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Adaptive Mutation in *Saccharomyces cerevisiae*

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ABSTRACT Adaptive mutation is a generic term for processes that allow individual cells of nonproliferating cell populations to acquire advantageous mutations and thereby to overcome the strong selective pressure of proliferation-limiting environmental conditions. Prerequisites for an occurrence of adaptive mutation are that the selective conditions are nonlethal and that a restart of proliferation may be accomplished by some genetic change in principle. The importance of adaptive mutation is derived from the assumption that it may, on the one hand, result in an accelerated evolution of microorganisms and, on the other, in multicellular organisms may contribute to a breakout of somatic cells from negative growth regulation, *i.e.*, to cancerogenesis. Most information on adaptive mutation in eukaryotes has been gained with the budding yeast *Saccharomyces cerevisiae*. This review focuses comprehensively on adaptive mutation in this organism and summarizes our current understanding of this issue.

KEYWORDS stress-induced mutagenesis, selection-induced mutations, replication-independent reversions, stationary phase, starvation, spontaneous mutation

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1. INTRODUCTION

Despite occasional reports on stationary-phase mutations (*e.g.,* Ryan, 1955; Grigg and Stuckey, 1966), it was long believed that spontaneous mutations generally arise in the course of genome duplication, either by DNA polymerase errors or by replication of damaged DNA. Intermittent times of environmentally enforced quiescence were believed to be merely resting stages, also regarding spontaneous mutagenesis. In the last two decades, however, it became increasingly evident that mutations occur readily during cell cycle arrest or slow proliferation. Since the majority of cells in nature spend large parts of their lifetimes under proliferation-limiting conditions, spontaneous mutations arising in cells in this state might even outnumber the incidence of proliferation-dependent mutations.

Many random mutations are deleterious and contribute to cellular defects and cell death. As a result, mechanisms have evolved to keep the frequency of spontaneous mutations at a certain "healthy" level. Intriguingly, this level was found to be fairly well conserved between a wide range of organisms (Drake *et al.*, 1998).

However, some of the mutations are beneficial, and that subset is most interesting in terms of evolution and several aspects of pathogenesis. By choosing appropriate experimental conditions, it is possible to study the emergence of such beneficial mutations in populations of cell cycle-arrested cells. Corresponding experimental systems consist of a combination of nonlethal selective pressure (mostly starvation) and the existence of at least one target gene whose mutation allows the respective cell to overcome the selective pressure.

This review specifically addresses the proliferation-independent formation of beneficial mutations in cell cycle-arrested populations of the budding yeast *Saccharomyces cerevisiae*. Unfortunately, this subset of beneficial mutations has been assigned a multitude of different designations, each with its specific pros and cons.

The term "stationary-phase mutation" (Babudri *et al.*, 2001; Halas *et al.*, 2002; Heidenreich *et al.*, 2006) is probably not appropriate because it is questionable whether the experimental conditions typically applied resemble a regular stationary phase initiated by carbon source limitation (see Section 6.1.). "Selection-induced mutation" (Hall, 1992; Heidenreich and Wintersberger, 1998) and "starvation-induced mutation" (Storchova *et al.*, 1997, 1998; Rojas Gil and Vondrejs, 1999) allege that the selective conditions start, rather than terminate, a mutagenic process, which putatively is not the case. "Starvation-associated mutation" (Storchova and Vondrejs, 1999; Cejka *et al.*, 2001) does not have this bias, but expresses a narrowing to the most common selective condition. "Replication-independent mutation" (Heidenreich *et al.*, 2003) reflects the crucial mechanistic difference to proliferation-dependent mutagenesis but was infrequently used. Additionally, all the above mentioned names do not reflect the benefit of the mutations. In contrast, "adaptive mutation" (Steele and Jinks-Robertson, 1992; Baranowska *et al.*, 1995; Heidenreich and Wintersberger, 1997; Greene and Jinks-Robertson, 1999; Heidenreich and Wintersberger, 2001; Heidenreich and Eisler, 2004; Heidenreich *et al.*, 2004, 2006) does so and is the most commonly used term in yeast. Adaptive mutation is also established as a designation for the analogous phenomenon

in *Escherichia coli* (early work reviewed by Foster, 1993; Rosenberg, 1994; Hall, 1998). In the beginning, adaptivity was interpreted in the strict sense, with the suggestion that exclusively beneficial mutations might arise. After dispute and reports of experimental evidence that other, unselected, mutations arise along with the beneficial ones (Foster, 1997; Torkelson *et al.*, 1997; Rosche and Foster, 1999; Godoy *et al.*, 2000), the definition of adaptive mutation in *E. coli* was extended to the present meaning of adaptive mutation in the broad sense, tolerating the additional occurrence of unselected mutations (Foster, 1999, 2000; Rosenberg, 2001).

Clearly, it would be helpful for future research to reach an agreement on a single designation in *S. cerevisiae*. Therefore, being aware that none of the above names is ideal and that "adaptive mutation" sometimes is used for the description of beneficial mutations during cell proliferation (*e.g.,* Zeyl, 2004), for reasons of name recognition I, nevertheless, suggest sticking to the designation "adaptive mutation" (in the broad sense), which I will use throughout this review. It should be mentioned that the term "adaptive mutation" is understood not only as a name for the sequence change, but also for the process that generates it.

Spurred by the revival of the adaptive mutation field in *E. coli*, which was stimulated by the controversial paper by Cairns *et al.* (1988), reports on adaptive mutation in *S. cerevisiae* as the first eukaryotic organism followed 4 years later (Hall, 1992; Steele and Jinks-Robertson, 1992). The emphasis of the first reports was the description of the characteristics of adaptive mutation and the discrimination of adaptive mutations from the canonical proliferation-dependent mutations. Later efforts were directed to an identification of the mechanisms behind this special kind of mutation formation.

Without DNA replication, the introduction of mutations into a genome nevertheless must result from some form of DNA processing or turnover. Therefore, errors occurring during the constantly necessary repair of spontaneous DNA damage as a consequence of a decay of the DNA or the attack by endogenous reactive molecules have been suspected as the origin of adaptive mutation. This assumption now has been supported at least partially (see Section 4).

The overall importance of adaptive mutation might be substantial. In populations of unicellular organisms, adaptive mutation provides the opportunity to adapt to growth-limiting environmental challenges *during* the resulting cell cycle arrest, given that i) the altered conditions do not kill the cells immediately, and ii) genetic changes in principle are able to result in a relief of the growth limitation. Adaptive mutation thus may considerably contribute to the evolution of microorganisms.

The situation is crucially different in multicellular organisms like humans. In adulthood, proliferation of the majority of somatic cells is arrested (despite sufficient nutrient supply) by inhibiting factors and/or the lack of growth factors. If, in somatic tissues, individual cells manage to escape this regulation by mutations that activate oncogenes or inactivate tumor suppressor genes, this may provide a proliferation advantage for the resulting tumorigenic clone, but such selfish behavior may eventually be fatal for the organism as a whole. It was noted that the number of distinct mutations required for multistage carcinogenesis is too large to be explainable by conventional mutation rates (*i.e.,* per cell division) derived experimentally (Loeb, 1991; Loeb *et al.*, 2003). Thus it was argued that many cancer-initiating and -promoting mutations might occur in a time-dependent manner during cell cycle arrest (Strauss, 1992; Hall, 1995).

It seems therefore promising to study the mechanisms that lead to mutations that allow the escape from cell cycle arrest in amenable model organisms like bacteria or yeast.

Scientific work on adaptive mutation in the budding yeast *S. cerevisiae* has so far only been reviewed in the form of small chapters in reviews dealing with adaptive mutation in general, but with an emphasis on *E. coli* (Foster, 1993; Rosenberg, 2001). This is the first comprehensive review devoted solely to adaptive mutation in *S. cerevisiae*.

2. EXPERIMENTAL SYSTEMS

2.1 Requirements

The experimental setup for a study of adaptive mutation in *S. cerevisiae* has to meet two main criteria. First, a population of cells has to be kept in a prolonged state of growth limitation by the application of nonlethal stress conditions. Ideally, the cells should completely arrest the cell cycle. Although it is conceivable that adaptive mutation mechanisms are also active during retarded proliferation, a cessation of the replicative cycle should be aspired to be able to separate the

process of adaptive mutation from DNA proliferation-dependent mutagenesis. Second, the possibility should exist that, as a matter of principle, the proliferation arrest is abolished by mutations. The growth-limiting stress applied represents a strong selective pressure favoring the prevalence of adaptive mutants that have acquired the ability to restart proliferation. The resulting emergence of a mutant clone amidst the majority of continuously starving cells allows the detection of adaptive mutation events.

In practice, two selective conditions that fulfill the above requirements have thus far been implemented in adaptive mutation experiments using *S. cerevisiae*. These are either starvation for an essential amino acid or starvation for an essential nucleobase. In both cases, auxotrophic cells are transferred to solid media with limiting amounts of, or completely lacking, the essential nutrient compound they are unable to synthesize themselves. After consumption of internal reserves, the cells enter a cell cycle arrest, and only cells that have already acquired prototrophy (*i.e.*, the ability to synthesize the essential compound on their own) manage to continue proliferation resulting in a detectable colony (or papilla). It is presently unclear whether the cells upon such one-component starvation enter a true G_0 phase (see discussion in Section 6.1). However, where examined, the vast majority of cells were reported to arrest unbudded, as characteristic for G_0 or G_1 cells (Heidenreich *et al.*, 2003). During prolonged cell cycle arrest, a certain type and localization of mutations is anticipated to occur due to the use of strains carrying more or less well known revertible auxotrophy alleles (Section 2.4). The revertants become the founders of papillae or colonies (Figure 1).

One of the inherent problems of adaptive mutation assays is that the experiment itself has to be preceded by a preculture phase, during which inevitably proliferation-dependent revertants arise that are carried over to the starvation plates. Since these pre-existing revertant cells continue proliferation and form colonies in the minimum time, it is possible to discriminate between proliferation-dependent revertants and adaptive revertants by their time of colony appearance.

It is advisable to determine the time necessary for a representative range of pre-existing revertants to grow into visible colonies under the conditions of competition with a bulk of arrested but metabolically active competitors by a reconstruction test (Section 3.1.1). Following the time span determined by such reconstruction tests, still further revertant colonies continue to arise, which are regarded to be the result of reversion events in starving cell cycle-arrested cells (thus, by definition, the result of adaptive mutation).

The experimental setup should also include a monitoring of cell number and cell viability during the starvation conditions. The former allows (if applicable) to rebut residual proliferation due to a possible leakiness of the allele or owing to nutrients released by dead cells or older revertant colonies. The latter helps to estimate the number of viable cells actually available for late-occurring mutations. Although starvation for a single amino acid or nucleobase is not acutely lethal, the viability of the starving cell population nevertheless progressively declines. The extent of this decline is quite diverse between different experimental systems and reports, ranging from a loss of only a few percent of viable cells per day (*e.g.*, Steele and Jinks-Robertson, 1992; Heidenreich *et al.*, 2003) to a loss of 50% or more per day (*e.g.*, Hall, 1992; Babudri *et al.*, 2001). Poor survival clearly impairs the significance of the experiment.

The principal experimental setup of all adaptive mutation studies adheres to either the papillae assay as established by Hall (1992) or the colony assay as established by Steele and Jinks-Robertson (1992). The next two sections introduce these assays, followed by a section covering alleles used.

