GENE DUPLICATION AS A MECHANISM OF GENETIC ADAPTATION IN *SACCHAROMYCES CEREVISIAE*[1]

PAUL E. HANSCHE

Department of Genetics, University of California, Davis, California 95616

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ABSTRACT

It has been shown that specific mutations of the gene that codes for the general acid monophosphatase (Aphtase) of *S. cerevisiae* can increase the affinity of this enzyme for β-glycerophosphate (BGP) and thereby provide this organism with the capacity to exploit extremely low concentrations of this organic phosphate (FRANCIS and HANSCHE 1973). In this report two additional avenues are demonstrated to be available to this organism for increasing its capacity to exploit low concentrations of organic phosphates. One avenue is through mutations that increase the amount of Aphtase that associates with the cell wall, where it catalizes the hydrolysis of exogenous organic phosphates. The other avenue is through duplication of the gene that codes for Aphtase, doubling the amount of Aphtase synthesized. —— The spontaneous duplication of the structural gene of Aphtase and the incorporation of the duplicate into this experimental population as a means of exploiting low concentrations of exogenous organic phosphates provides direct support for the first step of the mechanism through which new metabolic functions are postulated to evolve.

THE raw materials that sustain the evolutionary process are often indiscriminately referred to, by evolutionists, as mutations. However, the term mutation includes at least four classes of aberrant genetic information, each of which has a different potential effect on the course of evolution of genetic mechanisms and the biochemical systems they encode. They are: mutant nucleotide sequences of structural genes (e.g., missense mutations), mutant nucleotide sequences of the DNA that regulates the transcription of structural genes (e.g., promoter mutations), duplicates of the nucleotide sequences that comprise structural genes (e.g., the polymeric genes that code for d-glucosidase in *S. cerevisiae*), and duplicates of the nucleotide sequences comprising regulatory mechanisms (e.g., the multiply repeated nucleotide sequences demonstrated to comprise a major component of genomes of higher animals; BRITTEN and DAVIDSON 1973). The likelihood of any particular class having a determinant effect on the course of evolution depends on the frequency with which it arises, the fidelity with which it is transmitted from generation to generation, and its effect on fitness. For example, several studies have indicated that when bacterial fitness is limited by the affinity of a repressable enzyme for an essential substrate, regulatory mutants, con-

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stitutively synthesizing the crucial enzyme, rapidly replace normal genotypes (HORIUCHI, TOMIZAWA and NOVICK 1962; WU, LIN and TANAKA 1968; BROWN, BROWN and CLARK 1969; HEGEMAN and ROSENBERG 1970; FOLK and BERG 1971). Thus it appears that aberrant sequences of regulatory DNA arise relatively frequently in prokaryotes and apparently increase fitness in environments where the affinity of enzymes for substrates limits reproductive rate. Another class of genetic aberration that is believed to contribute to the adaptation of bacterial populations to such environments is comprised of duplications of structural genes. Apparently, genotypes with only one structural gene coding for the crucial enzyme are often replaced by genotypes with serial duplications of the structural gene coding for the crucial enzyme (HORIUCHI, TOMIZAWA and NOVICK 1962; FOLK and BERG 1971; RIGBY, BURLEIGH and HARTLEY 1974). Recent experimental evidence indicates spontaneous gene duplications may arise in bacteria almost as frequently as do gene mutations (JACKSON and YANOFSKY 1973; RIGBY, BURLEIGH and HARTLEY 1974). Duplicate genes that code for identical enzymes tend to double the potential rate and amount of enzyme synthesis (HORIUCHI, TOMIZAWA and NOVICK 1962; HEGEMEN and ROSENBERG 1970; JACKSON and YANOFSKY 1973; RIGBY, BURLEIGH and HARTLEY 1974). Still another, but much less common, class of aberrant genetic information that may contribute to the adaptation of populations to environments where essential enzyme substrates are in short supply is comprised of mutant codes for the crucial enzyme. Although mutations of structural genes arise relatively frequently, the vast majority of them appear to have a detrimental affect on fitness in such environments. Rarely, mutations of structural genes result in mutant enzymes that have increased affinities and specificities for the essential substrate that is in short supply (WU, LIN and TANAKA 1968; BROWN, BROWN and CLARKE 1969; FRANCIS and HANSCHE 1972, 1973).

However, duplicate genes that code for enzymes in prokaryotes are generally unstable in the laboratory (JACKSON and YANOFSKY 1973), and are unknown outside the laboratory (HEGEMAN and ROSENBERG 1970). Furthermore, constitutive enzyme synthesis is relatively uncommon in nature. Thus, even though mutations affecting constitutive enzyme synthesis and duplications of structural genes arise frequently in prokaryotic populations, they probably serve only as temporary determinants of adaptation under ordinary circumstances.

