Frameshift Mutagenesis: The Roles of Primer–Template Misalignment and the Nonhomologous End-Joining Pathway in Saccharomyces cerevisiae

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ABSTRACT Small insertions or deletions that alter the reading frame of a gene typically occur in simple repeats such as mononucleotide runs and are thought to reflect spontaneous primer–template misalignment during DNA replication. The resulting extrahelical repeat is efficiently recognized by the mismatch repair machinery, which specifically replaces the newly replicated strand to restore the original sequence. Frameshift mutagenesis is most easily studied using reversion assays, and previous studies in Saccharomyces cerevisiae suggested that the length threshold for polymerase slippage in mononucleotide runs is 4N. Because the probability of slippage is strongly correlated with run length, however, it was not clear whether shorter runs were unable to support slippage or whether the resulting frameshifts were obscured by the presence of longer runs. To address this issue, we removed all mononucleotide runs >3N from the yeast lys2ΔBgl and lys2ΔA746 frameshift reversion assays, which detect net 1-bp deletions and insertions, respectively. Analyses demonstrate that 2N and 3N runs can support primer–template misalignment, but there is striking run-specific variation in the frequency of slippage, in the accumulation of +1 vs. −1 frameshifts and in the apparent efficiency of mismatch repair. We suggest that some of this variation reflects the role of flanking sequence in initiating primer–template misalignment and that some reflects replication-independent frameshifts generated by the nonhomologous end-joining pathway. Finally, we demonstrate that nonhomologous end joining is uniquely required for the de novo creation of tandem duplications from noniterated sequence.

THE accumulation of mutations within genomic DNA is precisely regulated; mutations must be kept at a very low level to maintain genome integrity and yet must be frequent enough to support evolutionary change. Most spontaneous mutations are base substitutions or small insertions/deletions (indels) that reflect errors made either when replicating an undamaged DNA template or when synthesizing over a DNA lesion. Indels that are not a multiple of 3 bp are referred to as frameshift mutations because they change the reading frame of a translating ribosome, thereby altering all downstream amino acids and usually resulting in premature termination of translation. Given the very deleterious nature of frameshift mutations, it is critical that the corresponding mutational intermediates be efficiently recognized and removed.

Repetitive sequences such as mononucleotide or dinucleotide repeats are strong hotspots for frameshifts, and most intermediates arise through spontaneous, replication-associated strand slippage (Streisinger et al. 1966). As illustrated for a mononucleotide run in Figure 1A, misalignment between the primer and template strands generates an extrahelical repeat on one of the two strands. If not repaired, an extrahelical nucleotide on the primer strand will become a +1 frameshift mutation, while the persistence of an extrahelical nucleotide on the template strand will result in a −1 frameshift mutation. The frequency with which slippage occurs increases as a function of run length in vitro (Kunkel 1990) and in vivo (Tran et al. 1997). Our previous analyses in yeast suggested that only mononucleotide runs >3N accumulate more frameshifts than predicted by chance, indicating a threshold length of 4N for slippage in vivo (Greene and Jinks-Robertson
Frameshifts also occur, however, at low levels in smaller repeats and in noniterated sequence (Greene and Jinks-Robertson 2001).

In addition to the spontaneous strand slippage described above, in vitro studies have suggested two additional mechanisms of primer–template misalignment (reviewed in Garcia-Diaz and Kunkel 2006). First, frameshift mutagenesis can be initiated by the insertion of an incorrect nucleotide, which creates a mispaired primer terminus that is difficult for DNA polymerase to extend. Subsequent primer–template misalignment can restore proper base pairing, thereby promoting efficient primer extension (Bebenek and Kunkel 1990).

