Restriction map and α-amylase activity variation among *Drosophila* mutation accumulation lines

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Summary

The specific activities of α-amylase were measured for two sets of mutation accumulation lines, each set having originated from a different lethal-carrying second chromosome and *SM1(Cy)* chromosome and having been maintained by a balanced lethal system for about 300 generations. Significant variation was found to have accumulated among lines of both sets. Because of dysgenic crosses in the early generations of mutation accumulation, insertions or deletions of transposable elements in the *Amy* gene region were suspected of being the cause of this variation. In order to test this possibility, the structural changes in the 14 kb region of these chromosomes that includes the structural genes for α-amylase were investigated by restriction map analysis. We found that most part of the activity variation is due to replacements of a chromosomal region of *SM1(Cy)*, including the structural genes for α-amylase, by the corresponding regions of the lethal chromosomes. One line also contained an insertion in this region but this line has an intermediate activity value. Thus, insertions of transposable elements into the *Amy* gene region were not found to be responsible for the new variation observed in α-amylase activity. If we remove those lines with structural changes from the analysis, the genetic variance of α-amylase specific activity among lines becomes non-significant in both sets of chromosomes.

1. Introduction

Drosophila natural populations have been shown to have a large amount of genetic variation affecting the expression of enzyme-coding genes (reviewed in Laurie-Ahlberg, 1985). In order to understand the mechanisms by which this variation is maintained in natural populations, it is crucial to study the properties of new variation for enzyme activity produced by mutation. For this purpose, we need a large number of originally isogenic lines which have accumulated spontaneous mutations over a long period.

Mukai & Cockerham (1977) established two sets of 500 lines, each originated from a single heterozygous individual, and kept them by balanced-lethal systems to avoid contamination from outside sources. Although the original purpose of this experiment was to measure mutation rates of enzyme coding genes (see also Voelker, Schaffer & Mukai, 1980), other traits were also examined, i.e. chromosomal aberations (Yamaguchi & Mukai, 1974), visible mutations and mutator factors (Scobie & Schaffer, 1982*a*) and ADH activities (Mukai, Harada & Yoshimaru, 1984). Genetic variation considered to have arisen during the accumulation periods (150–300 generations) was found for all traits.

In the present study, we extend the study of Mukai, Harada & Yoshimaru (1984) to include the specific activity of α-amylase. Many genetic studies have already been carried out on the locus (*Amy*) which encodes this enzyme (summarized in Doane *et al.* 1983). It is located at map position 77.7 and polytene bands 54A;55 on chromosome 2R and is duplicated in many or perhaps all strains of *Drosophila melanogaster*. Furthermore, the region which includes the coding sequence of the gene has been cloned (Gemmil, Levy & Doane, 1985 and Levy, Gemmil & Doane, 1985). We measured specific activities of α-amylase in a subset of the 1000 mutation accumulation lines mentioned above using whole adult flies.

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Significant variation in α-amylase activity was detected among the lines.

Since mutator activities were suspected to exist among these lines, due probably to dysgenic crosses in the early generations (Yamaguchi & Mukai, 1974; Scobie & Schaffer, 1982*a*, *b*), it was speculated that this new variation might be caused by insertions or deletions of some transposable elements into the coding region or the regions close to the coding region. No relationship between the presence of large insertions and α-amylase activity was apparent among chromosomes sampled from natural populations (Langley *et al.* 1988). This absence may be due to the operation of natural selection. However, in the present case the chromosomes suffered from minimal natural selection during the mutation accumulation process. There are ample examples in which activities of genes are disturbed by insertions or deletions of transposable elements near the genes (see Finnegan & Fawcett, 1986). To investigate this possibility we also carried out restriction map analysis of the region containing *Amy*. It was found that the genetic variation of α-amylase activity observed was due to double crossing over or gene conversion between the balancer chromosome and the lethal chromosomes used, whose affected areas include the *Amy* coding region. Only one insertion into this region was found but its contribution to the variation was very small. Thus, it was concluded that most of the new variation observed was not due to insertions or deletions of transposable elements into the region of the *Amy* gene.