Whether mutations affecting constitutive synthesis of enzymes represent an ordinary mechanism through which eukaryotic populations adapt to environments in which fitness is limited by the affinity of an enzyme for an essential substrate is open to serious question. Constitutive mutations in eukaryotes have been observed only rarely (HEGEMAN and ROSENBERG 1970). Thus it appears that the reliability of the regulatory mechanism of eukaryotes is far greater than that of prokaryotes. The role of gene duplications in the process of genetic adaptation also remains unclear. Granted, highly replicated DNA sequences appear to be a common feature of higher eukaryotes (OHNO 1970; BRITTEN and DAVIDSON 1971, 1973); nevertheless, the proportion of these repeated DNA sequences that code for enzymes appears to be very small if not zero, and although it seems

likely that these highly repeated sequences are involved in the regulation of gene transcription, the proportion of them that actually are involved in the regulation of gene transcription is unknown. Furthermore, there exists little if any direct evidence bearing on the frequency with which these duplicate, or replicate, nucleotide sequences arise in eukaryotes (with the exception of polyploidy in higher plants). Resolution of this conundrum seems especially relevant, as gene duplications are postulated to have played a central role in the evolution of proteins with new metabolic functions (HOROWITZ 1965), and multiply repeated DNA sequences are postulated to comprise the major feature of mechanisms that regulate the transcription of the structural genes that encode these proteins (BRITTEN and DAVIDSON 1971, 1973).

FRANCIS and HANSCHE (1972, 1973) obtained neither constitutive mutants nor structural gene duplications in either of two extensive experiments in which the fitness of the eukaryote, *Saccharomyces cerevisiae*, was limited by the affinity of its general acid monophosphatase (Aphtase) for the only source of phosphate available in the environment, β-glycerophosphate (BGP). The only adaptations they observed resulted from mutations of the gene that codes for acid phosphatase. Genotypes with mutant acid phosphatases, hyperactive on β-glycerophosphate, were incorporated into the experimental populations, but only after about 600 generations (6×10^{11} cell generations).

Further experiments were designed to establish whether or not specific mutations of the structural gene of Aphtase comprise the only genetic means through which *S. cerevisiae* can increase its capacity to exploit novel organic forms of phosphate. The results reported here indicate there are at least two other avenues through which an enhanced capacity to exploit novel organic phosphates can arise. In addition, they provide direct support for the hypothesis that gene duplication can be an important determinant of the genetic-biochemical processes of eukaryotic evolution.

MATERIALS AND METHODS

Yeast strains: The M4 haploid strain of *Saccharomyces cerevisiae* of mating type α, with which this study of genetic adaptation was initiated, is a mutant selected from strain S288C in my laboratory (FRANCIS and HANSCHE 1973). It carries the dominant allele, *ACP-2*, of the acid phosphatase (Aphtase) structural gene, *ACP*, of strain S288C. The mutant allele, *ACP-2*, codes for an Aphtase hyperactive on β-glycerophosphate, BGP, (Figure 1). M4 also carries the mutant alleles ga_2, *ma*, and *me* of strain S288C. The haploid strain E1 is also a mutant selected from strain S288C by DR. J. C. KUHN (1969). Strain E1 carries the recessive mutant allele *acp* of the gene in S288C that codes for Aphtase, and has essentially no Aphtase activity (Figure 1). Strain E1 was used to test whether or not alleles that affected Aphtase hyperactivity in experimental strains belong to the same gene that codes for Aphtase in strain S288C.

The adaptation experiment: A chemostat population of approximately 10^9 cells of M4 was maintained at 30° for approximately 1,000 generations (10^9 cells $\times$ 1,000 generations $= 10^{12}$ cell generations). Reproductive rates of cells comprising this population were limited by the affinity (V_{max}/K_m) of the Aphtase of each cell for a low concentration of uridine 5′-monophosphate (UMP) in a culture medium buffered to pH 6. UMP is a secondary substrate of Aphtase, the *in vivo* rate of hydrolysis of this monophosphate being only 50% of that at which BGP is hydrolyzed. Hydrolysis of UMP by this enzyme was the only avenue through which these cells could obtain the essential nutrilite phosphate from the experimental environment. The mean

