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Genetics

Instability of dicentric plasmids in yeast

(spindle-mediated breakage/heterogeneous plasmid deletions/*RAD52* repair/*Saccharomyces cerevisiae*)

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ABSTRACT **Dicentric plasmids containing either two copies of centromere 4 or one copy of centromere 4 and one copy of centromere 3 in the yeast plasmid vector YRp17 were constructed *in vitro* and introduced into yeast cells by DNA transformation. The resulting colonies were heterogeneous for a mixed population of rearranged plasmids. The rearrangements always involved deletion of one or both centromere sequences originally present on the plasmid. Heterogeneity was due to the continued production of deleted plasmids from a pool of unrearranged dicentric plasmids maintained within some of the yeast cells in the colony. The *RAD52* gene product is known to be required for the repair of DNA double-strand breaks in yeast. Transformation of *rad52* mutant yeast cells with dicentric plasmids gave rearranged plasmids similar to those observed with RAD$^+$ yeast cells, but the transformation frequency was only 5–10% compared to transformation with monocentric plasmids. Also, the ratio of unrearranged dicentric plasmid to deleted plasmids was greatly reduced in the *rad52*-transformed cells. These observations are consistent with a model in which centromeric DNA sequences can interact independently with the yeast cell spindle apparatus. Occasional movement of centromeres to opposite poles may result in mechanical breakage of plasmid sequences. Plasmids deleted for one or both centromere sequences can be obtained from these broken molecules and are resistant to further rearrangement.**

DNA sequences putatively involved in centromere function have recently been isolated from the yeast *Saccharomyces cerevisiae* (1–4). Their classification as centromeric DNA sequences is based on their ability to stabilize mitotic segregation of autonomously replicating plasmids [plasmids containing an autonomously replicating sequence (ARS) or yeast replicating plasmid (YRp)] as well as to allow orderly meiotic segregation of these plasmids in yeast. In those cases in which the physical position of these sequences in the genome is known, it corresponds to the genetic map position for the centromere of a particular chromosome.

Classical cytogenetic studies in higher eukaryotes have shown that dicentric chromosomes are usually unstable (5–10). Independent attachment of the spindle apparatus to the two centromeres and movement to opposite poles results in bridge structures in mitosis or meiosis which can lead to breakage of the chromosomal fiber between the two centromeres. The resulting monocentric broken chromosomes are still unstable because they lack a telomere at their broken ends (11). In subsequent divisions, these chromosomes may undergo "breakage/fusion/bridge" cycles leading to eventual loss or stabilization of the chromosomes. Stabilization may involve the production of a monocentric chromosome with a "healed" end or of a chromosome that appears to be cytogenetically dicentric but shows some degree of stability (12, 13). The molecular mechanism of stabilization is not understood in either case.

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This report demonstrates that dicentric plasmid molecules are unstable upon introduction into yeast. In the experiments presented here, *CEN3* (CEN is centromeric DNA sequence) from chromosome 3 and *CEN4* from chromosome 4 of *S. cerevisiae* were used (4). Stably rearranged plasmids were produced at high efficiency from these dicentric plasmids. The rearrangements always involved deletion of one or both centromeric DNA sequences. This work provides additional evidence for the model that the sequences previously characterized as centromeric DNA (1–4) function by interaction with the yeast cell spindle apparatus.

MATERIALS AND METHODS

Bacterial and Yeast Strains. All bacterial transformations and plasmid constructions were with the *recA Escherichia coli* strain HB101 (our BNN6) *hsdS20* ($r_B^- m_B^-$), *recA13 supE44 lacY1 ara-14 proA2 galK2 rpsL20* (Sm^r) *xyl-5 mtl-1*. Construction of homogeneous populations of monomeric and duplication-containing plasmids requires the use of *recA* strains to prevent plasmid recombination in *E. coli*. DNA of the *CEN4* tandem duplication plasmid YC^2p3 (YC^2p denotes a yeast replicating plasmid containing two centromeres) (see Fig. 1) prepared from HB101 contained <0.1% of the monocentric plasmid that would result from mitotic recombination between the duplicated centromere sequences during the growth of this *recA* strain. The yeast strains used were YNN140 *a his3-532 trp1-289 ura3-1,2 ade2-1 can*^{r} $Inos^-$ and YNN209 *a rad52 his3Δ1 trp1-289 ade2-1*. DNA transformation of yeast (14) and electrophoretic procedures (15) were as described.

