

## Stability of YACs Containing Ribosomal or RCP/GCP Locus DNA in Wild-type *S. cerevisiae* and *RAD* Mutant Strains

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### Abstract

About 2% of human YAC clones, including tandemly repeated segments color vision pigment DNA, ribosomal DNA and alphoid DNA have been reported to be inherently unstable in yeast hosts, producing more stable deletion products. YACs containing color vision red pigment gene DNA or 1.5 rDNA tandem repeat units were transformed into hosts bearing lesions at the *RAD1*, *RAD6*, *RAD51*, or *RAD52* loci. YACs susceptible to deletion during outgrowth of wild-type cells (or in preliminary experiments, in *RAD6* transformants) were stable for up to 100 generations or more in the other strains. Thus both the *RAD1* and *RAD51/RAD52* epistatic pathways are apparently involved in the instability of YACs containing tandem repeat loci, presumably during recombination-based deletion formation; and a yeast host disarmed in these pathways will likely maintain YACs intact that are otherwise unstable.

**Key words:** YAC clone; instability; *rad* mutant

### 1. Introduction

Yeast artificial chromosomes (YACs) have proven an efficient cloning system for most human and other complex genomic DNA. Three problems have been encountered repeatedly, however, in studies of YACs from a number of chromosomal regions: first, a fraction of genomic DNA is not recovered in YACs; second, a considerable fraction of the YACs in a typical collection are chimeric or "cocloned," arising by the coming together of DNA fragments from more than one site in the genome; and third, about 2% of YACs are patently unstable, producing a series of smaller derivatives that lack internal DNA and can be rearranged.<sup>1</sup>

The extent to which these problems have a common or overlapping basis is unknown; but at least two of them, chimerism and instability, are related to recombination in yeast. Regarding chimerism, in two instances that have been analyzed at the sequence level, chimeric YACs were demonstrated to arise from a recombinational event.<sup>2</sup> In the first reported study of instability, Neil et al.<sup>3</sup> showed that tandem repeats of alphoid sequences were unsta-

ble in many YACs, and that consistent with linked recombination, the instability was partially alleviated in a recombination-deficient (*RAD52*) yeast strain. The notion is thereby strengthened that a strain disarmed in particular recombination capacities might be a favorable host for the construction and preservation of YACs.

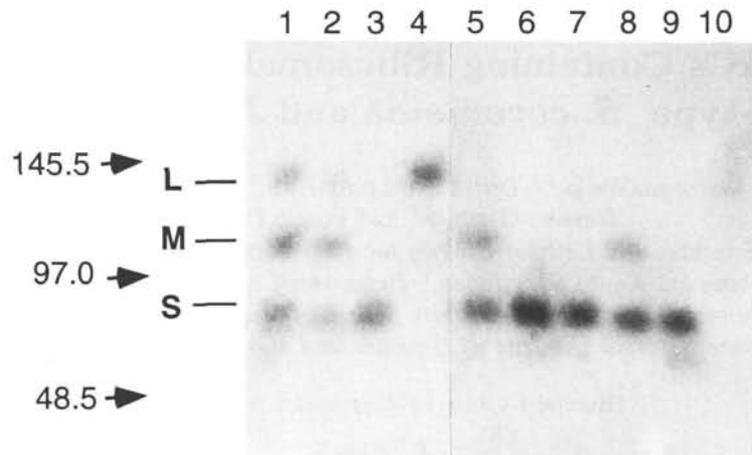
*RAD52* is one of a number of genes involved in the high recombinogenicity of yeast. Three epistasis groups have been defined for yeast genes based on cellular response to DNA damage.<sup>4,5,6</sup> The type organisms for these groups have lesions in *RAD3* or *RAD1*, *RAD52* or *RAD51*, and *RAD6*.

To extend the study of YAC stability in such yeast mutants, a major requirement is the availability of well-characterized YACs in which events can be examined in detail. Ideally, YACs should undergo well-defined deletional or recombinational events, but at a rate that permits both recovery of the initial intact YAC and observation of changes in the rate in mutant strains; an estimated rate of occurrence of about 1 in 1,000 cells would be appropriate. Here, we report results with two human gene clusters in which tandemly repeated units are unstable in YACs at an appropriate level. For both systems, YACs containing 18S-28S ribosomal DNA or the color vision locus, the use of recombination-affected yeast strains is extended to show essentially complete stabilization in *RAD1* and *RAD52* or *RAD51*, but apparently not in a

