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Saccharomyces cerevisiae Ku70 potentiates illegitimate DNA double-strand break repair and serves as a barrier to error-prone DNA repair pathways

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Ku, a heterodimer of polypeptides of ~70 kDa and 80 kDa (Ku70 and Ku80, respectively), binds avidly to DNA double-strand breaks (DSBs). Mammalian cells defective in Ku are hypersensitive to ionizing radiation due to a deficiency in DSB repair. Here, we show that the simple inactivation of the *Saccharomyces cerevisiae* Ku70 homologue (Yku70p), does not lead to increased radiosensitivity. However, *yku70* mutations enhance the radiosensitivity of *rad52* strains, which are deficient in homologous recombination. Through establishing a rapid and reproducible *in vivo* plasmid rejoining assay, we show that Yku70p plays a crucial role in the repair of DSBs bearing cohesive termini. Whereas this damage is repaired accurately in *YKU70* backgrounds, in *yku70* mutant strains terminal deletions of up to several hundred bp occur before ligation ensues. Interestingly, this error-prone DNA repair pathway utilizes short homologies between the two recombining molecules and is thus highly reminiscent of a predominant form of DSB repair that operates in vertebrates. These data therefore provide evidence for two distinct and evolutionarily conserved illegitimate recombination pathways. One of these is accurate and Yku70p-dependent, whereas the other is error-prone and Yku70-independent. Furthermore, our studies suggest that Yku70 promotes genomic stability both by promoting accurate DNA repair and by serving as a barrier to error-prone repair processes.

Keywords: DNA repair/recombination/DNA-PK/Ku/yeast

Introduction

Work in the early to mid-1980s identified Ku as a relatively abundant nuclear protein that is recognized by certain human autoimmune sera (Mimori *et al.*, 1981; Yaneva *et al.*, 1985; Francoeur *et al.*, 1986; Mimori and Hardin, 1986). These and subsequent studies revealed that Ku is a heterodimer of polypeptides of ~70 kDa and 80 kDa (Ku70 and Ku80, respectively) that binds with high selectivity to DNA double-strand breaks (DSBs) and to other discontinuities in the DNA double-helix (e.g. Mimori and Hardin, 1986; de Vries *et al.*, 1989; Paillard and Strauss, 1991; Falzon *et al.*, 1993). More recently, Ku was shown to function as the DNA targeting subunit of DNA-dependent protein kinase (DNA-PK; Dvir *et al.*, 1992; Gottlieb and Jackson, 1993). Consequently, DNA-PK is generally activated only in the presence of DNA DSBs (Carter *et al.*, 1990; Jackson *et al.*, 1990; Gottlieb and Jackson, 1993). The other component of DNA-PK, termed DNA-PK catalytic subunit (DNA-PK_{cs}), is a polypeptide of ~460 kDa (Hartley *et al.*, 1995).

The binding of DNA-PK to DNA DSBs suggested that this enzyme might function in DNA repair and/or recombination. This led to investigations into the Ku/DNA-PK_{cs} status of radiosensitive mutant mammalian cell lines that are defective specifically in the repair of DNA DSBs induced by ionizing radiation (IR) or radiomimetic chemicals (for reviews see Jeggo *et al.*, 1995; Zdzienicka, 1995). Since these cells are not markedly impaired in the repair of other forms of DNA damage, they are termed DSB repair mutants. Interestingly, mammalian DSB repair mutant cells are also severely deficient in the ligation of reaction intermediates generated during V(D)J recombination, the site-specific recombination process that generates the diversity of antigen binding sites of immunoglobulin and T-cell receptor proteins. This defect is evident in all mammalian DSB repair mutants analysed but is manifest most dramatically in the severe combined immune deficient (Scid) mouse, which essentially lacks B- and T-lymphocytes (see Lewis, 1994 for review). Recently, cells of the Scid complementation group were shown to lack DNA-PK activity due to a specific deficiency in DNA-PK_{cs} (Blunt *et al.*, 1995; Kirchgessner *et al.*, 1995; Peterson *et al.*, 1995). Similarly, the DSB repair mutant cell line *xrs-6* lacks DNA-PK activity due to a deficiency in Ku80 (Getts and Stamato, 1994; Rathmell and Chu, 1994; Smider *et al.*, 1994; Taccioli *et al.*, 1994; Boubnov *et al.*, 1995; Finnie *et al.*, 1995). These data therefore indicate that Ku80 and DNA-PK_{cs} are key components of a major DSB repair pathway in mammalian cells. This pathway is termed illegitimate DSB repair because it does not rely on homologies between the recombining DNA molecules.

Several possible roles for Ku/DNA-PK_{cs} in promoting illegitimate DNA repair have been proposed (for reviews, see Jackson and Jeggo, 1995; Jeggo *et al.*, 1995; Roth *et al.*, 1995). These include protecting DNA ends from nuclease attack, recruitment of DNA repair factors to sites of DNA damage, and triggering of DNA damage signalling systems that result in cell cycle arrest and/or the activation of DNA repair components. Because biochemical evidence indicates that Ku80 cannot be targeted to DNA DSBs in the absence of Ku70, and since Ku80 and Ku70 are invariably associated with one another within the cell, it is anticipated that cells deficient in Ku70 will also be defective in DSB repair and V(D)J recombination. To date, however, it has not been possible to test this prediction, as no Ku70-deficient mammalian cell line has been isolated.

Unlike vertebrate systems, where illegitimate DSB repair pathways predominate, lower eukaryotes such as *Saccharomyces cerevisiae* repair radiation-induced DNA

DSBs principally by homologous recombination, which relies on the exchange of genetic information between a damaged DNA molecule and an undamaged partner (for recent reviews, see Friedberg *et al.*, 1995; Haber, 1995; Shinohara and Ogawa, 1995). Two of the best-characterized components of the yeast homologous recombination system are Rad51p, which is related structurally and functionally to the eubacterial protein RecA (Aboussekhra *et al.*, 1992; Shinohara and Ogawa, 1995) and Rad52p, which appears to function in a complex with Rad51p (Milne and Weaver, 1993; Donovan *et al.*, 1994). Because homologous recombination is very efficient in *S.cerevisiae*, illegitimate DSB repair has not been studied extensively in this organism. However, it has been shown that DNA can integrate at low frequency into the yeast genome by illegitimate recombination if the introduced DNA does not bear extensive homologies with genomic sequences, and this illegitimate integration does not require the *RAD51* or *RAD52* gene products (Schiestl and Petes, 1991; Schiestl *et al.*, 1993, 1994). Furthermore, in situations where HO endonuclease-induced chromosomal DSBs cannot be repaired by homologous recombination, at least two types of illegitimate end-joining event occur in yeast cells, and recent investigations have begun to define the gene products that function in these pathways (Kramer *et al.*, 1994; Moore and Haber, 1996).

The above observations raise the possibility that Ku-dependent illegitimate DNA repair operates in *S.cerevisiae*. Consistent with this idea, the gene for the smaller subunit of the heterodimeric *S.cerevisiae* DNA binding protein HDF has been shown to encode a protein of ~70 kDa that displays weak but significant homology to mammalian Ku70 (Feldmann and Winnaker, 1993). Here, and in accord with very recent reports by Siede *et al.* (1996) and Mages *et al.* (1996), we show that yeast Ku70 (from now on referred to as Yku70p) is indeed involved in the repair of IR-induced or chemically induced DNA damage, but that heightened radiosensitivity of *yku70* mutant strains is only readily perceptible in the absence of a functional homologous recombination apparatus. More significantly, however, by establishing a plasmid-based *in vivo* DSB repair assay, we demonstrate that Yku70p is required for efficient and accurate DSB rejoining, even when the homologous recombination apparatus is operative. Finally, through characterizing the repair products generated in the presence and absence of Yku70p, we are led to conclude that Yku70p functions in at least two distinct ways to potentiate accurate and efficient DSB repair. These results provide significant insights into the mechanism of Ku action and help to explain the range of phenotypes associated with mammalian cell lines that lack functional Ku.

