Deoxyribonucleic Acid Plasmids in Yeasts

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INTRODUCTION

The eucaryotic genome is composed of chromosomes, various plasmid genomes, and, occasionally, miscellaneous stable extrachromosomal elements. These extrachromosomal components of an organism most probably have evolved a benignly parasitic or symbiotic relationship with their cellular host. As such, these elements have had to develop special processes and strategies to ensure their persistence without the benefit of an elaborate cellular structure, such as the mitotic apparatus, to effect their transmission from generation to generation. Successful strategies, i.e., those adopted by elements that have survived, achieve a delicate balance between usurping cellular resources for the replication and transmission of the element and not compromising the fitness of the host cell.

An evaluation of the deoxyribonucleic acid (DNA) plasmid species found naturally in yeasts presents an anthology of approaches to stable, extrachromosomal persistence. In this review we describe all the DNA plasmids that have been isolated and characterized from different yeasts, focusing our attention on the mechanisms underlying their successful existence as extrachromosomal elements. This not only emphasizes the diversity of approaches to filling a specific ecological niche but also provides a novel approach to an analysis of various aspects of DNA metabolism, cellular and nuclear architecture, and the process of partitioning of cellular components during division. Analysis of these elements has led to novel insights into such diverse areas as...
amplification of specific chromosomal domains, proteolytic processing of secreted proteins, and control of DNA replication. In addition, an appreciation of the biology of these elements has afforded the development of specialized vectors to harness the various yeast species for scientific and commercial applications of genetic engineering.

Clearly, in focusing on yeast DNA plasmids, we have excluded from consideration a rich diversity of other fungal extrachromosomal elements. These would include various transposon-like plasmids of Neurospora spp., the kalilo senescence factor of Neurospora spp., the Fusarium oxysporum host specificity factor, and the mitochondrial elements associated with the stopper phenotype of Neurospora spp., the ragged phenotype of Aspergillus spp., and senescence in Podospora spp. Fortunately, these topics have been treated in two comprehensive and recent reviews of eucaryotic extrachromosomal elements (27, 131). We have also excluded from consideration the various viruslike double-stranded ribonucleic acid (dsRNA) species found in yeasts such as Saccharomyces cerevisiae and Ustilago maydis, as well as retrotransposon species from S. cerevisiae. The former topic was also covered in reference 131, and both topics will be treated in depth in an upcoming volume of The Molecular Biology of the Yeast Saccharomyces (J. Pringle, E. Jones, and J. Broach, in press).

THE 2μM CIRCLE PLASMID OF S. CEREVISIAE

Biology of the 2μM Circle

The 2μm circle plasmid of Saccharomyces spp. was the first identified fungal cognate of bacterial plasmids. It is an extrachromosomal DNA species that has evolved molecular mechanisms to ensure its long-term autonomous survival. Its virtual ubiquity in its host species, S. cerevisiae and S. carlsbergensis, and the absence of any phenotype associated with its presence have led to the now-prevalent view that the 2μm circle and its relatives represent a form of benign genetic parasitism. The presence of 2-micron plasmids in yeast species widely divergent from Saccharomyces spp. (see below) implies that their adaptive strategies for molecular parasitism have been extremely successful over evolutionary time.

In most respects, the 2μm circle can be thought of as a tiny circular chromosome. It resides in the nucleus and behaves as a nuclear marker in cytoduction crosses (69, 110). Plasmid DNA is assembled into chromatin in vivo with a characteristic nucleosome phasing, using the same core histones as found in yeast chromatin (70, 80). The plasmid origin of replication functions as an autonomously replicating sequence (ARS) in transformation assays (9), has the consensus sequences typical of chromosomal ARSs (10, 133), initiates at a defined point in the S phase (138), and is dependent for its function on a number of gene products also required for chromosomal replication (66, 71, 72, 86).

The two most noticeable differences between the 2μm circle and a chromosome (other than size and topology) are precisely those features salient to its unique adaptive strategy for parasitic life in the yeast nucleus: maintenance of a controlled, high copy number (about 60 per G1-phase haploid nucleus) (18, 31) and the ability to partition daughter molecules uniformly at mitosis and meiosis without a centromere (54, 63, 77). Elucidation of the molecular mechanisms underlying these two aspects of the lifestyle of the 2μm circle currently dominates research on the plasmid, and in this section of this review we concentrate primarily on these lines of inquiry.

The 2μm circle has also been used as a substrate for studies of more general aspects of DNA metabolism, such as replication initiation (5, 14, 52, 55, 56, 67) and topoisomerase activity (23). In addition, the enzymology of its site-specific recombination reaction has been studied extensively (reviewed in references 20 and 90). Other reviews have dealt with these subjects in detail (6, 20, 90, 123, 133), and we will cite results of these studies only when they are germane to appreciating the mode of persistence of the plasmid.

Phenotypes associated with the 2μm circle. Because most bacterial plasmids encode gene products that endow their hosts with a selective advantage, evidence has been sought for such a role for the 2μm circle. Certainly, the plasmid is not required for normal mitotic growth: yeast strains completely devoid of the plasmid have been derived from plasmid-bearing strains, and such cells grow normally under routine conditions (24, 76). Earlier studies credited the plasmid with conferring various phenotypes on its host, such as resistance to oligomycin (39). Initial attempts to test these claims rigorously were thwarted by the absence of truly isogenic [cir+] and [cir-] strains (strains having a full complement of wild-type plasmid are denoted [cir+], and those lacking any plasmid are designated [cir-]). The subsequent availability of such isogenic strains led to the discovery that none of these phenotypes could be attributed to the 2μm circle. The fact that all four of the genes of the 2μm circle have now been shown to encode plasmid maintenance functions (see below) has considerably decreased the likelihood that the 2μm circle will be found to be a resistance factor.

The 2μm circle has a slight but reproducible inhibitory effect on the mitotic growth rates of strains in which it is resident. In very careful comparisons of isogenic [cir+] and [cir-] strains in continuous exponential growth on rich medium, Mead et al. (76) have shown that 2μm circle-bearing strains have a 1.5 to 3% longer generation time than plasmid-free strains. This weak parasitic effect may well be the full extent of the plasmid phenotype in normal yeast cells. As Futcher et al. (33) have pointed out, the observed stability of the plasmid and its ability to amplify copy number after the mating between [cir+] and [cir-] strains fully compensate for the slight deleterious effect on mitotic growth, as long as one assumes a reasonable frequency of outbreeding. Thus, the known behavior of the 2μm circle can completely account for its indefinite persistence in a yeast population, even in the absence of any selectable phenotype.

One curious phenotype that has been associated with the 2μm circle is that of nibbled colony morphology (51). This phenotype requires a recessive allele of a single chromosomal locus, NIB1. The nibbled phenotype is caused by clonal lethality, accompanied by excessively high plasmid copy number in moribund cells. This retards growth in sectors of colonies, giving them a ragged morphology. Recent findings indicate that in the susceptible genetic background, aspects of the nibbled phenotype are produced in the presence of any plasmid, whether or not it is related to the 2μm circle (V. Zakian, personal communication).

Structure of the 2μm Circle

Genome Organization. The primary structure and genome organization of the 2μm circle (Fig. 1) is representative of the most widespread class of yeast plasmids (40, 46). The sequenced isolate of the circle, designated 2μm Aspl, consists of 6,318 base pairs (bp) of double-stranded circular DNA, comprising 2 unique regions of 2,774 and 2,346 bp
The quires, ORF, extend surrounded each other bp, specific inverted (4x, etc.) strikingly (46). Restriction cis-acting distinguish the repeats, with a loss of the HpaI site. Scp3 has a deletion equivalent to four repeats, with a loss of both the HpaI and AvaI sites, and also an apparently unrelated loss of the EcoRI site in the RAFL gene (D coding region). In addition, Livingston (69) and Kikuchi (63) have described 2μm circle variants whose restriction maps are consistent with the loss of four repeats (like Scp3) and two repeats (like Scp2, but with retention of the HpaI site), respectively. Each variant probably represents an independent isolate. In each instance, the strain in which a variant is found carries only a single 2μm circle type. The viability of these variants suggests that, to a first approximation, the exact number of these repeats carried on a 2μm circle plasmid is inconsequential. Given the requirement in cis of this region for accurate plasmid partitioning (see below), a careful test of this hypothesis would seem to be in order.

Molecular Basis for Plasmid Persistence

Plasmid stability. The mitotic stability of the 2μm circle approaches that of a chromosome. The proportion of cells in an exponentially growing culture which lose the plasmid in any one generation has been measured as 10^-5 in a haploid strain, and less than 10^-5 in a diploid strain (31, 76). This is in sharp distinction to the instability of constructed high-copy yeast-Escherichia coli cloning vectors (i.e., YRp [yeast replicative plasmid] vectors, which consist of a selectable yeast gene and a chromosomal ARS, cloned in a bacterial vector). The general loss frequencies of these plasmids can be 10^-5 or even higher (133). Several components contribute to the long-term stability of the 2μm circle. The major factor underlying the stable persistence of the 2μm circle and of plasmids derived from it (YEp [yeast episomal plasmid] vectors) is its ability to distribute plasmid copies more or less evenly between the mother and bud cells at mitosis (54, 63, 77). This contrasts with artificial plasmids, whose partitioning is strongly biased toward the mother cell. In addition to promoting equi-partitioning, the 2μm circle is capable of sensing its cellular copy level and responding to that assessment by promoting copy number amplification in cells containing relatively few copies of the plasmid. Although the contribution of this process to stability is more difficult to document experimentally, the fact that this amplification system is shared by all circular yeast plasmids (see below) argues that amplification is an essential component of the survival strategy of these plasmids. The various processes contributing to plasmid persistence are discussed below.