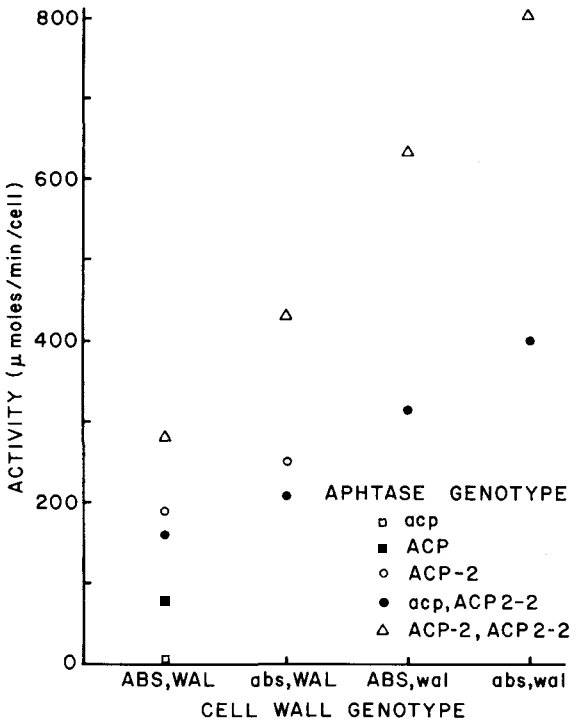

FIGURE 1.—Estimates of *in vivo* Aphtase activities on BGP (at pH 6) of 4 different cell wall and 5 different Aphtase genotypes. Strains E1, S288C, M4 and 409 have the following respective genotypes: *ABS, WAL, acp*; *ABS, WAL, ACP*; *ABS, WAL, ACP-2*; and *abs, wal, ACP-2*, and *ACP2-2*. The standard deviation of each estimate is approximately equal to 10% of the estimate.

specific reproductive rate of the population was 0.151. For details of the experimental apparatus see FRANCIS and HANSCHE (1972).

Population density was monitored during the experiment for increases indicating possible population change-overs due to replacement of the resident strain by a better-adapted mutant, i.e., a mutant more capable of exploiting the novel phosphate source, UMP. Estimates of *in vivo* Aphtase activities, Aphtase pH optima and Aphtase K_m's were estimated *via* the following procedures: Cells were grown in liquid culture to a stationary phase density imposed by the depletion of available phosphate. Aphtase activity assays were made on the second and third days after the populations reached stationary phase. Cells from aliquots taken from these liquid cultures were separated from the medium (identical to that described by FRANCIS and HANSCHE (1972) except that BGP was replaced by UMP) by vacuum filtration, washed and suspended in salt solution containing the macro and micronutrients as in the growth medium, except for molybdate. A given volume, x, of cell suspension was added to a volume, $2x$, of 1.5×10^{-1} M BGP (or UMP) in a pH 6, 0.15 M malate-tris buffer. The reaction mixture was sampled at regular intervals, and the orthophosphate concentration determined (FRANCIS and HANSCHE 1972). The concentration of cells in the reaction mixture was estimated with an electronic counter.

For pH optimum estimation, the organic phosphate was buffered with 0.15 M acetate buffer from pH 3.6 to 5.7, and with 0.15 M malate-tris buffer from pH 6 to 7.2. For K_m estimations, the enzyme activity was assayed with substrate ranging in molarity from 1.5×10^{-1} M to 1.5×10^{-3} M. Data were analyzed according to the Lineweaver-Burk procedure.

RESULTS

The strain extant in the chemostat after approximately 1,000 generations in an environment in which fitness was limited by the affinity of Aphtase for a low concentration of uridine 5′-monophosphate (UMP) was designated 409. Strain 409 is mating type α and is phenotypically ga^-, ma^-, and me^-, indicating it is descended from the strain M4 with which the experiment was initiated and that it is not a contaminant. Strain 409 has an *in vivo* Aphtase activity on UMP that is 4 times greater than that of M4 (Figure 1). Its activity on BGP is also 4 times that of strain M4 and about 8 times that of strain S288C (Figure 1), from which M4 was selected on the basis of its Aphtase hyperactivity (FRANCIS and HANSCHE 1973). About one-half of this increased activity can be attributed to mutant alleles of two independent segregating genes that affect cell wall structure. ADAMS and HANSCHE (1974) have previously shown that *in vivo* activity of Aphtase is limited by the amount of space in or on the cell wall available to this enzyme. Apparently only about one-half of the Aphtase normally synthesized by derepressed cells is actually associated with the cell wall at sites where it can contribute to *in vivo* Aphtase activity (Figures 1 and 2). These two mutant alleles arose and were incorporated into the population within the first 200 generations (2×10^{11} cell generations) of the experiment.

Haploid strains, derived from crosses of strain 409 with strain E1 (an Aphtase minus mutant of S288C), that carry the mutant allele symbolized *abs* (recessive to the wild-type allele *ABS*) have an *in vivo* Aphtase activity that is about 25% greater than that of strains with normal cell walls with genotypes *ABS*, *WAL*

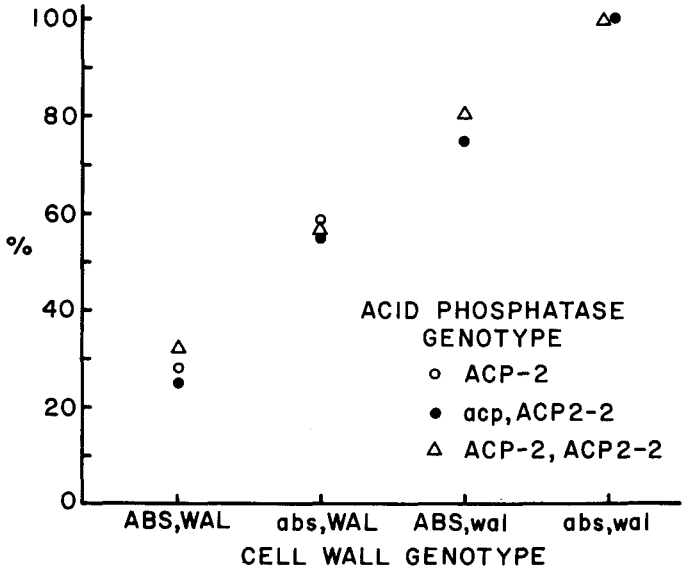

FIGURE 2.—Estimates of the effects of mutant cell wall genes on *in vivo* Aphtase activity. Estimates for each genotype are presented as percentages of the *in vivo* Aphtase activity measured on the double mutant, *abs*, *wal*, genotype.