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**Figure 1.** Instability of yWXD411 in wild-type yeast host. Original cells of strain AB1380 strain carrying yWXD411 DNA and AB1380 cells retransformed with the yWXD411-S DNA or yWXD411-L DNA species were grown aerobically in 10 ml of YPD for 4 days at 30°C, to a density of  $1 \times 10^8$  cells/ml. One hundred microliters of each culture were inoculated into 10 ml of YPD and were again incubated aerobically for 4 days at 30°C. Dilution and reculture was repeated 7 times (an estimated total of 105 generations). One-hundred microliters of each culture were then transferred into 10 ml of SD medium without uracil, and after growth for 4 days at 30°C, the cells were embedded in agarose plugs and lysed. After fractionation of the chromosomal DNA by pulsed-field gel electrophoresis, the gel was dried and hybridized with a  $^{32}\text{P}$ -labeled probe of *hs7* cDNA of the red pigment color vision gene.<sup>28</sup> yWXD411, YAC DNA of original clone cultured in SD medium (lacking uracil) (lane 1); the original clone yWXD411, cultured in complete medium (YPD) (lane 2); yWXD411-S DNA and yWXD411-L DNA from cells retransformed with small (lane 3) or large fragment (lane 4) after the first culture in complete medium (YPD); after the sixth culture (lanes 5, 6 and 7); and after the seventh culture (lanes 8, 9 and 10). Values shown on the ordinate are in kilobases. The L, M, and S DNA species are approximately 140, 115 and 90 kb.

*RAD6* host. Hosts incorporating lesions in the *RAD1* and *RAD52/RAD51* epistatic pathways may provide a route to create or preserve YACs that are otherwise unstable or unrecoverable.

## 2. Materials and Methods

### 2.1. Yeast strains and YAC clones

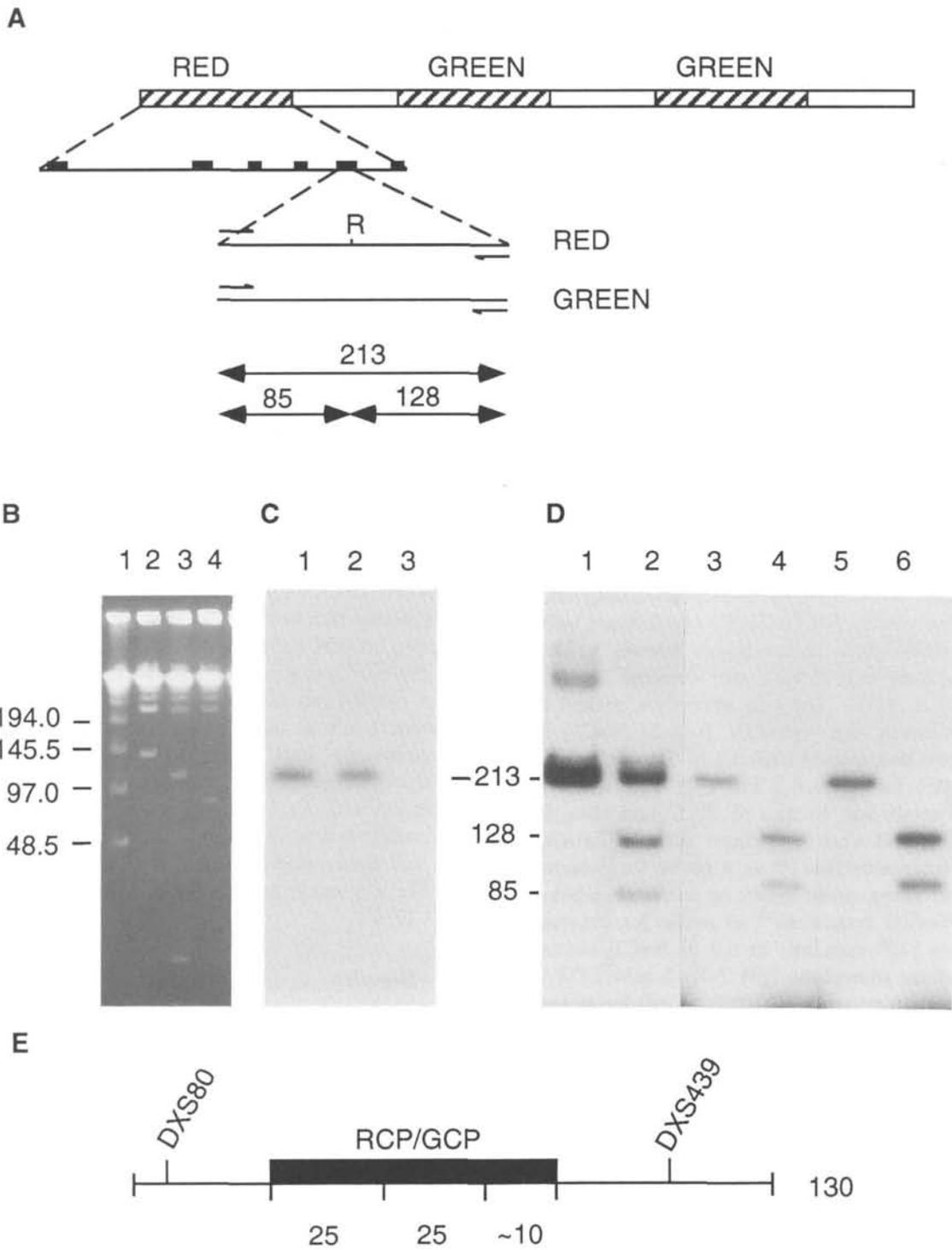
All yeast strains used in this study are shown in Table 1. YAC clones used for testing instability were yWXD411, containing the human color vision locus of