If the misinserted nucleotide is complementary to the next base of the template strand, relocation of the terminus will yield a +1 frameshift intermediate; if complementary to the previous base, realignment will produce a −1 frameshift intermediate (Figure 1B). Second, as an alternative to misinsertion/relocation, in vitro studies suggest that there can be dNTP-stabilized misalignment at the active site of polymerase, with the incoming dNTP base pairing with the next base in the template strand (Figure 1C) (Efrati et al. 1997). This mechanism generates only −1 frameshift intermediates and might be particularly relevant during the bypass of DNA lesions that lack base-pairing potential.

The first defense against polymerization errors derives from the inherent 3′→5′ exonuclease activity of replicative DNA polymerases, which “proofreads” mistakes as they are made (reviewed in Garcia-Diaz and Kunkel 2006). Mutation intermediates that escape proofreading become targets for the postreplicative mismatch repair (MMR) system, which recognizes distortions in the DNA helix (reviewed by Kunkel and Erie 2005). In the context of replication, the MMR system specifically removes a distortion-containing segment of the newly synthesized strand, providing another opportunity for error-free DNA synthesis using the original template. The role of MMR in removing frameshift intermediates is especially important in long runs, which support very high levels of spontaneous primer–template misalignment and are poor substrates for proofreading. In humans, hereditary nonpolyposis colorectal cancer (HNPCC) is associated with MMR defects, the diagnostic feature of which is highly elevated microsatellite instability (Shah et al. 2010).
Because of their association with human disease, most studies of frameshift mutagenesis in yeast have focused on highly repetitive sequences; little attention has been given to events that occur within short repeats or noniterated sequence. In the present study, we have focused on the latter events by removing mononucleotide runs >3N from model frameshift reversion assays used in our earlier analyses (Greene and Jinks-Robertson 1997; Harfe and Jinks-Robertson 1999). Analyses in wild-type (WT) and MMR-defective backgrounds demonstrate that runs of 2N or 3N can promote primer-template misalignment, but do so in a highly sequence-context-dependent manner. Significantly, we find that the nonhomologous end-joining (NHEJ) pathway contributes to frameshift mutations in both iterated and noniterated sequence and is uniquely required to generate de novo tandem duplications of noniterated sequence.

Materials and Methods

Strain constructions

The WT lys2ΔBgl and lysΔA746 strains (SJR357 and SJR922, respectively) were derived from SJR195 (MATα ade2-101oc his3Δ200 ura3Δ1) and were previously described (Greene and Jinks-Robertson 1997; Harfe and Jinks-Robertson 1999). The lys2ΔBgl,NR (NR, no run) and lys2ΔA746,NR alleles were created by site-directed mutagenesis of integrating plasmids pSR699 (lys2ΔBgl) (Steele and Jinks-Robertson 1992) and pSR585 (lys2ΔA746) (Harfe and Jinks-Robertson 1999), respectively. These plasmids contain HIS3 as a selectable marker and a 1.2-kb fragment of LYS2 that spans the reversion window monitored. The primers used to disrupt the 6A run within the reversion window were 5’-GCTAGCTGAAATCTTGG and 5’-CTTTGAAATGTACAGCTAGC (underlined bases reflect the extent of the original run, with mutated bases indicated in boldface type); the 5T and 4A runs were removed using primers 5’-CGTTTGCCGCTGAGTGATCCAGATTC and 5’-GAAATTTGATATCCAGAGGGCAAGAGCGT; and the 4C run was removed using primers 5’-GGAAGGGAGCGCTCAGTTG and 5’-CAACTGAGGCTCCTTCC. The resulting lys2ΔBgl,NR (pSR701) and lys2ΔA726,NR (pSR700) alleles were introduced at the LYS2 locus of strain SJR195 by two-step allele replacement (Rothstein 1991), generating strains SJR1468 and SJR1467, respectively. The MSH2 gene of SJR1467 and SJR1468 was disrupted using a hisG-URA3-hisG cassette (Greene and Jinks-Robertson 1997), yielding strains SJR1472 and SJR1473, respectively. The DNL4 gene of SJR1468 was disrupted using a dnl4Δ::loxP-URA3Kl-loxP cassette amplified from pUG72 (Guelden et al. 2002), yielding strain SJR3134. SJR3232 and SJR3296 were constructed by transforming SJR1467 with a dnl4Δ::kan and tcp1Δ::kan cassette, respectively, amplified from pFA6-kanMX6 (Longtine et al. 1998).

