclei. Assuming that the probability of a mutation of w^c is proportional to the number of chromosomes which carry w^c, the expected number of mutations at these different levels should be 1:2:4:8:16:16:16, because no significant chromosome replication occurs after meiosis commences. Because w^c is sex-linked, the cluster sizes produced by mutations at these different levels are 32, 16, 8, 4, 2, 1, and 1, respectively. Hence, counting the cluster of 33 obtained

from male number 8 as representing two clusters, one of size 32 and one of size 1, the 19 clusters in Table 1 should be distributed as 16.9 of size 1–7 and 2.1 of size 8–32. (The classes are pooled to increase the numbers in each one and to avoid the ambiguities produced by having clusters whose size is not an integral power of 2.) The observed distribution of cluster sizes is 11 of size 1–7 and 8 of size 8–32. A corrected χ^2 for goodness-of-fit produces $\chi^2 = 15.5$ ($df = 1$, $P < 0.001$** —the exact P value calculated from the binomial distribution is $P = 0.00054$). The overall conclusion is that there are far too many large-sized clusters. There are several ways to account for this discrepancy, but the easiest is to suppose that the cells remain for longer periods of time in the early stages of cyst maturation than in the final stages, thereby increasing the likelihood of a w^c mutation. This fits quite well with the picture of spermatogonial development summarized by HANNAH-ALAVA (1965). An equally plausible alternative is that w^c is more mutable in the early stages of cyst maturation than it is in the later stages.

One other interesting feature of the mosaic patches in Table 1 is that many more clusters were recovered from the first harem than the later ones. A discussion of this intriguing fact will be taken up later.

The second class of mosaic patches represents the true gonadal mosaics. In all, 6 such mosaics were detected, and their progeny records are tabulated in Table 2. The first point to note in Table 2 is that the size of the mosaic patches in the males falls into one of two groups, one having about 6% mutant progeny and the other having about 15%. It would be convenient to classify these as 1/16 and 1/8 mosaic, respectively, but the embryonic development of the germ cells disallows this simplification. The germ cells in *Drosophila melanogaster* develop as buds off the aboral end of the egg and are compressed between the posterior blastodermic wall and the vitelline membrane (HUETTNER 1923; SONNENBLICK 1941). For about 70 minutes after their formation these pole cells remain massed at the posterior of the egg; then they begin their tortuous and prodigious wanderings back into the embryo. Some migrate through the blastoderm (COUNCE 1963), others travel via the midgut (HATHAWAY and SELMAN 1961—see HANNAH-ALAVA 1965 for an extensive list of references). Eventually the pole cells migrate into the mesodermal bands which flank the midgut where they become surrounded by small mesodermal cells and are organized into the gonads.

Not all of them make it. RABINOWITZ (1941) counted an average of 55 pole cells at the end of the egg, with individual counts ranging from 36–73. Only 10–26 of these end up in the definitive gonad. On the basis of cell counts, young embryos fall into two groups, those which contain 5–7 cells per gonad and those which contain 9–13 cells (SONNENBLICK 1941, 1950). It has not yet been established whether the male or female gonads contain the larger number of germ cells, but most investigators have favored the view that the testes are the gonads with the larger number of cells (SONNENBLICK 1950; BODENSTEIN 1950; HANNAH-ALAVA 1965). MULLER *et al.* (1954) adopted the opposite point of view.

In view of the almost casual way in which the germ cells move to the gonads, it would come as no surprise if different males had different numbers of germ cells. One way to test this possibility is to determine whether the 15% mosaics

TABLE 2

The day-by-day progeny pattern of males which are stem-cell mosaics

Male number		Mutant females/Total female progeny Harem 1	2	3	4	5	6	7	8	9	Total mutant females	Total female progeny	Proportion of mutant females	Homogeneity χ^2
1	w^+	45/402	47/454	75/363	59/292	102/472	85/382	70/336	72/219	64/411	619	3331	.1858	75.8**
3	w	9/218	41/333	20/432	8/177	10/436	2/405	26/250	1/98	43/344	160	2693	.0594	98.9**
13	w	16/283	22/302	41/385	59/246	62/495	60/435	91/279	20/210	33/346	404	2981	.1355	145.5**
15	w	2/351	23/384	32/330	31/299	45/431	21/289	18/235	14/290	32/339	218	2948	.0739	42.1**
18	w	11/258	16/186	7/194	20/252	30/340	2/65	0/106	2/85	0/331	88	1817	.0484	13.9*†
29	w	5/279	41/293	0/219	17/324	36/625	43/513	0/332	55/374	19/332	216	3291	.0656	62.6**†

†These χ^2 values exclude the harems in which no mutant progeny appeared. To include them would make the χ^2's still more significant.
* $P < .05$.
** $P < .01$.