Xq28,<sup>7</sup> and yB3B3, containing the human rDNA units.<sup>8</sup>

### 2.2. Media and growth conditions

Synthetic medium (SD) contained 0.67% yeast nitrogen base without amino acids (Difco Laboratories), 0.5%  $(\text{NH}_4)_2\text{SO}_4$ , 2% glucose and the necessary amino acid supplements. Nutrient medium (YPD) contained 1% yeast extract, 2% peptone and 2% glucose. For solid media, 2% agar was added to the above media. Yeast cells were cultured aerobically in 10 ml medium in culture tubes on a rotating platform shaker (EYELA, MBS-1A) at 30°C.

**Figure 2.** Analysis of the deleted region in the intermediate-sized molecules of color vision DNA. A. Schematic representation of the color vision locus. The positions of red or green opsin genes are indicated by filled boxes on the top line, and filled rectangles along the expanded scale mark the exons of a red opsin gene. Small arrows represent the primers used for PCR amplification of exon 5 DNA of both red and green opsin genes, used as hybridization probes. Intact PCR-amplified DNA from intact yWXD411 DNA and restriction fragments of the PCR product digested with *RsaI* (R) are shown as bidirectional arrows. Values shown are in base pairs. B. Pulsed-field gel electrophoresis of yWXD411 DNAs from cells retransformed with purified L-, M- or S- yWXD411 DNA and cultured once. Lane 1 contains a size reference of concatemeric *lc 1857* DNA (Bio-Rad). yWXD411-L (lane 2), yWXD411-M (lane 3) and yWXD411-S (lane 4) DNAs from the transformants were fractionated by using CHEF gel electrophoresis with a switching time of 1–15 s for 22 h. Values on the ordinate are indicated in kilobases. C. Polyacrylamide gel electrophoresis of  $^{32}\text{P}$ -labeled PCR products. Lanes 1, 2 and 3 are the products from cultures retransformed with yWXD411-L, yWXD411-M and yWXD411-S DNAs, respectively. The PCR products detected with the labeled *hs7* probe in lanes 1 and 2 are 213 bp in length. D. Polyacrylamide gel electrophoresis of  $^{32}\text{P}$ -labeled PCR products from cellular DNA and *RsaI*-digested fragments of the PCR products. Lanes 1, 3 and 5 are  $^{32}\text{P}$ -labeled PCR products from genomic DNA of the human-hamster hybrid cell X3000.11, yWXD411-L DNA and yWXD411-M DNA, respectively. Lanes 2, 4 and 6 are the  $^{32}\text{P}$ -labeled PCR products from cellular DNA of the hybrid cell X3000.11, yWXD411-L DNA and yWXD411-M DNA, respectively, after digestion with *RsaI*. Values on the ordinate are in base pairs. E. Schematic of the possible structure of the 130 kb insert of yWXD411 (see text). Values on the ordinate are indicated in kilobases.