(Figures 1 and 2). The allele *abs* inhibits abscission of daughter from mother cells and thus leads to the formation of spherical clumps containing 40–60 cells in liquid cultures. This effect is similar to that displayed by wild-type strains of *S. cerevisiae* when grown in inositol-free media (GHOSH *et al.* 1960) and of some inositol minus mutants of *S. cerevisiae* (SNOW, personal comm. 1973). Abnormal inositol metabolism is known to have a major effect on cell wall composition (PHAFF 1971). Thus, I suspect the increase in *in vivo* Aphtase activity of strains with the *abs* allele arises from an increased capacity of the cell wall to accommodate Aphtase molecules. The suspicion is reinforced by the fact that my *abs* strains have growth rates in inositol-free media that are less than half those of the ABS strains. Furthermore, LAMPEN's strain FH4C, selected for invertase hyperactivity (another cell wall associated enzyme of *S. cerevisiae*), also clumps due to abnormal abscission of daughters from mother cells (LAMPEN 1968).

Haploid strains derived from 409 that carry the mutant allele symbolized *wal* have an *in vivo* Aphtase activity that is almost two times that of haploid strains with normal cell walls, i.e., with cell wall genotypes *ABS*, *WAL* (Figures 1 and 2). *ABS*, *wal* genotypes appear to have little, if any, cell wall, with whatever remains being insufficient to impose geometric integrity of cells grown under usual cultural conditions.

The effects of these two mutant alleles *abs* and *wal* on *in vivo* Aphtase activity appear to be independent, as the *in vivo* activities of haploid strains carrying both mutant alleles are about equal to the sum of the effects associated with each allele (Figures 1 and 2).

The additional Aphtase at the cell surface resulting from these cell wall aberrations accounts for about one-half of the increase in Aphtase activity that strain 409 has over that of strain M4 from which it was selected (Figure 1). The other half of the increase is apparently due to the acquisition of a second Aphtase coded by a "new" gene that does not exist in strain M4.

It has been reported that strains S288C and M4 in particular (FRANCIS and HANSCHE 1972), and *S. cerevisiae* in general (KUHN 1969), have only one structural gene that codes from this cell wall associated general acid monophosphatase. Consequently, crosses of strain S288C or strain M4 with strain E1, an Aphtase minus mutant of S288C with genotype *acp* (Figure 1), should always lead to asci with spores that segregate with respect to Aphtase activity (+:−) 2:2. However, analysis of 40 asci from crosses 409 with strain E1 revealed 8 asci with spores that segregated (+:−) 4:0, 24 asci that segregated 3:1 and 8 asci that segregates 2:2. These segregation ratios cannot be explained on the basis of 409 carrying only one Aphtase structural gene. The possibility of this segregation being due to an unlinked suppressor of the *acp* allele of strain E1 is ruled out by the fact that haploid strains carrying both genes have *in vivo* Aphtase activities approximately twice that of haploid strains carrying either one or the other of these two unlinked genes (Figure 1). The "new" Aphtase gene codes for an Aphtase that is associated with the cell wall, as is the Aphtase of strains M4 and S288C. The ratios of ascal types observed (see above and Table 1) indicates

TABLE 1

Segregation of spores from asci obtained from intercrosses among the four Aphtase-positive spores of an ascus from the cross 409 × El segregating (+:—), 4:0 for Aphtase activity

Matings among spores	Expected genotypes of parents (Disomic)	(Dimeric)	Observed			Ascal types (Disomic)			Expected (Dimeric)		
			4:0	3:1	2:2	4:0	3:1	2:2	4:0	3:1	2:2
1 × 3	*ACP-2* × *ACP-2*	*ACP-2* × *ACP-2*	59	..	..	59	..	..	59	..	..
2 × 4	*ACP-2/acp* × *ACP-2/acp*	*acp,ACP'-2* × *acp,ACP'-2*	61	..	..	27	27	7	61	..	..
1 × 4	*ACP-2/acp* × *ACP-2*	*ACP-2* × *acp,ACP'-2*	7	26	7	17	16	6	7	26	7
2 × 3	*ACP-2/acp* × *ACP-2*	*acp,ACP'-2* × *ACP-2*	6	16	4	11	10	5	4	18	4
			13	42	11	28	26	11	11	44	11

The expected genotypes of the four spores of this ascus are either *(ACP2)*, *(ACP2)*, *(ACP2/acp)* and *(ACP2/acp)* or *(ACP2)*, *(ACP2)*, *(acp,ACP'2)* and *(acp,ACP'2)*, depending upon whether strain 409 is a disomic haploid of genotype *(ACP2/ACP2)* or dimeric of genotype *(ACP2,ACP'2)*.