Results

Inactivation of YKU70 hypersensitizes rad52 mutant strains to IR

As an approach to address the function of Yku70p, we studied the phenotypic consequences of inactivating the *YKU70* gene in haploid yeast strains. Consistent with the previous report by Feldmann and Winnaker (1993), we found that *yku70* mutant strains were temperature-sensitive for growth at 37°C (Figure 1A). To verify that this phenotype is a direct consequence of a Yku70p deficiency, we cloned the wild-type *YKU70* gene into an episomal yeast vector and then introduced the resulting plasmid (p70FL) into *yku70* mutant cells. Importantly, transformation by p70FL, but not by the parental plasmid vector, results in complementation of the temperature-sensitive growth defect (Figure 1A and data not shown). Next, we assessed the effect of *YKU70* disruption on the sensitivity of yeast strains towards IR and a variety of other DNA-damaging agents. Notably, the inactivation of *YKU70* does not hypersensitize yeast detectably to either low or high doses of IR (Figure 1B). Furthermore, *yku70* mutant strains do not display enhanced sensitivity towards the DNA replication inhibitor hydroxyurea, nor towards agents such as ultraviolet light (UV) and methyl methanesulfonate (MMS; data not shown). However, we did note that Yku70p-negative cells grow more slowly than wild-type cells in the presence of 0.00063% to 0.005% MMS, suggesting some impairment in coping with DNA strand breaks that are induced by this chemical (data not shown).

Since IR-induced DNA damage is repaired predominantly by homologous recombination in *S.cerevisiae*, we reasoned that potential DNA repair defects associated with a lack of Yku70p might only be manifest in terms of significantly increased radiosensitivity in genetic backgrounds where homologous recombination repair is inoperative. To test this hypothesis, we generated strains that were either defective in Rad52p or Yku70p alone or were defective both in Yku70p and Rad52p, and then compared the radiosensitivities of these strains to each other and with parental strains. As expected, the inactivation of *RAD52* alone leads to dramatic increases in sensitivity towards IR (Figure 1C) and MMS (data not shown). Most importantly, however, strains defective in both Rad52p and Yku70p are clearly significantly more sensitive than *rad52* mutant strains to IR, and this is exhibited at all radiation doses tested (Figure 1C). Furthermore, *yku70/rad52* double mutants are also more sensitive than *rad52* single mutant strains to MMS (data not shown). Consistent with the IR and MMS hypersensitivity phenotypes being direct consequences of *YKU70* inactivation, similar results were obtained with a number of independently derived *yku70/rad52* mutant strains (data not shown). Moreover, the hypersensitivity of *rad52/yku70* mutant strains is reversed by the introduction of a yeast plasmid expressing full-length Yku70p (Figure 1C). In contrast to the results obtained with IR and MMS, *yku70/rad52* double mutants are not detectably more sensitive than *rad52* single mutants towards hydroxyurea or UV. These data are consistent with those reported recently by Siede *et al.* (1996) and imply that, in the absence of the *RAD52*-dependent homologous recombination system, Yku70p plays a key role in the repair of DNA strand breaks that are induced by IR or by MMS.

A plasmid-based repair system reveals that yku70 mutants are impaired in DSB repair in vivo

In mammalian cells, deficiencies in Ku function are manifest in decreased efficiencies of chromosomal DSB rejoining (e.g. Kemp *et al.*, 1984; Weibezahn *et al.*, 1985; for review, see Jeggo *et al.*, 1995). To assess whether Yku70p-deficient yeasts also display deficiencies in the rejoining of DSBs, we employed a plasmid repair assay.

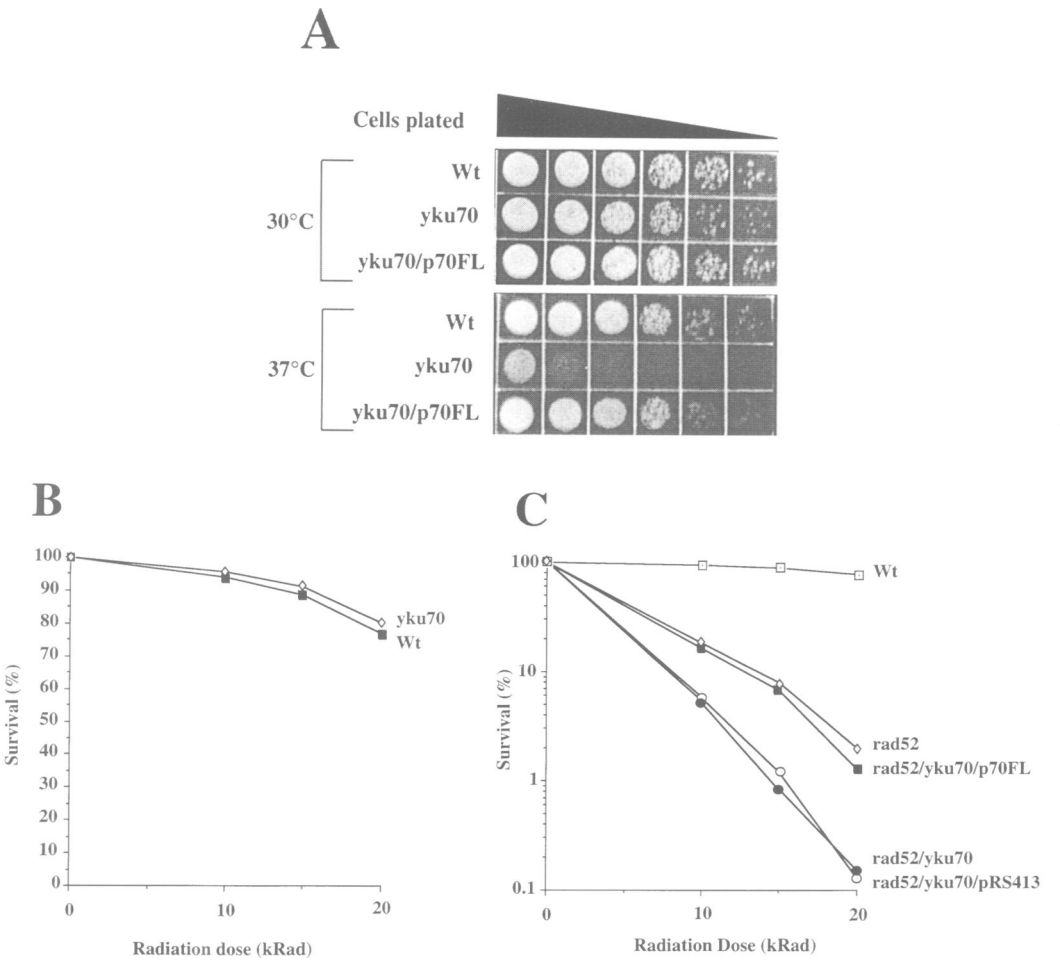
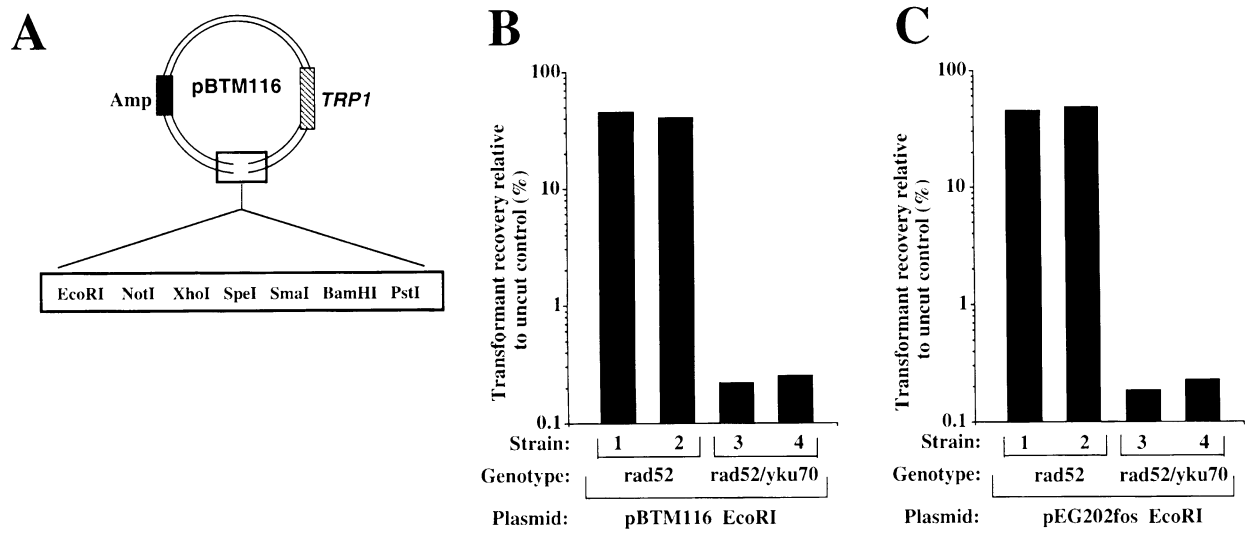


