STUDIES OF RECOMBINATION IN YEAST

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I was privileged to work with both of the men whose names are being memorialized in this Symposium. Lewis Stadler guided me to the Ph.D. degree at this University in 1942. E. G. Anderson was responsible for my obtaining a Gosney Fellowship at the California Insitute of Technology to enable me to revive an investigation of B-type chromosomes in maize that had been interrupted by World War II. I cherish memories of their friendship and encouragement.

When I was a graduate student, the subject of recombination was not at the forefront of research in genetics. The breakage-reunion hypothesis was accepted as a reasonable explanation of the mechanism of crossing over. The resurgence of interest in the topic in recent years is indicated by the number of titles dealing with it in this and past Stadler Symposia. This, it seems to me, is the consequence of the availability of techniques for the study of the molecular basis, both enzymologically and structurally, of recombination in bacteria and viruses, and the finding of gene conversion as a recombinational process that could not be explained by breakage and reunion alone (for earlier references see LINDEGREN 1953, ROMAN 1963).

We have been studying genetic recombination in mitotically-dividing diploid cells of the yeast *Saccharomyces cerevisiae*, using chemicals and radiation to enhance the frequency of recombinational events. The advantage of this organism is that single cells can be treated and the recombination products of the treatment can be recovered in the progeny cells (Figure 1). The starting genotype is heterozygous for the recessive tryptophan (*trp5*), leucine (*leu1*), and adenine (*ade6*) markers, and for the co-dominants *SUC1* and *MAL1*, for sucrose and maltose fermentation, respectively. The genes are located on chromosome VII (MORTIMER and HAWTHORNE 1966). Because the cell is also homozygous for the recessive gene *ade2* (chromosome XV), not shown in the diagram, the diploid produces a cell-limited red pigment and the colony derived from the cell is red. If the *ade6* allele becomes homozygous in progeny cells, the production of red pigment is blocked, and a white colony is obtained. Homozygosity for *ade6* can be achieved, as shown in the diagram, by a crossover in the

region between the centromere and the *ade6* locus, or by gene conversion that produces an extra *ade6* allele at the expense of one of the + alleles. As a result of crossing over, and

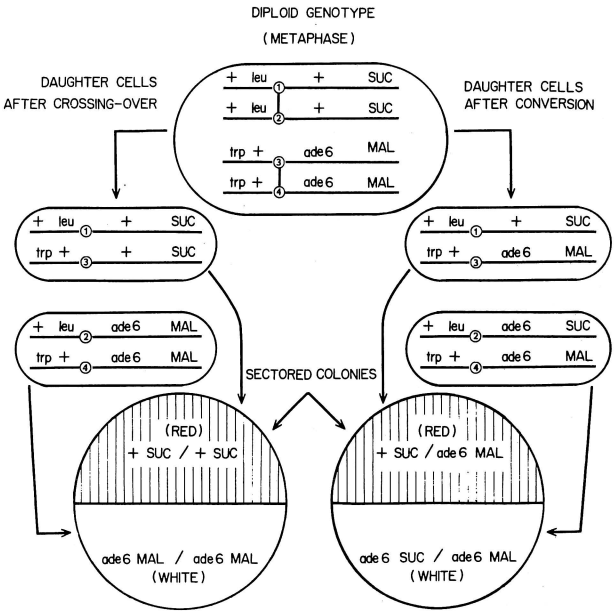


FIGURE 1. This diagram shows how sectored colonies are derived from crossing over (between the centromere and the *ade6* locus) and from gene conversion of + → *ade6*. Sister centromeres in the treated diploid cells are shown yoked by a vertical line.

the mitotic distribution of centromeres (shown as circles in the diagram), the daughter cells become homozygous for the + and *ade6* alleles, respectively. The fermentation markers are in homozygous condition as well. The resulting colony is sectored, theoretically half-red from the *+/+* daughter cell, and half-white from the *ade6/ade6* daughter cell. A similar half-red, half-white sectored colony is obtained following a gene conversion; the diagnostic feature of this event is the heterozygous *+/ade6* genotype of the red side of the sectored colony.

