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NOTES

Multiple, Tandem Plasmid Integration in *Saccharomyces cerevisiae*

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Nonreplicating plasmids transform *Saccharomyces cerevisiae* by recombining with a homologous site in the genome. Frequently, multiple copies of the plasmid integrate in a tandem array. We show that, after transformation with restriction enzyme-cut plasmids, most, if not all, multimers arise by sequential integration of plasmid molecules into the same genomic location.

Transformation of *Saccharomyces cerevisiae* by plasmids that lack an *ARS* element and are thus incapable of autonomous replication occurs by recombination between homologous regions on the plasmid and the *S. cerevisiae* genome, producing an integrated plasmid (3). We showed that when yeast cells are transformed with a plasmid containing a double-strand break or gap within sequences homologous to the *S. cerevisiae* genome, (i) the plasmid transforms at high frequency; (ii) the double-strand ends target the plasmid to integrate at the homologous locus in the genome; and (iii) the gap is repaired from chromosomal information during plasmid integration (4, 4a). As many as 50% of the transformants obtained from transformation with either circular or linear plasmid DNA contain multiple copies of the plasmid arranged in a tandem array (4, 6).

Multiple, tandem arrays of linear plasmids could result from ligation of the plasmid molecules into a multimer before integration. This mechanism was excluded by the following experiment. We made an 800-base-pair (bp) gap inside the *HIS3* fragment of pSZ62 by digesting it with the restriction enzyme *Bgl*II and transformed *S. cerevisiae* cells with the linear DNA. Analysis of the structure of the transformants by Southern blot restriction mapping revealed that the gap was repaired in every case (4). Moreover, in transformants with multiple copies every integrated copy was repaired and full length (4). If multimers were the result of ligation of plasmids before integration, we would have observed a fragment 800 bp smaller than the full-length *HIS3* fragment. Therefore, multimers are

Two alternative models for multimer formation remain: sequential integration of plasmid molecules into the same site, or replication of a plasmid during the integration event. The first model predicts that if a yeast strain is transformed with equal amounts of two different plasmids, both of which contain the same yeast gene, multimers that are mixtures of the two plasmids will be produced. If, however, multiple copies arise by the replication of a single plasmid during the process of integration, any one multimer transformant should contain only one of the plasmids. To distinguish between these models, we transformed yeast cells with two different plasmids containing *HIS3* and analyzed the structure of multimers by Southern blot restriction mapping.

pSZ63 consists of a 1.7-kilobase (kb) *Bam*HI fragment containing *HIS3* inserted into the *Bam*HI site of pBR322. pSZ505 was derived from pSZ63 by the insertion of a 2.3-kb *LEU2* fragment at the *Sal*I site (Fig. 1) and thus transforms *S. cerevisiae* cells to HIS^+ LEU^+. *Eco*RI digestion of pSZ63 yields a single fragment of 6.1 kb, whereas pSZ505 gives fragments of 5.1 and 3.2 kb. Each plasmid was digested with *Bgl*II, creating an 800-bp gap in the *HIS3* fragment, and strain LL20 (α *his3-11,15 leu2-3,112 can1*) was transformed with a mixture of 10 μg of each plasmid (4a). The *Bgl*II double-strand gap allowed us to detect any ligation of the two plasmids together before integration, because such an event would produce a restriction fragment with an 800-bp deletion. Selection was made for HIS^+ transformants; 24 of these were

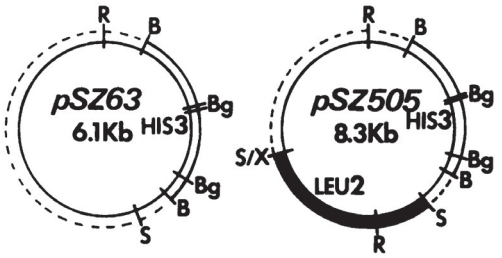

FIG. 1. Restriction map of pSZ63 and pSZ505. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; R, *Eco*RI; S, *Sal*I: X, *Xho*I. Symbols: - - - - -, pBR322 DNA; ═══, *HIS*3 fragment; ▬, *LEU2* fragment.

digested with *Eco*RI, transferred to nitrocellulose, and hybridized to nick-translated pBR322 (4a).

The LEU$^+$ transformants must have contained pSZ505; we determined whether they also contained pSZ63 by the pattern of *Eco*RI fragments present on the Southern blot. Figure 2 is a schematic diagram of the sizes of fragments predicted for transformants containing a single integrated copy of pSZ505, multiple copies of pSZ505, or mixed multimers containing both pSZ63 and pSZ505. These events were all distinguishable on the basis of the size of the *Eco*RI fragments that hybridized with pBR322. A single copy of pSZ505 gave 8.3-, 5.1-, and 4.9-kb *Eco*RI fragments (Fig. 2B). An additional 3.2-kb *Eco*RI fragment was characteristic of a pSZ505 multimer (Fig. 2C). The fragments from a mixed multimer differed depending on the order of the plasmid molecules within the tandem array. A mixed dimer with pSZ63 on the left-hand side gave 8.3-, 6.1-, 5.1-, and 4.9-kb fragments; with pSZ63 on the right, 8.3-, 7.9-, 5.1-, and 3.2-kb fragments were produced (Fig. 2D). When more than one copy of pSZ63 or pSZ505 was present in the mixed multimer, combinations of these fragments appeared. For example, a multimer containing two copies of pSZ505 and one copy of pSZ63 would have fragments of 8.3, 6.1, 5.1, 4.9, and 3.2 kb or of 8.3, 7.9, 5.1, and 3.2 kb depending on the arrangement. Of the 18 LEU$^+$ transformants, 4 contained a single copy of pSZ505 (Fig. 3). Of the 14 multimers, 10 contained both pSZ63 and pSZ505 (Fig. 3). None of the multiple integrations arose by ligation of the plasmids before the integration event, since no fragment contained an 800-bp deletion.