RESULTS

Fig. 1 shows the functional elements and restriction endonuclease sites for the three dicentric plasmids constructed for the experiments presented here. YC^2p1 and YC^2p2 both contained one copy of *CEN3* and one copy of *CEN4* but differed in the orientation of *CEN3* sequences relative to the *CEN4* sequence. YC^2p3 contained a head-to-tail tandem duplication of two *CEN4* sequences.

Instability of Dicentric Plasmids and Heterogeneity of Rearranged Plasmids. The three different dicentric plasmids were introduced into the haploid yeast strain YNN140 by DNA transformation selecting for the markers *TRP1* or *URA3* on the plasmids. The transformation frequencies (500–1,000 transformed colonies per μg of DNA) for the dicentric plasmids were indistinguishable from those of the monocentric plasmid YCp19 (4). Colonies resulting from transformation with these plasmids were streaked out to obtain clones of single cells under tryptophan or uracil selection. A marked heterogeneity in colony

Abbreviations: ARS, autonomously replicating sequence; CEN, centromeric DNA sequence; YRp, yeast replicating plasmid; YCp, yeast centromere plasmid; YC^2p, yeast replicating plasmid containing two centromeres; kb, kilobase(s).

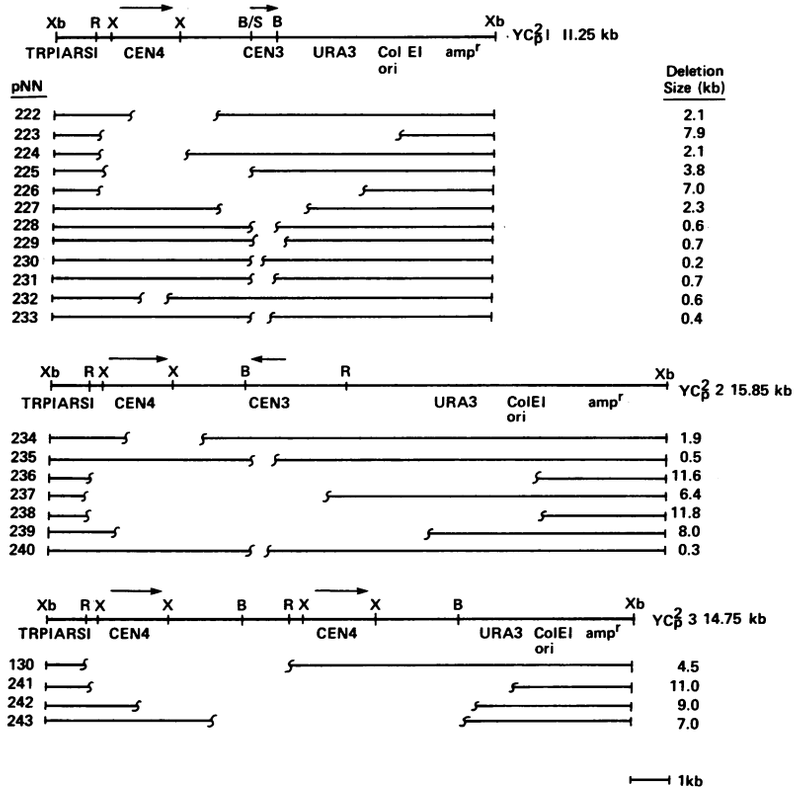

FIG. 1. Functional and restriction endonuclease maps of dicentric plasmids and the deleted plasmids obtained from them after yeast transformation. Shown are maps of the three different dicentric plasmids examined in this work: YC^2p1 (pNN244), YC^2p2 (pNN245), and YC^2p3 (pNN246). The isolation and delimitation of the functional centromeric DNA sequences for chromosomes III and IV have been described (4) or are from our unpublished data. All plasmids contain the 3.6-kilobase (kb) *CEN4* fragment present on the vector YCp19 (4). In addition, YC^2p1 contains a 0.627-kb *CEN3* (16) fragment and YC^2p2 contains a 2.6-kb *CEN3* fragment. Both possible orientations of the *CEN3* sequence relative to the *CEN4* sequence are represented in YC^2p1 and YC^2p2. YC^2p3 contains an additional 4.5-kb *CEN4* fragment present as a head-to-tail tandem duplication relative to the 3.6-kb *CEN4* fragment. Arrows above the centromeric DNA sequences represent the relative orientation of the two sequences to each other. Deletion derivatives of these plasmids are listed immediately below each dicentric plasmid. Open segments in the lines indicate the extent of the deletion in each case. The size and end points of deletions were estimated (±100 base pairs) from the size of small fragments containing the deletions. Restriction enzyme sites: B, *Bam*HI; B/S, *Bam*HI/*Sau* 3A fusion; R, *Eco*RI; X, *Xho* I; Xb, *Xba* I.

size was observed for these clones containing dicentric plasmids but not monocentric plasmids. Representative different-sized colonies were grown in small liquid cultures to obtain DNA for the recovery of plasmids in *E. coli*.