Mutation rates and spectra

Mutation rates were determined using at least 20 cultures from each of two independent isolates of each strain. Cultures were grown to saturation at 30°C in nonselective YEPGE medium (1% yeast extract, 2% Bacto-peptone, 2% glycerol, 2% ethanol, and 250 mg/liter adenine). Appropriate dilutions were plated onto YEPD medium (YEP plus 2% dextrose) to determine total cell number and onto lysine-deficient synthetic glucose medium to select Lys+ revertants. Mutation rates and 95% confidence intervals were determined by maximum likelihood using Salvador 2.0 software (Zheng 2005). Mutation rates for specific mutation types were calculated by multiplying the proportion of that event in the corresponding spectrum by the total Lys+ rate.

To generate mutation spectra, DNA was extracted from purified Lys+ colonies isolated from independent cultures (http://jinks-robertsonlab.duhs.duke.edu/protocols/yeast_prep.html). An appropriate portion of the LYS2 gene was amplified by PCR and sequenced by the Duke University DNA Analysis Facility (Durham, NC), using primer 5’-GTAA CGCGTGACGATG and 3N can promote primer

Results

The lys2ΔBgl allele was constructed by filling in BglIII-generated, 4-nt overhangs, which yields a direct duplication of the sequence GATC and creates the equivalent of a +1 frameshift mutation (Steele and Jinks-Robertson 1992). The lys2ΔA746 allele was constructed by deleting an adenine nucleotide located at position 746 (relative to the upstream XbaI site) of LYS2 and hence contains a −1 frameshift mutation (Harfe and Jinks-Robertson 1999). The lys2ΔBgl and lys2ΔA746 alleles have largely coincident, ~150-bp reversion windows that fall within a nonessential region of the Lys2 protein, allowing the detection of any compensatory frameshift mutation that restores the correct reading frame. Use of these two alleles thus allows a comparison of the relative locations, types, and rates of net +1 and −1 frameshift mutations that occur within a common region of DNA. The longest, naturally occurring mononucleotide run in this region is composed of six adenines (6A run), with an additional 5T, 4A, and 4C run.

We previously reported that most compensatory frameshifts in the lys2ΔBgl and lys2ΔA746 assays were deletions and insertions, respectively, of a single nucleotide within the mononucleotide runs noted above (Greene and Jinks-Robertson 1997; Harfe and Jinks-Robertson 1999). In a repair-proficient background, such mutations comprised 57% and 74% of the reversion spectra, respectively (see Figures 2A and 3A). Because these percentages greatly exceeded the proportion of the window occupied by these runs, and events at smaller runs or noniterated positions were
underrepresented, we concluded that the size threshold for spontaneous primer–template misalignment is 4N. In an msh2 background, which completely lacks the ability to recognize replication-generated mismatches (Kunkel and Erie 2005), the reversion rate of each allele was elevated several hundredfold and there was further skewing of events toward the longer runs. More than 98% of reversion events were in these runs, which completely obscured events that might be occurring at 3N runs, 2N runs, and noniterated sequence. To specifically examine these latter types of events, site-directed mutagenesis was used to remove the four mononucleotide runs >3N from the lys2ΔBgl and lys2ΔA746 reversion windows (highlighted in yellow in Figures 2 and 3). We refer to the resulting alleles as NR alleles, although there remain multiple 3N and 2N runs within the region monitored. As in analyses with the original lys2ΔBgl and lys2ΔA746 alleles, the lys2ΔBgl,NR and lys2ΔA746,NR alleles were located at the endogenous LYS2 locus on chromosome II in all analyses reported here.

