A Genomics-Based Screen for Yeast Mutants With an Altered Recombination/End-Joining Repair Ratio

Thomas E. Wilson

Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0602

Manuscript received May 3, 2002
Accepted for publication July 19, 2002

ABSTRACT

We recently described a yeast assay suitable for genetic screening in which simple religation nonhomologous end-joining (NHEJ) and single-strand annealing (SSA) compete for repair of an I-SceI-created double-strand break. Here, the required allele has been introduced into an array of 4781 *MAT***a** deletion mutants and each strain screened individually. Two mutants (*rad52* and *srs2*) showed a clear increase in the NHEJ/SSA ratio due to preferential impairment of SSA, but no mutant increased the absolute frequency of NHEJ significantly above the wild-type level. Seven mutants showed a decreased NHEJ/SSA ratio due to frank loss of NHEJ, which corresponded to all known structural/catalytic NHEJ components (*yku70*, *yku80*, *dnl4*, *lif1*, *rad50*, *mre11*, and *xrs2*); no new mutants in this category were identified. A clearly separable and surprisingly large set of 16 other mutants showed partial defects in NHEJ. Further examination of these revealed that *NEJ1* can entirely account for the mating-type regulation of NHEJ, but that this regulatory role was distinct from the postdiauxic/stationary-phase induction of NHEJ that was deficient in other mutants (especially *doa1*, *fyv6*, and *mck1*). These results are discussed in the context of the minimal set of required proteins and regulatory inputs for NHEJ.

EUKARYOTIC cells possess two enzymatically distinct pathways for double-strand break repair (DSBR; reviewed in Paques and Haber 1999; Jackson 2001). Repair by homologous recombination involves the concerted action of the *RAD52* epistasis group of genes (*RAD50–52*, *54–55*, *–57*, *–59*, *MRE11*, and *XRS2*). The products of these genes, along with other cellular factors, execute a resection of the 5′ ends at a DSB to create 3′ nucleoprotein filaments, followed by strand exchange with a homologous donor duplex, synthesis from the broken 3′ termini, and ultimately resolution of the extended D-loop. In the absence of a donor duplex, but where a sequence is repeated in tandem on either side of the break, Rad52 and Rad59 can also catalyze the direct annealing of the two resected 3′ ends independently of the other epistasis group members to create a deletion in a pathway known as single-strand annealing (SSA; Ivanov *et al.* 1996; Shinohara *et al.* 1998; Sugawara *et al.* 2000). In contrast, repair by nonhomologous end-joining (NHEJ) entails engagement and likely end-to-end bridging by the Ku heterodimer (Yku70/Yku80 in budding yeast; Jones *et al.* 2001) and ultimately ligation by the DNA ligase IV/XRCC4 complex (Dnl4/Lif1 in yeast; Wilson *et al.* 1997; Herrmann *et al.* 1998). Interestingly, the Mre11-Rad50-Xrs2 complex is also required for NHEJ, suggesting an early role for this complex in DSBR (Petrini 1999). Other genes are only variably required for NHEJ. *POL4* is required for yeast NHEJ only when the termini are incompatible and require processing prior to religation (Wilson and Lieber 1999). Artemis and DNA polymerase μ likely serve similar roles during mammalian NHEJ (Ma *et al.* 2002; Mahajan *et al.* 2002). Mutants of *SIR2-4* are NHEJ deficient, but only because they are functionally of the **a**/α mating type, which represses NHEJ by a mechanism now known to involve *NEJ1* (Lee *et al.* 1999; Frank-Vaillant and Marcand 2001; Kegel *et al.* 2001; Valencia *et al.* 2001). Finally, higher eukaryotic cells depend on DNA-PKcs for efficient NHEJ, but this gene is not conserved in budding yeast (Smith and Jackson 1999).

Many questions remain regarding the mechanism of each DSBR pathway, as well as how these seemingly competitive and redundant processes are coordinated to optimize the likelihood of genome restoration. Genetic screens for DSBR-deficient mutants have played an important role in the discovery process, but with limitations. In mammalian systems, studies of radiosensitive Chinese hamster cell lines (Thompson *et al.* 1980) and immunodeficient mice and human patients (Gennery *et al.* 2000) have revealed some components of both homologous and nonhomologous repair mechanisms, but the laboriousness of the genetic manipulations and diploid nature of the cells have left the picture incomplete. In yeast, early screens for radiosensitive mutants revealed the *RAD52* epistasis group genes (Resnick 1969; Game and Mortimer 1974), but uniformly failed to detect NHEJ components due to the relatively greater importance of recombinational repair. Indeed, the power of

Address for correspondence: Department of Pathology, University of Michigan Medical School, 1301 Catherine Rd., M4214 Med Sci I, Box 0602, Ann Arbor, MI 48109-0602. E-mail: wilsonte@umich.edu

the yeast genetic system for screening DSBR mutations has not been fully realized due to this and other technical limitations.

In this study, I capitalized on three recent technological developments to perform a comprehensive yeast genetic screen that had the ability to find not only those mutants deficient in the SSA and NHEJ repair pathways, but also those that changed the relative NHEJ/SSA repair ratio. These developments were, first, the availability of array sets of deletion mutants of nearly all genes of *Saccharomyces cerevisiae* (Winzeler *et al.* 1999); second, the description of an assay, termed suicide deletion, that not only can detect both SSA and NHEJ repair events in a simple plating format, but also can distinguish them and reveal their ratio by a simple color readout (Karathanasis and Wilson 2002); and third, the development of techniques to rapidly introduce the critical test allele into the mutant array (Tong *et al.* 2001; Vance and Wilson 2002). The screen revealed all known, but no novel, genes required for catalysis of NHEJ, as well as several novel genes that proved to serve two separable regulatory roles promoting NHEJ in the haploid and postdiauxic/stationary growth stages.

MATERIALS AND METHODS

Yeast strains, manipulation, and media: All strains were isogenic derivatives of BY4741 (Brachmann *et al.* 1998). The subarray of DNA damage response mutants will be described elsewhere (Vance and Wilson 2002). The *MAT***a** array was generated by the *Saccharomyces* Genome Deletion Project (Winzeler *et al.* 1999) and obtained from Research Genetics (Birmingham, AL). Media were as described (Karathanasis and Wilson 2002). Mutant arrays were manipulated using ethanol-sterilized manual replicators.

Plasmid construction: All PCR for the following constructions was performed using the Advantage HF high-fidelity PCR kit (CLONTECH, Palo Alto, CA). pMATa was constructed by ligating a PCR fragment corresponding to the *MAT***a** allele of BY4741 into the *Bam*HI and *Sal*I polylinker sites of the *CEN/HIS3* vector pRS413. pNEJ1 plasmid was made as follows. First, we constructed pTW367, a derivative of the *CEN/LEU2* plasmid pTW300 (Wilson and Lieber 1999) in which a single *Sma*I site was created downstream of the *ADH1* promoter and start codon. *Sma*I-digested pTW367 was then cotransformed into a *nej1*Δ strain with a PCR fragment corresponding to the *NEJ1* coding sequence that bore 5′ tails to direct gap repair with pTW367 (5′-ACCATGGCGTCCGAGCAAAAGCTCATTTC TGAAGAGGACTTGCGC and 5′-TTTATGTAACGTTATAGA TATGAAGGATTTCATTCGTCTGTCGAC for the amino- and carboxyl-terminal sides, respectively). The pNEJ1 plasmid was recovered from an isolate in which the *nej1* mutation had been complemented prior to reintroduction into other strains.

Allele and strain construction: Construction of the *ade2*::SD2+::*URA3* and *ade2*::SD0+::*URA3* alleles has been described previously (Karathanasis and Wilson 2002). The *ade2*::SD2+::*URA3* allele was further modified to facilitate high-throughput mating by adding to it the α-mating-type-specific marker gene *STE3-MET15*. This was achieved by creating PCR fragments corresponding to the *STE3* promoter region and *MET15* coding sequence and then fusing them in a second round of PCR by virtue of overlaps in the primers at the *STE3-MET15* junction. The product was then recombined into the *ADE2-RGA1* intergenic region by virtue of tails on the outside primers. The function of both *ADE2* and *RGA1* was preserved because no genomic sequence was deleted and the insertion point was in the 3′ untranslated region of both genes. The resulting strain, YW798 (*MAT*α *ade2*::SD2+::*URA3*::*STE3-MET15 his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0), was identical to BY4741 except at the *MAT* and *ADE2* loci.