Restriction endonuclease maps of a representative set of these plasmids are shown in Fig. 1. Each of the dicentric plasmids gave rise to a heterogeneous set of rearranged plasmids at high frequency after introduction into yeast. Thirty of 31 rearranged plasmids contained single colinear deletions of varying size which removed one or both sets of functional centromeric DNA. One plasmid contained a deletion which removed one of the two sets of *CEN4* sequences of YC^2p3 but also contained a complex rearrangement of other plasmid DNA sequences. No plasmids were found which contained rearrangements that did not involve deletion of centromeric DNA.

The heterogeneity in colony size observed when streaking out a colony obtained by transformation with a dicentric plasmid was due to the heterogeneous population of rearranged plasmids contained in the initial colony. Small deletions which removed one of the two sets of functional centromeric DNA sequences resulted in stable, monocentric plasmids. Cells containing these plasmids had a growth advantage, on media selecting for the presence of the plasmid, compared to cells containing plasmids with large deletions which removed both sets of centromeres. This results from the segregational instability of plasmids containing an ARS, but no CEN sequence (17). These results were confirmed by reintroducing the plasmids recovered in *E. coli* back into YNN140 by transformation. These transformants reproduced the characteristic colony size phenotypes of the clones from which the plasmids were obtained.

As expected, the type of rearranged plasmid that was recovered depended on the selections applied to introduce them into yeast and then into *E. coli*. The *ARS1* sequence is required for autonomous replication and high-efficiency transformation of the yeast cells. Rearrangements affecting the function of this sequence would not be recovered. *TRP1* or *URA3* or both remain intact because they are used to select for transformants containing a plasmid. Deletions that removed the ColE1 origin could have been maintained in yeast but would not have been recoverable in *E. coli*.

The plasmids obtained by transformation of YNN140 with YC^2p1 or YC^2p2 had deletions with numerous break points whose only constraint was that they remove centromere sequences but not eliminate functions necessary for their maintenance in yeast or recovery in *E. coli*. A striking difference was found for plasmids derived from transformation with the tandem duplication dicentric plasmid YC^2p3. Six of eight plasmids examined contained exact deletions of the 4.5-kb *CEN4* fragment (pNN130), as if they were formed by deletion of this fragment by mitotic recombination between tandemly duplicated sequences. The remaining two plasmids (pNN241 and -242) were large deletions of the type observed with YC^2p1 and YC^2p2.