Reversion of the lys2ΔBgl,NR allele in a WT background

The reversion rate of the lys2ΔBgl,NR allele was approximately twofold lower than that of the original lys2ΔBgl allele (Table 1), consistent with the elimination of events in runs >3N. Similar to the lys2ΔBgl spectrum, the lys2ΔBgl, NR spectrum was dominated by simple, 1-bp deletions (121/169 = 72%), but a greater variety of additional
mutation types and positions was evident (Figure 2, A and B). We expected that most 1-bp deletions in the lys2ΔBgl,NR spectrum would shift to the 3N runs (highlighted in pink), but only one of the nine 3N runs (indicated with the gray arrow) within the reversion window accumulated more −1 events than predicted by chance (P, 0.0001; expected number was based on proportion of reversion window occupied by the run). Although the overall number of events in the 3N runs (reflecting primarily events in a single 3T run) did not exceed that based on a random distribution of events (P = 0.57), there were many more 1-bp deletions in 2N runs (P < 0.0001) and many fewer events in noniterated sequence (P = 0.003) than expected. Almost 20% (22/121) of the 1-bp deletions occurred in a single 2G run (indicated by the yellow arrow), a run where only one event was observed in the lys2ΔBgl spectrum. We note that this 2G run is only 1 nt removed from the 4C run that was eliminated when constructing the lys2ΔBgl,NR allele (GGACC changed to GGagg), suggesting that local sequence context likely drives 2G hotspot activity. Even if one discounts the 2G hotspot, there was still an excess of 1-bp deletions within the remaining 2N runs (P = 0.0005).

Whereas sequence duplications were rare in the reversion spectrum of the lys2ΔBgl allele (7/145 = 5%), duplications of 2–20 bp accounted for 23% (39/169) of the lys2ΔBgl,NR spectrum. Significantly, more than half (23/39) of these duplications corresponded to the de novo creation of a repeat rather than the expansion of a preexisting repeat. Finally, there were a small number of events (9/169) within the lys2ΔBgl,NR spectrum that did not fall within either the duplication or the 1-bp deletion class, but these were too few in number to analyze in detail.

Figure 3 (A–D) lys2ΔA746,NR reversion spectra. The theoretical reversion window is shown, with runs >3N (or the original positions of these runs) highlighted yellow and 3N runs highlighted pink. All simple, 1-bp insertions are indicated by “+” and are below the sequence; all other mutation types are above the sequence. n, number of independent Lys+ colonies sequenced; cins, complex 1-bp insertion; DEL, deletion. The WT spectrum was published previously (Harfe and Jinks-Robertson 1999).
Table 1 Reversion of the lys2ΔBgl,NR allele in WT, msh2Δ, and dnl4Δ backgrounds

| lys2 allele | Relevant genotype | Lys⁺ rate at 10⁻⁹ (95% C.I.) | Lys⁺ rate relative to ΔBgl,NR WT strain |
|-------------|-------------------|------------------------------|----------------------------------------|
| ΔBgl       | WT                | 2.94 (2.13–3.85)             | 1.0                                    |
| ΔBgl,NR    | WT                | 1.79 (1.33–2.36)             | 1.0                                    |
| ΔBgl,NR    | msh2Δ             | 32.2 (27.5–37.0)             | 18                                     |
| ΔBgl,NR    | dnl4 Δ            | 0.97 (0.69–1.31)             | 0.54                                   |

| Rate of individual mutation type relative to ΔBgl,NR WT strain |
|---------------------------------------------------------------|
| 1-bp deletions | 3N runs | 2N runs | No run | Total | Small dups | Other events |
|----------------|---------|---------|--------|-------|------------|--------------|
| Small dup     | 0.44    | 0.72    | 0.32   | 0.58  | 0.21       | 1.44         |
| Other events  | 0.24    | 0.47    | 1.5    | 0.84  | 1.0        | 1.0          |

C.I., confidence interval; dups, duplications; ND, none detected.

