GENE CONVERSION IN UNSELECTED TETRADS FROM MULTIPOINT CROSSES

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In his last and posthumous contribution L. J. STADLER (1) brilliantly summarized the crucial issues involved in the experimental analysis of the gene. Throughout this memorable classic a recurrent theme emerges to the effect that our understanding of the genetic substance will be advanced meaningfully only to the extent that the distinction between operationalism and conceptualization is preserved rather than blurred. It is within this tradition of operationalism that the present summary or progress report on gene conversion in yeast is undertaken.

The Stadlerian doctrine of separation between fact and fancy is particularly relevant in the study of intragenic recombination or gene conversion, since presently, no fewer than five distinct hypotheses await testing against a comparative paucity of experimental data. To paraphrase L. J. STADLER, it is unfortunately true that the study of gene conversion is, at best, laborious. Accordingly, our presentation will be developed parallel to lines suggested by experimental evidence along with an evaluation of the various methodologies that were employed to obtain them. Only scant attention will be given to evaluating the distinctive merits inhering in the various theoretical models. Of course, this does not imply that theoretical considerations are not without importance, or that the contributions of the theoretician are any the less significant than those of the experimentalist. Surely, each provides an equivalent, albeit different measure of zest to the quest for understanding. However, at present, it would appear that fact might appropriately precede fancy in the interest of narrowing the chasm between theory and evidence.

WHAT IS GENE CONVERSION?

We may begin by asking, what is gene conversion? Since the phenomenon was first discovered in organisms characterized by life cycles in which analyzable meiotic tetrads are available, let us briefly review the life cycle of our experimental organism, a comparatively simple eucaryote, Saccharomyces cerevisiae. Yeast serves as a convenient model organism since it bridges the gap between viruses or bacteria and higher differentiated plants or animals. We shall confine our attention to the simple heterothallic cycle in Figure 1.

Yeast is typically haploid and haploid cells may exist in either of two mating types determined by a single gene difference. The haploid cells grow by budding which presumably reflects a typical

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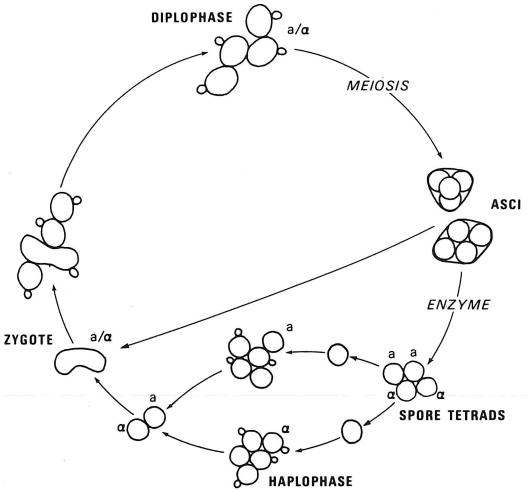
mitotic cycle since genetic continuity is maintained in marked strains even after repeated transfer. When cells of the opposite mating types are brought together, characteristic zygotes are generated in a matter of 3-4 hours. The cellular fusion is soon followed by a nuclear fusion. Buds produced from the zygote are stable diploid cells and these in turn may reproduce indefinitely by subsequent mitotic budding cycles.



Life cycles of heterothallic *Saccharomyces cerevisiae*

FIGURE 1

Unlike meiosis in higher plants and animals whose reproductive cycles are regulated by complex control mechanisms, meiosis may be induced at will in yeast. Thus, when richly nourished diploid cells are transferred to aerated 2% potassium acetate medium, meiosis is initiated within a few hours and a fully differentiated structure, an ascus containing the four products of a single meiosis, is formed within 48-72 hours. The four ascospores in the yeast tetrad are functionally homologous to the quartets of megaspores or microspores in higher plants, to the four spermatids derived from a single spermatocyte, and to an animal egg with its polar bodies. But in higher plants and animals cellular lineages are soon obscured in male germ lines during spermatogenesis or microsporogenesis, while in female germ lines only one of the four meiotic products remains viable. This situation does not prevail in yeast or many other fungi. Instead, the four ascospores resulting from meiosis in a single cell persist as a well-defined biological unit.

With the aid of appropriate enzymes (2) to digest the ascal wall and simple micromanipulation, the four spores of a yeast ascus may be individually isolated, cloned and analyzed by replica-plating or further crosses. In a sense then, formal yeast genetics is concerned mainly with the direct analysis of meiotic tetrads rather than the characterization of zygotes generated by random gametic unions. Thus, for a single heterozygous site, the fungal geneticist finds two wild type spores and two spores of mutant phenotype in each ascus. Clearly, this fundamental result is consistent with our concept of particulate inheritance. At the same time, it is an operational restatement of the primary Mendelian principle of segregation. Gene conversion represents an exception or departure from the expected intra-ascal 2+:2a segregation. Typically, gene conversions are

detected as 1+:3a or 3+:1a segregations in otherwise normal tetrads in contrast to expected 2+:2a segregation. The symbols + and a represent wild type and mutant alleles, respectively.

Irregular segregations of the 1+:3a and 3+:1a types as well as 0+:4a and 4+:0a types were reported almost forty-five years ago in fungi and mosses; e.g., BRUNSWICK (3), KNIEP (4), VON WETTSTEIN (5). They were later reported in yeast by LINDEGREN (6), MUNDKUR (7), WINGE and ROBERTS (8) and ROMAN (9). These aberrant segregations were explained as "allele-induced mutations" by LINDEGREN who adopted WINKLER's (10) term of gene conversion. WINKLER proposed that gene conversion accounted not only for irregular segregation, but he also suggested that gene conversion provided an alternative to the chiasmatype theory to account for recombination of linked genes as well. In the intervening years, it became abundantly clear that a wide variety of conventional genetic mechanisms could yield an operational result superficially indistinguishable from gene conversion (Table 1). In the present report we are concerned only with that residuum of irregular segregations not ascribable to such mechanisms.