We determined the number of integrated plasmids in each transformant and verified that all plasmids had integrated at *HIS3* by digesting the DNA from transformants with a restriction enzyme that cleaved only in flanking *S. cerevisiae* blots a single fragment was produced that was proportional in size to the number of integrated plasmid copies. These results confirmed that the four transformants contained a single integrated copy of pSZ505. One of the pSZ505 multimers had two copies of pSZ505, two had three copies, and one had four copies. Of the 10 multimers containing both pSZ63 and pSZ505, 2 contained two copies (one of each plasmid), 1 had three copies, 4 had four copies, and 3 had five or more plasmids (data not shown).

Two-thirds of the LEU$^+$ multimers were mixtures of the two plasmids. If the plasmids integrate independently, mixed multimers would be expected to occur at least twice as frequently as multimers containing only pSZ505. Therefore, these results strongly suggest that multiple, tandem integration of linear plasmids in *S. cerevisiae* results from the sequential integration of plasmids into the same site. We hypothesize that after the integration of one plasmid, a recombinogenic lesion is generated that stimulates the

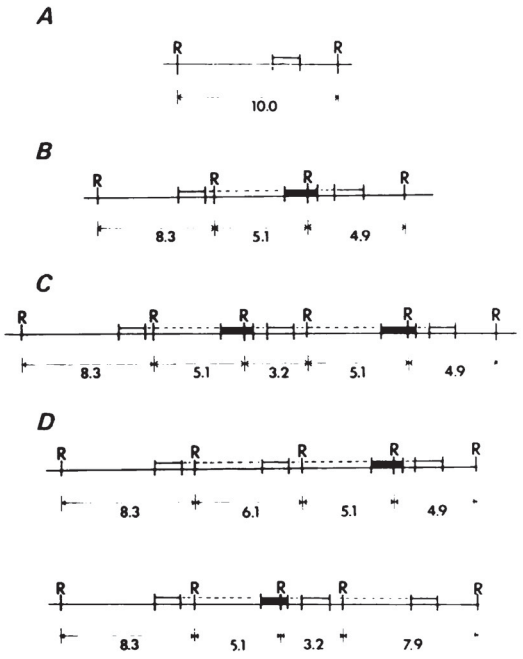

FIG. 2. Sizes of predicted *Eco*RI fragments from single or multiple plasmid integrations. (A) Genomic *HIS3* gene is present on a 10-kb *Eco*RI fragment. (B) Single integrated copy of pSZ505 produced three *Eco*RI fragments with homology to pBR322 of 8.3, 5.1, and 4.9 kb. (C) Multimer of pSZ505 contained an additional 3.2-kb *Eco*RI fragment. (D) Mixed multimers produced either a 6.1-kb fragment together with the pSZ505 fragments or 8.3-, 5.1-, 3.2-, and 7.9-kb fragments. Multimers containing more than one copy of pSZ63 and pSZ505 had combinations of these

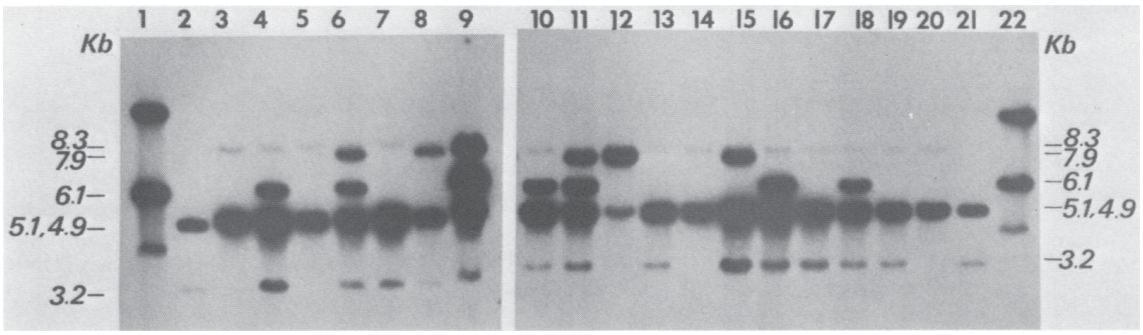

FIG. 3. Structural analysis of LEU$^+$ transformants. DNA was digested with *Eco*RI, transferred to nitrocellulose, and hybridized to nick-translated pBR322. Lanes: 1 and 22, standard of a transformant with multiple copies of pSZ62, showing 6.1-kb fragment (pSZ62 contained the same *HIS3* fragment in pBR322 as did pSZ63, but it was in the opposite orientation, resulting in flanking fragments of 12.0- and 4.1-kb rather than 8.3- and 4.9-kb); 2 and 21, *Eco*RI-cut pSZ505 standard with 5.1- and 3.2-kb bands; 3, 5, 14, and 20, single pSZ505 insert; 7, 13, 17, and 19, pSZ505 multimer; 8, 12, and 15, mixed multimer with one copy each of pSZ63 and pSZ505; 4, 6, 9 through 11, 16, and 18, mixed multimer with more than one copy of pSZ63 or pSZ505. Fragment sizes are given in kb and were determined relative to *Hin*dIII-digested λ DNA run as a standard.

integration of additional plasmid molecules. Multimers of circular plasmids may arise either by this mechanism or by recombination between the molecules before integration. Multiple, tandem integration of circular and linear plasmid DNAs has been described in mammalian cells (1, 2). However, mammalian cell multimers may be generated by a mechanism other than sequential integration, since ligation of transforming DNA occurs readily (2, 5), microinjected circular molecules recombine with each other (2), and integration of a plasmid into a homologous chromosomal site has not been observed.

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