In addition to the rearranged plasmids described above, ≈20% (7/38) of the plasmids recovered in *E. coli* from transformations of YNN140 with YC^2p1 or YC^2p2 were apparently unrearranged dicentric plasmids. When reintroduced into yeast, these plasmids were still unstable and generated the same types of deleted plasmids described above. In contrast, all of 11 plasmids recovered in *E. coli* after transformation of YNN140 with the tandem duplication plasmid YC^2p3 were rearranged.

As a control experiment, the frequency of rearrangement of monocentric plasmids after their introduction into yeast was examined. Twenty of 20 plasmids recovered in *E. coli* after transformation of YNN140 with YCp19 were identical to the starting plasmid by restriction mapping.

Maintenance of Unrearranged Dicentric Plasmids in Yeast. The distribution of rearranged and unrearranged dicentric plasmids was directly assayed within transformed yeast cells as a function of generations of growth. Fig. 2*A* shows an autoradiogram of uncut DNA isolated from five independent YNN140 colonies transformed with the *CEN4* tandem duplication plasmid YC^2p3 after ≈25 generations of growth. Four of five transformants (lanes 1–4) showed only a band corresponding to a

plasmid exactly deleted for the 4.5 kb *CEN4* fragment and trace amounts of a plasmid that comigrated with unrearranged YC^2p3. One of the transformants (lane 5) contained a small amount of the 4.5-kb *CEN4* deleted plasmid as well as a larger amount of a plasmid that contained an ≈1.5-kb deletion relative to YC^2p3. This pattern of bands remained unchanged with further mitotic growth except for a decrease in the amount of unrearranged YC^2p3 in the transformants.

Fig. 2*B* shows an autoradiogram of plasmids contained within five independent YC^2p1 transformants of YNN140 after ≈25 generations of growth. Four of five transformants appeared to contain only unrearranged YC^2p1 (lanes 1–3 and 5). One of the transformants contained two deleted plasmids (lane 4). Decreased hybridization to the band corresponding to unrearranged YC^2p1 occurred with further mitotic growth of these transformants. At ≈35 generations of growth, (Fig. 2*C*), novel deleted plasmids were seen in some of the transformants. After ≈45 generations of growth (Fig. 2*D*), little unrearranged plasmid was observed and only weak hybridization to any specific plasmid band was seen for the four transformants that contained unrearranged YC^2p1 at 25 generations of growth (lanes 1–3 and 5). Analogous results to those described for YC^2p1 were also obtained for cells transformed with YC^2p2 (data not shown).

