JOURNAL OF BACTERIOLOGY, Oct. 1992, p. 6703–6706
0021-9193/92/206703-04$02.00/0

Vol. 174, No. 20

Site-Specific Recombination of the Circular 2μm-Like Plasmid pKD1 Requires Integrity of the Recombinase Gene *A* and of the Partitioning Genes *B* and *C*

MICHELE M. BIANCHI

Department of Cell and Developmental Biology, University of Rome "La Sapienza," Piazzale Aldo Moro, 00185 Rome, Italy

Received 1 April 1992/Accepted 15 August 1992

In the circular plasmid pKD1, which stably replicates in *Kluyveromyces lactis*, the three open reading frames encode a site-specific recombinase (gene *A*) and two proteins involved in mitotic stability (genes *B* and *C*). A recombination analysis of plasmids in which gene *B* or *C* is inactivated reveals that unlike the 2μm plasmid of *Saccharomyces cerevisiae*, these genes are also required for the site specificity of plasmid recombination.

pKD1 is a circular plasmid isolated from *Kluyveromyces drosophilarum* (7) which is able to replicate stably in *Kluyveromyces lactis* (3) and other related *Kluyveromyces* species (6). pKD1 belongs to a family of yeast plasmids that includes the 2μm circle of *Saccharomyces cerevisiae* and other plasmids from the osmophilic yeasts of the *Zygosaccharomyces* genus. The stable maintenance of yeast plasmids in the cell population is possible because of efficient mitotic partitioning and copy number amplification. These yeast plasmids do not exhibit sequence homologies, but they have very similar genomic organizations and the functions of plasmid genes are analogous (16). In fact, in all plasmids analyzed, one gene codes for a site-specific recombinase while two other genes are involved in plasmid partitioning. Other plasmid elements, i.e., the replication origin, the inverted repeats, and a *cis*-acting stability locus (4, 9, 10), are required for plasmid replication, recombination, and partitioning, respectively. The site-specific recombinase (FLP) of the 2μm plasmid has been studied both in vivo and in vitro (1, 5) and has been shown to be involved in copy number amplification (8, 15).

In this work, I report an analysis of the stability, copy number, and recombination in *K. lactis* strains of an isomorphic set of vectors containing pKD1 genes which have been inactivated at the level of translation by frameshift mutations. The results revealed a new function of pKD1 partitioning genes in site specificity of recombination, which is the first important difference between pKD1 and the other circular plasmids.

The vectors used in this study were derived from pS13 (Fig. 1), which contains the integrative vector YIp5 inserted in the unique *Sph*I site of pKD1. This vector was chosen because of its high stability (Table 1). pKD1 genes (*A*, *B*, and *C*) in pS13 were subsequently modified by insertion of 4 bp within each open reading frame (vectors pKA, pKB, and pKC; Fig. 1). Doubly disrupted vectors (pKAB and pKAC)

TABLE 1. Plasmid stability and copy number in pKD1^0 and pKD1$^+$ strains of *K. lactis*

Vector	Stability[a] of the transformants after the indicated no. of generations:				Plasmid copy number[b] after the indicated no. of generations:			
	pKD1^0			pKD1$^+$, 8	pKD1^0			pKD1$^+$, 8
	8	50	100		8	50	100	
pS13	101.9 (± 11.7)	89.6 (± 16.6)	75.2 (± 47.8)	74.0 (± 1.4)	18.4 (± 1.8)	17.0 (± 5.0)	17.3 (± 5.8)	ND
pKA	79.3 (± 15.3)	36.1 (± 3.7)	17.1 (± 6.7)	66.3 (± 4.4)	6.9 (± 0.9)	8.4 (± 0.8)	5.1 (± 1.0)	ND
pKB	4.8 (± 2.4)	0.0	ND[c]	58.7 (± 7.4)	13.0 (± 5.9)	ND	ND	ND
pKC	4.2 (± 2.9)	0.0	ND	78.0 (± 4.4)	14.9 (± 11.4)	ND	ND	ND
pKAB	3.7 (± 5.9)	ND	ND	60.5 (± 4.6)	30.4 (± 29.6)	ND	ND	ND
pKAC	2.8 (± 2.5)	ND	ND	66.4 (± 15.3)	12.1 (± 7.6)	ND	ND	ND
pE1	98.1 (± 17.5)	ND	ND	ND				
pE11	29.4 (± 12.2)	ND	ND	26.7 (± 12.3)				

[a] Stabilities were measured as percentages of Ura$^+$ cells after growth in complete medium. Stability values are averages of three to six determinations on independent transformants. Standard deviations are reported in parentheses.