2. Materials and methods

(i) *Establishment of lines*

One thousand second chromosome lines were established using *In(2LR)SM1* (abbreviated as *Cy*) and two unrelated lethal-carrying chromosomes, *l(AW)* and *l(JH)*, derived from a cage population which had been started in 1954 from a wild population in Erie, Pennsylvania (Mukai & Cockerham, 1977). A single male, heterozygous for either *Cy* and *l(AW)* or *Cy* and *l(JH)*, was mated to a single C-160 [*In(2LR)SM1/In(2LR)bw^{V1}*] female. From the progeny of this mating, 500 lines heterozygous for *Cy* and *l(AW)* and 500 lines heterozygous for *Cy* and *l(JH)* were established by single-pair matings in three generations. These are numbered *AW-1*, *AW-2*, ..., *AW-500*, and *JH-1*, *JH-2*, ..., *JH-500*. For the first 200 generations of mutation accumulation, each line was maintained by a single-pair mating or a five-pair mating, for the next 100 generations these lines were maintained by mass mating. Occasionally a line became so weak that progeny of only one sex were available. In this case, progeny of some other line were crossed to this line and the name of the line was changed to one bearing both the parental line numbers. Up to this point, the lines were maintained at North Carolina State University. Early in 1980, about 100 lines, which were carefully chosen in order to avoid selecting duplicated lines in the process of accumulation of mutations, were transferred to Kyushu University. Their genetic backgrounds (*X*, *Y*, third and fourth chromosomes) were replaced by that of C-160, an isogenic line for the genetic background. From these lines, 26 *AW* and 22 *JH* lines were randomly chosen and used in the experiments. All lines used have the same electrophoretic phenotype, *AMY*^{1,6}, and share common ancestors only in the first three generations. Thus, all chromosomes of these lines are considered to have independently accumulated mutations for about 300 generations. Samples from the original cage population were found to act as P strains (Scobie & Schaffer, 1982*b*). However, tests of these lines later indicate that they are all of the M type of the P–M system (Mukai, Harada & Yoshimaru, 1984). A recent *in situ* hybridization experiment on these lines shows that these lines do not have P elements but have I and *hobo* elements and that *hobo* elements are responsible for the chromosome abberations observed by Yamaguchi & Mukai (1974) (Harada, Yukuhiro & Mukai, unpublished results).

(ii) *Experiment 1. Measurement of specific activity of α-amylase*

The activity of α-amylase was measured by a modification of the DNSA method (Hoorn & Scharloo, 1978) in the following way: Two different culture media were used to raise the flies. For each chromosome line, two *Cy/l* $\times$ *Cy/l* crosses were made with five flies of each sex in vials of $2{\cdot}5 \times 10$ cm on starch medium without living yeast. Another two crosses of the same type were made on corn meal medium. From each vial, five 4-day-old adult *Cy/l* males were sampled, were homogenized in 0·2 ml of distilled water and centrifuged. Fifty μl of supernatant was used to determine α-amylase activity by observing the colorimetric change of DNSA (3·5 dinitrosalicylic acid) due to the generation of reducing groups. The change was represented in enzyme *units*. The production of reducing groups equivalent to one micromole glucose per minute is defined as one unit. Twenty-five μl of supernatant from each vial was used for measuring the amount of total soluble protein according to the Lowry method (Lowry *et al.* 1951). The specific activities in units per milligram of soluble protein were calculated and these values were employed for the analyses.

(iii) *Experiment 2. Restriction map analysis*

Genomic DNA from each line was prepared by a method modified from Bender, Spierer & Hogness (1983) and was digested with *Eco* RI and *Sal* I together or with *Eco* RI and *Hin* dIII together, using the high or medium salt buffers of Maniatis, Fritsch &

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Sambrook (1982) and 5 mM spermidine for 2 h at 37 °C. Fragments were separated on 1·2% agarose gels using TBE buffer, denatured and neutralized (Smith & Summers, 1980) and blotted to Zetabind (AMF Cuno) in 1 M ammonium acetate/0·05 M-NaOH. The filters were prehybridized, hybridized and washed as described by the manufacturer (AMF Cuno). The phage λDm65 (Gemmil, Levy & Doane, 1985) containing an 14·2 kb genomic fragment from *Drosophila melanogaster*, including the duplicated *Amy* structural genes was used as nick-translated (Rigby *et al.* 1977) probe DNA. The filters were exposed to Kodak XAR films with intensifying screens.