**Table 1.** Experimental strains of *S. cerevisiae*

Strain	Genotype	Source
AB1380	Mat a <i>ade2-1 can1-100 his5 lys2-1 trp1 ura3</i>	L. Riles & M. V. Olson <sup>22</sup>
M3699-1A	Mat a <i>rad1-1 ade2 his3 leu2 trp1 ura3-52</i>	T. Saeki et al. <sup>a),23</sup>
YKK055	Mat a <i>rad6-1 his1-1 leu1-1 trp5-18 ura3</i>	This study <sup>b)</sup>
YKK054	Mat a <i>rad52 his1-1 leu1-1 trp5-18 ura3</i>	This study <sup>c)</sup>
XS3672-3c	Mat a <i>rad51-1 ade2 his4 leu2 lys2 ura3</i>	A. Shinohara et al. <sup>14</sup>
814-9/2d	Mat a <i>rad52::TRP1 ade2-1 his3-11 his3-15 leu2-3 leu2-112 lys2-1 trp1-1 ura3-1</i>	D. L. Neil. et al. <sup>3</sup>
814-7/4c	Mat $\alpha$ <i>rad1::LEU2 rad52::TRP1 ade2-1 leu2-3 leu2-112 lys1-1 trp1-1 ura3-1</i>	D. L. Neil. et al. <sup>3</sup>
846/11b	Mat a <i>rad52<math>\Delta</math> ade2-1 his3-11 his3-15 leu2-3 leu2-112 lys1-1 trp1-1 ura3-1</i>	V. Vilageline & C. Tyler-Smith <sup>24</sup>

<sup>a)</sup> The *rad-1* mutation of the M3699-1A strain donated from Saeki, T. is described in ref. 23. <sup>b)</sup> YKK055 harboring the *ura3* mutation was constructed from XS 1828-4C<sup>25</sup> according to the method described by Boeke et al.<sup>26</sup> <sup>c)</sup> YKK054 carrying the *ura3* mutation was constructed from XS 1882-4B<sup>27</sup> according to the method reported previously.<sup>26</sup>

### 2.3. Gel electrophoresis and southern analysis

DNA for conventional analysis and high-molecular-weight DNA for pulsed-field gel electrophoresis were prepared by standard procedures.<sup>9,10</sup> High-molecular-weight DNA samples were fractionated by a contour-clamped homogeneous electric field (CHEF) apparatus<sup>11</sup> using the switching times given in the figure legends. The gels were 1% agarose in 0.5XTBE and were run at 10 V/cm for 18–22 h at 14°C. The gels were then soaked in denaturing solution (0.5 N NaOH, 0.15 M NaCl) for 30 min and were neutralized with 0.5 M Tris-HCl (pH 7.5), 0.15 M NaCl for 30 min. The gels were dried under reduced pressure for 40 min at 25°C and then for 30 min at 60°C, and were hybridized with <sup>32</sup>P-labeled 1.0 kb *EcoRI* fragments (hs7)<sup>12</sup> as a probe for determining the red and green opsin genes or with <sup>32</sup>P-labeled 0.4 Kb *XbaI-HindIII* fragments<sup>13</sup> as probe for determining rDNA genes (10<sup>6</sup> cpm/ml) in 0.9 M NaCl, containing 0.05 M sodium phosphate (pH 7.0), 5 mM EDTA, 1% SDS and salmon sperm DNA (200  $\mu$ l/ml) for at least 12 h at 65°C. The gels were washed twice with 5XSSC at 25°C for 5 min, twice with 5XSSC at 65°C for 2 h, and were exposed to X-ray film at –70°C for 30 min –24 h.

