Epistatic interactions of spontaneous mutations in haploid strains of the yeast *Saccharomyces cerevisiae*

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Abstract

Several important biological phenomena, including genetic recombination and sexual reproduction, could have evolved to counteract genome contamination by deleterious mutations. This postulate would be especially relevant if it were shown that deleterious mutations interact in such a way that their individual negative effects are reinforced by each other. The hypothesis of synergism can be tested experimentally by crossing organisms bearing deleterious mutations and comparing the fitness of the parents and their progeny. The present study used laboratory strains of the budding yeast burdened with mutations resulting from absence of a major DNA mismatch repair function. Only in one, or possibly two, crosses out of eight did fitness of the progeny deviate from that of their parents in a direction indicating synergism. Furthermore, the distributions of progeny fitness were not skewed as would be expected if strong interactions were present. The choice of experimental material ensured that genetic recombination was extensive, all four meiotic products were available for fitness assays, and that the mutations were probably numerous. Despite this generally favourable experimental setting, synergism did not appear to be a dominating force shaping fitness of yeast containing randomly generated mutations.

Introduction

Deleterious mutations may affect fitness independently, so that the fitness of an individual carrying two mutations would be equal to the product of fitness of two individuals each carrying only one of them. Multiplicity of fitness effects is especially useful in the analysis of interactions (Kondrashov, 1988; Charlesworth, 1990). A synergistic interaction among mutations would be present if the proportional negative effect of a given mutation were not constant but tended to increase with increasing number of other deleterious mutations. The opposite would be true for an antagonistic interaction. Theoretical studies demonstrate that interactions between mutations, especially a synergistic interaction, can affect the evolution of genetic life-cycles (Kondra-

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shov & Crow, 1991), the magnitude of inbreeding depression (Charlesworth, 1998), the rate and mode of fitness decline due to mutation accumulation (Charlesworth *et al.*, 1993; Butcher, 1995), as well as the evolution of genetic recombination (Kimura & Maruyama, 1966; Feldman *et al.*, 1980; Barton, 1995) and sexual reproduction (Kondrashov, 1988).

Mukai (1969) found synergism of spontaneous deleterious mutations in his classical experiments with fruit flies, although they were later criticised as insufficiently controlled during long-term breeding (Shabalina *et al.*, 1997; Fry *et al.*, 1999; Keightley & Eyre-Walker, 1999). The authors of other studies, involving *Chlamydomonas* (de Visser *et al.*, 1997a), higher plants (de Visser & Hoekstra, 1998) and RNA-viruses (Elena, 1999), also detected synergism though they noted that their results are equivocal. As an alternative to accumulation of spontaneous mutations, Elena & Lenski (1997) inserted genetic markers into random positions of the *E. coli* genome. Similarly, de Visser *et al.* (1997b) focused on the

existing chromosomal markers in *Aspergillus*. In both studies, the results were mixed, indicating interactions in both directions but no clear overall trend. Further research is thus necessary not only to determine conclusively if synergism between deleterious mutations occurs, but also to help in answering a more general question about the evolutionary role of epistasis between fitness-affecting genes (Fenster *et al.*, 1997).

The budding yeast is especially suitable for testing interactions between spontaneous mutations. It can be grown mitotically either as haploid or diploid cells. A cross between two haploids of opposite mating types results in a diploid which, in turn, can be grown mitotically or induced to undergo meiosis after which four haploid spores are obtained. The two phases can be reliably maintained in the laboratory and the moment of mating or sporulation freely chosen by an experimenter. It is thus possible to work with haploids which ensures that dominance effects within loci do not interfere with between-loci interactions. Even more important is the ability to recover all four products of meiosis in the form of spores from a single diploid cell. The spores can then be used to generate vegetative haploid clones whose fitness can be assayed. This is critical to exclude the selection that can occur at the stage of gamete formation in other organisms. Such selection would result in the elimination of the least fit gametes and would generally go unnoticed. The strains used in this study came from previous experiments where they were subjected to rapid accumulation of spontaneous mutations (Korona, 1999b). The rate of accumulation was facilitated by the absence of a mismatch repair gene, *pms1* or *msh2*, which resulted in frequent frame-shifts and substitutions (Crouse, 1998). After approximately 20 serial transfers of such mutator strains, several dozen mutations decreasing in fitness have probably been accumulated (Korona, 1999a). Such an estimate is based on our recent study that indicates that at least one mutation decreasing fitness could arise during growth of a colony (D. M. Wloch, K. Szafraniec, R. H. Borts, & R. Korona, unpublished observations). A similar rate can be deduced from an earlier study (Reenan & Kolodner, 1992).