Removal of 1-bp deletion intermediates by the MMR machinery

In our previous analysis, elimination of Msh2 elevated the reversion rate of the lys2ΔBgl allele almost 200-fold, and all but one of 50 revertants analyzed contained a 1-bp deletion within the runs >3N (Greene and Jinks-Robertson 1997). While this demonstrated very efficient repair of −1 frame-shift intermediates that arise in these runs, it was not clear whether other types of events seen in the WT background were simply repaired less efficiently or escaped MMR altogether. This was addressed by examining reversion of the lys2ΔBgl,NR allele in an msh2Δ background. Loss of Msh2 was associated with an 18-fold increase in reversion rate of the no-run allele (Table 1), a 10-fold smaller increase than observed with the original lys2ΔBgl allele.

In contrast to the diversity of mutation types observed in the WT background, all of the 179 lys2ΔBgl,NR revertants sequenced from the msh2Δ background contained a simple, 1-bp deletion event (Figure 2C). The 1-bp deletions localized to discrete hotspots, some of which were prominent both in the WT and in the MMR-defective backgrounds (e.g., the 2G hotspot indicated with the yellow arrow in Figure 2, B and C) and some of which were evident only in the absence of MMR. For example, 70 events occurred at a single 3T run in the msh2Δ background (indicated by the pink arrow in Figure 2, B and C), whereas only one event was seen at this location in WT. The reverse pattern was also evident; the 3T run that was hottest in the WT background (gray arrow in Figure 2, B and D) contained only a single event in the msh2Δ background. Of the three 3A runs, one contained 10 events and the other two each contained only 1 event; of the six 3T runs, one contained 70 events, one contained 16 events, and the remaining four contained at most 2 events. Because the mutations that are elevated in an msh2Δ background presumably reflect replication errors, the data indicate that the probability of persistent primer-template misalignment varies dramatically between runs of the same size and composition.

Nonhomologous end joining produces small duplications in the lys2ΔBgl,NR assay

Given the large reversion-rate increase in the msh2Δ background, the absence of the small duplication class from the corresponding spectrum would be consistent either with dependence on functional MMR or with no change in rate. With regard to the former possibility, we previously reported that suppression of recombination by the MMR system promotes Polζ-dependent mutagenesis via the alternative translesion synthesis pathway, making such mutations dependent on functional MMR (Lehner and Jinks-Robertson 2009). We thus examined whether small duplications depend on the presence of Polζ. Deletion of the REV3 gene, which encodes the catalytic subunit of Polζ (Nelson et al. 1996), neither affected the rate of lys2ΔBgl,NR reversion nor reduced the proportion of small duplications in the corresponding spectrum (data not shown).

The lack of an effect of Msh2 or Polζ loss on small duplications suggests that most are generated outside the context of DNA replication. Because tandem duplications (as well as deletions) can arise when double-strand breaks (DSBs) are repaired via the NHEJ pathway (Daley et al. 2005), we examined the effect of deleting the DNL4 gene, which encodes the ligase required for NHEJ (Teo and Jackson 1997), on reversion of the lys2ΔBgl,NR allele. Relative to the WT background, the rate of lys2ΔBgl,NR reversion was reduced almost twofold in the dnl4Δ background (Table 1), and there were two notable changes in the reversion spectrum (Figure 2D). First, there was a significant reduction in duplications—from 39/169 mutations in WT to 10/113 in the dnl4Δ strain (P = 0.001). Second, there was a loss of simple deletions at two specific positions (indicated by gray arrows in Figure 2, B and D): the 3T hotspot noted previously in the WT background (16/179 vs. 1/113 events; P = 0.002), as well as a 2C run (8/169 vs. 0/113 events; P = 0.016). These data demonstrate that simple deletions within mononucleotide runs can result from error-prone end joining as well as from classical primer–template misalignment.