Plasmid partitioning. (i) Genetic components. Subcloning and mutational analysis have identified three parts of the sequences) determines the directionality (i.e., excision versus inversion of a sequence bounded by FRT sites) of the recombination, and recombination-proficient FRT sites with altered core sequences can recombine only with identical mutants (95).

2μm circle variants. Several structural variants of the 2μm circle have been observed frequently (6). Neither the mechanism responsible for their production nor their biological consequences have been examined. A common feature of most of them is deletion of sequences in the region of the 62-bp direct repeats. Although none of these has been sequenced, restriction analysis suggests that at least some represent precise excisions of copies of the repeats, possibly mediated by homologous recombination.

Sep1 and two of these variants constitute the canonical description of the structural repertoire of the 2μm circle (12).

The sequence of the 2μm circle reveals four methionine-initiated open reading frames (ORFs) greater than 500 bp (46). These were originally designated A, B, C, and D and encoded predicted proteins of 423, 373, 296, and 181 amino acids, respectively. Each unique region contains a pair of ORFs, whose 5' ends lie within several hundred base pairs of each other near the center of the region and which diverge on opposite DNA strands. The A, B, and C coding regions extend into the inverted repeats.

The two inversion forms of the 2μm circle that coexist in intracellular populations are interconverted by its own sitespecific recombination system (8). The enzymology of this reaction, which strongly resembles that of several bacterial systems, has been a very active research topic and is the subject of more specialized reviews (20, 90, 123). The only protein required for this reaction is the product of the A ORF, now called FLP. FLP-mediated recombination requires, and occurs within, a site called FRT within each 599-bp repeat (37, 75, 95). FRT consists of an 8-bp core surrounded by a pair of 13-bp repeats (92, 46). The repeats are 12- or 13-matches (the mismatched base has no mechanistic significance) and are in inverted orientation. Recombination proceeds via a pair of staggered strand cuts at the ends of the core, which make an 8-base 5' overhang (2, 95). Evidently, overhangs from the recombining partners anneal. The asymmetry of the core (not of the repeats or outside

FIG. 1. Structural organization of the 2μm circle. A diagram of the genomic organization of the yeast plasmid is shown, drawn to distinguish the inverted repeats (horizontal lines) from unique sequences (circular regions). The locations of the open coding regions ( ), arrows signifying the 5'-to-3' orientation, the origin of replication ( ), the FLP recombination target site ( ), the cis-acting stability locus ( ), and various restriction sites are indicated. The D coding region is sometimes referred to as RAF. Restriction site abbreviations: H, HindIII; Hp, HpaI; A, AvaI; E, EcoRI.

separated by a pair of exact inverted repeats each 599 bp long (46). Natural populations of the 2μm circle consist of about 80% monomeric plasmid by number, with the rest distributed among an arithmetic series of multimers (2x, 3x, 4x, etc.) whose frequencies decrease with increasing size (86). In such populations, the 2μm circle exists as an equal mixture of the two inversion isomers that would be formed by genetic recombination between the inverted repeats.

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REP2 protein is also a nuclear protein, although its localization to the matrix fraction is less certain (K. Armstrong and J. Broach, unpublished observations). In cir\(^3\) strains in which either or both of the REP proteins are expressed from an inducible promoter (97), REP1 protein is found in nuclear fractions with or without concurrent expression of REP2 protein. However, REP2 protein is found only in these fractions in cells expressing both proteins. This suggests that REP1 is required for either stabilization of REP2 protein or transport of REP2 protein to the nucleus (Armstrong and Broach, unpublished).

(ii) Possible mechanisms. How does the REP (STB) system promote plasmid stability? This is a difficult question to answer at the moment, since we do not yet understand why plasmids lacking REP (STB) components are unstable. As mentioned above, both YRp plasmids and 2μm circle plasmids lacking one or more of the partitioning components do not segregate well. As determined by pedigree analysis, poor segregation is a consequence of frequent failure to transmit plasmid copies to the daughter cell following mitosis and cell division (77). This is true even though plasmid molecules accumulate to high copy in the mother cell lineage. The idea that a high-copy plasmid should be unstable by default, owing to defective partitioning, is certainly counterintuitive. One assumes that such plasmids should diffuse throughout the nucleus, just as high-copy E. coli plasmids appear to do in the cell. Since very small circular plasmids and small to moderate-sized linear plasmids segregate reasonably well in yeasts even in the absence of a defined partitioning system (77, 139), we suspect that normal-sized circular plasmids fail to partition efficiently because they become topologically entangled, either in each other or in proteinaceous fibers of the nuclear architecture.

The nature of the internal architecture of yeast nuclei, which might account for the hindered migration of circular plasmids, is unknown. The existence of a biologically relevant, fibrous infrastructure of eucaryotic nuclei has been suggested from various experimental observations, although the presence of this structure is not unequivocally established (1, 135). Regardless of its precise nature, any internal nuclear architecture could constitute a barrier to free diffusion of plasmid molecules. Small circular DNA molecules and small to moderate-sized linear DNA molecules would be constrained less in their migration by such a structure than moderate-sized circular molecules would be. The obvious analogy is migration of DNA species through agarose or acrylamide gels, in which relaxed circular DNA migrates significantly more slowly than equivalent-sized linear DNA.

For restricted plasmid diffusion to lead to mother-biased inheritance, one has to postulate that protein elements of the nuclear substructure are synthesized in a nonrandom fashion during cell growth. New nuclear material would have to be localized predominantly to the new (i.e., bud cell) nucleus, leaving the old structures and their associated plasmids in the mother destined part of the nucleus. Although there is no evidence regarding the deposition of proteinaceous components of the nuclear interior, a protein of the nuclear envelope, the product of KARI, is inherited in precisely this way (M. Rose, personal communication).

One can imagine two general ways in which 2μm circle copies might be equipartitioned by the REP system in the context of restricted plasmid diffusion: active segregation and facilitated diffusion. In an active segregation scheme, the REP proteins might cause the 2μm circle to form a strong specific attachment via STB to some structure that is partitioned equally between mother and bud cells. This structure
could be the spindle apparatus or the chromosomes. In an extreme subset of this model, 2μm circle copies might be segregated pairwise like sister chromatids. The role of the REP proteins and STB in an active segregation model would be to mediate or induce the association of the 2μm circle with the segregating subnuclear structure. In this simplest case, this would involve the REP proteins forming a bridge between the STB sequence on the 2μm circle and the segregating subnuclear structure. In this case, one or both of the REP proteins might be expected to bind the STB sequence and subnuclear protein structures specifically. The above-mentioned association of REPI with the nuclear matrix-lamina-pore complex is consistent with this prediction. However, definitive evidence for binding of REPI or REP2 protein to STB has yet to be presented. Amati and Gasser (1) have shown that a yeast nuclear scaffold fraction specifically binds a restriction fragment carrying STB as well as the origin of replication. Whether this binding is specific for STB and whether it is relevant to partitioning await further experimentation.

In a passive partitioning model, specific binding of STB to subnuclear structures is not required. Rather, the REP proteins must somehow mediate or induce the free diffusion of STB-containing plasmids. One can imagine many ways they might do this. The REP proteins might direct a topoisomerase to STB-containing plasmids, or they might locally depolymerize proteins of the nuclear matrix, with the STB sequence serving as a triggering signal. Alternatively, the proteins might promote specific condensation of STB-containing molecules, effectively rendering them smaller and more diffusible.

Whatever the mechanism of plasmid partitioning, the process is not as efficient as is theoretically possible. If we assume random inheritance of each plasmid copy at cell division, the observed plasmid loss rate of 10⁻⁴ per generation would be consistent with a G2-phase plasmid population of only 12 or 13 (32). This is about one-fifth the number of 2μm circle molecules actually present in the average haploid yeast cell. This simple calculation ignores the effects of cell-to-cell variation in plasmid population size and of plasmid multimerization. Even granted these factors, though, one must still postulate that transmission of plasmids to daughter cells is a relatively inefficient process or that plasmids are inherited as clusters larger than a single molecule. Under the simple assumptions above, these clusters would have an average size of about five 2μm circle monomers.

**Plasmid amplification.** The second element in the strategy of the 2μm circle for persistence as a parasitic DNA species is its ability to raise its copy number. This property initially constituted a biological paradox. On the one hand, the 2μm circle is subject to strict cell cycle control of its replication. Replication of the plasmid proceeds bidirectionally from a single origin of replication (5, 52). Essentially every 2μm circle molecule (at least 95% of them) in a cell population acts as a replication template in every generation, and each undergoes one and only one round of replication (138). On the other hand, the 2μm circle can be demonstrated to increase its copy number in several different situations. This requires that the plasmid be able to duplicate itself at a rate greater than once every cell cycle. How the plasmid can increase its copy number without abrogating stringent cell cycle control of replication posed an intriguing dilemma.