To construct restriction maps of the chromosomal segments probed, one line from each group of lines which share the same restriction pattern was crossed to an isogenic wild strain W109 whose restriction map was already known (Langley *et al.* 1988). Curly and wild-type progeny, which are heterozygotes for *Cy* and W109 and for either *l*(*AW*) or *l*(*JH*) and W109 chromosomes, respectively, of these crosses were collected separately and their DNAs were extracted and analysed in the same way as above except that they were single- and double-digested with four restriction enzymes, *Eco* RI, *Sal* I, *Hin* dIII and *Bam* HI.

(iv) *Experiment 3. Measurement of specific activity of α-amylase in* Cy, AW *and* JH *chromosomes*

In order to see the effect of each chromosome on the specific activity of α-amylase, one or two lines from each group of lines which share the same restriction pattern were chosen. Five males from these lines were crossed to five females of a wild strain, Kaduna16, which had been inbred for about 20 generations. Six such crosses were made for each line and a pair of samples each of which consisted of five *Cy* and wild-type 4-day-old male progeny were obtained from each cross. In this experiment, only corn meal medium was used to raise the flies. The specific activity of α-amylase was measured for each sample in the same way as in Exp 1.

3. Results

(i) *Experiment 1*

A total of four measurements (2 media × 2 vials) was made for each line. The sequence of the measurements was randomized daily, so that the errors were randomized within and between media. Analyses of variance were made separately for the *JH* and *AW* lines, and the results are shown in Table 1. The effects of media and lines were significant but the interactions were not in both groups of lines. Thus, significant differentiation of α-amylase activity has accumulated among heterozygous lines (*Cy*/*l*) which originated from a single individual during 300 generations. Similar results were reported for ADH activity in the same material (Mukai, Harada & Yoshimaru, 1984).

(ii) *Experiment 2*

In the survey with double digestions *Eco* RI/*Sal* I and *Eco* RI/*Hin* dIII, four lines, *AW-52*, *JH-377*, *JH-397* and *JH-443* × *497*, were found to have fragment patterns different from the other *AW* or *JH* lines (Fig. 1). We call these mutant lines. *JH-397* and *JH-443* × *497* have the same pattern. By crossing these lines, together with representatives of the rest of *AW* and *JH* lines, to W109 and digesting DNA made from F1 progeny with several enzymes, we constructed restriction maps of this region of the chromosomes. Four haplotypes, *cy*, *aw*, *jh* and jh^{V} were identified (Fig. 2). The first three haplotypes differ from each other only at *Eco* RI recognition sites. The haplotype jh^{V} is the same as the haplotype *jh* except that it has an insertion, whose size is approximately 6 kb, between the *Hin* dIII site (0·3) and the *Bam* HI site (3·0). Non-mutant lines of *AW* and *JH* are heterozygotes, *aw*/*cy* and *jh*/*cy*, respectively. Mutant lines, *AW-52*, *JH-397* and *JH-443* × *497*, are homozygotes, *aw*/*aw*, *jh*/*jh* and *jh*/*jh*, respectively, and *JH-377* is a heterozygotes jh/jh^{V}, *jh* and jh^{V} being on *Cy* and *l*(*JH*) chromosomes, respectively. The haplotype *aw* has an additional *Eco* RI recognition site which is not in the *cy* haplotype at the right end of the region and lacks the *Eco* RI site of the *cy* haplotype at −2·1 (see Fig. 2). The haplotype *jh* has two additional *Eco* RI sites on the right end and at −8·1 which are not in the *cy* haplotype and lacks the *Eco* RI site at −2·1. Since both haplotype pairs, *aw-cy* and *jh-cy*, are differentiated by more than one site and since the probability of gaining the same restriction site at the same position is very low, we

Table 1. *Analysis of variance of specific activities of α-amylase using all lines in* D. melanogaster

Source	D.F.	Sum of squares	Mean square	*F*
(*a*) *JH* lines				
Media	1	0·175319	0·175319	30·77***
Lines	21	0·280429	0·013354	3·52***
Interaction	21	0·119650	0·005698	1·269
Duplicates	44	0·167017	0·003796	
Total	87	0·742415		
(*b*) *AW* lines				
Media	1	0·132253	0·132153	89·43***
Lines	25	0·106439	0·004257	1·77*
Interaction	25	0·036945	0·001478	0·61
Duplicates	52	0·125305	0·002410	
Total	103	0·400843		

*** Significant at 0·1% level; ** significant at 1% level; * significant at 5% level. The same notation applies for other tables.

