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Hanna Baranowska · Zofia Policińska
Witold J. Jachymczyk

Effects of the *CDC2* gene on adaptive mutation in the yeast *Saccharomyces cerevisiae*

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Abstract We have studied the influence of a temperature-senstitive *cdc2-1* mutation in DNA polymerase δ on the selection-induced mutation occurring at the *LYS-2* locus in the yeast *Saccharomyces cerevisiae*. It was found that in cells plated on synthetic complete medium lacking only lysine, the numbers of Lys^+ revertant colonies accumulated in a time-dependent manner in the absence of any detectable increase in cell number. When *cdc2-1* mutant cells, after selective plating, were incubated at the restrictive temperature of 37 °C for 5 h daily for 7 days, the frequency of an adaptive reversion of $lys^- \rightarrow Lys^+$ was significantly higher than the frequency in cells incubated only at the permissive temperature, or in wild-type cells incubated either at 23 °C or 37 °C. Therefore, when the proofreading activity of DNA polymerase δ is impaired under restrictive conditions, the frequency of adaptive mutations is markedly enhanced.

Key words DNA polymerase δ · *CDC2*
Adaptive mutation · *S. cerevisiae*

Introduction

When populations of microorganisms are subjected to certain forms of strong, non-lethal, selection pressure, changes in DNA sequence cause an appropriate change in phenotype (such as reversion of an amino-acid auxotrophy). If selection is being applied on a plate, useful revertants will form colonies among the non-growing cells and continue to appear for several days after plating, whereas useless mutants do not. This phenomenon has been variously called directed, adaptive, or selection-induced mutation (Foster 1993) and represents a direct challenge to the dogma that mutations occur only randomly and without regard to utility (Lederberg and Lederberg 1952). In recent years the occurrence of adaptive reversion has been well established in *Escherichia coli* (Cairns et al. 1988; Hall 1990, 1991) and subsequently in yeast (Steele and Jinks-Robertson 1992; Hall 1992). Repeating his previous experiments with *E. coli*, Hall found that revertants of a histidine auxotrophy arose after 5 days and continued to arise for an additional 25 days after cells were plated on histidine-limited medium. Steele and Jinks-Robertson investigated the reversion of a defined frameshift mutation at the *LYS2* locus. They studied the reversion behaviour of a *LYS2* frameshift allele in order to determine whether reversion events in yeast occur in a random manner before selective plating or whether they can occur as an adaptive response after selective pressure has been imposed. In genetic and physical analyses of approximately 100 independent Lys^+ revertants, roughly 90% contained an additional, compensatory frameshift in the *LYS2* coding sequence. The remainder were true revertants, none resulted from extragenic suppression. The authors showed in a conclusive manner that when cells were plated on synthetic complete medium lacking only lysine, revertant colonies accumulated in a time-dependent manner, in the absence of any detectable increase in cell number. The revertants of lysine auxotrophy continued to appear for 8 days during lysine selection, but did not accumulate when cells were starved for tryptophane, leucine, or both lysine and tryptophane. The early appearing Lys^+ revertants occurred randomly during non-selective pregrowth of the cultures, while others, late appearing Lys^+ revertants, occurred only after selective plating. Statistical analyses, as well as reconstruction and transformation experiments, indicate in both studies that the late-appearing colonies result from genetic events occurring after selective plating. In the reconstruction

H. Baranowska (✉) · Z. Policińska · W.J. Jachymczyk
Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 5a Pawińskiego Street, PL 02-106 Warsaw, Poland

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experiments Hall used a medium without inositol and Steele and Jinks-Robertson used canavanine; in both cases growing cells would have been killed and in neither case were death rates significantly increased.

At present there is little information concerning the origin of these mutations: whether they result from stress-specific changes in cellular metabolism or whether they reflect normal cellular processes. Several models have been proposed to explain the origin of adaptive mutations after selective pressure has been imposed (for review see Foster 1993). Among them the model presented by Stahl (1988), and subsequently by Boe (1990), drew our special attention. According to their suggestion, selection-induced mutations are the consequence of the slow action of the mismatch repair system in nutritionally deprived cells. When the error-correcting enzymes act slowly, the errors in the transcribed strand may persist long enough to encode an useful protein. The cell could then replicate its DNA before the mismatch was corrected and in turn would fix the new, selectively advantageous, mutations.