The most striking demonstration of the ability of the 2μm circle to overreplicate arose during matings of a [cir⁺] and a [cir⁻] strain under conditions in which the parent nuclei did not fuse after cytoplasmic mating (for example, in crosses involving a karl-1-containing strain (19)). Such matings yield heterokaryons, from which emerge haploid progeny cells, composed of a haploid nucleus from one strain and cytoplasmic markers from both parents. In addition, plasmids molecules are occasionally transferred from one haploid nucleus to the other within the initial heterokaryon (26, 96). Sigurdson et al. showed that when such transfers of the 2μm circle from the [cir⁺] to the [cir⁻] nucleus occurred, the progeny cells contained the appropriate plasmid complement for a haploid cell (96). Since only a few plasmid copies were transferred to the recipient nucleus in the particular experimental protocol used, attainment of normal copy number in the recipient strain must have arisen via overreplication.

Futcher (29) proposed an ingenious solution to the dilemma presented by 2μm plasmid amplification. He noted that a topological change in the usual theta-form replication intermediate, catalyzed by the plasmid site-specific recombinase, could yield plasmid amplification without having to invoke multiple replication initiation events.

The Futcher model (29) for plasmid copy number amplification is diagrammed in Fig. 2. The model assumes that production or activity of the recombinase is induced in cells in which the 2μm circle copy number is depressed. This activation, in turn, increases the probability that a plasmid molecule will recombine between its FRT sites at a crucial stage of DNA replication. This stage is reached after one of the diverging replication forks has passed the FRT site near the origin but before the other fork has arrived at the other FRT site. Recombination of the unreplicated site with either of the replicated ones rearranges the replication intermediate so that the forks, formerly converging, now move around the circular genome in the same direction. This allows indefinite
DNA chain extension from a single replication initiation event. The product of this chain elongation is a head-to-tail plasmid multimer, which can be reduced to monomeric units via either FLP-mediated or general recombination between directly reiterated sequences. The net effect of this maneuver is plasmid copy number amplification without the need for reinitiation of DNA synthesis at an origin already used once in an S phase.

The double-rolling-circle (DRC) amplification model has generated a number of hypotheses, some of which have been tested and confirmed. One fruitful avenue of research has been to identify the structural requirements of the amplifiable substrate and the gene products necessary for amplification (88, 122). In these experiments a test plasmid is introduced synchronously into most of the cells in a population by induced recombinational excision from a chromosome. This permits the observation of copy number changes over short time courses, which minimizes the contribution of plasmid segregation and outgrowth to the observed copy number.

Experiments of this sort have shown that for amplification, a 2μm plasmid must be an extrachromosomal circle with a pair of active FRT sites in inverted orientation (88, 122). The presence of FLP recombinase is required continuously for amplification, implying that the act of recombination, not the simultaneous presence of both inversion isoforms, is required (122). Additionally, amplification occurs only in growing cells, in agreement with the assumption of the model that replication initiation occurs by the normal mechanism. Finally, these experiments showed that plasmid genes other than FLP were neither necessary (88) nor sufficient (122) for copy number amplification.

A key prediction of the DRC model of amplification is the existence of characteristic amplification intermediates. Direct observation by electron microscopy of these intermediates in the DNA of vegetatively growing cells has been unavailing (B. Futcher and C. Newlon, personal communication). This is not particularly surprising, given the expected rarity of the species, its large size, and its branched structure. However, several experimental results hint at the production in vegetatively growing cells of head-to-tail 2μm circle multimers, which might have arisen by the DRC mechanism. First, the steady-state population of 2μm circle multimers is mostly head to tail (30). Second, multimers are enriched in the light-light shoulder of the density peak of DNA purified from cells transferred for one generation from medium containing heavy isotope derivatives to medium composed of light isotopes (30). Finally, a large majority of 2μm circles selected for their presence in cells in which FLP recombination had occurred recently are multimeric (F. C. Volkert and J. R. Broach, unpublished results). Although none of these results proves that FLP amplification produces multimers in vegetatively growing cells, all of them are at least consistent with the prediction.

Regulation of plasmid gene expression and control of plasmid copy number. The preceding model for FLP-mediated copy number amplification implies that the 2μm circle must sense its copy number in order to correct it. The most intuitive assumption is that some plasmid gene product varies in concentration, in proportion to the plasmid copy number, and that this product regulates the production or activity of the recombinase.

To test this hypothesis for plasmid copy control, several groups conducted systematic studies of the transcript regulation of the 2μm circle (79, 88, 97, 121). In these studies, 2μm circle gene products were produced in vivo from high-level, inducible promoters, and their regulatory activities were assessed both by using lacZ fusions to various plasmid coding regions and by directly measuring transcript levels from various 2μm circle genes. These studies yielded essentially the same major conclusion: the REPI and REP2 gene products coordinately repress transcription from most of the 2μm circle genes studied, including FLP, REPI, and RAF (or D coding region) but not REP2. In addition, REP1-REP2 proteins repress transcription of a 1,950-base transcript of unknown function, which begins distal to the 3’ end of the RAF gene, proceeds through that gene in an antisense orientation and through REP1 in a sense orientation, and has the same 3’ terminus as the minimal REP1 transcript (97, 109). Repression of the FLP and 1,950-base transcripts can be as much as 100-fold. Finally, Murray et al. (79) showed clearly that hyperexpression of RAF antagonized the repression effect of REP1 and REP2 on the FLP promoter. This observation confirmed prior indications of a role for RAF protein in control of FLP expression (13). A summary of this regulatory circuitry is presented in Fig. 3.

These data can be used to synthesize a coherent model for stable maintenance of 2μm circle copy levels. This model has been described in detail previously (4) and is only briefly outlined here. The primary stabilizing factor of the plasmid is equal mitotic partitioning, mediated by the two REP proteins acting on STB. The REP proteins also repress transcription of REP1, FLP, RAF and the 1,950-base transcript. We assume that production and degradation of the REP proteins are in steady-state equilibrium when the plasmid is at its normal high copy level. When the plasmid copy number falls (owing to unequal partitioning, errors in plasmid replication, or mating of a [cir+] strain with a [cir-] one), the concentration of the REP proteins would also fall, relieving repression of the regulated promoters. FLP would be expressed and the copy number deficit would be corrected by the amplification of
scheme described above. As the copy number rose, so would the REP protein concentration, resulting in reestablishment of repression and an end to amplification. As discussed previously (4), autogenous regulation of REP1 expression by the REP1-REP2 complex and antagonism of FLP and REP1 repression by RAF protein serve to sharpen the responsiveness of plasmid gene expression to changes in repressor concentration. In theory, this enhances the sensitivity of the plasmid to fluctuations in plasmid copy levels.

Several observations are consistent with this model of plasmid copy control. First, by using an in vivo recombination assay, more FLP activity could be detected in cells with a single copy of the gene, chromosomally inserted in a [cir+] strain, than is present in [cir-] cells (88, 97). That is, a single copy of FLP, in the absence of other plasmid proteins, yields more total recombination activity than the combined expression of all 60 copies of the gene in a [cir+] strain. This strongly suggests that FLP expression is substantially repressed by plasmid-encoded components. Second, production of high-level FLP expression raised the copy number of endogenous 2µm severalfold. This indicates that the availability of FLP is copy number limiting under ordinary conditions (79, 88, 97). Third, mutation of REP1 or REP2 increases the copy level of 2µm circle-based plasmids with an intact FLP-FRT recombination system, compared with plasmids with wild-type REP1 and REP2 or with repI/rep2 plasmids in which the FLP-FRT system has been inactivated (120, 121). This is consistent with the proposed role of REP1 and REP2 proteins as repressors of FLP-mediated amplification. In addition, Veit and Fangman (120) observed shifts in the nucleosome protection patterns in nucleosome-assembled plasmids upon mutational inactivation of REP1 or REP2. These mutations changed the nucleosome phasing patterns around STB and in the intergenic region immediately 5’ to FLP, indicating that 2µm circle stabilization and expression regulation might have a common mechanism. Finally, the STB locus and the regions immediately 5’ to the genes regulated by REP1 and REP2 all encompass one or more copies of a specific non-nucleotide sequence (4). This conserved element could represent the site through which these proteins effect partitioning and repression.

Other, more speculative thoughts concerning aspects of plasmid copy control should be considered. As noted above, concurrent with derepression of FLP and RAF, expression of the 1,950-base transcript is substantially induced. Certainly, this event could damp the hypothesized RAF-activation of FLP expression and prevent runaway induction, via hybridization of the 1,950-base and RAF messenger RNA (mRNA). This would rationalize the existence and regulation of the 1,950-base transcript, which otherwise remains unexplained. Also remaining to be explained is the regulatory distinction between the REP1 and REP2 genes (the former is regulated, whereas the latter is constitutive). A possible clue to this is the observation that overexpression of REP1 in the absence of the 2µm circle is quite toxic to yeasts (J. R. Broach, unpublished results); tight regulation of this gene might be necessary to prevent the 2µm circle from becoming a destructive parasite. From an evolutionary standpoint, becoming a destructive parasite could be self-defeating.