the "new" gene segregates independently of the original previously reported Aphtase structural gene. The "new" Aphtase structural gene is repressed by PO^{-}_{4} and its Aphtase is inhibited by PO^{-}_{4}, as is the case with the gene of strain S288C that codes for Aphtase and its product (KUHN 1969). Estimates of the activities of the enzymes coded by these two genes were obtained over a wide range of pH values on the substrate BGP (Figure 3). The activities of these two enzymes do not differ significantly from each other, nor do they differ from the activity of the hyperactive Aphtase of the mutant strain M4, with which this experiment was initiated. However, the activities of the enzymes coded by these two genes both differ significantly from the activity of the "wild-type" Aphtase of strain S288C (Figure 3) from which strain M4 was selected on the basis of its Aphtase hyperactivity. Neither do the K_m's of the enzymes coded by these two genes, isolated from strain 409, differ significantly from one another nor from that of the mutant Aphtase of strain M4, being $(9.3 \pm 1.6) \times 10^{-3}$, $(9.1 \pm 1.8) \times 10^{-3}$ and $(9.0 \pm 1.2) \times 10^{-3}$ M, respectively, on BGP. However, they all differ significantly

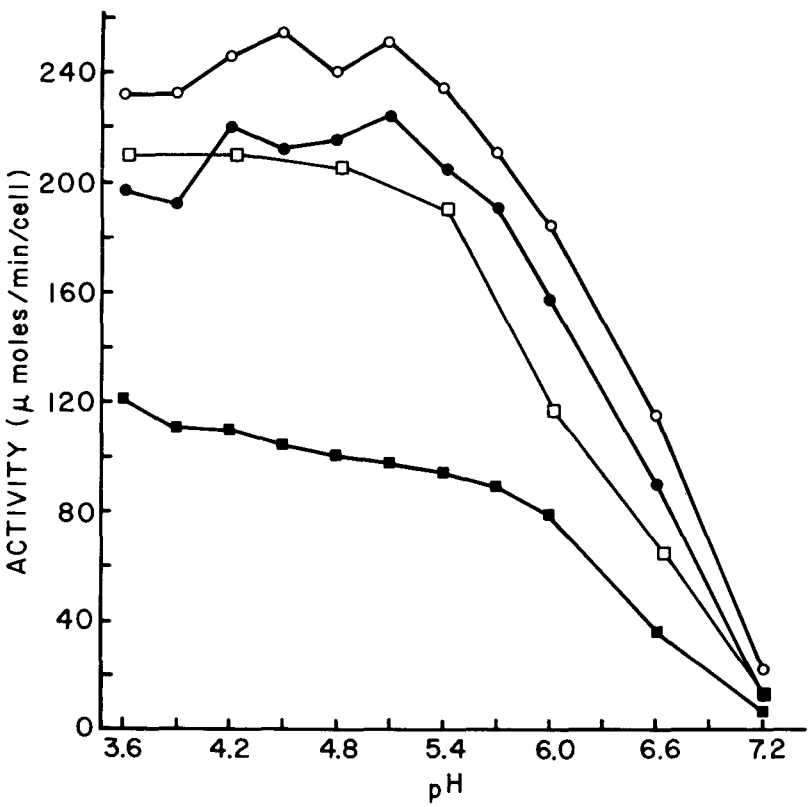

FIGURE 3.—pH optimum curves for the Aphtase of strain S288C, ■ (genotype *ABS*, *WAL*, *ACP*); of strain M4, □ (genotype *ABS*, *WAL*, *ACP-2*); of strain 13, ○, a derivative of strain 409 with an Aphtase gene that is allelic to *ACP* and *ACP-2*; and of strain 24, ●, a derivative of strain 409 with an allele *acp* (which codes for a nonfunctional Aphtase) of the original Aphtase gene, and with the "new" Aphtase gene that segregates independently of *ACP* or *ACP-2*.

from the K_m of $(3.4 \pm .46) \times 10^{-3}$ M, on BGP, of the Aphtase of strain S288C. The picture is essentially the same with respect to the substrate UMP. The maximum activities, at pH 6, of the Aphtase coded by these two genes, 161 and 138 μmoles/min/10^{10} cell, and K_m's $(2.8 \pm .83) \times 10^{-2}$ and $(3.6 \pm 1.1) \times 10^{-2}$ M do not differ significantly from one another. However, they both differ significantly from the maximum activity (61 μmoles/min/10^{10} cell) and K_m of $(6.2 \pm .9) \times 10^{-3}$ M of the Aphtase of strain S288C.