Reversion of the lys2ΔA746,NR allele in a WT background

The reversion rate of the lys2ΔA746,NR allele was approximately threefold lower than that of the original lys2ΔA746 allele (Table 2), a decrease consistent with the loss of simple 1-bp insertions within the runs >3N (Figure 3A). Simple 1-bp insertions comprised 38% (64/169) of the lys2ΔA746,NR reversion spectrum and were primarily clustered in a subset of the 3N runs (Figure 3B; 3N runs are highlighted in pink). In addition to +1 events, the spectrum contained a large number of 2-bp deletions and 4-bp duplications (20 and
16 events, respectively), neither of which was associated with repetitive sequence elements. Finally, large (144 bp) deletions accounted for a much larger proportion of the *lys2ΔA746,NR* than of the original *lys2ΔA746* spectrum (46/169 and 6/104, respectively), which is consistent with the Lys⁺ rate differences. These large deletions have endpoints in 10-bp direct repeats and are affected by the direction of DNA replication (Abdulovic et al. 2007), suggesting that most reflect repeat-mediated realignment of a blocked 3' end during replication.

**The MMR system efficiently removes +1 frameshift intermediates in 3N runs**

Deletion of the *MSH2* gene was associated with a 6.7-fold increase in the reversion rate of the *lys2ΔA746,NR* allele (Table 2). This increase was accompanied by a proportional increase in +1 events in the corresponding spectrum: from 38% in the WT background to 85% (149/175) in the MMR-defective background (Figure 3C). Most of the simple +1 events were within only three of the nine 3N runs, however, again suggesting that the frequency of replication-associated strand misalignment within individual runs is highly variable. As reported previously, the rate of large deletions was also elevated 3- to 4-fold upon loss of MMR (Harfe et al. 2000). In contrast to the increases in 1-bp insertion and large-deletion rates upon loss of MMR, the 2-bp deletion and 4-bp duplication classes were almost completely absent in the *msh2Δ* background.

**Loss of NHEJ alters the *lys2ΔA746,NR* reversion spectrum**

Given the dependence of 2-bp insertions on NHEJ in the *lys2ΔBgl,NR* assay, we examined the relevance of this pathway to the 2-bp deletion and 4-bp duplication classes detected in the *lys2ΔA746,NR* assay. Deletion of *DNL4* did not change the overall reversion rate of the *lys2ΔA746,NR* allele (Table 2), but it did significantly alter the reversion spectrum in several important ways (Figure 3D). Significant decreases in 2-bp deletions (P = 0.048) and especially 4-bp tandem duplications (P < 0.001) were associated with Dnl4 loss, indicating that both types of events are predominantly produced via NHEJ. There was also a decrease in the proportion of 1-bp insertions (P < 0.001), with reductions being distributed across the spectrum rather than concentrated in specific locations. Finally, there was a twofold proportional increase in the large deletion class (P < 0.001), indicating that, in addition to a DNA polymerase-based realignment mechanism, large deletions with endpoints in direct repeats can result from a DSB repair mechanism that is an alternative to NHEJ. We suggest that the single-strand annealing pathway, which specifically generates deletions between direct repeats (Symington 2002), is the most likely NHEJ alternative.

In a plasmid-based NHEJ assay, 4-bp duplications arise at a low frequency following transformation with linear molecules containing complementary, 4-nucleotide 5’ overhangs. Such events are specifically elevated in the absence of *Tdp1*, a 3’ nucleosidase whose action presumably blocks the filling in of the recessed ends (Bahmed et al. 2010). We thus examined whether loss of *Tdp1* affects reversion of the *lys2ΔA746,NR* allele. Neither the total rate of Lys⁺ revertants nor the proportion of 4-bp duplications in the corresponding spectrum was elevated in a *tdp1Δ* background (Table 2).