The present work was initiated by crossing a mutated haploid clone with a standard laboratory strain of yeast in which DNA-repair functions had never been absent. The resulting diploid clone was induced to go through meiosis producing four haploid clones from a single diploid cell. At this stage, parental mutations were extensively rearranged due to the segregation of the 16 yeast chromosomes and approximately four cross-overs per chromosome (Roeder, 1997). The average fitness of these progeny was then compared with the parental mean. Under the hypothesis of multiplicity, the same average number of mutations in the parents and offspring would result in their average log-fitness being equal. In a cross between two individuals of similar fitness, synergism is found when the average log-fitness
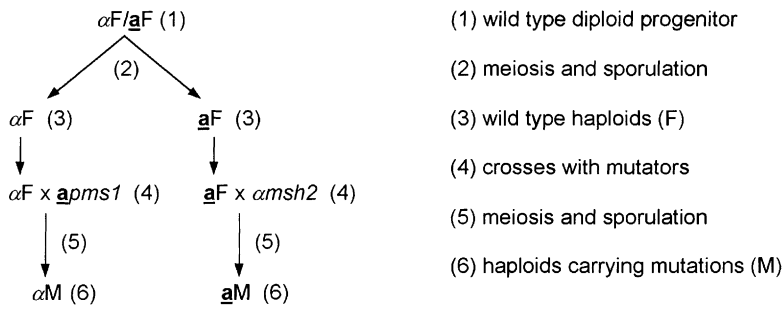
of progeny is lower than that of the parents. The reason is that some individuals among the progeny carry more deleterious mutations than their parents and, due to the reinforcing effect of synergism, their fitness is exceedingly low, which lowers the average fitness of all offspring. (For the sake of simplicity we assume that deleterious mutations always interact in the same way regardless of their particular combinations.) When the parents are very different in the number of deleterious mutations, synergism is marked by the average log-fitness of offspring being higher than the parental mean. This is because the progeny will usually have half as many mutations as the more mutated parent. Therefore, a negative epistatic interaction will be less severe in the offspring, and, as a result, the offspring mean will be relatively high. The second parameter studied was the skew in the distribution of log-fitness of progeny, g_1. A skew to the left ($g_1 < 0$) would mark synergism. A simple intuitive argument might be helpful here. Imagine a population with an average of n mutations per individual. Under multiplicity, fitness of an average individual would be proportionally as much lower than fitness of an individual with $n - 1$ mutations as fitness of an individual with $n + 1$ would be lower than its own fitness. After log-transformation, equality of proportions converts to equality of linear distances and this leads to the symmetry of log-fitness distribution. Under synergy, however, the benefit of subtracting a mutation would be smaller than the harm done by adding it because every additional mutation is more damaging. As the individuals with higher than average number of mutations constitute the left tail of log-fitness distribution, this side of distribution would be elongated more than the opposite one. (This argument is introduced more formally by de Visser *et al.*, 1997a.) In tests of both means and skews, antagonism would be inferred if the biases were opposite to those anticipated for synergism.

Material and methods

Parental strains

The 'parental' strains were of two classes. Half of them were normal haploid strains that have never been subject to intentional accumulation of mutations. They were obtained from a single diploid clone with two heterozygous marker loci: *lys ADE/LYS ade*. The derived haploids were either *MATa lys ADE* or *MATα LYS ade*, where *MATα* and *MATa* denote the yeast mating types. In the following description of crosses, these haploids will be designated by an 'F' – meaning free of new mutations. The marker loci were used for identification of clones and screening against contaminants.