Contribution of plasmid copy control and amplification to stable plasmid persistence. Despite the demonstration of 2µm circle copy number amplification and copy number regulation under a variety of experimental circumstances, the adaptive significance of these processes to the plasmid in nature is only speculative. One function of amplification could be to readjust copy levels during mitotic growth to compensate for inexact plasmid segregation or occasional plasmid replication failures. If REP partitioning involves random allocation of plasmid to daughter cells, it would be expected to be imprecise. In this case, daughter cells with suboptimal plasmid copy levels would arise occasionally during normal mitotic growth. Additionally, since the majority of plasmid copies are monomers and hence rely on a single origin to initiate replication, a finite rate of replication failure may occur. This would necessitate copy number correction as well. One should note that if amplification repairs copy number deficits due to inexact segregation, some mechanism must come into play to deal with cells that receive excess plasmids during segregation, otherwise the mean plasmid copy number of the cell population would rise indefinitely.

Although not conclusive, available evidence supports a role for FLP-mediated amplification in vegetative plasmid maintenance. The presence of detectable multimers in the light-light DNA fraction following a density shift (30) indicates that amplification occurs in vegetatively growing cells. In addition, several lines of evidence indicate that FLP is active during vegetative growth. FLP mRNA is present in growing cells (97). FLP recombination acts on plasmids transformed into [cir+] cells. It inverts or excises FRT-bounded segments and integrates FRT-containing plasmids into each other or into endogenous 2µm circle (9, 88). Finally, a simple prediction of the involvement of FLP in vegetative plasmid maintenance is that a flp 2µm circle mutant will exhibit a gradual decrease in mean copy number in a cell population as well as a lower stability than the wild type. This hypothesis is currently being tested.

A second possible role for FLP amplification is to prevent dilution of the plasmid in natural matings between [cir+] and [cir-] cells. Certainly, corrective amplification occurs when such matings are performed in the laboratory. Furthermore, Futer et al. (33) have demonstrated that the 2µm circle can in fact spread in artificial populations via repeated rounds of induced sporulation and mating. Plasmid spread in the occasional natural outcross may also occur. The existence of genetic polymorphism in natural 2µm circle isolates suggests that field population genetics studies (in Bordeaux, perhaps) should be performed to test this hypothesis.

2µm CIRCLE-LIKE PLASMIDS OF YEASTS

Distribution and Structures of Circular dsDNA Plasmids of Yeasts

Although the 2µm circle is virtually ubiquitous in S. cerevisiae and S. carlsbergensis strains, few other yeast strains have been found to harbor identifiable DNA plasmids. Toh-e et al. (113) surveyed 100 yeast strains other than S. cerevisiae and found only 2 strains, both osmophilic yeasts of the species Zygosaccharomyces that contained plasmids. Similarly, Gunge et al. (44) examined 57 yeast strains and found no circular plasmids, but did identify two linear plasmids. These are discussed below. By focusing their attention on osmophilic yeasts, Toh-e et al. found three additional unique circular DNA plasmids among 20 different strains of several Zygosaccharomyces species (112). Finally, Chen et al. (16) adventurously identified a circular dsDNA plasmid in a strain of Kluyveromyces drosophilae. From all this effort to date, only the 2µm circle and six other distinct circular DNA plasmids have been identified in yeasts. Five of these have been sequenced, and all have been character-
ized to some extent (3, 16, 114, 118). Diagrams of the genome organizations of these plasmids are provided in Fig. 4, and a summary of some of their features is presented in Table 1.

The most striking feature of these plasmids is their uncanny similarity to the 2μm circle. All of the plasmids are small (4,757 to 6,615 bp), circular, dsDNA species, and all are composed of two regions that are precise inverted repeats of each other and that divide the genome approximately in half. All of the plasmids encompass either three or four extended open coding regions, the largest of which most probably encode a recombinase that catalyzes recombination at specific sites within the inverted repeats (112, 119). All of the plasmids carry an ARS, either within the inverted repeat or just adjacent to it (112, 116, 114). These similarities exist, even though, except for the recombinase-encoding region, the sequences of these seven plasmids are completely unrelated.

All of the features shared by yeast circular plasmids are precisely those that are required for amplification via the recombination mechanism described above for the 2μm circle. This provides compelling circumstantial evidence that all of these plasmids exploit recombination-promoted plasmid amplification as an integral component of their life cycle. In addition, the fact that these are the only circular DNA plasmids identified in yeasts to date argues that site-specific recombination is essential to the survival of circular plasmids in yeasts, most probably as a means of amplification.

Functional Analysis of 2μm Circle-Like Plasmids

ARS elements. Various functions encoded by 2μm circle-like plasmids both in S. cerevisiae and in the yeast species from which each plasmid was isolated have been analyzed. One or more ARS elements functional in S. cerevisiae have been identified and mapped in every plasmid examined to date (16, 112, 114). The approximate locations of these elements are shown in Fig. 4. For plasmids pKD1 and pSB3, ARS elements have been mapped by using strains of the same genus as those from which the plasmid was originally isolated (K. lactis for pKD1 and Z. rouxii for pSB3). In both cases, the site of the ARS as judged by propagation in S. cerevisiae is exactly the same as that as determined by propagation in the normal host species. Therefore, the same sequence that functions as an ARS in S. cerevisiae also serves as an ARS in Kluyveromyces and Zygosaccharomyces spp., suggesting some degree of conservation in the replication apparatuses among these yeasts. Surprisingly, the ARS elements from pKD1 and pSB3 do not encompass the consensus sequence characteristic of most ARS elements isolated from S. cerevisiae. In addition, although most of these plasmids will replicate in S. cerevisiae, the 2μm circle does not replicate in Z. rouxii (3) or K. lactis (20a).

Site-specific recombination. All of the plasmids identified to date undergo high-frequency intramolecular recombination between inverted repeat domains (16, 112, 118). This recombination can occur both in the normal cellular environment of the plasmid and in S. cerevisiae. For pSR1 and pSB3, this recombination has been shown to require the product encoded by the largest ORF in the plasmid, and, for pSR1, to occur at a specific site within the inverted repeat (73, 119). For other sequenced 2μm circle-like plasmids, the extensive homology to FLP protein of the predicted product of the largest coding region of each plasmid strongly suggests that these products also function to catalyze site-specific recombination. The site in pSR1 at which recombination initiates exhibits a sequence organization similar to that of the analogous site in the 2μm circle (73). An analogous sequence

TABLE 1. Structural features of 2μm circle-like plasmids from yeasts

| Plasmid Source | U1 | U2 | IR | T | Size of following ORF a,b | Reference(s) |
|----------------|----|----|----|---|--------------------------|--------------|
| pSR1 Z. rouxii | 2,654 | 1,679 | 959 | 6,251 | 490c | 410 | 233 | 3, 113 |
| pSB1 Z. bailii | 2,300 | 2,900 | 675 | 6,550 | 414 | 357 | 158 | 112, 118 |
| pSB2 Z. bailii | 2,457 | 2,004 | 477 | 5,415 | 568c | 322 | 178 | 112, 114 |
| pSB3 Z. rouxii | 3,168 | 2,665 | 391 | 6,615 | 568c | 322 | 178 | 112, 114 |
| pSM1 Z. fermentati | 2,552 | 2,160 | 352 | 5,416 | 372c | 260 | 224 | 200 | 118 |
| pKD1 K. drosophilarum | 2,137 | 1,928 | 346 | 4,757 | 447c | 415 | 212 | 30 | 16 |
| 2μm S. cerevisiae | 2,774 | 2,346 | 599 | 6,318 | 387c | 373 | 295 | 180 | 46 |

a Lengths (in nucleotide base pairs) of the larger unique region (U1), smaller unique region (U2), inverted repeat (IR), and the entire plasmid (T).

b Sizes in amino acid codons of the open coding regions identified by sequence analysis of the plasmids.

Plasmids pSR2 (113) and pSB4 (112) have identical restriction maps to plasmid pSR1.

c Encodes the plasmid site-specific recombinase as judged by mutational analysis.

d Encodes a protein whose predicted amino acid sequence is homologous to FLP and the site-specific recombinases of pSR1 and pSB3.

Formerly classified as Z. bisporus.
can also be seen in the inverted repeats of all the plasmids sequenced to date, suggesting that initiation of recombination is restricted to a specific site in each of these plasmids as well (118). The amino acid sequences of the recombinases encoded by the 2μm circle and the five sequenced plasmids are highly homologous, suggesting a common origin for these genes (see below). Despite these similarities, plasmid-encoded recombination systems are generally not cross-functional. The 2μm circle FLP gene will not catalyze recombination between the inverted repeats of any of these plasmids, and vice versa (112, 119). Similarly, the pSB3 and pSR1 FLP proteins will not act on the heterologous recombination site (119). The cross-functionality of the two most closely related recombinases, encoded by pSR1 and pSB2, has not been examined to date.

The mechanisms of recombination of the various FLP recombinases are similar but not necessarily identical. The 2μm circle FLP protein promotes recombination, with little associated gene conversion of outside markers within the inverted repeats (8, 75). On the other hand, recombination in *S. cerevisiae* catalyzed by the pSR1-encoded FLP protein is attended by a high level of gene conversion of outside markers (73).

