Mol Gen Genet (1984) 194:211–218

MGG

Recombination-dependent recircularization of linearized pBR322 plasmid DNA following transformation of *Escherichia coli*

E.C. Conley and J.R. Saunders
Department of Microbiology, University of Liverpool, Liverpool, L69 3BX, U.K.

Summary. Monomeric pBR322 DNA that had been linearized at its unique *Sal*I site transformed wild-type *Escherichia coli* with 10^2 to 10^3 times less efficiency than CCC plasmid DNA. Dose-response experiments indicated that a single linear plasmid 'molecule' was sufficient to produce a transformant. Transformation with linearized pBR322 DNA was reduced 10 to 40 fold in *recA*$^-$, *recBC*$^-$ or *recF*$^-$ backgrounds. In contrast, transformation with CCC DNA was unaffected by the *rec* status of the host. Transformation with linear pBR322 DNA was increased 3-fold in a DNA ligase-overproducing (*lop11*) mutant and decreased to a similar degree by transient inactivation of ligase in a *ligts7* mutant.

A proportion (ranging from about 9% in the wild-type to 42% in a *recBC*, *lop11* mutant) of the transformants obtained with *Sal*I-linearized pBR322 monomeric DNA contained deleted plasmids. Deletion rates were generally higher in *rec*$^-$ strains. Dephosphorylation of the termini on linear DNA or the creation of blunt-ended pBR322 molecules (by end-filling the *Sal*I 5′ protrusions or by cleavage with *Pvu*II) decreased the transformation frequency whilst increasing the deletion rate.

Linear pBR322 dimeric DNA gave transformation frequencies in *recA*$^+$ and *recA*$^-$ strains that were reduced only 3 to 7 fold respectively relative to frequencies obtained with dimeric CCC DNA. Furthermore, in contrast to transformation with linear monomeric DNA, deletions were not observed.

We propose that the majority of transformants arise, not by simple intracellular reannealing and ligation of the two cohesive *Sal*I-termini of a linear molecule, but by intramolecular recombination. Deleted plasmids could be generated therefore during recyclization caused by recombination between short directly repeated sequences within a pBR322 monomer. We suggest that perfectly recircularized monomeric pBR322 molecules, which are found in the majority of transformants, arise primarily by intramolecular recombinational resolution of head-to-tail linear pBR322 dimers. Such linear oligomeric forms are created during preparation of linearized plasmid DNA by annealing of the *Sal*I cohesive termini and constitute a variable proportion of the total molecules present.

Offprint requests to: J.R. Saunders

Introduction

Plasmid DNA that has been linearized at a unique site by restriction endonuclease cleavage transforms Ca^{2+}-treated *Escherichia coli* with much lower efficiency than the corresponding covalently-closed circular DNA form (Cohen et al. 1972; Thompson and Achtman 1979). A high proportion of the transformants that do arise with linearized plasmid DNA contain plasmids bearing deletions that extend various distances away from the cleavage site used for linearization. Thus transformation of *E. coli* with linearized plasmid DNA has been used as a method of obtaining in vivo deletions for mapping or trimming of DNA sequences that have been cloned in plasmid vectors (Chang and Cohen 1977; Thompson and Achtman 1979; Manning et al. 1979). The fate of linearized plasmid DNA is also of interest because such molecules, either alone or linked to passenger DNA, occur in recombinant ligation mixtures even under conditions favouring circularization (Dugaiczyk et al. 1975; Mertz and Davis 1972). Certain of the deletions and rearrangements that are often observed in cloning experiments may well be due to processing and subsequent establishment of such linear molecules.

The establishment of linearized plasmid molecules as replicons must of necessity involve recyclisation at an early stage. This report describes experiments to determine the mechanisms by which recircularization and subsequent establishment of linearized plasmid pBR322 (Bolivar et al. 1977) and its deletion derivatives occur. pBR322 was chosen because it is a widely used cloning vector and its entire nucleotide sequence is known, (Sutcliffe 1979; Peden 1983) hence aiding the analysis of transformants. pBR322 also contains no *chi* sites (Smith et al. 1981) and no gross regions of internal homology which might lead preferentially to recombination events at particular regions of the molecule.

Materials and methods

Bacterial strains and media used. The bacterial strains used were all derivatives of *Escherichia coli* K12. AB1157 is a *thr-1*, *leuB6*, *thi-1*, *lacY1*, *galK2*, *ara14*, *xyl-5*, *mtl-1*, *proA2*, *rpsL31*, *tsx-33*, *his-4*, *argE3*, λ^-, F$^-$ strain (Bachmann 1972). The following derivatives of AB1157, JC2924, a *recA* strain (Clark 1973); JC5519, a *recBC* strain (Willetts and Clark 1969; Stahl and Stahl 1977); JC9239, a *recF* strain

(Clark 1973) and JC8679, a *recBC, sbcA* strain (Gillen et al. 1981) were kindly provided by A.J. Clark. C600 is a *thi-1, thr-1 leu6, lacY, tonA21, supE44*, λ^-, F^- strain (Bachmann 1972). Derivatives of C600, N1323, a *lop11* mutant (Gellert and Bullock 1970) and N3098, a *ligts7, supF* mutant (Gottesman et al. 1973) were kindly provided by N.E. Murray who also supplied SF8, an *hsdR*$^-$, *hsdM*$^-$, *recBC, lop-11, supE44, gal-96, rpsL31, leuB6, thi-1, thr* strain (Davis et al. 1980).

Bacteria were grown in Lab M No. 2 nutrient broth solidified where necessary with 1% w/v Lab M agar No. 2. For selection of antibiotic-resistant bacteria, ampicillin (Beecham) at 100 μg/ml or tetracycline (Sigma) at 10 μg/ml were added to the agar.