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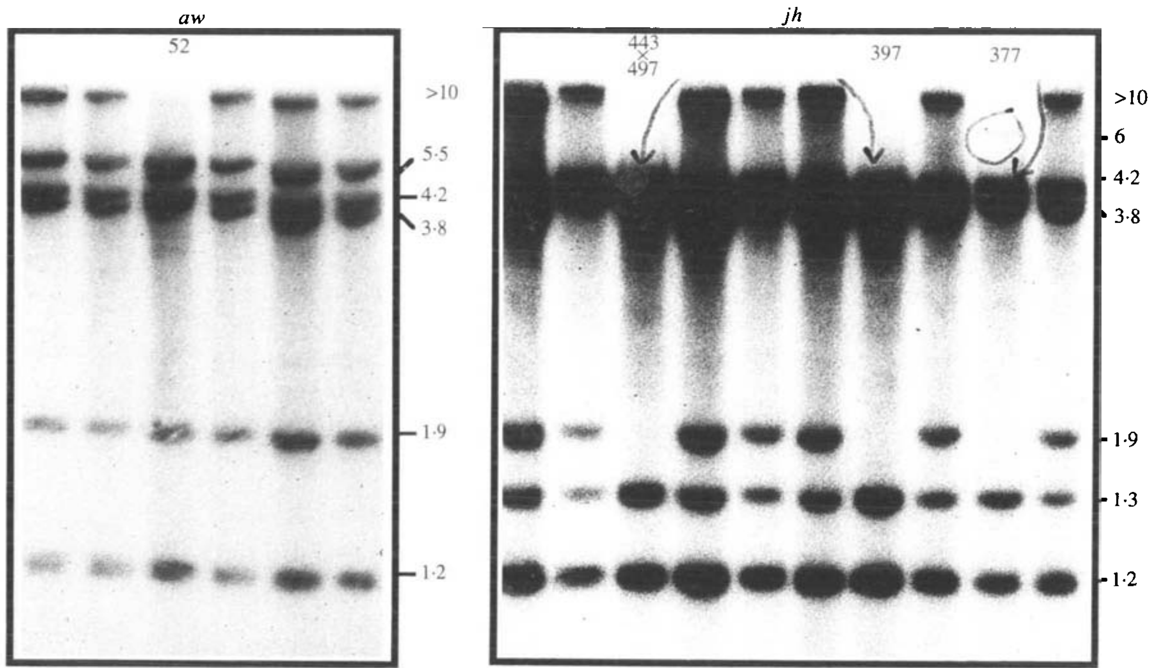

Fig. 1. Southern blot of genomic DNAs from *AW* and *JH* lines digested with *Eco* RI/*Hin* dIII and probed with λDm65. Lanes containing mutant lines are numbered. Unnumbered lanes are example of non mutant lines. Approximate lengths of fragments in kb are indicated on the right. The largest fragment labeled as > 10 is the right most fragment of *cy* haplotype (see Fig. 2). All mutant lines, *AW-52*, *JH-377*, *JH-397* and *JH443* × *497*, lack this large fragment and the 3·8 kb fragment. *JH* mutant lines also lack the 1·9 kb fragment which is found in non-mutant *JH* lines and all *AW* lines. *JH-377* acquired a new fragment about 6 kb in size.