[b] Copy numbers were determined as follows: total DNAs from the transformants were digested with *Bgl*II endonuclease, electrophoresed, and blotted onto membrane filters (Hybond-N; Amersham). Gel slots were loaded with increasing amounts of the same digestion mixture. Filters were then hybridized with a probe composed of pBR322 and *KEX1* (17) sequences. Intensities of the signals belonging to the vector and hybridizing with the pBR322 moiety of the probe were compared with the reference scale of signals deriving from the single yeast chromosomal gene *KEX1*. Values were corrected by the percentages of Ura$^+$ cells in the cultures: the *Bgl*II fragment hybridizing with pBR322 contained the marker gene *URA3* (Fig. 1). Copy numbers of pKB and pKC are probably lower than actual values as a result of the loss, on part of these vectors, of bacterial sequences (see text). Copy number values are averages of three to six determinations on independent transformants. Standard deviations are reported in parentheses.

[c] ND, not determined.

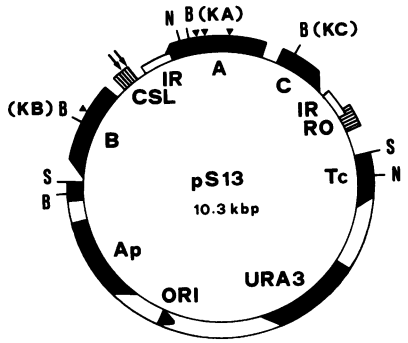

FIG. 1. Map of pS13, composed of pKD1 (upper part) and YIp5 (pBR322 plus the marker gene *URA3*; lower part). Genes (black boxes), inverted repeats of pKD1 (IR) (open boxes), and the *cis*-acting stability locus (CSL) and putative replication origin (RO) of pKD1 (striped boxes) are shown. Restriction sites: S, *Sph*I; N, *Nru*I; B, *Bam*HI; small triangles, *Bgl*II; small arrows, *Mst*II. Inactivation of pKD1 genes was obtained as follows: pS13 was partially digested with *Bam*HI, and the protruding ends were filled in by Klenow enzyme and religated. The resulting vectors, named pKA, pKB, and pKC, had frameshift mutations in the corresponding genes, and stop codons occurred a few nucleotides downstream of the 4-bp insertions. The doubly disrupted vectors pKAB and pKAC were obtained from pKA in a manner similar to that of vectors described above. YIp5 insertion at *Bam*HI sites had already resulted in gene inactivation (4). Vectors pKA, pKB, and pKC, obtained by frameshift inactivation at *Bam*HI sites of the corresponding genes, are indicated in parentheses (KA, KB, and KC, respectively). The *Mst*II sites were used to obtain vector pE11 (a gift of H. Fukuhara) from vector pE1 (see text).

