

DNA Inversion in the 2 μ m Plasmid of *Saccharomyces cerevisiae*

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I. THE 2 μ m PLASMID: HISTORICAL OVERVIEW

The isolation of closed circular DNA from the yeast *Saccharomyces cerevisiae* yields a population of DNA molecules with limited size heterogeneity. The major component is approximately 2 μ m in circumference (10, 22, 28, 60). The minor species are approximately 3, 4, and 6 μ m long (10, 22). The 2- μ m circle, referred to as the 2 μ m plasmid, is present at 60 to 100 copies per cell (11). Restriction analysis showed that there are two major species, related by a large inversion of approximately 40% of the molecule (29). Denaturation and renaturation of nicked or cleaved 2- μ m DNA produced DNAs with a dumbbell structure in which two single-stranded loops were separated by a double-stranded stem, indicating the presence of long inverted repeats. The inversion could be explained by recombination between these repeats (23). The 4- and 6- μ m DNAs are dimers and trimers of the 2- μ m circles, and these

could also be accounted for by recombination (55). Recombination between the repeats occurs efficiently in vivo during plasmid propagation (6) and is mediated by a plasmid-encoded site-specific recombination system (7, 8, 17).

The sequence of the 2- μ m circle (24) revealed important details of its structure (Fig. 1). The plasmid consists of 6,318 base pairs (bp), with perfect inverted repeats of 599 bp separating unique regions of 2,774 and 2,346 bp. Within the 599-bp repeats are two sets of shorter repeats, a pair of 16-bp inverted repeats and three repeats of a 13-bp sequence, concentrated within 122 bp. The 13-bp repeats are binding sites for the plasmid-encoded site-specific recombinase (FLP, see below). Another set of short repeats, a tandem array containing 5.5 copies of a 62- to 63-bp sequence, is found in the large unique region designated REP3. The 2- μ m circle contains four significant open reading frames, designated A, B, C, and D (or FLP, REP1, REP2, and RAF) in order of decreasing size. These encode proteins with molecu-

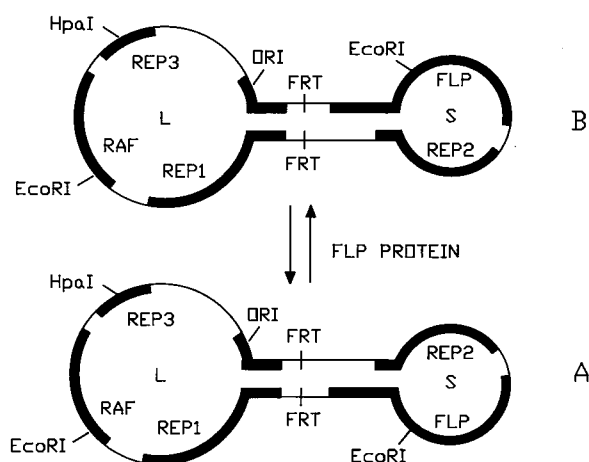


Figure 1. The *S. cerevisiae* 2 μ m plasmid. Major genetic loci described in the text are designated by filled boxes. The FRT site (Fig. 3) spans the *Xba*I cleavage sites. Interconversion between the A and B forms involves site-specific recombination at the FRT site. The 599-bp inverted repeats are presented as horizontal lines. FLP, REP1, REP2, and RAF correspond, respectively, to open reading frames A, B, C, and D (see text). REP3, also called STB, is a DNA site recognized by REP1/REP2.

lar weights of 48,619, 43,231, 33,196, and 21,289, respectively (24). The origin of replication (ORI) is about 75 bp long and overlaps the end of one of the 599-bp repeats. The plasmid confers no known phenotype on its host other than a slight decrease in the growth rate (14). It may be regarded simply as an intracellular parasite (14).