Agarose gel electrophoresis. Plasmid DNA molecules and restriction fragments were analysed by electrophoresis (typically 140 V, 50 mA for 6 h or 40 V, 20 mA for 16 h) through 1% (w/v) horizontal agarose gels in 40 mM tris-OH, 20 mM sodium acetate, 2 mM EDTA, pH 8.2. Gels were stained by inclusion of ethidium bromide in the gel solution and running buffer at 1 μg/ml and were photographed using Polaroid type 665 film under long wave ultraviolet illumination. Molecular weights were calculated by determining mobilities relative to *Eco*RI digested SPP1 DNA (Ratcliff et al. 1979). These were determined directly from projections of photographic negatives using an Apple Graphics Tablet coupled to an Apple II microcomputer with software written for this purpose by Dr. P. Miller of this department.

Topological forms of plasmid DNA were quantified from negatives (with exposures not exceeding the linear response range of the film) of photographs of agarose gels using a Unicam SP1800 Spectrophotometer fitted with an SP1809 scanning densitometer at 540 nm, 0.2 mm slit width. Areas of traces proportional to the amount of DNA present, were calculated using software running on an Apple II microcomputer/graphics tablet combination.

Preparation of circular plasmid DNA. All pBR322 (Bolivar et al. 1977) plasmid DNA used for transformation experiments was prepared from cultures of C600 (pBR322) grown overnight at 28° C by the PEG-precipitation method of Humphreys et al. (1975). CCC-DNA purified from CsCl-Ethidium bromide density gradients was dialysed extensively against TE buffer (10 mM Tris pH 8.0, 0.1 mM Na_2 EDTA) in visking bags or using a purpose-built continuous flow cell apparatus.

Analytical scale preparation of plasmid DNA was by the rapid boiling method of Holmes and Quigley (1981) or the alkaline denaturation method of Birnboim and Doly (1981). A scaled-up version of the latter method provided enough DNA for several restriction digests for testing whether a restriction site had been lost or retained in deletants.

Large-scale preparation of linear plasmid DNA molecules. CCC monomeric DNA prepared as above was linearized by complete digestion with an appropriate restriction endonuclease (1 unit/μg) cleaving pBR322 at a unique site. Digestions were terminated by heating to 65° C for 10 min. Ten to twenty μg samples were then subjected to preparative electrophoresis (10 V/cm in 0.75% w/v agarose gels containing 0.5 μg/ml ethidium bromide). DNA that migrated with the mobility of linear monomers was isolated by electrophoresing into a slot in the agarose cut ahead of the appropriate band, filled with electrophoresis buffer and lined with dialysis tubing (Yang et al. 1979). For isolation of greater quantities (> 30 μg) a purpose-built preparative apparatus was used. This consisted of a loading trough and a marginally wider and deeper elution trough (6 × 30 × 3 mm) cast in a horizontal agarose gel. The passage of a particular DNA species was halted as desired using a tailored acetate film template. Electrophoresis was continued until the migration of the DNA into the buffer in the slot or trough was judged complete by UV illumination. Immediately prior to collecting the buffer, polarity was reversed for approximately one minute to loosen any DNA bound to the membrane or acetate film. Following centrifugation through siliconized cotton fibres to remove agarose particles (unnecessary with trough electroelution) DNA was recovered from the buffer by sequential isopropanol and ethanol precipitations. The integrity of the termini of purified linear plasmid DNA was assessed by ensuring that T4 DNA ligase could rejoin > 95% of the linearized molecules and that the resulting circular molecules could be re-cut with the original linearising enzyme.

CCC dimeric DNA was prepared as for monomers from an isolate of JC2924 (*recA*$^-$) that exclusively propagates pBR322 dimers. (This strain was obtained by transforming JC2924 with pBR322 DNA from JC8679, *recBC, sbcA* strain in which plasmid oligomerization occurs readily (Fishel et al. 1981).) Dimeric DNA was linearized by partial digestion with *Sal*I and molecules of linear dimeric length were purified on agarose gels as described above.

Transformation. Transformation of *E. coli* strains was as described previously (Brown et al. 1979; Saunders et al. 1984) but with omission of the expression period. Transformants were selected by spreading on nutrient agar containing ampicillin (100 μg/ml). (The *ligts7* mutant required a special protocol which is outlined in the results section.) Deletions of the *tet* gene of pBR322 were detected by patching Ap^r transformants on to agar containing tetracycline (10 μg/ml).

Restriction digests. Restriction enzymes were obtained from The Boehringer Corporation, London, or P and S Biochemicals Ltd., Liverpool and were used as recommended by the manufacturers typically to a final concentration of 1 unit/μg DNA. All reactions were terminated by heating at 65° C for 10 min.

Ligations. DNA fragments that possessed compatible cohesive-ends were generally ligated together at a concentration of 1 μg = 100 μl in a mixture consisting of 20 mM Tris-HCl, 0.6 mM ATP, 10 mM DTT, 10 mM $MgCl_2$, pH 7.6. T4 DNA-ligase (Boehringer) was added at 0.1 units per μg DNA. Blunt ended ligations were carried out at a concentration of 1 μg = 20 μl with the addition of 1 unit T4 DNA ligase per μg DNA. Incubations were started in a beaker of water at 22° C which was allowed to cool overnight to +4° C. The ligase was inactivated by heating the mixture to 65° C for 10 min.

Dephosphorylation of 5′ phosphate groups on linear plasmid DNA. Calf intestinal alkaline phosphatase (Boehringer) was purified and activated as recommended by the manufacturers. Three units of this prepared enzyme were added to

10 µg samples of phenol-extracted/ethanol-precipitated linearized DNA (previously heated to 65° C for 10 min and quickly cooled) in a solution containing 1 mM EDTA (total volume 50 µl). After incubation at 37° C for 30 min a second amount of enzyme (3 units) was added and the reaction was continued for a further 30 min. Following inactivation, by addition of 10 µl 10 × S.T.E. buffer, 10 µl 5 × SDS and heating at 68° C for 15 min, the DNA was recovered by two sequential phenol-chloroform and ether-extractions and ethanol-precipitation.