**Discussion**

In this study, we have used the complementary *lys2ΔA746, NR* and *lys2ΔBgl,NR* alleles to identify net +1 and −1 frameshift mutations, respectively, within a common, ~150-bp segment of yeast genomic DNA. A key feature of the region monitored is that it contains no mononucleotide runs >3N, thereby allowing detection of rare indels and other mutation types that are normally masked by frequent, spontaneous slippage in longer runs. In a WT background, the total rates of 1-bp insertions vs. 1-bp deletions were similar in the region monitored, but their distributions were very different. This is evident in the compiled spectrum presented in Figure 4A, where events in the eight common 3N runs are highlighted pink to facilitate comparisons. Whereas 70% of 1-bp insertions were in 3N runs, <20% of 1-bp deletions were in these runs. The 1-bp deletions were not randomly distributed, however, but clustered at several 2N hotspots (highlighted in yellow).

Mutations elevated upon loss of MMR reflect errors made by the replicative DNA polymerases that fail to be removed by the associated proofreading activity. In an *msh2Δ* background, 1-bp insertion and deletion rates increased 15-
24-fold, respectively; relative to the WT strain, there was an enrichment of each within 3N runs. Although this demonstrates that 3N runs can promote primer–template misalignment during replication, there was dramatic run-to-run variation with respect to the accumulation of +1 and/or −1 events (Figure 3B). Two of the 3N runs were hotspots for insertions and deletions, one accumulated only insertions, and one accumulated only deletions. Because of the strong context effects observed, we suggest that 1-bp indels in these small runs are most likely derived from misinsertion/primer relocation or dNTP-stabilized misalignment rather than from spontaneous primer–template misalignment. Misinsertion/relocation is expected to generate both 1-bp insertions and deletions, while dNTP-stabilized misalignment is predicted to produce only 1-bp deletions. The accumulation of 1-bp insertions and deletions in 3N runs, but only 1-bp deletions in 2N runs, is intriguing and may indicate that 3N is the lower threshold for misinsertion/relocation. An alternative explanation for the highly variable distribution of the 1-bp indels among 3N runs in the msh2∆ background is that the efficiency of polymerase-associated proofreading is dependent on local sequence context.

Changes in the spectra of spontaneous 1-bp indels upon elimination of MMR are most simply interpreted as site-specific differences in the efficiency of MMR. The efficiency of MMR could be affected, for example, by glycosylase-associated shielding of extrahelical nucleotides (Klapacz et al. 2010). An alternative possibility, however, is the existence of additional mutagenic processes that act outside the context of DNA replication and/or do not generate mismatch-containing intermediates. Indeed, data from the dnl4∆ background indicate that ~50% of the 1-bp indels in a WT background are generated via the NHEJ pathway. Although there appeared to be a general deficit of 1-bp insertions at all positions, two examples of NHEJ-dependent, 1-bp deletion hotspots were evident in the lys2∆Bgl, NR assay (indicated by the arrows in Figure 4). Such NHEJ-associated deletions presumably reflect the removal of nucleotides from one or both ends of the initiating DSB, which may or may not be associated with inappropriate annealing between overhangs and gap-filling reactions. The possible origins of NHEJ-generated insertions as well as duplications are discussed in more detail below.

In addition to facilitating examination of 1-bp indels in very short mononucleotide runs and noniterated sequence, use of the complementary lys2∆Bgl, NR and lys2∆A746, NR alleles allowed the efficient detection of larger insertions and deletions. In the lys2∆Bgl, NR spectrum, de novo tandem duplications, most of which were 2 bp, were frequent and were clearly NHEJ dependent. In addition to de novo duplications, there were a small number of the 2-bp insertions that expanded a preexisting repeat. Similar insertions in
mononucleotide runs were previously reported among lys2ΔBgl revertants isolated in one WT strain background (Heidenreich et al. 2003), but this particular class was not observed in at least two other backgrounds (Marsischky et al. 1996; Greene and Jinks-Robertson 1997). In the lys2ΔA746, NR assay, 2-bp deletions and 4-bp tandem duplications each comprised ~10% of the reversion spectrum, and each class was significantly reduced in the dnl4Δ background.