Table 1

CAUSES OF IRREGULAR SEGREGATION

A. Chromosomal	
Polyploidy:	most genes, 4:0, 3:1 and 2:2
Polysomy:	genes on one chromosome, 4:0, 3:1 and 2:2
Meiotic nondisjunction:	genes on one chromosome, occasional 4:0, 3:1 and 2:2
Mitotic crossing over before meiosis:	genes distal to crossover, 4:0 or 0:4
B. Multiple gene control	
Polymeric:	single traits, 4:0, 3:1 and 2:2
Complementary:	single traits, 2:2, 1:3 and 0:4
Suppressors:	suppressible traits, 4:0, 3:1 and 2:2
C. False tetrad	
Four spores from more than one meiosis:	most genes, irregular segregation
Binucleate spores:	most genes, irregular segregation
D. Gene conversion	any gene, occasional 3:1 and 1:3

When and how frequently in the cell cycle does gene conversion occur? From ROMAN's (11) illuminating pioneer efforts it became apparent that conversion or non-reciprocal recombination could occur in both the mitotic and meiotic phases of the life cycle. However, he found that meiotic frequencies were at least 3 or 4 orders of magnitude higher than mitotic frequencies. Moreover, recent evidence by WILDENBERG (12) and FOGEL and HURST (13) strongly suggests that gene conversion is independent of major DNA synthesis since it may occur both prior or subsequent to the major round of DNA replication in mitosis or meiosis.

PARITY

Some 200-300 marker genes have been identified in yeast (14, 15). These include genes affecting a) biosynthetic pathways of amino

acids, lipids, purines and pyrimides; b) transfer RNA's; c) resistance or sensitivity to temperature, U.V., X-rays, various metabolic poisons and antagonists; d) fermentation or utilization of various sugars; e) mitochondrial integrity and cytochromes. From this abundance of marker diversity, it is comparatively simple to synthesize hybrids that contain ten to twenty heterozygous sites. Moreover, each segregation is unambiguously scorable in every tetrad by means of replica-plating or subsequent diagnostic crosses. Phenotypic overlaps or ill-defined phenotypic reactions constitute rare exceptions amounting to about 0.1% of all segregations or less. Moreover, these are easily resolved by further physiological or genetic tests.

Table 2 displays a partial summary of our experience with about 21,000 meiotic segregations. Several significant generalizations may

Table 2. Number of irregular segregations at miscellaneous loci.

Locus	3:1	2:2	1:3	Percent conversion
a/α	2	2225	6	0.4
ade2-1	0	748	4	0.5
arg4-19	1	184	1	1.1
arg4-4	5	686	6	1.6
arg4-1	22	602	16	5.9
arg4-2	54	1563	56	6.6
arg4-17	53	1460	60	7.2
arg4-16	12	180	6	9.0
leu1-1	10	2038	15	1.2
leu1-12	1	1239	1	0.2
lys1-1	27	1170	22	4.0
met1-1	8	1490	7	1.0
pet1-1	2	1755	2	0.2
thr1-1	56	1935	54	5.4
trp1-1	3	1355	2	0.4
trp5-48	3	536	5	1.5
ura1-1	15	806	15	3.6
ura3-1	4	1060	3	0.7
	278	21032	281	2.59

Data from Roman (1963), Fogel and Mortimer (1969), Mortimer (unpublished).

be drawn from these data. First, for miscellaneous markers, gene conversion occurs with an average frequency of about 1.5%. The frequencies may vary from about 0.5% for the mating type alleles to about 20% for a super-suppressor, SUP6 (16). Also, the different alleles of a cistron, as in arg4 (argininosuccinase) may differ in their conversion frequencies by nearly an order of magnitude. It would therefore appear that the frequency of gene conversion is independent of the properties of the locus (11, 17). While different alleles, for example at the arg4 locus, show widely different conversion frequencies, these differences can be correlated only with the position of the alleles in the locus and not with any other properties of the alleles. For example, alleles arg4-4, arg4-2 and arg4-17 are nonsense mutations of the ochre variety, while alleles arg4-19, arg4-1 and arg4-16 are missense mutations. Among the nonsense

mutation alleles arg4-4 and 4-17 are readily distinguishable from each other by a fourfold difference in conversion frequency. The allele arg4-17, characterized by the higher meiotic conversion frequency is particularly susceptible to reversion by U.V. However, it fails to complement with allele arg4-3 as does arg4-4. Allele arg4-2 displays a conversion frequency not s nsibly different from arg4-17 or the missense mutation arg4-16. Mutant arg4-19 complements vigorously with arg4-16 but it has a conversion frequency intermediate to that of alleles arg4-4 and arg4-1. No correlation can be found between the conversion frequency and any of the specific properties intrinsic to the different alleles. Finally, from the totals, as well as in those subsamples of adequate size, a parity principle is evidently operative: the likelihoods of conversion in either direction are equally probable.

While parity seems to be the general rule in Saccharomyces cerevisiae, a significant number of investigations based on other fungi report instances of disparity; i.e., when 3+:1a segregations significantly exceed 1+:3a or vice versa. Without exception, these data were obtained from systems in which the potentially variable developmental attributes, spore color or colony color, were scored (18, 19, 20, 21, 22, 23, 24). The scoring procedures utilized in these studies implicitly assume that a complete correlation exists between phenotype and genotype. An example of the scoring reliability in the cases involving spore color mutants is as follows: GAJEWSKI et al. (25) found that 30% of their 5+:3a and 3+:5a asci were in fact normal 4+:4a segregations upon subsequent genetic testing. Additionally, it is assumed that rare events of all types are detected with equal facility and reliability. Can these assumptions be justified experimentally? Clearly, what is required but lacking in these studies are wholly objective tests of genotypic ascertainment in every spore of every tetrad. Also, at best, only a fraction of the aberrant tetrads were verified. In addition, there are the unfulfilled requirements of internal controls or safeguards that monitor against the possibilities of subtle interascal selection resulting from differential survival or development among ascogenous cells. Moreover, the conventional mechanisms (Table 1) known to generate apparent, although spurious, disparity must be routinely excluded. In the absence of such systematic, rigorous testing, the mere observation of disparity cannot be considered as unquestioned prima facie evidence for its existence or validity. Yet, it would be unreasonable to suppose that bona fide instances of disparity do not exist. However, a convincing demonstration that constitutes an exception to the rule of parity has not been established with the customary rigor encountered in gene mutation studies.

