Involvement of ArgR and PepA in the pairing of ColE1 dimer resolution sites

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Dimer formation and associated copy number depression is an important cause of multicopy plasmid instability. Natural multicopy plasmids employ site-specific recombination to convert dimers to monomers, thus maximizing the number of independently segregating molecules at cell division. Resolution of dimers of *Escherichia coli* plasmid ColE1 requires the plasmid *cer* site and at least four chromosome-encoded proteins: the XerC and XerD recombinases, and accessory factors ArgR and PepA. It has been suggested that ArgR has a role in the initial pairing of recombination sites and we describe here an attempt to detect this process *in vivo*. Our approach exploits a previous observation that a *cer*-like site known as the type II hybrid supports inter-molecular recombination and causes extensive multimerization of plasmids. We report that type-II-mediated multimerization can be repressed by a *cer* site *in cis* or *in trans* and propose that this is due to a physical interaction between the sites. If this hypothesis is correct, suppression of multimer formation provides an assay of site pairing. Our results demonstrate that the putative pairing interaction is independent of the topological relationship of the sites and that both PepA and ArgR are involved. Although most recombination-deficient mutant derivatives of ArgR are unable to pair recombination sites, we have found two (ArgR110 and ArgR115*) which retain pairing activity. The validity of the pairing hypothesis is discussed in the light of alternative explanations for our data.

Keywords: *cer*, plasmid stability, recombination, nucleoprotein complexes, site-specific recombination

INTRODUCTION

Multicopy plasmids of *Escherichia coli* are distributed randomly at cell division and the frequency of plasmid loss increases with decreasing copy number. Plasmid multimer formation reduces the number of independent plasmid molecules in the cell (Summers, 1993; Summers & Sherratt, 1984) and is an important cause of cloning vector instability. Natural, multicopy plasmids such as ColE1 avoid this problem because multimers are resolved to monomers by site-specific recombination (Summers & Sherratt, 1984). Resolution of ColE1 dimers requires the 280 bp *cer* site (Fig. 1a) and at least four host-encoded proteins: ArgR, PepA, XerC and XerD (Stirling *et al.*, 1988, 1989; Colloms *et al.*, 1990; Blakely *et al.*, 1993). Consistent with its biological role, *cer*-Xer recombination is restricted to sites in tandem repeat within the same molecule so that it resolves, but does not generate, multimers.

Of the four proteins required for ColE1 multimer resolution, the roles of XerC and XerD are best understood. They are the subunits of a heterodimeric site-specific recombinase which footprinting experiments have shown to bind to a region of approximately 30 bp between the *Mlu*I and *Taq*I sites in *cer* (Blakely *et al.*, 1993; Fig. 1a). The roles of ArgR and PepA are less clearly defined but ArgR has been shown to bind specifically to an Arg-box sequence in *cer* (Stirling *et al.*, 1988; Fig. 1). PepA, a leucine aminopeptidase, is essential for dimer resolution but there is no evidence that it binds to DNA, nor is its peptidase activity required for recombination (McCulloch *et al.*, 1994).

Although *cer*-mediated recombination is abolished in *argR* and *pepA* mutant hosts, the related type II hybrid site (generated by recombination between *cer* and a multimer resolution site from CloDF13; Fig. 1b) does not require ArgR, PepA, or DNA sequences beyond the XerC-D

Fig. 1. Recombination sites used in this study. Vertical bars mark restriction sites and the end-points of deletions described in the text (the co-ordinate of the first undeleted base is shown). The positions of the Arg box and XerCD binding sites, and the sequence of the Arg box (in bold type) are shown. (a) The 281 bp *HpaII*-*TaqI* *cer* fragment of ColE1. (b) Type II hybrid site generated by recombination between *cer* and *CloDF13 parB*. This site contains the left-hand end of *cer* up to co-ordinate 3921 and the right-hand end of *parB* (heavy line). (c) The minimal type II site.