The strategy used to introduce the suicide deletion allele into the arrays will be described in detail elsewhere (Vance and Wilson 2002). Briefly, YW798 was mated to an array in liquid culture in microtiter dishes and then sporulated on plates. Spore cultures were transferred into microtiter dishes, diluted, and then spotted back to glucose germination plates lacking methionine and uracil and containing 200 μg/ml G418. Red colonies on these plates must be haploid *MAT*α *ade2*::SD2+::*URA3*::*STE3-MET15 mutx*Δ::kanMX4 (where *MUTx* refers to any of the genes deleted in the different array strains) because the *STE3* promoter is active only in cells of the α mating type. These were picked and passaged twice in the same liquid medium prior to spotting. All screen positives were repurified prior to quantitative testing, and the identities of the *doa1*, *fyv6*, and *mck1* mutants were all verified by allele-specific PCR.

The *nej1 mutx* double-mutant strains were constructed by first isolating the *nej1*Δ::kanMX4 strain from the *MAT***a** array and changing its marker from kanMX4 to *LEU2* by PCR-mediated replacement. The resulting *nej1*Δ::*LEU2* allele was then introduced into a mini-array of the desired *MAT*α *ade2*::SD2+::*URA3*::*STE3-MET15 mutx*Δ::kanMX4 strains by the above method except that leucine was also omitted from the media.

Suicide deletion screening assay: Three assay plates were routinely spotted (all additionally lacked methionine and contained 200 μg/ml G418): glucose complete (as a growth control), galactose complete (to detect mutants with an increased NHEJ/SSA ratio), and galactose lacking adenine (to detect NHEJ-deficient mutants; see Figures 1 and 2 for phenotype scoring). In retests, a fourth glucose plate lacking adenine was also spotted to rule out the presence of rare contaminating diploid cells that had somehow managed to become Met^+; when detected, these mutants were purified prior to further testing. Scoring of plates was performed by scanning them into computer image files. These were magnified and examined using a Microsoft Access database where all spots for an individual strain could be readily compared. Numerous parameters were recorded for each strain that were subsequently used to assign one of the following screen outcomes: failure (insufficient growth to score), negative (no significant difference from the typical spot appearance), or positive (spot growth or color differed on galactose but not on glucose plates). All primary data from the screen can be viewed at http://tewlab.path.med.umich.edu/SDScreen/frames.html.

Suicide deletion quantitative assay: Two different methods, which differ by whether DSB induction occurred on plates or in liquid medium, were used. For each, source cultures were routinely grown in synthetic complete glucose medium lacking uracil and containing 40 μg/ml adenine (further lacking histidine and/or leucine as required for plasmid maintenance) for 48 hr to ensure that even slow-growing mutants had achieved early stationary phase. When indicated, exponential-phase source cultures were instead grown from high dilution in the same medium overnight so that the OD_{600} was <1.0 in the morning. In method 1, 10-fold serial dilutions of the glucose source cultures were made in water, and appropriate volumes plated to synthetic defined medium. The absolute frequencies of SSA and NHEJ were determined by the percentage survival on galactose plates relative to parallel platings to glucose, where completely red colonies on galactose complete were counted as SSA events, and all colonies on galactose lacking adenine were counted as NHEJ events. The NHEJ/

SSA repair ratio was calculated from these. In method 2, 5×10^6 cells from the glucose source cultures were used to inoculate 2.5 ml of synthetic complete galactose medium containing 40 μg/ml adenine (further lacking histidine and/or leucine as required for plasmid maintenance), followed by shaking at 30° for 48 hr. Tenfold serial dilutions of these cultures were made and appropriate volumes plated to synthetic defined glucose plates with and without adenine. The NHEJ/SSA ratio was calculated by dividing the corrected colony count from the plate lacking adenine by the corrected red colony count from the plate containing adenine.

Viability and thermotolerance in stationary phase: Exponential-phase cultures were diluted to a calculated starting OD_{600} of 0.005 in 20 ml YPAD and allowed to grow with shaking in 50-ml tubes for 13 days. After most cultures had reached an $OD_{600} \sim 5$ (empirically determined to correspond to the diauxic shift), the colony-forming units (cfu) per milliliter of the cultures were determined at various time points by plating appropriate dilutions to YPAD and counting colonies formed after 3 days at 30°. Because the *fyv6* strain never achieved as high a density as the other mutants tested, the cfu per milliliter values obtained for each strain were normalized to the average of the values obtained for the first three time points (*i.e.*, before the onset of stationary phase) to facilitate comparison of strains. At the last three time points, a 500-μl aliquot was heat-shocked in a 55° water bath for 5 min, followed by cooling on ice for 5 min, prior to diluting and plating to determine the thermotolerant cfu per milliliter.

RESULTS

Sensitivity of the suicide deletion screen: The DSBR assay used throughout this study is based on the *ade2*::SD2+ suicide deletion allele diagrammed in Figure 1A and described in detail in Karathanasis and Wilson (2002). Briefly, the I-SceI endonuclease creates two DSBs when its expression is induced with galactose, allowing repair in which the two chromosome ends are either ligated by NHEJ or joined by SSA via 28-bp terminal direct repeats. These DSBs result in the excision of the *GAL1*-I-SceI gene cassette from the chromosome that terminates I-SceI expression and largely prevents the recleavage of the newly created I-SceI site that would otherwise prevent outgrowth of the efficient simple religation NHEJ event. SSA and NHEJ events are distinguished by *ADE2* status, allowing for both selection for the Ade^+ NHEJ events as well as color readout in both screening (*i.e.*, spotting) and quantitative plating formats. NHEJ repairs ~3% of the broken chromosomes in this system whether or not the direct repeats are present, with SSA being ~10-fold more efficient (Karathanasis and Wilson 2002).

To estimate the sensitivity of the *ade2*::SD2+ suicide deletion assay in screening format, I spotted the wild-type strain as well as strain mixtures in which varying percentages had been replaced with either *rad52* or *yku70* mutant cells (Figure 1B). When spotted to galactose-complete plates, the mixture containing 90% *rad52* cells could reliably be detected as SSA-deficient as evidenced by increased whiteness of the spot. When spotted to galactose plates lacking adenine, the mixture containing 80% *yku70* cells could reliably be detected as

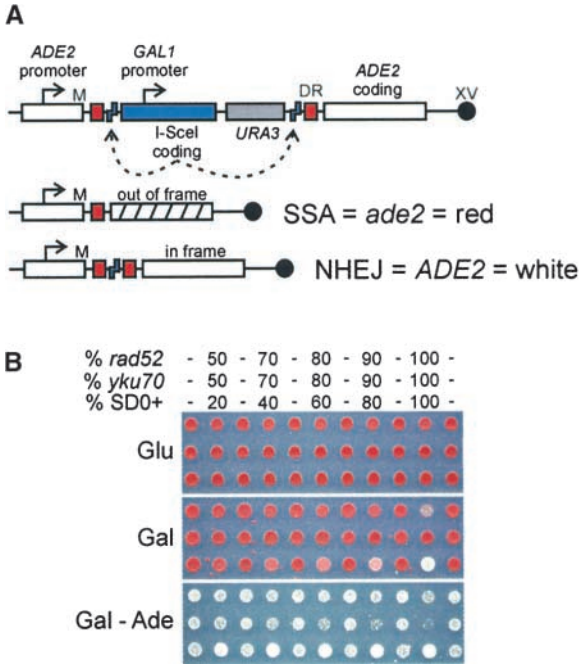

Figure 1.—Sensitivity of the suicide deletion screen in spotting format. (A) Diagram of the *ade2*::SD2+ suicide deletion allele used in these experiments. After induction with galactose, I-SceI is expressed and cuts its gene from the genome, along with the *URA3* marker, via two flanking cleavage sites. The resulting chromosome ends can be fused by SSA via the 28-bp direct repeat (red box, DR) or by simple religation NHEJ, which for this allele give *ade2*/red and *ADE2*/white yeast, respectively. (B) A test mini-array was spotted to a glucose complete control plate (top) as well as galactose indicator plates with and without adenine (middle and bottom, respectively). Positions marked "-" all contained pure cultures of wild-type yeast bearing the *ade2*::SD2+ allele shown in A. Positions marked with numbers contained the indicated percentages of the following strains: *rad52* and *yku70* yeast bearing the same *ade2*::SD2+ allele and wild-type yeast bearing the *ade2*::SD0+ allele (not drawn) for which SSA events are *ADE2*/white and NHEJ events are *ade2*/red. This last strain served as a sensitivity indicator for *ade2*::SD2+ mutants in which the NHEJ/SSA (*i.e.*, color) ratio was reversed (see text). The remaining fraction of the mixtures was made of wild-type yeast bearing the *ade2*::SD2+ allele.

NHEJ deficient as evidenced by decreased spot density. These correspond to detection limits of an ~10-fold decrease in SSA and a 5-fold decrease in NHEJ. The screen was also designed to detect putative regulatory mutants in which the total repair rate was preserved but the NHEJ/SSA repair ratio was increased. Since no such mutant was known, this sensitivity was estimated by mixing the wild-type *ade2*::SD2+ strain with a wild-type strain bearing the *ade2*::SD0+ allele (Karathanasis and Wilson 2002), for which SSA events are *ADE2*/white and NHEJ events are *ade2*/red, thus mimicking a partial to complete reversal of the color ratio. The screen was in fact most sensitive to this mutant class, being able to consistently detect a mixture containing only 40% *ade2*::SD0+ cells, corresponding to a <2-fold change in the color ratio.