To obtain strains containing mutations, single F strains (F_1–F_8) were mated with different mutator strains of yeast (Fig. 1). The latter strains were mismatch repair-deficient and had accumulated mutations during a period

(1) wild type diploid progenitor

(2) meiosis and sporulation

(3) wild type haploids (F)

(4) crosses with mutators

(5) meiosis and sporulation

(6) haploids carrying mutations (M)

Fig. 1 Derivation of 'parents': free of mutations (F) and bearing new mutations (M). The α*msh2* and **a***pms1* are haploid clones deficient in mismatch repair. Other details are described in Materials and methods in the section 'Parental strains'.

of several hundred generations of mitotic growth. The resulting diploids were sporulated and the haploid products of sporulation were screened to have the same genotypes as their parental F strains and to be nonmutators (Korona, 1999b). These strains, whose known genotypes (including nonmutators) were the same as their ancestral F strains, but had the new random mutations, are designated by M_1–M_8. Here, and through the whole paper, we use the term 'genotype' to describe the known genetic markers, although the random mutations also contribute to the genotype. The M strains contained different sets of 'new' mutations because they were acquired independently from different mutator strains.

Crosses

The F and M clones are the 'parents' in the following crosses. All comparisons between parents and progeny involved strains with the same genetic markers. As mating is possible only between opposite mating types, it was necessary to switch the mating type of one parent (Fig. 2). This was accomplished by transformation of the F strains with a plasmid containing a galactose inducible *HO* gene (Herskowitz & Jensen, 1991). These F′ clones, in which only the *MAT* locus had been changed, were mated with M clones of the same known genotype. In total, eight independent diploid clones were obtained:


(1) mating type conversion

(2) cross between parents of opposite mating types

(3) diploid

(4) meiosis and sporulation

(5) selection of **a** progeny haploids

(6) fitness assays of **a** parents and progeny

Fig. 2 Experimental crosses carried out to derive progeny clones (P). Other details are described in Materials and methods in the section 'Crosses'.

five *MATa lys ADE/MATα lys ADE* (numbered here from 1 to 5) and three *MATα LYS ade/MATa LYS ade* (6–8). A single colony of each diploid strain was transferred to sporulation media. Tetrads were selected and the four single spores were separated from each other on a YPD (1% yeast extract, 2% peptone, 2% glucose) plate using a microscope with a micromanipulator. For each of the eight crosses 40 tetrads were dissected. After germination and growth, the spore colonies were tested for mating type. One progeny clone, P, of the same mating type as their M and F (not F′) parents was chosen from each tetrad for further experiments. This could result in overrepresentation of genes linked to the mating type locus. However, the mentioned earlier large number of chromosomes and high frequency of crossing-over ameliorate this problem.

Fitness assay

Maximum growth rate (MGR) in liquid YPD at 30° was used as a measure of fitness. Each clone to be assayed was first grown to stationary phase. This was then used to inoculate a new culture. Growth of the latter was monitored by measuring optical density every 20 min. The data on absorbence were converted using a lognormal transformation, and a regression analysis was performed. The last eight points before reaching a predetermined optical density were considered to be the exponential growth phase and used in the regression. This density was defined as a point after which the growth rate tended to slow down. The measurements were done in blocks in which the F, M and P clones came from the same cross. (Comparison between Fs from different crosses was done in a separate block.)

Statistical analysis

Estimates of fitness should be log-transformed before testing for epistasis (measured as a departure from multiplicity) when they are expressed as an expected number of offspring or related traits (Kondrashov, 1988; Charlesworth, 1990; de Visser & Hoekstra, 1998). Here, the estimates of maximum growth rate were used and they need not be transformed because $m = \log w$, where
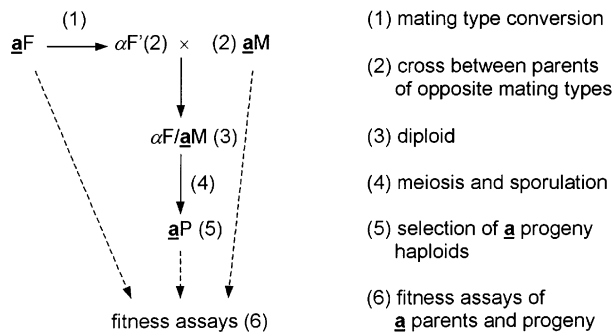
m and w denote the growth rate and expected number of progeny per generation, respectively (Crow & Kimura, 1970).