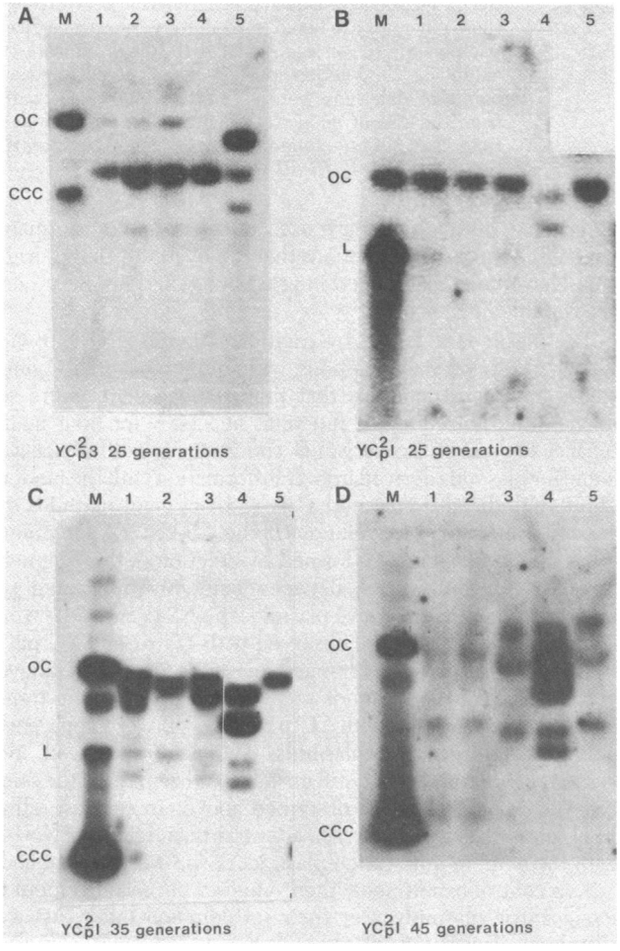

FIG. 2. Hybridization analysis of plasmids present in yeast cells transformed with different dicentric plasmids. (*A*) Plasmids present in five independent transformants of YNN140 with the dicentric plasmid YC^2p3 after 25 generations of growth. (*B–D*) Plasmids contained in five independent YC^2p1 transformants of YNN140 after 25, 35, and 45 generations of growth, respectively. In each case, marker plasmid is present in lane M. OC, L, and CCC are open circular, linear, and closed circular forms of the plasmid. In these rapid lysate DNA preparations, extensive nicking had converted most of the plasmid DNA to open circular form. ^{32}P-Labeled pBR322 DNA was used as the hybridization probe.

Fig. 2 *B–D* shows that transformed yeast cells contain either unrearranged dicentric plasmid or a limited number of plasmids containing small deletions. In contrast to this, plasmids recovered in *E. coli* from similarly transformed cells include a heterogeneous collection of deleted plasmids. We interpret these results to mean that the colonies obtained from transformation of yeast with dicentric plasmids are heterogeneous for the population of plasmids they contain. If, upon entering the yeast cell, a dicentric plasmid undergoes an early deletion event that creates a monocentric plasmid, the resulting stable plasmid will selectively outgrow the unstable dicentric plasmid it was derived from. An example of this would be the transformant in lane 4 of Fig. 2*B*. If no early deletion event occurs, a heterogeneous set of deleted plasmids is continuously generated from a pool of unrearranged dicentric plasmids maintained within the transformed yeast cells. Eventually, the heterogeneous set of deleted plasmids will overwhelm the population of unstable dicentric plasmids. The heterogeneous set of deletions is seen as a smear of hybridization. This accounts for the reduction of hybridization to unrearranged dicentric plasmids during the growth of transformants shown in Fig. 2 *B–D*. The autoradiograms do not have the sensitivity to delineate clearly the heterogeneous set of deleted plasmids that can be observed by recovery and amplification in *E. coli*.

To confirm this explanation for the apparent lack of heterogeneity in the autoradiograms of Fig. 2, clones of single cells were isolated from the heterogeneous population of cells present after 35 generations of post-transformation growth. DNA was prepared from clones and used for hybridization analysis as well as for transformation of *E. coli* to recover plasmids. These results (presented in Fig. 3) show that hybridization analysis after electrophoretic separation of plasmids is a poor indicator of the heterogeneity present in colonies transformed with dicentric plasmids. However, it is a sensitive indicator of the presence of unrearranged dicentric plasmid or of the major deleted plasmids in the transformation colony.

***RAD52*-Mediated Repair of Broken Dicentric Plasmids.** The *RAD52* gene product is required for meiotic recombination (18, 19) and for the repair of DNA double-strand breaks in yeast (20, 21). Mutant *rad52* yeast strains also have decreased levels of mitotic gene conversion but apparently normal levels of mitotic reciprocal recombination (22, 23). The effect of the *RAD52* gene product on the stability of dicentric plasmids was examined by introducing YC^2p1 and YC^2p3 into the haploid *rad52* mutant strain YNN209. The transformation frequency for both YC^2p1 and YC^2p3 was 5–10% of that for the monocentric plasmid YCp19. The plasmids contained in these colonies were examined by hybridization and autoradiography, as for the RAD^+ transformants shown in Fig. 2.