2.2 The Papillae Assay

Hall (1992), in his first report on adaptive mutation in *S. cerevisiae*, used a modification of an experimental design he previously applied to *E. coli* (Hall, 1990). The basic features of this papillae assay are depicted on the left side of Figure 1. Rather small aliquots of a preculture of auxotrophic yeast cells (about 125 colony-forming units [CFU] per plate) are spread on selective plates containing an excess of all nutrients, but only a limiting amount of one essential nutrient that the cells cannot synthesize on their own. This limiting amount is optimized in a way that small colonies of about 3.5×10^5 cells form before the cells are forced into a starvation-induced cell cycle arrest. This continued proliferation takes about 3 days and reversions to prototrophy occurring during that phase (*i.e.*, proliferation-dependent revertants) result in an overgrowth of the small colonies by revertant papillae until about day 5 (unless the situation is complicated by the use of suppressible alleles, see Sections 2.4

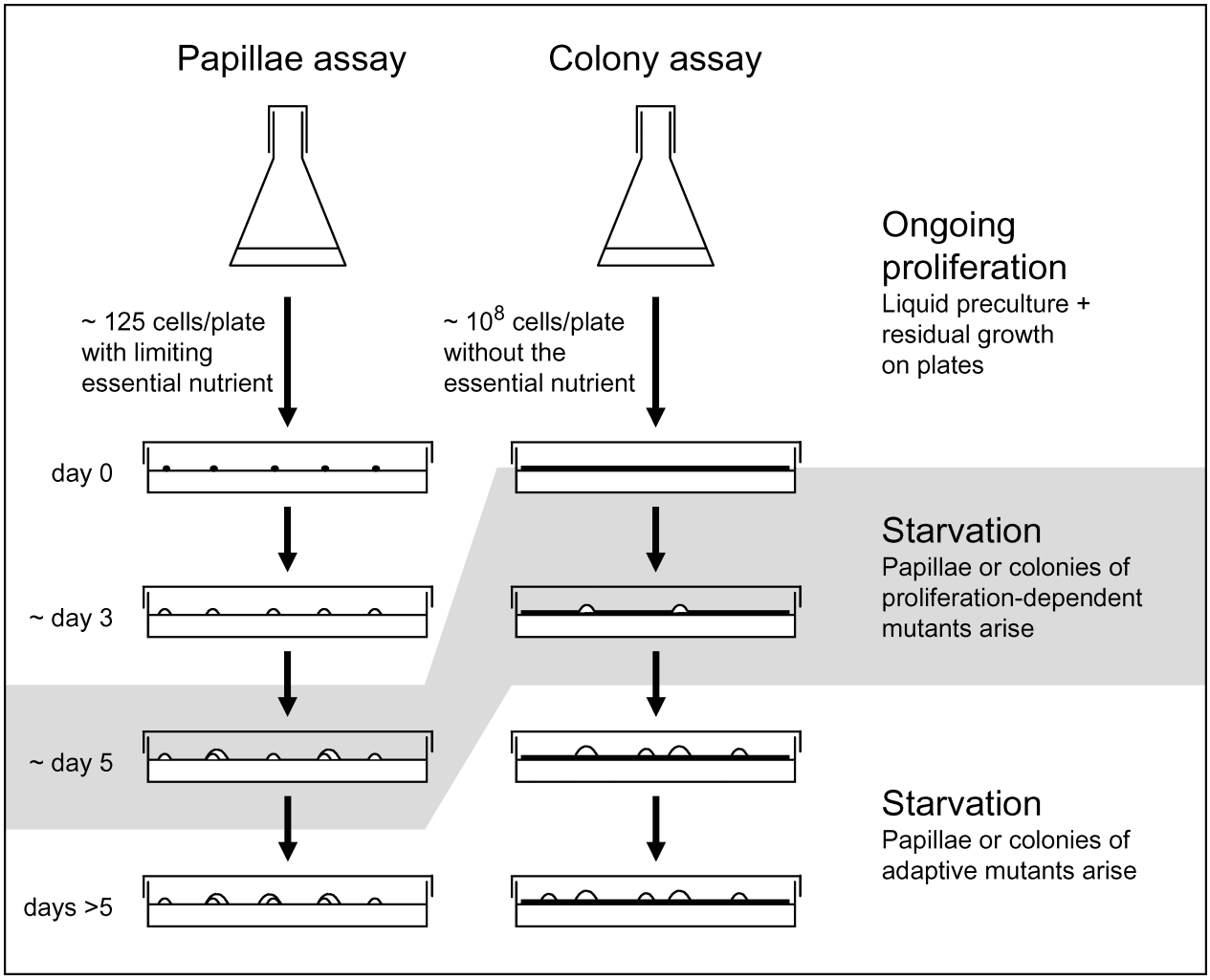

Figure 1 **Comparison of the two best-established adaptive mutation assays, the papillae assay and the colony assay. A time course of papillae and colony appearance is schematically depicted on lateral views of petri dishes. Details of the assays are described in Sections 2.2. and 2.3. Cell and colony sizes are exaggerated to aid recognition.**

and 3.1.1). Papillae making their debut later than day 5 should indicate adaptive reversion events. The continuing appearance of new papillae is monitored for at least 1 week.

It is an advantage of the papillae assay in comparison to the colony assay that almost all cell divisions preceding the starvation/selection phase occur on the plates (between day 0 and day 3). Unlike during the preculture phase of the colony assay, the progeny of a single proliferation-dependent revertant is not physically separated. Therefore, one reversion event results in only one papilla, whereas in the colony assay early occurring reversions provoke a high number of CFU (*i.e.*, a so-called jackpot). The papillae assay therefore is of some benefit when the proliferation-dependent mutation rate of an allele is high (Babudri *et al.*, 2001).

However, the papillae assay has some disadvantages as well. The major one is that the passage from proliferation to starvation is slow due to a progressive shortage of the essential compound, and not as abrupt as for the colony assay. This effect is probably amplified by the inherent zoning within colonies of microorganisms. Because of the nutrient supply from the medium below, the cells at the base of the colony might still proliferate by means of diffusion of nutrients from unpopulated areas of the plate, while the cells that have been lifted to the top of the colony already have lost access to scarce nutrients and have entered a cell cycle arrest. At least during the transition from proliferation to starvation, the colonies therefore do not represent a homogenous population with regard to cell cycle status, progression into stationary phase, gene expression, and so on (Minarikova *et al.*, 2001; Palkova and Vachova,

2006). Thus, the danger of a "contamination" of the incidence of late-arising adaptive mutant papillae with proliferation-dependent mutant papillae persists longer than with the colony assay.

The papillae assay in the original setup by Hall (1992) does not allow a detection of more than one papilla per colony due to an overgrowth of the small colony of starving cells by the revertant clone. Others tried to optimize a variation of the original papillae assay by producing a smaller number of larger colonies that can accommodate a number of up to ≈30 distinguishable revertant papillae (Rojas Gil and Vondrejs, 1999). However, with their adenine starvation system, in contrast to a colony assay with the same strain (Storchova *et al.*, 1997), they encountered the problem that about 25% of the isolated papillae subclones did not show the expected Ade^+ phenotype.

2.3 The Colony Assay

The experimental setup of the colony assay was introduced by Steele and Jinks-Robertson (1992). In contrast to the papillae assay, almost all cell divisions necessary to provide the required high cell numbers take place in liquid preculture in complete medium (Figure 1, right side). Typically, overnight precultures are grown to late exponential phase and 10^8 cells per plate are then spread evenly on medium lacking the essential nutrient compound. This spreading results roughly in a monolayer of cells. The cell inoculum therefore encounters quite uniform conditions in contrast to the inherent differences within the small colonies of the papillae assay. Although the inoculum cells are asynchronous at the beginning, the abrupt lack of the essential nutrient causes the transition to starvation-induced cell cycle arrest during the first few hours after plating. Residual proliferation due to internal reserves of the essential nutrient may lead to a doubling of the cell number but is limited to the first day of incubation (Heidenreich and Wintersberger, 1997; Storchova *et al.*, 1998). Pre-existing proliferation-dependent revertants normally form colonies no later than day 3 (excluding extragenic suppressors, see Sections 2.4 and 3.1.1). Depending on the allele used, adaptive revertants can be expected to make their appearance as colonies from day 3 or 4 onward. This continuing appearance of colonies is monitored for at least 1 week.

Essentially, the only disadvantage compared to the papillae assay is the fact that randomly arising jackpots of proliferation-dependent revertants (caused by a reversion arising early during liquid preculture) may lead to a overcrowding of early-arising colonies on the plates, which might constrain the growth of late-arising adaptive revertant colonies. However, this seems to be a problem only with proliferation-dependent mutation rates higher than 1×10^{-7} per cell division (Babudri *et al.*, 2001, Heidenreich, unpublished observation).

It is common that the necessary preculture phase is arranged in the form of a fluctuation test, *i.e.*, in several independent subcultures, which are transferred to separate series of plates (Luria and Delbrück, 1943). This arrangement not only allows an accurate estimation of the proliferation-dependent mutation rate for comparison with the adaptive mutation frequency but also a monitoring of a switch from a Luria-Delbrück distribution to a Poisson distribution (see Section 3.1.2).

Overall, the colony assay seems to be superior to the papillae assay in most cases (directly compared, *e.g.*, by Rojas Gil and Vondrejs, 1999; Babudri *et al.*, 2001) and consequently was more often used for adaptive mutation studies.

2.4 Test Alleles

The suitability of a certain allele for adaptive mutation studies is influenced by many factors, not all of which are knowable by the experimenter ahead of establishing a new system. An ideal allele should allow experiments with a quick and tight cell cycle arrest, good long-term viability and a best-possible lack of artifacts caused by cryptic proliferation. Table 1 lists, to the best of my knowledge, all alleles used in adaptive mutation studies in *S. cerevisiae*. It should be mentioned that several additional reports on observations of adaptive mutation may exist that have not been recognized or appreciated as such by the authors. Since in these cases this was usually not the objective of the reported experiments, mostly no clear separation of what we now regard as adaptive mutation from proliferation-dependent mutations has been made and controls like those described in Sections 3.1 and 3.2 are lacking. Unfortunately, observations of adaptive mutation in *S. cerevisiae* may therefore not have received the proper attention in the past and are now difficult to evaluate for their significance.

TABLE 1 **Alleles used in adaptive mutation studies**

Allele	Mutation type	Reference
ade2-1	Nonsense, ochre suppressible	(Storchova *et al.*, 1997, 1998; Rojas Gil and Vondrejs, 1999)
ade2-101	Nonsense, ochre suppressible	(Cejka *et al.*, 2001)
his1-7	Missense	(Marini *et al.*, 1999)
his4-303	Nonsense of start codon	(Hall, 1992)
his6	Unknown	(Hall, 1992)
his7-2	Frameshift	(Babudri *et al.*, 2001)
hom3-10	Frameshift	(Heidenreich and Wintersberger, 1998; Heidenreich *et al.*, 2003)
lys2	Unknown	(Storchova and Vondrejs, 1999)
lys2ΔBglII	Frameshift	(Steele and Jinks-Robertson, 1992; Heidenreich and Wintersberger, 1997, 1998; Greene and Jinks-Robertson, 1999; Heidenreich and Wintersberger, 2001; Halas *et al.*, 2002; Heidenreich *et al.*, 2003; Heidenreich and Eisler, 2004; Heidenreich *et al.*, 2004, 2006)
lys2-1	Nonsense, ochre suppressible	(Baranowska *et al.*, 1995)
trp1	Unknown	(Storchova and Vondrejs, 1999)
trp1-1	Nonsense, amber suppressible	(Steele and Jinks-Robertson, 1992; Storchova *et al.*, 1998)
trp5-48	Nonsense, ochre suppressible	(Marini *et al.*, 1999)

Given these restraints, the work of Ilynia *et al.* (1986) for example, using an *ade2–192* allele, may in fact be considered one of the first reports on adaptive mutation in *S. cerevisiae*, predating the 'official' discovery of yeast adaptive mutation in the year 1992 (Hall, 1992; Steele and Jinks-Robertson, 1992).

Auxotrophy systems with reversible deficiencies in the pathways for adenine, histidine, homoserine, lysine, and tryptophan biosynthesis have been employed. Not all pathways and genes belonging to certain pathways are equally well suited, but adequate comparisons are not available. Tryptophan starvation systems might be comparatively less convenient than the others, since they were repeatedly reported to be rather problematic because of growth artifacts (Steele and Jinks-Robertson, 1992; Storchova *et al.*, 1997; Rojas Gil and Vondrejs, 1999; Storchova and Vondrejs, 1999).

Another important factor is the type of auxotrophy-causing mutation. Frameshift mutations and nonsense mutations may be regarded as more practical than missense mutations because they lead to a truncation of the encoded protein and therefore normally completely abolish its function and avoid the danger of leakiness.