Fig. 1. Phenotypic effects of *yku70* mutations. (**A**) Disruption of *YKU70* function leads to temperature-sensitivity. Aliquots (15 µl) of serial 10-fold dilutions of yeast cultures were spotted onto YEPD plates and were grown for 36 h at 30°C or 37°C, as indicated. Strain abbreviations: Wt, wild-type *YKU70* strain W303-1B; yku70, mutant *yku70* strain yku70a; yku70/p70FL, *yku70* mutant strain complemented by a plasmid that expresses full-length Yku70p. (**B**) Inactivation of *YKU70* does not hypersensitize yeast to IR in a wild-type *RAD52* background. Yeast cultures were subjected to increasing doses of IR and cell viability was assessed after growth on YPED plates as described in Material and methods. Strain abbreviations are as in (A). (**C**) Mutations in *YKU70* hypersensitize *rad52* mutant strains to IR. The IR-sensitivity of strains was assessed as in (B). Strain abbreviations: Wt, wild-type *RAD52/YKU70* strain W303-1A; rad52, mutant *rad52* strain DWY176; rad52/yku70, *rad52/yku70* double-mutant strain rh10b; rad52/yku70 /p70FL, *rad52/yku70* double-mutant strain rh10b complemented by a plasmid that expresses full-length Yku70p; rad52/yku70 /pRS413, *rad52/yku70*, double-mutant strain rh10b containing plasmid pRS413 that does not contain the *YKU70* gene. These results were also obtained using *rad52* strains DWY85, DWY86 and DWY91; *rad52/yku70* strains rh8b and rh12b, with and without the complementing plasmid p70FL.

In this assay, restriction endonucleases are used to generate DSBs within a region of a *S.cerevisiae–Escherichia coli* shuttle plasmid that is not homologous to chromosomal sequences (Figure 2A). Samples of the linearized plasmid are then transformed into yeast strains and the number of transformants obtained are ascertained by counting the number of colonies arising after growth on selective medium. To normalize for potential minor differences in transformation efficiencies between various yeast strains, parallel transformations are performed using a supercoiled version of the same plasmid. Importantly, the plasmids used in these experiments can only be propagated efficiently in *S.cerevisiae* cells after they have been recircularized and ligated. Thus, the efficiency of transformation using linearized plasmid normalized to the efficiency of transformation using uncleaved DNA provides a quantitative readout of the DNA DSB repair capacity of the yeast strain under investigation.

In light of the radiosensitivity data described above, we initially compared the plasmid repair capacities of the *yku70/rad52* double mutants with those of strains disrupted for *RAD52* function alone. To begin, we utilized the restriction endonuclease *Eco*RI, which cleaves at a single site within a non-essential region of the yeast episomal plasmid pBTM116 and thus yields a linear DNA molecule bearing two complementary 5′ overhanging ends (Figure 2A). As shown in Figure 2B, *rad52* mutant strains are very effective at repairing *Eco*RI-linearized plasmids, in that transformants are recovered at levels of between 30% and 50% of those obtained with undigested vector. A striking point, however, is that the fraction of repaired plasmids is reduced by ~100-fold when *yku70/rad52* mutant strains are employed (Figure 2B; repaired plasmid yields are typically 0.2–0.3%). To determine whether this dramatic effect is specific to pBTM116, we carried out similar assays using a variety of other plasmids that had been cleaved with *Eco*RI. In each case, *yku70/rad52* double-mutant strains were found to display hugely depressed transformant yields with linearized DNA compared with *rad52* single mutants (Figure 2C and data not shown).

To test whether the effects described above are specific



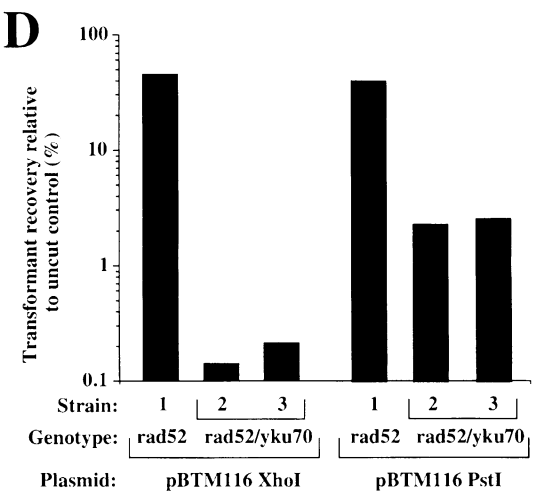


Fig. 2. An *in vivo* plasmid repair assay reveals deficiencies in DSB rejoining in *yku70* mutant strains. (**A**) Map of plasmid pBTM116 showing relevant restriction enzyme cleavage sites. (**B**) *yku70* mutations lead to decreased transformant yield of *Eco*RI-linearized pBTM116 in *rad52* mutant backgrounds. Competent cells for yeast strains of the indicated genotype were transformed, in parallel, with supercoiled pBTM116 and with pBTM116 that had been cleaved with *Eco*RI. For each strain used, the value plotted is the number of transformants obtained with *Eco*RI-linearized vector expressed as a percentage of the number obtained with supercoiled vector. Strains and transformant numbers (number for linearized plasmid/supercoiled plasmid) are as follows: 1, *rad52* mutant strain DWY91 (35510/78564); 2, *rad52* mutant strain DWY176 (36059/85246); 3, *rad52/yku70* double-mutant strain rh8b (1014/72418); 4, *rad52/yku70* double-mutant strain rh10b (1187/69854). (**C**) *yku70* mutations lead to decreased transformant yield of *Eco*RI-linearized pEG202Fos in *rad52* mutant backgrounds. Plasmid repair assays and strains were performed as in (B). Transformant recoveries (*Eco*RI-linearized/supercoiled vector) are as follows: 1, (43020/92518); 2, (38641/81694); 3, (709/78864); 4, (1053/75216). Essentially identical results were obtained using linearized vectors of the pRS series (see Materials and methods). (**D**) In a *rad52* mutant background, defects in *YKU70* lead to dramatically reduced transformant yields for linearized plasmids bearing various types of complementary 5′ and 3′ overhanging DNA ends. Transformations were conducted as in (B) using *Xho*I- or *Pst*I-linearized pBTM116, as indicated.

for *Eco*RI-generated DNA ends, we also performed assays with pBTM116 that had been cleaved with other types of restriction enzyme that generate complementary overhanging DNA ends. In each case, *YKU70* inactivation was found to lead to large (25- to 400-fold) reductions in transformant recoveries (Figure 2D), this being the case for 5′ overhanging DNA ends generated by enzymes such as *Xho*I and *Eco*RI, and also for 3′ overhanging DNA ends that are produced by enzymes such as *Pst*I. Consistent with these plasmid repair defects being attributable specifically to *YKU70* inactivation, similar results were obtained using a variety of independently derived *yku70* mutant strains (data not shown). Moreover, this phenotype is complemented fully by p70FL, which directs the synthesis of wild-type Yku70p, but is not complemented by the parental vector pRS413 (Figure 3B; right-hand panel). Since inactivation of *YKU70* has no appreciable effect on transformation efficiencies obtained with supercoiled plasmid DNAs (e.g. see legend to Figure 2B and C), these data suggest strongly that the reduction in transformant yields with restriction enzyme-digested linear plasmid molecules reflects decreased efficiencies of plasmid DSB repair in the absence of *YKU70* function.