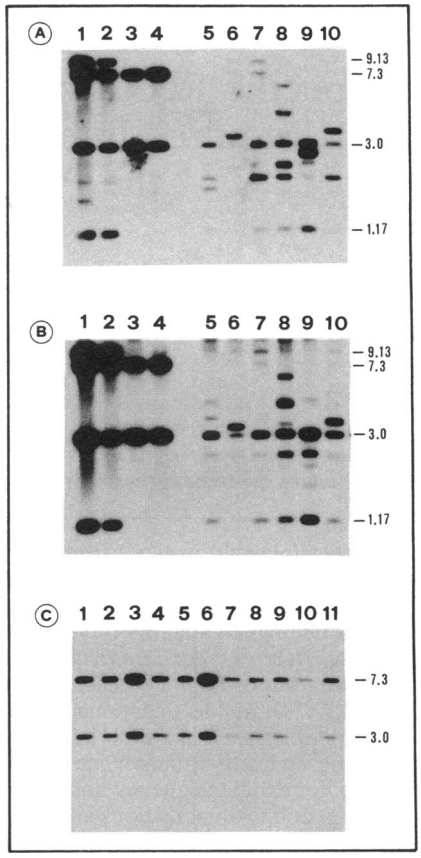

FIG. 2. Southern analysis of *Nru*I digestion of DNAs extracted from independent pKD1^0 transformants. (A and B) The filters with pS13-derived vectors were hybridized with labelled pS13 (A) and pKD1 (B). Lanes 1 and 2, pS13; lanes 3 and 4, pKA; lanes 5 to 7, pKB; lanes 8 to 10, pKC. (C) The filter with doubly disrupted vectors was hybridized with labelled pS13. Lanes 1 to 5, pKAB; lane 6, pKA (control); lanes 7 to 11, pKAC. The molecular sizes of the regular *Nru*I fragments of pS13 are marked to the right of the gels (form B, 3.00 and 7.30 kbp; form A, 1.17 and 9.13 kbp).

were also constructed. Two *K. lactis* strains, VD1 (α *argA lysA uraA* K$^-$ pKD1^0) and the isogenic MD2/1 (pKD1$^+$) strain, were transformed with all the vectors described by the protoplast procedure (3). Stabilities of the transformants and vector copy numbers per Ura$^+$ cell were measured; results are reported in Table 1.

Results concerning the stability of the vectors confirmed the relevance of genes *B* and *C* in plasmid partitioning, although inactivation of gene *A* also resulted in lower stability with respect to the parental pS13 plasmid, especially after prolonged growth. On the other hand, copy number was significantly more affected by the inactivation of gene *A* than by that of gene *B* or *C*. The results suggest a possible role of recombinase A in copy number amplification, as has also been indicated for the FLP enzyme of 2μm. The mitotic loss of pKA can probably be explained by the low copy number of pKA, instead of as a consequence of a direct involvement of the recombinase in partitioning. The large standard deviations of pKB, pKC, pKAB, and pKAC copy numbers arose from the low but variable percentages of Ura$^+$ cells caused by the uneven segregation of the plasmid molecules during cell division.

Recombination properties of the vectors were tested by Southern analysis of plasmid DNA from transformants (Fig. 2). Digestion with *Nru*I endonuclease made it possible to distinguish the input form of the vector (form B) from its isomeric form (form A) produced by site-specific recombination. Both isomers were found in pS13 transformants (Fig. 2A and B, lanes 1 and 2), while only the input form was present in pKA transformants (Fig. 2A and B, lanes 3 and 4). Unexpected results were obtained with vectors pKB and pKC. In fact, in addition to the four regular fragments, additional molecules of different sizes which varied from one transformant to the other were detected (Fig. 2A and B, lanes 5 to 10). The additional fragments, which were detected with labelled pS13 (Fig. 2A), yielded signals of a different intensity when hybridized to pKD1 alone (Fig. 2B) or to pBR322 (not shown). On the other hand, the large *Nru*I fragments of the regular isomers, which did not contain the pKD1 origin of replication (Fig. 1), yielded only very faint signals with all the probes used. These observations suggested that bacterial and/or pKD1 sequences present in vectors pKB and pKC were lost in the transformants. Figure 3 shows the Southern analysis of undigested DNAs from the transformants. The higher electrophoretic mobility of the circular molecules detected in pKB and pKC transformants (lanes 5 to 10) confirmed that deletion products of the input vectors were present in these transformants.

To determine whether these circles were produced by the plasmid recombinase or by some host activity, DNAs from the doubly disrupted pKAB and pKAC transformants were analyzed (Fig. 2C and 3). Only the input form of the vectors was present in these transformants, a fact which indicates that the appearance of aberrant molecules had in fact been due to the plasmid recombinase. The low stability of the

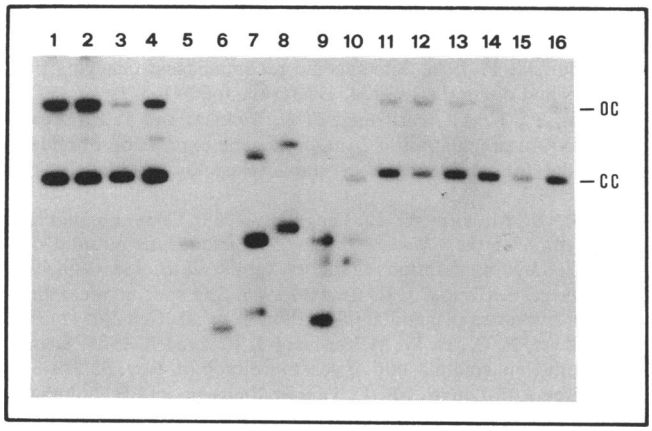

FIG. 3. Southern analysis of undigested DNAs from independent pKD1^0 transformants hybridized to labelled pS13. Lanes 1 and 2, pS13; lanes 3 and 4, pKA; lanes 5 to 7, pKB; lanes 8 to 10, pKC; lanes 11 to 13, pKAB; lanes 14 to 16, pKAC. CC, closed circular DNA; OC, open circular DNA.