Nonsense alleles allow a study of adaptive base substitution mutations. However, reversions not only occur by locus mutations that inactivate the improper stop codon, but frequently also by extragenic suppression through the mutation of tRNA genes. Unfortunately, suppressor mutants can only proliferate at a reduced rate. Thus, suppressible nonsense alleles give rise to a mixed population consisting of rapidly proliferating locus revertants and slowly proliferating suppressors, which clearly complicates the interpretation of adaptive mutation experiments. When employing a nonsense allele, it is essential to determine the time required to form a visible colony or papilla not only for the locus revertants but also for nonsense suppressors. A classification of locus versus suppressor mutants by means of a second suppressible marker allele is instrumental (Hall, 1992; Storchova *et al.*, 1998; Storchova and Vondrejs, 1999).

Frameshift alleles generally revert by compensating frameshifts within a stretch of coding sequence surrounding the original frameshift. Most experience and knowledge on adaptive mutation in *S. cerevisiae* has been collected with the aid of the *lys2ΔBglII* frameshift allele. It has to be noted, that Jinks-Robertsons group used a *lys2ΔBglII* allele controlled by the heterologous cytochrome c1 promoter (Steele and Jinks-Robertson, 1992; Greene and Jinks-Robertson, 1999), whereas Halas *et al.* (2002) and my group (since 1997) used the *lys2ΔBglII* gene under control of its native promoter.

3. EVIDENCE FOR THE GENUINENESS OF ADAPTIVE MUTATION

The hallmark of adaptive mutation is that mutations and resulting colonies or papillae continue to arise during prolonged nonlethal stress. To ensure the genuineness of adaptive mutation, it is essential to ensure that putative adaptive mutant clones, on the one hand, do not represent slowly growing proliferation-dependent revertants and, on the other hand, are not the consequence of cell turnover on the plates. Corresponding controls are described in the next two sections, followed by a section on genetic evidence for independence of the adaptive mutation phenomenon and a collection of data pro and contra adaptivity in the strict sense.

3.1 Late-Arising Revertant Colonies Result from Post-Plating Mutations

3.1.1 Reconstruction Tests

Reconstruction tests are routinely used to determine the time span needed for a single revertant cell to grow into a visible colony (or papilla). In an attempt to mimic the conditions of the original assay (either the papillae or the colony assay), a small number of characterized revertant cells is mixed with a vast majority of nonreverting competitor cells (*e.g.*, cells with a deletion of the test allele) and transferred to selective media plates.

Locus revertants of nonsense alleles and frameshift revertants typically give rise to colonies (or papillae) after 2 to 3 days, whereas suppressor mutants of nonsense alleles need 3 to 4 days for colony (or papilla) formation (Hall, 1992; Steele and Jinks-Robertson, 1992; Heidenreich and Wintersberger, 1997; Cejka *et al.*, 2001; Heidenreich and Wintersberger, 2001; Halas *et al.*, 2002). Simple determinations of the time required for colony formation on selective media without an excess of competitors are less meaningful, but yield similar results (Baranowska *et al.*, 1995; Storchova *et al.*, 1997, 1998). Consequently, one can plausibly assume that more than 4 days after cessation of proliferation only colonies or papillae arise that originate from adaptive reversions.

The time frames determined by reconstruction tests were indistinguishable whether the revertant cells were derived from colonies (or papillae) early-arising or late-arising in the original adaptive mutation assay (Steele and Jinks-Robertson, 1992; Heidenreich and Wintersberger, 1998, 2001; Halas *et al.*, 2002).

A refinement of the method was made possible by DNA sequencing of isolated revertant clones. Sequencing allowed the identification of pairs of proliferation-dependent revertants and adaptive revertants with an identical reversion genotype. Reconstruction tests revealed that such pairs of revertants resulted in simultaneously fast-appearing colonies, although one of them, the adaptive revertant, made its appearance in the original adaptive mutation assay several days later (Heidenreich and Wintersberger, 1998, 2001, and unpublished results). The late appearance of adaptive revertants can thus only be plausibly explained by fresh mutations generated in starving cells.

In order to exclude a potential phenotypic lag due to the need to produce a sufficient amount of gene product, Steele and Jinks-Robertson (1992) employed a different approach. They determined the time required by *lys2ΔBglII* cells freshly transformed with a *LYS2* plasmid to form colonies on lysine-free medium. The observation that 90% of the transformants formed colonies by day 3 prompted the authors to conclude that there is no significant phenotypic lag after acquisition of a functional *LYS2* gene.

In summary, it is well established that the late-appearing revertant colonies (or papillae) in these assays cannot be explained by an occurrence of slow-growing pre-existing revertants. For each new assay developed, similar reconstruction tests are needed to determine whether this is the case.

3.1.2 Distribution Effects

A specific feature of the colony assay is that by incorporating a fluctuation analysis, colonies originating from pre-plating reversions may be distinguished from colonies originating from post-plating reversions by means of their distribution among subcultures (Cairns *et al.*, 1988; Steele and Jinks-Robertson, 1992). If the inoculum for the colony assay is arranged as a series of independent subcultures, then some of the subcultures randomly experience early proliferation-dependent reversions. Early revertants have a head start in exponential proliferation and due to physical separation by liquid perturbation finally end up as a high number of CFU on the selective plates (representing a so-called jackpot). If the first reversion occurs late in a subpopulation, very low numbers of

colony-forming units are the consequence. This results in a broad variation of revertant numbers among different subpopulations as described by Luria and Delbrück (1943) (from now on referred to as Luria-Delbrück distribution).

Mutation events happening under selection on solid medium also allow the cells to divide exponentially. These cells do not, however, separate and consequently are regarded as one colony. Since all subpopulations find the same conditions, the resulting distribution should be random (from now on referred to as Poisson distribution).

Distribution analyses with the *lys2ΔBglII* and the *hom3-10* frameshift alleles revealed that the overall distribution of all revertants scored from day 3 to day 8 was intermediate between a Luria-Delbrück and a Poisson distribution, indicating a mixture of pre- and post-plating events. However, the distribution of revertant colonies arising later than day 4 closely approximated the corresponding predicted Poisson distribution (Steele and Jinks-Robertson, 1992; Heidenreich and Wintersberger, 1998).

A similar result was also obtained by a statistical analysis of variance (ANOVA). From day 4 onwards, the variance among independent subpopulations was not significantly different from the mean variance within subpopulations, indicating that all late-arising revertants are members of a single population (Steele and Jinks-Robertson, 1992).

Although revertants of the *lys2ΔBglII* frameshift typically formed colonies by day 3 in reconstruction tests whereas the shift to a pure Poisson distribution was diagnosed one day later, both approaches agree that colonies arising later than day 4 may not be the consequence of slowly proliferating pre-existing revertants.

3.2 Exclusion of Artifacts

Some experimental setups for the study of adaptive mutation in *S. cerevisiae* are not completely free of shortcomings concerning the strictness of the prolonged cell cycle arrest. In order, therefore, to rule out a contamination of the adaptive mutant counts with unsolicited proliferation-dependent mutants, appropriate controls must be designed.

Three types of potential artifact sources can be distinguished: residual proliferation, crossfeeding, and cryptic proliferation.

Residual proliferation is the initial proceeding of proliferation owing to internal reserves of the essential nutrient, and resulting from the endeavor to complete ongoing rounds of replication and reach a certain position of the cell cycle (typically G_1/G_0). The extent of residual proliferation varies from system to system. In the papillae assay, the inherent transit from feeding on the limiting nutrient in the medium to feeding on internal reserves is hardly discernible, and residual proliferation is therefore essentially not measurable. In colony assays, residual proliferation can be monitored by washing the cells from plates immediately after plating and 24 hours later, respectively, and by comparing cell counts. Residual proliferation may account for up to a doubling of the cell number during the first 24 hours after transfer to selective medium (von Borstel, 1978; Heidenreich and Wintersberger, 1997; Storchova *et al.*, 1998). A lower rate of residual proliferation (less than one doubling) seems to be achievable by using more saturated precultures (Steele and Jinks-Robertson, 1992). However, a complete lack of residual proliferation seems unusual since the cells accommodate pools of internal reserves during growth in complete medium. For example, with regard to the two most commonly used starvation systems, it is known that pools of the basic amino acids histidine and lysine are accumulated in the vacuole (Kitamoto *et al.*, 1988). Nevertheless, residual proliferation in colony assays is no major problem since, typically, no increase in cell number is observed after the initial 24 hours, and revertants originating from residual proliferation quickly form colonies that are hardly distinguishable from colonies founded by pre-existing revertants.

Crossfeeding is the ability to restart proliferation feeding on nutrients excreted by older revertant colonies. It is easily diagnosed by the resulting halo of microcolonies surrounding older revertant colonies (colony assay) or by colony swelling in the vicinity of old papillae (papillae assay). Crossfeeding was reported to be troublesome when using tryptophan auxotrophy-mediating alleles (Storchova *et al.*, 1997; Rojas Gil and Vondrejs, 1999; Storchova and Vondrejs, 1999) or the *his1–7* allele (Marini *et al.*, 1999; see also Lax *et al.*, 1979; von Borstel *et al.*, 1998). In other auxotrophy systems, crossfeeding only infrequently occurs at a later date (*e.g.*, after 2 weeks of starvation). The standard remedy in the case of crossfeeding is an advanced termination of the experiment some days before an emergence of

proliferation artifacts (Heidenreich and Wintersberger, 1997; Marini *et al.*, 1999; Babudri *et al.*, 2001). The absence of crossfeeding during experiments may not only be verified by visual inspection but also by a lack of correlation of the numbers of early-appearing and late-appearing revertant colonies (Steele and Jinks-Robertson, 1992).

Cryptic proliferation might be a consequence of an availability of nutrients released by dying or dead cells. Due to a progressive decline in the viability upon starvation, it is conceivable that, at late stages of the experiment, some cell turnover by cryptic proliferation might occur. Unlike crossfeeding, cryptic proliferation is not detectable by visual inspection, but by washing off and counting cells from control plates periodically throughout the experiment. The persistence of dead yeast cells as a consequence of their thick cell wall rules out a hidden turnover of cells when cell counts are constant. Overall, the extent of cryptic proliferation reported was within the range of sampling error. It thus appears that cryptic proliferation does not generally represent a relevant problem during adaptive mutation experiments in *S. cerevisiae*.

3.3 Sequence Spectra of Adaptive Mutations

A completely different approach to the question whether adaptive mutagenesis is distinct from proliferation-dependent mutation formation was provided by obtaining and comparing sequence spectra of revertants. So far, sequence spectra of reversions of the frameshift alleles *hom3-10* and *lys2ΔBglII* have been investigated (Heidenreich and Wintersberger, 1998; Greene and Jinks-Robertson, 1999; Heidenreich and Wintersberger, 2001; Heidenreich *et al.*, 2003). In order to restore translation of a full-length protein, compensatory frameshifts must occur in a reversion window of the gene bounded by two out-of-frame stop codons upstream and downstream of the original frameshift site.

The original frameshift of the *hom3-10* allele is a gain of one T in a mononucleotide run of six Ts (Marsischky *et al.*, 1996). Unfortunately, the *hom3-10* allele was found to exhibit a strong bias to revert at the original position (by a deletion of one T in the allele-specific 7T-stretch) not only during proliferation, but also during starvation. In total, 38 of 52 sequenced reversions restored the wild-type DNA sequence and additional 10 restored the wild-type amino acid sequence of the encoded protein aspartokinase (Heidenreich and Wintersberger, 1998). Thus, in the comparison of reversion spectra of proliferation-dependent revertants and adaptive revertants, in both cases deletions within mononucleotide runs resembling a polymerase-slippage type of mutation dominated. However, since the relevant region of the *hom3–10* allele evidently tolerates little variation, we reasoned that a meaningful difference in the mutation spectra might be concealed by this hindrance (Heidenreich and Wintersberger, 1998).

Subsequent analyses of reversion spectra of the *lys2ΔBglII* allele revealed that this lesion is much more appropriate for a diagnosis of mechanistic differences in mutation formation. The *lys2ΔBglII* allele was constructed by filling-in the overhangs of a BglII restriction site, thereby creating a +4 frameshift. The key advantage of the *lys2ΔBglII* allele is that reversion events occur scattered all over the 146 base-pair reversion window, although almost all of them result in an altered amino acid sequence in this region of the encoded protein (Greene and Jinks-Robertson, 1997; Flores-Rozas and Kolodner, 1998; Greene and Jinks-Robertson, 1999; Heidenreich and Wintersberger, 2001; Heidenreich *et al.*, 2003). This diversity of potential reversion events facilitates a diagnosis of differences in mutational spectra.