Filling-in of cohesive termini on linear plasmid DNA using DNA polymerase I (Klenow fragment). Linear DNA (heated to 65° C for 10 min) was added to give a concentration of 5 µg/25 µl in a reaction mixture consisting of 2 nmoles each of the four dNTPs, 6 mM $MgCl_2$, 1 mM 2-mercaptoethanol pH 7.4 and 5 units of Klenow fragment. After 3 h incubation at 25° C reactions were terminated by addition of 1 µl 0.5 *M* EDTA. Unincorporated dNTPs were removed by phenol-chloroform-ether extractions followed by centrifugation through a short column of Sephadex G-50 (Maniatis et al. 1982). The DNA was then recovered from the eluate by ethanol precipitation.

Results

Transformation with linearized pBR322 monomeric DNA

Linearization of plasmid DNA at a unique site reduces the transformation frequency relative to circular molecules by 10^2 to 10^3-fold (Cohen et al. 1972; Thompson and Achtman 1979). However, it is not clear whether the residual transformation activity observed is due in part to molecules that have escaped cleavage and remain circular. Therefore monomeric length linear plasmid molecules purified from agarose gels were used in order to demonstrate unequivocally whether such molecules have transforming activity. Most of the experiments outlined in this paper involved linearizing pBR322 by digestion with *Sal*I which cuts this plasmid approximately in the middle of the tetracycline-resistance determinant (Peden 1983). Transformants could therefore be selected by plating on ampicillin-agar. A gross measure of deletions arising during the recircularization process and extending away from the linearization site could then be obtained by determining whether Ap^r transformants were Tc^r or Tc^s.

One of the problems of measuring the efficiency of transformation is the variability observed both in transformation ability of particular plasmid DNA preparations and in competence levels achieved with *E. coli* (Brown et al. 1979). Therefore, efficiencies of transformation with linearized plasmid DNA were expressed using the ratio C/L: this is the transformation frequency (transformants per viable cell) obtained with CCC (form I) DNA divided by the transformation frequency obtained with linear (form III) plasmid DNA measured at the same time with equimolar amounts of DNA and the same preparation of competent cells. The C/L ratio is therefore a characteristic of the two DNA preparations involved. Use of the C/L ratio obviates the effects of differences in viability and competence levels obtained with different batch cultures of the same strain (Brown et al. 1979; Saunders et al. 1984). It also normalizes the effects of differential viability caused by the genotype of some strains, particularly those that are recombination-deficient.

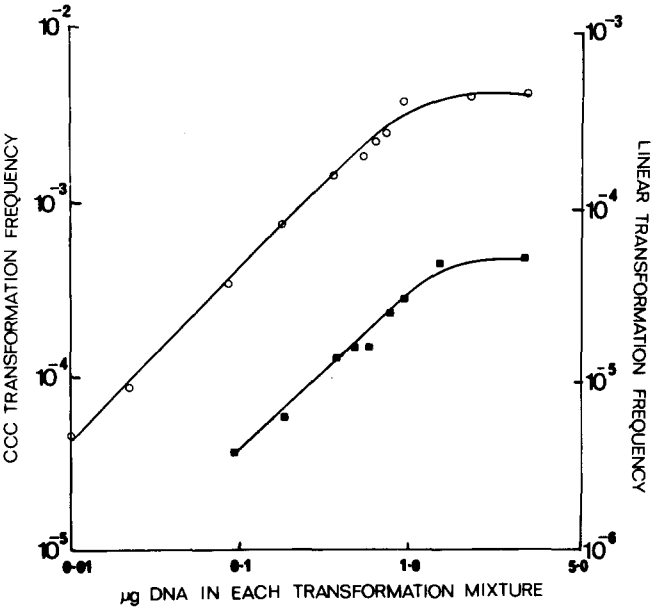

Fig. 1. Dose-response kinetics for the transformation of *E. coli* AB1157 with CCC and linear pBR322 DNA. Transformation was carried out as described in Materials and Methods. o ampicillin-resistant transformants obtained with CCC pBR322 DNA; ■ ampicillin-resistant transformants obtained with *Sal*I-linearized pBR322 DNA

In using the C/L ratio it is assumed that circular or linear molecules are neither damaged differentially prior to DNA uptake nor taken up preferentially into transformants. A comparison of the dose-response relationships for CCC pBR322 DNA and pBR322 DNA that had been linearized with *Sal*I was therefore performed using wild type *E. coli* as a recipient (Fig. 1). In the transformation system used here the dose-response slope for CCC DNA at non-saturating DNA concentrations is about 1 and saturation occurs at about 0.5 to 1 µg DNA per approximately 5×10^8 viable cells (Fig. 1 and Weston et al. 1981). Linearized pBR322 DNA also produced a dose-response slope of about 1 but the efficiency of transformation overall was reduced approximately 100-fold relative to CCC DNA (Fig. 1). In replicate experiments it was found that two to three times more linear plasmid DNA than circular plasmid DNA was generally required to produce saturation depending on the genetic background. This is consistent with the behaviour of linear fragments of chromosomal DNA which saturate at higher concentrations than CCC plasmid DNA (H.E.N. Bergmans, personal communication) and may reflect the topological differences between linear and circular molecules. However, we believe that this is unlikely to affect our experiments since the non-saturating regions of the dose-response slopes are parallel and any slight preferential uptake of linear molecules would be outweighed by their inherently poor transforming ability. In order to standardise experiments, subsequent transformation mixtures contained 1 µg of either circular or linear DNA which was at the point of saturation or slightly subsaturating respectively.