Replication of the 2- μ m circle is under stringent cell cycle control during normal growth (68). Each molecule replicates just once early in S phase. The origin of replication is structurally related to the chromosomal *ars* sequences (45, 66). Replication proceeds bidirectionally and depends completely on host functions (41). Plasmid-encoded proteins function in plasmid segregation during cell division and in the regulation of copy number as outlined below.

II. STABLE MAINTENANCE OF THE 2 μ m PLASMID: ROLE OF PLASMID GENES

The plasmid possesses two systems that ensure its own stable maintenance. The first facilitates equal partitioning of the plasmid population at mitosis. This system consists of two *trans*-acting factors, the REP1 and REP2 proteins (open reading frames B and C, respectively), and a *cis*-acting element, designated REP3 or STB (34, 37, 65), that appears to be the site of action of REP1 and REP2 proteins (35, 46). REP3 is a centromerelike locus consisting of a series of 62- to 63-bp repeats (described above). The REP1 protein is highly homologous in its carboxy one-half

with vimentin, tubulin, and myosin heavy chain (65). It is found in the nucleus and cofractionates with an *S. cerevisiae* nuclear skeletal structure. These and other results led to a model in which the REP proteins form a microtubulelike structure that connects the 2 μ m plasmid to some nuclear structure that partitions equally between daughters at mitosis (65). The REP system can stabilize plasmids with replication origins other than the 2 μ m ORI (37).

Copy number is also controlled by a second system, designated FLP, in addition to the REP system. The FLP system is responsible for copy number amplification (54, 64). FLP protein is the only 2 μ m gene product required for amplification (54). This protein promotes site-specific recombination at two sites designated FRT within the 599-bp inverted repeats. This recombination inverts the two unique sequence regions of the plasmid, and, as described below, this inversion often leads to plasmid amplification.

REP1 and REP2 also regulate copy number by acting in concert to reduce FLP gene transcription (47, 54). The RAF protein, in contrast, increases FLP expression by relieving the inhibition caused by REP1 and REP2 (47). RAF expression is itself decreased by the REP1 and REP2 proteins (47). Many of the details about how this complicated regulatory circuit maintains and fine tunes plasmid copy number remain to be worked out.

III. THE ROLE OF DNA INVERSION IN COPY NUMBER AMPLIFICATION

The role of the FLP DNA inversion system in causing 2 μ m plasmid amplification has only recently been elucidated. Its function was initially obscured by the observation that deletion of the FLP gene did not reduce the high plasmid copy number (8, 15, 18). These FLP⁻ plasmids, however, are much less stable than the FLP⁺ plasmid (14, 15), and the high copy number was probably due to the strong selection for cells that contained plasmids in this study (15).

A role for the FLP system in copy number amplification was first suggested by Futcher (13) in an elegant explanation of how cell cycle control of replication could be circumvented by site-specific recombination (Fig. 2). Initiation of bidirectional replication at the origin results in two replication forks traveling in opposite directions. In the absence of recombination, these forks meet on the other side of the plasmid, creating two daughter molecules. The 599-bp repeat nearest the origin is replicated before the second 599-bp repeat. If recombination involving these repeats occurs between the replication of the

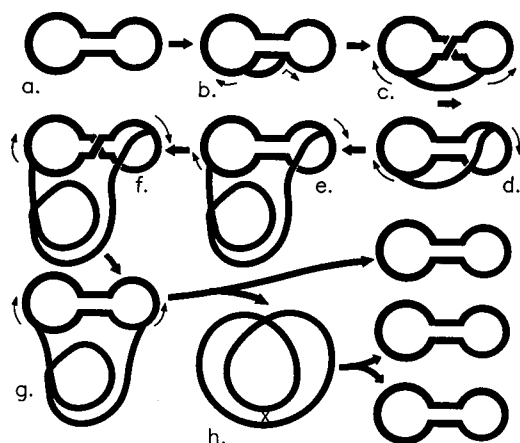


Figure 2. Futcher's (13) model for amplification of 2- μ m circle copy number. Inversion following replication of one 599-bp repeat (step c) results in a double rolling circle (d). This results in multiple copies of the 2- μ m plasmid even though replication is initiated only once. (Adapted from reference 64.)