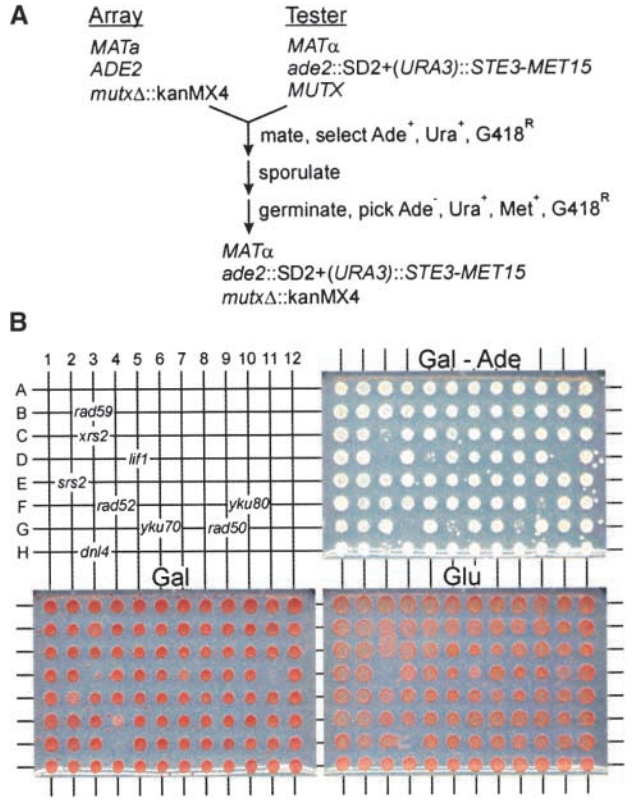

FIGURE 2.—Validation of the suicide deletion spotting screen using a "subarray" of DNA damage response mutants. The *ade2*::SD2+ allele was introduced into an array of 96 mutants by the method diagrammed in A and described in MATERIALS AND METHODS. The final strains then were spotted to the control and indicator plates (B). Grid positions that contain mutants known to be defective in SSA or NHEJ are labeled. The identities of the remaining mutants are omitted here for clarity; the complete searchable data set may be viewed at http://tewlab.path.med.umich.edu/SDScreen/frames.html. Two strains could not be picked in this experiment and so their grid positions are empty (grid positions D3 and G4, corresponding to *rad6* and *mre11*, respectively). Grid position D1 corresponds to *msh1*; this strain illustrates the phenotype typical of a petite/very slow-growing mutant and does not reflect a DSBR deficiency of this strain.

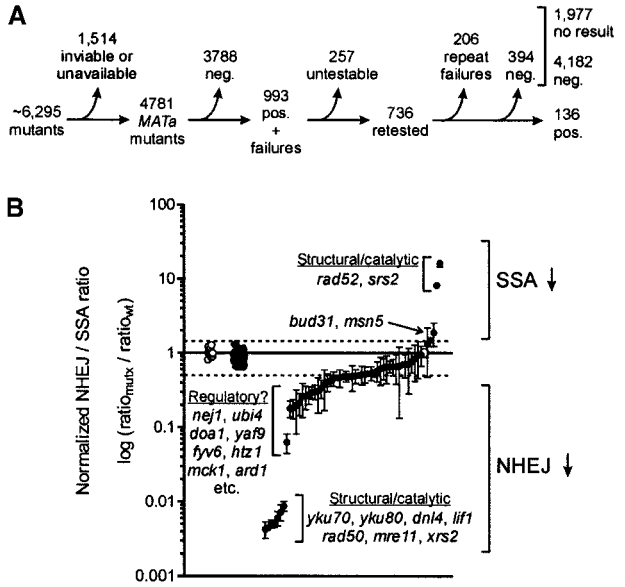

FIGURE 3.—Results of the screen of the complete *MAT*a array. (A) The chart shows the number of mutants removed from consideration at various stages of the screening process. Untestable mutants and screen failures are described in the text; the majority of these were petite. The 736 retested strains were picked as new segregants from fresh mating and sporulation reactions. (B) The 136 mutants positive by spotting were subjected to quantitative analysis by method 1. The data are plotted here as the average mutant NHEJ/SSA ratio expressed relative to the average wild-type NHEJ/SSA ratio. The cluster of open circles to the left in the chart represents replicates of the wild-type strain as a demonstration of the inherent variability in the assay (the average of these points is 1.0). The cluster of solid circles to the right of this corresponds to the majority of mutants, tested only once since they showed no defect. The remaining points are the mean ± standard deviation for the wild-type strain (open circle) and those mutants tested in replicate (solid circles), ordered from lowest to highest NHEJ/SSA ratio. The horizontal dashed lines indicate the approximate NHEJ/SSA ratios that corresponded to the statistical significance thresholds of Tables 1 and 2.

Validation of the suicide deletion screen with a panel of DNA damage response mutants: Prior to embarking on large-scale screens, the approach was further validated using a single-plate "subarray" of deletion mutants known to be deficient in the spectrum of damage response functions. To perform this test screen, as well as the full screen described below, it was of course necessary to introduce the *ade2*::SD2+ allele into the mutant strains. This was accomplished by a high-throughput mating strategy as described in MATERIALS AND METHODS and Figure 2A. The final subarray contained three mutants known to be deficient in SSA and six deficient in NHEJ. All were readily scored as positive except *rad59* (Figure 2B). This mutant also proved negative on further screening, which likely reflects the atypical nature of the suicide deletion SSA event (see DISCUSSION). No other mutants showed a defect, demonstrating the power of examining strains individually and the specificity of the phenotypes for the predicted changes in DSBR efficiency.

Suicide deletion screen of 4781 haploid deletion mutants: The *ade2*::SD2+ suicide deletion allele was next introduced into an array of nearly all viable haploid yeast deletion mutants (WINZELER *et al.* 1999), and the resulting strains were scored for the phenotypes described above. The screen progress is diagrammed in Figure 3A. A total of 136 strains were ultimately scored as positive. This number is large because the inclusion threshold was deliberately kept low to maintain high sensitivity. Quantitative analysis provided a facile and precise way of subsequently eliminating the false positives. This was initially performed using method 1, in which cells were plated to galactose so that both absolute and relative repair frequencies could be assessed (see MATERIALS AND METHODS). The NHEJ/SSA repair ratio was examined first. This parameter provides the greatest power

TABLE 1

Mutant classes observed

Mutant class	NHEJ/SSA	NHEJ	SSA	No. found
I	↑	No Δ	↓	1[a]
II	Variable	↓	↓	1[b]
III	↑	↑	No Δ or ↓	2?[c]
IV	↓	↓	No Δ	24[d]

[a] Cutoff criteria were $P < 0.0001$ for the NHEJ/SSA ratio and a >1.5-fold decrease in SSA as compared to wild type. This mutant is *rad52*.

[b] Cutoff criteria were $P < 0.0001$ for the NHEJ/SSA ratio, a >1.5-fold decrease in SSA, and a >2-fold decrease in NHEJ as compared to wild type. This mutant is *hpr5/srs2*.

[c] Cutoff criteria were $P < 0.0001$ and a 1.5-fold increase for the NHEJ/SSA ratio with any increase in NHEJ as compared to wild type. The question mark indicates that these two mutants, *msn5* and *bud31*, showed a pattern that makes it difficult to assign a clear defect. They are nonetheless tabulated because only four mutants displayed a consistent increase in the NHEJ/SSA ratio.

[d] Cutoff criteria were $P < 0.0001$ for the NHEJ/SSA ratio and a >2-fold decrease in NHEJ as compared to wild type.

of the suicide deletion assay, since all counted events must have induced *GAL* genes and grown in the presence of galactose, which in turn means that they must have all broken and repaired chromosome XV. This ratio is therefore largely independent of potential growth biases. As shown in Figure 3B, most screen-positive mutants showed no alteration in the NHEJ/SSA ratio. This was expected and represents the fact that growth defects in many strains had caused them to be scored as falsely positive in the screen. These strains indeed provided essential controls demonstrating the reproducibility of the quantitative assay, its insensitivity to general growth defects, and the significance of those mutants that did deviate from the wild-type range. Among the DSBR-deficient strains, a nearly 4-log range of NHEJ/SSA ratios was observed (Figure 3B). Considering absolute repair frequencies (*i.e.*, percentage survival), the wild-type strain showed NHEJ and SSA frequencies of 4.5 and 58%, respectively (Table 2), similar to previous results (Karathanasis and Wilson 2002). Mutant strains with altered NHEJ/SSA ratios nearly always showed a decrease in only one of these frequencies with the other falling in the normal range and with *hpr5/srs2* being a notable exception (Table 2; see discussion). The combined data allowed these mutants to be categorized into four classes of DSBR defect (Table 1; see discussion).