In the first two studies dealing with comparisons of *lys2ΔBglII* reversion spectra between proliferation-dependent and adaptive mutants, some conflicting evidence was obtained (Greene and Jinks-Robertson, 1999; Heidenreich and Wintersberger, 2001). Greene and Jinks-Robertson (1999) reported that the sequence spectra of 98 sequenced proliferation-dependent revertants and 93 adaptive revertants were alike with a probability of $p > 0.6$ (using the algorithm of Adams and Skopek provided as a computer program by Cariello *et al.*, 1994). In both reversion subsets, the predominant type of mutations observed was single-base deletions in mononucleotide repeats, with a preference for long mononucleotide repeats. Their adaptive reversions as well as their proliferation-dependent reversions therefore resembled a sequence spectrum typical for microsatellite instability (MSI), a condition normally ascribed to polymerase slippage errors that escaped correction due to a deficiency in the major repair pathway concerned, terms the mismatch repair (MMR) pathway.

In contrast, a similar comparison of *lys2ΔBglII* reversion spectra in my lab (Heidenreich and Wintersberger, 2001) yielded the result that the adaptive reversion spectrum differed significantly from the spectrum of proliferation-dependent reversions ($p = 0.002$, obtained with the same algorithm). Only the adaptive reversion subset displayed a bias toward -1 deletions in mononucleotide repeats, whereas in the proliferation-dependent subset, nucleotide gains and losses in a variety of sequence contexts were reasonably balanced. The reason for this discrepancy between the two reports is unclear, but a closer look at the data revealed that the adaptive revertant spectra of Greene and Jinks-Robertson (1999) and, that of my lab (Heidenreich and Wintersberger, 2001) were not actually significantly different (with a probability of $p = 0.18$ for being identical). The difference lies in the nature of the proliferation-dependent mutation spectra. Whereas during proliferation, the strain of Greene and Jinks-Robertson exhibited a MSI-resembling spectrum from the start, our strain exhibited quite a manifold spectrum of *lys2ΔBglII* reversions. We presently do not know why the proliferation-dependent spectra were so different and can attribute this only to unknown factors in the strain background.

It is in any case evident that a shift to a MSI-like sequence spectrum is not observable when the corresponding spectrum of proliferation-dependent revertants is already MSI-like, as seen with the *hom3-10* allele (Heidenreich and Wintersberger, 1998) or the data of Greene and Jinks-Robertson (1999). For this reason, we regard our *lys2ΔBglII* data as more instructive, since in that case the shift to an MSI-typical reversion spectrum obviously revealed an adaptive mutation-specific characteristic.

Our data thus provided a first molecular distinguishing feature for adaptive mutation in *S. cerevisiae* and a strong indication that adaptive mutation relies on different mechanisms than proliferation-dependent mutations. Furthermore, it is interesting that a MSI-like spectrum of mutations is generated at all in cells expected not to be undergoing full-scale DNA replication, since such mutations are generally attributed to polymerase slippage errors during replication. This finding suggests that length alterations in monotonous repeats may occur not only during replication, but also between rounds of replication (with possible implications for pathogenesis). A similar prevalence of adaptive frameshift mutations to occur in monotonous mononucleotide repeats has been reported previously with a detection assay for episomal frameshift mutations in *E. coli* (Foster and Trimarchi, 1994; Rosenberg *et al*., 1994) although it remains to be established to what extent the underlying mutagenic mechanisms may be comparable.

Further *S. cerevisiae* results obtained by sequencing of DNA ligase IV-deficient revertants are discussed in Section 4.2.1.

3.4 Evidence for Adaptivity in the Strict Sense

An interesting, albeit controversial, point is the question of whether adaptive mutations arise as a specific response to the selective condition applied (adaptivity in the strict sense) or rather as a consequence of a generally elevated frequency of mutagenesis not specific to the selection (unspecific mutagenicity of starvation). As a practical example, this would for instance correspond to the question of whether Lys^+ revertants of a Lys^-Trp^- double mutant strain would arise not only during lysine starvation, but also during tryptophan starvation (or double starvation for lysine and tryptophan, or glucose starvation), when a reversion does not confer an immediate growth advantage. Since this issue is important for evolutionary considerations, it requires careful examination. Unfortunately, the technical realization turned out to be challenging, and conflicting evidence was reported (Hall, 1992; Steele and Jinks-Robertson, 1992; Storchova *et al.*, 1998; Marini *et al.*, 1999). Already the first two papers on adaptive mutation in *S. cerevisiae* included assays to answer this question. Together with a later report, these papers put forward the idea that adaptive mutation in the respective systems is adaptive in the strict sense.

Hall (1992) determined the number of reversion events at the nonselected *ino1-13* locus during starvation for histidine, thus under conditions that select for a reversion of the *his4-303* allele. The results prompted him to conclude that "the mutation rate to *INO1* under conditions of histidine starvation is at least an order of magnitude lower than the rate of mutation to *HIS4*". Disadvantages of the papillae assay and a high rate of cell death prevented a more accurate measurement.

Steele and Jinks-Robertson (1992), employing the colony assay, determined the incidence of *lys2ΔBglII* revertants after 3 days of prestarvation either for tryptophan or leucine, or tryptophan and lysine

simultaneously. In all three approaches, following the transfer of the cells to lysineless selective media the number of Lys^+ revertant colonies arising subsequent to these nonspecific starvation conditions was not increased in comparison to control experiments without such nonspecific prestarvation. This led the authors to the notion that "adaptive reversion events do not accumulate unless they afford an immediate selective advantage." However, they observed some unexplained loss of expected Lys^+ revertants during nonspecific starvation compared with controls, leaving open the possibility that the numbers observed might be the result of a higher incidence of reversions to Lys^+ combined with a higher rate of cell death of revertants during nonspecific starvation.

Likewise, Storchova *et al.* (1998) observed in three of the four allele/nonspecific starvation combinations they used a substantially lower number of nonspecific revertants than expected based on controls. Although they also concluded that the reversions are specific to the selection applied, the mysterious loss of expected revertants remains a worry. It should also be mentioned that Storchova *et al.* (1998) used exclusively a repair-deficient *rad6* strain for their adaptivity analysis but no repair-proficient wild-type strain.

A study in disagreement with the above conclusions was reported by Marini *et al.* (1999). The authors noted a tenfold increase in the number of Trp^+ revertants between day 3 and day 7 of starvation for histidine. These results are in direct contrast to the results of Hall (1992), who also monitored the occurrence of a second reversion during histidine starvation in a papillae assay. Unfortunately, Marini *et al.* (1999) failed to show how much the residual proliferation they suspected to occur between the third and the fourth day might account for this increase. Nonetheless, they interpreted their results as an indication that the reversions arising in their system are nonadaptive (in the strict sense).

Although three of four studies support adaptivity in the strict sense, owing to technical shortcomings of the assays applied, it cannot be ruled out that the adaptivity in the strict sense is just apparent because carriers of nonbeneficial reversions simply perish sooner or later. To avoid ambiguities, more sophisticated methods have to be developed, which include rigorous controls if cells with pre-existing or new reversions of gene "A" on medium selecting for reversions of gene "B" display increased, similar or decreased degrees of residual proliferation and viability loss compared with the bulk of non reverted cells. As also confirmed by experience obtained in our lab (unpublished observations), these parameters clearly influence the outcome of adaptivity assays and might explain different results in different approaches. For the time being, the matter of adaptivity in the strict sense has not been conclusively resolved. In the light of the emerging insight that more than one pathway might produce adaptive mutations, it may as well turn out that part of the reversions are adaptive in the strict sense and part of them unspecific.

4. MECHANISMS OF ADAPTIVE MUTATION

Most information on mechanisms that govern the formation of adaptive mutants stems from experiments with genetically modified strains deficient in selected genes of interest. The focus was put on the multitude of DNA repair pathways since it was evident that the introduction of mutations into the genome in the absence of apparent DNA replication nevertheless requires some sort of DNA processing. Representatives of all major DNA repair pathways (with the noteworthy exception of the base excision repair pathway) have been analyzed for their impact on adaptive mutation in an attempt to gain information on the mechanisms behind this (these) replication-independent mode(s) of mutation formation. In addition, the roles of DNA polymerases and one signal transduction enzyme have been studied.

A valuable tool for corresponding screening attempts was the employment of genetically modified strains for adaptive mutation assays. To avoid ambiguities, it is favorable, if possible, to use complete knockouts of selected genes of interest, rather than point mutation alleles. In the case of essential genes, however, the use of partially or conditionally deficient alleles such as temperature-sensitive alleles was necessary (Baranowska *et al.*, 1995; Babudri *et al.*, 2001).

It turned out that impairments among many different pathways actually increased the incidence of adaptive mutation (Section 4.1), which in turn implies that these pathways contribute to a suppression of adaptive mutation, as long as they are fully functional (Figure 2). This does not necessarily mean that the corresponding genes and pathways directly counteract adaptive mutation-producing pathways. Instead, their

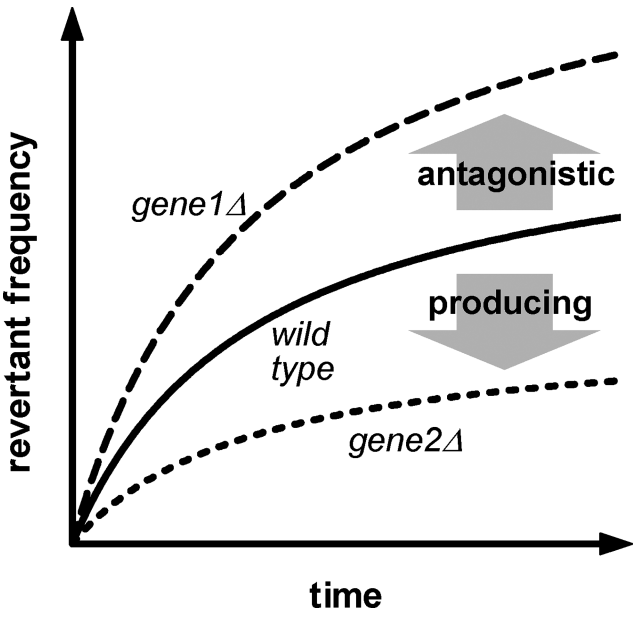

Figure 2 **Diagram showing possible consequences of gene knockouts. The curves illustrate the time-dependent accumulation of adaptive revertants. A start of the time course with, *e.g.*, day 4 ensures that no pre-existing proliferation-dependent revertants enter the record. If deletion of a certain gene results in an increase of the frequency of adaptive revertants (*gene 1*Δ) in relation to the wild-type, this indicates that the gene product is acting in an antagonistic pathway, *e.g.*, in a high-fidelity repair pathway. If, on the other hand, the incidence of adaptive revertants is decreased in relation to the wild-type by the gene knockout (*gene 2*Δ), it can be inferred that the gene product either directly produces or at least positively regulates adaptive mutations.**

deficiency might cause a higher incidence of some sort of DNA damage that is subsequently rechanneled into a mutagenic pathway.

In contrast, it turned out to be a more challenging task to find genes whose knockout decreased the frequency of adaptive revertants, indicating that, in reverse, the functional gene contributes to the production of adaptive mutants. Only a small minority of the gene knockouts analyzed so far resulted in a decline of the adaptive mutation curve (Figure 2) and, therefore, allowed an identification of protein components in adaptive mutation mechanisms (section 4.2).

In the search for adaptive mutation-producing mechanisms, it must be considered that an increase in the adaptive mutation frequency brought about by a gene defect may also reflect a combination of a strong antagonistic effect with a weaker simultaneous adaptive mutation-promoting effect of the gene product. With this in mind, a rating as an antagonistic mechanism should be regarded as tentative.

4.1 Antagonistic Mechanisms

4.1.1 Replicative Polymerases

The majority of DNA repair pathways involve DNA resynthesis ranging from a single nucleotide to several kilobases. The replicative DNA polymerases Pol δ and Pol ε of eukaryotic organisms not only operate in DNA replication but also in the fill-in synthesis of gaps generated during nucleotide excision repair, mismatch repair and long-patch base excision repair (Wood and Shivji, 1997; Burgers, 1998). Pol δ and Pol ε, both are very accurate owing to their intrinsic proofreading activities. Therefore, repair synthesis executed by Pol δ and Pol ε is assumed to be largely error-free, in particular in the case of repair stretches of limited length.