Fig. 2. Multimer profiles of plasmid DNA extracted from DS941 (lanes 1 and 2), DS941*argR* (lanes 3 and 4) or DS941*argR* pAG115* (lanes 5 and 6), and run on a 0.85% agarose gel. For each host strain, the first track is pKS2000 (which contains a full-length type II site) and the second is pKS2001 (which contains a minimal type II site). Monomer (1^m) and dimer (2^m) supercoiled forms of the plasmids are marked. In lanes 5 and 6, pAG115* runs just ahead of pKS2000 and pKS2001 dimers, respectively.

binding site (Summers, 1989). We refer to a type II site from which non-essential sequences have been removed as a minimal site (Fig. 1c). Similarly, recombination at *dif*, a chromosomal site implicated in the resolution of chromosomal dimers, requires XerC and XerD but is independent of ArgR and PepA (Blakely *et al.*, 1993). The observation that recombination at both *dif* and the type II hybrid site is unconstrained (i.e. both inter- and intra-molecular) implies that ArgR and PepA may be required only for reactions where recombination is restricted to

sites within the same molecule (Summers, 1989; Blakely *et al.*, 1993).

The ability of the type II hybrid site to support inter-molecular recombination means that it causes plasmids to multimerize extensively. Fig. 2 shows that in DS941 (our standard Xer-*cer* recombination-proficient host) different profiles of multimeric forms are produced by plasmids containing the full-length and minimal sites (pKS2000 and pKS2001, respectively). This is seen most easily by comparing the ratio of monomers to dimers. For a plasmid with a full-length site, the monomer band is significantly brighter than the dimer band. When the plasmid contains a minimal site the dimer band is similar in intensity to, or slightly brighter than, the monomer band (Fig. 2, lanes 1 and 2). We believe that this difference results from the ability of the full-length site to support both constrained and unconstrained recombination while the minimal site supports only unconstrained recombination (Summers, 1989). In an *argR* background, this distinction is lost (Fig. 2, lanes 3 and 4) and both sites behave as though they support only unconstrained recombination.

These observations suggest that collisions between minimal type II sites with bound XerCD result in recombination, whether or not the sites are on the same molecule. In contrast, the site selectivity apparent in *cer-mer* recombination implies that *cer* sites complexed with ArgR, XerC-D and possibly PepA, approach recombination via a route which is sensitive to the topological relationship of the sites. The full-length type II site falls somewhere between these two extremes and appears able to use both the constrained and unconstrained pathways in a wild-type host. An important difference when pairs of *cer* and minimal type II sites recombine may lie in the nature of their initial pairing. Stirling *et al.* (1988) have suggested that since ArgR brings together chromosomal operators by looping-out the intervening DNA (Charlier *et al.*, 1992), it may be involved in the pairing of *cer* sites during ColE1 dimer resolution. This cannot be the case for recombination between minimal type II sites which have no Arg box and

are presumably paired by interactions between XerC-D heterodimers.

Clearly it is pertinent to investigate whether ArgR is involved in the pairing of *cer* sites. Impressive progress has made towards an understanding of the mechanism of strand exchange during dimer resolution through the development of an *in vitro* system (Blakely *et al.*, 1993) and the mechanism of pairing might also seem suited to an *in vitro* approach. Nevertheless, it is important not to underestimate the importance of studies *in vivo* when detailed mechanistic issues are being addressed. We describe here the development of a technique to detect the pairing of protein-DNA complexes *in vivo*. The assay is based upon our proposal that intra-molecular pairing between *cer* and a full-length type II site prevents the type II site from participating in inter-molecular recombination. The inhibition of plasmid multimerization can thus be used as an assay of pairing between sites. The work described here tests the validity of this assay system and provides evidence that both ArgR and PepA play an important role in the pairing process.

METHODS

Bacteria, plasmids, media and transformation. The strains used in this work are derivatives of DS941 (AB1157 *recF lacI^qlacZΔM15*) and have been described previously (Summers & Sherratt, 1988). Plasmid constructs are based upon pUC8 or pUC9 (Vieira & Messing, 1982). pKS2000 and pKS2001 are pUC8 derivatives containing a full-length or minimal type II site, respectively. They were made by exchanging the short *MluI*-*HindIII* fragment from the type II product of pAP451 recombination (Summers, 1989) with the corresponding *MluI*-*HindIII* fragment from pKS490 (for pKS2000) or pKS3888 (for pKS2001) (Summers & Sherratt, 1988). Enzymes were used according to the manufacturers' recommendations. DNA was prepared by the method of Holmes & Quigley (1981). Media preparation and routine procedures for transformation and electrophoresis were as described previously (Summers & Sherratt, 1988).

