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DNA transcription and repressor binding affect deletion formation in *Escherichia coli* plasmids

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Chimeric plasmids containing phage M13 and plasmid pBR322 sequences undergo deletions in *Escherichia coli* with a high frequency. In all plasmids one deletion end-point is the M13 replication origin nick site. We examined the effects of transcription on the position of the other deletion end-point, by inserting in the plasmids an inducible promoter followed by a transcription terminator. Transcription dramatically affected deletions in an orientation-dependent way, such that >95% of end-points were localized downstream from the inserted promoter when it faced the major plasmid transcripts. The end-points were not constrained to the transcribed region and were not affected by the orientation of pBR322 DNA replication. We propose that deletion events occur preferentially in a plasmid domain which is rendered positively supercoiled by convergent transcription. We also show that interaction of LacI repressor with the cognate operator generates a localized deletion hot spot. This hot spot is dependent on pBR322 replication, and therefore probably acts by arresting progression of DNA replication.

Key words: illegitimate recombination/M13 bacteriophage/operator/promotor/replication origin

Introduction

Transcription affects various DNA processes, such as replication, recombination and repair. For instance, initiation of replication at the *Escherichia coli* chromosomal origin is facilitated by transcription of adjacent sequences (Skarstad *et al.*, 1990), while maintenance of certain plasmids is adversely affected by transcriptional read-through into the replication region (Stueber and Bujard, 1982; Bujard *et al.*, 1985; Brown *et al.*, 1990). Transcription stimulates homologous recombination in *E.coli* and yeast and site-specific recombination in yeast and eukaryotic cells (Thomas and Rothstein, 1991). Preferential repair of the transcribed strand has been reported for *E.coli*, yeast and mammalian cells (Mellon and Hanawalt, 1989; Smerdon and Thoma, 1989) and it was suggested that transcription might play a role in the increased mutation rate in stationary phase cultures of *E.coli* (Davis, 1989). Transcription induces changes in DNA topology; positive supercoils accumulate in front of the transcription complex and negative supercoils behind it (Liu and Wang, 1987; Pruss and Drlica, 1989; Rahmouni and Wells, 1992). Such changes might affect the accessibility of DNA to various proteins and thus its capacity to undergo different transactions. In this work, we show that transcriptional activity of a DNA region can influence illegitimate recombination events.

Hybrid plasmids composed of phage M13 and pBR322 have been used previously for studying illegitimate recombination (Michel and Ehrlich, 1986; Michel *et al.*, 1989; Bierne *et al.*, 1991). These plasmids are structurally unstable in *E.coli* and frequently undergo deletions. The deletions result from the joining of the nucleotide adjacent to the nick in the M13 replication origin to a nucleotide elsewhere in the molecule, and require no homology between the origin and the sequence encompassing the distant nucleotide. Here we show that transcription dramatically affects localization of the end-points. In addition, we show that a repressor-bound operator can be a deletion hot spot.

Results

Experimental approach

The chimeric plasmid pU3D is composed of phage M13mp2, the replication origin of plasmid pC194, the Em^r resistance gene of plasmid pE194 and plasmid pBR322, which lacks sequences between bp 1425 and 2066 (Figure 1A). This plasmid is stably maintained in *Bacillus subtilis* but undergoes deletions in *E.coli* (Michel *et al.*, 1989). To test whether transcription affects formation of deletions we inserted three different transcription units, named P_{Tac}, P_L and GP_L, in the *Nru*I site of pU3D (Figure 1B). P_{Tac} and P_L were inserted in pU3D in two orientations, signalled by superscripts − and + for transcription proceeding in the direction of main pU3D transcripts and in the opposite direction, respectively.

pU3D and derivatives were introduced by transformation into restrictionless rep^- *E.coli* cells (JJC42, Table I), selecting for ampicillin resistance. Restrictionless cells are required to be transformed with DNA coming from *B.subtilis*, and rep^- cells were chosen to prevent elongation of M13 replication (Takahashi et al., 1978) and thus simplify the system. Transformation efficiencies comparable with that of a control plasmid stably maintained in this host ($\sim 10^6$ transformants/μg of DNA) were observed in all cases. All of the 350 transformants analysed contained one or more plasmids smaller than the parental and therefore generated by deletion. One deletion end-point (named 'first' in this text) was at the nick site in the M13 origin in all the plasmids characterized by sequence and/or fine restriction analysis (202 in this study), as previously reported (Michel *et al.*, 1989). The position of the other deletion end-point (named 'second') could therefore be deduced to within 50−100 bp simply from plasmid size. The propensity of a given plasmid region to undergo deletion can be deduced from the distribution of second deletion end-points.