This situation stands in contrast to our experience in yeast where scoring unselected tetrads for mating type or stringent nutritional requirements on a chemically defined medium is much more reliable. Under these circumstances, no violations of the parity rule have been detected. The limited data from unselected tetrads in Neurospora (26, 27) provide a partial confirmation of the parity rule.

FIDELITY

Since conversion is most simply detected as 3+:1a or 1+:3a segregations in otherwise normal tetrads, it might be assumed that gene conversion is a process akin to spontaneous mutation and therefore represents a rich source of allelic diversity. What can be said of the identity or non-identity between parental and converted alleles? ROMAN (9) attempted to resolve this question by employing a recombinational test of rather high resolving power. When he crossed each of the three mutant spores derived from a 1+:3a convertant ascus with cells containing the parental allele, he found that

the mitotic reversion frequencies of the three resultant diploids were not sensibly different from each other or from the characteristic spontaneous haploid or homoallelic reversion frequency. If conversion generated new alleles the combination of parental and derived alleles would usually be expected to exhibit significantly higher mitotic reversion rates than the corresponding homoallelic combinations. Compared to their parental forms, alleles emerging from conversion show no alterations in their recombinational behavior with other known alleles. Nor are they altered in their complementation responses, U.V. or X-ray induced reversion rates, osmoremediability or temperature sensitivity. Similar results were obtained by ZIMMERMANN (29) who examined convertants at the gene product level. Thus, it was reasonable to suppose that the mutant allele derived by conversion was indistinguishable from the input or parental mutant allele. A similar conclusion was reached by CASE and GILES in their studies on unselected tetrads in Neurospora (29).

Another critically sensitive genetic test of conversional fidelity is possible (30). Certain mutants in yeast have been identified as protein chain-terminating mutants (31) in which the original DNA alteration resulted in a nonsense mutation of the ochre, amber or umber type (32). Respectively, these correspond to the anticodons UAA, UAG and UGA. Such mutants are readily distinguished one from another in terms of their suppressibility by external suppressors or structural genes which probably code for an altered tRNA (33, 34, 35). FOGEL and MORTIMER (36) have demonstrated that when conversion occurs in crosses heterozygous for amber or ochre mutants, the three mutant spores in 1+:3a convertant asci display equivalent responses to different classes of suppressors. This test is capable of detecting single base changes in the mutant codon. Thus, if the converted allele differed from the parental mutant in one or more base pairs of the mutant codon, the converted allele should have changed to a missense mutation, or to a mutant with an altered response pattern to specific suppressors. In more than 60 independent cases analyzed, no alterations were found. This finding leads to the view that gene conversion is an essentially conservative process that neither creates nor destroys genetic information. Rather, it may be inferred that gene conversion operates with complete fidelity and involves replacement of the genetic information in the relevant DNA segment with information that is identical to that carried in the corresponding segment of the homologous non-sister chromatid.

It may be noted in passing that the hybrid DNA repair model proposed by HOLLIDAY (37, 38) suggests the possibility of generating new alleles via gene conversion which is not consonant with the data presented above. Admittedly, this feature is not central to HOLLIDAY's model along with those of WHITEHOUSE (39), EMERSON (40) and STADLER (41) which attribute considerable significance to the role and functions of the mismatched base pairs. On the other hand, mismatched base pairs are not integral to STAHL's (42) model. In the section that follows, we shall see that little or no significance can be attached to the specific mispairings. What then are the regularities characterizing the informational transfer process?

INFORMATIONAL TRANSFER OR CO-CONVERSION

Gene conversion or interallelic recombination is also analyzable in tetrads derived from diploids that carry in repulsion two or more independent mutants at a single locus, $\frac{a1\ +}{+\ a2}$. Ideally, the heteroallelic site is flanked by closely linked markers. In addition, the genetic system should contain sufficient and adequate monitoring devices that would signal the occurrence of events that lead to a spurious diagnosis or misclassification. Such perturbations, among

others, include polyploidy, non-disjunction, preferential segregation, gross chromosomal losses or rearrangements, mitotic recombination, polymeric or complementary gene interactions, interascal selection, dominant suppressors (see Table 1). Of course, every spore of every tetrad must be tested for a complete, objective ascertainment of its genotype (17, 43). We regard the above as a set of minimal requirements essential to any study of intragenic recombination, for it is within the matrix of these criteria that our, or any other, data must be evaluated.

Normal segregations from heteroallelic diploids of the type $\frac{a1\ +}{+\ a2}$ yield two a1 + spores and two + a2 spores in each tetrad. Yet, prototrophic or revertant spores (++) occur among the meiotic products of such heteroallelic diploids. For the most part, these wild type recombinants arise as gene conversions, or 3+:1a segregations for either parental allele and to a much lesser extent from reciprocal recombination between the input alleles (61). Prototrophs, it is generally assumed, represent the consequence of some recombinational event, and their meiotic frequency among random spores has been widely utilized as a measure of the genetic distance between the mutant sites. On the basis of a metric that is essentially an index of non-reciprocal recombination, reasonably consistent genetic fine-structure maps have been elaborated. Clearly, this is a paradoxical situation. The paradox is all the more apparent when we consider that rather stringent selective procedures are typically employed in these studies, although they cannot and do not detect all intragenic events. Therefore, in an attempt to resolve the paradox we (17) conducted a comprehensive survey of all intragenic events in a random population of tetrads not selected on the basis of events occurring in the locus under consideration or on any other known basis. Similar studies were undertaken earlier by MITCHELL (44), ROMAN (9), STADLER (26), CASE and GILES (27, 29). However, in each of these studies the limited sample of convertant tetrads detected did not permit the development of generalizations. Our previous work involving hybrids heterozygous for various allelic mutant pairs has now been extended to hybrids heterozygous for three and even four mutant sites within a cistron (43).

At the focus of our studies is the arg4 cistron, the structural gene for argininosuccinase. It was chosen for intensive analysis because MORTIMER's unpublished preliminary studies indicated that some arg4 alleles were characterized by a meiotic gene conversion frequency greater than 5%. He also provided a tentative fine-structure map and assembled a collection of some 57 independently derived mutants. These included missense, nonsense, complementing, osmoremedial and temperature sensitive mutations. In addition, from the behavior of certain complementing nonsense mutants, the direction of translation was also known. Beyond these desirable attributes, closely linked outside markers had been mapped (45). The fine-structure map and the outside markers are shown in Figure 2. The mutant sequence in Figure 2 was established by two, and in some instances, three independent methods.