Construction of pAG3733IR, pAG3733M and pAG3733DR.

The structures of these plasmids are shown in Fig. 3. A source plasmid for the type II hybrid site was constructed by replacing the *EcoRI*-*MluI* fragment of pKS2001 with the corresponding fragment from pKS490(D3733) (Summers & Sherratt, 1988) which carries ColE1 *cer* sequence from co-ordinate 3733 to the *MluI* site at 3899. The *EcoRI*-*HindIII* polylinker fragment from this construct was cloned into the polylinkers of pKS450 and pKS451 (Summers & Sherratt, 1988) yielding pAG3733IR and pAG3733DR, respectively. pAG3733M was constructed by cutting the *cer* site of pAG3733IR with *MluI*, filling-in the sticky ends with Klenow polymerase and religating.

Construction of deletion derivatives of the type II site. We have described previously the construction of a series of pUC18 derivatives containing *cer* sites with exonuclease-III-generated deletions extending into their left-hand boundary regions (Summers & Sherratt, 1988). In these constructs there is an *EcoRI* site adjacent to each deletion end-point (the co-ordinates of the end-points are shown in Fig. 1a). The *EcoRI*-*MluI* fragment of pKS2001 was replaced with *EcoRI*-*MluI* fragments from these *cer* deletion derivatives to give a series of type II site deletion derivatives: pAG3733II, pAG3748II and pAG3800II. *EcoRI*-*HindIII* fragments carrying these type II sites were

transferred from each of these plasmids to pKS451 giving pAG3733DR, pAG3748DR and pAG3800DR, respectively (Fig. 3).

Construction of pAG489. The 300 bp *EcoRI*-*HindIII* fragment containing the type II site from pAG3733II was inserted into the polylinker of pCH5 (which is compatible with pUC8/9 and related plasmids) to give pAG489. pCH5 was constructed in this laboratory by ligating the 1002 bp *PvuII*-*Clal* fragment of pACYC184 (Chang & Cohen, 1978) to the 2917 bp *StyI*-*AatII* fragment of pSELECT1 (Promega). The pACYC184-derived *XbaI* site was subsequently removed by cutting, filling-in the sticky end with Klenow polymerase and religating (T. C. Hodgman, unpublished).

RESULTS AND DISCUSSION

The site pairing hypothesis

Fig. 4 illustrates the principle of our *in vivo* assay for protein-mediated pairing of recombination sites. We propose that the establishment of a protein-DNA complex between *cer* and a full-length type II site (either in *cis* or in *trans*) renders the type II site unavailable for recombination with a third site. In particular, it cannot recombine with a type II site on another plasmid and multimerization is therefore inhibited. If any of the components necessary for site pairing are absent, the *cer*-type II complex cannot form and plasmid multimerization is restored.

There is ample circumstantial evidence to support this hypothesis. *cer*-like sites must come together before they recombine and the pairing process is likely to depend upon protein-protein interactions. There are many examples of protein-mediated juxtaposition of distant sites on DNA (Schlief, 1992) and protein-mediated pairing between *incA* and *incC* in the basic replicon of the P1 prophage appears to have important consequences for the control of DNA replication. *incC* contains the replication origin and it is proposed that RepA-mediated pairing of this site with *incA* prevents the initiation of replication (Abeles & Austin, 1991).