One potential bias of method 1 was that the NHEJ frequency of a mutant would be underestimated if it had a specific growth defect on medium lacking adenine, where there was already an inherent colony size heterogeneity due to limited I-SceI recleavage (Karathanasis and Wilson 2002). For this reason, many positives were repeated by quantitative method 2, in which galactose induction of all cells, and therefore DSB induction and repair, occurs in the same nonselective liquid culture. While this allows only estimation of the NHEJ/SSA ratio, it is largely free of growth biases. Method 2 generally agreed with method 1, but with a few exceptions. Specifically, the *yaf9*, *ubi4*, *htz1*, and *ard1* mutants showed a lesser NHEJ deficiency with method 2, although *yaf9*, *ubi4*, and *ard1* mutants still showed a significant and reproducible defect. Of these, *ubi4* was surprising because this mutant grew at wild-type rates, so the basis for the method dependence is not clear.

***NEJ1* mediates mating-type regulation of NHEJ:** At least two yeast cell-cycle states influence DSBR pathway utilization by enhancing NHEJ: haploid mating types (Lee *et al.* 1999) and the transition to postdiauxic/stationary phase (Karathanasis and Wilson 2002). Identification of genes that contribute to this regulation was a major goal of the screen. Indeed, among the class of mutants with deficient NHEJ, all novel genes showed only a partial deficiency as compared with the previously known mutants such as *yku70*, consistent with the notion that they may fulfill regulatory roles. At this point in my work, other groups reported that *nej1* mutant strains were NHEJ deficient because they were unable to induce NHEJ in the haploid state (Frank-Vaillant and Marcand 2001; Kegel *et al.* 2001; Ooi *et al.* 2001; Valencia *et al.* 2001). Indeed, the *nej1* array mutant had been independently identified in my screen and verified as specifically deficient in NHEJ (Figure 3, Table 2). I noted a critical difference, however, in that the *nej1* mutant was clearly only partially NHEJ defective in suicide deletion while it had showed a deficiency equivalent to Ku and DNA ligase IV mutants in others' transformed plasmid assays (Kegel *et al.* 2001; Ooi *et al.* 2001; Valencia *et al.* 2001). The residual *ADE2* events in the *nej1* mutant were NHEJ-dependent suicide deletion by all previously described criteria (Karathanasis and Wilson 2002) and by the observation that they were still dependent on *RAD50* (not shown). This afforded me the opportunity to ask to what extent the mating-type and *nej1* effects overlap and whether the stationary-phase effect is mediated through *NEJ1*.

As seen in Figure 4A, adding a plasmid-borne *MAT***a** gene to *MAT*α strains caused the same partial (as compared to *rad50*) ~10-fold decrease in NHEJ efficiency as did the *nej1* mutation, with *MAT***a**/*MAT*α *nej1* strains being no more deficient, demonstrating an epistatic relationship of the *MAT***a**/*MAT*α and *nej1* genotypes with regard to NHEJ deficiency. Further, both the *nej1* and *MAT***a**/*MAT*α NHEJ defects were corrected by a plasmid expressing *NEJ1* from the strong constitutive *ADH1* promoter, confirming that regulated loss of *NEJ1* expression is in fact responsible for the *MAT***a**/*MAT*α effect (Figure 4A). It is thus apparent that *NEJ1* expression can entirely account for the mating-type effect on NHEJ efficiency, but that Nej1 is not an obligatory participant in chromosomal NHEJ.

TABLE 2

Quantification of verified positives

Gene	Description	Method 1 fold change			Method 2 fold change
		NHEJ	SSA	NHEJ/SSA	NHEJ/SSA
YKU80	Ku component	−320 ± 80	−1.2 ± 0.2	−240 ± 70	ND
DNL4	Lig4 component	−250 ± 70	−1.0 ± 0.2	−220 ± 20	ND
MRE11	MRX complex	−160 ± 20	1.1 ± 0.2	−200 ± 20	ND
YKU70	Ku component	−220 ± 20	−1.1 ± 0.1	−200 ± 20	ND
LIF1	Lig4 component	−200 ± 30	−1.2 ± 0.2	−170 ± 40	ND
RAD50	MRX complex	−120 ± 0	1.2 ± 0.2	−140 ± 40	−113 ± 25
XRS2	MRX complex	−110 ± 10	1.0 ± 0.0	−120 ± 20	ND
NEJ1	Lif1 interacting protein	−19 ± 8	−1.1 ± 0.2	−17 ± 4	−13 ± 2.1
YAF9	Similar to human AF-9	−5.1 ± 3.4	1.3 ± 0.1	−6.9 ± 5.0	−2.7 ± 0.8
DOA1	Required for ubiquitin proteolysis	−5.8 ± 1.6	−1.0 ± 0.1	−5.9 ± 1.5	−6.0 ± 1.3
UBI4	Stress-induced polyubiquitin	−8.0 ± 3.0	−1.4 ± 0.4	−5.9 ± 1.6	−2.0 ± 0.5
FYV6	Mutant hypersensitive to killer toxin	−3.9 ± 1.5	1.3 ± 0.2	−5.2 ± 2.0	−7.4 ± 2.6
HTZ1	Variant histone H2A.Z	−3.9 ± 2.1	1.2 ± 0.1	−4.7 ± 2.3	−1.5 ± 0.2
MCK1	GSK-3-related protein kinase	−4.0 ± 1.6	1.0 ± 0.0	−4.2 ± 1.8	−6.3 ± 1.3
ARD1	N-terminal acetyltransferase subunit	−3.6 ± 1.3	1.1 ± 0.2	−3.9 ± 1.1	−2.1 ± 0.4
LAG1[a]	Endoplasmic reticulum protein	−4.4 ± 2.5	−1.1 ± 0.4	−3.7 ± 1.3	−7.9 ± 3.4
CSE2	Mediator complex subunit	−2.5 ± 0.3	1.4 ± 0.3	−3.5 ± 0.6	−3.9 ± 1.1
YPL181W	Contains a PHD finger domain	−2.8 ± 0.6	1.2 ± 0.0	−3.4 ± 0.7	−1.2 ± 0.6
YPL055C	Unknown function	−2.8 ± 0.8	1.1 ± 0.2	−3.2 ± 0.8	−3.1 ± 0.6
YIL040W	Unknown function	−2.3 ± 0.8	1.1 ± 0.2	−2.6 ± 1.0	−3.0 ± 0.8
ARP6	Actin-related protein	−2.2 ± 0.3	1.1 ± 0.1	−2.4 ± 0.5	−1.8 ± 0.2
YOL071W	Unknown function	−3.0 ± 1.5	−1.3 ± 0.2	−2.3 ± 0.9	ND
AOR1	Actin-overexpression resistant	−2.1 ± 0.9	1.1 ± 0.2	−2.3 ± 0.8	ND
BRE5	Mutant hypersensitive to brefeldin A	−2.1 ± 0.3	−1.0 ± 0.1	−2.1 ± 0.2	ND
Wild type		1.0 ± 0.1 (4.5 ± 0.4%)	1.0 ± 0.1 (58 ± 5%)	1.0 ± 0.1 (0.079 ± 0.010)	−1.0 ± 0.3 (0.039 ± 0.007)
MSN5	Nuclear exportin	1.2 ± 0.2	−1.2 ± 0.1	1.5 ± 0.1	1.3 ± 0.2
BUD31	Influences bud site selection	1.3 ± 0.7	−1.5 ± 0.2	1.9 ± 0.7	2.7 ± 0.1
HPR5	DNA repair helicase	−2.1 ± 0.7	−16 ± 7	8.2 ± 0.5	ND
RAD52	Recombination protein	−1.5 ± 0.3	−26 ± 7	16 ± 2	34 ± 0.6

For method 1, the fold change in the percentage survival by NHEJ, the fold change in the percentage survival by SSA, and the fold change in the NHEJ/SSA ratio are shown for each strain verified as positive by the criteria listed in Table 1. Mutants are sorted by the fold change in the NHEJ/SSA ratio. Negative numbers represent an *x*-fold decrease relative to wild type, positive numbers an *x*-fold increase. Values are the means ± standard deviations of at least three independent measurements. Values that are statistically significant to $P < 0.0001$ are underlined. Numbers in parentheses for the wild-type strain are the actual value of the measurement prior to normalization and are the mean ± standard deviation of 15 independent measurements. Method 2 data are presented in the same way except that the wild-type sample was repeated 10 times. ND, not done.

[a] This *lag1* mutant is suspect because the strain failed to show the expected α mating despite being Met^+; it is tabulated for completeness, but was not analyzed further.