A red-white sectored colony would also be obtained if the centromeres disjoined as in meiosis, that is, the distribution of the sister centromeres 1 and 2 to one daughter cell and of 3 and 4 to the other daughter cell. The role of the leucine and tryptophan markers is to monitor such a distribution since the daughter cells would as a consequence become homozygous for *leu* and *trp* and would exhibit leucine and tryptophan requirements, respectively. Such cells are found only

rarely and are probably not even then due to a meiotic-like disjunction. It is safe therefore to assume that the chromosomes are distributed as we have shown them to be in the diagram.

It should be further pointed out that the color signal we are using does not allow us to detect all of the instances of either crossing over or conversion. Following crossing over in the specified interval, a sectored colony is obtained only if the centromeres are distributed as shown, that is 1 with 3 and 2 with 4. If 1 should segregate with 4 and 2 with 3, each of the daughter cells would receive a + allele and therefore the colony would be wholly red, indistinguishable from the original diploid by phenotype. In the case of gene conversion, the direction of the conversional event must be from + → *ade6* (and not in the reverse direction) in order to be detectable. If the nondetectable events occur as frequently as those that are detectable, the frequency of sectored colonies represents only half the number of crossover and conversional events that have taken place in the treated cells.

The results of treatment with ethylmethane sulfonate, nitrosoguanidine, and ultraviolet light are shown in Table 1. In untreated controls, the frequency of sectored colonies is of the order of 1 in 10,000 cells plated. There is a considerable enhancement of the frequency of sectored colonies with each of these agents even at doses that have little or no effect on survival. In addition to sectored colonies, whole white colonies are obtained. These turn out on genetic analysis to be like the white side of the sectored colonies. We are of the opinion, therefore, that the white colonies are the result of the same events that produce the sectored colonies and that the red side has been lost because of the failure of the progenitor cell to multiply. The reciprocal event, loss of the white side, would of course be undetectable.

The range in sector size is shown in Table 2. The sector emanates from the center of the colony and may comprise from less than 1/8 to more than 7/8 the circumference of the colony. The half-sectored colony is the largest class in the distribution. We have done reconstruction experiments in which white cells and red cells have been placed side by side with a micromanipulator and have observed a similar range and frequency distribution of sector size among colonies obtained from the pairings. We therefore conclude that sector size is variable because of uncontrolled factors in colony formation, such as a lag in the start of growth of one of the two daughter cells, and therefore that in treated cells the segregation of the recombination products takes place mainly in the first division of the treated cell, as shown in Figure 1. There is a tendency toward smaller sectors in colonies induced by the chemical mutagens. This tendency may reflect either the residual effects of the mutagens that are trapped in the cell or a delay in sectoring until the second division of the treated cell.

For the genetic analysis of the sectored colonies, six red and six white isolates are tested for leucine and tryptophan requirements and for sucrose and maltose fermentation.

Table 1. Frequencies of red-white sectoring induced by ultraviolet light (UV), nitrosoguanidine (NG) and ethylmethane sulfonate (EMS) in typical experiments.

Inducing Agent		No. of Colonies	Survival %	Sectored Colonies %	White Colonies %	Total %
UV	ergs/mm^2					
	500	10,133	100	1.49	0.25	1.74
	1000	5,465	54	3.53	1.19	4.72
	1500	1,584	16	4.93	2.84	7.77
EMS[1]	1 hr	5,490	100	1.70	0.06	1.76
	2	5,760	100	2.52	0.28	2.80
	3	5,088	91	2.85	0.43	3.28
NG[2]	1 hr	2,504	100	1.80	0.12	1.92
	2	1,887	71	3.39	0.26	3.65
	3	1,326	54	3.39	0.60	3.99

[1]In reaction mix containing 8.77 ml phosphate buffer (pH 8.0), 0.23 ml EMS, and 1.0 ml washed cells (2 x 10^8).

[2]In reaction mix containing 8.4 ml acetate buffer (pH 5.0), 0.6 ml nitrosoguanidine (10 mg/10 ml acetate buffer) and 1.0 ml washed cells (2 x 10^8).

The red isolates are tested in addition for the presence of the *ade6* allele. Since the genotype *+/ade6* produces sectored colonies in response to ultraviolet light and the *+/+* genotype does not, the colonies can be classified according to whether they are in heterozygous or homozygous condition in the red sector.

Table 3 summarizes the results obtained from the different treatments with reference to the distribution of the adenine alleles. I can be seen that the majority colony type induced by the chemical mutagen is heterozygous for the *ade6*

allele on the red side, and is therefore likely to be of conversional origin (the contribution of mutation to this class will be discussed further below). Ultraviolet light, by contrast, induces a majority class that is homozygous for the

Table 2. Frequencies of sectored colonies of various sizes in the experiments of Table 1.