We have tested the involvement of DNA polymerase δ(III) in the repair of lesions induced in DNA by MMS in *Saccharomyces cerevisiae*. The catalytic subunit of this polymerase is encoded by the *CDC2* gene. According to Simon et al. (1991) and to our results (Suszek et al. 1993), the temperature-sensitive mutation *cdc2-1* simultaneously affects the DNA polymerase site and the 3′ →5′ exonuclease site, which represents the proofreading activity of the enzyme. Incubation of *cdc2-1* mutant cells at the restrictive temperature of 37 °C resulted in a decrease in survival and in an increase in the frequency of reversion of *meth*, *his* and *trp* mutations. Taking into account this highly error-prone ability, we wanted to study the influence of the *cdc2-1* mutation in DNA polymerase δ on adaptive reversion of the *LYS2* locus in yeast, the same locus for which adaptive reversion had been carefully elaborated earlier by Steele and Jinks-Robertson (1992). We expected that the *cdc2-1* mutation would increase the adaptive reversion of *LYS2* locus under conditions when 3′ →5′ exonuclease activity was affected in mutant cells by restrictive conditions.

Materials and methods

Yeast strains. Two haploid strains were used in this work:
(1) Strain HB80-2A (*MAT***a** *ade2-1 lys2-1 his3-Δ200 trp1-Δ901 ura3-52 meth can1-100 cdc2-1*), which was constructed by standard techniques of yeast genetics (Sherman et al. 1971) from strain MKP-o (*MATα can1-100 ade2-1 lys2-1 leu2-3, 112 his3-Δ200 trp1-Δ901*) kindly donated to us by Dr. Benard Kunz, and strain HB71-18D (*MAT***a** meth his *cdc2-1*).
(2) Strain HB80-2A/1, a phenotypic temperature-resistant revertant of the *cdc2-1* mutation.

Media and growth conditions. Yeast strains were grown non-selectively in YPD medium (1% yeast extract, 2% bactopeptone, 2% dextrose and 2% agar for plates). Strains were grown selectively on synthetic minimal medium (WO) containing all amino acids except lysine. The strains were incubated at 23 °C.

Isolation of revertants.
Colonies were excised from YPD plates and grown overnight in 5 ml of liquid YPD. Cells were harvested from 5-ml cultures by centrifugation, washed with 5 ml of sterile water and resuspended in 1 ml of sterile water. Aliquots of 100 µl, containing approximately 10^8 cells per ml, were plated on selective media to select revertants. Appropriate dilutions were also plated non-selectively on YPD to determine the total number of viable cells in each culture. One part of each plate was incubated immediately at 23 °C and the second part pre-incubated at 37 °C for 5 h of each day of incubation. The number of prototrophic colonies appearing daily on a selective medium was counted, beginning at 3–10 days after plating. For determination of the growth rate of the revertants obtained, the prototrophic Lys^+ revertants were isolated, grown overnight in liquid YPD medium, centrifuged, washed with sterile water and plated selectively in appropriate dilutions on W0-lys medium to identity revertants.

Results

Reversion of a *lys2* frameshift mutation

Steele and Jinks-Robertson (1992) studied the reversion behaviour of a *LYS2* frameshift allele in order to determine whether reversion events in yeast occur in a random manner before selective plating or whether they can occur as an adaptive response after selective pressure has been imposed.

Using approximately similar conditions for studying the appearance of Lys^+ revertants in the temperature-sensitive *cdc2-1* mutant of DNA polymerase δ in yeast, we have examined *lys2* →Lys^+ reversion in 30 independent cultures. Data from one experiment in which five cultures were plated are presented in Table 1. As can be

Table 1 Time-dependent appearance of Lys^+ revertants in five independent cultures of HB80-2A, a *cdc2-1* mutant strain. Data obtained from five independent cultures are given. Each number given represents the sum of the colonies on all six plates for a given day post-plating. The food increase for each culture is the ratio of the cumulative number of colonies through day 10, to the number of colonies counted on the 3rd and 4th day ("each appearing" revertants)