### 2.4. Polymerase chain reaction with yWXD411 YAC DNA

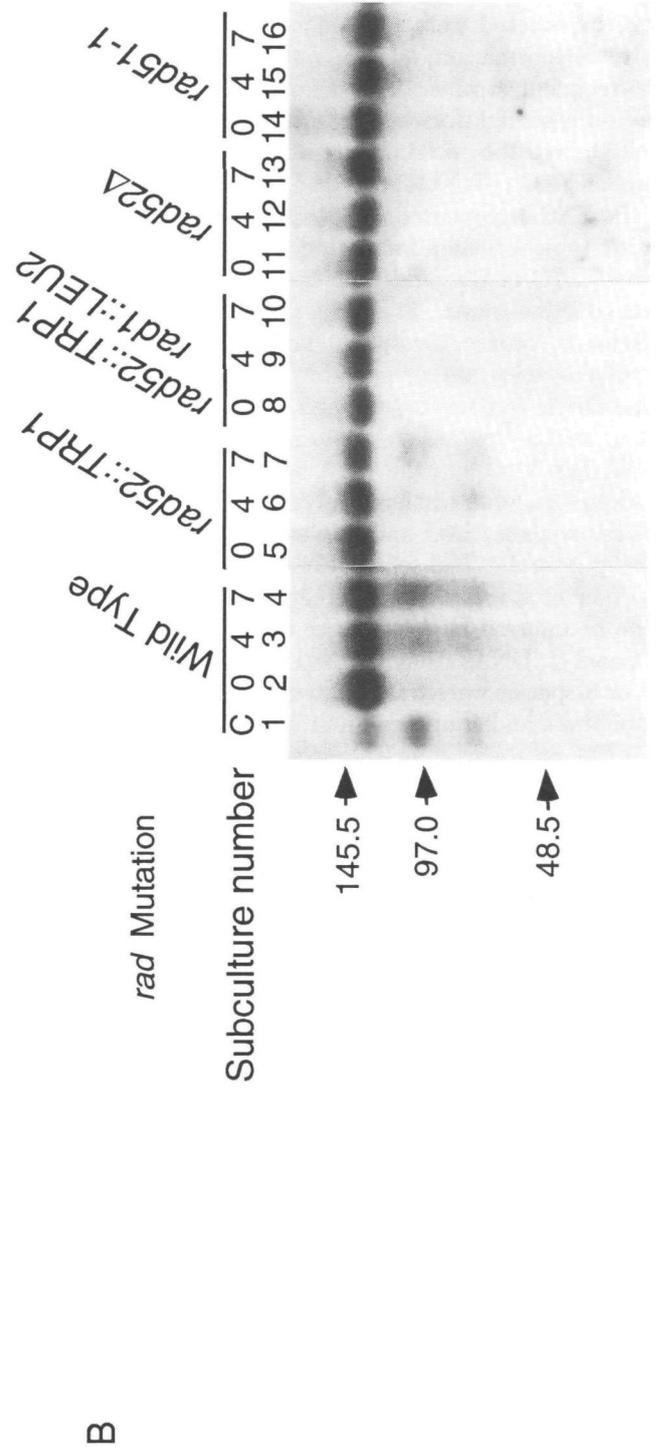
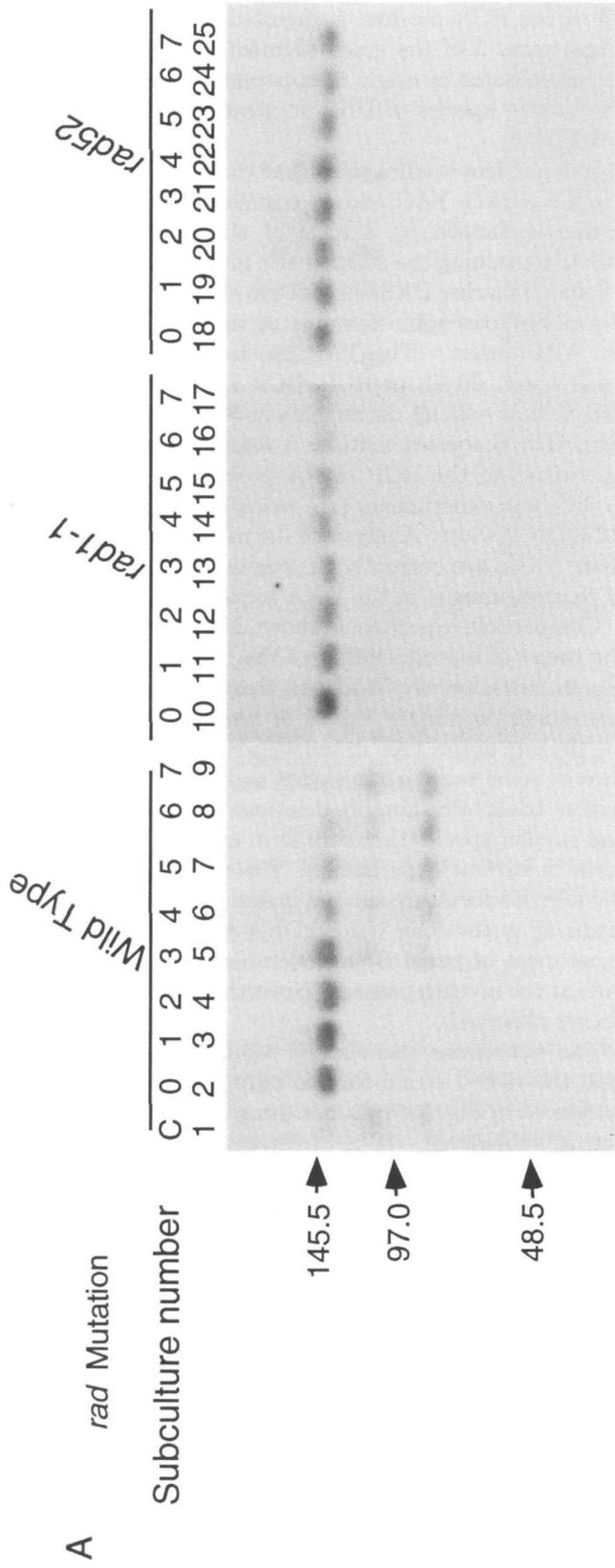
Amplification was carried out on 0.2–0.5  $\mu$ g of YAC