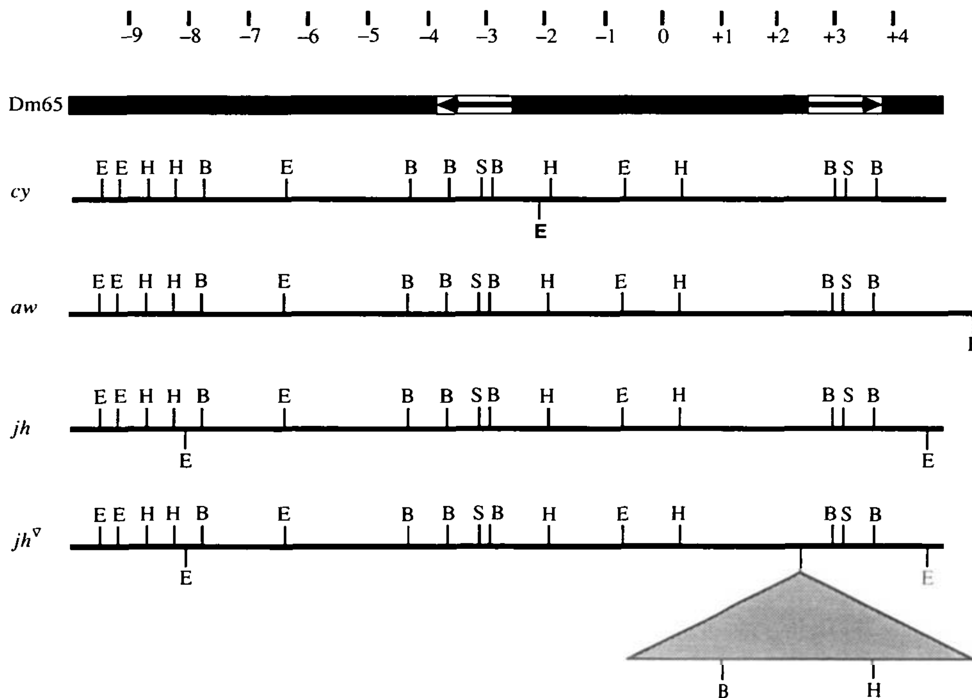

Fig. 2. Restriction maps of the four haplotypes *cy*, *aw*, *jh* and jh^{∇}. Coordinate centred between the two coding regions is given at the top. Other recognition sites can be referred by designating how many kb left (minus) or right (plus) of this reference point they are located at. Duplicated coding regions of *Amy* are indicated as arrows. Four types of recognition sites, *Eco* RI, *Hin* dIII, *Sal* I and *Bam* HI, are designated by E, H, S and B, respectively. Recognition sites common to all haplotypes are shown above the bars and those specific to haplotypes are shown below the bars. An *Eco* RI site beyond 10·0 exists in the *cy* haplotype, but it is omitted in the figure. The insertion in jh^{∇} is designated by a shaded triangle and is located at some place between the *Bam* HI site at 3·0 and the *Hin* dIII site at 0·3. Non-mutant lines of *AW* and *JH* are heterozygotes of haplotypes, *aw*/*cy* and *jh*/*cy*, respectively. Mutant lines, *AW-52*, *JH-397* and *JH-443* × *497*, are homozygotes of haplotypes, *aw*/*aw*, *jh*/*jh* and *jh*/*jh*, respectively, and *JH-377* is a heterozygote of haplotypes jh/jh^{∇}, *jh* and jh^{∇} being on *Cy* and *l*(*JH*) chromosomes, respectively.

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Table 2. *Analysis of variance of specific activities of α-amylase excluding mutant lines*[a] *in* D. melanogaster

Source	D.F.	Sum of squares	Mean square	F
(*a*) *JH* lines				
Media	1	0·102072	0·102072	22·13***
Lines	18	0·109889	0·006105	1·48
Interaction	18	0·083039	0·004613	1·12
Duplicates	38	0·156454	0·00417	
Total	75	0·451453		
(*b*) *AW* lines				
Media	1	0·134851	0·134851	95·36***
Lines	24	0·069799	0·002908	1·38
Interaction	24	0·033940	0·001414	0·67
Duplicates	50	0·104981	0·002100	
Total	99	0·343570		

[a] The mutant lines are *AW-52*, *JH-377*, *JH-397* and *JH-443 × 497*.

concluded that the whole or a part of *Amy* region of the *Cy* chromosome of each mutant line was replaced by the corresponding region of *l*(*AW*) or *l*(*JH*) chromosome. In *AW-52*, the replaced region includes at least the segment between the right end at 5·4 and the *Eco* RI site at −2·1, and in *JH* mutant lines, it includes at least that between the right end at 4·6 and the *Eco* RI site at −8·1. All replaced regions include both coding regions of *Amy*.

If we look at the specific activities of these mutant lines, we find that *AW-52* has the highest value in the *AW* lines and *JH-397* and *JH-443 × 497* have the highest and the second highest values in *JH* lines. *JH-377* has an intermediate value among *JH* lines. After the removal of these mutant lines, the genetic variances among *AW* lines and among *JH* lines become non-significant, both at the 5% level (Table 2).