Colonies ($\times 10^7$/ml)	Number of newly arising colonies on day:							Fold increase
	3	4	5 + 6	7	8	9	10	
74	5	16	127	11	11	7	7	8.8
96	26	8	92	5	6	2	5	4.2
55	9	24	69	9	4	2	3	3.6
66	5	23	141	5	4	4	2	6.6
63	23	74	157	12	9	2	0	2.9
Mean								5.2
	Temp 37°C (5 h)							
17	17	63	75	21	7	0	16	2.5
16	12	40	43	12	3	5	2	2.3
16	5	16	20	7	8	5	1	2.9
18	5	87	62	9	6	5	0	1.8
18	16	144	50	7	0	3	2	1.4
Mean								2.2

Table 2 Time-dependent apearance of Lys^+ revertants in all 30 independent cultures of HB80-2A, a *cdc2-1* mutant strain

No. of CFU[1] ($\times 10^7$/ml)	Temperature	Total number and frequency ($\times 10^{-7}$) of newly arising colonies on day:					
		3	4	5	6	7	8
38	23 °C	109	3084	1517	426	179	43
survival 100%		0.3	5.2	2.6	0.7	0.3	0.1
14	37 °C	426	2684	1090	318	186	75
survival 36%		4.3	11.2	4.5	1.3	0.7	1.5

[1] CFU – colony forming unit

seen, the number of Lys^+ revertants continued to increase after the initial appearance of prototrophic colonies on day 3. Thus by day 10, the average increase in the number of revertants per culture was 5.2-fold for 23 °C, and 2.2-fold for 37 °C. It should be noted that the number of late-appearing revertants is independent of the number of early appearing revertants, as was observed previously by Steele and Jinks-Robertson (1992). A summary of the results of the appearance of time-dependent Lys^+ revertants in all 30 independently tested cultures is presented in Table 2.

Role of the *CDC2* gene in adaptive mutation

We studied the distribution of the total numbers and frequencies of Lys^+ revertants among all 30 independent cultures of *cdc2-1* mutant cells, which appeared at permissive (23 °C) and restrictive (37 °C) temperatures. As can be seen from Fig. 1, the total frequency of newly arising colonies from day 3 to day 8 is always higher at the restrictive temperature than that obtained at the permissive temperature. This increase in the frequency of reversion is accompanied by a decrease in the survival of cells (Table 2). Therefore, in *cdc2-1* mutant cells, the restrictive temperature of 37 °C has an influence not only on the activity of DNA polymerase δ but also on the efficiency of the time-dependent appearance of Lys^+ revertants. This dependence is even more evident in the comparative experiment, when mutant strain HB80-2A (*cdc2-1*) and its revertant HB80-2A/1 with a wild-type activity of the gene *CDC2* were tested simultaneously. (Fig. 2). In contrast to the HB80-2A strain, no increase in the frequency of reversion at 37 °C was observed for the HB80-2A/1 strain. The cumulative frequencies of prototrophs on days 5 through 8 post-plating for strain 2A/1 at both temperatures are the same. Moreover, the level of reversion frequency obtained for strain 2A/1 at 23 °C and 37 °C was similar to that obtained for strain 2A at the permissive temperature of 23 °C. It should be noted that the *cdc2-1* mutation also affects mutations occurring during pre-growth of colonies on selective media. As can be seen from Table 2 and Fig. 1, the frequency of early revertants appearing on day 3 was markedly higher in