DNA or genomic DNA of the human-hamster hybrid cell X3000.11 in a 50- $\mu$ l reaction mixture containing 0.8 mM dNTPs, 370 KBq of [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol), 300 ng of primers 5'-GGGGTTGTAGATAGTGGCAC and 5'-GTGGCAAAGCAGCAGAAAGA, and 0.25 U of Taq polymerase in a buffer of 10 mM Tris-HCl (pH 8.3), containing 50 mM KCl, 12 mM MgCl<sub>2</sub> and 0.01% gelatin. Thirty-one cycles of polymerase chain reaction (PCR) were carried out as follows: each cycle of the reaction was for 1 min at 90°C for denaturation, 2 min at 65°C for annealing, and 2 min at 72°C for extension. PCR products were analyzed by electrophoresis after complete digestion with *RsaI* restriction enzyme; 2 ml of the reaction mixture was loaded onto a 16-cm 8% polyacrylamide gel and electrophoresed at 8 V/cm for approximately 2 h. The gel was exposed to X-ray film for 30 min –24 h at –70°C.

### 3. Results

Table 1 lists the strains that have been used to assess the effects of deficiencies in recombination on the stability of YACs. The strain with a lesion in *RAD51*, whose protein product interacts with the *RAD52* gene product, most likely corresponds to *recA* in *E. coli*,<sup>14–17</sup> would make the gene especially central in recombination. In addition to *RAD51* and point or deletional

**Figure 3.** Stabilization of color vision DNA in YACs in *rad1*, *rad51*, and *rad52* hosts. AB1380 and *rad* strains (Table 1) were retransformed with yWXC411-L DNA and cultured and analyzed as described in the legend to Fig. 1. Panel A. Lane 1 is yWXD411 DNA in the original transformant in AB1380. Lanes 2–9, DNA from retransformed AB1380 after the first to seventh culture, respectively; Lanes 10–17, DNA from retransformants of M3699-1A (*rad1-1*) after the first to seventh culture, respectively; lanes 18–25, comparable DNA preparations from strain YKK054 (*rad52*). Panel B. Lanes 1–4, as in panel A, lanes 1, 2, 6, and 9; lanes 5–7, comparable DNAs from cultures of strain 814-9/2d (*rad52::TRP1*); lanes 8–10, DNAs from cultures of the double mutant 814-7/4c (*rad1::LEU2 rad52::TRP1*); lanes 11–13, from strain 846/11b (*rad52* deletion); and lanes 14–16, from strain XS3672-3c (*rad51-1*). Values on the ordinate are in kilobases, as in Fig. 1.



lesions in *RAD52*, standard mutants in *RAD1* and *RAD6*, representative of the other two epistasis groups, were tested.

YACs containing color vision pigment genes extracted from their original host, AB1380, were already heterogeneous in size (Fig. 1, lane 1), showing instability of these clones in the wild-type yeast host. The YACs extracted were introduced into the other strains by retransformation, and their stability was measured in the recipient strains. The primary assay for instability has been straightforward: to start from a transformant that showed the "original" larger YAC (yWXD411-L) or smaller YAC (yWXD411-S) and then follow the stability of the YAC on repeated regrowth of the culture. Figure 1 shows typical results indicating the instability of a color vision YAC in the wild-type strain after different numbers of subcultures. The reduction in size of the largest species to others 25 and 50 kb smaller is clear. The smaller species are presumably deleted for RCP/GCP gene DNA; but the repeat unit of color vision genes is about 39 kb<sup>12</sup> rather than the 25 kb units lost from the YAC. The YAC was therefore studied further.

Figure 2 shows further analysis of the likely structure of the original YAC and the smaller species produced during growth. The schematic of panel A indicates the location of a red and two green pigment genes in tandem, as inferred by Vollrath et al.<sup>12</sup> for uncloned DNA. In panel B, DNAs from AB1380 transformed with the L, M, or S species were fractionated by pulsed-field gel electrophoresis and stained with ethidium bromide. In panel C, PCR products from the strains containing L, M, or S species were amplified with a primer pair for exon 5 of the color vision gene. The 213-bp product expected for either green or red color vision pigment genes is seen from the L or M species, but not from the S species; apparently, the smallest derivative of the YAC lacks most or all of the color vision gene exon 5 that is amplified by the PCR primer, though it still hybridized to a cDNA probe (Fig. 1).