(iii) *Experiment 3*

Since the mutant lines except for *JH-377* have two copies of *aw* or *jh* haplotypes, it was hypothesized that *Amy* segments of these two haplotypes have factors which produce higher specific activity of α-amylase than that of the *cy* haplotype. To test this hypothesis, we carried out a third experiment. *AW-4* representing the *AW* lines, *JH-60* representing the *JH* lines, and the four mutant lines were crossed to Kaduna16, and the specific activities of their *Cy*/Kaduna and wild-type *l*/Kaduna offspring were compared. Within each cross-type there were six vials and two samples of each phenotype, Curly or wild type, were taken from each vial. Analysis of variance was performed within each cross type and the main effects of the phenotypes on the specific activity of α-amylase were tested using the

Table 3. *Comparisons of specific activities between wild-type and* Cy *offspring*

Line[a]	Phenotype	Mean	*F*(1, 5)
(1) *AW-4*	Wild	0·504	20·85**
	Cy	0·245	
(2) *AW-52*	Wild	0·550	0·031
	Cy	0·566	
(3) *JH-60*	Wild	0·518	20·81**
	Cy	0·312	
(4) *JH-377*	Wild	0·342	2·86
	Cy	0·436	
(5) *JH-397*	Wild	0·386	0·282
	Cy	0·338	
(6) *JH-443 × 497*	Wild	0·560	6·15
	Cy	0·353	

[a] Lines crossed to Kaduna16.

F test (Table 3). As expected, wild-type offspring of the crosses between Kaduna16 and non-mutant lines (*AW-4* or *JH-60*), which had one copy of the *aw* or *jh* haplotype, have significantly higher specific activities than the Curly offspring of the same cross, which had one copy of the *cy* haplotype, whereas there is no significant difference between two types of offspring in mutant lines, both of which have one copy of the *aw* or *jh* haplotype.

4. Discussion

(i) *Structural changes*

The following five events are thought to have happened among our 48 lines during 300 generations of mutation accumulation: the regions including all or part of the *Amy* gene of the *Cy* chromosomes were replaced by the corresponding regions of the lethal chromosomes in *AW-52*, *JH-377*, *JH-397* and *JH-443 × 497*. In addition, an insertion approximately 6 kb in size was inserted into this region of the *JH* chromosome of *JH-377* after the replacement. This is the only insertion we found. In the restriction site analysis, the largest fragment in this region was a 4·2 kb *Eco* RI/*Sal* I fragment except for the very right end fragment of the *cy* haplotype. If there were other insertions or deletions of more than 200 to 300 bp, they could have been detected. The rates of replacement and of insertion in this region are estimated to be $4/(300 \times 48) = 0{\cdot}00028$ per line per generation and $1/(300 \times 48) = 0{\cdot}000069$ per line per generation, respectively. No such structural changes were observed in the *Adh* region in a companion study (Aquadro *et al.* unpublished results).

Since the *Cy* chromosome has multiple inversions to suppress recombination with the standard chromosomes such as *l*(*AW*) or *l*(*JH*), there are two possible explanations for the replacement of the whole or a part of the region. One is gene conversion. In

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Drosophila melanogaster, the rate of gene conversion ranges roughly from 10^{-5} to 10^{-4} (Lamb, 1984). Our estimate of 0·000 28 is close to the upper bound of this range. However, Hilliker & Chovnick (1981) reported that two sites more than 0·001 map unit apart in the *rosy* locus have a low probability of being converted together (co-conversion). Using Chovnick, Gelbart & McCarron's (1977) conversion rate, 0·01 map unit = 8·8 kb, at this locus, gene conversion of segments of more than one kilobase are considered to be unlikely. In our case, converted segments are probably more than 10 kb long, arguing against the possibility of gene conversion in our case. Another possibility is double crossing over. Since the inversion which includes the *Amy* locus (located at 54A;55) spans from polytene band 42A3 to polytene band 58A4 in the *Cy* chromosome (Lindsley & Grell, 1968), double crossing over could occur within this region. The lines used in this study are known to have some hybrid dysgenic characteristics, one of which is a high frequency of male recombination in some of the lines (Yamaguchi & Mukai, 1974; Scobie & Schaffer, 1982*a*). Furthermore, in the lines which showed male recombination, extremely high frequencies of uni-directional double crossing over were observed (Scobie & Schaffer, 1982*b*). Although our lines do not include those found to have this property at the time of that experiment, the uni-directionality fits our observations. In all four mutant lines, segments of the *Cy* chromosome were replaced. The agents which might have existed in these lines and caused male-recombination may have been lost. Alternatively, the replacement may be caused by crossing over in females. To our knowledge, there is no estimate of the rate of double crossing over within this inverted region between the *Cy* and the standard chromosomes. However one may obtain some ideas from the estimate of double crossing over between the *In(2L)t*, the break points of which are at 22D and 34A, and the standard chromosomes. Mukai & Voelker (1977) estimated the rate of double crossing over within the area spanned by *In(2L)t* between the *In(2L)t*-carrying and the standard chromosomes to be 0·000 22 using α-GPDH (located between 25F and 26B) as a marker. Again this is close to what we obtained for the *Amy* gene. At this point, since we are looking at the end products only, we can not decide on which mechanism, gene conversion or double crossing over, is responsible for the replacements.