The likelihood of this second Aphtase being a product of an Aphtase gene that is ordinarily repressed in "wild-type" strains (e.g., in strain S288C) seems unlikely since on the one hand the "new" enzyme is unlike the "wild-type" Aphtase of S288C with respect to its K_m and pH optimum on both substrates tested, BGP and UMP; but on the other hand, it is indistinguishable from the previously selected (FRANCIS and HANSCHE 1973) hyperactive mutant enzyme of strain M4, from which strain 409 was subsequently selected, with respect to its K_m and pH optimum on both BGP and UMP, with respect to its association with the cell wall, with respect to its inhibition by PO_4^- and with respect to the gene being repressed by PO_4^-. Consequently, I concluded that the "new" structural gene that codes for Aphtase in strain 409 is a *duplicate* of the previously evolved Aphtase allele of strain M4, *ACP-2*, which codes for an Aphtase hyperactive on BGP (FRANCIS and HANSCHE 1973 and Figure 1).

Three tests were devised to determine whether strain 409 is aneuploid, a haploid disomic for the chromosome carrying *ACP-2*, and consequently carrying a copy of *ACP-2* in each of two homologous chromosomes, being of genotype *ACP-2/ACP-2*; or whether it is a dimeric haploid with a copy of *ACP-2* in each of two independent nonhomologous linkage groups, being of genotype *ACP-2*, *ACP′-2*, *the prime denoting the transpositioned duplicate of ACP-2.*

The first test is based on the fact that both dimeric and disomic haploids that carry two independent dominant wild-type alleles, whether they exist in homologous or nonhomologous linkage groups (i.e., whether of genotype *ACP-2/ACP-2* or *ACP-2*, *ACP′-2*) are expected to produce some asci that segregate (+:−) 4:0 with respect to Aphtase activity when crossed with a haploid with the recessive (−) phenotype (i.e., of genotype *acp*). The genotypes of spores in such asci would be (*ACP-2*), (*ACP-2*), (*ACP-2/acp*), and (*ACP-2/acp*); or (*ACP-2*), (*ACP-2*), (*acp,ACP′-2*), and (*acp,ACP′-2*)—depending on whether the parental strain were disomic or dimeric. However, intercrosses among the spores of such 4:0 asci should produce asci whose spores would segregate according to different expectations depending on whether the original wild-type parent was disomic or dimeric.

If the original wild-type parent carrying two structural genes was a dimeric haploid of genotype *ACP-2*, *ACP′-2*, with two identical alleles each existing in nonhomologous independent linkage groups, 4:0 asci should contain two dimeric haploid spores of identical genotype, both *acp,ACP′-2* and two monomeric haploid spores of genotype *ACP-2*. Crosses between the two dimeric spores with identical genotypes, both *acp,ACP′-2*, should result in dimeric diploid zygotes of genotype *acp/acp,ACP′-2*, which upon sporulation should produce nothing but

4:0 asci; all spores being dimeric haploids with identical genotypes, all *acp, ACP'-2* (see column 3 of Table 1). Crosses between the remaining two monomeric spores, of the genotype *ACP-2*, will also lead to 4:0 asci, all spores being monomeric with genotype *ACP-2* (column 3, Table 1).

However, if the original wild-type parent were a disomic haploid of genotype *ACP-2/ACP-2*, 4:0 asci would carry two *ACP-2* monosomes and two *ACP-2/acp* heterozygous disomes. Crosses between the two ACP-2 monosomes from such asci would lead only to 4:0 asci. This would also be expected in the case of a dimeric parent. However, contrary to these expectations, crosses between the remaining two disomic haploid spores (of genotype *ACP-2/acp*) should lead to duplex tetrasomic diploids of genotype *ACP-2/ACP-2/acp/acp*, (see column 2, Table 1) and asci with spores that would segregate 4:0, 3:1, and 2:2 in the ratio 4:4:1, since the gene coding for Aphtase has been shown to segregate independently of its centromere (KUHN 1969). Therefore, a minimum of 5/9ths of asci dissected would be expected to segregate 3:1 or 2:2.

Strain 409, a disomic haploid of genotype *ACP-2/ACP-2* or dimeric haploid of genotype *ACP-2, ACP'-2*, was crossed with strain E1, of genotype *acp*. The spores from one of the asci that segregated 4:0 were arbitrarily numbered 1, 2, 3 and 4. Fifty-nine asci from crosses between asexual descendants of spores 1 and 3, of a 4:0 ascus obtained from the cross of strain 409 with strain E1, as described above, were dissected and found to segregate 4:0 (Table 1), indicating spores 1 and 3 could either be monosomic haploids of genotype *ACP-2* or dimeric haploids of genotype *acp,ACP'-2*. They could not be disomic haploids of genotype *ACP-2/acp*, nor could one be disomic of genotype *ACP-2/acp* and the other monomeric of genotype *ACP-2*, since in either case, with the probability greater than 99%, some asci segregating 3:1 or 2:2 should have been observed in the large sample analyzed.