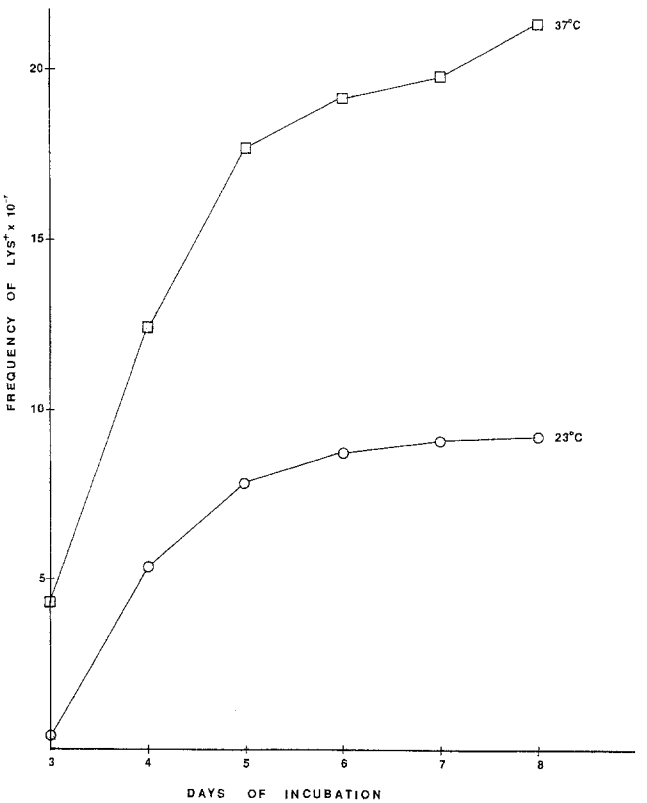

Fig. 1 Total frequency of revertants ($Lys^+ \times 10^{-7}$) in the HB80-2A, *cdc2-1* mutant strain. *Open circles* incubation at 23 °C. *Open squares* pre-incubation at 37 °C for 5 h daily

colonies incubated at 37 °C than at 23 °C. Therefore, mutation *cdc2-1* causes an alteration in the pathway(s) by which DNA errors are generated and fixed during selection pressure.

Special tests were done to determine whether the differential appearance of Lys^+ revertants is the result of a direct response to selection or to the growth rate of the revertants. According to the second possibility an early appearing colony could correspond to a fast-growing revertant, while the late-appearing colony would have a slow growth rate (Hall 1990; Steele and Jinks-Robertson 1992). This was tested by experiments with revertants which appeared at defined times (day 3, day 4 ... till day 8) during reversion assays at 23 °C. In these experiments approximately 100 cells of each

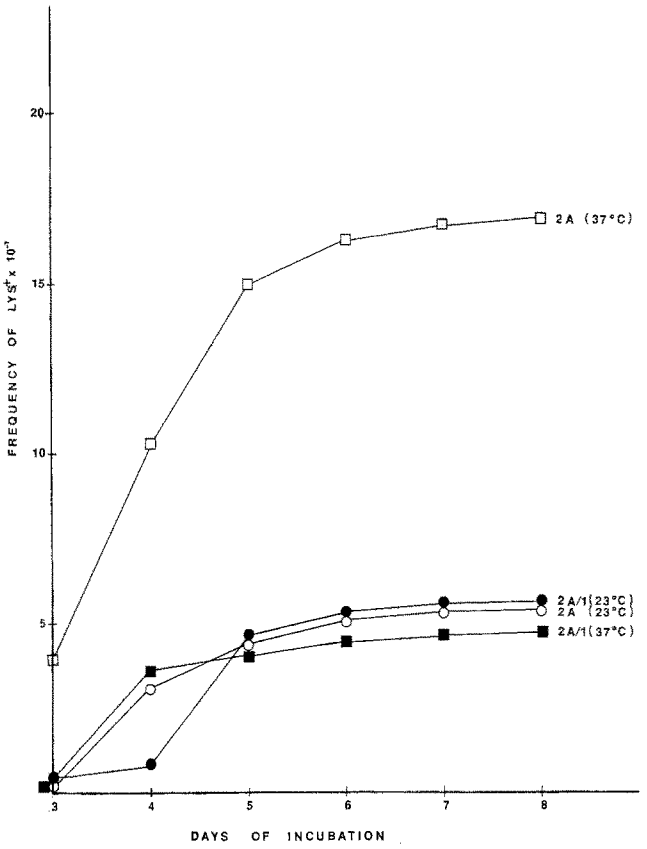

Fig. 2 Total frequency of Lys^+ revertants ($\times 10^{-7}$) in the HB80-2A, *cdc2-1* mutant and HB80-2A/1, wild-type strain. *Open symbols* *cdc2-1* mutant strain, *closed symbols* wild-type strain. *Circles* incubation at 23 °C; *squares* pre-incubation at 37 °C for 5 h daily

revertant were plated on selective medium and the time frames in which colonies appeared were determined. All revertants appeared within 3 days after plating, regardless of the time when the individual revertants initially appeared in the original reversion assay. Thus, all Lys^+ revertants have an approximately similar rate of growth, independently of their original time of appearance.