Figure 2, panel D extends the analysis to determine whether the PCR products from the L and M YAC species arise from red, green, or both red and green pigment gene DNA. The products from the red and green genes can be distinguished, since only the red pigment gene contains a restriction site for *RsaI*, which would generate two products, 128 and 85 bp, from the 213-bp PCR product. Lane 1 shows the PCR product from the cellular DNA containing the X chromosome from which yWXD411 was made, with a predominant 213-bp product (and a somewhat slower-moving species that appeared in variable amounts in different experiments). As expected, lane 2 shows that the major product is partially digested into two fragments, so that both green and red pigment DNA are present. Lanes 3 and 5 show that the intact PCR product of 213-bp is also produced

from the L and M species; but lanes 4 and 6 show that all of the PCR product is digested into two fragments. Thus, exon 5 of the green pigment gene present in the X chromosome of origin is apparently already absent in the largest species of DNA in strains transformed with yWXD411.

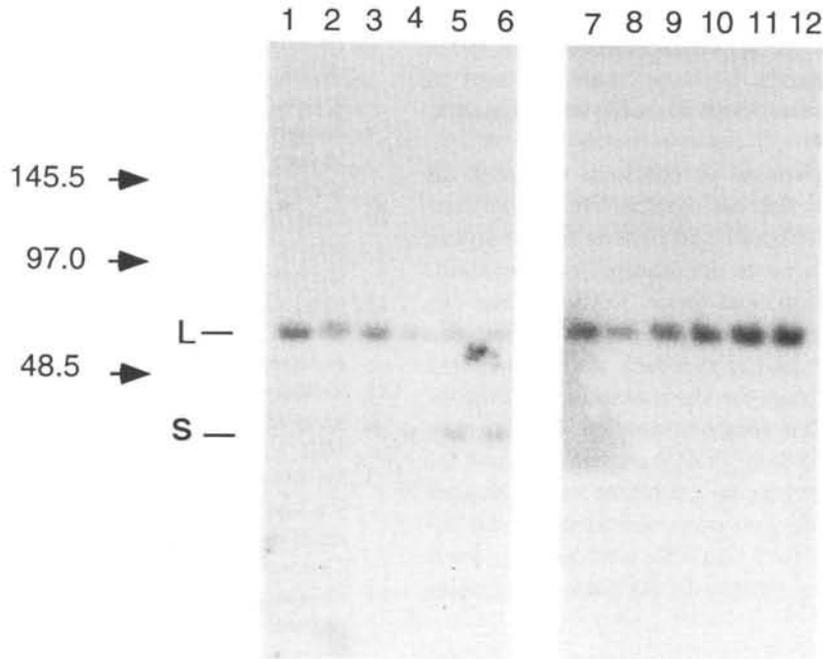
Independent studies show that the RCP/GCP DNA in the yWXD411 YAC and in cosmids covering the same region is flanked by a total of about 70 kb of other DNA, containing the centromeric marker DXS80 and the telomeric marker DXS439 (D'Urso et al., in preparation). Panel E shows some features of the likely structure of the YAC insert. The YAC species L apparently contains about 60 kb of RCP/GCP DNA, and species M and S show ~25 kb decrements in RCP/GCP DNA content. The S species contains a remaining fragment that hybridizes to the RCP cDNA probe, but it lacks exon 5. In some experiments (see below) even the remaining RCP DNA is lost. Analysis of the molecular structures of these YACs are currently in progress to clarify the sites of rearrangements in the DNA sequence.

Comparable experiments shown in Fig. 3 indicate both the range of reproducibility of the results and the striking stabilization of yWXD411 that is seen with strains containing the *rad1-1*, *rad52*, or *rad51* lesions. Concerning the fate of the YAC in the wild-type strain, instability showed some variation in extent and timing over five successive trials. Presumably deletions occur stochastically, and smaller species therefore form at somewhat different rates in various experiments. For example, in Fig. 3A, the fourth subculture showed massive loss of material hybridizing to the color vision cDNA probe, whereas in the experiment of panel B, considerable species L remained even at the seventh passage. Nevertheless, instability was always observed.

The *rad* strains also showed some variability. In general, the *rad1-1* strain showed only partial stabilization, evidenced in Fig. 3A by a declining level of the L species during subculture. It is of interest that a *rad1* allele showed no appreciable stabilization of alphoid sequences in YACs,<sup>3</sup> consistent with a weaker and perhaps locus-dependent effect.