An analysis of recombination between *cer* and a type II hybrid site

In the first part of the study we looked for evidence of pairing between recombination sites *in cis*. The first plasmid used was pAG3733DR, a pUC9 derivative containing *cer* in direct repeat with a type II site (Fig. 3). A potential problem with pAG3733DR is that if recombination occurs between *cer* and type II, any pairing interaction will be transient and difficult to detect. Our fears were realized when we transformed pAG3733DR into DS941 (our standard *cer*-Xer recombination-proficient host) and intra-molecular recombination led to the deletion of the DNA between the sites and a reduction in plasmid size of approximately 440 bp. In *argR* and *pepA* backgrounds, intra-molecular recombination between *cer* and type II was abolished but the appearance of a ladder of plasmid multimers was evidence of continuing inter-molecular recombination between type II sites. In a *xerC* host, both inter- and intra-molecular recombination were

Fig. 3. Plasmid constructs used in this study. The figure shows only those parts of each plasmid containing *cer* or type II recombination sites. Arrows show the relative orientation of sites, following the convention that the arrow points in the direction of transcription from the P_{cer} promoter (Patient & Summers, 1993).

Fig. 4. Inhibition of plasmid multimerization by *cer*-type-II pairing. The box represents a complex within which the type II site is unable to participate in inter-molecular recombination. (a) Pairing *in cis* between *cer* (black) and type II (white) sites; (b) pairing *in trans*.

absent (Fig. 5, lanes 1–4). The result demonstrates that *cer* and type II sites in direct repeat recombine efficiently and is consistent with previous observations that type II recombination requires XerC but is ArgR- and PepA-independent (Summers, 1989).

Clearly pAG3733DR is not a suitable substrate for a study of site pairing. What is needed is a plasmid in which sites associate but do not recombine. One possibility is to have the sites in inverted repeat, because studies of the Tn3 cointegrate resolution system have shown that *res* sites in inverted repeat form a synaptic complex even though they cannot recombine (Benjamin & Cozzarelli, 1988). We therefore examined the behaviour of plasmid pAG3733IR in which the *cer* and type II sites are in inverted orientation.

Fig. 5. Multimer profiles of plasmids containing *cer* and a type II hybrid site. Lanes 1–4: pAG3733DR isolated from *xerC*, wild-type (DS941), *argR* and *pepA* hosts, respectively; lanes 5–8: pAG3733IR isolated from *xerC*, wild-type (DS941), *argR* and *pepA* hosts, respectively.

Because the type II site can undergo unconstrained recombination, there exists the possibility of recombination between *cer* and type II in pAG3733IR, which would invert the DNA between the sites. We looked for evidence of recombination by digesting plasmid DNA from DS941 with *EcoRI* and *HindIII*. In the absence of recombination, pAG3733IR yields two fragments, the smaller of which is approximately 300 bp. Intra-molecular

Fig. 6. Inhibition of plasmid multimerization by a recombination-defective *cer* site. Lanes 1–4: pAG3733M isolated from *xerC*, wild-type (DS941), *argR* and *pepA* hosts, respectively.

recombination will result in the inversion of the DNA between the sites and generate a restriction fragment polymorphism with an extra band at 450 bp. There was no 450 bp band in a digest of pAG3733IR from DS941 (data not shown), suggesting that recombination between *cer* and a type II site in inverted repeat is rare or non-existent. The absence of recombination between *cer* and a full-length type II site means not only that pAG3733IR is a suitable substrate for a study of site pairing but also that recombination between these two sites is subject to the same topological constraints as *cer*–*cer* recombination (Summers, 1989).

A *cer* site inhibits type-II-mediated plasmid multimerization

We looked for evidence of pairing between *cer* and the type II site in pAG3733IR by preparing plasmid DNA from wild-type and recombination-deficient backgrounds and analysing its multimeric state on an agarose gel. It was striking that multimers were absent from plasmid DNA isolated from DS941 as well as from the recombination-deficient *xerC* host which served as a negative control (Fig. 5, lanes 5 and 6). According to the pairing hypothesis (Fig. 4a), the absence of multimers in DS941 is due to pairing between recombination sites. Their restoration in an *argR* or *pepA* host (Fig. 5, lanes 7 and 8) implies that the products of these genes play a key role in the suppression of plasmid multimerization.

pAG3733IR contains an active *cer* site and a trivial explanation for the absence of pAG3733IR multimers in

DS941 is that as soon as they form, they are resolved by *cer*. To test this we constructed pAG3733M, which is identical to pAG3733IR except that the unique *Mlu*I site in *cer* has been cut, filled-in and religated. This creates a site totally deficient in dimer resolution (unpublished data from this laboratory). If *cer*-mediated recombination is responsible for the absence of pAG3733IR multimers, pAG3733M should multimerize in DS941 as extensively as pKS2000 (which contains only a full-length type II site). Although pAG3733M produced slightly more multimers than pAG3733IR (compare Fig. 6, lane 2 with Fig. 5, lane 6), there were significantly fewer than with pKS2000 (Fig. 2, lane 1). This result demonstrates that *cer* does not need to be recombinationally active to suppress plasmid multimer formation.