Having identified a highly significant effect of inactivating *YKU70* in *rad52* mutant backgrounds, we next tested whether inactivation of *YKU70* yields discernible plasmid repair deficiencies in strains where the homologous recombination apparatus is operative. Thus, we compared the plasmid repair capacities of the *RAD52*-positive strain W303-1B with its derivatives that are disrupted in *YKU70*. As shown in Figure 3A, for W303-1B (Wt), the transformation efficiency for *Eco*RI-, *Xho*I- or *Pst*I-cleaved plasmid DNA is ~70% of that obtained with undigested vector. In marked contrast, however, for each of the three restriction enzymes used, this value is reduced to <8% for *yku70* mutant strains (Figure 3A). Importantly, this dramatic reduction in transformant yields with linearized plasmids is complemented to essentially wild-type levels by p70FL that expresses the *YKU70* gene, but is not complemented by the parental plasmid pRS413 (Figure 3B). Taken

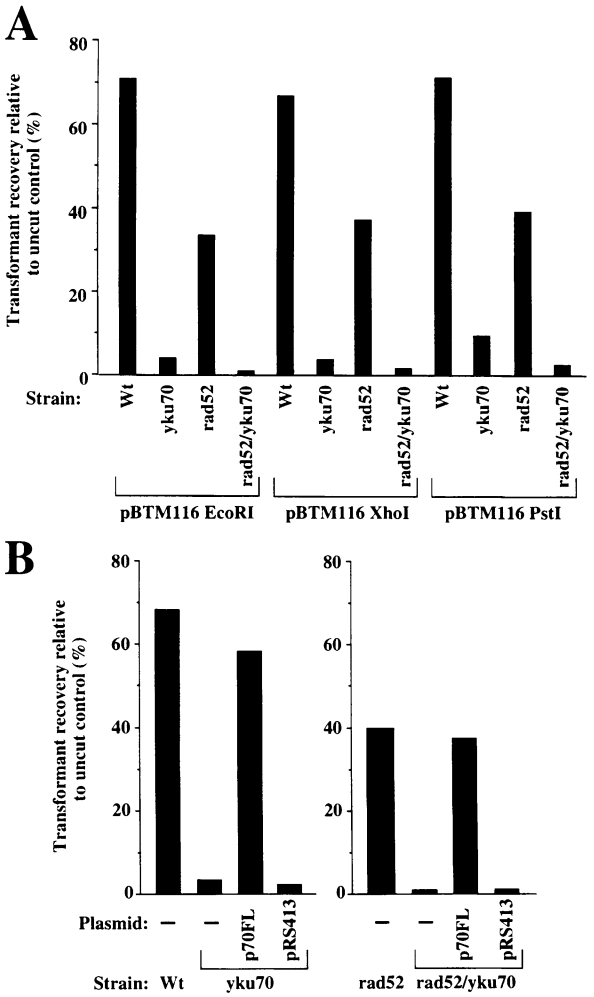


Fig. 3. Disruption of *YKU70* function leads to decreased plasmid repair efficiencies even in the presence of a functional homologous recombination repair system. (**A**) Yeast plasmid repair assays were performed as described in the legend to Figure 2, using pBTM cleaved with *Eco*RI, *Xho*I or *Pst*I, as indicated. Strains used are as follows: Wt, *RAD52/YKU70* strain W303-1α; yku70, *yku70* mutant strain yku70a; rad52, *rad52* mutant strain DWY176; rad52/yku70, *rad52/yku70* double-mutant strain rh12a. (**B**) The plasmid repair defect of *yku70* mutant strains is complemented by a plasmid expressing wild-type Yku70p. Plasmid repair assays were conducted as in (A) using the following yeast strains: Wt, *RAD52/YKU70* strain W303-1a; yku70, *yku70* mutant strain yku70α; rad52/yku70, *rad52/yku70* double-mutant strain rh12a. These strains either did not contain a plasmid (–), contained plasmid p70FL that expresses the full-length wild-type *YKU70* gene, or contained the parental vector pRS413, as indicated. These strains were transformed, in parallel, with supercoiled pBTM and *Eco*RI-linearized pBTM, and transformant yields from the linearized vector were plotted as described in Figure 2.

together, these results indicate that, in *S.cerevisiae*, the dominant pathway for repairing restriction enzyme-generated DSBs that do not lie in regions homologous to chromosomal sequences utilizes Yku70p and does not require the *RAD52* gene product. Nevertheless, it is important to note that inactivation of *RAD52* does lead to an ~2-fold reduction in transformant yields in the plasmid repair assay, and that this effect is observed both in the presence and in the absence of functional Yku70p (Figure 3A and B). The most simple interpretation of these observations is that, in addition to a Yku70p-dependent mechanism for plasmid repair, there exists a back-up pathway that utilizes the *RAD52*-dependent homologous recombination apparatus.

In the absence of Yku70p, residual plasmid repair operates through an error-prone pathway that involves the deletion of terminal DNA sequences

To gain insights into the mechanisms by which Yku70p and Rad52p participate in the repair of restriction enzyme-digested plasmid DNA, individual yeast transformants resulting from the transformation experiments described above were grown up and the plasmids that they contained were rescued into *E.coli*. As a first step to investigate the repaired products, we analysed the ability of recovered plasmids to be cleaved with the restriction enzyme that had been used to cut the plasmid before it was introduced into the yeast strain. Notably, of the ~120 plasmids recovered from *YKU70*-positive strains, every one was capable of being re-cleaved by the appropriate enzyme (e.g. Figure 4B; see Figure 4A for restriction map of pBTM116 which was used in these experiments). A 100% success rate for re-cleavage was observed for plasmids that had been digested originally with *Eco*RI, *Xho*I or *Pst*I and was the case irrespective of whether or not the yeast strain employed was mutated in *RAD52* (data not shown). Furthermore, in all cases tested, digestion of these re-covered plasmids with the restriction enzyme *Hin*dIII, which cuts at sites flanking the site of original cleavage (see Figure 4A), yielded a DNA fragment that was indistinguishable in size from the 1075 bp *Hin*dIII fragment derived from the original plasmid (Figure 4B). Together, these data suggest strongly that, in the presence of functional Yku70p, all plasmids are repaired by the precise religation of complementary DNA ends. In striking contrast, plasmids recovered from *yku70* mutant strains could not be cleaved by the restriction enzyme that had been used to linearize the vector before transformation (Figure 4C and D). In addition, *Hin*dIII digestion of these plasmids liberated fragments of varying sizes that generally migrated significantly faster on agarose gels than the corresponding fragment derived from the original plasmid preparation (Figure 4C and D). Interestingly, careful inspection of restriction digests of plasmids derived from *yku70/RAD52* strains suggested that only two types of product are generated from *Eco*RI-cut pBTM116 (Figure 4C and data not shown). In contrast, *yku70/rad52* double-mutant strains were found to yield a larger spectrum of products (e.g. Figure 4D; see below for more details).