first and the second copies, the plasmid is converted to a "double rolling circle" (Fig. 2). In this, the two replication forks move in the same direction and produce a multimeric form of the 2- μ m plasmid. This multimer can be resolved to monomers by FLP-mediated recombination between directly repeated 599-bp repeats. It is not clear if homologous recombination can substitute for the FLP system in the resolution step. All key features of this model have now been substantiated experimentally, with a clear link established between copy number amplification and the FLP system (47, 54, 64). Overexpression of the FLP protein in *S. cerevisiae* results in abnormally high plasmid copy numbers and causes increased cell size and lethality (54). Similar effects are observed with strains of mutants in the chromosomal NIB genes (30, 31). This suggests that the NIB product, along with REP1 and REP2 proteins, regulates FLP gene expression.

IV. THE FLP SITE-SPECIFIC RECOMBINATION SYSTEM

A. Fundamental Properties

The FLP system carries out both intramolecular and intermolecular recombination in vivo (6, 12, 55). No evidence has been found for the involvement of any protein besides the plasmid-encoded FLP protein. The FLP gene has been cloned and expressed in *Escherichia coli* (12, 63). The FLP system functions well in vivo in *E. coli*, carrying out intra- and intermolecular recombination at the specific FRT sites (12). Since no *S. cerevisiae* gene other than FLP

was transferred to bacteria in these experiments, it was clear that FLP protein is the only *S. cerevisiae* protein required for recombination.

In vitro recombination systems that utilize extracts from *E. coli* cells expressing FLP protein have been developed in several laboratories (44, 51, 63). Recombination in vitro requires only FLP protein, a substrate, buffer (pH of ~6 to 9), and appropriate concentrations of salt. No divalent cations or high-energy cofactors are needed. The substrate DNA can be relaxed or supercoiled, although the rate of the reaction is increased with supercoiled DNA (see below). The system promotes intramolecular (inversions, deletions) or intermolecular recombination given appropriate substrates, and all results obtained in vitro are consistent with observations made in vivo.

In vitro systems have been developed for several other site-specific recombination systems, primarily from procaryotic sources (56; see J. F. Thompson and A. Landy; H. Varmus and P. Brown; D. Sherratt; and A. C. Glasgow, K. T. Hughes, and M. I. Simon, all this volume). The FLP system ranks among the simplest of these. Unlike bacteriophage λ integration or procaryotic DNA inversion, the FLP system requires no proteins other than the recombinase itself. Unlike the procaryotic invertases and the resolvases from the Tn3 class of transposable elements, the FLP system will carry out both inversions and deletions (depending on the orientation of the FRT sites). The FRT recombination site employed by the FLP system is among the smallest (about 30 bp; see below). The only known procaryotic system that appears to be analogous to FLP is the Cre-Lox system of bacteriophage P1 (25-27). The added complexity of most of these other recombination systems probably reflects regulation of the rate and direction of the reaction imposed on the fundamental chemistry of site-specific recombination.

B. The Recombination Site: Primary Structure

The recombination site used by the FLP system has been designated FRT (42), and its sequence is given in Fig. 3. It consists of three 13-bp repeats. The first and second are inverted and separated by an 8-bp spacer. The third is a direct repeat, in tandem to the second. Deletion analysis (3, 58) demonstrated

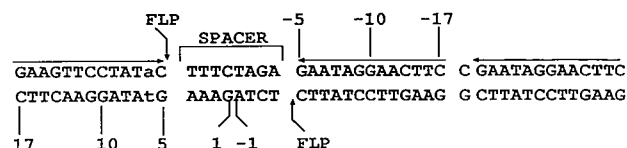


Figure 3. The FRT site. Position numbers are as in reference 9.