Transformation of *E. coli* strain AB1157 (Fig. 1) and a variety of *rec*$^-$ derivatives of this strain with *Sal*I-linearized pBR322 DNA all gave dose-response slopes of approx-

imately 1 (data not shown). This suggests that a single linear plasmid molecule is sufficient to produce a transformant and that the majority of recyclizations result from intramolecular events.

The effect of DNA ligase mutations on transformation with linear monomer DNA

Whatever the mechanism(s) involved in recyclisation of linear plasmid molecules there must at some stage be a joining reaction to reform a functional covalently closed circular plasmid replicon. The most obvious candidate for joining the ends of a linearized molecule would be the *E. coli* DNA ligase (Sgaramella 1972). The effect of DNA ligase on the establishment of pBR322 transformants was studied by using ligase-deficient (*ligts7*) and ligase-overproducer (*lop11*) mutants. The *ligts7* mutation is lethal to host cells at the restrictive temperature (Gottesman et al. 1973). Therefore the optimum time of incubation at this temperature necessary to inactivate ligase without greatly reducing viability was first determined (Fig. 2). The *ligts7* strain was first grown at the permissive temperature for a sufficient time to optimize competence. The cells were then shifted to the restrictive temperature, monitoring the critical A_{660} at which to transform (representing the minimum intracellular ligase concentration before rapid cell death occurred). Following the heat pulse step and initial establishment, the cells were returned to the permissive temperature and incubated, to allow survival of the transformants and de novo synthesis of ligase.

The relative efficiency (C/L ratio) of establishing linear plasmid molecules was about 3 times lower when ligase was inactivated than the wild-type grown under similar conditions (Table 1). Absolute frequencies of transformation of *ligts7* for both CCC and linear DNA molecules were higher with this mutant than for the parental *E. coli*. This can be attributed to distortions caused by low survival of the recipient after the heat-pulse stage of transformation and is a common occurrence with strains of low viability (and see also Table 2). This apparent variation in competence levels illustrates the value of using the C/L ratio as a comparator of the transformation efficiency of different strains using linear plasmid DNA.

Overproduction of ligase in a *lop11* mutant resulted in a 3-fold increase in the efficiency of transformation with linear pBR322 DNA. These results suggest that the amount of intracellular ligase is critical in the recyclization of linear plasmid molecules.

Effect of rec mutations on transformation with linearized plasmid DNA

The potential role of recombination functions in the recircularization and establishment of linearized plasmid monomers was investigated by transforming a series of derivatives of *E. coli* AB1157. Mutations in *recA*, *recBC* or *recF* increased the C/L ratio (and therefore reduced the relative recovery of transformants with linear DNA) by between 20 and 30 fold relative to the wild-type (Table 2). The *sbcA* mutation which activates exonuclease VIII (Gillen et al. 1981) partially reversed the effects of a defective *recBC* product (exonuclease V). *recBC lop11* double mutants had C/L ratios that approached the wild-type, suggesting that the detrimental effect of losing exonuclease V could be

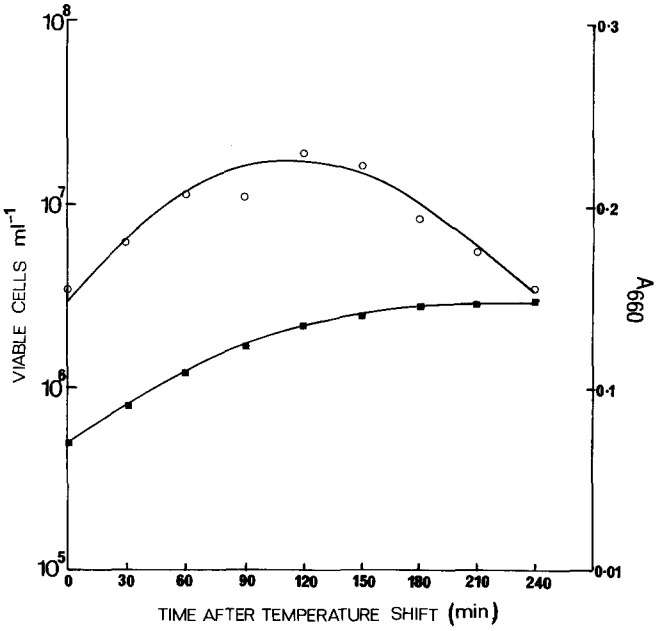

Fig. 2. Effect on a culture of *E. coli ligts7* of shifting growth temperature from 28° C to 42° C. *E. coli* N3098 was grown overnight in nutrient broth at 28° C with shaking (150 rev/min), diluted 1:100 in fresh prewarmed broth and incubation was continued until $A_{660}=0.05$. The culture was then shifted to 42° C ($t=0$) and incubation continued at this temperature. At the times indicated samples were removed for the determination of o, viable cells/ml and ■, A_{660}

Table 1. Effect of DNA ligase mutations on plasmid transformation

Strain	Mean transformation frequency		C/L ratio
	CCC DNA	linear DNA	
C600	7.8×10^{-4}	2.2×10^{-6}	355
N3098 (*ligts7*)	2.6×10^{-1}	2.4×10^{-4}	1083
N1323 (*lop11*)	2.3×10^{-3}	1.9×10^{-5}	121

Results are the average of two separate experiments. All strains were rendered competent by growth overnight in nutrient broth at 28° C, diluted 1:100 in fresh broth and grown at 28° C with shaking until A_{660} 0.1, cells were shifted to 42° C for 1 h to A_{660} 0.2. All strains were transformed simultaneously with the same DNA stock

Table 2. Transformation of *E. coli* AB1157 Derivatives with pBR322 DNA

Strain	Relevant genotype	Mean transformation frequency		Mean C/L ratio
		CCC pBR322 (C)	*Sal*-I linearized (L)	
AB1157	Wild type	3.7×10^{-3}	5.6×10^{-5}	66
JC2924	*recA*	3.6×10^{-2}	1.9×10^{-5}	1894
JC5519	*recBC*	2.3×10^{-3}	1.5×10^{-6}	1533
JC8679	*recBC, sbcA*	2.3×10^{-2}	2.4×10^{-5}	930
JC9239	*recF*	3.4×10^{-3}	1.6×10^{-6}	2102