Treatment	Fraction White in Sectored Colonies		
	$\leq$ 1/4	1/3-2/3	$\geq$ 3/4
UV	20	51	28
EMS	44	35	21
NG	35	40	25

Table 3. Results of tests for genotype of red side of sectored colonies, with reference to the distribution of the adenine alleles.

Treatment	GENOTYPE OF RED SECTOR	
	+/ade6	*+/+*
UV	90 (28%)	228 (72%)
EMS	101 (69%)	46 (31%)
NG	70 (84%)	13 (16%)

+ allele and is therefore likely to be the product of crossing over.

These interpretations receive further support from the relationship of the distribution of the fermentation markers to the distribution of the adenine alleles. As is shown in Figure 1, a crossover in the region between the centromere and the adenine locus would result in a sectored colony that is homozygous for the *+* allele on the red side, and also homozygous for the *SUC* allele on the same side. The white side of the colony would be homozygous for the *MAL* gene.

The data concerning this relationship are given in Table 4. It is evident that those colonies that are +/+ on the red side, and therefore candidates for a crossover origin,

Table 4. Distribution of fermentation markers and adenine alleles in sectored colonies. The ability of cells in the sector to ferment sucrose *(SUC)* or maltose *(MAL)* is indicated by +, inability by -.

Treatment	FERMENTATION MARKERS Red Side *SUC*	*MAL*	White Side *SUC*	*MAL*	ADENINE ALLELES IN RED SIDE* *+/ade6*	*+/+*	TOTAL
UV	+	+	+	+	79 (88%)	48 (21%)	127 (40%)
	+	-	-	+	11 (12%)	180 (79%)	191 (60%)
EMS	+	+	+	+	98 (97%)	4 (9%)	102 (69%)
	+	-	-	+	3 (3%)	42 (91%)	45 (31%)
NG	+	+	+	+	65 (93%)	2 (15%)	67 (81%)
	+	-	-	+	5 (7%)	11 (85%)	16 (19%)

* White side is *ade6/ade6* in both cases.

also show in the main the distribution of the fermentation markers expected from crossing over. A fraction of these colonies, particularly those obtained from ultraviolet light, remain heterozygous for the fermentation markers in both sectors. We interpret these latter to be the result of a second crossover between the adenine locus and the fermentation markers. Meiotic tetrad analysis indicates that the map distance of this region is much larger than the map distance from the centromere to the adenine locus. Therefore, it is not surprising that an effective inducer of mitotic crossing over such as ultraviolet light is capable of inducing multiple exchanges. No such correlation is found among the colonies that are heterozygous for the adenine alleles on the red side. Among these, the fermentation markers remain in heterozygous condition in both sectors in 90% or more of the colonies.

Because the inducing agents we are using, and especially the chemical mutagens, are also inducers of gene mutation, we investigated the mutational contribution to colony sectoring. It should be obvious from Figure 1 that the mutation of the + allele to a new *ade6* allele different from the input *ade6* allele would also yield a sectored colony that would be indistinguishable from the conversional type except by special test. Similarly, a deletion of a part or of all of the + locus should have a similar result.

We have measured the frequency of gene mutation from + to *ade6*, in response to treatment with nitrosoguanidine. A haploid red *ade2* strain was used in these experiments. Red-white and whole white colonies were obtained from the treatments (Table 5). An isolate from the white side of the sectored colonies was tested for the presence of the *ade6* allele.

Table 5. Results of nitrosoguanidine treatment of red (*ade2*) haploid yeast for frequencies of white and red-white colonies and for frequencies of mutation of + → *ade6*.

Treatment	No. of Colonies	Survival %	Sectors %	Whites %	+ → *ade6* %
Control	14971	100	0	0.006	-
NG 1 hr	10723	22	0.21	0.35	-
NG 2 hr	6942	5	0.61	1.24	0.27
NG 3 hr	2330	1	0.82	3.80	0.56

Since it is known that the production of red pigment can be blocked by mutation at a number of different loci that affect the synthesis of adenine (ROMAN 1956), the test consisted of making crosses of the isolate with strains known to carry one or another of the established mutational blocks. An isolate was identified as having an *ade6* mutation if the diploid from the cross with a known *ade6* tester strain failed to produce the red pigment, whereas crosses with all other tester strains did produce the pigment. There are no known instances of complementation between *ade6* mutants and, therefore, the test procedure should detect all of the mutational changes to *ade6* that were identified by white sectoring. Mutations to *ade3*, *ade4*, *ade5*, *ade7*, and *ade8* were also found in the course of these tests, thus confirming the efficacy of the testing procedure.