The role of Pol δ and Pol ε in adaptive mutagenesis was studied using temperature-sensitive or proofreading-deficient alleles of the essential genes coding for the catalytic subunits of these polymerases.

One study employed a *cdc2-1* (also known as *pol3-1*) strain, which has a temperature-sensitive mutation in the gene coding for the catalytic subunit of Pol δ. The frequency of adaptive reversions of a base substitution test allele was higher in *cdc2-1* cells incubated repeatedly at the restrictive temperature of 37°C than in the corresponding $Cdc2^+$ control strain or upon incubation at the permissive temperature of 23°C (Baranowska *et al.*, 1995). However, the lack of knowledge of the exact consequences of a temporary lack of Pol δ activity and the recurrent temperature shift treatment make these results difficult to interpret.

In another study, Babudri *et al.* (2001) analyzed reversions of a frameshift allele in strains deficient for the 3′,5′ proofreading exonuclease function of either Pol δ (*pol3-01*) or Pol ε (*pol2-4*). The reversion rate during proliferation was considerably elevated (44-fold and 17-fold, respectively) in these strains. An evaluation of adaptive mutagenesis revealed that the elimination of the proofreading activity in Pol δ or Pol ε resulted in a clear mutator phenotype in nonreplicating cells as well. A comparison of the increase in adaptive mutagenesis in this study was complicated by the fact that the incidence of adaptive revertants in the two proofreading-deficient strains was determined with papillae assays, whereas the revertant numbers of the corresponding wild-type were determined with a colony assay, for technical reasons. However, the relation of

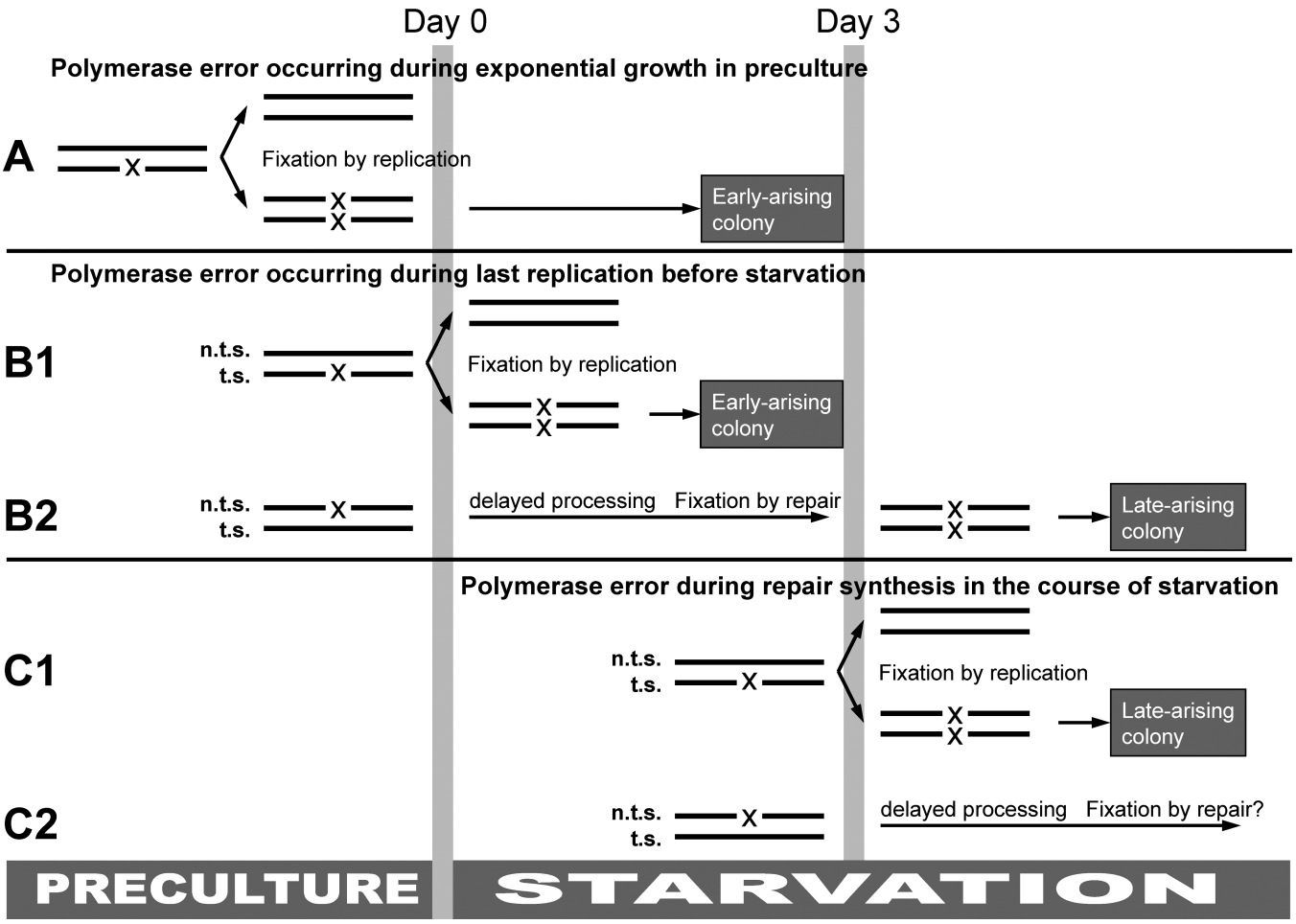

Figure 3 **Model of polymerase error-based mutation formation in the course of an adaptive mutation assay. For the sake of clarity, only the case of a colony assay is considered. The two strands of the relevant region of the test allele are symbolized by a double line. The transcribed strand (*t.s.*) and the nontranscribed strand (*n.t.s.*) are labeled where necessary for a better understanding. A mismatch introduced by occasional infidelity of a DNA polymerase is symbolized by an "x". In this model, we only consider configurations, where "x" results in an advantageous sequence alteration (*e.g.*, reversing an auxotrophy). The further fate of the resulting mismatched structure is depicted depending on the time and strand of the polymerase error. For the development of the phenotype, it is sufficient if the beneficial alteration is on the transcribed strand, irrespective of the situation on the other strand. In this case, transcription and subsequent translation of a functional protein enables the synthesis of the essential nutrient compound, a continuation (A, B1) or a restart (C1) of proliferation, and thereby a fixation of the mutation on both strands. If the polymerase error occurs on the nontranscribed strand, some additional process is required to mirror the alteration to the transcribed strand. During exponential growth, this happens inevitably by replication (A), but during cell cycle arrest some sort of repair process has to require the altered strand as a template, resulting in a conversion of the alteration to the transcribed strand (B2, C2). In scenario C2, mutation fixation and resultant colony appearance might fail to occur within the duration of the assay. It has to be emphasized that a polymerase-independent pathway and DNA damage-induced pathways of adaptive mutagenesis have been identified (see Section 4.2), which are not included in the above model.**

the mutation incidences between these three strains observed during proliferation was largely retained also during non-growth conditions. This suggests that the proofreading exonuclease activities of Pol δ, and to a lesser degree Pol ε, play a role in the avoidance of mutation formation not only during replication, but also during cell cycle arrest.

Since a knockout of these essential polymerase genes is technically not feasible, it remains elusive whether occasional infidelities of Pol δ and Pol ε in spite of functional proofreading are a relevant source of adaptive mutation in wild type strains (see also next section). A model for the possible contribution of uncorrected polymerase errors to the incidence not only of early-arising, but also of late-arising revertant colonies in adaptive mutation assays is shown in Figure 3.

4.1.2 Mismatch Repair

The major pathway for correction of errors that escaped correction by the proofreading activities of the replicative polymerases is the mismatch repair

(MMR) pathway. Most of the eukaryotic MMR proteins were named after the homologous bacterial proteins either MutS-homolog (Msh) or MutL-homolog (Mlh). The MMR pathway starts with a recognition of the mismatch and proceeds with a exonucleolytic removal of a portion of the altered strand, finalized by a resynthesis of that strand (Jiricny, 1998; Kolodner and Marsischky, 1999; Marti *et al.*, 2002; Schofield and Hsieh, 2003; Jiricny, 2006). The crucial factor for the usefulness of this process is the strand-specificity, *i.e.*, the ability to discriminate between the old pristine strand and the new altered strand. In eukaryotes, the necessary information most likely is provided by direct interaction with the DNA replication machinery and/or the presence of DNA termini in the newly synthesized strand (Pavlov *et al.*, 2003). If this information is not available, as supposed in G_1-arrested cells, an operation of MMR proteins might potentially just as well lead to a fixation of a mutation instead of a correction to the original sequence. Furthermore, a transient deficiency in the MMR capacity during prolonged starvation was associated with adaptive or stress-induced mutagenesis in *E. coli* (Longerich *et al.*, 1995; Harris *et al.*, 1997, 1999; Bjedov *et al.*, 2003) and *Bacillus subtilis* (Pedraza-Reyes and Yasbin, 2004).

In view of these considerations, it seemed promising to evaluate the potential influence of MMR on adaptive mutation in *S. cerevisiae*.

A comprehensive collection of MMR-deficient yeast strains, including knockouts of all basic MutS-homologs and MutL-homologs, was analyzed by Halas *et al.* (2002). They found that the level of adaptive frameshift mutations was increased in all MMR-deficient strains, with the smallest effect observed in the *mlh3* and the *pms1* strain, followed with increasing effect by the *mlh1*, the *msh3*, and the *msh6* strain, ending with the strongest mutator effect in the *msh2* strain. Thus, the role of the MutS family members in avoidance of adaptive frameshift mutations was more pronounced than that of the MutL family members, and the obligatory constituents of MMR protein complexes (Msh2 and Mlh1) were more important than the constituents that can substitute for each other (*i.e.*, Msh3 for Msh6 and Mlh3 for Pms1, respectively).

There is a possibility that DNA mismatches arise by means of heteroduplex formation in the course of recombination between < 100% homologous sequences. Without presently being able to reject this potential source of mismatches by experimental results, it seems unlikely that it plays a relevant role in the adaptive mutation experiments with haploid strains reported. Another possibility, the generation of a mismatch by the spontaneous deamination of 5-methylcytosine to thymine, might only be a source of base substitutions, but not of frameshifts. If we consequently assume that DNA polymerase errors are the main source of mismatches relevant for adaptive mutagenesis in the reported frameshift system, then the results obtained with MMR-deficient strains (Halas *et al.*, 2002) are in line with the results obtained with Pol δ or Pol ϵ proofreading-deficient strains (Babudri *et al.*, 2001), presented above.

In both approaches, an excess of uncorrected polymerase errors somehow triggered increased levels of adaptive mutation. Figure 3 summarizes the different scenarios how polymerase errors might result in revertant colonies in the course of an adaptive mutation assay.

Unfortunately, the present data cannot answer several important questions. Most prominently, it remains unclear whether the hypothetical premutagenic mismatches are predominantly generated during limited repair DNA synthesis in the cell cycle-arrested cells (scenarios C1 and C2 in Figure 3) or whether they are predominantly relics from the last replication cycle, which are slowly processed during the subsequent state of starvation-induced cell cycle arrest (scenario B2 in Figure 3). Thus, for the case of polymerase errors, it is presently unclear whether mutagenic errors are constantly produced during starvation, or whether mutagenesis relies on a limited reservoir of premutagenic lesions from the last replication cycle.

If we consider these two possibilities in more detail, we find that for the former models (C1 and C2 in Figure 3) we do not know whether repair-associated DNA synthesis is more error-prone than usual, *e.g.*, due to a decreased fidelity of the concerned polymerases or due to a transiently decreased MMR capacity. In the latter model (B2 in Figure 3), we do not know the putative processes that result in a duplication of the alteration to the transcribed strand. In that special case, a delayed MMR might even actively trigger the production of mutations, if the information about which strand should be retained was lost since the last replication.

Furthermore, we do not know at the moment whether uncorrected polymerase errors only are responsible for the increased incidence of adaptive mutations

in proofreading-deficient or MMR-deficient cells, or whether they also contribute to the basal level of adaptive mutation in wild-type strains. Clearly, we need to understand a lot more about the role of polymerase errors in adaptive mutation. Appropriate experiments have yet to be devised and conducted to be able to answer these questions.