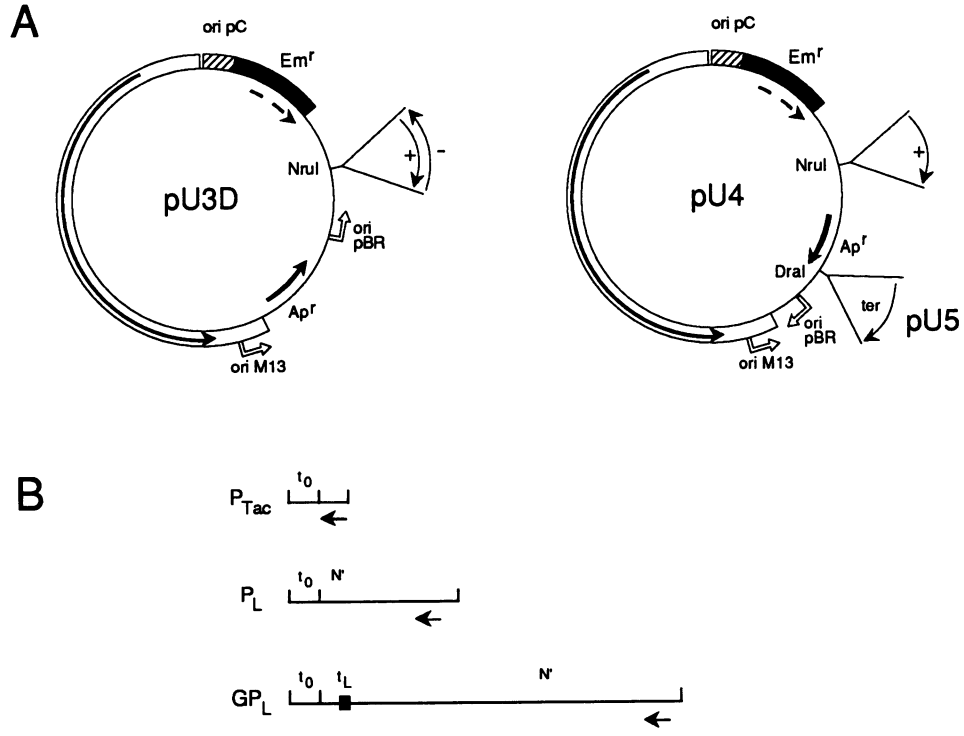

Fig. 1. (**A**) Schematic representation of pU3D, pU4 and pU5 plasmids. pBR322 sequences are represented by a thin line, pE194 *Em*^{r} gene by a closed box, pC194 origin by a hatched box and M13mp2 sequences by an open box. ori M13, ori pBR and ori pC stand for replication origins of M13, pBR322 and pC194, respectively, and ter for the *E.coli terB* replication terminator present in pU5. Genes conferring resistance to ampicillin and erythromycin are symbolized by *Ap*^{r} and *Em*^{r}, respectively. The *Nru*I restriction site, used for the insertion of the transcription units, and the *Dra*I site, used for insertion of the replication terminator *terB*, are represented. Direction of leading strand synthesis initiated at the origins functional in *E.coli* is indicated by bent arrows. Direction of transcription is indicated by curved arrows and, within the transcription units inserted in the *Nru*I site, also by + and − symbols. (**B**) Transcription units P_{Tac}, P_L and GP_L. Direction of transcription and the promoter location are indicated by arrows. P_{Tac} unit (195 bp) contains promoter P_{Tac} and the λ transcription terminator t_0, P_L unit (565 bp) is composed of P_L promoter, a part of the λ N′ gene and t_0 and GP_L unit (1315 bp) contains P_L promoter, the N′ gene inactivated by a frameshift mutation and the λ t_L and t_0 transcription terminators.

Transcription affects deletion end-points in an orientation-dependent manner

Distribution of the second deletion end-points in 73 plasmids derived from pU3D (Figure 1) is displayed in Figure 2A. End-points were scattered over the region extending between 2.25 and 7.5 kb from the M13 replication origin. Deletions ending closer to M13 origin would inactivate pBR322 replication required for plasmid maintenance, whereas deletions ending much further could possibly preserve toxic M13 sequences and give non-viable transformants. The slight clustering of end-points between 4.5 and 5 kb from the M13 origin (Figure 2A), is probably not significant.

A different distribution was obtained when pU3DP$^+{}_{Tac}$ (Figure 1) was used to transform *E.coli* cells in the presence of the P_{Tac} inducer IPTG, since 95% of deletion end-points were clustered between the pBR322 replication origin and the P_{Tac} promoter (Figure 2B). Such clustering was not observed with pU3DP$^-{}_{Tac}$ (Figure 2C), only 30% of second deletion end-points mapping in the region between the pBR322 replication origin and the promoter, which resembles the distribution seen with pU3D. These results indicate that transcription affects deletion formation in an orientation-dependent manner.