A striking difference was seen for plasmids contained in the YC^2p1-transformed *rad52* strain compared to RAD^+ transformed cells after 25 generations of post-transformation selective growth. Nine of 10 independent transformants of the *rad52* mutant strain contained predominant deleted plasmids in the mass culture, with variable smaller quantities of unrearranged YC^2p1. Seven of these transformants are shown in Fig. 4. One transformant apparently contained no single homogeneous plasmid species at a high enough concentration to show a strong hybridization band (lane 1). In contrast, only one of five YC^2p1 transformants of the RAD^+ yeast strain showed the presence of any deleted plasmids by autoradiography after 25 generations of post-transformation growth. The remaining four transformants showed only the presence of unrearranged YC^2p1 by autoradiography (Fig. 2*B*).

For YC^2p3-transformed cells, there was no difference in the

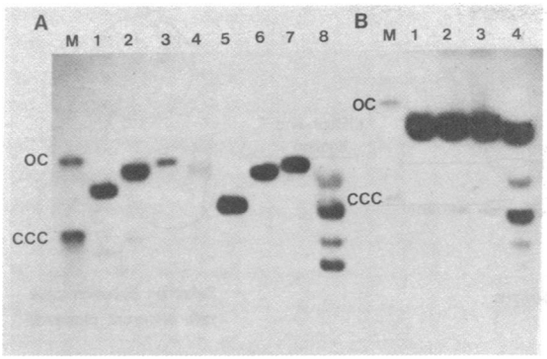

FIG. 3. Homogeneous clones from heterogeneous mass transformation colonies. (*A*) Lanes: 1–3, plasmids contained in clones obtained from a YC^{2}p1-transformant of YNN140; 4, plasmids contained in the original transformation colony from which these clones were derived. One clone contained a deleted plasmid (lane 1) that was not seen in the DNA for the mass transformation colony. Another clone contained a deleted plasmid (lane 2) that may correspond to the deleted plasmid seen in the original transformation colony. Only a single type of deleted plasmid was recovered in *E. coli* from transformations using DNA from these clones. The size of the deleted plasmids recovered from these clones (pNN229 and -230) was in agreement with the estimated size of the deleted plasmids seen in lanes 1 and 2. The results show that these clones, obtained from the heterogeneous mass transformation colony, were now homogeneous for a single stable plasmid derived from the original dicentric plasmid by deletion. One clone contained unrearranged YC^{2}p1 (lane 3) as shown by its comigration with YC^{2}p1 marker and by the recovery of unrearranged YC^{2}p1 from this clone in *E. coli*. Lanes 5–7 show plasmids within clones of cells obtained from a second YC^{2}p1 transformant. Two clones (lanes 5 and 6) contained plasmids that comigrated with the two major deleted plasmids observed in the DNA of the mass transformation colony (lane 8). One clone contained a deleted plasmid (lane 7) that was not observed in the DNA of the mass transformation colony (lane 8). These clones had a homogeneous plasmid population in that plasmids recovered from them in *E. coli* (pNN227, -231, and -232) were of a single deleted type that corresponded to the size seen in the autoradiogram. (*B*) Lanes: 1–3, plasmids present in clones of cells from the YC^{2}p3 mass transformation colony containing plasmids shown in lane 4. These clones contained a single deleted plasmid that corresponded to the major deleted plasmid seen in the DNA of the mass transformation colony (lane 4). M lanes, marker YC^{2}p1 and YC^{2}p3 for *A* and *B*, respectively.

predominant deleted plasmid obtained for RAD$^+$ and *rad52* mutant cells despite the difference in transformation frequencies for the two strains. Ten of 10 independent transformants of the *rad52* strain contained only the 4.5-kb precisely deleted plasmid (pNN130) that was seen in all of the RAD$^+$ transformants in Fig. 2*A*.