Further crosses were made to discriminate between possibilities of both spores 1 and 3 being monosomic of genotype *ACP-2* or both 1 and 3 being dimeric of genotype *acp, ACP'-2*. Thirteen asci from crosses of asexual descendants of spore 1 with strain M4a were dissected and all 13 were found to segregate 4:0. Similarly 6 asci from crosses of asexual descendants of spore 3 with strain M4α and 9 asci from crosses of asexual descendants of spore 3 with strain S288C were dissected and all were found to segregate 4:0. Since no 3:1 nor 2:2 ascal types were observed, it was concluded that spores 1 and 3 do not carry the *acp* allele, but, in fact, are monosomic of genotype *ACP-2*. This being the case, then spores 2 and 4 should carry two Aphtase structural genes, one of which was inherited from strain 409 and codes for the hyperactive enzyme of strain M4, and one of which was inherited from strain E1 (of genotype *acp*), and codes for a nonfunctional enzyme. Both spores 2 and 4 should either be disomic haploids, of genotype *ACP-2/acp*, or they both should be dimeric haploids of genotype *acp,ACP'-2*. If spores 2 and 4 are both dimeric rather than disomic, the genotype of the dimeric diploid zygote produced should be *acp/acp,ACP'-2/ACP'-2* and only 4:0 asci should be produced, all spores being identical of genotype *acp,ACP'-2*. No 3:1 nor 2:2 asci should be produced (Table 1).

Of the 61 asci that were analyzed from crosses among asexual descendants of spores 2 and 4, all segregated 4:0. None segregated 3:1 or 2:2 (Table 1). These results are contrary to the expectation that 5/9ths of these asci segregating 3:1 or 2:2 were strain 409 disomic; but they are consistent with expectations if strain 409 is a dimeric haploid carrying two copies of *ACP-2* in independently segregating nonhomologous linkage groups.

Although improbable, it is possible that spores 2 and 4 were both disomic of genotype *ACP-2/acp*, but that their asexual descendants used in the above cross had both lost the extra chromosome; both lost chromosomes by chance carrying the *acp* allele. If this were the case, then asci resulting from the cross would all segregate 4:0, as was observed (Table 1). The prediction that the descendants of both spores 2 and 4, utilized in this cross, carry the *acp* allele (which codes for a nonfunctional enzyme) of the original Aphtase gene and that they also both carry a gene that codes for a functional Aphtase (whether or not it is allelic to *acp*) was tested by crossing asexual descendants of spores 2 and 4 with asexual descendants of 1 and 3 (which were demonstrated above to be of genotype *ACP-2*). Since spores 1, 2, 3 and 4 all have Aphtase activity, crosses of the types 1×4 and 2×3 are expected to produce asci that segregate 4:0. They can also be expected to produce some 3:1 and some 2:2 asci *only* if asexual descendants of spores 2 and 4 carry the *acp* allele. In fact, both types of cross 1×4 and 2×3 did yield asci that segregated 4:0, 3:1 and 2:2 (Table 1), indicating that, as predicted, asexual descendants of spores 2 and 4 both carry one gene, *acp*, that codes for a nonfunctional Aphtase and one gene that codes for a functional Aphtase.

A more detailed analysis of the crosses of type 1×4 and 2×3 provides a second test of the hypothesis that spores 2 and 4 are not disomic but are dimeric. Were these two spores heterozygous disomic haploids of genotype *ACP-2/acp*, the zygotes resulting from these crosses would be duplex trisomic diploids of genotype *ACP-2/ACP-2/acp* and upon sporulation the ascal types 4:0, 3:1 and 2:2 should occur in the ratio of 12:10:5. However, if spores 2 and 4 were dimeric of genotype acp,ACP′-2 the zygote diploids *monosomic for the transpositioned duplicate Aphtase gene* would be of genotype *ACP-2/acp, ACP′-2* and should produce the ascal types 4:0, 3:1 and 2:2 in the ratio of 1:4:1. The ascal segregation observed from crosses of the type 1×4 and 2×3, 13:42:11 (Table 1) represent a highly significant statistical deviation from the expected ratio of 28:26:11 (Table 1), were spores 2 and 4 disomic haploids of genotype *ACP-2/acp*. In fact, the probability of observing this result were spores 2 and 4 disomic of genotype *ACP-2/acp* is less than one in one thousand. On the other hand, the segregation of ascal types observed, 13:42:11, fits extremely well to the expectation of 11:42:11, were spores 2 and 4 dimeric of genotype *acp, ACP′-2* (Table 1).

A third test of the hypothesis that spores 2 and 4 are aneuploid disomic haploids of genotype (*ACP-2/acp*) rather than dimeric haploids of genotype *acp, ACP′-2* was made by backcrossing the asexual descendants of spores 2 and 4 with strains E1 (of Aphtase genotype *acp*). If, on the one hand, spores 2 and 4 were aneuploid, haploid disomics of genotype (*ACP-2/acp*), then the zygotes resulting from this