The tandem, 4-bp duplications seen here are of particular interest as they are similar to those recently reported using a plasmid-based NHEJ assay (Bahmed et al. 2010, 2011). Because such duplications were observed only following transformation of linear molecules with cohesive 5’ overhangs, it was proposed that they are generated by the precise ligation of filled-in, blunt ends (Figure 5A). In the plasmid-based assay, tandem duplications were elevated upon loss of either Tdp1 (Bahmed et al. 2010) or Exo1 (Bahmed et al. 2011). It was suggested that the 3’-nucleosidase activity of Tdp1 converts the recessed 3’-OH to a recessed 3’-phosphate, thereby preventing the filling in of the enzyme-generated end (Bahmed et al. 2010). In the case of Exo1, either its 5’ > 3’-exonuclease or its 5’-flap endonuclease activity could remove the complementary sequence following the fill-in reaction (Bahmed et al. 2011). Although a similar, end-filling mechanism could be generating tandem duplications in the lys2ΔA746, NR assay, we saw no increase in these events in either a tdp1Δ or an exo1Δ background (Table 2 and data not shown). This could reflect a plasmid–chromosome difference in how similar ends are processed (e.g., the ends of spontaneous chromosomal breaks are not accessible to Tdp1 or Exo1), but we think it more likely that the ends are different. Duplications of the sort seen here can be generated, for example, by a misannealing of 3’ (or 5’) overhangs, followed by the filling in of gaps (Figure 5B). This type of mechanism has been proposed to explain the creation of small duplications following the cleavage of yeast genomic DNA with the HO endonuclease, which creates 4-nt, 3’ overhangs (Moore and Haber 1996).

Spontaneous primer–template misalignment requires at least two copies of a repeat unit and so expands only pre-existing repeats. Although the alternative misalignment models presented in Figure 1, B and C, are, in principle, capable of creating 2N mononucleotide runs from noniterated sequence, they cannot be used to generate larger repeat units. For repeat units ≥2 bp, NHEJ can provide a mechanism for creating tandem duplications from noniterated sequence. Bioinformatic studies support this type of mechanism for the origin of microsatellites (Zhu et al. 2000; Leclercq et al. 2010), and data presented here demonstrate that NHEJ-mediated duplications do indeed arise spontaneously in yeast genomic DNA. Finally, we note that NHEJ could provide a mechanism for adding (or deleting) multiple repeat units in a single step. This could, for example, contribute to trinucleotide expansions and may be especially relevant in slow-growing or post-mitotic cells.

While the frequency of primer–template misalignment within mononucleotide runs is strongly correlated with the number of repeat units in vivo (Tran et al. 1997), whether a lower threshold exists has been unclear. While early studies using the original lys2ΔBgl and lys2ΔA746 frameshift-reversion assays suggested that 4N is the likely threshold in yeast (Greene and Jinks-Robertson 1997; Harfe and Jinks-Robertson 1999), more recent bioinformatic studies have concluded that even 2N is sufficient for slippage in yeast (Pupko and Graur 1999) as well as humans (Leclercq et al. 2010). By limiting the current analyses to a region where there are no mononucleotide runs >3N, we have been able to confirm that smaller repeats can be hotspots for indels in yeast, but are not universally so. Importantly, we have shown that the replication-independent mechanism of NHEJ also contributes to 1-bp indels in very short runs and additionally provides a mechanism for the de novo creation of tandem duplications of variable size. Given the high conservation of DNA metabolic processes, the results obtained in the yeast system will likely be of relevance to issues of genome stability and evolution in higher eukaryotes.

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**Figure 5 (A and B) Generation of tandem duplications by NHEJ.** The original DNA is black and newly synthesized DNA is red. Yellow triangles indicate the positions of nicks that create DSBs; the 4-bp sequence duplicated is highlighted gray.
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