**Stability functions.** Functions required for the stable propagation of plasmids pSR1 and pSB3 in *Z. rouxii* have been examined by mutational analysis (57, 58, 114). Insertional inactivation of either the second or third coding region in each plasmid (genes P and S for pSR1; genes B and C for pSB3) leads to diminished stability of the hybrid plasmid in a [circ] *Z. rouxii* strain compared with that of the same plasmid with all reading frames intact. Stability is restored to these mutant plasmids by propagation in a *Z. rouxii* strain harboring the cognate wild-type plasmid. This indicates that the destabilizing mutations occur in trans-acting functions. In addition, Jearnpipatkul et al. (58) have identified a site within pSR1 that is required in *cis* for stable propagation. This site encompasses a series of repeated elements in direct and inverted orientation with low (65 to 80%) cross-homology. The role of these repeated elements in promoting stability has not been examined.

Even less is known at present regarding the mechanism of plasmid-promoted stability enhancement for pSR1 and pSB3 than is known for the 2μm circle system. For instance, it has not been determined whether mutant plasmids with reduced stability exhibit mother cell bias in segregation, as is the case for 2μm-based plasmids. It is noteworthy, though, that the pSR1 stability system has measurable effects on the stability of pSR1-based plasmids when propagated in *S. cerevisiae*. This would indicate either that at least a component of the stability machinery acts independently of host components or that host components with which the stability system interacts are sufficiently conserved to maintain some level of heterologous function.

**Origin of 2μm Circle-Like Plasmids**

The origin of the seven circular dsDNA yeast plasmids is puzzling. The conserved structure of recombine in the six sequenced plasmid species argues strongly that these genes diverged from a common ancestral gene. Application of a simple algorithm to the sequences of six genes yields a family tree that indicates their relative relatedness (Fig. 5). It is clear from this family tree that genes of plasmids in the same genus are more closely related to each other than to genes of plasmids in different genera. This would suggest that the plasmids have remained restricted to individual yeast lineages and have undergone little or no horizontal transmission between species. This is consistent with the observation that the 2μm circle shows no detectable horizontal transmission between laboratory strains in the absence of sexual mating.

Despite the close similarities among the plasmid recombinases, the plasmids are otherwise completely unrelated by sequence. The only exception is a limited homology between the B coding region of pSB2 and the P coding region of pSR1. Two explanations could account for the conservation of recombinase sequences in otherwise unrelated plasmids. First, all of the plasmid could have arisen from a single progenitor plasmid. In this case, the rate of divergence of the recombinase gene could have been significantly lower than that of the rest of the plasmid. This evolutionary constraint could account for the significantly higher constraint on possible variations in the structure of the protein that could occur without diminishing its function. Alternatively, within the context of a single progenitor plasmid, sections of the plasmid could have undergone wholesale replacement in the various lineages. This could occur through illegitimate recombination between the plasmid and chromosomal sequences. In this context, it is noteworthy that Utatsu et al. (117) identified a gene in one strain of *Z. rouxii*, but not in others, that shows a high degree of homology to the P gene of pSR1. This model would argue that the recombinase was the only component of these plasmids whose function was irreplaceable, at least by any sequence which any of the plasmids could have encountered during their evolutionary journey.

A second model of the origin of the various plasmids is based on the assumption that they emerged independently, after the various genera in which they are found had diverged. This model would postulate that the recombinase existed as a nuclear gene within the progenitor yeast strain. Excision of the recombinase and flanking repeat element could have occurred by a mechanism similar to that proposed below for amplification of chromosomal sequences. Subsequent capture of other chromosomal sequences that rendered the inchoate plasmid more stable would have completed the emergence of mature plasmid.

**LINEAR KILLER PLASMIDS OF *K. lactis***

The Killer Phenotype of *K. lactis* Is Encoded by Plasmids

**Killer phenotype and killer toxin.** Certain strains of the lactose-fermenting budding yeast *K. lactis* exhibit a killer phenotype (44). When picked onto a freshly plated lawn of *S. lactis* strain 307, colony formation is extensive, suggesting that a homologous recombinase is present in the cells. This is most likely to be true, since the plasmids in the cells are closely related to the plasmids isolated from the cells. The presence of a homologous recombinase in the cells suggests that the killer phenotype is encoded by the plasmids.
cerevisiae or one of several other yeast species and incubated, colonies of K. lactis become surrounded by a turbid halo of S. cerevisiae cells which have ceased to grow. This killer phenotype is distinct from the clear killing zone manifested by dsRNA-containing strains of S. cerevisiae (21), since sensitive cells are not lysed, but appear to be arrested at the G1 stage of the cell cycle (106). A wide range of yeasts are sensitive to the killer effect, including S. cerevisiae, S. italicus, Z. rouxii, K. thermotolerans, K. vanuendenii, Torulopsis glabrata, Candida utilis, C. intermedia (44), and nonkiller strains of K. lactis.

K. lactis killer cell secretes a protein toxin into the surrounding growth medium. This toxin is composed of three subunits, α (99 kDa, including a single asparagine-linked core oligosaccharide unit), β (30 kDa), and γ (27.5 kDa) (102). The toxin is most active at pH 6.5 and, when measured in plate assays, against sensitive cells growing on galactose, glycerol, or maltose as the carbon source (84). The mode of action of the killer toxin is currently unknown. Recent studies (Broach, unpublished) have demonstrated that it does not inhibit adenylate cyclase of sensitive cells as previously reported (106). The mechanism by which killer K. lactis strains exhibit immunity to toxin is also unknown.

Identification of killer plasmids. Comparison of killer and nonkiller derivatives of K. lactis (44, 82) (the latter prepared by irradiation of killer strains with ultraviolet light) revealed that the killer phenotype is strictly correlated with the presence of two DNA species, of 13.4 kilobase pairs (kb) (called pGKL2 or k2) and 8.9 kb (pGKL1 or k1). Densitometric measurements of total DNA isolated from killer strains and subjected to agarose gel electrophoresis indicated that the elements are present at between 50 and 100 copies per haploid genome (42). Strains lacking both of these killer plasmids do not express a killer phenotype and lack the α, β, and γ toxin subunits in their culture supernatants. Moreover, such strains are sensitive to killer toxin produced by plasmid-bearing (k1k2) K. lactis strains. Crosses between [k1- k2+] and [k00 k2+] strains (lacking both plasmids), followed by meiosis, result in 4:0 segregation of k1, k2, and the killer and toxin-immunity phenotypes (44). Toxin immunity and toxin production are therefore plasmid-specific characteristics, making k1 and k2 the first yeast DNA plasmids known to confer a phenotype upon their host.

Irradiation of [k1- k2+] strains with ultraviolet light occasionally gives rise to strains which have lost the smaller plasmid, k2. These [k00 k2+] strains are both nonkillers and toxin sensitive, indicating that both phenotypes are wholly or partly dependent upon functions encoded by k2 (82). Deletion derivatives of k1 have also been found; k1-NK2 (and a similar element, k1-S) lacks a 3-kb region of k1 DNA, and [k1-NK2 k2+] strains are nonkillers but are immune to K. lactis toxin (82, 127). This suggests that the killer and immunity phenotypes reside within distinct parts of k1. No strains have been described which lack k2 but retain k1, suggesting a role for k2 in the maintenance of the smaller plasmid.

Biological Organization of Killer Plasmids

Structure and localization. Electron microscopy and restriction analysis (99) have revealed that k1 and k2 are linear dsDNA molecules, and analytical density gradient centrifugation has indicated that they are extremely rich in adenine and thymine nucleotide residues. The 5′ termini of the plasmids are resistant to digestion by lambda exonuclease (a 5′-to-3′ exonuclease) but not to exonuclease III (a 3′-to-5′ exonuclease) (65). This suggests that the 5′ termini of the plasmids are blocked, a result confirmed by failure to radio-label plasmid DNA with T4 kinase and [γ-32P]ATP after treatment by alkaline phosphatase. Treatment of terminal fragments of killer plasmid DNA with pronase E, proteinase K, or trypsin leads to a shift in their electrophoretic mobility through polyacrylamide gels (65), and full-length plasmids do not migrate into agarose gels unless previously treated with proteinase K (100). Together, these data indicate the presence of a protein molecule associated with the 5′ termini of each killer plasmid. Following iodination and deoxyribonuclease treatment of terminal plasmid fragments, k1 and k2 terminal proteins of 28 and 36 kDa, respectively, were identified (100).

k1 and k2 plasmids have inverted terminal repeats (ITRs) of 202 and 184 bp, respectively, although no sequence similarity is evident between the termini of k1 and k2 (50, 99). With their ITRs and terminal proteins, K. lactis killer plasmids are quite reminiscent of a number of previously characterized replicons, such as adenosvirus (87), bacteriophage ϕ29 (34), plasmids of Streptomyces rochei (48, 49), mitochondrial plasmids S1 and S2 of Zea mays (62), and a variety of plasmids found in filamentous fungi (for a summary, see reference 131). For many of these replicons, the terminal structure has been shown to play an integral role in the mechanism of replication. This is discussed further below.

Unlike the yeast DNA plasmids described above, K. lactis killer plasmids are extranuclear. Following transfer of killer plasmids to strains of S. cerevisiae that lack mitochondrial DNA (mtDNA), staining with the fluorescent DNA-binding dye 4',6-diamidino-2-phenylindole (DAPI) revealed the presence of cytoplasmic DNA in [k1- k2+] but not [k00 k2+] strains (42). Fractionation of killer plasmid-bearing cells of K. lactis showed that 95% of killer plasmid DNA was present in the cytoplasmic fraction (100). This extranuclear location is consistent with the high A+T content of killer plasmid DNA. It has been suggested that the high A+T content of mtDNA is a consequence of a lack of access to uracil excision and repair mechanisms present within the nucleus (25, 103).