The above results suggest that, whereas the precise religation of complementary DNA ends of pBTM116 occurs efficiently in strains possessing functional Yku70p, this reaction is essentially abolished in *yku70* mutant strains. Instead, the low level of residual plasmid repair that operates in *yku70* mutant cells appears to be mediated by mechanisms that result in the loss of variable amounts of plasmid DNA. To verify that this is the case and to determine the precise nature of the repaired products generated in *YKU70*-positive and *yku70*-negative yeast backgrounds, we subjected representative sets of recovered plasmids to DNA sequence analyses. Significantly, for every plasmid recovered from strains containing functional Yku70p, no nucleotide loss or addition had taken place and the two DNA ends were found to have been repaired by direct ligation. Furthermore, this was the case for all

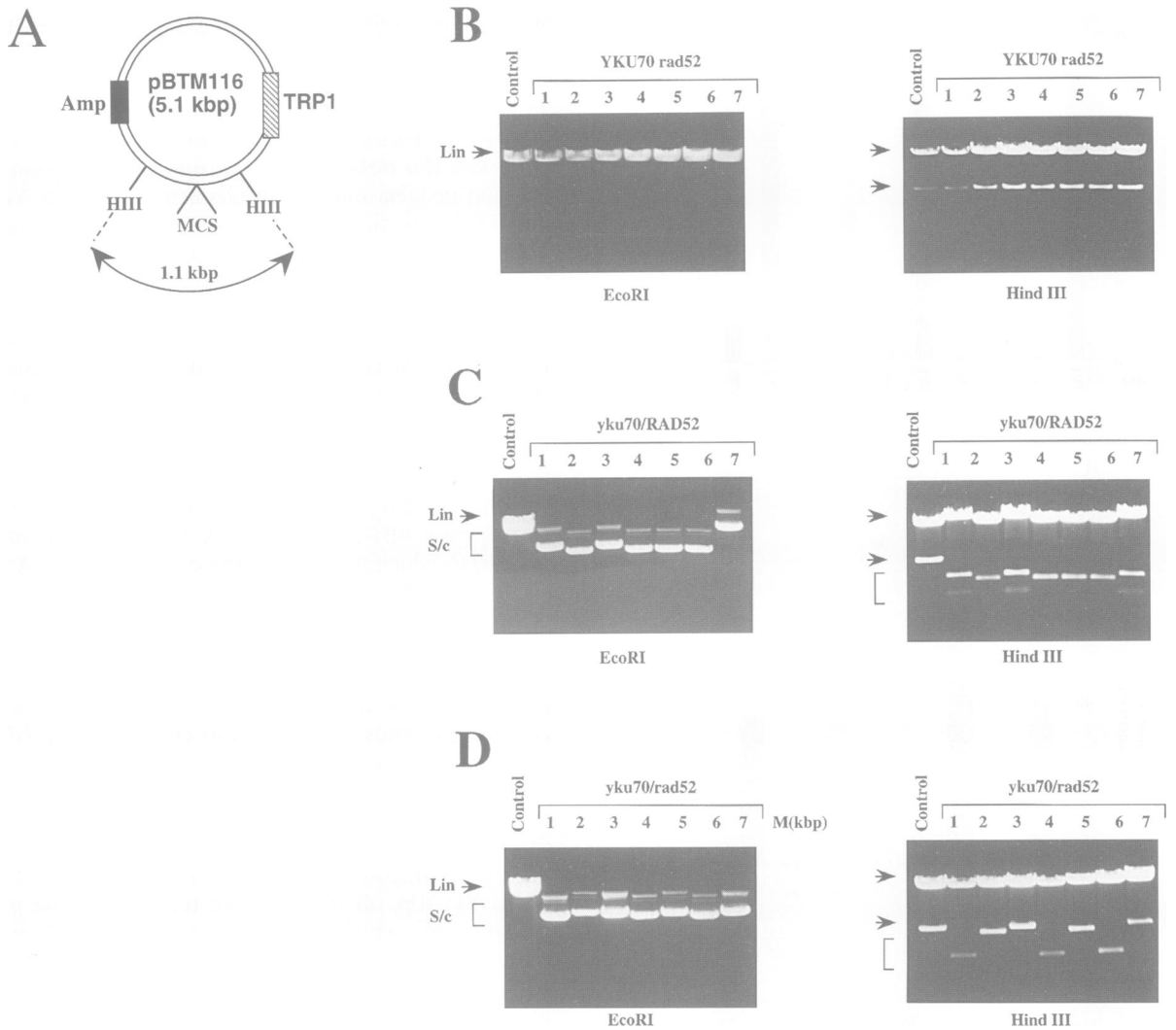


Fig. 4. Analyses of plasmid repair products demonstrates that error-prone DSB repair pathways operate in *yku70* mutant backgrounds. (**A**) Restriction enzyme map of pBTM116 indicating the location of the multiple cloning site (MCS) and the *Hin*dIII cleavage sites that flank this region and are separated from one another by 1.1 kbp of sequence. For details of the MCS, which contains the sites used to cleave pBTM116 before transformation into yeast cells, see Figure 2A. (**B**) Individual transformants arising after the *rad52* mutant strain DWY176 had been transformed by *Eco*RI-linearized pBTM116 were isolated and the repaired pBTM116 plasmid molecules that they contained were rescued into *E.coli*, then were amplified and purified. After cleavage of seven representative plasmid preparations (1–7) with *Eco*RI (left-hand panel) or *Hin*dIII (right-hand panel), the digestion products were separated by electrophoresis on an 1.0% agarose gel and then were visualized by ethidium bromide staining and UV irradiation. As controls, virgin pBTM116 was digested with *Eco*RI or *Hin*dIII and was run as the far left-hand lane ('Control'). In the left-hand panel, the position of linearized (Lin) full-length pBTM116 is indicated by an arrow. In the right-hand panel, the two arrows indicate the locations of the 1.1 kbp and the 4.0 kbp *Hin*dIII digestion products of pBTM116. (**C**) Assays were conducted as in (B) using the *yku70* mutant strain yku70a. Annotations are as in (B) except for the following: in the left-hand panel, none of the plasmid repair products is cleavable with *Eco*RI and the position of the uncleaved supercoiled (S/c) monomeric plasmid is indicated by a bracket. The uncleavable plasmid molecule in lane 7 corresponds to a dimeric version of the plasmids in lanes 1 and 3. In the right-hand panel, the bracket indicates the locations of aberrant *Hin*dIII cleavage products derived from the repaired plasmids. (**D**) Assays were performed as in (B) using the *yku70/rad52* double-mutant strain rh7a. Annotations are as in (C).

three restriction enzymes used. In marked contrast, every one of the plasmids recovered from *yku70* mutant strains had suffered deletions from one or both of the DNA ends before ligation had taken place (Figure 5B). These deletions varied from 1 bp to over 800 bp. Analyses of plasmids derived from *yku70/rad52* double-mutant strains revealed a large diversity of repaired products (e.g. Figure 5B and C). Strikingly, in every instance, ligation was found to have occurred through short, direct repeat sequences in the DNA (Figure 5C). In the majority of cases, the repeat lengths ranged from 4 to 8 bp, although one product (rh7) was generated utilizing a repeat of only 3 bp and one (rh4) employed a region of homology of 16 bp. The plasmid repair products generated in the absence of Yku70p and Rad52p therefore resemble closely certain types of illegitimate DSB repair product that are generated frequently in vertebrate systems (see Discussion).