The mutagenic effect of nitrosoguanidine is recorded in Table 5. If the response of haploid and diploid cells to this agent is substantially the same, it is clear that the frequency of mutation to *ade6* can account for only a small

fraction of the sectored colonies in the diploid (Table 1).

A more direct examination of the contribution of mutation to sectoring and also of deletion of the *ade6* locus, was achieved in a diploid especially designed for the purpose. This diploid had a genotype identical to that described in Figure 1 except that the *ade6* allele was mutant at two sites in the cistron. ELIZABETH W. JONES (1964) has mapped 22 mutations of spontaneous origin in the *ade6* locus by fine-structure recombination analysis. Two of these mutations, *ade6*-21 and *ade6*-45, lie at opposite ends of the cistron. They recombine with each other in meiosis with a high frequency; of 90 asci dissected, 5 asci contained one spore that proved to be doubly-mutant, designated *ade6*-21,45,by genetic test.

Such a double-mutant was crossed with a strain carrying the + allele for the locus. Sectored colonies were induced in the resulting diploid with ultraviolet light and nitrosoguanidine. Cells obtained from the two halves of the sectored colony were then tested as described above and, in addition, were sporulated for a complete analysis of the segregation products. The rationale for the experiment was as follows: If a conversional event has occurred to produce the sectored colony, the red side of the colony should be heterozygous for the + allele and the *ade6*-21,45 allele. This follows from the schematic in Figure 1. The white side of the same colony should have the *ade6*-21,45 allele as well as a converted allele, namely, either *ade6*-21,45 or *ade6*-21 or *ade6*-45, since conversion of one or both sites is sufficient to produce a white sector. If mutation is the reason for sectoring, the red side should be *+/ade*-21,45 and the white side should carry the new mutation, *ade6*-x and *ade6*-21,45. If a crossover has occurred between the centromere and the *ade6* locus, the red side should be *+/+* and the white side *ade6*-21, 45/*ade6*-21,45.

The identification of the *ade6* allele can be accomplished by crosses of each spore segregant with strains carrying *ade6*-21 alone and *ade6*-45 alone. The diploids from these crosses were tested for recombinational reversion to adenine prototrophy (ROMAN and JACOB 1958). If the alleles in the diploid share a common mutant site (i.e., the diploid is homoallelic), recombinational reversion does not occur. If the mutant sites are at different locations in the two cistrons, reversion does occur. If a new allele, *ade6*-x, has been produced, the odds are that the mutant site will be at a different point in the cistron than *ade6*-21 and *ade6*-45 and will recombine with both. Of 22 spontaneous mutations mapped by E. W. Jones, only two were indistinguishable from each other by recombinational mapping. The results of the tests for reversion are shown in Table 6.

To detect deletions of the cistron that could mimic a conversional or mutational event, an additional test was performed. As mentioned before the sites of *ade6*-21 and *ade6*-45 are at opposite ends of the cistron. The mutation *ade6*-1

lies nearly midway in recombinational distance between *ade6*-21 and *ade6*-45. All spore segregants that were white were crossed to a third tester, carrying *ade6*-1. If the spore segregant carries a deletion for the cistron the diploid heterozygous for the deletion and *ade6*-1 should not yield +

Table 6. Tests for the identification of the *ade6* allele in the two sides of sectored colonies induced by nitrosoguanidine. Diploids obtained from the crosses are tested for their ability or inability to produce revertants to adenine prototrophy (+ or -). See text for details.

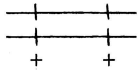
Proposed genotype of segregants	Crossed with tester strains carrying:			
	21	45	21,45	1
ade6-21	-	+	-	+
ade6-45	+	-	-	+
ade6-21,45	-	-	-	+
ade6-x	+	+	+	+
ade6-del	-	-	-	-

recombinants (Table 6). None such was found; all of the diploids that were heterozygous for *ade6*-1 produced + revertants by recombination. Thus there is no evidence in this harvest of segregants from sectored colonies that any of them originated from the deletion of the locus.