Table 3. Gross deletion frequencies as determined by tetracycline-sensitivity of transformants

Relevant genotype	Original transforming pBR322 DNA	Total number of transformants patched	Total number Tc-sensitive	% Deletion
Wild type (AB1157)	*Sal*I purified linear	870	78	9.0
Wild type (AB1157)	Alkaline phosphatased *Sal*I linear	200	28	14.0
Wild type (AB1157)	Klenow-filled termini *Sal*I linear	200	68	34.0
Wild type (AB1157)	CCC	200	0	0
recA	*Sal*I purified linear	642	152	23.7
recBC	*Sal*I purified linear	352	83	23.5
recF	*Sal*I purified linear	836	143	17.2
recBC, sbcA	*Sal*I purified linear	568	32	5.6
Wild type (C600)	*Sal*I purified linear	704	63	8.9
Wild type (C600)	CCC	88	0	0
lop11	*Sal*I purified linear	220	69	31.4
recBC, lop11	*Sal*I purified linear	220	93	42.2

counter-balanced by elevated levels of ligase (data not shown). These results indicate that >95% of the transformants with *Sal*-I-linearized, pBR322 DNA arise by events involving some form of homologous recombination.

Deletion formation following transformation with SalI linearized plasmid DNA

Previous workers have reported that high frequencies of deletion are obtained when transforming linearized plasmid or recombinant DNA molecules (Thompson and Achtman 1979; Manning et al. 1979; Garaev et al. 1982). The results presented here confirm these observations (Table 3). The overall frequency of deletions extending from the *Sal*I linearization site was obtained by determining the number of Ap^r transformants that were Tc^s. Of necessity only deletants that retained the *bla* gene and origin of replication could be detected in this system meaning that at greatest approximately 2.6 kb could be deleted from the wild type sequence whilst still retaining a viable replicon with a selectable phenotype.

Since the unique *Sal*I site lies approximately in the middle of the tetracycline-resistance determinant of pBR322, removal of nucleotides from either *Sal*I terminus prior to recircularization should produce a Tc^s phenotype. It is conceivable that small numbers of bases could be removed from these termini whilst still maintaining the correct reading frame and/or activity of the reconstituted tetracycline-resistance determinant. However, such events are considered to be so infrequent as to be unlikely to affect the results. In any case, such Tc^r transformant plasmids should have lost the *Sal*I site and in all cases Ap^r Tc^r transformants contained plasmids that retained this site when their DNA was analysed by agarose gel electrophoresis (data not shown). In contrast, as would be expected, >95% of Ap^r Tc^s transformants contained plasmids that were less than monomeric length and had lost the *Sal*I site. Occasionally Tc^s transformants contained plasmids that were partially cleavable with *Sal*I but were additionally greater than unit monomeric length. The characterization of these and other deletant molecules obtained will be published separately. Our results are in contrast to those of Garaev et al. (1982) who were unable to obtain deletants with *Sal*I linearized pBR322 DNA.

Deletant plasmids were obtained with both recombination proficient and -deficient recipients (Table 3). The proportion of transformants that contained deletants was somewhat higher for recombination-deficient strains but the absolute numbers of transformants obtained were substantially lower in these backgrounds (data not shown). The highest frequencies of deletion (42%) were obtained in the *recBC lop11* mutant whereas the lowest were found in the (*recBC*, *sbcA*, (exo $VIII^+$)) mutant and the wild types, at approximately 6% and 9% respectively.

Restructuring the ends of linearized pBR322 DNA by 'filling in' of the *Sal*I termini (and to a lesser extent by dephosphorylating these termini) increased the proportion of deletants obtained (Table 3).

Table 4. Effect of storage at +4° C of purified *Sal*I cleaved pBR322 monomers

	Absolute transformation frequencies at	
	$t=0$	$t^*=+10$ weeks
CCC	4.8×10^{-3}	2.6×10^{-3}
Linear	1.2×10^{-5}	1.0×10^{-4}
C/L ratio	400	26

Transformations of *E. coli* C600 were carried out with the same stocks of linear and CCC DNA

Effect of preparation and storage of linear plasmid DNA on transformation ability

During the course of these experiments it was noticed that the transformation ability of *Sal*I-linearized monomeric pBR322 DNA varied from preparation to preparation and the ability of individual preparations increased on storage at 4° C (Table 4). This increase correlated with the appearance of linear dimer, trimer, and higher oligomer forms in the purified preparations of linear DNA as judged by agarose gel electrophoresis (in preparation). No such forms could be detected in preparations of blunt-ended molecules that had been linearized with *Pvu*II. Moreover pBR322 DNA that had been completely linearized by *Sal*I digestion and then used immediately (without ethanol precipitation)

Table 5. Transformation of *E. coli* AB1157 derivatives with linear plasmid DNA

Transforming pBR322 DNA (1 μg)	AB1157		JC2924 (*recA*)		JC5519 (*recBC*)	
	Frequency	C/L	Frequency	C/L	Frequency	C/L
CCC	6.4×10^{-3}	–	7.6×10^{-3}	–	9.7×10^{-4}	–
*Sal*I linear (pure)	7.1×10^{-5}	90	8.2×10^{-6}	933	7.4×10^{-7}	1,310
Klenow-filled *Sal*I linear	1.5×10^{-5}	428	3.2×10^{-6}	1,180	9.2×10^{-8}	>10,000
Alkaline-phosphatased *Sal*I linear	7.1×10^{-6}	900	1.6×10^{-6}	2,346	3.2×10^{-8}	>30,000