Consistent with the restriction enzyme cleavage data (Figure 4C), sequence analyses of plasmids derived from *RAD52/yku70* strains revealed that only two types of product are generated from *Eco*RI-digested pBTM116 (Figure 5B and C). One of these (represented by clones yku1, yku3 and yku7) was found to have arisen through a gap-repair mechanism, in which the *ADH1* promoter

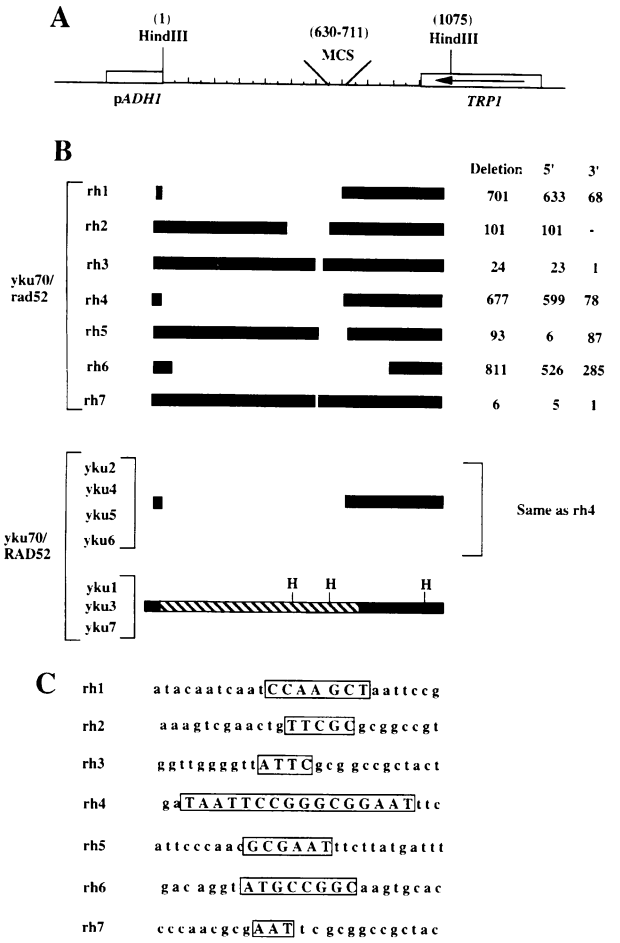


Fig. 5. Sequence analyses of repaired plasmids recovered from *yku70* mutant strains reveal that they have been generated by error-prone DNA repair processes. (**A**) Restriction map of the region of pBTM116 showing the locations of the multiple cloning site (MCS; see Figure 2A), the *ADH1* promoter region (p*ADH1*) and the *TRP1* gene. (**B**) Diagrammatic representation of representative products (see Figure 4) generated from repair of *Eco*RI-linearized pBTM116 in the *yku70/rad52* double-mutant strain rh10b or the *yku70* single mutant strain yku70α, as indicated. The plasmid repair products are denoted rh1–rh7 and yku1–yku7, respectively for these two strains. Black bars represent DNA and align with the restriction map of pBTM in (A), and gaps represent deletions of plasmid sequences. On the right are indicated the total size of the deletion and the numbers of bp that have been lost from the DNA to the left hand (5′) and right hand (3′) of the *Eco*RI cleavage site. Analysis of clones yku1, yku3 and yku7 revealed that they had been generated by a gap repair process involving the chromosomal *ADH1* gene; the striped region represents DNA derived from the genomic locus. (**C**) DNA sequences of junction regions of clones rh1–rh7. In all cases, the two sides of the DNA had become joined through regions of short, direct repeat homologies, which are boxed and are indicated in upper-case letters.

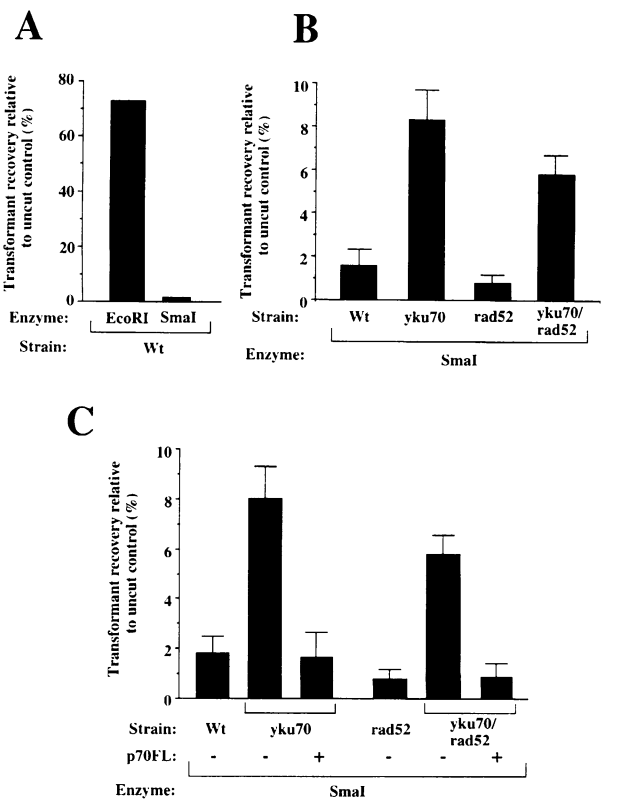


Fig. 6. Effects of YKU70 inactivation on the repair of linearized vectors bearing blunt non-cohesive ends. (**A**) Transformant yields are low with *Sma*I-cut pBTM116 even in wild-type strains. Yeast plasmid repair assays were performed on the YKU70/RAD52 strain W303-1a as described in the legend to Figure 2, using *Eco*RI- or *Sma*I-linearized pBTM116. (**B**) Inactivation of YKU70 results in increased yields of transformants using *Sma*I-linearized pBTM116 in both RAD52 and *rad52* backgrounds. Assays were conducted as in (A) and strains used are as follows: Wt, W303-1a and W303-1b; *yku70*/RAD52, yku70a and yku70α; YKU70/*rad52*, DWY91 and DWY176; *yku70/rad52*, rh7a, rh10b and rh12a. The experiment was repeated at least three times for each strain and the error bars represent the range of values obtained from these experiments. (**C**) The increased transformant yield with *Sma*I-cleaved pBTM116 upon inactivation of *YKU70* is reversed by expression of Yku70p from the episomal vector p70FL. Assays were performed as in (A). Strains used were as in (B) and either lacked (–) or contained (+) p70FL.

and transcription terminator regions of pBTM116 had been engaged in homologous recombination with the yeast chromosomal *ADH1* locus (Figure 5B). The other product (represented by clones yku2, yku4, yku5 and yku6) is identical to the product rh4 obtained from *yku70/rad52* double-mutant strains, and corresponds to a molecule generated as the result of recombination between two perfect 16 bp direct repeat elements that fortuitously flank the multiple cloning site of pBTM116. Although this latter product can form in the absence of Rad52p, the fact it arises much more frequently in *RAD52/yku70* strains than in *rad52/yku70* strains clearly indicates that its formation is potentiated by a functional homologous recombination system. Therefore, despite the fact that regions as short as 16 bp are not generally effective at directing homologous recombination, it appears that such sequences can engage in *RAD52*-dependent recombination to some degree, at least when they are present in direct repeat orientation on the same plasmid molecule.

Inactivation of YKU70 leads to enhanced repair rates of plasmids bearing blunt termini

Having demonstrated that Yku70p is required for the efficient repair of overhanging complementary DNA ends, we next assessed the potential effects of *yku70* mutations on the repair of linear plasmid molecules bearing two 'blunt' termini, which had been generated through digestion with the restriction enzyme *Sma*I. As shown in Figure 6A, this type of DNA end architecture leads to very low transformant yields, even in wild-type yeast backgrounds (transformant yields range from 1.5% to 2.5% of the values obtained using the uncut plasmid). Perhaps surprisingly,

however, and in marked contrast to the results obtained using plasmids bearing complementary DNA termini, disruption of *YKU70* actually results in significant and reproducible 5- to 10-fold increases in transformant yields with *Sma*I-linearized plasmid DNA (Figure 6B). Importantly, this effect is evident both in the presence or absence of a functional *RAD52* gene product (Figure 6B), and is observed with several independently derived *yku70* mutant strains (data not shown). Furthermore, the increased rates of repair of *Sma*I-cleaved DNA in *yku70* mutant backgrounds is reversed when these strains are complemented by plasmid p70FL that expresses full-length wild-type Yku70p but is unaffected by the parental vector pRS413 (Figure 6C).