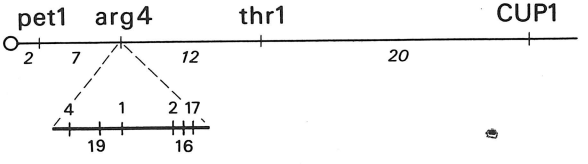


Figure 2. Linkage map of chromosome 8 and fine structure map of arg4.

Using the mitotic X-ray mapping technique developed by MANNEY and MORTIMER (46) we found the sequence and distances between mutants consistent with their earlier unpublished and published fine-structure maps. The ordering of various alleles relative to the centromere and flanking markers was also inferred from asci in which a conventional reciprocal exchange had occurred between the mutant sites. In some cases, tetrad analysis of mitotic revertants from three and four point crosses provided additional confirmation on the relative spatial separation between mutants and their order.

With the X-ray mapping technique, the slopes of the linear dose-response curves are calculated in X-ray map units. An X-ray map unit, defined as 1 prototroph/10^8 survivors/roentgen, is equivalent to 43 amino acids or 129 nucleotides (47). Furthermore, it is reasonable to suppose that this unit is constant across the yeast genome, since molecular weight estimates based on biochemical data and X-ray data are in full agreement for three distinct proteins, viz. cytochrome c (47), tryptophane synthetase (31) and ATP-PRPP pyrophosphorylase (48). Figure 2 displays the linkage relations of arg4 and the tentative fine-structure map of the various mutants employed. Some uncertainty attaches to our estimates of the nucleotide distance between mutants. This will be clarified by a detailed X-ray mapping study (in progress) involving two point crosses of various arg4 alleles.

Our experience involving some 1600 asci from two point crosses will be summarized now (17).

TWO POINT CROSSES

Figure 3 shows the various ascal types that are encountered among unselected tetrads of heteroallelic diploids. These include

MAIN TYPES OF ASCI RECOVERABLE FROM HETEROALLELIC DIPLOIDS

(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)
a_1 +	a_1 +	a_1 +	a_1 +	a_1 +	a_1 +	a_1 +	a_1 +
a_1 +	+ +	a_1 +	a_1 +	a_1 a_2	+ a_2	a_1 +	a_1 a_2
+ a_2	+ a_2	a_1 a_2	+ +	+ a_2	+ a_2	a_1 +	+ +
+ a_2	+ a_2	+ a_2	+ a_2	+ a_2	+ a_2	+ a_2	+ a_2
Normal	Conversion of +/a_1		Conversion of +/a_2		Symmetrical conversion of +/a_1 and +/a_2		Reciprocal recombination

FIGURE 3

single site conversions, symmetrical double site conversions and reciprocal recombinants. Single site conversions are characterized by a 3+:1a or 1+:3a segregation for either the proximal or distal allele accompanied by a normal 2:2 segregation of the adjacent allele. Reciprocal recombinants display all the features expected to ensue from conventional reciprocal exchange between the mutant sites. Double site conversions, almost without exception, fall into two subsets that involve a 3+:1a1 segregation of one allele accompanied by a symmetrical 1+:3a2 segregation of the second allele. It is most unlikely that these double site conversions represent independent although simultaneous events, since conversion of one allele is characteristically associated with conversion of the second allele on the same strand. A similar interpretation was reported by CASE and GILES (29) in their earlier study. If double site symmetrical conversions (Figure 4), aptly termed co-conversions by DAVID STADLER,

were merely consequences of coincident events, six additional convertant ascal types are predicted. Of the six types (Figure 4),

ASCAL TYPES EXPECTED IF HETEROALLELIC SITES CONVERT INDEPENDENTLY

(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)
++	$a_1 a_2$	++	$a_1 a_2$	++	++	$+a_2$	$+a_2$
++	$a_1 a_2$	++	$a_1 a_2$	$+a_2$	a_1+	a_1+	a_1+
++	$a_1 a_2$	a_1+	a_1+	$+a_2$	a_1+	a_1+	$+a_2$
$a_1 a_2$	++	$+a_2$	$+a_2$	$a_1 a_2$	$a_1 a_2$	a_1+	$+a_2$

FIGURE 4

five should contain one or more prototrophic spores, and of these four should contain either one or three double mutant spores. The last category contains two double mutant spores along with two spores representing each of the input parental alleles. On the assumption that double site conversions represent coincident events we would expect all categories to occur with equal frequencies as a first approximation. This did not occur. Thus, we are led to the conclusion that co-conversion represents a single primary event. Within a total sample of more than 6500 unselected tetrads, in which every spore was crossed to each allele present in the cross, such asci, with apparent coincident conversion events, were conspicuously rare. In fact, their frequencies are below the predicted values obtained by multiplying the appropriate separate probabilities. The analogy to chiasma interference is apparent. Hence, we may infer that conversion involves a sizeable segment of DNA rather than a narrowly restricted point.

When mutants are at opposite ends of the cistron they behave in conversion as though they were essentially independent entities (Table 3). Thus, for the widely separated allele pair arg4-4/arg4-17,

Table 3. Frequency and types of gene conversion in two point crosses

Diagnosis	Diploid: / Locus: / Allele pair a_1/a_2: / Nucleotide distance:	BZ34 arg_4 4/17 1060	X841 X901* arg_4 1/2 520	BZ140 arg_4 2/17 128
Single-site conversions				
Proximal allele 3+:1a	a_1+, $+a_2$, $+a_2$, + +	3	3	1
Proximal allele 1+:3a	a_1+, a_1+, $+a_2$, a_1a_2	5	3	3
Distal allele 3+:1a	a_1+, a_2+, $+a_2$, + +	18	10	3
Distal allele 1+:3a	a_1+, $+a_2$, $+a_2$, a_1a_2	20	11	2
Double-site conversions				
Prox. 3+:1a, distal 1+:3a	a_1+, $+a_2$, $+a_2$, $+a_2$	2	13	14
Prox. 1+:3a, distal 3+:1a	a_1+, a_1+, a_1+, $+a_2$	1	10	13
Reciprocal recombinants	+ +, a_1a_2, a_1+, $+a_2$	9	5	0
Exceptional tetrads		1	1	0
Total aberrant segregations		59	56	36
Total tetrads analyzed		697	502	544

*Pooled data from two closely related hybrids.
From Fogel and Mortimer 1969 (17).