Mating-type and growth-phase regulation of NHEJ are separable phenomena: We have previously considered whether *NEJ1* might be responsible for mediating the stationary phase as well as the mating-type effects on NHEJ (Karathanasis and Wilson 2002). Each of these effects, like the *nej1* mutation, leads to only partial loss of chromosomal NHEJ, however, which makes it more likely that these effects are separable and that NHEJ-stimulating genes other than *NEJ1* exist. Indeed, when tested in combination using wild-type yeast, the **a**/α mating type and exponential growth phase combined to drive chromosomal NHEJ to levels even lower than those of each parameter individually (Figure 4B). The combination showed an almost 100-fold decrease in the NHEJ/SSA ratio relative to *MAT*α stationary-phase yeast, very nearly the same defect seen in *rad50* yeast. While yeast bearing the *nej1* mutation were again insensitive to the mating-type effect, both *MAT***a** *nej1* and *MAT***a**/*MAT*α *nej1* yeast showed a significantly decreased NHEJ/SSA ratio when tested in exponential phase. It is thus clear that unlike the mating-type effect, *NEJ1* is not required for postdiauxic/stationary-phase stimulation of NHEJ.

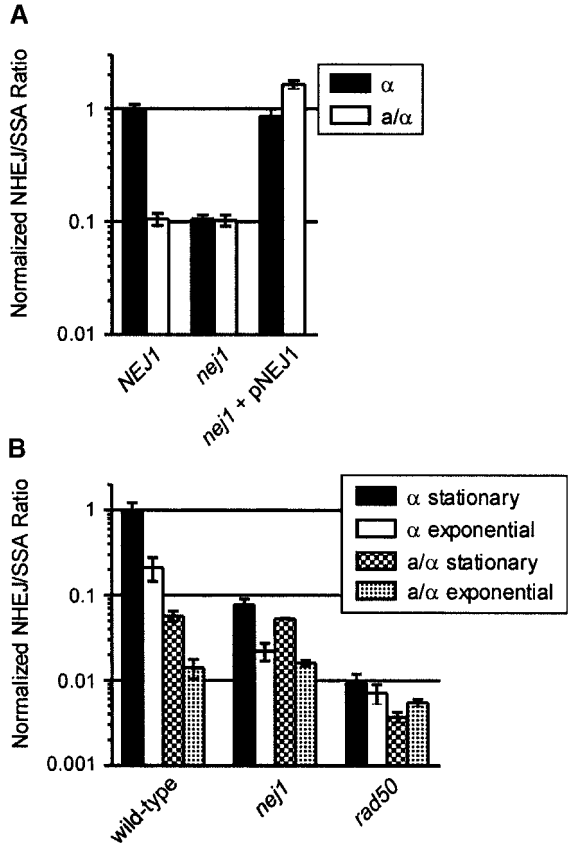

FIGURE 4.—*NEJ1* expression can completely account for the mating type but not the growth-phase regulation of NHEJ efficiency. (A) Haploid yeast of the indicated genotypes were tested by suicide deletion quantitative method 2. All strains were chromosomal *MAT*α, but where indicated also bore the *MAT***a** allele on a plasmid (a/α, open bars). pNEJ1 expresses *NEJ1* from the constitutive *ADH1* promoter. (B) Similar experiment to A, except that the growth stage of the yeast used to inoculate the galactose medium was varied between active glucose exponential phase and early stationary phase.

Identification of genes required for growth-phase regulation of NHEJ: The identity of other partially NHEJ-deficient mutants suggested that they may in fact play a role in coordinating growth-phase-dependent induction of NHEJ (see DISCUSSION). To further explore this possibility I examined their phenotypes and relationships with *nej1* in detail. Although many strains were tested in part, this discussion focuses on those strains that showed the largest consistent decrease in the NHEJ/SSA ratio, namely *doa1*, *mck1*, and *fyv6*. Each of these mutants showed a six- to sevenfold decrease in the NHEJ/SSA ratio by method 2 that was similar in magnitude to the decrease observed in wild-type exponential cells (Table 3). This similarity proved to be more than coincidental on the basis of several observations. First, the *doa1*, *mck1*, and *fyv6* mutants were each largely insensitive to a further exponential-phase decrease in the NHEJ/SSA ratio (Table 3), parallel to the manner in which *nej1* mutant cells were insensitive to the mating-type effect. Second, the *doa1*, *mck1*, and *fyv6* mutations all showed synthetic decreases in the NHEJ/SSA ratio when combined with the *nej1* allele (Table 3), indicating that, like the exponential-phase regulation of NHEJ, these genes act separately from *NEJ1*. It was thus not surprising that none of these mutants was corrected by the pNEJ1 plasmid (Table 3). In total, the data are fully consistent with the hypothesis that *DOA1*, *MCK1*, and *FYV6* promote fully efficient NHEJ by a mechanism activated in postdiauxic/stationary phase that is consequently distinct and separable from the action of *NEJ1*.

Finally, I measured two parameters to determine whether the defect of the *doa1*, *mck1*, and *fyv6* cells is in the induction of stationary phase *per se* (*i.e.*, in the global response to nutritional deprivation) or in a downstream signaling of this response to the NHEJ apparatus. These parameters were maintenance of viability and induction of thermotolerance over many days in culture. As seen in Figure 5, three different patterns were observed. The *doa1* mutant was deficient in stationary-phase induction in that it progressively lost viability from ~4 days after the diauxic shift and never achieved wild-type levels of thermotolerance. In contrast, the *mck1* mutant behaved as wild type in this analysis, showing both maintenance of viability and induction of thermotolerance, thus differentiating its growth-dependent NHEJ defect from stationary-phase induction. The *fyv6* mutant was intermediate in that it did not lose viability but had poor induction of thermotolerance, further demonstrating that the various physiological changes that occur during prolonged nutrient deprivation are independent.

DISCUSSION

Recent observations have suggested that unknown regulatory genes influence DSBR pathway utilization, in particular NHEJ, in *S. cerevisiae* (LEE *et al.* 1999; KARATHANASIS and WILSON 2002). Further, the lack of a comprehensive genetic screen capable of detecting NHEJ mutants has left the possibility that unknown genes participate structurally in this repair event. The screen described here was undertaken to search for these genes. As it was being completed, OOI *et al.* (2001) reported their screen, using an approach in which a pool of deletion mutants was transformed with linearized plasmids with microarray analysis used to identify those with NHEJ deficiencies. Because of the unique aspects of each approach (see below), results here complement and extend their findings. This discussion is organized according to the classes of DSBR-deficient mutants that were detected (Table 1).

Class I mutants, isolated SSA deficiency: Although not a primary goal, the screen described here was able to detect mutants with a deficiency in SSA via short terminal direct repeats. The only mutant identified in this class was *rad52* (but note *srs2*, below). This is consistent with previous findings that SSA, unlike true recombination, is independent of *RAD52* epistasis group mem-

TABLE 3

Three mutants exhibit defective growth-phase regulation of NHEJ

mutx	Standard assay	Exponential	*nej1*	pNEJ1
Wild type	1.0 ± 0.2	0.21 ± 0.07**	0.086 ± 0.019**	0.86 ± 0.04
doa1	0.17 ± 0.04*	0.11 ± 0.04	0.018 ± 0.003*,**	0.23 ± 0.12
mck1	0.16 ± 0.03*	0.11 ± 0.03	0.022 ± 0.003*,**	0.15 ± 0.002*
fyv6	0.15 ± 0.05*	0.23 ± 0.09	0.032 ± 0.006*,**	0.14 ± 0.004*
nej1	0.077 ± 0.013*	0.027 ± 0.010*,**	NA	0.96 ± 0.05**

The normalized NHEJ/SSA ratio of various strains, either wild type or bearing the indicated mutation (*mutx*), was determined by the standard method 2 assay (Standard assay), by varying the growth conditions so that cells were in an active exponential growth phase at the time of DSB induction, by first deleting *NEJ1*, or by first introducing pNEJ1, a constitutive *NEJ1* expression plasmid. NA, not applicable. *$P < 0.01$ (column significance) as compared with wild type (*i.e.*, top row) for that column. **$P < 0.01$ (row significance) as compared with the standard assay (*i.e.*, left column) for that row.

bers required for strand invasion (Ivanov *et al.* 1996). As with NHEJ (see below), this does not mean that no other genes are involved, however. For example, Smith and Rothstein (1999) found that specific mutations of the essential gene *RFA1* act as suppressors of the *rad52* SSA defect, indicating that RPA and Rad52 interact in the process of single-strand coating and annealing. It is noteworthy that *rad59* was not among the class I mutants based on several spotting screens, since recent studies have demonstrated a role for Rad59 in SSA both *in vitro* and *in vivo* (Petukhova *et al.* 1999; Sugawara *et al.* 2000; Davis and Symington 2001). I have not tested the *rad59* mutant by quantitative analysis and so cannot rule out a minor SSA defect, and indeed the screen was least sensitive for this mutant class. Nonetheless, it seems that it is not strongly impaired in suicide deletion SSA, a conclusion supported by observations that this same mutant displays a recombination-defective phenotype when treated with various replication inhibitors (J. R. Vance, A. Iacco and T. E. Wilson, unpublished results). This likely reflects the fact that the suicide deletion repeats are unusual in that they are short (28 bp) and at the termini, so that the enzymatic requirements for repair are relaxed due to a relative ease of homology searching (note that even the *rad52* defect is comparatively modest). Finally, an important negative in all SSA screens to date is the failure to find a mutant deficient in 5′ resection, which is required for SSA and recombination alike. This likely reflects an enzymatic redundancy in 5′ resection (Tsubouchi and Ogawa 2000; Moreau *et al.* 2001; Lewis *et al.* 2002), and consequently more involved genetic analyses will be required to elucidate these mechanisms.