Aspects of the recombinogenic behavior of k1 and k2 are reminiscent of the high frequency of recombination seen between molecules of mtDNA. A number of spontaneous and induced plasmid mutations affect both the killer and immunity phenotypes and the gross structure of the plasmids (e.g., plasmid k1-NK2, described above). Another class of killer-negative mutations do not result in structural changes to the plasmids and are presumed to be point mutations (126). Genetic studies have shown these mutations reside within k1. When haploid strains bearing two nonallelic killer-negative mutations were crossed, 85% of the resulting diploid cells were killer positive (126). This suggests that homologous recombination between k1 plasmids occurs at high frequency. The killer plasmids also appear toparticipate in illegitimate recombination events. Transformation of [k1- k2+] strains of K. lactis with a fragment of DNA unrelated to the killer plasmid but containing an appropriate selectable marker resulted in recombination with k1, and the formation of circular YRp-like molecules (89). Transfornants were also obtained when similar experiments were conducted with a [k00 k2+] strain of K. lactis (A. Boyd, personal communication).

Gene organization. Both k1 and k2 have been sequenced (50, 98, 101, 103, 116, 134). Plasmid k1 is 8,874 bp long (although the precise nature of the terminal, presumably
protein-linked nucleotide, is unknown) and has an overall base composition of 73.2% A+T. Analysis of the k1 sequence (98, 103) indicates the presence of four large ORFs, ORF1 (2,987 bp), ORF2 (3,440 bp), ORF3 (1,286 bp), and ORF4 (749 bp), all of which are transcribed. ORF3 is encoded on the opposite DNA strand to that encoding ORF1, ORF2, and ORF4, and the eight 3'-proximal codons of ORF3 overlap the eight 3'-proximal codons of ORF2 (Fig. 6). The plasmid is organized in a highly compact fashion. ORFs make up 95% of the plasmid DNA, with intergenic regions correspondingly quite short.

The amino-terminal regions of the predicted gene products of ORF2 and ORF4 resemble signal sequences and are followed by consensus cleavage sites for signal peptidase or the KEX2 protease of S. cerevisiae (K. lactis has been shown to contain a Kex2-like protease that is required for the processing of k1-encoded killer toxin [17, 129]). Also, the predicted ORF2 product contains several consensus sequences for attachment of N-linked core oligosaccharides. Amino-terminal sequencing of purified killer toxin subunits (102) has shown that the α and β toxin subunits arise by KEX2-like cleavage of the ORF2 protein, whereas the γ toxin subunit derives from signal peptidase-cleaved ORF4 protein.

The predicted product of the ORF1 gene has homology to the class B family of DNA polymerases (59). Other members of the group include the DNA polymerases of adenovirus, herpesvirus, vaccinia virus, and bacteriophages 629, PRD1, and T4. An ORF of the mitochondrial linear plasmid S1 of Z. mays also encodes a product belonging to this family.

On this basis, we expect that the ORF1 product is required for replication of plasmid k1. Consistent with this hypothesis, two deletion derivatives of k1, pk92L and pk92S, lack an intact ORF1 gene and are unable to persist in the absence of intact k1 (41). Reciprocally, F1 and F2, deletion derivatives of k1 that have lost all ORFs other than ORF1, are able to replicate in the absence of k1 (64).

Assignment of killer immunity determinants is not yet complete. The k1-NK2 deletion derivative of plasmid k1 lacks most of ORF2 (Fig. 6) and fails to induce killing. This is consistent with the assignment of toxin subunits α and β to ORF2. However, strains carrying k1-NK2 are immune to exogenously applied toxin, whereas those carrying plasmids F1 and F2 (which lack ORF2, ORF3, and ORF4) are toxin sensitive. This indicates that ORF3 or ORF4 or both encode a component of the toxin immunity apparatus. ORF3, cloned into an autonomously replicating yeast nuclear vector, confers toxin immunity upon [k1] but not [k1] strains of K. lactis (115). This suggests that toxin immunity results from interaction of the ORF3 product with at least one k1-encoded factor. The alternative explanation, i.e., that plasmid k1 may be required for expression of the vectorborne ORF3, is unlikely for reasons discussed below.

Plasmid k2 is 13,457 bp long (116) and has a base composition (74.7% A+T) similar to that of k1. Plasmid k2 encompasses 10 potential ORFs (Fig. 6), at least 8 of which have been shown to be transcribed and all of which have the same codon usage as the k1 genes. The k2 ORFs are organized even more compactly than those of k1. ORFs make up 97% of the k2 genome, with reading frames overlapping extensively not only on opposite strands but also on the same strand (116; D. W. Wilson, Ph.D. thesis, Leicester University, Leicester, England, 1988).

ORF2 of k2 shares homology with ORF1 of k1 and other class B DNA polymerase genes (116). It seems reasonable to presume that this gene product is involved in the replication of k2. Another ORF, termed ORF6 or ORF974 (on the basis of the number of amino acids in its predicted product), encodes a protein with homology to both the β and β' subunits of E. coli RNA polymerase (134) and the two largest
subunits of eucaryotic RNA polymerases. Functions have not yet been ascribed to the other ORFs of \( k_2 \).

**Gene expression.** A protein with the hybrid RNA polymerase subunit structure of the ORF974 (\( k_2 \) ORF6) product (134) has not previously been described, although possession of a novel RNA polymerase is consistent with the atypical organization of killer plasmid genes. None of the ORFs of \( k_1 \) or \( k_2 \) is preceded by recognizable yeast promoter elements. The transcriptional start sites of each of the ORFs of \( k_2 \) have been determined (89) and lie approximately 14 bp downstream of a conserved sequence motif, which has been termed the upstream conserved sequence (UCS). The consensus UCS is \((\text{AT\!/AT\!/AT\!/AT\!/C\!/TGA}) (89, 103)\). Each UCS is separated from the initiation codon of its cognate ORF by approximately 20 nucleotides (for ORF1, ORF2, and ORF3) or 50 nucleotides (for ORF4). This intervening sequence is capable of forming a stem-loop structure (98, 103). The ATG codon of each \( k_2 \) ORF is immediately preceded by three consecutive adenine residues.

The upstream sequences of several \( k_2 \) ORFs resemble those of the \( k_1 \) ORFs (89, 134). The initiation codons of \( k_2 \) ORF2, ORF7, and ORF10 are immediately preceded by three consecutive adenine residues, whereas ORF4 and ORF6 each possess a second in-frame ATG downstream of the first (18 and 8 codons, respectively). These downstream ATG codons, but not the upstream ones, reside downstream of a UCS-like sequence. Accordingly, these may be the true sites of translational initiation for these ORFs (134). Intervening hairpin structures have been reported only for ORF1 and ORF10 (89).

\( k_1 \) transcripts terminate approximately 100 nucleotides downstream of the termination codon of each ORF (103), although no consensus sequences can be identified that might serve as a termination signal. Whether plasmid transcripts are polyadenylated is currently unknown (103). No transcriptional start or stop sites have been defined for plasmid \( k_2 \). However, if each ORF is fully encompassed by a transcript, some transcripts must terminate within the coding domain of an adjacent ORF, since several of the \( k_2 \) genes overlap. If this is the case, a mechanism must exist to distinguish between transcripts that terminate at an internal site and those that must continue to a more distal site. Alternatively, \( k_2 \) expression might involve polycistronic mRNAs, with internal translation initiation of downstream coding regions.

A number of attempts have been made to express cloned killer plasmid genes in yeasts. Several workers have introduced the entire \( k_1 \) sequence, cloned on a circular replicating plasmid, into \( K. \text{lactis} \) strains lacking \( k_1 \). Such transformants failed to produce toxin (89, 100) (Wilson, Ph.D. thesis). In such constructs the \( k_1 \) genes were not accurately transcribed. Transcription of each of \( k_1 \) ORF1, ORF2, and ORF4 initiated at several aberrant positions and terminated prematurely within the ORF (89). This occurred even with plasmids \( k_2 \) and \( k_1-Nk_2 \) in the strain, introduced to provide any trans-acting expression factors that might be encoded by these elements. Similarly, no \( \beta \)-galactosidase activity was detected when a lacZ fusion was used to quantitate the level of expression of cloned ORF2 in \([k_1^+ k_2^+] \) strains of \( K. \text{lactis} \) and \( S. \text{cerevisiae} \) (134). In light of these observations, it is surprising that cloned ORF3 conferred toxin immunity upon \( k_2 \)-containing strains (see above). Perhaps ORF3 contains no fortuitous internal termination signals. No transcripts (normal or aberrant) were detected by Northern (RNA) blotting from strains carrying a cloned ORF3 (89), even though similar strains were phenotypically immunity positive (115).