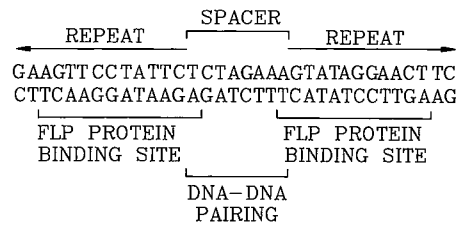


Figure 4. Summary of structure and function within the minimal FRT site.

that the third 13-bp repeat was not necessary for recombination *in vitro*, although this repeat appears to increase the efficiency of intermolecular recombination slightly *in vivo* (33). Further deletions from the outer ends of the first and second 13-bp repeats produce little or no effect *in vitro* until more than 3 bp is removed. As additional base pairs are removed, a gradual reduction in FRT site function is observed, and deletion of 8 bp inactivates the site. The minimal sequence for a fully functional FRT site therefore consists of 28 to 30 bp, including the spacer and 10 bp of each adjacent repeat (3, 58).

The FLP protein cleaves the FRT site at the boundaries of the spacer and adjacent repeats (3, 58), making 8-bp staggered cuts and leaving overhangs with 5' hydroxyl termini. The protein forms a transient covalent intermediate with the DNA via a 3' phosphotyrosyl linkage (21).

The functional organization of the minimal FRT site is summarized in Fig. 4. The inverted repeats are binding sites for FLP protein. Use of the spacer is homology dependent, but not strictly sequence dependent. This implies that DNA-DNA pairing occurs

between spacer sequences of two reacting FRT sites during recombination. These functions are described below.

1. The FLP-binding sites: protein-DNA interactions

FLP protein-purine contacts, defined by methylation protection experiments, occur within two 12-bp regions, the base pair at either side of the spacer and the proximal 11 bp in the adjacent repeat (9). FLP protein-phosphate contacts are clustered near the point at which the DNA is cleaved (9).

This work is complemented by the extensive mutational analyses of the FLP binding site (2, 42, 51, 59a). The effects of base pair substitutions in the FLP-binding site on recombination *in vitro* are summarized in Fig. 5. The effects at different positions fall into three classes: either (i) all three changes have small effects, (ii) all three changes have large effects, or (iii) one change has a large effect, while the others have small effects (59a). Interestingly, eight positions fall into class (i), with 31 of 37 mutations tested having effects of 10-fold or less. Only one position (11) falls into class (ii). Most interesting are positions 5 to 7, which fall into class (iii). One change at each position, in each case of transversion, has a large deleterious effect on recombination. The other two changes have either small or no detectable effects. These patterns may provide important clues as details of the interaction between FLP protein and this site are elucidated.

Other patterns observed in these studies suggest functional interactions between FLP-binding sites

FLP PROTEIN BINDING SITE																	
INVERTED REPEAT																	
A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
A	A	G	A	A	T	A	G	G	A	A	C	T	T	C			WILD TYPE DNA SEQUENCE
	A	G	G	C	G	A	A	G	G	T	C	C					MUTATIONS
	T	T	C	C	G	C	T	C	C	G	A	G					
	C	T	T	A	T	T	C	T	T	A	G	A					
	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		BASENUMBER
		1	4	5-10	5-10	10	1	>100	5	10	5	5	2				MUTANT X MUTANT RECOMBINATION
	3-5	5	5	2-5	5	5-10	2-5	20	2	2	2	2	2	2			
		>100	40	>100	10	10	5	50	5	5	5	5	2				
		1	4	5	1	5	1	5	2-5	5	2	2	1				MUTANT X WILD TYPE RECOMBINATION
	2-5	2.5	1-2	2	5	2	2	2	2	2	2	2	2				
	10	10	10	5	5	2	5	2	2.5	2	2	2	2				

Figure 5. Effects of mutations in the FLP protein-DNA-binding site. Asterisks denote purine contacts identified in methylation protection studies. Numbers denote the factor by which the FLP protein concentration must be raised to produce a minimal reaction relative to a normal recombination site; i.e., higher numbers correspond to less reactive sites.

both within a single FRT and between different FRT sites. An interaction within a single FRT is indicated by the greater than additive effect of mutations at certain positions when present in both rather than in only one of the FLP-binding sites within an FRT site. An interaction between different FRT sites is indicated by the improvement in the reaction efficiency of mutant sites (with single mutations in one FLP-binding site) observed generally when the second FRT site participating in a recombination event is wild type (Fig. 5) (51, 59a).