Table 6. Transformation with dimeric pBR322 DNA

DNA	AB1157		JC2924 (*recA*)		C600		C600 *lop11*	
	Frequency	C/L	Frequency	C/L	Frequency	C/L	Frequency	C/L
CCC monomer	1.2×10^{-3}	–	1.0×10^{-2}	–	8.3×10^{-4}	–	2.9×10^{-4}	–
Linear monomer	1.3×10^{-5}	92	7.8×10^{-6}	1,282	1.5×10^{-6}	553	1.1×10^{-6}	263
CCC dimer	1.3×10^{-3}	–	1.5×10^{-2}	–	1.5×10^{-3}	–	3.2×10^{-4}	–
Linear dimer	3.5×10^{-4}	3.7	2.4×10^{-3}	6.3	4.3×10^{-4}	3.5	8.9×10^{-5}	3.6

to transform *E. coli* AB1157 had a transformation ability that was up to 1,000 fold lower than similar DNA that had been purified from an agarose gel and precipitated. In contrast precipitation procedures had no detectable effect on the transformation ability of CCC pBR322 or of blunt ended pBR322 molecules created by digestion with *Pvu*II. Precipitated and stored *Sal*I linearized DNA that had been heated to 65° C for 5 min prior to transformation and which presumably melted out any end-annealed linear oligomers also had a reduced transformation ability. Presumably the high concentration of *Sal*I cohesive ends caused by ethanol-precipitation either increased the chances of end-to-end oligomer formation or in some other way caused more than one linear monomer to become aggregated so as to enter transformed cells with single-hit kinetics.

In all cases tested, linear molecules with cohesive termini, whether purified from agarose gels or not, gave lower C/L ratios than blunt-ended molecules. Blunt-ended pBR322 molecules created by cleavage with *Pvu*II, which should have no ability to associate at their termini, still retained some transforming ability despite having C/L ratios of about 5,000. Interestingly, >80% of transformants obtained with such DNA had lost the *Pvu*II site. Furthermore, about 15% of the transformants obtained were Tc^s even though the *Pvu*II linearization site at base no. 2066 lies well outside the *tet* region. This suggests that deletion of blunt-ended plasmid molecules occurs at higher frequency than molecules possessing cohesive-termini.

Blunt-ended molecules of pBR322 were also prepared by filling in the single stranded *Sal*I terminal protrusions with the Klenow fragment of DNA polymerase I. Such molecules gave very high C/L ratios particularly in a *rec*BC background (Table 5). Removal of the terminal phosphate groups of *Sal*I linear pBR322 DNA by treatment with alkaline phosphatase gave 2–3 times yet higher C/L ratios than blunt-ended molecules created by filling-in (Table 5). Establishment of such molecules was also dramatically decreased in $recA^-$ or $recBC^-$ backgrounds.

Transformation with dimeric DNA

In view of the possibility that a proportion of the transformants observed resulted from uptake of plasmid oligomers, the ability of purified linear dimeric pBR322 DNA to transform *E. coli* was tested. Linear dimer molecules were found to be about thirty times more efficient than linear monomers and gave transformation frequencies that were only 3 to 4 fold less than those obtained with CCC dimers (Table 6). Interestingly the $recA^-$ mutation had a less dramatic effect (2-fold as opposed to 14-fold) in reducing transformation with linear dimers when compared with linear monomers (Table 6). We have no explanation of this finding as yet except that alternative, *recA*-independent pathways of plasmidic recombination (Doherty et al. 1983) may be involved in the establishment of such linear dimers. No Tc^s deletants were obtained when 220 of each type of transformant were tested. This is in contrast to the high proportion of deletants obtained with preparations of monomeric DNA (Table 3). There was no stimulation of transformation with linear dimers in a *lop11* background (Table 6). This in contrast to linear monomers where overproduction of ligase produced a 2–3 fold increase in transformation efficiency (Tables 1 and 6). This finding may suggest that the stimulatory effect of ligase overproduction arises as a consequence of the need to seal the nicks in hydrogen bonded linear oligomers.

Discussion

Transformation of *E. coli* with linearized plasmid DNA is evidently a much less efficient process than with circular DNA. Furthermore, presumably as a consequence of in vivo processing by the host cell, a proportion of the transformants ranging from 5–80% (depending on the genetic background and the termini of the plasmid molecules involved) are deleted to varying extents. It is perhaps surprising, given the number of nucleases in wild-type *E. coli* that such a high proportion (about 95%) of transformants obtained with linearised DNA actually contain perfectly recir-

cularized plasmid molecules. Dose-response experiments indicate that a single 'molecule' of linearized plasmid DNA is sufficient to produce a transformant. It is therefore concluded that the vast majority of transformants arise by intramolecular events. The most naive interpretation of these findings would be that the majority of linear plasmid monomers recircularize by reannealing between their compatible cohesive termini with subsequent ligation in vivo before such processing could occur. Indeed, we have shown that the efficiency of transformation with linear DNA is dependent on the amount of ligase in recipient cells. However, this explanation probably does not account for the formation of the majority of transformants obtained with linear DNA. The first reason for proposing an alternative mechanism is the marked effect of recombination-deficiency (i.e. *recA*, *recBC* and *recF*) in the host in reducing the recovery of transformants with linear as opposed to circular DNA. Thus AB1157 (*recA*$^+$) is approximately 10–40 times more proficient at establishing linear molecules than its *recA*$^-$ or *recF*$^-$ derivatives. Since mutations in *recA* and *recF* reduce plasmidic recombination by 40–100-fold (Doherty et al. 1983; Fishel et al. 1981; Laban and Cohen 1981), this implies that >95% of transformants arise by some form of homologous recombination event. It is difficult to see why molecules recircularized by simple annealing of cohesive termini would require recombination functions. It is also interesting that recombinant DNA molecules formed by dA-dT hompolymer-tailing and which are not normally ligated in vitro prior to transformation, give about 10 times fewer recombinant clones in *recA*$^-$ than in *recA*$^+$ derivatives (Peacock et al. 1981). This implies that homologous recombination is also involved in the establishment of these molecules.