The unusual upstream and downstream sequences flanking killer plasmid genes and the capacity of \( k_2 \) to encode a unique RNA polymerase-like protein suggest that \( k_1 \) and \( k_2 \) are replicated and transcribed in a nonnuclear location. This is consistent with DNA fluorescence data mentioned above. Since killer plasmids are able to persist within \( S. \text{cerevisiae} \) strains devoid of mtDNA (42, 43) and yield proteins that enter the secretory pathway, the plasmids most probably exist free within the cytoplasm of host cells, as suggested by results of cell fractionation studies (100). However, unlike dsDNA killer plasmids of \( S. \text{cerevisiae} \), no evidence exists to suggest that these \( K. \text{lactis} \) plasmids are assembled into virusslike particles.

**Maintenance of Killer Plasmids**

**Replication of \( k_1 \) and \( k_2 \).** Linear DNA molecules face a number of problems not encountered by the circular plasmids described above. Linear molecules are prone to degradation by cellular exonucleases, and unprotected termini are highly recombinogenic (consider integrative transformation of \( S. \text{cerevisiae} \) [93] and the breakage-fusion-bridge cycle of \( Z. \text{mays} \)). Second, all known DNA polymerases synthesize DNA in a template-directed manner by the addition of 2'-deoxynucleoside-5'-triphosphate residues to the 3' terminus of a nucleic acid primer strand. Usually, this primer is an RNA molecule synthesized onto upstream of the point at which DNA synthesis is initiated. However, an RNA primer cannot be synthesized upstream of the most terminus-proximal nucleotides of a linear molecule. Without some special priming mechanism, this would result in the loss of terminal sequence information during replication (125). One solution to this problem is the telomere-telomerase apparatus of eucaryotic chromosomes (36, 47, 124). A second solution, apparently adopted by the killer plasmids of \( K. \text{lactis} \) and other rewiths with a similar structure, is priming terminal replication by using a nucleotide-terminal protein complex.

Studies of adenovirus (87, 104, 105, 108) and bacteriophage (9, 12) have provided the clearest understanding of protein-mediated terminal priming. By analogy with the models developed for these systems, it appears likely that during killer plasmid replication, a free terminal protein molecule (or terminal protein precursor) becomes attached to a deoxynucleotide, possibly via a phosphodiester linkage between the 5' position of the deoxyribose sugar and a serine residue of the protein. The nucleotide-protein complex then binds to the terminus of the killer plasmid, possibly by recognition of the ITR or the terminal protein already present. The free 3' OH of the bound nucleotide provides the primer for DNA synthesis (Fig. 7). The terminally attached protein may also serve to protect at least the 5' terminus of each DNA strand from exonuclease activity (65).

Studies of adenovirus have implicated particular regions of the ITRs in the process of terminal recognition, binding of the terminal protein, and replication (15, 108). Since \( k_1 \) and \( k_2 \) have dissimilar ITRs, they are most probably recognized by different terminal proteins and replicated by different DNA polymerases. This is consistent with differing sizes for the \( k_1 \) and \( k_2 \) terminal proteins (100) and the capacity of each plasmid to encode its own class B DNA polymerase molecule (see above and Fig. 6) (59, 116). The DNA replication apparatus of the two plasmids are apparently not interchangeable. The \( k_1 \) derivatives pk92L and pk92S lack
ORF1 and cannot be maintained in the absence of k1, despite the presumed presence of the k2-encoded DNA polymerase (41). Adenovirus encodes its own terminal protein, and one assumes that the killer plasmids do so as well. Of the killer plasmid ORFs with no assigned function, three (k2 ORF3, ORF4, and ORF9) are sufficiently large to encode either the k1 (28-kDa) or k2 (36-kDa) terminal protein.

No published reports concerning the mechanism of replication of the killer plasmids have appeared. However, indirect evidence about plasmid replication has emerged from investigation of several naturally occurring rearranged forms of k1. Following transformation of S. cerevisiae with the killer plasmids, two novel k1-derived plasmids, F1 (7.8 kb) and F2 (3.9 kb), were obtained (42). Restriction analysis and Southern blotting demonstrated that F2 is identical to the left-hand (ORF1-containing) terminus of plasmid k1 (Fig. 6) and is attached to a protein molecule. The other terminus of F2 consists of a perfect hairpin loop, such that denaturation of this double-stranded plasmid generates a single-stranded head-to-head dimer of the terminal region of k1 (64). Plasmid F1 is the double-stranded form of this molecule. An adenovirus-like model for killer plasmid replication has been used to explain the origin and interconversion of F1 and F2 (64). Other such hairpin plasmids have been described, including ones derived from plasmid k2 (41).

Both k1 and k2 contain sequences with ARS activity in S. cerevisiae and K. lactis, although the plasmids are extranuclear. The discovery of ARS elements was a consequence of early attempts to develop techniques for the genetic manipulation of the killer plasmids. Transformation of S. cerevisiae and K. lactis with circular or linear DNA molecules, containing fragments of k1 DNA ligated with a suitable marker, led to the recovery of circular autonomously replicating plasmids (22, 111). Such circular YRp-like molecules were even recovered when a yeast nuclear marker was ligated to k2 termini bearing terminally attached proteins (28). The circular derivatives were, like YRp plasmids, extremely unstable, and k1-derived recombinants were not dependent upon plasmid k2 for their maintenance. Such molecules are maintained by an apparatus distinct from that used by native, linear k1. These studies, and earlier ones demonstrating illegitimate recombination between native killer plasmids and transforming DNA in vivo (89), suggested that ARS elements are present within k1 and k2. The position and sequence of k1 regions which confer autonomous replicative ability upon recombinant plasmids in both K. lactis and S. cerevisiae have been mapped (111).

What is the significance of the existence of ARS elements within k1 and k2? One possibility is that although the termini of the killer plasmids are replicated by protein-mediated priming, the internal regions of the plasmids are replicated by using ARSs and RNA primers. Such a situation would be analogous to that found in yeast chromosomes, in which telomeric replication is distinct from replication of internal DNA. However, such a suggestion is at odds with an extranuclear location for the killer plasmids. It is more likely that the occurrence of these sequences is fortuitous. Consensus ARS elements are A+T rich and could be expected to occur by chance, an event not without precedent (133).

The mechanism of replication of k1 and k2 may have bearing upon the timing of plasmid replication during the cell cycle. If the elements are indeed cytoplasmic and use a nonnuclear replication apparatus, they may replicate throughout the cell cycle, as is the case for mtDNA (81). This property would sharply distinguish them from the other yeast DNA plasmids so far described. Continuous replica-
ion would explain the high copy number of the killer plasmids in the absence of any apparent recombination mechanism for copy number amplification. No investigation of the cell cycle dependence of killer plasmid replication has yet been reported.

**Segregation of \( k_1 \) and \( k_2 \).** Nothing is known about the mechanism by which \( k_1 \) and \( k_2 \) are segregated to daughter cells at division. It is known that the plasmids do not share homology with *K. lactis* nuclear DNA or mtDNA (128) and so do not use other replicons as carriers. In this way they differ from the S plasmids of *Z. mays* mitochondria, which integrate into the mitochondrial chromosome (35). Additionally, no chromosomal mutations have been defined that affect the maintenance of these killer elements, analogous to the *MAK* genes responsible for segregation and replication of the cytoplasmic dsRNA killer system in *S. cerevisiae* (130, 132). Neither \( k_1 \) nor \( k_2 \) contains sequences resembling the *CEN* regions of chromosomes or the *STB* partitioning locus of the 2\( \mu \)m circle. In any case, it is unlikely that cytoplasmic elements could gain access to the partitioning apparatus of such nuclear genetic material. The simplest model for segregation is that these high-copy-number cytoplasmic elements are partitioned randomly at cytokinesis. Resulting fluctuations in copy number could easily be corrected if killer plasmid replication occurs independently of nuclear replication, as postulated above.

Although the mechanism of killer plasmid maintenance remains obscure, several observations have bearing upon it. One is the apparent portability of the killer plasmids between yeast species, perhaps indicative of a maintenance apparatus completely independent of the host cell. Spheroplast fusion and direct transformation have been used to introduce the plasmids into *S. cerevisiae*, *K. fragilis*, and *C. pseudotropicalis* (42, 43, 107). Although stable within certain isolates of each of these strains, some plasmid-containing cells lose the plasmids at high frequency. Surprisingly, many [rho\(^{-}\)] strains of *S. cerevisiae* maintain the plasmids stably, whereas all [rho\(^{+}\)] and [rho\(^{-}\)] strains tested do not (45) (yeast strains containing wild-type mitochondrial genomes are designated [rho\(^{-}\)], and those with no mitochondrial DNA are termed [rho\(^{+}\)], and those with mutant mitochondrial genomes causing respiratory deficiency are termed [rho\(^{-}\)]. The absence of functional mitochondrial ribosomes in [rho\(^{-}\)] cells indicates that the killer plasmids are probably incompatible with mtDNA itself, rather than with its products. Whether incompatibility occurs because killer plasmids and mtDNA have some common replication or segregation factors or whether it is an indirect effect arising from the heterologous nature of the host is unclear. The killer plasmids are certainly not incompatible with *K. lactis* mtDNA.