The MGR of each parent was assayed 20 times. The expected mean MGR of the progeny (P) from a single cross was calculated as the mean of the means of their parents, i.e. the F and M clones from which they were derived. The variance of this estimate was calculated as $(1/n) \Sigma [(f + m)/2 - (\bar{f} + \bar{m})/2]^2 = (1/4n) \Sigma [(f + m) - (\bar{f} + \bar{m})]^2$, where f and m stand for individual MGR measurements of the F and M clone, respectively. The variance of the sum (on the right side of equation) was assumed to be equal to the sum of the variances, each based on the 20 MGR measurements. In comparisons between the expected and observed ($n = 40$, because 40 tetrads were dissected) means of progeny, the Welch's approximate t' was used because both the size of samples and their variances were not equal (Zar, 1999, p.128).

The experiment involved eight planned tests of significance. To obtain an experiment-wide type I error of 0.05, the critical t-values were calculated using the Dunn–Šidak procedure when testing both the skews of progeny distributions and the differences between the expected and the obtained progeny means (Sokal & Rohlf, 1995, p. 239).

Although the sample size was intended to be 20 for the parental clones and 40 for the progeny, small deviations from this pattern resulted owing to the accidental loss of a few clones. The statistical tests performed were based on the actual sample sizes.

Results

The first question asked was whether the parental clones that did not carry new mutations (the F clones derived from a single F/F diploid, Fig. 1) were indeed a neutral 'background' for the introduced new mutations. This condition would not be satisfied if their MGRs (maximum growth rates) were different. The F-test for comparisons between regression lines did not show such differences ($F_{7,52} = 1.959$, $P = 0.079$; Sokal & Rohlf,

1995, p. 495). The estimates of growth rates were accurate because the average standard error of the regression slope was only 1.4% of the slope itself, i.e. $SE_b/b = 0.014$.

The parental and progeny MGRs are compared in Table 1. The left side of the table shows the differences between the parents, e.g. MGR of M_1 minus MGR of F_1 is equal to $0.428 - 0.725 = -0.297$ $[1/h]$. Significant differences were found in five out of eight cases. On the right side of Table 1, an observed average MGR of offspring (e.g. 0.691 $[1/h]$ for P_1) is compared with its expected value, that is, the parental mean $(0.428 + 0.725)/2 = 0.577$ $[1/h]$ for F_1 and M_1. The presence of an interaction among deleterious mutations would be marked by a significant difference between the observed and expected values for the progeny. Out of eight crosses, there were two significant deviations: one positive when parents differed ($F_1 \times M_1$), and one negative when the parents were similar ($F_6 \times M_6$). Note that this result was obtained after correction for multiple comparisons. It means that in one-quarter of the crosses interactions were indeed strong. Progeny from the remaining six crosses did not differ significantly from the mean of the parents. These data should be interpreted as a lack of evidence of interactions rather than corroboration of the model assuming multiplicity. The power of the test in those comparisons where the hypothesis was not rejected was low, 0.55 for the $F_4 \times M_4$ and even lower for other crosses.

The distributions of the offspring's MGR are presented in Fig. 3. The skew was estimated by calculating the third central moment, g_1, and tested for significance with the Student's t-test using the exact formula for standard error (Sokal & Rohlf, 1995, p.138). The statistics are presented on the relevant graphs (Fig. 3). No clear tendency was found; five skews were positive, and three were negative. Only one distribution, P_3, was skewed significantly ($t_{39} = -4.436$, $P < 0.001$), but this skew was apparently produced by only one offspring clone.

Another test was done to examine whether the MGR of the most and the least fit individuals was estimated

Table 1 The results of Student's t-test for difference in MGR (maximum growth rate) between parents and between parents and progeny.