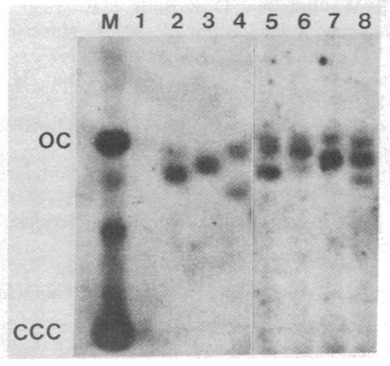

FIG. 4. Transformation of a *rad52* strain with YC^{2}p1. Shown are plasmids contained in eight independent YC^{2}p1 transformants of the haploid *rad52* strain YNN209 after 25 generations of growth. Marker YC^{2}p1 is in lane M. Equal amounts of total cellular DNA were applied to each lane. The transformant shown in lane 1 apparently has no single homogeneous plasmid species present at a high enough concentration to give a strong hybridization signal.

DISCUSSION

Model of Processing Pathway of Dicentric Plasmids in Yeast. Fig. 5 illustrates the various fates of dicentric plasmids after introduction into yeast and presents a model of the cellular mechanisms that act on these plasmids. The initial and final products of transformation are known, but we have no direct evidence for the postulated intermediates. However, the model is in accord with earlier work on the processing of dicentric chromosomes in higher eukaryotes and describes the simplest route to the observed end products.

Competent yeast cells initially take up many plasmid molecules during DNA transformation (17). Some of the dicentric plasmids taken up during transformation must eventually engage the yeast cell segregation apparatus. Coorientation of centromeres allows the orderly segregation of sister chromatid plasmids from each other (path a). Alternatively, independent attachment of the yeast cell spindle apparatus to the two centromeres and movement to opposite poles can lead to mechanical breakage of the dicentric plasmid (path b). Two breaks of plasmid DNA sequences are necessary to separate the two centromeric DNA sequences from each other. The resulting linear molecule may be subject to DNA exonuclease action (path c). This would explain our recovery of plasmids containing large deletions which remove both centromeric DNA sequences. Plasmids of this type could not be generated by mechanical breakage and religation alone. The plasmids we have recovered contain deletion break points in nonhomologous DNA, so we have postulated a blunt-end ligation of broken linear molecules to reform a circular molecule (path d). In addition to plasmids with simple colinear deletions, 1 plasmid of 31 examined had a complex rearrangement of plasmid sequences associated with deletion of one of its centromere sequences. Complex rearrangements may result if the ends of broken linear molecules catalyze further plasmid rearrangements. For example, the broken molecules may undergo fusion/bridge cycles after replication (7–10).

Precise Excision of Tandem Duplication Dicentrics. Dicentrics containing one copy of *CEN3* and one copy of *CEN4* gave rise to plasmids containing deletions of centromere sequences with numerous break points. In contrast, a plasmid containing a tandem duplication of *CEN4* sequences gave rise mainly to plasmids containing a precise deletion of the duplicated *CEN4* sequences (Fig. 5, path g). This is the expected result if the deletion had occurred by mitotic recombination between duplicated sequences. This high-frequency deletion was specific for tandem duplications of centromeric DNA sequences. A monocentric plasmid that contained a tandem duplication of noncentromeric DNA sequences did not undergo high-frequency deletion of the duplicated sequences upon transformation or during mitotic propagation after transformation (data not shown). One explanation for all these results is that breakage within the tandemly duplicated sequences produces linear DNA ends which are known to be recombinogenic in yeast (24). These linear DNA ends would stimulate reciprocal recombination between the duplicated sequences. This process did not require the *RAD52* gene product. Interestingly, the increased integration of linear versus circular plasmids during DNA transformation, a process that is thought to be mediated by mitotic gene conversion, does require the *RAD52* gene product (24). This difference might reflect the requirement of the *RAD52* gene product for mitotic gene conversion but its dispensibility for mitotic reciprocal recombination (22–24).