The effect of *recBC*$^-$ mutations in dramatically reducing transformation with linear plasmid DNA is in direct contrast to their effect on transformation of *E. coli* with linear chromosomal DNA fragments where the *recBC* nuclease is known to be inhibitory (Cosloy and Oishi 1973; Wackernagel 1973). Furthermore, the *recBC*$^-$ mutation has no apparent effect on intra- or interplasmidic recombination during vegetative replication (Fishel et al. 1981; Laban and Cohen 1981). The reduction in linear plasmid transformation is similar to the effect of *recBC* mutations on conjugation, where recipient frequencies are reduced about 100-fold relative to the wild-type (Emmerson and Howard-Flanders 1967). The *recBC* nuclease functions in vitro as a potent endo-and exonuclease (Barbour and Clark 1979; Oishi 1969) but probably also acts in vivo as a helicase to unwind duplex DNA and hence initiate genetic recombination by exposing single-stranded DNA (Taylor and Smith 1981; Rosamond et al. 1979). *recA* protein could bind to such exposed single-stranded regions of a DNA molecule (in the case of linearized plasmid DNA this would be one 'intact' or 'processed' end of the molecule) and traverse intact duplex regions of the same or a different molecule to search for regions of homology and form a recombination intermediate (Dressler and Potter 1982). Recircularization could therefore occur by recombination between short regions at one end of a linearized plasmid molecule and homologous regions either at the other end or internally. Recircularization can occur by recombination at sites with as little as 3 b.p. homology between partially homologous cloned sequences located on either end of a linearized plasmid molecule (Weber and Weissman 1983). *recA*-dependent and -independent recombination between direct repeats as short as 5 b.p. has also been shown to occur and to lead to deletions both in the chromosome of *E. coli* (Edlund and Normark 1981) and in plasmids (Jones et al. 1982). Deletions occurring during transformation with linear DNA would arise if a short sequence on one end of the incoming molecule was able to recombine with another homologous sequence anywhere in the remainder of the molecule. *rec* mutations decrease the frequency of transformation but increase the proportion of deletant plasmids obtained. This might suggest that recircularization and deletion of linear molecules occur by different mechanisms. Alternatively deficiency in one or more *rec* functions might increase the likelihood of exonucleolytic processing prior to recircularization.

A problem arises, however, to explain 'perfect' *rec*-dependent recircularization recreating the original linearizing site since there is no homology (apart from the cohesive protrusions) at the termini of a pBR322 monomer. Indeed filling in of *Sal*I termini with Klenow fragment to create 4 base pair regions of duplex homology at either end of linear pBR322 molecules actually decreases the transformation frequency of a linear pBR322 monomer. This might indicate that a proportion of perfect monomers do actually result from in vivo annealing and subsequent ligation of the *Sal*I termini of a monomeric molecule by a mechanism requiring *rec* functions. However, perfect circular molecules could also arise by recombinational resolution and recyclization in vivo of a head-to-tail linear dimer (or higher order oligomer). Purified linear pBR322 dimer DNA transforms *E. coli* efficiently with frequencies that approach those obtained with circular DNA. Furthermore, in contrast to linear monomers, deletions are not observed. A small proportion of linear oligomeric molecules transforming with high efficiency in a mixture with linear monomers would therefore have a disproportionate effect on the overall transformation frequency. End-annealed linear oligomers are generated in vitro during the preparation and storage of linear pBR322 monomers and the presence of such oligomeric forms in a DNA preparation is correlated with high transformation frequencies (in preparation). Conversely, if steps are taken to reduce the formation of linear oligomers, by heating DNA prior to use or by end-filling cohesive termini or by using plasmid DNA that has been linearized with a restriction endonuclease producing flush termini, then transformation efficiency is substantially reduced. Head-to-tail dimers could be recircularized by homologous recombination between the two directly repeated copies of the sequence to produce perfect length monomeric molecules. Such a dimeric substrate for transformation could tolerate substantial exonucleolytic damage before the formation of a perfect monomeric recombinational product would be prevented.

Acknowledgements. EC is grateful for a postgraduate scholarship from the Medical Research Council. We are grateful to A.J. Clark and N. Murray for providing strains, to P. Strike for helpful discussions and P. Miller with computing assistance. JRS acknowledges the support of a grant from the Royal Society for computer equipment.

References

Bachmann BJ (1972) Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol Rev 36:525–557

Barbour SD, Clark AJ (1970) Biochemical and genetic studies of recombination proficiency in *Escherichia coli*. I. Enzymatic activity associated with *rec*B$^+$ and *rec*C$^+$ genes. Proc Natl Acad Sci USA 65:955–961

Bolivar F, Rodriguez RL, Greene PJ, Betlach MC, Heyneker HL, Boyer HW, Crosa JH, Falkow S (1977) Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2:95–113

Brown MGM, Weston A, Saunders JR, Humphreys GO (1979) Transformation of *Escherichia coli* at different phases of growth. FEMS Microbiol Lett 5:219–222

Chang S, Cohen SN (1977) *In vivo* site-specific genetic recombination promoted by the *Eco*RI restriction endonuclease. Proc Natl Acad Sci USA 74:4811–4818

Clark AJ (1973) Recombination-deficient mutants of *Escherichia coli* and other bacteria. Annu Rev Genet 7:67–85

Cohen SN, Chang ACY, Hsu L (1972) Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc Natl Acad Sci USA 69:2110–2114

Cosloy SD, Oishi M (1973) Genetic transformation in *Escherichia coli* K12. Proc Natl Acad Sci USA 70:84–87