2. The spacer: homology and directionality

Initial work on the spacer focused on its two most obvious features: size and sequence. These studies at first indicated that the size was important but the sequence was not.

The unaltered spacer contains a cleavage site for the restriction enzyme *Xba*I. Destruction of this *Xba*I site (removal of 4 bp) was found to eliminate inversion in *S. cerevisiae* (7). Similar results were obtained in vivo in *E. coli* (12) and in vitro in *S. cerevisiae* (44), providing an important experimental link with the normal system in *S. cerevisiae*. The importance of spacer size was suggested by the fact that the *Xba*I site mutations altered the size of the spacer (adding or subtracting 4 bp at this *Xba*I site had the same effect [8, 12, 44]). Subsequent tests showed that FRT function was impaired when the spacer size was increased or decreased by 1 bp and inactivated by the addition of 2 bp to the spacer (58).

The spacer sequence, in contrast, initially appeared to be unimportant provided that the spacers of the two sites undergoing reaction were homologous. This was first suggested by the increase in reaction efficiency observed when FRT sites with spacers altered by ± 1 bp were reacted with identical mutant sites rather than with a wild-type site (58). Base substitutions at each of the central six positions in the spacer were subsequently found to have no effect on the reaction as long as reactants were homologous (1, 59).

The homology requirement implies that the spacers of two reacting FRT sites are paired at some point in the reaction. This might reflect the formation of a four-strand intermediate prior to DNA cleavage, as suggested for bacteriophage λ integration (38), or events subsequent to DNA cleavage. Similar homology requirements have been noted in other site-specific recombination systems (5, 27, 36, 38, 48). The role of homology has been most thoroughly analyzed in the λ integration reaction. Here, it has been demonstrated that the cleavage events are ordered. DNA cleavage and reunion first occur on one

side of the crossover region, creating a "Holliday structure" intermediate. This intermediate is resolved by DNA cleavage and reunion in the complementary strands 7 bp away on the other side of the crossover region (40, 49). Homology in the crossover region (spacer) is required for the branch migration that occurs before the second set of cleavage and reunion events. A possible additional requirement for homology at an earlier step in the reaction (i.e., a four-stranded intermediate) has not been precluded by experimental tests. The evidence argues against an alternative model in which all four cleavage events are simultaneous and homology is required to reanneal the resulting cohesive ends before religation (48). The recombination events mediated by the Cre-Lox system (26) and the FLP system (43a; M. Jayaram, K. L. Crain, R. L. Parsons, and R. M. Harshey, *Proc. Natl. Acad. Sci. USA*, in press) also proceed via Holliday intermediates. In both cases, the intermediates have been isolated and characterized, and they are resolved by the respective recombinase in vitro.

The spacer homology requirement serves an additional special function in the FLP system, the alignment of the two FRT sites in the same orientation during recombination. The spacer is the only asymmetric segment of the FRT site, if the third 13-bp repeat is removed, and is sufficient to define the directionality of the intramolecular reaction (inversion versus deletion). An FRT site with a symmetrical spacer (and two FLP-binding sites) is still functional in vitro, but directionality is abolished (59). A similar observation has been made in the Cre-Lox system (27). Directionality is not restored by the third 13-bp FLP-binding site if the spacer is symmetrical (X. Qian and M. Cox, unpublished results).

The sequence flexibility in the spacer, combined with the homology requirement, makes it possible to construct numerous different functional FRT sites that do not cross-react, and therefore to set up two discrete reactions in the same tube. Abolishing directionality by using a symmetrical spacer has the effect of increasing the number of possible products generated in a given experiment. This ability to establish two distinct reactions in the same tube has proven useful experimentally in the study of reactant turnover (15a) and the internal comparison of several effects of FLP-binding-site mutations in a single experiment (59a).