ArgR and PepA may be required for pairing of recombination sites

There was extensive multimerization of pAG3733M in *argR* and *pepA* strains (Fig. 6, lanes 3 and 4). This, together with the reappearance of pAG3733IR multimers in the same mutant strains, implies a role for both accessory proteins in the establishment or maintenance of pairing between recombination sites.

To investigate further the implication that ArgR is involved in site pairing, we examined the pairing proficiency of mutant ArgR proteins which were identified originally because they exhibit serious defects in *cer*-mediated dimer resolution (Guhathakurta, 1994). Details of the isolation and characterization of these mutants are being prepared for publication elsewhere. Thirteen pCH5-based plasmids carrying mutant *argR* genes were introduced in turn into DS941 *argR* and screened for their ability to suppress multimerization of pAG3733M. Two mutant proteins (ArgR110 and ArgR115*) which fail to complement *cer*-mediated dimer resolution in DS941 *argR*, continued to suppress multimerization of pAG3733M as effectively as wild-type ArgR. The mutant ArgR proteins produced by the remaining 11 plasmids were deficient in the suppression of multimerization (data not shown). Mutant allele *argR115** has a C to T base change at position 544 of the 920 bp *Sph*I–*Acc*I fragment described by Stirling *et al.* (1988). This corresponds to a change from threonine (ACC) to isoleucine (ATC) at position 130 in the amino acid sequence of ArgR. *argR110* has a G to A change at position 522 which corresponds to a change from glycine (GGC) to serine (AGC) at amino acid 123. These two residues form part of a nine amino acid motif (positions 123–131) which is strongly conserved in ArgR from *E. coli* and *Salmonella typhimurium*, and the related AhrC protein from *Bacillus subtilis* (Fig. 7).

Sherratt and co-workers have studied two ArgR derivatives with amino acid changes in this conserved nine amino acid motif (Burke *et al.*, 1994). ArgRDH (which has amino acid 128 changed from D to H) and ArgRNV (which has amino acids 128 and 129 changed from DD to NV) bind DNA in an arginine-independent manner. Under gel filtration, ArgRNV and wild-type ArgR co-migrate as hexamers. However, evidence from band shift

Fig. 7. The C-terminal amino acid sequences of *E. coli* and *S. typhimurium* ArgR and *B. subtilis* AhrC. Conserved amino acids are enclosed in boxes and the residues altered in ArgR110 and ArgR115* are labelled. Amino acids 128 and 129 altered in ArgR derivatives described by Burke *et al.* (1994) are in bold type.

Fig. 8. Plasmid DNA isolated from DS941 *argR* pAG115* transformed with pAG3733DR (lane 1), pAG3748DR (lane 2) or pAG3800DR (lane 3). pAG (1^m), pAG (2^m) etc. indicates the various multimeric forms of these plasmids. Bands labelled D are deletion derivatives of pAG3733DR and pAG3748DR formed by *cer*-type-II recombination.

assays (where the protein concentration is significantly lower) suggests that ArgRNV binds the *cer* Arg box as a trimer. In contrast to ArgR110 and ArgR115*, ArgRNV and ArgRDH both support dimer resolution *in vivo*. Burke *et al.* (1994) suggest that when the protein is expressed from a multicopy plasmid, it is at a high intracellular concentration and can interact with a *cer* site as a hexamer. The inability of ArgR110 and ArgR115* to support dimer resolution suggests that they may not form hexamers, even at high intracellular concentration.