Davis RW, Botstein D, Roth JR (1980) Advanced bacterial genetics. Cold Spring Harbor Laboratory, New York

Doherty MJ, Morrison PT, Kolodner R (1983) Genetic recombination of bacterial plasmid DNA. Physical and genetic analysis of the products of plasmid recombination in *Escherichia coli*. J Mol Biol 167:539–560

Dressler D, Potter H (1982) Molecular mechanisms in genetic recombination. Annu Rev Biochem 51:727–761

Dugaiczyk A, Boyer HW, Goodman HM (1975) Ligation of *Eco*RI endonuclease-generated DNA fragments into linear and circular structures. J Mol Biol 96:171–184

Edlund T, Normark S (1981) Recombination between short DNA homologues causes tandem duplication. Nature 292:269–271

Emmerson PT, Howard-Flanders P (1967) Cotransduction with *thy* of a gene required for genetic recombination in *Escherichia coli*. J Bacteriol 93:1729–1731

Fishel RA, James AA, Kolodner R (1981) *rec*A-independent general genetic recombination of plasmids. Nature 294:184–186

Garaev MM, Bobkov AF, Bobkova AF, Kalinin VN, Smirnov VD, Khudakov VE, Tikchonenko TI (1982) The site specific deletion in plasmid pBR322. Gene 18:21–28

Gellert M, Bullock ML (1970) DNA ligase mutants of *Escherichia coli*. Proc Natl Acad Sci USA 67:1580–1587

Gillen JR, Willis DK, Clark AJ (1981) Genetic analysis of the RecE pathway of genetic recombination in *Escherichia coli* K12. J Bacteriol 145:521–532

Gottesman MM, Hicks ML, Gellert M (1978) Genetics and function of DNA ligase in *Escherichia coli*. J Mol Biol 77:531–547

Holmes DS, Quigley M (1981) A rapid boiling method for the preparation of bacterial plasmids. Anal Biochem 114:193–197

Humphreys GO, Willshaw GA, Anderson ES (1975) A simple method of the preparation of large quantities of pure plasmid DNA. Biochim Biophys Acta 383:457–463

Jones IM, Primrose SB, Ehrlich SD (1982) Recombination between short direct repeats in a *rec*A host. Mol Gen Genet 188:486–489

Laban A, Cohen A (1981) Interplasmidic and intraplasmidic recombination in *Escherichia coli* K12. Mol Gen Genet 184:200–207

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning – a laboratory manual. Cold Spring Harbor Laboratory, New York

Manning PA, Thompson R, Achtman M (1979) Generation of deletions during transformation: An analysis of the surface exclusion genes of the F sex factor. In: Glover SW, Butler LO (eds) Transformation 1978. Cotswold Press, Oxford, pp 319–329

Mertz JE, Davis RW (1972) Cleavage of DNA by RI restriction endonuclease generates cohesive ends. Proc Nat Acad Sci USA 69:3370–3374

Oishi M (1969) An ATP-dependent deoxyribonuclease from *Escherichia coli* with a possible role in genetic recombination. Proc Natl Acad Sci USA 64:1292–1299

Peacock Sl, McIver CM, Monohan JJ (1981) Transformation of *E. coli* using homopolymer linked plasmid chimeras. Biochim Biophys Acta 655:243–249

Peden KWC (1983) Revised sequence of the tetracycline resistance gene of pBR322. Gene 22:277–280

Ratcliff SW, Luh J, Ganesan AT, Behrens B, Thompson R, Monengro MA, Morelli G, Trautner TA (1979) The genome of *Bacillus subtilis* phage SPP1: The arrangement of restriction endonuclease generated fragments. Mol Gen Genet 168:165–172

Saunders JR, Docherty A, Humphreys GO (1984) Transformation of bacteria by plasmid DNA. In: Richmond MH, Bennett PM, Grinsted J (eds) Volume in series 'Methods in Microbiology'. Academic Press, London, in press

Sgaramella V (1972) Enzymatic oligomerisation of bacteriophage P22 DNA and of linear simian virus 40 DNA. Proc Natl Acad Sci USA 69:3389–3393

Smith GR, Schultz DW, Crasemann JM (1981) Generalized recombination nucleotide sequence homology between *chi* recombinational hotspots. Cell 19:785–794

Stahl FW, Stahl MM (1977) Recombination pathway specificity of chi. Genetics 86:715–725

Sutcliffe JG (1979) Complete nucleotide sequence of the *E. coli* plasmid pBR322. Cold Spring Harbor Symp Quant Biol 43:77–90

Taylor A, Smith GR (1981) Unwinding and rewinding of DNA by the RecBC enzyme. Cell 22:447–457

Thompson R, Achtman M (1979) The control region of the F sex factor DNA transfer cistrons: physical mapping by deletion analysis. Mol Gen Genet 169:49–57

Wackernagel W (1973) Genetic transformation in *E. coli*: the inhibitory role of the *rec*BC DNase. Biochem Biophys Res Commun 51:306–311

Weber H, Weissman C (1983) Formation of genes coding for hybrid proteins by recombination between related cloned genes in *E. coli*. Nucl Acids Res 16:5661–5669

Weston A, Brown MGM, Perkins HR, Saunders JR, Humphreys GO (1981) Transformation of *Escherichia coli* with plasmid DNA: calcium-induced binding of DNA to whole cells and to isolated membrane fractions. J Bacteriol 145:780–787

Willetts NS, Clark AJ (1969) Characteristics of some multiply recombination-deficient strains of *Escherichia coli*. J Bacteriol 100:231–239

Yang RCA, Lis J, Wu R (1979) Elution of DNA from agarose gels after electrophoresis. In: Wu R (ed) Methods in enzymology, vol 68. Academic Press, New York, pp 176–182

Communicated by P.T. Emmerson

Received July 22 / November 3, 1983