Analyses of the repaired products of *Sma*I-digested pBTM116 indicated that, unlike the situation with DNA molecules bearing overhanging complementary ends, no detectable direct end ligation takes place. Instead, all of the products analysed had sustained deletions of varying lengths, and had been joined through short, direct repeat motifs. However, although inactivation of *YKU70* increases the efficiency of the repair of *Sma*I-cut DNA, it does not influence the type of repair product generated; the same set of products are generated in the presence of functional Yku70p as are generated in its absence. Moreover, the products generated appear to be identical with those obtained from *Eco*RI-linearized pBTM116 in strains lacking *YKU70* function. Thus, irrespective of *YKU70* status, in wild-type *RAD52* backgrounds, two types of product are generated from the *Sma*I-cut plasmid, and these correspond to products yku1 and yku2 in Figure 5C. Similarly, in *rad52* mutant backgrounds, the repair of *Sma*I-linearized DNA in both *yku70* and *YKU70* strains yields molecules akin to products rh1 to rh7 of Figure 5C. Together, these data indicate that DNA molecules bearing blunt termini cannot be repaired by Yku70p-dependent direct end-joining and, instead, rely on the less efficient error-prone pathway(s) that results in deletion of terminal sequences. Furthermore, the fact that inactivation of *YKU70* leads to increased rates of transformant recovery using *Sma*I-digested plasmid DNA indicates that one role for Ku *in vivo* is to suppress such error-prone repair mechanisms.

Discussion

In mammalian systems, Ku80 exists as a heterodimeric complex with Ku70 and plays crucial roles in DNA DSB repair and V(D)J recombination. Previous work has established that the ~70 kDa subunit of *S.cerevisiae* HDF is ~22% identical in sequence to mammalian Ku70 (Feldmann and Winnaker, 1993). Here, we have shown that this yeast protein is involved in the illegitimate recombination of DNA DSBs and, thus, appears to be a functional homologue of mammalian Ku70. We have therefore renamed this factor Yku70p (yeast Ku70 protein). Significantly, we find that the simple inactivation of *YKU70* does not sensitize yeast cells to IR or to other DNA-damaging agents. However, when the homologous recombination apparatus is rendered inoperative by mutations in *RAD52*, the inactivation of *YKU70* leads to significantly enhanced sensitivity to IR and MMS. These results are consistent with those of Siede *et al.* (1996) and Mages *et al.* (1996), and indicate that Yku70p is involved in DSB repair in *S.cerevisiae*. Furthermore, they imply that Yku70p-dependent repair is distinct from repair that is mediated by the well-characterized homologous recombination system. The available evidence therefore suggests that the mechanisms for both homologous and illegitimate DSB repair are highly conserved from yeast to man. These organisms differ significantly, however, in the relative importance of the two systems in repairing radiation-induced DNA DSBs; whereas homology-driven processes are preferred in yeast, illegitimate repair pathways appear to predominate in higher eukaryotic systems. Although the reason for this difference is unclear, one attractive possibility is that higher eukaryotes, with larger genomes, are forced to rely on illegitimate methods in many circumstances due to kinetic or physical barriers to a broken chromosome entering into synapsis with its partner.

To investigate the type of DNA repair that involves Yku70p, we have devised an *in vivo* assay to measure the repair of linearized plasmid molecules whose ends are not homologous to chromosomal sequences. Strikingly, we find that strains defective in *YKU70* are transformed efficiently by closed circular plasmid molecules but are severely impaired in yielding transformants when the plasmid has been cleaved by restriction endonucleases that yield overhanging complementary ends. This 25- to 100-fold reduction in plasmid repair that is caused by Yku70p defects is evident both in the presence or absence of functional *RAD52*. The establishment of a simple, highly sensitive and reproducible plasmid repair assay for defects in *YKU70*, together with an ability to complement such defects with the wild-type *YKU70* gene, should greatly facilitate the rapid identification of important functional domains of Yku70p. Such an assay system for Yku70p should prove to be particularly valuable in light of the current lack of a Ku70-deficient mammalian cell line.

In order to gain insights into Yku70-dependent and Yku70-independent DNA repair, we retrieved plasmid repair products generated in various strains and subjected them to molecular analyses. Most notably, we find that, in the presence of functional Yku70p, every plasmid is rejoined by direct ligation and does not suffer deletion or insertion of DNA sequences. In contrast, other than one type of repair product that is generated by *RAD52*-dependent recombination with chromosomal DNA, in the absence of Yku70p every plasmid is repaired by an error-prone system that results in the deletion of terminal sequences. Together, these findings indicate that *S.cerevisiae* possesses at least three distinct pathways for repairing DNA DSBs: (i) *RAD52*-dependent homologous recombination; (ii) *YKU70*-dependent illegitimate end-joining; and (iii) *YKU70*-independent illegitimate recombination (Figure 7). Our data are consistent with a model in which yeast cells generally use homologous DSB repair and only resort to the other repair pathways when the damaged DNA is unable to undergo pairing with an undamaged template. Potential natural situations where illegitimate DSB repair systems might operate are when two homologous chromosomes are not closely apposed, or when homologous pairing is impeded by proteins bound to chromosomal DNA. Another is when haploid yeast cells traverse the G_1 phase of the cell cycle.

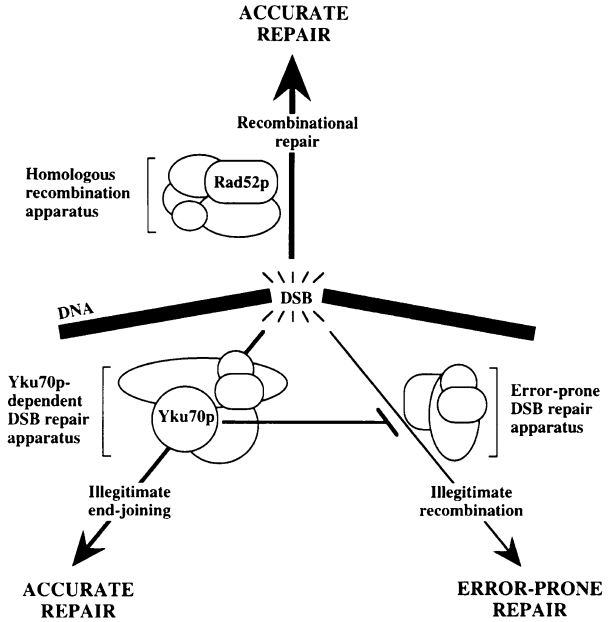


Fig. 7. Schematic representation of the three types of DSB repair that operate in *S.cerevisiae* (see text for details).

In this regard, it is noteworthy that *yku70* mutations lead to slightly enhanced radiosensitivity when haploid *RAD52* strains are irradiated in stationary-phase cultures, where a large fraction of cells exist in G_1 (Siede *et al.*, 1996; S.J.Boulton and S.P.Jackson, unpublished data).

In circumstances where DNA ends bearing complementary overhanging ends cannot be repaired by homologous recombination, they appear to be repaired almost exclusively by Yku70p-dependent illegitimate end-joining. At first, the requirement of Yku70p for the repair of complementary DNA termini is surprising; one might anticipate that such reactions could be mediated by a DNA ligase alone. However, it is known that purified ligase enzymes are very ineffective at rejoining DSBs at physiological temperatures, even when cohesive DNA termini are present. The fact that DNA end-joining operates extremely efficiently in eukaryotic cells and in crude eukaryotic cell lysates, therefore, implies the existence of additional DNA repair components. In light of our findings, it is tempting to speculate that Ku might function as an end-alignment factor in DSB repair by recognizing DNA termini and juxtaposing them to facilitate their ligation. Although it is possible that the Yku70p-dependent repair system comprises just Ku and a ligase, it is probable that other factors are also required. One attractive candidate for such a factor is Rad50p, which plays a crucial role in the illegitimate integration of DNA into the yeast genome (Schiestl *et al.*, 1994). Also in this regard, it is noteworthy that, in mammalian systems, DNA-PK_{cs} associates with Ku and is an important component of the DSB repair apparatus (Jackson and Jeggo, 1995; Jeggo *et al.*, 1995). Although no DNA-PK_{cs} homologue appears to exist in *S.cerevisiae* by strict sequence criteria (S.J.Boulton and S.P.Jackson, unpublished data), *S.cerevisiae* cells do possess the DNA-PK_{cs}-related proteins Mec1p and Tel1p (for reviews, see Jackson, 1995; Zakian, 1995; Carr, 1996). Given the fact that Tel1p and Mec1p have been linked to DNA damage recognition pathways, it will clearly be of great interest to determine whether *tel1* or *mec1* mutant yeasts display deficiencies in the efficiency or accuracy of plasmid DSB repair. Because IR-induced DNA damage is repaired predominantly by homologous recombination in yeast, it is easy to see why components of the Yku70p-dependent DNA DSB repair system might not have been identified by previous genetic screens for radiosensitive yeast mutants. It may therefore prove fruitful to reconduct such screens in *rad52* mutant backgrounds.