4.1.3 Rad6 Epistasis Group

The heterogeneous Rad6 epistasis group comprises at least three different sub-pathways of high-fidelity and error-prone postreplication repair (Xiao *et al.*, 2000). The common function of these pathways is the accomplishment of a bypass of replication-blocking lesions. Since the lesions are not actually repaired during such bypass, these are DNA damage tolerance pathways rather than repair pathways (reviewed by Lawrence, 1994; Broomfield *et al.*, 2001; Friedberg *et al.*, 2005). The bypass of lesions occurs either by a poorly characterized template-switching mechanism or by specialized translesion synthesis (TLS) polymerases. Members of the Rad6 group are of crucial importance for the level of damage-induced mutagenesis. Also, part of spontaneous mutagenesis in proliferating cells is determined by error-prone activities within this group.

Although the Rad6 group is generally associated with replication-inflicted problems, several attempts were made to define a potential role of Rad6 group members in spontaneous mutagenesis in cell cycle-arrested cells (Storchova *et al.*, 1998; Rojas Gil and Vondrejs, 1999; Cejka *et al.*, 2001; Heidenreich *et al.*, 2004). Different point mutation alleles as well as a complete knockout of the name-giving *RAD6* gene resulted in increased frequencies of reversions of base substitution test alleles (Storchova *et al.*, 1998; Rojas Gil and Vondrejs, 1999; Cejka *et al.*, 2001). A more comprehensive study additionally included *rad5*, *rad18*, *rev3*, and *mms2* strains, covering different aspects of the Rad6-dependent pathways (Cejka *et al.*, 2001). The authors classified the strains into three groups according to their influence on adaptive base substitution mutation. The strongest increase in adaptive mutation was observed in the *mms2*, *rad5*, *rad6* and *rad18* single mutants, the *rad5*/*rad6*, *rad6*/*rad18* and *rev3*/*rad6* double mutants and the *rad5*/*rad6*/*rad18* triple mutant. A moderate mutator phenotype was conferred by the *rad5*/*rad18* double mutant. In contrast, a knockout of *REV3* and a double deficiency for *REV3* and *RAD5* resulted in a wild type-like level of adaptive mutation. Thus, whereas most members of this collection putatively antagonize adaptive base substitution formation when functional, the (mostly error-prone) activity of Rev3p, which is the catalytic subunit of the TLS polymerase Pol ζ, was neutral in this assay. Interestingly, although not interpreted by Cejka *et al.* (2001), a functional *REV3* gene was essential for the mutator phenotype of a Rad5 deletion strain (but not a Rad6 deletion strain). This was a first indication that Pol ζ has the potential to act promutagenically in resting cells.

However, the result that a Rev3 deficiency did not influence the frequency of adaptive mutation in the wild-type genetic background was also confirmed in a frameshift detection assay (Heidenreich *et al.*, 2004). In the same study, it was noted that knockouts of the other TLS enzymes present in yeast, Rev1p and polymerase η (encoded by the *RAD30* gene) did not significantly change the incidence of adaptive frameshift mutations either (nor did the triple knockout of all three TLS enzymes simultaneously).

Intriguingly, additional data attest that the translesion synthesis enzymes may contribute to the production of adaptive mutations under certain circumstances (see Section 4.2.2).

4.1.4 Nucleotide Excision Repair

Nucleotide excision repair (NER) is the major pathway for repair of UV-induced DNA damage and other helix-distorting DNA lesions. Repair proceeds by excision of an oligonucleotide comprising the lesion, followed by gap-filling DNA resynthesis with the complementary undamaged strand as a template (de Laat *et al.*, 1999; Prakash and Prakash, 2000; Friedberg *et al.*, 2005). There are two sub-pathways of NER, namely global-genome NER and transcription-coupled NER, which differ in the mode of damage recognition. NER is generally a high-fidelity repair process. If NER fails for some reason, persisting lesions represent hurdles for replication and may trigger mutagenesis by means of error-prone TLS requiring Pol ζ and Rev1p (Lawrence, 2004).

Adaptive mutation assays with NER-deficient strains revealed an unexpected antagonistic function of NER in nonreplicating cells (Heidenreich *et al.*, 2004). This adaptive mutation-counteracting activity was diagnosed for NER in general (represented by the xeroderma pigmentosum A homolog Rad14) but also for the global-genome subpathway alone (represented

by Rad16) and, to a lesser extent, for the transcription-coupled subpathway alone (represented by the Cockayne syndrome B homolog Rad26). It has to be emphasized that these results were obtained without UV irradiation treatment of the cells. We interpret these results to be an indication that even without UV irradiation, a nameable activity of NER is required in cell cycle-arrested cells to repair poorly understood types of spontaneously occurring lesions, which, in the case of a failure of NER, can provoke the formation of adaptive mutations (Heidenreich *et al.*, 2004).

4.1.5 Homology-Dependent Double-Strand Break Repair

DNA double-strand breaks (DSBs) can be repaired by at least three principally different repair pathways: nonhomologous end joining (NHEJ), homologous recombination (HR), and single-strand annealing (SSA) (Haber, 2000; Pastink *et al.*, 2001; Jackson, 2002). NHEJ requires no sequence homology. This pathway is discussed separately in Section 4.2.1.

In contrast, HR and SSA both rely on the existence of homologous sequences, yet they differ in other important aspects (Paques and Haber, 1999; Symington, 2002). HR is a versatile mechanism that uses the sequence information of a nonbroken homologous template molecule to mend the DSB. Depending on the nature of the template, this is usually a high-fidelity repair process. SSA requires tandem repeats on either side of the DSB for an anneal and seal type of reaction. However, this pathway should be regarded as an emergency strategy since it necessarily leads to a deletion of all intervening sequences between the repeats.

An examination of a possible influence of homology-dependent DSB repair pathways on adaptive mutation has been made by the employment of *rad52* and *rad54* strains in a frameshift detection assay (Heidenreich *et al.*, 2003). The Rad52 protein has a dual function in both HR and SSA, whereas Rad54p only is a HR factor (Symington, 2002). Any potential difference between a *rad52* and a *rad54* strain may therefore be tentatively interpreted as an effect of SSA. In haploid strains, the frequency of adaptive reversions was similar to the wild type in a *rad54* knockout strain and even increased in a *rad52* knockout strain (Heidenreich *et al.*, 2003). However, in haploid cells arrested in the G_1 phase, no homologous template for HR is available, which impairs the evaluation of a potential influence of HR on adaptive mutation. To overcome this limitation, we, for the first time, employed diploid strains. In corresponding adaptive mutation assays, it turned out that the influence of the *rad52* and *rad54* deletions was similar in diploid strains and haploid strains. Together, the results with haploid and diploid *rad52* and *rad54* strains suggest that neither HR nor SSA contribute to the formation of adaptive frameshift mutations.

The cause for the mutator phenotype of haploid and diploid *rad52* strains is poorly understood. An interpretation is handicapped by the fact that the experimental design of adaptive mutation assays only allows a detection of reversions, but not of error-free repair (as indicative of HR), repair producing large deletions (as indicative of SSA) or a failure of repair, eventually causing cell death. Additional experiments with deficiencies for further DSB repair factors such as Rad51, and combinations of repair deficiencies are necessary for an understanding of the interplay of different DSB repair mechanisms in resting cells. HR and SSA most likely influence the level of adaptive mutations by competing with NHEJ for the common substrate, DSBs (see Section 4.2.1).

4.1.6 Ras2

One study was devoted to the effect of the *RAS2* gene product on adaptive mutation. *RAS2* and the highly related *RAS1* gene are homologs of the mammalian ras proto-oncogene (Powers *et al.*, 1984; Toda *et al.*, 1986). Ras2 constitutes a plasma membrane-bound molecular switch in a signal transduction pathway that regulates diverse processes like nutritional sensing, UV response, control of lifespan, sporulation, and filamentous growth (Gibbs and Marshall, 1989; Weeks and Spiegelman, 2003).

The study yielded the result that an inactivation of the *RAS2* gene caused an increase in the frequency of adaptive mutation in comparison to the wild type strain and to a strain with a constitutively active version of Ras2 (Storchova and Vondrejs, 1999). Based on the available information on the function of Ras2, the influence of Ras2 on adaptive mutation has to be quite indirect and is unlikely to hint at the identity of adaptive mutation-producing mechanisms.

4.2 Mutation-Producing Mechanisms

4.2.1 Nonhomologous End Joining

Nonhomologous end joining (NHEJ) is one of the major pathways for the repair of DSBs. It is

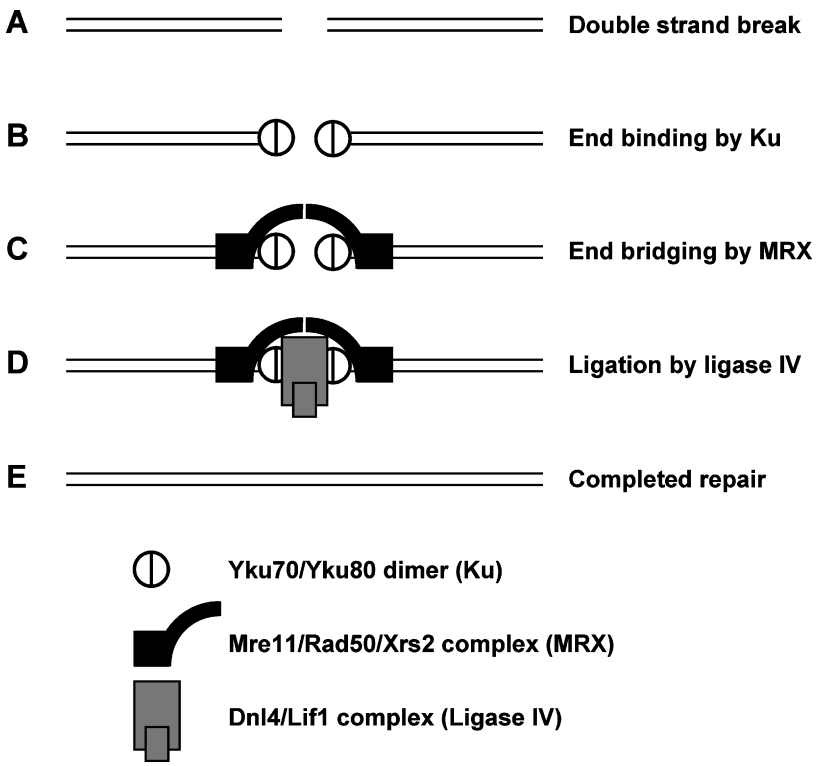

Figure 4 **Sequence of the NHEJ repair process. Since the order of Ku and MRX complex binding is currently unknown, steps B and C could be reversed. Depending on the nature of the double-stranded DNA ends, the activity of nucleases or polymerases, not shown here, may optionally be necessary between steps C and D to obtain ligatable ends.**

able to handle a diverse set of double-stranded end configurations and results in a direct rejoining of ends (Lewis and Resnick, 2000; Daley *et al.*, 2005; Hefferin and Tomkinson, 2005). In *S. cerevisiae*, the repair reaction is mediated by the DNA double-stranded end-recognizing and end-binding activities of theYku70/Yku80 (Ku) heterodimer, the end-bridging function of the Mre11/Rad50/Xrs2 (MRX) protein complex and the ligase activity of the Dnl4/Lif1 complex (Figure 4). Additional proteins might be necessary for a processing of the ends before ligation. In contrast to DSB repair by homologous recombination, no template DNA molecule is required for NHEJ, which makes NHEJ especially valuable in the G_1 (or G_0) phase of the cell cycle.

For an analysis of the influence of NHEJ on adaptive mutation, strains deleted either for the yeast Ku70 gene *YKU70* or the DNA ligase IV gene *DNL4* were employed. Each one of these gene knockouts resulted in an about 50% decreased incidence of adaptive frameshift reversions in haploid *lys2ΔBglII* strains (Heidenreich *et al.*, 2003). Since both genes are essential for NHEJ, the conclusion was drawn that about half of all spontaneous adaptive frameshift mutations depend for their formation on the NHEJ pathway. This was the first time that a gene knockout-induced decrease of spontaneous adaptive mutations (compare Figure 2) provided an insight into an adaptive mutation-producing mechanism in yeast.