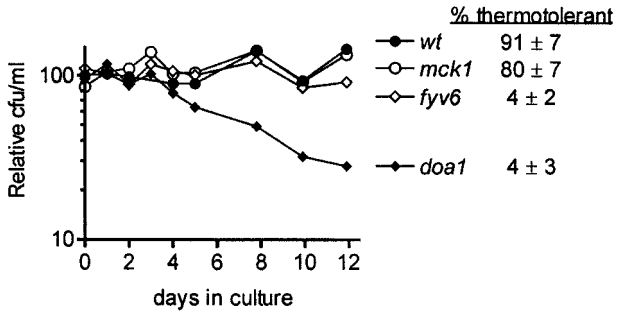

Figure 5.—Delineating the stationary-phase deficiency of *doa1*, *fyv6*, and *mck1* mutants. The graph shows the relative cfu per milliliter in prolonged cultures of wild-type, *doa1*, *fyv6*, and *mck1* strains. The zero time point corresponds to cultures at or just after the diauxic shift in YPAD. To the right are the percentages of cells showing tolerance to a 55° heat shock; values are the means ± standard deviations of measurements made at the last three time points.

Class II mutants, combined NHEJ and SSA deficiency: The array *hpr5* mutant (more commonly known as *srs2*) was initially scored in the screen as SSA deficient. While a minor SSA deficiency has indeed been observed for *srs2* by the Haber laboratory (Sugawara *et al.* 2000), the phenotype here is surprisingly severe, especially given the *rad59* result above. Again, this may reflect the nature of the suicide deletion repeats, whose short length may increase the need for Srs2 to unwind nonproductive strand associations. On quantitative analysis the *srs2* mutant proved to have an additional modest but reproducible twofold deficiency in NHEJ. This is entirely consistent with a previous report from the Klein laboratory (Hegde and Klein 2000) and, importantly, verifies that the *srs2* NHEJ defect is observed in the repair of chromosomal as well as plasmid DSBs. It remains unclear why the Srs2 helicase would be required for NHEJ via fully compatible 3′ overhangs, however. Importantly, *rad50*, *mre11*, and *xrs2* mutants are known to be deficient in both homologous recombination and NHEJ. The fact that these (and others?) were not detected as class II mutants reflects the fact that SSA is independent of the Rad50/Mre11/Xrs2 complex.

Class III mutants, increased NHEJ efficiency: The screen described here was especially sensitive not only to DSBR pathway deficiencies, but also to mutants in which NHEJ efficiency was increased, which would be manifested as an increased *ADE2/ade2* ratio. In particular, I anticipated that mutants showing altered pathway regulation or deficient 5′ resection might favor NHEJ

over SSA. The *msn5* and especially *bud31* mutants did show a reproducible and significant increase in the NHEJ/SSA ratio, but this was modest and not accompanied by an obvious increase in the absolute NHEJ frequency. Msn5 is an exportin required in various nuclear transport cycles (Gorlich and Kutay 1999), while Bud31 is a protein of uncertain molecular function that might be involved in bud site selection (Ni and Snyder 2001). It is conceivable that either of these might affect DSBR pathway utilization, although it seems equally likely that these mutant phenotypes could be explained by secondary effects. Finally, it is noteworthy that *rad5* was not scored as positive in the screen (and so was not tested further in the quantitative assay), as others have found Rad5 to function in avoidance of NHEJ of incompatible ends (Ahne *et al.* 1997). Although I again cannot rule out a minor effect below the sensitivity of the screen, my result is more consistent with data from Hegde and Klein (2000) that simple religation NHEJ is neither increased nor decreased in *rad5* mutants.

Class IVa mutants, structural/catalytic NHEJ deficiency: At the time that this work was initiated, seven mutants were known to show NHEJ deficiencies consistent with structural or enzymatic participation in rejoining of compatible overhangs: *yku70*, *yku80*, *dnl4*, *lif1*, *rad50*, *mre11*, and *xrs2*. Each of these, but no novel genes in this class, was readily uncovered in the suicide deletion screen. This is in contrast to the results of Ooi *et al.* (2001) in which *mre11* and *xrs2* mutants were not identified due to the fact that they gave insufficient signal in the pool due to growth deficiencies. This demonstrates a particularly powerful feature of the spot-screening approach used here and also recently by Bennett *et al.* (2001) in a screen for radiosensitive mutants, namely that strains are examined individually and so the array is screened comprehensively. It is thus relevant to ask whether Ku, DNA ligase IV, and the Mre11/Rad50/Xrs2 complex in fact represent the complete set of genes required to execute a simple-religation NHEJ event. Answering this requires a consideration of the technical and genetic limitations of my approach.

To ultimately be scored as positive, any array strain needed to be mating proficient, Met^+, Ura^+, Ade^+, Gal^+, and not petite (most petite mutants did not grow sufficiently on galactose to be scored). In general, it is unlikely that mutants in these failure classes would be structurally required for NHEJ, although some may be deficient in mating type and nutritional regulation of NHEJ. For example, sterility prevented the recovery of *sir2-4* mutants, and possibly others, that are not structurally required for NHEJ but nonetheless lead to impairment of NHEJ via loss of *HMR* and *HML* silencing (Lee *et al.* 1999). Beyond this, these technical classes of screen failure in fact proved to be quite helpful. In spotting analysis strains are identified only by well position, in contrast to the positive identification provided by "molecular bar codes" in pooled microarray analysis, and so it was possible that cross-contamination could lead to misidentification. In total, >350 mutants with predictable phenotypes behaved as expected during screening, which in conjunction with PCR verification of novel DSBR-deficient mutants demonstrated that the array had not decayed to a measurable degree during the handling steps.

Mutants of true NHEJ genes may also have been missed for genetic reasons if they were inviable (or otherwise absent from the array), highly redundant with other genes, or linked to either the *ADE2* or *MAT* loci. Linkage was of surprisingly little concern, given that a great many asci could be spotted; only ~10 open reading frames (ORFs) to either side of the *ADE2* and *MAT* loci needed to be discounted from consideration. It is of course impossible to judge the likelihood of redundancy, but importantly the screen sensitivity would have allowed detection of partially redundant functions. Regarding inviability, it is clear that NHEJ is not an essential function in yeast. However, the finding that histone modifications are required for fully efficient NHEJ makes it clear that repair must of course occur in the context of chromatin, components of which frequently are essential or redundant (Downs *et al.* 2000). In total, the properties of my screen make it increasingly likely (although not completely certain) that Ku, DNA ligase IV, and the Mre11/Rad50/Xrs2 complex represent the complete set of proteins required specifically for catalysis of simple religation NHEJ, although these almost certainly interact with unidentified chromatin components during repair. This interpretation is supported by recent biochemical studies in which purified fractions of Ku, DNA ligase IV, and the Mre11/Rad50 complex appear to effectively reconstitute NHEJ (Chen *et al.* 2001; Huang and Dynan 2002). Finally, I note that the present suicide deletion assay and this discussion address only simple religation NHEJ. Alternative approaches are being developed to identify genes that collaborate with *POL4* and so participate in NHEJ only when ends are incompatible.

Class IVb mutants, partial/regulatory NHEJ deficiency: The high sensitivity of the suicide deletion screen also allowed for detection of partially NHEJ-defective mutants that proved, as hypothesized, to serve regulatory roles. Somewhat surprisingly, some mutants had less severe defects than the initially estimated detection threshold of a fivefold decrease in NHEJ. This reflects the fact that the inclusion criteria were deliberately relaxed during the screening phase. A necessary and important corollary is that this class of mutants is almost certainly incomplete due to false negatives, in contrast to the discussion above regarding frank catalytic NHEJ deficiency. For example, checkpoint mutants previously reported to have minor deficiencies in NHEJ of transformed plasmids (de la Torre-Ruiz and Lowndes 2000) were consistently scored as negative. It is thus apparent that a large number of genes contribute to promoting maximal NHEJ efficiency. At the same time, it is difficult

to be certain that the smallest defects are biologically meaningful. My attention thus focuses on those mutants with greater than fivefold NHEJ deficiencies or functions that suggest a role in NHEJ regulation.