The general interpretation of studies of simple substitution mutations was that the spacer sequence was not important if homology was retained. However, further analysis has shown that the spacer sequence can affect FRT site function and has led to a reevaluation of spacer function, as described in the next section.

C. The Recombination Site: Higher-Order Structure

While generating mutant FRT sites, several that differed from wild type at several rather than just one position were found that nearly inactivated the site even in homologous reactions. These results do not appear to reflect undetected DNA-protein contacts in the spacer region, because many multiple-base-pair changes (up to five in a single site) had no effect regardless of which positions were altered. No individual base pair substitution in the central 6 bp of the 8-bp spacer had a large effect on recombination. A comparison of many different spacer sequences (62a) indicates that there are at least two features of the general sequence structure of the FRT site that are essential for its function. The first is the set of two nearly symmetrical pyrimidine tracts that begin on each strand in the spacer and continue in opposite directions through the first 9 bp of the adjacent repeats. The second is the predominance of A · T base pairs in the spacer (six of eight). Disruption of the polypyrimidine tract or an increase in spacer G+C content decreases the recombination efficiency.

Significantly, the spacer alterations that inactivate the FRT site do **not** have a deleterious effect on *in vitro* binding by FLP protein and therefore provide a clear distinction between protein-DNA recognition and site function. A recombination step subsequent to protein binding and prior to product dissociation is affected. The effects of G+C content, and the additional observation that the deleterious effects are moderated if the DNA is supercoiled, suggest that the affected step involves strand separation within the spacer (62a).

These results are relevant to the study of DNA structure-function relationships. Specific DNA sequences take up a variety of non-B DNA structures where the biological functions are still obscure in many cases. In the most extreme case, the FRT site could simply represent a highly degenerate recognition sequence that localizes recombination to that site. The results of the spacer mutations described above, however, demonstrate that the higher-order sequence structure of the FRT site (as opposed to the primary sequence itself) is essential not only for recognition but for site function. The FRT site is hypersensitive to cleavage by S1 nuclease in the absence of FLP protein (S. Umlauf and M. Cox, unpublished results). The FRT site therefore appears to adopt a non-B DNA structure. The effects of A + T content in the spacer and the fact that DNA supercoiling enhances recombination efficiency (62a) indicate that this structure facilitates DNA unwinding during recombination in a manner as yet undefined at the molecular level. Enhanced sensitivity of polypy-

rimidine tracts to S1 nuclease digestion has been observed in several other studies (39, 53). Polypyrimidine tracts are not standard features of recombination sites used by site-specific systems. The sequences found at these sites, however, do exhibit other motifs (Thompson and Landy; Sherratt; and Glasgow et al., all this volume). Features represented include poly(A) tracts (lambda integration, bacterial DNA invertases) and alternating purine-pyrimidine tracts (Cre-Lox system [27]). Most of these recombination sites do feature A+T-rich regions. The results obtained for the FLP system indicate a need for further study of the functional significance of such DNA sequence arrangements.

D. The FLP Protein

The FLP protein has recently been obtained from extracts of *E. coli* cells harboring appropriate recombinant DNA plasmids and has been purified to near homogeneity by using site-specific DNA chromatography (16, 43). The pure protein is free of detectable nuclease activity and is stable for many months at -70°C . Stability under reaction conditions at 30°C is greatly enhanced by the addition of bovine serum albumin and glycerol. When substrates with two FRT sites are employed, reactions are almost entirely intramolecular. Intermolecular recombination is facilitated by the addition of polyethylene glycol (16).