Cross	Difference in MGR between parental F and M clones				Observed – Expected MGR of progeny clones			
	MGR_{M-F}	n_M, n_F	t	P	MGR_{O-E}	n_O, n_E, v†	t	P
$F_1 \times M_1$	**−0.297**	20, 20	−16.453	<0.001**	**0.114**	40, 20, 26.3	11.061	<0.001**
$F_2 \times M_2$	**−0.247**	20, 20	−15.070	<0.001**	−0.005	40, 20, 44.0	−0.353	0.728
$F_3 \times M_3$	−0.016	20, 19	−0.568	0.575	0.010	40, 19, 20.6	0.621	0.549
$F_4 \times M_4$	**−0.140**	20, 20	−8.603	<0.001**	−0.024	40, 20, 52.5	−2.039	0.049
$F_5 \times M_5$	**−0.162**	20, 20	−8.536	<0.001**	−0.012	40, 20, 43.5	−0.708	0.486
$F_6 \times M_6$	0.011	19, 20	0.623	0.537	**−0.049**	40, 19, 20.7	−3.840	<0.001**
$F_7 \times M_7$	−0.056	20, 20	−2.162	0.037	−0.034	40, 20, 52.9	−1.811	0.079
$F_8 \times M_8$	**−0.103**	20, 18	−3.162	0.003*	−0.029	40, 18, 31.7	−1.347	0.185

* and ** show which probabilities remain lower than 0.05 and 0.01, respectively, when corrected for multiple comparisons. †v are the number of degrees of freedom when corrected for unequal sample sizes and variances.
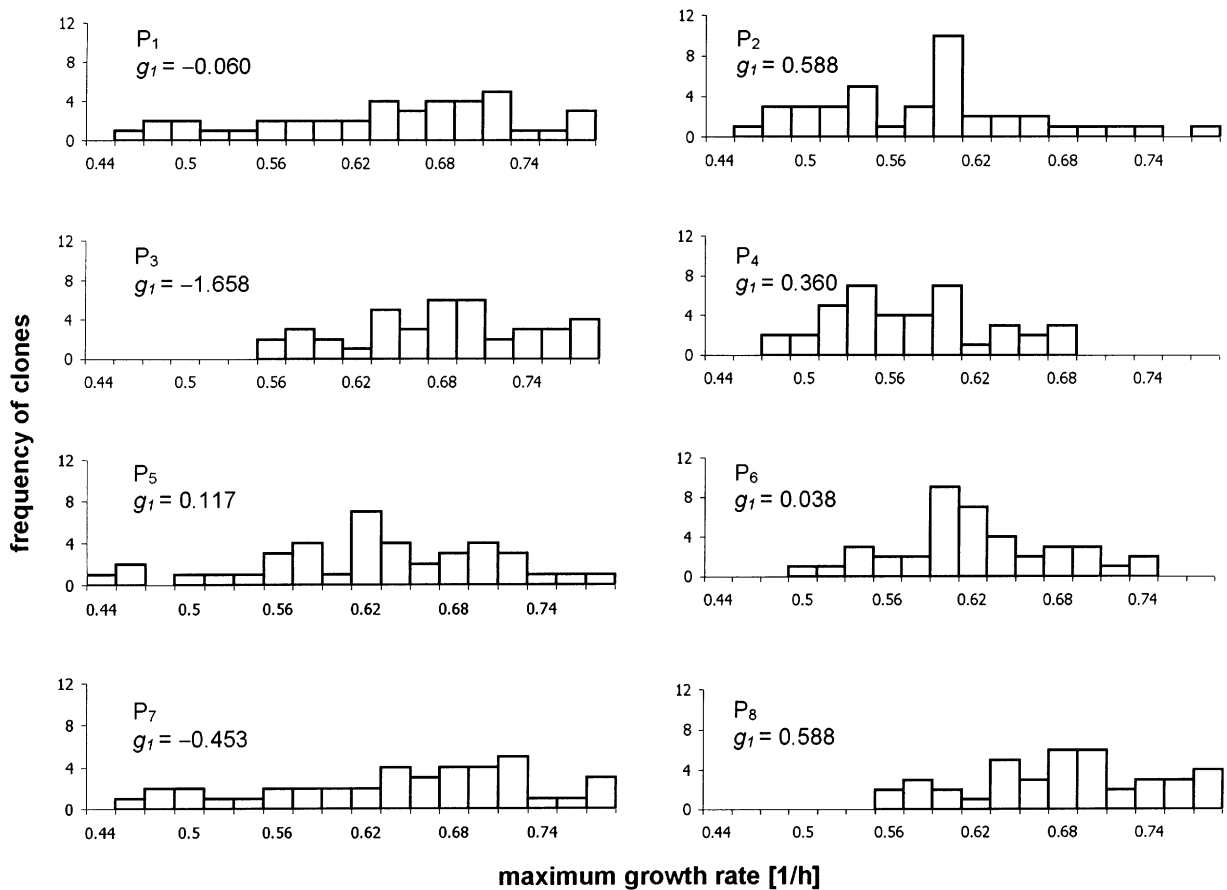
Fig. 3 Frequency distributions of the progeny fitness (maximum growth rate).

with similar accuracy. To assess this, individual MGR estimates were correlated with their standard errors. There was no correlation between the value of growth rate and accuracy of its estimation ($r = -0.087$, $n = 320$, $t = -0.134$, $P = 0.894$) which suggests that the skews of the progeny distributions were not affected by measurement errors.