The results of the various tests just described are shown in Table 7. The sectored colonies that were subjected to sporulation and tetrad analysis were of two types, those that were sucrose fermenters on the red side and maltose fermenters on the white side, and those that were fermenters for both on both sides. The first type, as discussed above, is the candidate for recombination between the centromere and

the adenine locus. Sixteen sectored colonies obtained from ultraviolet light and fourteen sectored colonies obtained from nitrosoguanidine treatment all turned out to be homozygous for the + allele on the red side and homoallelic for *ade6*-21,45 on the white side, exactly as expected from crossing over in the specified region. Among colonies of the second type, the

Table 7. Gene conversion and mutation in a diploid heterozygous for *ade6*-21,45:



Treatment	Conversion to 21	45	Both	Mutation + → *ade6*-x	Deletions of Locus
UV	2	3	2	0	0
NG	1	4	3	7	0

seven that were UV-induced and the fifteen that were nitrosoguanidine-induced were all heterozygous for *ade6* on the red side. No new mutations were identified in the sample from ultraviolet light. However, in the case of nitrosoguanidine, seven of the fifteen sectored colonies were of mutational origin. It is of interest to note in passing that subsequent recombinational analysis revealed that the seven new mutations were distributed throughout the cistron. The remaining eight events were those expected from conversion either at the *ade6*-21 site, the *ade6*-45 site, or at both sites. The proportion of new mutations to conversional events is higher than was expected from the measurement of mutation in the haploid. Additional experiments are needed to distinguish between sampling error and a higher mutational contribution than was originally suspected.

The finding of gene mutation as a factor in the production of sectored colonies, especially when a chemical mutagen such as nitrosoguanidine is used, does not alter the conclusions reached earlier. Conversions are induced by both nitrosoguanidine and ultraviolet light, as is crossing over. Crossing over is favored by ultraviolet light relative to conversion. The converse is true of nitrosoguanidine and ethylmethane sulfonate. Thus one can experimentally enrich for one type of event versus the other by using the appropriate inducing agent. Conversion is not intimately associated with crossing over in mitosis, as it appears to be in meiosis (see FOGEL and MORTIMER 1971, also FOGEL, HURST and MORTIMER 1971).

Molecular models have been proposed that seek to account for the correlation in meiosis (HOLLIDAY 1964, WHITEHOUSE and HASTINGS 1965); they should be generalized to take into account the mitotic data, in which this correlation is not found.

With reference to molecular explanations, it is essential to know the stage in the cell cycle when the cells are being subjected to the inducing agent. We have generally used cells in the stationary phase of growth, that is, at a cell concentration of approximately 5 x 10^8 per ml in a culture medium containing 2% glucose, 1% yeast extract, and 2% peptone. At this stage, upwards of 80% of the cells are single and the rest have a bud of varying size. The relative proportions depend on the strain that is used. After sonication of the typical culture, less than 5% of the cells still have buds. It has been our assumption in previous papers dealing with this subject that stationary phase cells have replicated their DNA so that no further replication synthesis is needed during sporulation. Measurements by the diphenylamine technique showed that asci have the same DNA content as cells in stationary phase. It turns out, however, that these measurements were probably in error, most likely because of extraction difficulties. Most stationary phase cultures exhibit replication synthesis in sporulation medium, the extent of the synthesis depending on the strain, the time of harvest, and the frequency of asci after sporulation. Although we do not yet know the precise makeup of cells in the stationary phase, with reference to DNA replication, we have been able to separate out an essentially pure suspension of cells that have not replicated their DNA, that is, G1 cells. These are separated following the method of DARLAND (1969): a stationary phase culture is sonicated, washed, and resuspended in 20% Renografin-76 (Squibb), at a concentration of approximately 10^8 cells per ml. Two ml of this suspension is layered on the surface of 10 ml of a 35% solution of Renografin. The step-gradient is centrifuged at moderate speeds (2500 rpm for 15 minutes). Two cell populations can be separated in this way. The population at the interface of the step-gradient consists of the larger, vacuolated cells of the culture. We will refer to this sample as Fraction I. The cells in the pellet at the bottom of the tube (Fraction II) are smaller, non-budded whether sonication has preceded the separation or not, and devoid of vacuoles. Most of the cells that are capable of sporulation are found in Fraction I, as noted previously by Darland. Fraction I also has a higher DNA content per cell than Fraction II. After sporulation of Fraction I, the asci are separated from the vegetative cells that did not sporulate, again by Renografin step-gradient centrifugation. The step-gradient that we used in this separation contained 35% of Renografin layered on a 45% solution, and was centrifuged at 10,000 rpm for 20 minutes. The pellet after centrifugation consists almost entirely of asci; the cells at interface, designated Fraction IA, are nearly free of asci. (It should be mentioned that the concentrations of Renografin that have been given for these separations are not necessarily applicable to other strains and usually must be adjusted