A general strategy for isolating FLP⁻ mutations has been described that uses a selection based on loss of antibiotic resistance in bacteria by FLP-mediated deletion unless the cell is FLP⁻ (19). Site-directed mutagenesis has also been used (52). Mutations that change Tyr-343 to Ser or Phe inactivate the protein but do not decrease its binding to the FRT site. This provides evidence that this is the tyrosine involved in the covalent link to the DNA (52). Mutations at His-305 cause an accumulation of DNA cleavage products that do not complete the recombination process (R. Parsons, P. Prasad, R. M. Harshey, and M. Jayaram, submitted for publication). These early results emphasize the importance of mutational analysis of the FLP gene in dissecting this site-specific recombination reaction pathway.

E. The Recombination Reaction

Site-specific recombination reactions provide an opportunity to examine important principles of protein-DNA interactions. The recombination proteins must locate specific binding sites, juxtapose two sites, cleave a DNA within the two sites, and then religate the resulting ends to new partners with high fidelity. Each of these recombinases must act both as a

specific endonuclease and as a DNA ligase. Because of its relative simplicity, the FLP system seems ideal for probing the chemical details of such processes. Until recently, however, many properties of the system were elucidated with partially purified preparations, and, unless noted, the information presented below was obtained with FLP protein fractions that were approximately 5% pure (1, 44).

A DNA-binding assay based on gel retardation has been used to resolve three binding complexes, which apparently correspond to the binding of one, two, or three FLP monomers, respectively, to one, two, or three 13-bp repeats (1, 51, 59a). If an FRT site with a strong deleterious mutation in one FLP-binding site is employed, FLP protein binds and cleaves only the DNA adjacent to the unaltered FLP-binding site (59a). The efficiency of intramolecular recombination decreases as the distance between FRT sites increases, suggesting that tracking may be involved in site juxtaposition (20). This relationship was abolished, however, if KCl was omitted from the reaction, suggesting that under at least some conditions, site juxtaposition may involve random collisions (57). The cleavage of the DNA and formation of a covalent adduct has been described above. As with other site-specific recombination systems and topoisomerases, this covalent adduct preserves the high-energy bond and obviates the need for high-energy cofactors in the reaction.

A common observation with site-specific recombination systems is that levels of recombinase that are at least equimolar with the concentration of available recombination sites are generally required to produce an optimum reaction *in vitro* (56). This suggests that these proteins do not turn over, and therefore are not true enzymes. Similar observations were made with the early *in vitro* experiments with FLP protein. However, recent results with purified FLP protein using conditions that enhance its stability indicate that this protein turns over slowly when relaxed DNA substrates are used (15a). The turnover rate is increased 1.5- to 3-fold if supercoiled substrates are used. The apparent turnover number is low, $\sim 0.12 \text{ min}^{-1}$ with linear substrates under one set of conditions, and the slow step in the process has not yet been identified.

V. THE EFFECTS OF OTHER PROTEINS ON FLP PROTEIN-PROMOTED RECOMBINATION

Purification of FLP protein was hampered to some degree by variation from one fraction to the next in the relative efficiencies of inter- and intramolecular recombination and by increases and decreases

in overall activity (20, 44). Increasing levels of many FLP-protein-containing fractions led first to a general enhancement of intermolecular recombination, while inhibition of the reaction was observed when higher concentrations of the same fraction were used. These effects have been traced to *E. coli* proteins that were present as prominent contaminants at early stages of the FLP protein purification (43). These proteins do not include factors known to play a role in other procaryotic site-specific recombination systems, such as IHF, HU, or FIS (Glasgow et al., this volume; Thompson and Landy, this volume). At least four *E. coli* proteins can inhibit FLP protein action, and each of these has recently been purified to homogeneity (R. Bruckner and M. Cox, unpublished results). One (M_r 27,000) is the H protein (32), a histonelike protein that cross-reacts immunologically with histone H2A. The *in vitro* properties of the other three proteins (M_r 26,000, 24,000, and 16,000) suggest that they also are DNA-binding proteins. The M_r 26,000 protein is a common contaminant of H-protein preparations (32). N-terminal sequence analysis of each of these proteins has shown, surprisingly, that all four are ribosomal proteins (Bruckner and Cox, unpublished results). H protein is ribosomal protein S3, and the other proteins are S4, S5, and L2. Interestingly, histones produce similar effects on FLP protein-promoted recombination (Bruckner and Cox, unpublished results). The significance of this result is not clear, although these effects almost certainly do not reflect specific interactions with the FLP system. It is worth pointing out, however, that the 2 μ m plasmid is packaged as chromatin in *S. cerevisiae* and it is conceivable that histones indirectly help regulate or modulate this recombination system in some manner *in vivo*.