For the insertion found in *JH-377*, we know that it is inserted between two coding regions of *Amy* and that its size is about 6 kb. However, two facts are worth mentioning. First, this occurred in one of the *JH* lines for which a higher rate of chromosomal abberation (mostly inversion) was observed (Yamaguchi & Mukai, 1974). Hence, the insertion might reflect the stronger dysgenic property of *JH* lines. Since the size of the insertion is about 6 kb, the insertion is not a P, I or *hobo* element whose size is known to be less than 6 kb (see Finnegan & Fawcett, 1986). Second, the specific activity of α-amylase of *JH-377* is lower than those of the two other mutant *JH* lines at face value although the difference is not significant at the 5% level. Since the only difference between *JH-377* and *JH-397* or *JH-443* × *497* is the presence of the insertion, the insertion between two duplicated genes may have caused the decrease. Further studies on this line are necessary to pursue this possibility.

We observed no evidence of base substitution among the lines examined. There are five or six *Eco* RI, four *Hin* dIII and two *Sal* I sites recognized in the survey experiment. All enzymes recognize six base pairs and there is no overlap, which means that we are looking at about 70 base pairs in this 14 kb region. There will be roughly equal numbers of base pairs at which mutation would have created new recognition sites. Thus, we can say the average mutation rate of a base pair is possibly less than $1/(300\times48\times2\times2\times70) = 2{\cdot}5\times10^{-7}$ per generation. This upper limit is far above the estimate $(3{\cdot}72-18{\cdot}6)\times10^{-9}$ per base pair per generation, obtained indirectly from the electrophoretic data of the whole 1000 lines by Mukai & Cockerham (1977). We would need to expand the size of the experiment by more than 20- to 100-fold to obtain a direct estimate of the base-pair mutation rate.

(ii) *Change in specific activity of α-amylase*

We found significant genetic variation for α-amylase specific activity among the *AW* and the *JH* lines, each set of lines originating from a single individual 300 generations ago. There are several possible ways in which this variation might have been created, (1) point mutations in the coding region of the *Amy* gene, (2) insertions or deletions of transposable elements in the vicinity of the *Amy* gene, (3) gene conversion (Chovnick, Gelbart & McCarron, 1977) or double crossing over (Scobie & Schaffer 1982*a*, *b*) between the balancer and lethal chromosomes and (4) mutations including structural changes in modifier loci (Mukai, Harada & Yoshimaru, 1984). Variations in modifier loci are found in natural populations (see Abraham & Doane, 1978; Yamazaki & Matsuo, 1984; Maroni & Laurie-Ahlberg, 1983). The possibility (1) seemed unlikely on the same ground as that of ADH case (see the argument in Mukai, Harada & Yoshimaru, 1984). Because of the presence of mutator activities in some of the lines (Yamaguchi & Mukai, 1974; Scobie & Schaffer, 1982*a*, *b*) and since many mutations are considered to be due to insertions or deletions of transposable elements (Finnegan & Fawcett, 1986), (2) was suspected as a likely cause for the variation. However, by examining the restriction maps of the region containing the *Amy* gene, we found that most of the variation was due to (3) in this region. Insertions or deletions of transposable elements in the

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vicinity of the *Amy* gene [(2)] was found to contribute little to the variation observed in our activity assay. Finally, although non-significant, the mean squares for line effects are greater than those for duplicates even after the removal of the lines with structural changes in both sets of lines (Table 2). This suggests accumulation of genetic variation among the lines, which is not explained by the mechanism, (2) or (3). This part of variation, if it really exists, is more interesting from the evolutionary standpoint and further study of this material is necessary to determine the nature of this variation.

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