46 of 49 conversional asci among 697 tetrads were single site events. This stands in contrast to the situation involving alleles in close proximity, as in the allele pair arg4-2/arg4-17 where of 36 total conversions 27 were symmetrical co-convertants in a total sample of 544 tetrads. In the intermediate situation, with alleles arg4-1/arg4-2, single site and co-conversional events were almost equally frequent.

What conclusions can be adduced from these findings? Clearly, the most significant clue is provided by the co-conversion category. The magnitude of this component is patently an inverse function of the physical distance between the mutant alleles. Thus, we may say that when alleles are widely separated they behave as essentially independent units in conversion. However, as the proximity between mutant sites is increased, the probability of co-conversion rises in a predictable fashion at the expense of single site events. The frequency of co-conversion as a function of the second allele's distance from arg4-17 is shown in Figure 5.

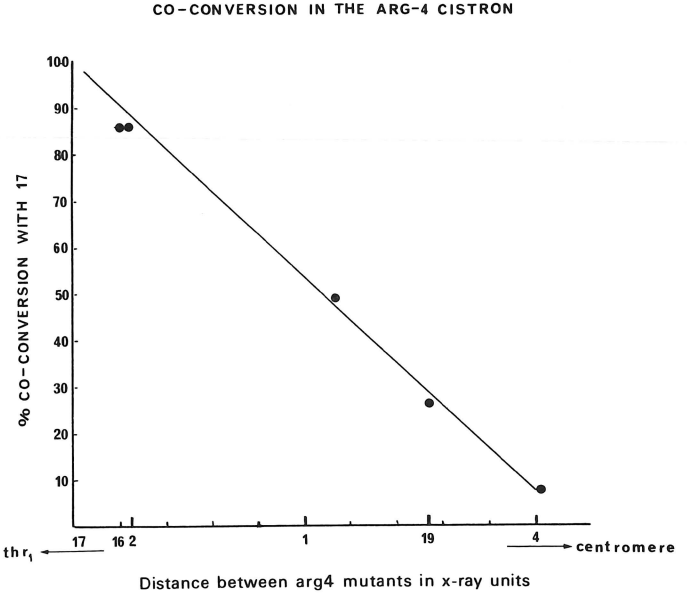


FIGURE 5

Co-conversion is most simply explained as the consequence of a single event involving the replacement of a segment of information in one homologue with information identical to that contained in the corresponding section of a non-sister homologue. The segment has a variable length, but modally it is of the order of several hundreds of nucleotides. Moreover, the informational transfer occurs with complete fidelity.

In connection with the paradox relating to fine-structure mapping via estimates of prototrophic spores, it is important to note that symmetrical double site conversions do not yield wild type or revertant spores. Since the relative frequency of such double site conversions rises at the expense of single site conversions with increasing proximity of alleles, consistent fine-structure maps can be expected even though the recombinational events are non-reciprocal.

Similarly, the double site symmetrical conversions taken alone provide a partial explanation for the genetical effect known as map expansion. Map expansion and polarity of distribution for outside markers among allelic recombinants are typically based either on data derived from random spores assayed for their prototroph frequency or on the frequency of asci found to contain prototrophs. Mutants in close proximity to each other will typically experience co-conversion and prototrophs will be generated only rarely as a consequence of conversion at either single site. Hence, with prototrophic spore frequency as the metric of "distance" between the sites, we assign a value to the interval that is too low by an amount equal to the co-conversion factor. For longer intervals this "error" is relatively less than that for shorter intervals. Upon summing the length of such short intervals we should consistently find that the sum is less than the observed recombination frequency for the two termini (via prototrophic spores frequency). Thus, the resultant map expansion is only apparent for, in part, it is a manifestation of co-conversion.

The significance of co-conversions must also be considered in evaluating the distributions of outside marker arrays among prototrophic convertants obtained by any selective procedure. Again, only limited assertions concerning the polarity relations in conversions are warranted by data of this sort, since they reflect, at best, only 50% of the single site conversions and the rarer reciprocal exchanges between alleles. But double or multiple site events do not yield prototrophs. Consequently, although they may represent the bulk of all intragenic events, they are nonetheless excluded from consideration by the selective procedures typically employed in genetic fine-structure analysis. Unquestionably, selective tactics can yield useful information; however, they do not generate an unambiguous or undistorted reflection of the underlying events in intragenic recombination (49).

Perhaps, the only justifiable assertion that can be drawn from the analysis of prototroph frequencies among random spores is that the allele associated with the most frequent parental strand converts with the highest frequency. However, no estimates of relative conversion frequency are possible because the relative frequencies of double site conversions cannot be determined among random prototrophic spores. In fact, it may be pointed out that co-conversion actually accentuates the polarity as a result of the differences in conversion. For example, in Table 3 we observe that allele arg4-1 was involved in six single site events and 23 double site events, while allele arg4-2 participated in 21 single site and 23 double site events. Thus, we may contrast the ratios of 29:44 vs. 6:21 and find that co-conversion produces more than a twofold magnification of the real differences.

FOUR AND THREE POINT INTERALLELIC CROSSES

Preliminary studies indicated that we could objectively score each spore in unselected tetrads with an ease and reliability equivalent to that obtained in simple heteroallelic crosses. Thus, for three and four point crosses of the type

$$\frac{+ \quad a_2 \quad +}{a_1 \quad + \quad a_3} \text{ and } \frac{a_1 \quad a_2 \quad + \quad +}{+ \quad + \quad a_3 \quad a_4}$$

respectively, each spore of every ascus is routinely crossed to each of the three or four alleles involved. For every tetrad 12 or 16 independent scorings are required for complete genotypic ascertainment at the arg4 locus alone. Typically, recombinational or complementation reactions are involved, although additional tests for X-ray

or U.V. induced revertibility, or suppressibility tests are also utilized. Temperature sensitivity and osmoremediability provide additional criteria. At least one additional test is required to score each of the other linked and unlinked markers. Taken collectively, these assays constitute an objective, reliable and internally consistent genotypic diagnosis for each spore of every tetrad. What does analysis of such complex systems reveal?