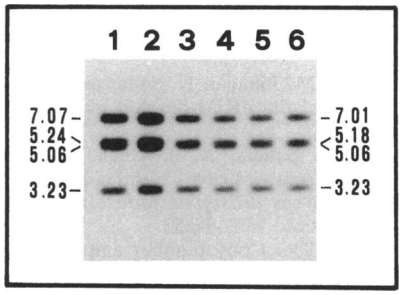

FIG. 4. Southern analysis of DNAs from pE1 (lanes 1 and 2) and pE11 (lanes 3 to 6) transformants of the pKD1^0 strain, digested with *Nru*I nuclease. The filter was hybridized with labelled pE11. The sizes of the *Nru*I fragments of the input form (pE1, 5.24 and 5.06 kbp; pE11, 5.18 and 5.06 kbp) and of the other isomer (pE1, 7.07 and 3.23 kbp; pE11, 7.01 and 3.23 kbp) of the vectors are indicated at the sides of the gel.

pKB and pKC transformants cannot have been due to marker loss as a result of plasmid rearrangements, because the pKAB and pKAC transformants, in which no rearranged molecules were found, showed a similar instability. DNAs from MD2/1 (pKD1$^+$) transformants of all the vectors described yielded the regular four-signal pattern after *Nru*I digestion (data not shown).

The results indicated that anomalous recombination events took place in the absence of the *B* or *C* gene product; these events were the result of the activity of the plasmid recombinase and gave rise to two smaller circular molecules. The circles bearing the pKD1 origin of replication could then be selectively maintained in host cells. That there had been large deletions of DNA from the transforming vectors was also suggested by the fact that all attempts to recover the rearranged molecules from the yeast transformants by *Escherichia coli* transformation consistently failed. The presence of low amounts of the regular isomers, on the other hand, indicated that the loss of specificity of the plasmid recombinase was not complete.

However, to exclude the possibility that the anomalous recombination was an indirect consequence of faulty segregation, the recombination properties of a vector defective in partitioning because of a deletion in the *cis*-acting stability locus (*CSL*) were tested. This vector, called pE11, was derived from the stable vector pE1 (already described in references 3 and 6) by digestion with *Mst*II endonuclease and religation, which yielded a 60-bp deletion within the *CSL* gene (Fig. 1). This deletion resulted in a substantial decrease in the stability of pE11 (Table 1). Recombination analysis of these vectors (Fig. 4) showed that only the expected recombination events took place in pE11 transformants, thus excluding the above-mentioned possibility. Another possibility was that the anomalous recombination was due to an overexpression of gene *A* in the absence of the *B* and *C* gene products which might have regulatory activity, as in the 2μm system. This possibility, however, was not supported by Northern (RNA) analysis of poly(A)$^+$ transcripts from vectors bearing disrupted *B* and *C* genes (2).

I suggest that *B* and *C* gene products are required by the plasmid recombinase to promote site-specific recombination. The excision of DNA sequences of variable length from the replicative circles indicates that the loss of site specificity of the reaction was also accompanied by a loss of directionality. Actually, in the different site-specific recombination systems studied, excisions occur when the recognition sequences, which impart directionality because of their inherent asymmetry, are directly arranged on genomes (12). Thus, the excision of DNA sequences from pKB and pKC vectors might have been the consequence of the absence of elements that could have imparted directionality to the sequences unspecifically recognized by the plasmid recombinase. The recognition of the specific recombination targets in the presence of *B* and *C* gene products might be derived from a direct protein-protein interaction with the plasmid recombinase or DNA-protein interaction with the specific recombination sites. Other possibilities, such as interaction of the partitioning proteins with cryptic recombination sites or conformational effects on DNA, are not excluded.