Pairing between sites in direct repeat

The pairing-proficient, recombination-deficient phenotype of ArgR115* provides an opportunity to study pairing in pAG3733DR, where *cer* and type II are in direct repeat. We have seen already that 100% of plasmid DNA extracted from DS941 transformed with pAG3733DR had undergone *cer*-type-II-mediated deletion (Fig. 5, lane 2). When DS941 *argR* containing pAG115* (a pCH5 derivative expressing *argR115**) was transformed with pAG3733DR and plasmid DNA was extracted from the transformants, only about 10% of the input plasmid had undergone *cer*-type-II recombination (Fig. 8, lane 1). It is not unusual in our experience for mutant proteins or recombination sites which do not support dimer resolution to show a low level of recombination when the sites are moved close together in a two-site assay plasmid such as pAG3733DR. The paucity of pAG3733DR multimers in the transformants implies the existence of pairing between the *cer* and type II sites. The experiment was repeated with derivatives of pAG3733DR in which the left-hand end of the type II site had been deleted up to co-ordinate 3748 (pAG3748DR) or 3800 (pAG3800DR). pAG3748DR showed very few multimers whereas pAG3800DR multimerized extensively (Fig. 8, lanes 2 and 3). This result suggests that pairing occurs between sites in direct repeat and that some part of the type II site between 3748 and 3800 is essential for the interaction. A candidate sequence is the Arg box, which is intact in pAG3748DR but which has its left-hand end deleted in pAG3800DR (Fig. 1b).

Type-II-mediated multimerization is inhibited by a *cer* site *in trans*

In the experiments described above, *cer* and the type II site were always on the same plasmid. To investigate whether *cer* can inhibit type-II-mediated multimerization *in trans* (Fig. 4b), we constructed pAG489, a plasmid of relatively low copy number which contains a type II site and is compatible with pUC9 and its derivatives. pAG489 multimerized in DS941; monomer and dimer bands were of approximately equal intensity and trimers, tetramers and higher forms were also visible (Fig. 9). In the presence of pUC9, dimers of pAG489 were the predominant form. When pKS451 (a derivative of pUC9 with *cer* in the unique *NarI* site) was introduced, pAG489 became almost

Fig. 9. Inhibition of plasmid multimerization by a *cer* site in *trans*. Plasmid DNA was isolated from the following hosts. Lanes: 1, DS941 pAG489; 2, DS941 pUC9 pAG489; 3, DS941 pKS451 pAG489; 4, DS941 *argR* pKS451 pAG489; 5, DS941 *pepA* pKS451 pAG489.

entirely monomeric. In *argR* or *pepA* hosts containing pKS451, multimers of pAG489 reappeared (Fig. 9, lanes 4 and 5), so the accessory proteins are essential for *trans* suppression of multimerization.

The results of experiments with pAG3733IR (Fig. 5) and pAG489 (Fig. 9) demonstrate that *cer* can act both *in cis* and *in trans* to inhibit multimerization of a plasmid containing a type II site. Interpreting these results in the light of the pairing hypothesis, site pairing appears to be a promiscuous process which contrasts strikingly with the rigid constraints which apply to recombination itself. Formally, a *trans* (rather than a *cis*) interaction could be responsible for the suppression of multimerization with multicopy substrates such as pAG3733IR where *cer* and the type II site are on the same molecule. We suspect, however, that the higher effective concentration of sites within the same molecule means *cis*-pairing will predominate. There is a parallel between these results and the observations of Chatteraj *et al.* (1988), who found that the Rep-protein-mediated pairing of the *incA* and *incC* sequences in the P1 basic replicon occurred readily both *in cis* and *in trans*.

The validity of the pairing hypothesis

In this section we consider a number of alternative models or objections to the pairing hypothesis in the light of the experimental observations presented in this paper.