Discussion

Sexual reproduction disseminates mutations present in parents among their offspring. The relation between the fitness of parents and progeny can then be used to identify epistatic interactions among the mutations. In the present study, in six out of eight crosses the fitness of progeny, estimated as the maximum growth rate, did not differ significantly from the means of parents, which suggests that interactions were weak or absent. Two other crosses showed relatively strong interactions: the P_1 progeny were more fit and the P_6 less fit than expected. Because the parents of P_1 differed in fitness while those of P_6 did not, both crosses pointed toward synergism. The distributions of progeny fitness were generally symmet-

rical and therefore did not indicate any interactions. A general conclusion is that neither antagonism nor synergism was a dominating pattern although there was some evidence for the latter.

When the fitnesses of parents are similar and higher than that of the progeny (e.g. P_6), there is an alternative explanation to synergism that cannot be ruled out. Fitness of the mutated parental strain could have been relatively high not because deleterious mutations were not accumulated but because compensatory mutations arose and hid the negative effects of the former. These specific associations would be lost in crosses, and this could lower the fitness of progeny. Such effects are probably unavoidable in studies involving large numbers of mutations and extensive propagation.

Both the tests of means and skews require that the parents free of deleterious mutations do not contain beneficial mutations interacting among themselves (West *et al.*, 1998). For example, antagonism between beneficial mutations would produce effects similar to those predicted for synergism between deleterious ones. In the present experiment, such parents were haploids that came from a single diploid cell. The only known

heterozygous loci in the diploid were those containing genetic markers (including the mating type). If there were other loci which were heterozygous and affected fitness, the progeny haploids would vary in fitness. This was not found (reported in the first paragraph of the Results) and therefore it can be assumed that the observed interactions in the progeny were associated with alleles introduced by the mutated parents.

The distributions of progeny fitness were generally not skewed, even in the two crosses in which synergistic interactions were suggested by the test of means. West et al. (1998) have predicted in their formal analysis that skews would be difficult to show experimentally. Their results were obtained under specific assumptions: the selection coefficient of deleterious mutations was small and fixed, and the form of synergism was stable and followed a quadratic function of the number of mutations. However, if the synergistic interaction were more threshold-like, then individuals carrying more mutations than some critical value would show much lowered fitness and this would likely produce skew to the left. Therefore, we suggest that not only the means but also skews should be studied whenever possible given the dearth of knowledge about the nature of genetic interactions. Unfortunately, a negative result of the skew test is ambiguous, either interactions are absent or they are present but too weak to detect through the shape of the fitness distribution.

Several problems present in other studies were avoided here. For example, although some of the former studies of interactions between spontaneous mutations involved meiotic segregation of spontaneous mutations (Mukai, 1969; de Visser et al., 1997a), it was impossible to obtain all the four meiotic products in the organisms used (Drosophila and Chlamydomonas), and therefore haploids (gametes) containing many mutations may not have been recovered, biasing the results. In research done with haploid organisms, Aspergillus (de Visser et al., 1997b) and Escherichia (Elena & Lenski, 1997), only few mutations were accumulated and they were obtained by inserting genetic markers which themselves affected fitness. None of these problematic factors was present in this study, but conclusive evidence that synergism is a dominating feature of interactions among deleterious mutations was still not found. These results conform to an overall picture suggesting that the average effect of interactions is not much different from multiplicity. However, there is variation among individual interactions, and there may be some bias towards synergism. Theoretical studies suggest that not only the strength but also the variability of genetic interactions between deleterious mutations can be critical for understanding the evolution of genetic recombination (Barton, 1995; Otto & Feldman, 1997; Otto, 1997), and further research should help to quantify these two parameters.

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