In conclusion, these results demonstrate that unlike the situation in the 2μm system, where the FLP enzyme alone is sufficient for site-specific recombination and the partitioning genes *REP1* and *REP2* act only as transcription regulators (11, 13, 14), in pKD1 the partitioning genes *B* and *C* are also directly involved in plasmid recombination. The possible roles in transcription regulation of genes *B* and *C* of pKD1 still remain to be investigated, but the absence of the fourth open reading frame suggests that the mechanism of transcription control which is at work is different from the one in the 2μm circle.

I thank L. Frontali for helpful discussions.

This work was supported by Consiglio Nazionale delle Ricerche (Progetto Finalizzato Biotecnologie e Biostrumentazioni) and by Rhône-Poulenc Santé (contracts with the University of Rome PES 5871289 and PES 0370591).

REFERENCES

1. **Babineau, D., D. Vetter, B. J. Andrews, R. M. Gronostajski, G. A. Proteau, L. G. Beatty, and P. D. Sadowski.** 1985. The FLP protein of the 2-micron plasmid of yeast. J. Biol. Chem. **260**:12313–12319.
2. **Bianchi, M. M.** Unpublished data.
3. **Bianchi, M. M., C. Falcone, X. J. Chen, M. Wésolowski-Louvel, L. Frontali, and H. Fukuhara.** 1987. Transformation of the yeast *Kluyveromyces lactis* by new vectors derived from the 1.6 μm circular plasmid pKD1. Curr. Genet. **12**:185–192.
4. **Bianchi, M. M., R. Santarelli, and L. Frontali.** 1992. Plasmid functions involved in the stable propagation of the pKD1 circular plasmid in *Kluyveromyces lactis*. Curr. Genet. **19**:155–161.

5. **Broach, J. R., V. R. Guarascio, and M. Jayaram.** 1982. Recombination within the yeast plasmid 2μ circle is site-specific. Cell **29:**227–234.
6. **Chen, X. J., M. M. Bianchi, K. Suda, and H. Fukuhara.** 1989. The host range of the pKD1-derived plasmids in yeast. Curr. Genet. **16:**95–98.
7. **Falcone, C., M. Saliola, X. J. Chen, L. Frontali, and H. Fukuhara.** 1986. Analysis of a 1.6 μm circular plasmid from the yeast *Kluyveromyces drosophilarum*: structure and molecular dimorphism. Plasmid **15:**248–252.
8. **Futcher, A. B.** 1986. Copy number amplification of the 2 μm circle plasmid of *Saccharomyces cerevisiae*. J. Theor. Biol. **119:**197–204.
9. **Jearnpipatkul, A., R. Hutacharoen, H. Araki, and Y. Oshima.** 1987. A cis-acting locus for the stable propagation of yeast plasmid pSR1. Mol. Gen. Genet. **207:**355–360.
10. **Kikuchi, Y.** 1983. Yeast plasmid requires a *cis*-acting locus and two plasmid proteins for its stable maintenance. Cell **35:**487–493.
11. **Murray, J. A. H., M. Scarpa, N. Rossi, and G. Cesareni.** 1987. Antagonistic controls regulate copy number of the yeast 2μ plasmid. EMBO J. **6:**4205–4212.
12. **Sadowski, P.** 1986. Site-specific recombinases: changing partners and doing the twist. J. Bacteriol. **165:**341–347.
13. **Som, T., K. A. Armstrong, F. C. Volkert, and J. R. Broach.** 1988. Autoregulation of 2μm circle gene expression provides a model for maintenance of stable plasmid copy levels. Cell **52:**27–37.
14. **Veit, B. E., and W. L. Fangman.** 1988. Copy number and partition of the *Saccharomyces cerevisiae* 2μm plasmid controlled by transcription regulators. Mol. Cell. Biol. **8:**4949–4957.
15. **Volkert, F. C., and J. R. Broach.** 1986. Site specific recombination promotes plasmid amplification in yeast. Cell **46:**541–550.
16. **Volkert, F. C., D. W. Wilson, and J. R. Broach.** 1989. Deoxyribonucleic acid plasmid in yeasts. Microbiol. Rev. **53:**299–317.
17. **Wésolowski-Louvel, M., C. Tanguy-Rougeau, and H. Fukuhara.** 1988. A nuclear gene required for the expression of the linear DNA-associated killer system in the yeast *Kluyveromyces lactis*. Yeast **4:**71–81.