Assertion 1: the suppression of type-II-mediated multimerization is due to the rapid resolution of plasmid multimers by *cer*. It may seem naïve that we expected multimers of pAG3733IR in DS941 since the plasmid contains a functional multimer resolution site. We have long suspected, however, that multimer resolution by *cer* is neither very rapid nor efficient. This conclusion is based

on observations that while multicopy cloning vectors multimerize extensively in an *shcA* host, the introduction of *cer* reduces the proportion of multimers rather than eradicating them (Summers & Sherratt, 1984). Two experiments described here refute the idea that the missing multimers have been resolved by *cer*-Xer recombination. Firstly the suppression of multimerization is seen with pAG3733M in which the *cer* site has been inactivated by filling-in the *Mlu*I site. Secondly, suppression is also seen to occur *in trans* when *cer*-mediated multimer resolution could not possibly be responsible.

Assertion 2: the suppression of type-II-mediated multimerization is due to sequestration of recombinase by the *cer* site. As long as sequestration of the recombinase is ArgR and PepA-dependent, this proposal appears to provide a valid explanation for the suppression of plasmid multimerization both *in cis* and *in trans*. It cannot, however, explain the results depicted in Fig. 8, where a dramatic difference in multimerization of pAG3748DR and pAG3800DR is seen in the presence of ArgR115*. These two plasmids contain identical *cer* sites so the effect of sequestration of the recombinase by *cer* should be identical in both cases.

Assertion 3: the increase in plasmid multimers seen in an *argR* or *pepA* background, or when the left end of a site is deleted (as in pAG3800DR) is due not to a loss of pairing but to a change in the topological properties of the type II site. We have known for some time that a type II site produces more multimers in an *argR* host than in a wild-type, and that the removal of the left end of the site (to produce a minimal site) also increases the production of higher forms (Summers, 1989; Fig. 2). The idea that the reappearance of multimers of our assay plasmids in *argR* and *pepA* hosts (Figs 5 and 6), or when the left end of the type II site was removed (Fig. 8), was due simply to an

increased rate of multimer production thus appeared to provide a plausible alternative to the pairing hypothesis. However, we noticed that in the presence of ArgR115*, plasmids containing full-length and minimal type II sites show indistinguishable multimer profiles (Fig. 2, lanes 5 and 6). The dramatic difference between multimerization of pAG3748DR and pAG3800DR in DS941 *argR* pAG115* (Fig. 8) cannot, therefore, be attributed to a change in the topological properties of the type II site. It is, however, entirely consistent with the pairing hypothesis if pairing requires a DNA sequence between co-ordinates 3748 and 3800.

Conclusions

A physical interaction between the recombination sites provides the most plausible explanation for the ability of *cer* to suppress type-II-mediated plasmid multimerization. In order for suppression of multimer formation to be a useful assay of site pairing, the interaction must not lead rapidly to recombination. This was achieved in our experiments either by putting the sites *in trans* or in inverted orientation (topological constraints prevent recombination in these cases), or by using a mutant derivative of ArgR which supports pairing but is defective in recombination. In these situations, we were able to examine the conditions necessary for the pairing of protein-DNA complexes *in vivo*; something which has previously been extremely difficult to achieve.

Assuming the correctness of the pairing hypothesis, our results suggest that the *cer*-type-II interaction requires ArgR, PepA and a DNA sequence between co-ordinates 3748 and 3800 (which includes part of the Arg box). A role for ArgR in pairing was anticipated but more surprising is the role of PepA. Although our results suggest that PepA is essential for site pairing, there is no evidence that it binds to *cer* or related sites. What could it be doing? We propose that its role may be analogous to the yeast co-activator GAL11 which forms a protein bridge to stabilize the interaction of the GAL4 transcriptional activator with general transcription factors (Stanway, 1991). PepA might, for example, form a bridge between ArgR and XerCD, stabilizing the single-site complex and facilitating ArgR-mediated pairing of sites.

Our results suggest that pairing is independent of the topological relationship between the sites even though recombination is limited strictly to sites on the same molecule and in direct repeat. This implies that progress towards recombination consists of promiscuous pairing (detected in our assay) followed by a topologically sensitive stage. After the initial pairing of sites, a topological filter of the type discussed by Gellert & Nash (1987) may prevent maturation of the synaptic complex where sites are in inverted repeats or on different DNA molecules. The function of the ArgR- and PepA-mediated pairing interaction would be to stabilize an initial complex which can subsequently be tested for geometrical and topological correctness.

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