to achieve the same results). DNA measurements by the fluorometric technique (KISSANE and ROBINS 1958) show that the cells of Fraction IA contain half as much DNA as the asci, and the asci in turn have more DNA than the starting Fraction I cells but not twice as much. We conclude from these data that cells in Fraction IA, exclusive of the small contamination of asci, are in G1 and that Fraction I may be a mixture of pre-replicated cells, post-replicated cells, and perhaps cells in various stages of DNA synthesis.

Since our preconceptions had lead us to the view that the chromosomes of stationary phase cells had replicated, as shown in Figure 1, we conducted experiments with Fraction IA cells with certain expectations. First, crossing over must take place at the four-strand stage in order to be detected by our methods. Second, if the molecular hybridization explanation for gene conversion is valid (see review by HOLLIDAY 1968), there is no *a priori* reason that conversion could not occur at the two strand stage (i.e., prior to replication). Therefore, since ultraviolet light induces crossing over in preference to gene conversion, we might expect that treatment of pre-replicated cells with this agent would produce fewer sectored colonies, and these only of conversional origin, than if post-replicated cells were treated, from which crossover products would also be harvested. We recognize a number of parameters that have not been stated that would also affect the expectation, most notably the possibility that the effect of the inducing agent is not expressed immediately but may be delayed until after replication. In point of fact, the expectation was not realized in the experimental results. Treatment of stationary phase cells, Fraction I cells, and Fraction IA cells yielded sectored colonies with essentially similar frequencies for the same levels of survival, and with the same characteristics with reference to the distribution of the fermentation markers and the adenine alleles. Experiments with ethyl methanesulfonate as the inducing agent gave similar results.

We are therefore confronted with two broad possibilities. First, the time of treatment may have nothing to do with the results of treatment, that is, all recombinational events take place after replication whether or not the cells are pre- or post-replication at the time of treatment. The second and more general hypothesis is that the onset of the pairing and recombinational process takes place prior to replication as an interaction between two DNA duplexes and continues until the chromosomes have replicated for segregation to the daughter cells. A number of chromosomal models have been proposed to account for both gene conversion and crossing over, and the relationship between the two. I will leave a further discussion of mechanisms to Rollin Hotchkiss, the next speaker in this symposium. An excellent summary of molecular models and their implications can also be found in a paper by SIGAL and ALBERTS (1972).

The nature of the physical interaction of DNA molecules and the timing of the duration of the recombination process

are questions that are now approachable by direct observation of the molecules with the electron microscope. For this purpose yeast offers unusual advantages. The haploid cell of *Saccharomyces cerevisiae* is estimated to have 17 chromosomes (MORTIMER and HAWTHORNE 1973) and a DNA content of approximately 10^{10} daltons. The average chromosome therefore contains about 6×10^8 daltons of DNA (compared with 1.2×10^8 daltons for bacteriophage T4 and about 25×10^8 daltons for E. coli). Chromosomal DNA from protoplast lysates has been partially separated by sucrose gradient centrifugation (PETES and FANGMAN 1972; BLAMIRE, CRYER, FINKELSTEIN and MARMUR 1972) and visualized with the electron microscope (PETES, BYERS and FANGMAN, personal communication). The molecules are linear duplexes, ranging in size from 50 microns to about 350 microns (T4, for comparison, is 52 microns long). With the availability of procedures for substantially enhancing the frequencies of crossing over and gene conversion in mitotic cells, and the technical progress that has been made in the isolation and visualization of yeast chromsomal DNA, there appear to be no formidable obstacles remaining to a direct test of the models that have been proposed for genetic recombination. The efficacy of this approach has already been demonstrated in T4 by BROKER and LEHMAN (1971).

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