(males number 1 and 13) and the 6% mosaics are heterogeneous among themselves in respect to the overall fraction of mutant progeny. Indeed they are. The 15% mosaics generate a $\chi^2 = 29.3$ (df = 1, P $\ll$ 0.0001**) and the 6% mosaics give $\chi^2 = 13.4$ (df = 3, P $\approx$ 0.004**). Hence the extent of mosaicism in the males is significantly different. The reciprocal of the fraction of mutant progeny gives a consistent estimate of the number of germ cells. The number of germ cells at the time of the mutation of w^e in the 15% mosaics is estimated as 6–7; the number of germ cells at the time of the mutation of w^e in the 6% mosaics ranges from 14–21. This latter figure provides an estimate of the maximum number of germ cells in a male. The reason is that the more germ cells there are, the more likely a mutation of w^e will occur, and the more likely a mosaic at this level will be detected. Note, for example, that there were twice as many 6% mosaic males as 15% mosaic. So the finding of 1/21 as the lowest level of mosaicism strongly suggests that there are no more than 21 germ cells in a male, and the heterogeneity in the 6% mosaic class suggests that the number of germ cells in a typical male is in the range of 7–10 per testis.

This number agrees very well with the germ cell counts made in immature testes (SONNENBLICK 1941, 1950) if it is assumed that the gonads with 9–13 cells are indeed testes. One problem arises with this interpretation, however; the 9–13 germ cells per testis undergo one or two additional mitoses even before the egg hatches (SONNENBLICK 1941), and BODENSTEIN (1950) has suggested an even greater amount of cell proliferation. Our genetic results, along with those of ALEXANDER (1954) and KHISHIN (1955), suggest that however much proliferation occurs, the number of germ cells in the stem line remains the same (see HANNAH-ALAVA 1965). One way to account for the non-stem line cells would be to suppose that there are a fairly large number, say 10 per testis, which behave as indefinitive spermatogonia in prepupal stages but which cease to divide thereafter. If from among these cells some definitive spermatogonia are selected, then one would expect more of the "single cyst" clusters to occur in the first or second harems than in the later ones because the short-lived germ cells, if mutant, would lead to an early cluster which would be classified as a mutation in a single cyst. Of the 18 clusters observed (Table 1), 14 occurred in the first or second harem.

This hypothesis is consistent with the suggestion of HANNAH-ALAVA (1965) that the number of stem cells is progressively reduced throughout the lifetime of a male. She suggests that a reduction occurs primarily in aged males (over 2 weeks) and uses this to account for the progressive reduction of offspring from increasingly older males. We are suggesting that there is also a reduction in pupal or very young males. The harem-by-harem pattern of mosaicism in Table 2 gives no evidence for progressive reduction of stem cells during the period when the males are 0–14 days old, but this does not invalidate HANNAH-ALAVA's hypothesis.

The day-by-day pattern of mosaicism in Table 2 is heterogeneous for any individual male (cf. χ^2 values in the last column). This probably reflects the asynchrony of germ cell divisions (STERN 1941; COOPER 1950; KAUFMANN and

GAY 1963). The germ cells must also undergo some periods of quiescence because a gonadal mosaic male will sometimes produce no mutant progeny over a 24- or 48-hour period (see males number 18 and 29 in Table 2).

The overall picture which arises from these data is that 0–14 day old males have 7–10 germ cells per testis, a conclusion in excellent agreement with that reached by SONNENBLICK (1950) and HATHAWAY and SELMAN (1961).

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SUMMARY

24 gonial mutations of the mutable w^e gene in *Drosophila melanogaster* males have been analyzed. 18 of these evidently occurred within single cysts and the remaining 6 occurred in stem cells. The distribution of cluster sizes among the 18 cyst mutations is not that expected from the cytological evidence of testis differentiation. There are far too many large clusters; these occur predominantly in very young males. We suggest that there is a population of indefinitive spermatogonia which are active as such only in the pre-pupal stages. Some of these are selected as definitive spermatogonia and give rise to a fraction of the sperms ejaculated by very young males. Analyses of the 6 stem cell mutations lead to the conclusion that males aged 0–14 days have effectively 7–10 stem cells per testis and that different males may have different numbers of stem cells. The stem cells divide asynchronously and may individually undergo quiescent periods of 24 to 48 hours.

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