The only known substrate of NHEJ is DSBs. Therefore, the model that emerges for the formation of about 50% of the adaptive frameshifts reads as follows: by some unknown mechanism(s), DSBs arise spontaneously in starvation-stressed cells. Since the majority of cells are arrested in the G_1/G_0 phase and thus no second copy of the genome is available in these haploid cells, NHEJ is the preferred DSB repair pathway. NHEJ then rejoins the broken ends, but occasionally introduces a frameshift at the joint, maybe promoted by damaged DNA termini.

In contrast to the hypothetical pathways of mutation formation during starvation shown in Figure 3, this concrete mutagenic process does not require any DNA synthesis, at least for the prevailing reversion type observed, small deletions (see Section 3.3). Experimental evidence supporting this presumption also comes from the analysis of Pol4 knockout strains. Pol4p is the yeast ortholog of mammalian polymerase λ (Burgers *et al.*,

2001) and is an accessory constituent of the NHEJ machinery for the joining of not fully compatible DNA ends. Pol4 not only acts as a polymerase, it also stimulates the processing of 5′ or 3′ terminal mismatches and ligation (Wilson and Lieber, 1999; Tseng and Tomkinson, 2002). Since the adaptive mutation frequency was not decreased in the Pol4-deficient strain, it is likely that none of the activities of yeast polymerase λ is necessary for the formation of adaptive *lys2ΔBglII* allele reversions (Heidenreich and Eisler, 2004).

Unlike mammalian organisms, *S. cerevisiae* has a strong bias to repair DSBs by HR, whenever possible. To be able to evaluate a possible competition of NHEJ with HR, diploid strains were tested for their adaptive mutation properties. Employing the *lys2ΔBglII* frameshift assay again, diploid strains exhibited a 50% reduced frequency of adaptive reversions in comparison to the isogenic haploid strain (Heidenreich *et al.*, 2003). Thus, the diploid wild type resembled the haploid NHEJ-deficient strains regarding the frequency of adaptive mutations. This might be interpreted as a consequence of the well known downregulation of NHEJ in diploid yeast strains. Using the heterozygosity of the mating type locus as a determinant of diploidy, NHEJ is suppressed in diploid cells by a regulatory pathway that results in a lack of nuclear localization of the regulatory subunit of ligase IV, Lif1 (Frank-Vaillant and Marcand, 2001; Kegel *et al.*, 2001; Valencia *et al.*, 2001). To see whether a suppression of NHEJ by this regulatory pathway is responsible for the decreased incidence of adaptive frameshift reversions in the diploid strain, we tested a diploid strain engineered to be homozygous for the mating type locus (Heidenreich *et al.*, 2003). Indeed, the *lys2ΔBglII* reversion frequency in this strain was increased to somewhat more than the level of the corresponding haploid strain, providing support for the hypothesis that the regulation of NHEJ determines the difference in adaptive frameshift frequencies between haploid and diploid *S. cerevisiae* strains.

Besides this regulation of NHEJ in response to ploidy, the effect of competition with the alternative DSB repair pathways HR and SSA has to some extent been examined (Heidenreich *et al.*, 2003). A deficiency for HR and SSA by a knockout of the *RAD52* gene resulted in an increased adaptive frameshift frequency in diploid and haploid strains, but it remains to be demonstrated whether this increase was effected by the NHEJ pathway.

Since it has previously been shown that adaptive reversions of the *lys2ΔBglII* allele predominantly occur as -1 deletions in mononucleotide repeats (Greene and Jinks-Robertson, 1999; Heidenreich and Wintersberger, 2001), it was interesting to ask whether this MSI-like mutation spectrum was also typical for the NHEJ-dependent reversion events. A comparative sequence analysis of reversions in a Dnl4-deficient and a Dnl4-proficient background was performed and showed that the NHEJ-dependent fraction of spontaneous frameshift reversions in the wild-type strain was even slightly more enriched in simple deletions in mononucleotide repeats (Heidenreich and Wintersberger, 2001). This allowed the novel assessment that a MSI-like mutational spectrum might not only be a consequence of unrepaired polymerase slippage errors but also a consequence of NHEJ. MSI is a characteristic feature of various human malignancies, especially colorectal cancers (Speicher, 1995; Karran, 1996; Pawlik *et al.*, 2004; Oda *et al.*, 2005). Although a deficiency for MMR is frequently identified as the most likely source of MSI, part of the microsatellite-unstable tumors apparently are MMR proficient and their genesis awaits another explanation. NHEJ could contribute to such an explanation.

The observed bias for -1 deletions in mononucleotide repeats in the *lys2ΔBglII* reversion system could be caused either by a higher incidence of strand breaks in such repeats or by a random occurrence of breaks, but an enhanced mutagenic repair at such microsatellite sequences. These possibilities have not been sorted out experimentally, but considering the mechanism of NHEJ, it could be meaningful that microsatellite sequences, unlike noniterative sequences, still provide complementarity after a shift in the alignment of DNA ends with small single-stranded overhangs.

4.2.2 Translesion Synthesis Enzymes

The translesion synthesis (TLS) enzymes of *S. cerevisiae* belong to the Rad6 epistasis group briefly introduced in Section 4.1.3. These enzymes are specialized in the bypass of a variety of noncoding lesions.

Rev1 is a deoxycytidyl transferase that is able to incorporate nucleotides opposite of non-coding lesions (Nelson *et al.*, 1996a; Haracska *et al.*, 2001; Lawrence, 2002). The TLS polymerase Pol ζ is a heterodimer composed of the catalytic subunit Rev3 and the regulatory subunit Rev7. Pol ζ is quite tolerant regarding unusual structures at the primer terminus and cooperates with

Rev1 in a mostly mutagenic bypass of noncoding lesions (Nelson *et al.*, 1996b; Lawrence and Maher, 2001; Lawrence, 2002).

In Section 4.1.3, it was reviewed that the wild-type level of spontaneously occurring adaptive mutations was not altered by a deletion of the *REV1* gene or the *REV3* gene.

In contrast, in one study the increase in adaptive nonsense-allele reversions brought about by a Rad5-deficiency (and thus a defect in high-fidelity bypass mechanisms) was abolished in a *rad5*/*rev3* double mutant (Cejka *et al.*, 2001), pointing to a mutation-promoting role of Rev3 in this genetic background. Furthermore, it was noticed that in instances when NER-specific lesions became abundant in the genome of starvation-arrested cells, either by NER-deficiency or by UV-irradiation, the frequency of adaptive frameshift mutations increased significantly (Sections 4.1.4 and 5.1). Intriguingly, corresponding experiments showed that these increased frequencies were dependent on the cooperative function of Rev1 and Rev3 (Heidenreich *et al.*, 2004, 2006).

These results therefore imply that a bypass of noncoding DNA lesions, unexpectedly, is not only necessary in proliferating cells, but also during starvation, at least in the case of an excess of unrepaired NER-specific damage.

It is unclear whether and how a typical substrate for TLS (most likely a gap in one strand opposite of a non-coding lesion on the other strand) might arise in nonreplicating cells. Different models are discussed in Heidenreich *et al.* (2006). Irrespective of this uncertainty, an error-prone bypass of certain structures accomplished by Rev1 and Pol ζ is apparently able to introduce mutations into the genome of cell cycle-arrested cells. An alternative, although purely hypothetical, explanation would be that TLS polymerases introduce premutagenic mismatches during error-prone repair DNA synthesis along an undamaged template, in a manner analogous to scenarios C1 and C2 in Figure 3.

Independently of adaptive mutation assays, it has become evident in the last years that Pol ζ, besides the well established bypass function during replication of damaged DNA, is also required for mutagenesis in the course of a wide range of different cellular processes that are not connected to replication. Pol ζ was in particular identified as required for enhanced mutation frequencies during transcription (Datta and Jinks-Robertson, 1995), recombinational double-strand break repair (Holbeck and Strathern, 1997; Rattray *et al.*, 2002), and interstrand crosslink repair (McHugh *et al.*, 2000; Sarkar *et al.*, 2006).

In summary, the growing number of occasions where Pol ζ gets implicated in an apparent replication-independent occurrence of mutations might augment an emerging recognition of Pol ζ as a quite general mutator in quiescent cells during stressful conditions.

5. INDUCED ADAPTIVE MUTATION

Adaptive mutations arise spontaneously in populations of starving cells without additional environmental mutagenic influences. Most efforts were directed to an elucidation of the mechanisms behind this basal level of endogenously determined mutations. However, in some studies, an increase in the adaptive mutation frequency was induced by exogenous mutagens. The rationale behind this was, on the one hand, an evaluation of the mutagenic potential of the treatment itself, and on the other, the employment of an artificially produced excess of defined DNA lesions as a tool to facilitate the elucidation of adaptive mutation mechanisms.

5.1 UV-induced Adaptive Mutation

The exposure of amino acid-starved cell populations to UV light and a subsequent inhibition of photoreactivation resulted in a dramatic increase of the frequency of adaptive frameshift reversions (Steele and Jinks-Robertson, 1992; Heidenreich *et al.*, 2004, 2006). The predominant type of DNA lesions induced by the short-wavelength UV light used are pyrimidine dimers. Therefore, the conclusion can be drawn that an abundance of pyrimidine dimers in resting cells is somehow converted to an increase in mutations, independently of replication.

Pyrimidine dimers are replication-blocking lesions. In proliferating cells, unrepaired pyrimidine dimers trigger mutagenesis during the next round of replication in that they evoke a partially error-prone bypass. It is less clear, how UV-induced mutations arise in a replication-independent way. However, studies with genetically modified strains showed that the TLS enzymes Rev1 and Rev3 (but not Rad30) are required for the increased levels of adaptive frameshifts following UV irradiation (Heidenreich *et al.*, 2004, 2006). Thus, in terms of their dependency on Rev1 and Rev3, UV-induced

adaptive mutations resemble UV-induced mutations in proliferating cells as well as adaptive mutations occurring in NER-deficient cells (see discussion in Section 4.2.2).

5.2 IR-induced Adaptive Mutation

The influence of ionizing radiation on adaptive mutation was studied by exposing cell cycle-arrested cells to a low dose (25 Gy) of γ-irradiation from a ^{60}Co source (Heidenreich and Eisler, 2004). This treatment resulted in a substantially increased frequency of adaptive reversions of the *lys2ΔBglII* frameshift allele.

Ionizing radiation induces strand breaks in DNA, including double-strand breaks. Since previous work has revealed the importance of the NHEJ DSB repair pathway for the formation of adaptive frameshifts in yeast (Heidenreich *et al.*, 2003), the consequences of a disruption of the NHEJ pathway on the frequency of γ irradiation-induced adaptive mutations have been examined. It was expected that NHEJ is not only essential for a mutagenic processing of spontaneously occurring DSBs but also for a mutagenic processing of an artificially imposed excess of DSBs. Indeed, this assumption was supported by a significantly reduced reversion frequency in a γ-irradiated Dnl4-deficient strain (Heidenreich and Eisler, 2004).

6. UNRESOLVED ISSUES

The mechanisms of adaptive mutation formation are only partially understood, especially for mutation types other than the most frequently studied frameshift mutations (Section 4). However, irrespective of the need for an acquisition of further knowledge on the mechanisms that actually introduce sequence alterations into the genome of nonreplicating cells, there are other open issues that are of interest. These will be pointed out in the following sections.

6.1 The Poorly Defined State of Starving Cells

With respect to its importance for the evolution of microorganisms and, possibly, later stages of carcinogenesis, it is interesting to study adaptive mutation as a response to stressful conditions. However, with regard to a potential role for early stages of carcinogenesis, it would instead be desirable to be able to study adaptive mutation under least-possible stressful G_0 conditions. The latter conditions are obviously not easy to achieve experimentally.

It is not clear to what extent the unusual one-sided starvation conditions typically applied for adaptive mutation experiments (*i.e.*, starvation for a single amino acid or nucleotide precursor) are comparable to a canonical stationary phase, initiated by an exhaustion of carbon source (Herman, 2002; Gray *et al.*, 2004).

A major indication that the conditions during adaptive mutation experiments hitherto in use in yeast are different from a regulated quiescence comes from the observation that in all adaptive mutation studies reported the number of viable cells declines progressively in the course of the experiment.

Generally, only fragmentary evidence is available for the state in which the starving cells are exist. For example, it was noted that during the course of adaptive mutation assays employing amino acid starvation, a remarkable accumulation of mitochondrial rho$^-$ mutants took place (Heidenreich and Wintersberger, 1997). This was interpreted as an indication that the mitochondrial genome is prone to some detrimental effect during one-sided starvation. However, no details of this process have been elucidated.