VI. BIOLOGICAL RELEVANCE: COPY NUMBER AMPLIFICATION AND GENE AMPLIFICATION

The mechanism employed by the 2 μ m plasmid to amplify its copy number may be widespread among eucaryotic plasmids. A number of plasmids with similar overall sequence arrangement have been found in several *Zygosaccharomyces* species, and each of these also possesses a site-specific inversion system (4, 62). These plasmids exhibit little or no homology to the yeast 2- μ m circle, except for limited homology at the amino acid level within the FLP genes. If these plasmids evolved independently, their common structural features may have general adaptive significance.

Chromosomal gene amplification can occur in

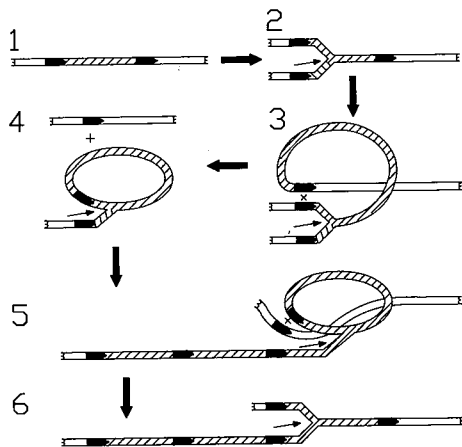


Figure 6. A model for gene amplification. A recombination event (homologous, site specific, illegitimate) occurring between one site in front of a replication fork and one site behind the replication fork will result in the formation of a rolling circle (4). This will result in amplification of the cross-hatched DNA sequences in one of the daughter DNA molecules. This amplified sequence will be present in a tandem array. Shaded sequences represent potential recombination sites. Small arrows denote the movement of the replication fork.

many species as part of a normal developmental process (e.g., rRNA genes, chorion), cell transformation (e.g., oncogenes such as *myc*, *sis*, *ras*), or under strong selection pressure (for a review, see reference 61), and may involve similar recombination events. Mechanisms previously proposed to explain amplification include (i) unequal crossing over following replication or (ii) a cycle of excision of a DNA sequence, its extrachromosomal replication, and then reinsertion into the chromosome as amplified DNA. These models do not satisfactorily explain the inverted duplication structure found in many amplified DNAs. The model for 2 μ m copy number amplification (13) (Fig. 2) has suggested alternative mechanisms to explain such chromosomal gene amplification events (50). In this model, illegitimate recombination immediately following replication would produce an extrachromosomal circular DNA molecule with the DNA to be amplified in inverted repeats. The second step would involve recombination events to produce a double-rolling-circle-replicating species (as in Fig. 2). After amplification, this DNA would be reinserted into the chromosome.

A more straightforward model (Fig. 6) to explain amplified DNA sequences in tandem arrays again uses features of the Futcher proposal (67; U. Hornemann, personal communication). Its key feature is a recombination event (illegitimate, site specific, or homologous) between two sites, one in front of and one behind the replication fork. This produces a single rolling circle and tandem repeats of the DNA

within the circle. A similar recombination event restores the original replication fork, with DNA sequences now amplified on one of the daughter DNA molecules.

In conclusion, the properties of the FLP system outlined above recommend it for eucaryotic site-specific genetic recombination, as a model system for dissection of the basic chemistry of site-specific recombination reactions in general, and as a vehicle to test ideas about the biological functions of diverse DNA rearrangements.

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