Another important point is that \( k_2 \) is completely dependent upon \( k_2 \) for its maintenance, whereas \( k_2 \) is capable of an independent existence. Since the genes of \( k_2 \) are organized similarly to those of \( k_2 \), it seems likely that \( k_1 \) is dependent upon the \( k_2 \)-encoded putative RNA polymerase. In the absence of \( k_2 \), \( k_1 \) might be unable to express its DNA polymerase-like product and be unable to replicate. Similarly, \( k_2 \) is likely to encode the \( k_1 \)-terminal protein, which is essential for \( k_1 \) replication. Lastly, since \( k_2 \) may encode a product essential for immunity to killer toxin, strains which have lost \( k_2 \) would become toxin sensitive and hence be unable to grow.

### Perspectives and Conclusions

**Yeast Present a Limited Repertoire of Plasmids**

As is evident from this review, only two types of DNA plasmids have been identified in yeasts to date: linear plasmids and circular plasmids structurally similar to the 2\( \mu \)m circle. This paucity of plasmids in yeasts is surprising, given the plethora of plasmids that have been identified to date in bacterial species. This may reflect a true difference in the relative diversity of intracellular fauna in yeasts versus bacteria. On the other hand, the screening procedures traditionally used to identify yeast plasmids may have missed a variety of such elements. Plasmids that are fairly large or exist at low copy number could easily have escaped detection.

Even in a limited sampling of bacterial plasmids, the variety of replication and partitioning strategies they use is striking. In contrast, all of the circular DNA plasmids of yeasts appear to use an identical amplification process and probably use similar partitioning mechanisms. Thus, the possible avenues to achieving high-level, stable persistence in yeasts as a circular DNA molecule appear to be limited. However, within the known examples of all eucaryotic and prokaryotic plasmid species, with possible exceptions listed below, yeasts are the only class of organism in which plasmids that use this particular amplification mechanism have been found. This suggests either that this mechanism is effective only in yeast cells or that 2\( \mu \)m circle-like plasmids represent a novel evolutionary experiment that simply has not been attempted in other organisms.

The collection of 2\( \mu \)m circle-like plasmids provides a potential resource for identifying salient features of plasmid amplification and partitioning. As noted above, the conserved recombination system in all these plasmids argues forcibly that the DRC mode of amplification is a critical feature for the survival of these plasmids, even though mutational analysis of 2\( \mu \)m circle-based plasmids has yet to confirm a role for this amplification mechanism in the persistence of the 2\( \mu \)m circle. In addition, comparative analysis of the recombinases and their targets may prove useful in identifying the nature of the DNA interaction domains of the recombinases. An even more fruitful use of these plasmids may be in exploring the nature of the partitioning system. Assuming that each of these plasmids faces essentially the same obstacle to equipartitioning, and given that the components of the plasmid segregation apparatuses show little conservation, an exploration of the nature of these partitioning elements could prove valuable. Already, the absence of observable STB-like repeats in any of the plasmids suggests that the repetition of elements within the 2\( \mu \)m circle STB locus may not be critical to its activity.

The other class of DNA plasmids in yeasts—the linear plasmids encoding killer toxin and immunity—are noteworthy for their adoption of a very widespread mechanism for replication of linear DNA species. Like replication of a number of eucaryotic viruses and bacterial plasmids, replication of the killer plasmids of *K. lactis* most probably uses a terminal protein as a primer for DNA synthesis. One can only surmise whether this represents a solution to the problem of replication of linear molecules that has been discovered multiple times during evolution or whether these various species evolved from a common ancestor.

The persistence strategy of the killer plasmids embraces an often-used selection theme. Cells that harbor the plasmid are capable of suppressing the growth of cells that lack the
FIG. 8. Possible mechanisms for chromosomal amplification via recombination. (A) Formation of multiple tandem repeats. Line a shows a chromosomal segment during early S phase, with a replication bubble lying between regions A and B. The circle to the left represents the centromere. In line b, recombination, either by homology or as an illegitimate event, occurs between a site within the replication bubble and an unreplicated site located centromere distal to the bubble. In line c, resolution of the combination event yields a deletion of the B-C segment from one daughter strand and formation of a circular B-C segment attached by a replication fork to the other daughter strand. In line d, continued replication yields one daughter strand with a deletion of B-C and a second strand with an extended tandem direct repeat of B-C.

(B) Formation of acentric palindromes. The structure at the top of the figure represents the region of a chromosome lying near a telomere (designated by the arrowhead). Letters along the chromosome represent stretches of sequences, the orientations of which are indicated by the orientations and positions of the letters. Within this region, a single origin of replication (1) lies asymmetrically between a pair of inverted repeats ([ ] and [ ], labeled C). Initial replication elongation from the origin yields duplication of one repeat but not the other. Recombination between a duplicated repeat and the nonduplicated repeat results in inversion of the sequences between them, as well as inversion of the direction of progression of the replication fork. Continued elongation of each fork to the end of the chromosome generates the acentric palindromic fragment shown at the bottom of the figure. The chromosomal template is left unaltered, except for inversion of the sequences lying between the repeats. Reprinted from Cancer Cells: Eukaryotic DNA Replication (4) with permission.
plasmid. Although this is an effective means of ensuring the maintenance of plasmid-bearing cells, it seems to some extent to be icing on the cake. Given the apparent ability of the plasmid to undergo unrestricted replication, to partition simply by passive diffusion through the cytoplasm, and, like 2 μm, to spread by mating to plasmid-free strains, expression of a selectable phenotype seems redundant. The redundant nature of this selection scheme is emphasized by the fact that, at least in a laboratory setting, the k₂ plasmid can persist for an extended time in the absence of the killer-toxin-encoding k₁ plasmid. However, no natural isolates of strains containing the k₂ plasmid alone have been identified. This raises the possibility that the killer system is critical for survival in the wild.

Yeast Plasmids as Vectors

The yeast plasmid 2 μm has been mobilized effectively and often as the basis for vectors for propagation and expression of cloned genes in S. cerevisiae (7, 11; A. B. Rose and J. R. Broach, Methods Enzymol., in press). Although this has proved quite valuable for harnessing brewers' yeast for production of biologically and commercially relevant proteins, the inability of the 2 μm circle to propagate efficiently in other yeast genera has limited its general utility. This void has been filled to a large extent by the more recent availability of the other plasmid species described in this report. For instance, Chen et al. (17) have adapted plasmid pKD1 for use as a vector for the introduction and propagation of sequences in K. lactis. Similarly, the pSR and pSB plasmid-based vectors transform and persist in various Zygosaccharomyces species. Given the commercial importance of these osmophilic yeasts in the fermentation industry, this capability could prove a valuable resource for strain improvement or other genetic engineering feats. Finally, the linear killer plasmids seem to exhibit a very broad host range (42, 43, 107). As proposed above, this may be the consequence of a maintenance apparatus that is completely independent of the host cell. With further refinement of the molecular genetics of killer plasmid gene expression, vectors based on these plasmid could become highly versatile shuttle vectors.

Generalization of DRC Model of Gene Amplification

The amplification mechanism used by the 2 μm circle and its relatives is an exceedingly elegant solution to the problem of obtaining multiple copies of a defined segment of DNA, even when normal regulation restricts the replicon to a single initiation event per cell cycle. Given the normal parsimony of nature, it seems unlikely that such an elegant solution would be used only for yeast plasmids. In fact, although other procarycotic and eucaryotic plasmids have eschewed this avenue, other cases of DNA amplification appear to proceed through this mechanism. For example, DRC amplification could be part of the survival strategies of other stable eucaryotic genomes that encompass inverted repeats (4). Chloroplast genomes are, in overall structure, giant replicas of the 2 μm circle. They have a pair of long inverted repeats delimiting two unique regions, and they exist as invert isomer mixtures (83). The organization of the repeats and the replication origin in these genomes suggest that a DRC mechanism could be involved in proliferation of these plastid genomes.

Amplification of some chromosomal sequences could proceed by a variation of this mechanism. A number of loci in mammalian cells can, under certain circumstances, exhibit local amplification. The favored mechanism to account for this process invokes an onion skin model, in which a single replication origin is activated multiple times within a single cell cycle (94). Although this may account for some programmed amplification events, such as chorion gene amplification (60, 61), it may not be as universally applicable as presumed. For example, Cox (20) has suggested that reiterated tandem repeats could arise by formation of a rolling-circle intermediate, attendant on recombination between sequences lying in direct orientation on either side of a chromosomal replication fork (Fig. 8A). In addition, amplified loci in higher eucaryotes frequently have been shown to feature DNA segments reiterated in inverted orientation (85). These molecules could result from DRC replication of DNA that had been excised from a chromosome as a circle with an inverted repeat (85). In both these cases, it is not generally assumed that the DRC is formed by site-specific recombination; general homologous recombination would have the same effect, albeit at a lower efficiency.

Similarly, acentric palindromic fragments, such as the amplified ribosomal DNA repeat in the macronucleus of ciliated protozoa (136, 137), could arise by recombinational amplification from a chromosomal site. A model for this process (Fig. 8B) postulates recombination between a pair of inverted repeats in one arm of a chromosome to yield a linear acentric minichromosome bearing all the genetic information between the centromere-proximal copy of the repeats and the telomere on that chromosome arm. This reaction can proceed without destruction of the original chromosome and is therefore a route to excision, amplification, and mobility of chromosomal sequences. The DRC mechanism certainly does not account for all examples of specific gene amplification. Nonetheless, it certainly warrants inclusion in the panoply of processes by which selective expansion of chromosomal domains occurs.

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