NEJ1 mediates mating-type regulation of NHEJ: NEJ1 has now been identified by several independent means as a regulator of NHEJ efficiency, including the functional screen described here (Frank-Vaillant and Marcand 2001; Kegel *et al.* 2001; Ooi *et al.* 2001; Valencia *et al.* 2001). That this gene in fact mediates the mating-type regulation of NHEJ was suggested by the fact that it is expressed only in haploid cells (Frank-Vaillant and Marcand 2001; Kegel *et al.* 2001; Valencia *et al.* 2001). Indeed, expression of *NEJ1* from a mating-type-independent promoter (in our case the *ADH1* promoter) has in all hands proven to relieve the inhibition of NHEJ seen in *MAT***a**/*MAT*α cells (Figure 4 and Kegel *et al.* 2001; Valencia *et al.* 2001), thereby demonstrating that mating-type regulation is dependent on transcriptional regulation of *NEJ1*. In the case of the chromosomal suicide deletion assay used here, it was further evident that the NHEJ defects seen in *nej1* and *MAT***a**/*MAT*α cells were quantitatively equivalent and epistatic. This conclusion was made possible by the fact that these were only partial defects (~10- to 20-fold) as reflected in both the absolute and relative NHEJ frequencies (Table 2). This first makes clear that Nej1 is not an obligatory participant in NHEJ catalysis in the same fashion as, for example, Ku (although this does not rule out that Nej1 might participate in the NHEJ structural complex). Further, it appears likely that *NEJ1* is itself the sole mediator of mating-type regulation and that the other partially NHEJ-deficient mutants affect a different regulatory input. This conclusion is based on the facts that the *nej1* and *MAT***a**/*MAT*α effects are equivalent, that no mutant in the screen behaved epistatically to *nej1*, and that no mutant (except *nej1* itself) was complemented by pNEJ1 (Figure 4 and Table 3). Indeed, previous observations strongly suggest that both the upstream regulation of *NEJ1* and the downstream effect of Nej1 are mediated directly. Specifically, the *NEJ1* promoter has sites for the Mat**a**1-Matα2 repressor encoded by the *MAT* alleles of *MAT***a**/*MAT*α cells (Kegel *et al.* 2001; Valencia *et al.* 2001), and Nej1 itself interacts strongly with Lif1 (Frank-Vaillant and Marcand 2001; Kegel *et al.* 2001; Ooi *et al.* 2001).

Multigenic NEJ1-independent growth-phase regulation of NHEJ: The partial defect of *nej1* in suicide deletion contrasts with its Ku- or DNA ligase IV-equivalent defect seen in plasmid transformation assays (Kegel *et al.* 2001; Ooi *et al.* 2001; Valencia *et al.* 2001). In addition to the chromosomal nature of the suicide deletion break and enhanced sensitivity resulting from limited recleavage of ligated I-SceI sites, this difference can be accounted for at least in part by the use of early stationary-phase cells in the standard suicide deletion methods. We have previously argued that there is a stimulation of NHEJ in the postdiauxic/stationary phase that is masked in plasmid assays using cells growing exponentially in rich glucose medium; simply transforming cells from late stage cultures causes a substantial increase in NHEJ event recovery (Karathanasis and Wilson 2002). Enhancement of NHEJ in postdiauxic/stationary cultures is also evident in the suicide deletion assay. Although we had initially considered that *NEJ1* might mediate this effect, experiments here consistently demonstrated that this component of NHEJ regulation is still active in the absence of Nej1. In contrast, the screen did identify other mutants deficient in growth-phase regulation of NHEJ. Specifically, the *doa1*, *fyv6*, and *mck1* mutants themselves not only were NHEJ deficient to approximately the same degree as exponential wild-type cells (approximately fivefold), but also were insensitive to a further significant decrease in NHEJ when assayed in the exponential phase. A difficulty in the initial description of the growth-phase regulation of NHEJ was that it might be an experimental artifact reflecting a growth-dependent change in suicide deletion dynamics independent of a true change in the DSBR efficiency (Karathanasis and Wilson 2002). The fact that this second input to increased NHEJ efficiency can also be abrogated by genetic alteration of equivalently grown early stationary-phase cells demonstrates that the phenomenon is not a growth artifact.

The nature of these genes provides the final evidence that they are specifically defective in a postdiauxic/stationary-phase induction of NHEJ. Doa1 (also known as Ufd3) is a protein required for the degradation of ubiquitin-tagged proteins (Johnson *et al.* 1995; Ghislain *et al.* 1996). Its precise function is unknown, but mutants have very low levels of free ubiquitin, and at least some mutant phenotypes are known to be corrected by increased expression of *UBI4*. *UBI4*, also identified here as a class IVb mutant, is the yeast polyubiquitin gene known to be induced in numerous stress responses including nutritional deprivation (Ozkaynak *et al.* 1987). Although the mechanism is not established, *ubi4* mutants do not establish stationary phase and die after ~4 days in culture (Peck *et al.* 1997), a phenotype shared by *doa1* mutants (Figure 5). The fact that the *ubi4* NHEJ deficiency was significantly less than that of *doa1* (at least by method 2), despite being profoundly deficient in stationary-phase induction, provides a first indication that these are overlapping but separable phenomena, however.

MCK1 encodes a dual-specificity protein kinase of the glycogen synthase kinase-3 (GSK-3) family that affects a surprisingly large number of cellular processes. Though too numerous to list here (see Rayner *et al.* 2002), the collection has suggested a role for Mck1 in mediating nutritional/stress responsiveness, to which we add enhancement of NHEJ in postdiauxic/stationary phase. But again, this regulation is distinct from stationary-phase induction *per se*, because the *mck1* mutant was able to

maintain viability and achieve thermotolerance during prolonged culture.

The reserved gene name *FYV6* stands for function required for yeast viability in response to K1 killer toxin, although no report has appeared to date. It is induced during stationary phase (Planta *et al.* 1999). This coupling might suggest a role in stress responsiveness. Strikingly, it presents yet a third pattern of stationary-phase deficiency, since it maintained viability but failed to achieve thermotolerance.

Summary: The screen described here has provided a clear picture of the catalytic/structural requirements for NHEJ and suggests that Ku, DNA ligase IV, and the Rad50/Mre11 complex are likely to provide all of the obligatory functions for simple religation NHEJ. Moreover, the screen has revealed a dual regulatory input into the regulation of NHEJ. *NEJ1* mediates an input dependent on mating type, with no other genes identified as cooperating with *NEJ1* in this regard. In contrast, a series of genes, most notably *DOA1, FYV6*, and *MCK1*, mediate a separate input dependent on the nutritional status of the culture. This latter input is correlated with the passage into stationary phase, but is distinct from it. Although Doa1, Fyv6, or Mck1 might modify the function of the NHEJ machinery, their action need not be direct and in fact no such interactions have been identified by systematic screening (Uetz *et al.* 2000).

I thank the members of my laboratory for many helpful discussions during the development of this project and Sarah Sutter and Elissa Karathanasis for technical help. pTW367 was constructed by Jamuna Thimmarayappa. This work was supported in part by the Pew Scholars Program in the Biomedical Sciences of the Pew Charitable Trusts and Public Health Service grant CA-90911 (T.E.W.).

LITERATURE CITED

Ahne, F., B. Jha and F. Eckardt-Schupp, 1997 The *RAD5* gene product is involved in the avoidance of non-homologous end-joining of DNA double strand breaks in the yeast *Saccharomyces cerevisiae*. Nucleic Acids Res. **25:** 743–749.

Bennett, C. B., L. K. Lewis, G. Karthikeyan, K. S. Lobachev, Y. H. Jin *et al.*, 2001 Genes required for ionizing radiation resistance in yeast. Nat. Genet. **29:** 426–434.

Brachmann, C. B., A. Davies, G. J. Cost, E. Caputo, J. Li *et al.*, 1998 Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast **14:** 115–132.

Chen, L., K. Trujillo, W. Ramos, P. Sung and A. E. Tomkinson, 2001 Promotion of Dnl4-catalyzed DNA end-joining by the Rad50/Mre11/Xrs2 and Hdf1/Hdf2 complexes. Mol. Cell **8:** 1105–1115.

Davis, A. P., and L. S. Symington, 2001 The yeast recombinational repair protein Rad59 interacts with Rad52 and stimulates single-strand annealing. Genetics **159:** 515–525.

de la Torre-Ruiz, M., and N. F. Lowndes, 2000 The *Saccharomyces cerevisiae* DNA damage checkpoint is required for efficient repair of double strand breaks by non-homologous end joining. FEBS Lett. **467:** 311–315.