The *rad52* and *rad52::TRP1* strains also showed some decrease in hybridization to the L species as well as variable amounts of smaller YACs in several experiments. In contrast, the *rad52::TRP1*, *rad1::LEU2* double mutant and the *rad52Δ* and *rad51-1* strains showed no detectable smaller species during subculture (Fig. 3B). Of the three *rad52* strains tested, the deletion strain showed rather greater stabilization over five experiments.

Concordant results were obtained with the YAC containing ribosomal DNA tandem repeats. In this case, the smaller species produced during growth in AB1380 (Fig. 4) has been demonstrated to represent a 44-kb tandem repeat unit, and most probably represents the last stage



**Figure 4.** Stabilization in a *rad52* host of rDNA cloned in a YAC. Cells of strains AB1380 and YKK054 retransformed with YAC yB3B3 were cultured and analyzed as described in the legend to Fig. 1, except that cells were cultured in SD medium. Lanes 1–6 are yB3B3 DNA from transformed AB1380 after the first to sixth cultures, respectively. Lanes 7–12 are the corresponding DNAs from transformed YKK054 after the first to sixth cultures. Values shown in the left ordinate are kilobases. Sizes of the DNA of L and S are approximately 70 and 23 kb, respectively.

in successive loss of a large number of equivalent repeat units.<sup>13</sup> Sample data are given for stabilization of the YAC in a *rad52* strain (YKK054) (Fig. 4).

#### 4. Discussion

YAC instability and the use of recombination-deficient strains have been reported before.<sup>3,18,19</sup> Extending the earlier work, YACs that contain genes rather than satellite sequences have been studied here. Also, a more extensive series of *rad* mutants has been examined, and in contrast to earlier work, partial stabilization of these YACs is seen in a *rad1* strain.

Apparently, lesions in two epistatic pathways involved in radiation repair and recombination, represented by *rad51/rad52* and *rad1*, can more or less stabilize the YACs.

Building on the observations of Neil et al.<sup>3</sup> of partial stabilization of alphoid DNA in YACs in a *rad52* strain, Chartier et al.<sup>18</sup> have created a mouse YAC library in such a strain background. No studies have been reported of the stabilization of tandem repeats in that library; but the results reported here suggest that such collections of YACs will more faithfully preserve tandemly-repeated DNA regions that are otherwise zones of instability. In that regard, it is not clear why the stabilization of YACs

reported here is more complete than that observed by Neil et al.<sup>3</sup> but of course both the numbers and sizes of repeated elements differ at color vision and rDNA loci compared to alphoid sequences, and some nonisogenicity in the strains used may also contribute to differences.

Their nonisogenicity in fact requires an important qualification of comparisons of results with various strains. To facilitate the rapid search for strains that stabilize YACs, experiments were done by simply transforming a YAC into each strain. Strain background could therefore influence the degree of stabilization, and is superimposed on some variation from one experiment to another as a possible source of apparent differences (cf. results with AB1380 in Figs. 3A and 3B). Enough isogenic pairs of strains show effects, however (Table 1), to make the inference of stabilization reliable; and in particular, it is intuitively reasonable that a *rad52* deletion or a *rad52 rad1* double mutant should be more effective than single point mutants.

The results indicate that, in contrast to the *RAD1* and *RAD51/52* pathways, the third epistatic group of *rad* strains, represented here by *RAD6* (data not shown), is not involved in YAC dynamics; but the result is a negative one, and the strain has not been checked for possible reversion of the *rad6* allele, so the result should be considered tentative.

Other genes may of course affect YAC stability. Those of possible interest include the *DST* genes,<sup>20</sup> which are reported to inhibit deletion formation. Lesions that block the function of those genes, however, may also lead to slower growth of cells, lower levels of stable recombinants, and poor survival.

It seems useful at present to continue to focus on the *RAD* strains to develop an optimal recombination-deficient strain as a YAC host. To further the analysis, we have begun to use a more demanding assay system. YACs containing an additional locus, *DXS49*, show instability with more variable deletion products than are seen for the *RCP/GCP* and *rDNA* loci, and with a less well-defined structural basis for the instability.<sup>21</sup> Preliminary results indicate that single mutants in *RAD* genes are also effective in stabilizing YACs containing that locus. Currently, we are trying to construct viable strains blocked in both relevant pathways, preferably with lesions in three genes (*RAD51*, *RAD52*, and *RAD1*). Such hosts may offer the best chance to stabilize marginally recovered YACs.

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