cross would be simplex trisomic diploids of genotype (*ACP-2/acp/acp*). Since the Aphtase structural gene is not centromere-linked (KUHN 1969) the theoretical expectation of the segregation of spores within asci of such a trisomic is 2/27ths 1:3 asci and 25/27ths 2:2 asci. On the other hand, if spores 2 and 4 were dimeric haploids of genotype *acp,ACP'-2* then the zygotes should be dimeric diploids monosomic for the transpositioned duplicate Aphtase genes of genotype *acp/acp,ACP'-2* and all asci should segregate 2:2. Spores from the 40 asci of this cross that were analyzed all segregated 2:2. None segregated 1:3. Since the probability of obtaining at least one ascus that segregated 1:3 in a sample this size is greater than 95% if one of the parents were aneuploid, being a disomic haploid of genotype (*ACP-2/acp*), the results of this experiment are contrary to expectations were spores 2 and 4 disomic; but are consistent with expectations if spores 2 and 4 were dimeric haploids carrying two Aphtase genes, one coding for a functional Aphtase and the other for a nonfunctional Aphtase, i.e., of genotype *acp,ACP'-2*.

The results of all three of the above genetic tests are incompatible with strain 409 being aneuploid of genotype *ACP-2/ACP-2* but are compatible with strain 409 carrying two Aphtase genes, one a duplicate of the other. The results all indicate that the duplicate has been transpositioned into a linkage group that segregates independently of and is nonhomologous with the linkage group that carries the original Aphtase gene, *ACP*. Therefore, I have symbolized this transpositioned duplicate gene as *ACP2*, according to the standard procedure for symbolizing the several other known polymeric genes of *S. cerevisiae*. The particular allele of this duplicate gene existing in strain 409 is symbolized *ACP2-2*, as our results indicate it is a duplicate of *ACP-2*, an allele selected by FRANCIS and HANSCHE (1973) that is hyperactive on BGP.

The fidelity with which the transpositioned duplicate gene is transmitted asexually from generation to generation in haploid populations was tested by growing the haploid genotype (*acp,ACP2-2*) on a nonselective medium (containing inorganic phosphate), plating on phosphate-free agar and checking for Aphtase minus colonies. None were found among the 8,500 colonies tested, indicating this duplicate gene is transmitted asexually with a fidelity that is at least 100 times that of the typical duplicated enzyme structural gene in bacteria (JACKSON and YANOFSKY 1973; RIGBY, BURLEIGH and HARTLEY 1974).

Genetic tests with sublines derived by sampling periodically from the chemostat throughout this adaptation experiment indicated the allele *ACP-2* was transpositioned, duplicated and incorporated into the population by the 400th generation of the experiment (about 4×10^{11} cell generations), or some 200 generations after the incorporation of the mutations affecting cell wall structure.

DISCUSSION AND CONCLUSIONS

One hypothesis concerning the evolution of proteins with new enzymatic properties postulates as major determinants of this process mutations of permeases and regulatory genes, duplication of structural genes, and, finally, mutation and differential selection of the structural genes and their duplicates

(HOROWITZ 1965; HEGEMEN and ROSENBERG 1970; OHNO 1970; KOCH 1972). The core of this hypothesis, sometimes referred to as retrograde evolution, envisions, for example, the evolution of a biosynthetic pathway as being an adaptive response to an environment in which fitness depends on the joint affinity of an enzyme for two substrates, one normal, the other novel. The enzyme's normal substrate considered to have existed in a very low concentration, the environment having become depleted, whereas the novel substrate (an immediate precursor of the normal substrate) is postulated to have existed in higher concentration. The sequence of adaptive genetic responses to such environmental constraints on fitness is hypothesized to have been initiated by duplication of the enzyme structural gene. This is followed by mutation of one of the duplicates and the subsequent differentiation of the duplicate gene, guided by natural selection, to increase affinity and specificity of the mutant enzyme for the novel substrate. By the same rationale, evolution of a step in an independent pathway is postulated to follow when fitness depends on the joint affinity of an enzyme for substrates essential to two independent pathways.

To the author's knowledge, duplication of the Aphtase structural gene, and its incorporation into this experimental population as an adaptive response to the short supply of an essential nutrilite, is the first direct experimental demonstration in a eukaryote that gene duplication may play its postulated role in evolution, i.e., as an intermediate adaptive step in the evolution of a new enzyme function (HOROWITZ 1965).

These results indicate the gene that codes for Aphtase was transpositioned, duplicated, and incorporated into the experimental population within about 4×10^{11} cell generations. Thus, spontaneous gene duplication in haploid eukaryotes may occur less frequently than in prokaryotes. JACKSON and YANOFSKY (1973) provided evidence that spontaneous gene duplication transposition events occur in *E. coli* at a frequency between 10^{-8} and 10^{-7} chromosomal replications. FRANCIS and HANSCHE (1973) observed two independent mutations of the Aphtase structural gene that increased *in vivo* activity on low concentrations of BGP which arose at about the same frequency as the adaptive gene duplication, i.e., 10^{-11}. When taken together, the results of that study and the study reported here support the contention that gene duplications and specific gene mutations may be equally likely adaptive responses of haploid eukaryotes subjected to environments where fitness depends on the affinity of enzymes for scarce substrates; i.e., the two classes of adaptive aberrant genetic information may arise with approximately equal frequency.

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Corresponding editor: R. W. ALLARD