It is interesting to consider situations in yeast where a DSB cannot be repaired either by homologous recombination or by Yku70p-dependent end-joining. Our results indicate that, in such circumstances, products are generated that have lost terminal sequences and in which recombination has occurred through short, direct repeat sequences. Although these products could arise through strand-invasion mechanisms, we currently favour a model in which they are produced through a single-stranded annealing-type (SSA) pathway that has been implicated previously in DSB repair processes in *S.cerevisiae* (e.g. Kramer *et al.*, 1994; Mezard and Nicolas, 1994; Moore and Haber, 1996). In this model, single-stranded regions are generated from the DNA ends by strand-specific exonucleases; complementary strand pairings then occur between short repeat elements and, after the removal of unpaired single-stranded tails, repair synthesis by DNA polymerase and ligation ensue. Interestingly, DSB repair events involving deletional mechanisms that result in joining via short regions of homology have been described in many instances in vertebrate systems (e.g. Roth and Wilson, 1986; Thacker *et al.*, 1992; King *et al.*, 1993), suggesting that, as with homologous recombination and Ku-dependent end-joining, this pathway is highly conserved throughout the eukaryotic kingdom.

Because of the apparent inefficiency of error-prone DSB repair and since this process is inherently mutagenic, it is tempting to speculate that, at least in yeast, this is a salvage pathway that operates only when DSBs cannot be repaired by other means. Consistent with this, we find that cohesive DNA ends are only repaired by this mechanism in *S.cerevisiae* when repair by homologous recombination or Yku70p-dependent end-joining is not possible. Furthermore, we have found that Yku70p actually suppresses the error-prone repair of blunt-ended linearized plasmid molecules. Together, these findings suggest that, in addition to functioning positively in promoting direct end-joining events, Yku70p may have the secondary role of serving to suppress error-prone DSB repair. One way that this could occur is by Ku simply preventing components of the error-prone repair system from gaining access to the DNA termini. This model is particularly appealing in light of the involvement of exonucleases such as the Rad1p/Rad10p complex in SSA pathways (Ivanov and Haber, 1995), and in view of the fact that Ku can protect DNA ends from exonuclease attack (Getts and Stamato, 1994). It is tempting to speculate that mammalian Ku also has a dual role in controlling DSB repair processes. Indeed, the available evidence indicates that mammalian Ku-deficient cells are impaired both in the efficiency and accuracy of DSB repair; e.g. analyses of the rare V(D)J recombination signal sequence junctions that form in Ku80-defective hamster cells has revealed that they bear terminal deletions and tend to map to short, direct repeat sequence elements

Table I. Yeast strains

Strain	Genotype	Constructed by:
W303-1A	*Matα ade2 his3 leu2 trp1 ura3 can1-100*	Feldmann and Winnacker (1993)
W303-1B	*Mata ade2 his3 leu2 trp1 ura3 can1-100*	Feldmann and Winnacker (1993)
yku70α	W303-1a *yku70::URA3*	Feldmann and Winnacker (1993)
yku70a	W303-1b *yku70::LEU2*	Feldmann and Winnacker (1993)
DWY85	*Matα ho::LYS2 leu2::hisG rad52::URA3*	D.Weaver
DWY86	*Mata l ho::LYS2 leu2::hisG rad52::URA3*	D.Weaver
DWY91	*Matα arg4.RV leu2-3-112 cyhR trp1-289 rad52::URA3*	D.Weaver
DWY176	*Mata his3 cyhS ade2 ura3-52 trp1-289 rad52::URA3*	D.Weaver
rh7a	*Mata arg4.RV leu2.3-112 trp1 ade2 his3 yku70::LEU2 rad52::URA3*	this study
rh8b	*Mata ade2 his3 trp1 ura3 can1-100 yku70::LEU2 rad52::URA3*	this study
rh10b	*Matα ade2 his3 trp1 ura3 can1-100 yku70::LEU2 rad52::URA3*	this study
rh12a	*Mata ade2 trp1 his3 yku70::LEU2 rad52::URA3*	this study
rh16b	*Matα ade2 trp1 his3 yku70::LEU2 rad52::URA3*	this study

(Pergola *et al.*, 1993). Upon considering these points, it seems likely that future investigations into Ku function will reveal yet further fundamental similarities between illegitimate DSB repair pathways in yeast and mammalian systems.

Materials and methods

Yeast strains

Yeast strains used are listed in Table I.

Yeast media and growth conditions

Non-selective medium (YPED), selective media (YNB), pre-sporulation and sporulation media were as described by Sherman *et al.* (1979). Sensitivity towards MMS was measured as described by Milne and Weaver (1993). Briefly, yeast colonies were picked into distilled water, and diluted six times by serial 10-fold dilutions. Aliquots (15 μl) of each dilution were spotted in duplicate onto YPED plates with or without the appropriate concentration of MMS (0.00063%, 0.0025%, 0.005%), and were then incubated at 30°C for 3–4 days. The temperature-sensitivity of yeast strains was determined by spotting 15 μl aliquots of a serially diluted culture (as for the MMS phenotype above) in duplicate onto non-selective media which were then incubated at 30°C or 37°C, for 3–4 days.

X-ray cell survival assays

Yeast colonies were used to inoculate 10 ml of YPED and were grown overnight by incubation at 30°C. The cultures were then diluted in water to an OD_{600} value equivalent to 1×10^7 cells/ml. Aliquots (1 ml) were then irradiated using a ^{137}Cs source for various time points at a dose of 0.18 krad/min. Irradiated and non-irradiated control samples were then diluted serially in water, plated in duplicate on YPED, and incubated at 30°C for 3–4 days.

S.cerevisiae plasmid repair assays

Undigested plasmid DNA (5 μg) or an equivalent amount of plasmid DNA that had been digested to completion with the appropriate enzyme(s), was transformed into competent yeast cells by the method of Schiestl and Gietz (1989). Transformation reactions were then plated as serial dilutions onto selective media and colonies were counted after plates had been incubated for 3–4 days.

Plasmids and vectors

All yeast–*E.coli* shuttle vectors used have CEN/ARS sequences for stable maintenance in yeast, an auxotrophic yeast selectable marker, an OriC for high copy number in *E.coli* and a β-lactamase gene for ampicillin selection in *E.coli*. Plasmids used are as follows: p70FL; selectable marker is *HIS3* (see Cloning of full-length *YKU70*). pBTM116; selectable marker is *TRP1* (see Figure 2A). pEG202.fos; selectable marker is *HIS3* and was constructed by inserting a 1.1 kb fragment of the human c-Fos cDNA into the polylinker of pEG202 (Bennetzen and Hall, 1982). pRS413/4/5/6 (Stratagene); contain the *HIS3*, *TRP1*, *LEU2* or *URA3* selectable markers, respectively (Stratagene).

Cloning of full-length YKU70

Full-length *YKU70* was cloned by a PCR approach using the Bio-X-act proof-reading DNA polymerase (Bioline). The oligonucleotide primers used were yKu70-3 (GAGATTTCTATGCTCGAGGAGAACTTC) and yKu70-5 (GGGACCCACAAAGGATTCTCAGGAAGTGG) and generated a product of 2.667 kbp that contained the entire *YKU70* open reading frame and its 5′ and 3′ flanking regions. This was cloned into the pGEM-T vector (Promega), then was sub-cloned into the yeast–*E.coli* shuttle vector pRS413 (Stratagene), which is an episomal plasmid containing the *HIS3* gene. The sequence of the *YKU70* insert in the resulting plasmid, p70FL, was then verified by automated sequencing using an Applied Biosystems sequencer.

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