In the four point cross, the distance between alleles 1 and 17 includes the distal half of the arg4 cistron. Alleles 2, 16, 17 represent a cluster spanning a distance of some hundreds of nucleotides (Figure 6). Evidently, the most common single event is a

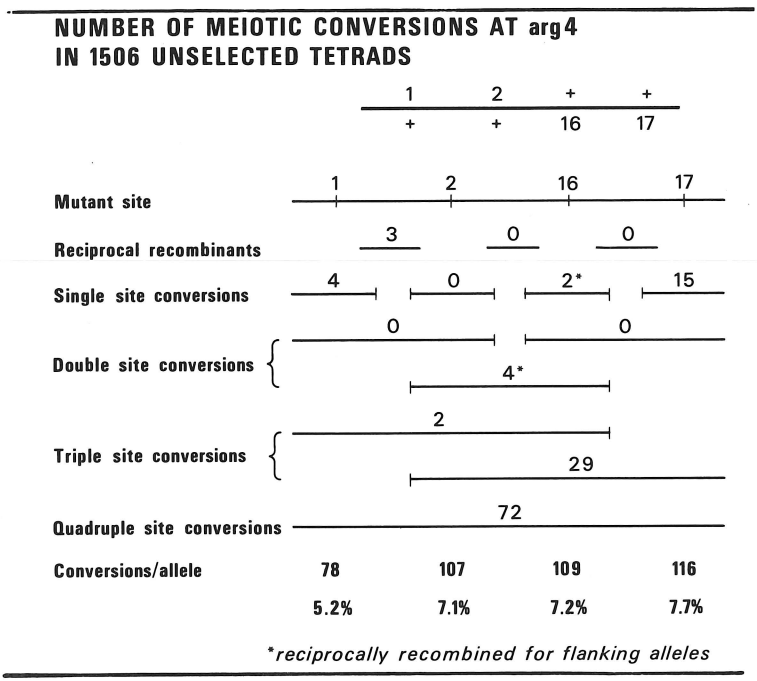


FIGURE 6

symmetrical co-conversion embracing all four alleles in the marked segment. These represent more than half the intragenic events in the sample of 1506 unselected tetrads. The next most frequent single event is represented by the triple site co-conversion category involving the clustered alleles 2, 16 and 17. These occur about half as often as the quadruples. Taken together, the triple and quadruple co-conversions account for 101 out of 131 total intragenic events. Clearly, the informational segment transferred during gene conversion is a sizeable one amounting to several hundreds of nucleotides. The limits of the converted segment are indicated by short vertical bars.

Special attention is drawn to the items marked with an asterisk. These represent two single site conversions of arg4-16 and four co-conversions of the adjacent alleles 2 and 16. In all six instances, the conversional events were accompanied by a reciprocal recombination for the flanking marker alleles within the arg4 cistron. Considered in another way, the data suggest that a

region of non-reciprocity exists between reciprocally recombined markers. Thus, we may entertain the notion that if recombination is a consequence of some breakage-reunion event, the underlying process must be deemed to be essentially non-reciprocal in the sense that the breaks in homologues may occur at different nearby points and result in the production of an overlap region or hybrid DNA segment.

The three point cross may be considered now. The total marked interval is somewhat longer than the spanned region in the previous four point cross and it represents almost two-thirds of the arg4 cistron. It should be noted that arg4-19 lies some distance proximal to arg4-1, the terminal proximal marker in the four point cross. In the present cross (Figure 7) the most common event is represented

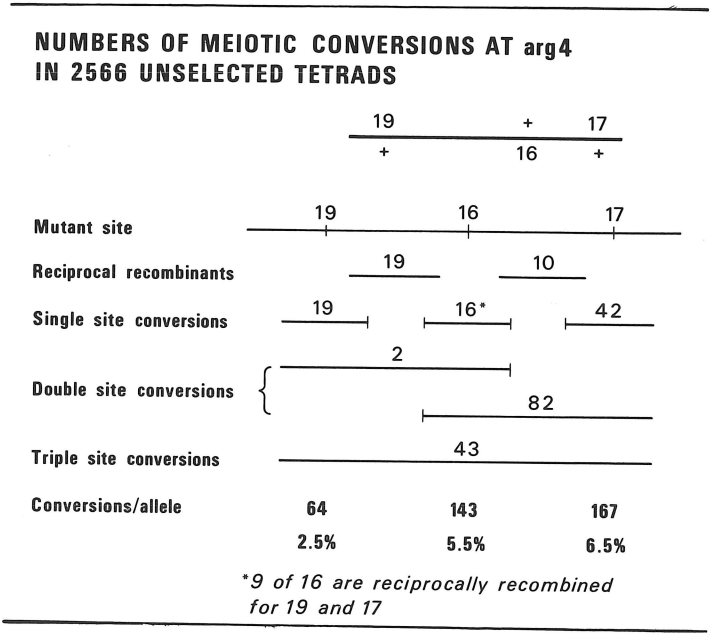


Figure 7. Frequency and types of conversions in a three point cross.

by the class of double site co-conversions involving alleles arg4-16 and arg4-17. The proximal terminus of the informational transfer in these events falls somewhere between arg4-16 and arg4-19. Often, it might be presumed to terminate in a region to the left of allele arg4-1. Triple site co-conversions spanning the entire marked segment occur about half as frequently as double site events. Again, special attention is drawn to the 16 single-site conversions of the central allele. Of these, nine are reciprocally recombined for the outside alleles. What conclusion can be drawn from these data? It would appear that conversional events are frequently associated with reciprocal recombination for the outside markers. Alternatively, we may state that contained between reciprocally recombined markers is a segment of non-reciprocity with an average length of 100 or more nucleotides. A critical test of this view is now in progress. The test is as follows: If we place a conditional mutant, say,

midway between the alleles 4 and 17, then upon selecting for recombinants between them, we should find that the internally situated conditional mutant is converted regularly.