In the special case of starvation for the nucleotide precursor adenine, it was reasoned that an imbalance of the nucleotide pool might develop, and consequently enhance misincorporation of nucleotides during repair DNA synthesis (Ilyina *et al.*, 1986). However, no definite proof of this assumption has been determined.

One important general point is that glucose (as the standard carbon source) is consistently an ingredient of selective media to allow outgrowth of colonies (or papillae) and thus detection of reversion events. An availability of glucose, though, foils a coordinated transition to stationary phase, since glucose is a major stimulus for an exit from stationary phase (Granot and Snyder, 1991). It has even been shown that the presence of glucose, in the absence of other essential nutrients, drives yeast cells into apoptosis (Granot *et al.*, 2003). This finding is in agreement with results obtained by an analysis of apoptotic markers in *S. cerevisiae* cells starved in the long-term for a single amino acid on glucose-containing media, as is typical for most adaptive mutation assays. In that study, evidence was presented that the observed decline in the number of viable cells largely results from apoptotic cell death (Eisler *et al.*, 2004).

Although technically challenging, it would be worthwhile to develop an adaptive mutation assay in *S. cerevisiae* employing carbon source starvation as a selective condition, analogous for example to the *lacI33* system in *E. coli*. In this system, the sole carbon source supplied is lactose, which can only be used by cells that have acquired an anticipated mutation in the *lac* operon (reviewed by Hall, 1998; Foster, 1999; Rosenberg, 2001).

Additional evidence for the stress imposed by imbalanced nutrients came from the observation of high levels of intracellular reactive oxygen species (ROS) in cells starved for a single amino acid (Eisler *et al.*, 2004). Taken together, these findings imply that the one-sided starvation conditions typically applied in adaptive mutation experiments are quite stressful for the cells and are associated with an increased burden of endogenous mutagenic factors like ROS.

6.2 Is There a Hypermutable Subpopulation?

Whereas genetic stability is of paramount interest during times of opulence, it may be a good strategy to enter a transient hypermutable state during times of nonlethal stress to be able, eventually, to overcome growth-limiting environmental challenges by means of an occasional beneficial mutation (Rosenberg *et al.*, 1998; Rosenberg and Hastings, 2003). However, since considerable portions of random mutations are detrimental, it may be an even better strategy if only quite a small subpopulation of cells enters such a hypermutable state, allowing the majority of cells to wait for better times with an unaltered genome.

The existence of such a hypermutable subpopulation has been proposed by Hall (1990) for the case of adaptive mutation studies in *E. coli*. A testable prediction of this hypothesis is that the frequency of nonselected additional mutations should be higher in adaptive revertant clones than in clones derived from nonreverted cells. Indeed, several studies confirmed this prediction (reviewed by Rosenberg, 1997; Hall, 1998; Foster, 1999), supporting the occurrence of a hypermutable subpopulation during adaptive mutation assays in *E. coli*. Presently, it is not known what makes a cell hypermutable in contrast to isogenic siblings.

It would be interesting to know, whether a hypermutable subpopulation also emerges when eukaryotic cells like yeast cells experience starvation stress. Regrettably, little data are available. Steele and Jinks-Robertson (1992) reported that they failed to detect additional auxotrophies in over 1500 adaptive revertants. However, since it is uncertain whether an effect could have been expected with this number of probes, it is hard to estimate the significance of this result and it is too early to reject the possibility of a transiently hypermutable subpopulation in starving yeast populations.

An exciting new aspect to this issue comes from the recent discovery of distinct subpopulations within stationary-phase yeast cultures (Allen *et al.*, 2006). The authors report that one subpopulation consists of truly quiescent, dense, unbudded cells, whereas another subpopulation of cells obviously has not managed to achieve an ordered transition to quiescence. The latter subpopulation consists of less dense, heterogeneous, replicatively older cells, which are plagued by ROS, prone to apoptosis, and thus resemble in some properties the amino acid-starved cells described by Eisler *et al.* (2004). This differentiation opens up the possibility that such genetically identical but epigenetically differing subpopulations also differ in their adaptive mutability.

6.3 Which DNA Lesions Are Important for Adaptive Mutation and How Do They Arise

Our state of knowledge about adaptive mutation-relevant sorts of DNA damage is regrettably incomplete. Since adaptive mutations arise spontaneously without obvious external influences other than nutritional limitation, correspondingly, spontaneous DNA damage appears to be entirely sufficient for induction of mutagenic processes in cell cycle-arrested cells. Whereas a plethora of different spontaneous lesions affects the genome, we do not know how broad the spectrum of potentially adaptive mutation-triggering lesions is.

DSBs are the only known substrate of the NHEJ repair pathway. Therefore, the identification of NHEJ as an adaptive mutation-producing mechanism (Section 4.2.1) indirectly identifies spontaneously occurring DSBs as a potential source of adaptive mutations, at least for the case of frameshift mutations. The increased incidence of adaptive mutation after γ irradiation and its partial NHEJ dependency further supports this relevance of DSBs (Section 5.2.). Intriguingly, previous

work with *E. coli* also has implicated DSBs in the genesis of adaptive mutations (reviewed by Rosenberg, 2001). However, *E. coli* K12 strains like those used in those studies apparently have no NHEJ-like repair system (Bowater and Doherty, 2006). Instead, DSB repair by homologous recombination seems to trigger adaptive mutagenesis in at least some of a couple of distinct adaptive mutation pathways in *E. coli* (Harris *et al.*, 1994; Ponder *et al.*, 2005).

So far, other types of DNA lesions have only been implicated in yeast adaptive mutagenesis when the cells were predisposed to adaptive mutation by the introduction of a deficiency in mutation avoidance mechanisms. The Rev3- and Rev1-dependent increased incidence of adaptive frameshift mutations in NER-deficient strains suggests some unknown bulky or helix-distorting non-coding lesions as an initiator of adaptive mutation. Furthermore, the increase in adaptive mutation as a consequence of deficiencies in the proofreading activities of polymerases or in MMR suggests an excess of polymerase errors as influential (if we consider polymerase errors as "honorary members" of the big family of DNA lesions).

However, it is unlikely that this listing of potentially adaptive mutation-triggering DNA lesions is complete, especially if we consider that the generating mechanisms of types of adaptive mutations other than frameshifts are poorly studied. It is likely that a future addendum to the adaptive mutation-producing mechanisms will also uncover new types of adaptive mutation-triggering lesions.

Several indications point to a prominent role of oxidative damage as a trigger of adaptive mutation. Reactive oxygen species (ROS) are inevitably generated as byproducts of aerobic metabolism. The increased level of ROS observed in amino acid-starved cells (Eisler *et al.*, 2004) might cause considerable amounts of oxidative damage in the nuclear genome. Among many other types of DNA damage, ROS also produce DSBs and might therefore be the causative agent of NHEJ-mediated adaptive mutation. ROS-induced DSBs have also been proposed as a major source of genetic instability of somatic cells in mammalian pathogenesis (Barnes, 2002) and aging (Karanjawala and Lieber, 2004). Whereas DSBs could plausibly be generated by unavoidable background ionizing radiation from the cosmos or the ground (Thorne, 2003), on the other hand, an addition of the radical scavenger N-acetyl cysteine to the medium resulted in a decreased frequency of adaptive mutation in the *lys2ΔBglII* frameshift assay (Amiri and Heidenreich, unpublished results). Since about 50% of the reversions in this assay were dependent on NHEJ, these results could be interpreted as indication for an oxidative origin of DSBs.

6.4 Is Adaptive Mutation Pathological or an Evolutionary Strategy?

One related issue is the question whether adaptive mutation is just a pathologic byproduct of genotoxic stress or perhaps a kind of evolutionary strategy for overcoming selective barriers by a regulated temporary increase of spontaneous mutagenesis. A mutator phenotype is regarded as useful in times of strong selective pressure by increasing the probability of beneficial mutations (Chicurel, 2001; Giraud *et al.*, 2001; Matic *et al.*, 2004).

In terms of economics, a mutator phenotype would result with the least energy expense from a suboptimal level of repair of the continuously occurring endogenous DNA damage. On the other hand, examples are known where a mutator phenotype is conferred by a regulated induction of additional DNA damage. For instance, a high degree of local variation is specifically induced in the human genome by the mechanisms of V(D)J recombination and somatic hypermutation.

During V(D)J recombination of immunoglobulin and T-cell receptor genes, DSBs are at first produced by an endonuclease and then repaired by NHEJ (Lieber *et al.*, 2004).

During somatic hypermutation, a succession of transcription, deamination of cytosine residues by the activation-induced deaminase AID, and several possibilities of further processing (including TLS pathways) of the resulting uracil residues leads to frequent mutations in the variable region of immunoglobulin genes (Seki *et al.*, 2005; Casali *et al.*, 2006; Martomo and Gearhart, 2006).

Although it is notable that in *S. cerevisiae* the NHEJ process and TLS have been identified as potential sources of genetic variation in the form of adaptive mutations, the evidence at hand does not presently allow an assessment as to whether damage induction and mutagenic repair during adaptive mutagenesis occurs spontaneously or as a regulated response.

7. CONCLUSION

Fifteen years of adaptive mutation research in *S. cerevisiae* have provided interesting insights into that special kind of spontaneous mutagenesis in cell cycle arrested cells. The genuineness of the adaptive mutation phenomenon is now firmly established in this eukaryotic model organism. One finding of fundamental importance was that not only one, but several mechanisms are able to generate spontaneous adaptive mutations. Most information was acquired on the occurrence of frameshift mutations. In their case, the NHEJ pathway of DSB repair stands out as a source of about 50% of the adaptive mutations in haploid strains. The close homology of this pathway in yeast and mammals and the fact that it is the major pathway of DSB repair in human G_1/G_0 cells opens up the possibility that its importance for mutagenesis in cell cycle arrested cells might also apply for human cells.

Other nameable fractions of adaptive mutations most likely arise by errors introduced during limited repair DNA synthesis along either a normal or a damaged template. The coordinated function of the TLS polymerases Rev1 and Pol ζ seems to emerge as a quite universal mutator function in stationary-phase cells, at least when the amount of non-coding lesions is increased above usual (endogenously originating) levels.

The importance of error-prone bypass mechanisms mediated by a group of "sloppier copier" DNA polymerases received a lot of attention recently, especially with regard to the de-blocking of stalled replication forks (Goodman and Tippin, 2000; Lawrence, 2004). However, results obtained with *rev1* and *rev3* strains in adaptive mutation experiments suggest that lesion bypass mechanisms in non-replicating cells might be more relevant than traditionally thought.

For unicellular organisms, the crucial advantage of adaptive mutation in evolutionary terms is that an adaptation to altered environmental conditions does not depend solely on a collective of randomly generated genetic variants that are already present in the population before the onset of an environmental challenge. Instead, adaptive mutation provides a means to generate successful genetic variants subsequent to the start of selective conditions. Whereas a sudden exposure to lethal stress still can only be overcome by the prior existence of mutants in the population, adaptive mutation provides a valuable supplement to the conventional process of mutation and selection when the selective stress is nonlethal (Wintersberger, 1991). Experimental data substantiate that the concept of adaptive mutation has been implemented in prokaryotic as well as in eukaryotic microorganisms (although the degree of conservation is currently unclear).

For multicellular organisms, adaptive mutation is not likely to contribute to evolution of the germ line, since selective forces do not act on the germ cells but on the organism as a whole. However, the analogy of adaptive mutation to carcinogenesis is striking. Carcinogenesis is increasingly recognized as an evolutionary process (Nowell, 1976; Merlo *et al.*, 2006). On the way from the first breakout from tight regulation to full-blown malignancy, tumor cells have to pass several selective barriers (Hanahan and Weinberg, 2000). Overcoming these restraints usually coincides with mutations in certain key genes. Here, it would be likewise interesting to know if genetic variants arise before or after onset of selection. A nameable contribution of time-dependent but replication-independent adaptive mutation mechanisms could be to provide an explanation as to why mutation rates per cell division derived experimentally are not sufficient to account for the observed numbers of specific mutations in individual cancer cells.

In the light of these considerations, adaptive mutation research looks forward to an exciting future.

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