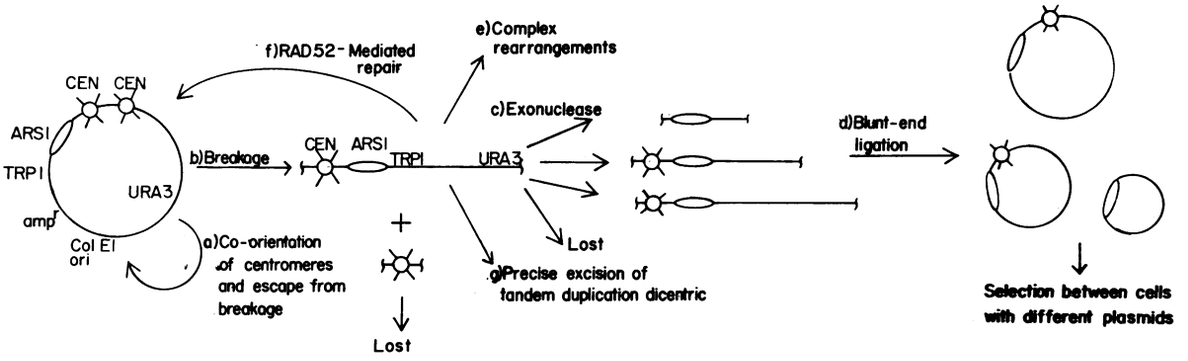

FIG. 5. Model for the processing of dicentric plasmids in yeast.

***RAD52*-Mediated Repair of Dicentric Plasmids.** We observed a 5–10% reduction in the transformation frequency of mutant *rad52* yeast cells when dicentric instead of monocentric plasmids were used. The transformation frequency of RAD^+ yeast cells is indistinguishable for dicentric and monocentric plasmids. These results imply that 90–95% of the transformation-competent *rad52* yeast cells which take up dicentric plasmids are unable to produce a viable colony. The *RAD52* gene product is required for the recombinational repair of broken yeast chromosomes (20, 21). It has been postulated that this repair process involves the recovery of lost information from a homologous DNA template in a process akin to mitotic gene conversion (24, 25). One explanation for the decreased transformation frequency is that *rad52* strains are unable to repair broken dicentric molecules back to unrearranged dicentric plasmid (path f). Instead, these broken molecules would be degraded most of the time, thereby precluding the formation of a transformed colony. In 5–10% of the cells that take up plasmid, the broken molecules go through one of the pathways that create a deleted plasmid that is stable to further spindle-mediated rearrangement. Cells containing these plasmids would then form a colony. Consistent with this explanation is the observation of a much reduced ratio of unrearranged to deleted plasmid within *rad52* mutant cells transformed with YC^2p1. Another possible explanation for the reduced transformation frequency involves the known dominant lethality of broken yeast chromosomes in *rad52* yeast cells (26).

Frequency of Rearrangement of Monocentric Plasmids in Yeast. Are monocentric plasmids undergoing high-frequency rearrangement in yeast? This might occur if *in vivo* recombination produced dimer dicentric plasmids that were then subject to spindle-mediated breakage and plasmid rearrangement. We have not yet observed the rearrangement of any monocentric plasmid within transformed yeast cells in the absence of a selection for rearrangements (23). In addition, we have prepared a pure population of dimer dicentric plasmid in a *recA* strain of *E. coli*. This pure population of dimer dicentric plasmid was used to transform the RAD^+ yeast strain YNN140. Six of six transformants examined by autoradiography contained only the original monocentric plasmid from which the dimer dicentric was obtained. Apparently, the major processing pathway of dimer dicentric plasmids in yeast involves a resolution back into monomeric centromere plasmid. This is similar to the high frequency deletion of duplicated sequences after introduction of the tandem duplication dicentric YC^2p3 into yeast.

Linear Versus Circular Dicentric Molecules. This work involved only circular dicentric molecules. A crucial difference between circular plasmids and linear chromosomes is the absence of telomeres on plasmids. Breakage of a small dicentric plasmid yields linear molecules with two free ends in close proximity to each other. These ends apparently can be efficiently religated to reform a plasmid. Breakage of a linear dicentric produces two linear molecules, each containing one telomere and one free end. These molecules cannot circularize because a telomere caps one end. Stabilization must involve a religation of broken molecules or a "healing" of the broken ends (7–12). Because of these differences, the processing of linear and circular dicentric chromosomes in yeast is likely to differ.

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