Again, the significance of double and multiple site conversions must be emphasized for another reason. They have a special bearing on several hybrid DNA repair models--particularly on those aspects of the models of HOLLIDAY, WHITEHOUSE and STADLER that assign a special significance to the specificity of the mismatched base pairs. At issue here are the attempts to explain the observed differences in conversion frequencies in terms of particular base pair mismatches. The models suggest that a conversional event is initiated by a base pair mismatch and each of the four possible mismatches may be assigned a characteristic probability for initiating the excision-repair cycle. Let us reconsider the findings of the four point system.

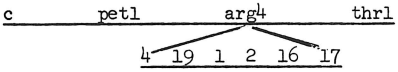
We observed (Figure 6) that the most common intragenic event involved all four markers simultaneously. This observation on symmetrical co-conversion places severe restrictions on the possibilities for excision and replacement. Clearly, the excision and replacement in one heteroduplex cannot be independent of the repair cycle in the corresponding heteroduplex, nor can the correction of each mismatch be considered independently of other mismatches. If random repair were operative, for only the terminal alleles arg4-1 and arg4-17, we would expect to encounter the six additional ascal classes in Figure 4. However, these are conspicuously rare. The difficulties are even greater when four markers are considered in detail. Clearly, these alleles are not independent with regard to conversion events. We may consider that quadruple conversions are initiated either at arg4-17 or to the right of this marker. Once initiated, the recombination event embraces all four markers simultaneously. But since the conversion frequencies of alleles arg4-17, arg4-16, arg4-2 and arg4-1 are approximately equal, it is difficult to assign any significance to the particular base pair mismatches that may be involved. Rather, we would predict that any allele within the region embraced by alleles 1 and 17 would be characterized by the same meiotic gene conversion frequency regardless of its properties.

To fully account for co-conversion, it is sufficient to propose that the converted alleles are located on the same side of the conversion-initiating site and that once initiated, conversion can extend to include several markers. Also, since co-conversion may extend across two and even three cistrons encoding distinctly different proteins (50, 51), it would appear that the conversional mechanism does not recognize the beginnings or ends of genes. Under the weight of the total evidence presented, it is clear that if the above models are to be preserved, they will require additional elasticity or further modification.

What then is the relationship between gene conversion and classical crossing over? Table 4 summarizes our collective experience on this question. Data from different hybrids are presented and in each instance recombination is scored for the markers immediately flanking the converted segment. Two major generalizations emerge from the evidence. First, it is apparent that half the conversions in all categories are associated with reciprocal recombination of the adjacent outside markers. This may be restated by saying that two conversions are equivalent to one crossover. Second, the quantitative relationship between crossing over and conversion prevails regardless of the interval length in which outside marker recombination is scored (Table 5). Therefore, it is not unreasonable to suppose that events leading to recombination must occur very close to the converted segment or that conversion and crossing over are simply expressions of the same fundamental event.

TABLE 4

ASSOCIATION OF RECOMBINATION WITH CONVERSION IN *arg4*



Diploid	*arg4* Genotype	No. of Asci	Allele(s) Converted	Interval	No. of Conversions	No. of Conversions with Recombination in Interval
BZ34	4 + + 17	690	4	petl - 17	8	7
			17	4 - thrl	42	19
			4-17	petl - thrl	5	3
Z4049 Z3932	4 + + 2	517	4	c - 2	1	0
			2	4 - thrl	18	10
			4-2	c - thrl	2	2
BZ28	+ 17	313	17	petl - thrl	22	10
Z3956 Z3957 Z3958	+ 17 2 17	243	2	petl - thrl	22	13
BZ140	2 + + 17	544	2	petl - 17	1	1
			17	2 - thrl	7	3
			2-17	petl - thrl	28	14
X841	1 + + 2	367	1	petl - 2	3	1
			2	1 - thrl	14	8
			1-2	petl - thrl	19	8
X901	1 + + 2	116	1	c - 2	2	2
			2	1 - thrl	6	3
			1-2	c - thrl	5	4
X2961	19 + 17 + 16 +	2566	19	petl - 16	19	12
			16	19 - 17	16	9
			17	16 - thrl	41	21
			19-17	petl - thrl	10	5
			19-16	petl - 17	2	2
			16-17	19 - thrl	82	38
			19-16-17	petl - thrl	42	21
X2988 X2976	1 2 + + + + 16 17	1505	1	c - 2	4	1
			16	2 - 17	2	1
			17	16 - thrl	15	3
			2-16	1 - 17	4	4
			1-2-16	c - 17	2	1
			2-16-17	1 - thrl	31	11
			1-2-16-17	c - thrl	74	31
Total		6861			549	268

TABLE 5

THE FREQUENCY OF CONVERSION-ASSOCIATED RECOMBINATION RELATIVE TO LENGTH OF THE OUTSIDE MARKER INTERVAL

Interval	Interval Length (cM)	Number of Conversions	Number of Conversions with Crossover	Fraction of Conversions with Crossover	X^2 (1:1)
c - thr1	20.5	81	37	0.46	0.6
pet1 - thr1	17.1	148	74	0.50	0.0
arg4 - thr1	14.2	256	116	0.45	2.3
c - arg4	6.3	9	4	0.40	-
pet1 - arg4	2.9	33	23	0.70	5.1*
arg4-a - arg4-6	< 1	22	14	0.60	1.6
Total		549	268	0.49	0.31

*Significant at the 5% level.

At this juncture some cautious speculation is warranted. If we assume that classical crossing over is simply a manifestation of an otherwise cryptic conversional event, then several logical consequences or predictions may be generated. Primarily, these concern chiasma or chromosomal interference. On the assumption that conversion and crossing over are expressions of the same primary event in DNA, we may predict that conversion in one cistron should interfere with conversion in a closely linked cistron. Also, conversion should interfere with recombination in an adjacent marked segment. In addition, it may be predicted that "conversionless" mutations would also be recombination deficient. Experiments along these lines are in progress, but the results to date are too fragmentary to merit presentation.

The foregoing considerations prompt us to inquire whether conversion-associated exchange alone is sufficient to account for all of the recombination observed in the yeast genome. Organized as follows, the calculation is probably correct only within the limits of an order of magnitude.