Downs, J. A., N. F. Lowndes and S. P. Jackson, 2000 A role for *Saccharomyces cerevisiae* histone H2A in DNA repair. Nature **408:** 1001–1004.

Frank-Vaillant, M., and S. Marcand, 2001 NHEJ regulation by mating type is exercised through a novel protein, Lif2p, essential to the ligase IV pathway. Genes Dev. **15:** 3005–3012.

Game, J. C., and R. K. Mortimer, 1974 A genetic study of x-ray sensitive mutants in yeast. Mutat. Res. **24:** 281–292.

Gennery, A. R., A. J. Cant and P. A. Jeggo, 2000 Immunodeficiency associated with DNA repair defects. Clin. Exp. Immunol. **121:** 1–7.

Ghislain, M., R. J. Dohmen, F. Levy and A. Varshavsky, 1996 Cdc48p interacts with Ufd3p, a WD repeat protein required for ubiquitin-mediated proteolysis in *Saccharomyces cerevisiae*. EMBO J. **15:** 4884–4899.

Gorlich, D., and U. Kutay, 1999 Transport between the cell nucleus and the cytoplasm. Annu. Rev. Cell Dev. Biol. **15:** 607–660.

Hegde, V., and H. Klein, 2000 Requirement for the *SRS2* DNA helicase gene in non-homologous end joining in yeast. Nucleic Acids Res. **28:** 2779–2783.

Herrmann, G., T. Lindahl and P. Schar, 1998 *Saccharomyces cerevisiae LIF1*: a function involved in DNA double-strand break repair related to mammalian XRCC4. EMBO J. **17:** 4188–4198.

Huang, J., and W. S. Dynan, 2002 Reconstitution of the mammalian DNA double-strand break end-joining reaction reveals a requirement for an Mre11/Rad50/NBS1-containing fraction. Nucleic Acids Res. **30:** 667–674.

Ivanov, E. L., N. Sugawara, J. Fishman-Lobell and J. E. Haber, 1996 Genetic requirements for the single-strand annealing pathway of double-strand break repair in *Saccharomyces cerevisiae*. Genetics **142:** 693–704.

Jackson, S. P., 2001 Detecting, signalling and repairing DNA double-strand breaks. Biochem. Soc. Trans. **29:** 655–661.

Johnson, E. S., P. C. Ma, I. M. Ota and A. Varshavsky, 1995 A proteolytic pathway that recognizes ubiquitin as a degradation signal. J. Biol. Chem. **270:** 17442–17456.

Jones, J. M., M. Gellert and W. Yang, 2001 A Ku bridge over broken DNA. Structure **9:** 881–884.

Karathanasis, E. K., and T. E. Wilson, 2002 Enhancement of *Saccharomyces cerevisiae* end-joining efficiency by cell growth stage but not by impairment of recombination. Genetics **161:** 1015–1027.

Kegel, A., J. O. Sjostrand and S. U. Astrom, 2001 Nej1p, a cell type-specific regulator of nonhomologous end joining in yeast. Curr. Biol. **11:** 1611–1617.

Lee, S. E., F. Paques, J. Sylvan and J. E. Haber, 1999 Role of yeast *SIR* genes and mating type in directing DNA double-strand breaks to homologous and non-homologous repair paths. Curr. Biol. **9:** 767–770.

Lewis, L. K., G. Karthikeyan, J. W. Westmoreland and M. A. Resnick, 2002 Differential suppression of DNA repair deficiencies of yeast *rad50, mre11* and *xrs2* mutants by *EXO1* and *TLC1* (the RNA component of telomerase). Genetics **160:** 49–62.

Ma, Y., U. Pannicke, K. Schwarz and M. R. Lieber, 2002 Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination. Cell **108:** 781–794.

Mahajan, K. N., S. A. Nick McElhinny, B. S. Mitchell and D. A. Ramsden, 2002 Association of DNA Polymerase μ with Ku and ligase IV: role for pol μ in end-joining double-strand break repair. Mol. Cell. Biol. **22:** 5194–5202.

Moreau, S., E. A. Morgan and L. S. Symington, 2001 Overlapping functions of the *Saccharomyces cerevisiae* Mre11, Exo1 and Rad27 nucleases in DNA metabolism. Genetics **159:** 1423–1433.

Ni, L., and M. Snyder, 2001 A genomic study of the bipolar bud site selection pattern in *Saccharomyces cerevisiae*. Mol. Biol. Cell **12:** 2147–2170.

Ooi, S. L., D. D. Shoemaker and J. D. Boeke, 2001 A DNA microarray-based genetic screen for nonhomologous end-joining mutants in *Saccharomyces cerevisiae*. Science **294:** 2552–2556.

Ozkaynak, E., D. Finley, M. J. Solomon and A. Varshavsky, 1987 The yeast ubiquitin genes: a family of natural gene fusions. EMBO J. **6:** 1429–1439.

Paques, F., and J. E. Haber, 1999 Multiple pathways of recombination induced by double-strand breaks in *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. **63:** 349–404.

Peck, V. M., E. K. Fuge, P. A. Padilla, M. A. Gomez and M. Werner-Washburne, 1997 Yeast *bcy1* mutants with stationary phase-specific defects. Curr. Genet. **32:** 83–92.

Petrini, J. H., 1999 The mammalian Mre11-Rad50-nbs1 protein complex: integration of functions in the cellular DNA-damage response. Am. J. Hum. Genet. **64:** 1264–1269.

Petukhova, G., S. A. Stratton and P. Sung, 1999 Single strand DNA binding and annealing activities in the yeast recombination factor Rad59. J. Biol. Chem. **274:** 33839–33842.

Planta, R. J., A. J. Brown, J. L. Cadahia, M. E. Cerdan, M. de Jonge *et al.*, 1999 Transcript analysis of 250 novel yeast genes from chromosome XIV. Yeast **15:** 329–350.

Rayner, T. F., J. V. Gray and J. W. Thorner, 2002 Direct and novel regulation of cyclic AMP-dependent protein kinase by Mck1p, a yeast GSK-3. J. Biol. Chem. **4:** 4.

Resnick, M. A., 1969 Genetic control of radiation sensitivity in *Saccharomyces cerevisiae*. Genetics **62:** 519–531.

Shinohara, A., M. Shinohara, T. Ohta, S. Matsuda and T. Ogawa, 1998 Rad52 forms ring structures and co-operates with RPA in single-strand DNA annealing. Genes Cells **3:** 145–156.

Smith, G. C., and S. P. Jackson, 1999 The DNA-dependent protein kinase. Genes Dev. **13:** 916–934.

Smith, J., and R. Rothstein, 1999 An allele of *RFA1* suppresses *RAD52*-dependent double-strand break repair in *Saccharomyces cerevisiae*. Genetics **151:** 447–458.

Sugawara, N., G. Ira and J. E. Haber, 2000 DNA length dependence of the single-strand annealing pathway and the role of *Saccharomyces cerevisiae RAD59* in double-strand break repair. Mol. Cell. Biol. **20:** 5300–5309.

Thompson, L. H., J. S. Rubin, J. E. Cleaver, G. F. Whitmore and K. Brookman, 1980 A screening method for isolating DNA repair-deficient mutants of CHO cells. Somatic Cell Genet. **6:** 391–405.

Tong, A. H., M. Evangelista, A. B. Parsons, H. Xu, G. D. Bader *et al.*, 2001 Systematic genetic analysis with ordered arrays of yeast deletion mutants. Science **294:** 2364–2368.

Tsubouchi, H., and H. Ogawa, 2000 Exo1 roles for repair of DNA double-strand breaks and meiotic crossing over in *Saccharomyces cerevisiae*. Mol. Biol. Cell **11:** 2221–2233.

Uetz, P., L. Giot, G. Cagney, T. A. Mansfield, R. S. Judson *et al.*, 2000 A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*. Nature **403:** 623–627.

Valencia, M., M. Bentele, M. B. Vaze, G. Herrmann, E. Kraus *et al.*, 2001 *NEJ1* controls non-homologous end joining in *Saccharomyces cerevisiae*. Nature **414:** 666–669.

Vance, J. R., and T. E. Wilson, 2002 Yeast Tdp1 and Rad1-Rad10 function as redundant pathways for repairing Top1 replicative damage. Proc. Natl. Acad. Sci. USA (in press).

Wilson, T. E., and M. R. Lieber, 1999 Efficient processing of DNA ends during yeast nonhomologous end joining. Evidence for a DNA polymerase β (Pol4)-dependent pathway. J. Biol. Chem. **274:** 23599–23609.

Wilson, T. E., U. Grawunder and M. R. Lieber, 1997 Yeast DNA ligase IV mediates non-homologous DNA end joining. Nature **388:** 495–498.

Winzeler, E. A., D. D. Shoemaker, A. Astromoff, H. Liang, K. Anderson *et al.*, 1999 Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science **285:** 901–906.

Communicating editor: L. S. Symington