We also examined the possibility of growth of *lys*$^-$ cells after selective plating, since the occurrence of late-appearing Lys^+ revertants described above could be explained if the auxotrophic cells continued to divide after selective plating. Growth on W0-lys medium was studied by washing cells off the plates immediately after plating (day 0) and at various days post-plating. Cells were counted in a haemacytometer to determine the total number of cells and appropriate dilutions were plated on YPD plates at the permissive temperature to determine the total number of viable cells. After 6 days of incubation on W0-lys plates at 23 °C the survival rate ranged from 60% to 25% of that observed at day 0. After 8 days of incubation the survival rate ranged from 54% to 24%. When plates were incubated at 37 °C during 5 h of each day of incubation, the survival was 22–7%. Therefore, no cell growth was detected on W0-lys selective medium in these experiments and the numbers of viable cells decreased with the time of incubation. These data were similar to those obtained earlier for Lys^+ revertants in *S. cerevisiae* by Steele and Jinks-Robertson (1992) and for His^+ revertants by Hall (1992).

Discussion

The occurrence of adaptive mutation seems to be well established in *S. cerevisiae*, although the studies on this organism were not as vigorous as those on *E. coli*. Results obtained by Hall (1992) and by Steele and Jinks-Robertson (1992) are very convincing and provide evidence for adaptive mutation in a simple eukaryote as well as in bacteria. The question of whether mutations can be induced under starvation conditions when cells are not dividing is not particularly original. Even taking into account that DNA synthesis is required to produce most types of mutation, it is known that cells in stationary phase sporadically replicate or repair their DNA, although the amount of "de novo" DNA synthesis that occurs in starving cells is small. According to a few studies that have been done with stationary phase cells, the amount of DNA synthesis in non-dividing cells is in the range of 0.5–5% per genome per day (Ryan 1961; Boe 1990; Foster 1993). Taking into account a high rate of mutations arising in non-dividing cells, it seems that the DNA turnover (i.e. continuous DNA degradation and re-synthesis without a net change in cellular DNA content) that takes place during starvation should be more error-prone that normal DNA synthesis. Starvation undoubtedly depletes the cells for deoxyribonucleotide substrates, which may interfere with the regulation of the ratios of the four bases (Davis 1989). Such metabolic changes might be expected to affect both replication errors and the fidelity of DNA repair mechanisms. Also the loss or malfunctioning of repair enzymes may be expected among the changes in protein composition that have been observed during starvation. Errors that appear in DNA under the influence of all such changes in cellular metabolism may help explain the appearance of spontaneous mutations in starving cells. What is surprising, however, is that the observed mutations seem to arise only when they are directly advantageous to the cell, or else that the selective pressure fixes those mutations which otherwise might only be transient.

Between several models which attempt to explain the phenomenon of adaptive mutation, we pay special attention to that suggested by Stahl (1988) and subsequently by Boe (1990). According to this model, the main cause of the appearance of adaptive mutations in starving cells is the slow repair of lesions formed in DNA during starvation. The authors suggested that

during continuation of DNA metabolism under starvation, chromosomal nicks and gaps accumulate. Repair synthesis of lesions by DNA polymerase(s) would be expected to introduce mismatches into DNA which, when incorporated into the transcribed strand, would occasionally result in the production of a functional gene product and thus would allow cell growth to begin. If the mismatch repair system functions poorly or particularly slowly, or alternatively if DNA polymerase is particularly error-prone under starvation conditions, then errors may persist in the newly synthesised strand. If this DNA is transcribed and produces a useful protein, the cell will replicate its DNA, fixing the error and immortalising the selectively advantageous mutation.

Taking together this idea and our earlier observation that the *cdc2-1* mutation affects the proof-reading !activity of DNA polymerase δ in yeast, we studied the influence of the *cdc2-1* mutation on the rate of adaptive mutations in yeast. As was indicated in this work, when after selective plating, mutant cells, were incubated at the restrictive temperature 37 °C for 5 h daily during 7 days, the frequency of an adaptive reversion of $lys^- \rightarrow Lys^+$ was significantly higher than the equivalent frequency in cells incubated only at the permissive temperature, or in wild-type cells incubated either at 37 °C or 23 °C. Therefore, when the proof-reading activity of DNA polymerase δ is impaired under restrictive conditions, the frequency of adaptive mutations is markedly enhanced. We believe that these results reinforce in some way the slow DNA repair model for the appearance of adaptive mutation in cells, as proposed by Stahl in 1988.

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