Given 1) The average meiotic conversion frequency = 1.5%
2) Total DNA/genome = 1.5×10^7 nucleotide pairs (N.P.)
Then, total DNA converted/meiosis = $1.5 \times 10^{-2} \times 1.5 \times 10^7$
$= 2.25 \times 10^5$ N.P.
If we assume average length of the converted segment = 1000 N.P.
Then, the number of conversions/meiosis

$$= \frac{2.25 \times 10^5}{7.50 \times 10^3} = 225 \text{ conversions/meiosis}$$

Now, if 2 conversions = 1 crossover = 50 map units, then the total number of crossovers/meiosis

$$= \frac{225}{2} = 112.5 \text{ crossovers}$$

and 112.5 crossovers x 50 map units = 5625 c.o. map units.

This result compares favorably with an estimated total map length of 3600 units yielded by the mapping studies of HAWTHORNE and MORTIMER (45). Therefore, we are encouraged to the outlook that reciprocal and non-reciprocal recombination are both genetic consequences of a single process.

What is the molecular mechanism that leads to gene conversion and recombination in general? At present, this cannot be fully specified despite an embarrassment of riches provided by a growing body of diverse theoretical models. The various models (cited above) differ widely in their assumptions. Yet they are not readily distinguishable one from another in terms of a priori predictions. Nevertheless, they are heuristically valuable, and therefore prized, since they conduce to our thinking about purely genetic data within an invigorating framework of constructs drawn from the molecular biology of DNA and its enzymatically mediated alterations.

Basic to most models are several shared features. Among them is the notion that recombination entails a DNA repair cycle initiated by enzymatic breakage in single stranded DNA helices of different parental origin. Depending on the particular hypothesis, the single helices are assumed to be of the same or opposite polarities. Broken single strands then anneal by complementary base pairing. A heteroduplex, or region of hybrid DNA is generated which contains mismatched base pairs at the included heterozygous sites. The various base pair mismatches might be imagined to provide signals of varying efficiency responsible for initiating excision of the offending mismatched base pairs. Resynthesis along the remaining template and ligation of the breaks completes the cycle. STAHL's model (42) is constructed on different assumptions and principles. It is a refreshing challenge for it provides one more way to look at gene conversion--. The model proposed by STAHL does not depend on the repair of mismatched base pairs in a DNA heteroduplex. Rather it envisions that following the major round of premeiotic DNA replication, an additional synthesis occurs in homologous segments of synapsed chromatids. This gives rise to "sex circles" that are formally equivalent to the fixed pairing regions considered by MURRAY (49). Then, two breakage-reunion exchanges occur in the DNA molecules within the sex circle. These generate short regions of hybrid DNA in the vicinity of the breaks. Only a single product of the exchange is conserved. The strands involved in the second exchange are determined by a rule (the Rule of Good Sense) that allows for the recovery of a continuous chromatid. As a consequence of these restrictions, the DNA between the two non-reciprocal exchanges originates from a single parent. If this segment involves heterozygous sites, they are expected to exhibit gene conversions in the resulting tetrads. Because the region that is converted contains a segment of transferred information entirely derived from one parent, this scheme readily accounts for co-conversion.

In addition, STAHL's model predicts that 50% of the conversional events will be associated with outside marker exchange if the markers fall outside of the sex circles. It is also supposed that conversion-associated marker exchange will generate chromosome interference in the adjacent region. Finally, it should be noted that the model implies short regions of hybrid DNA at the breakage-reunion sites. Mutant sites within such regions are expected to exhibit postmeiotic segregation. At the present moment, our findings on parity, fidelity, co-conversion, postmeiotic segregation and interference accord more closely with STAHL's model than any other, although if constraints are placed on the other models they can account for the data equally well.

Attractive and plausible as the varied schema might be, we must ask what are the supportive items that comprise direct, compelling evidence. At best, they are few in number compared to the sizeable body of indirect genetic investigations. In procaryotes, breakage-reunion process at the DNA level has been demonstrated by MESELSON and WEIGLE (52). Also, repair synthesis mechanisms responsible for repairing abnormal or damaged DNA have been elucidated by HOWARD-FLANDERS and BOYCE (53). In Neottiella, ROSSEN and WESTERGAARD (54) demonstrated that chromosomal replication essential to meiosis occurred in haploid nuclei prior to syngamy. Thus, meiosis may occur in a cell that is not committed to a round of DNA replication. The studies of SUEOKA (55) and his co-workers indicate that a similar situation exists in Chlamydomonas. Earlier evidence of DNA doubling in the premeiotic interphase has been critically summarized by RHOADES (56) who pointed out that DNA replication and recombination were probably separable events. STERN and HOTTA (57), and subsequent studies by STERN's group, identified two minor periods of DNA synthesis which occur at zygonema and pachynema after the major round has been completed. Conceivably, the zygonemal synthesis is involved in chromosome pairing, while pachynemal synthesis attended by a rise in the activity of intracellular endonuclease might provide the repair synthesis.

If we are to understand the recombinational process at the molecular level, several issues require clarification. Among these are the following questions: are heteroduplex formation, or sex circles a necessary prelude to recombination? If they occur, is their extent sufficient to account for all crossing over? What are the enzymological details involved in these processes? Do specialized recombinator regions exist and if they do, what is their chromosomal distribution? Are these regions characterized by specific base sequences?

How might we address ourselves to these issues in the near future? A promising approach would entail a biochemical-genetical analysis of mutants known to affect recombination and gene conversion. The direct experimental modification of chiasma formation by heatshock as illustrated by PEACOCK's (58) achievements are equally promising.

In 1954 L. J. STADLER (1) wrote, "The difficulties in the study of the genic substance are obvious. It cannot be isolated for chemical analysis or pure culture. The possibility of direct analysis of specific segments or individual genes is, of course, even more remote. The properties of genes may be inferred only from the results of their actions." To those of us who were privileged to experience the impact of L. J. STADLER's insatiable concern with the gene, we sense, and know, that his statement was a legacy framed as a challenge. The prophetic challenge of this "geneticist extraordinary" has been met in the equally extraordinary and illuminating work of MILLER and BEATTY (59, 60). They have provided us with portraits of genes and techniques for the direct visualization of DNA, messengers, RNA polymerases, polysomes, and promotor sites. Clearly visible are structural genes, induced genes, repressed genes and derepressed genes, and they have been caught in "flagrante transcripto." Surely, the time for the direct examination of genetic recombination events is close at hand.

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