To test whether transcription units other than P_{Tac} also affect deletion formation, we used the transcription unit P_L (Figure 1). The second deletion end-point was located between pBR322 replication origin and P_L in 98 and 24% of plasmids derived from pU3DP$^+{}_L$ and pU3DP$^-{}_L$, respectively (144 and 25 plasmids were analysed respectively; not shown), which is similar to the results obtained with pU3DP$^+{}_{Tac}$ and pU3DP$^-{}_{Tac}$ (Figure 2B and C). These results indicate that transcription convergent with the main pU3D transcripts and pBR322 replication directs deletion end-points to the region downstream of the promoter.

Transcription-directed deletions were not constrained to the region delimited by the promoter and the transcription terminator, but also ended downstream from the terminator (cf. Figure 2B). To examine whether this could be due to inefficient termination of transcription, we used plasmid pU3DGP$^+{}_L$ (Figure 1). This plasmid contains P_L and two λ terminators, t_L and t_0, to reduce the amount of potential transcriptional read-through. Distribution of second deletion end-points in pU3DGP$^+{}_L$ is shown in Figure 2D. All the end-points were located between the pBR322 replication origin and the P_L promoter but only 35% were found in the transcribed region. The density of the deletion end-points in this region, which represents about one half of the distance between the pBR322 origin and P_L, is therefore slightly lower than in the remainder of the deletogenic region. Since it is unlikely that the read-through past two terminators was significant we conclude that deletions are not constrained to the transcribed region.

Sequence analysis of end-points of 16 transcription-

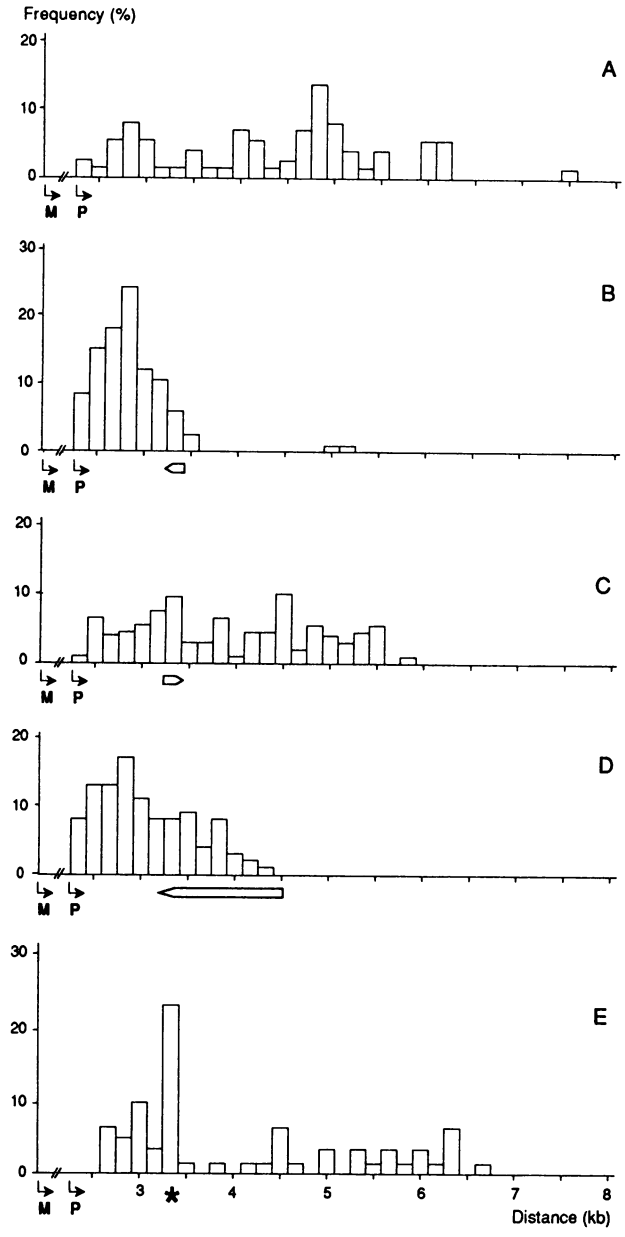

Fig. 2. Localization of the second deletion end-points. Bent arrows indicate the positions of the M13 (M) and pBR322 (P) replication origins, empty arrows the position and orientation of the transcription units, star the position of *lac* operator. Bars show the frequency of deletion end-points within each 170 bp interval situated at the indicated distance from the M13 replication origin. (**A**) pU3D, 73 plasmids were analysed; (**B**) pU3DP$^{+}_{Tac}$, 84 plasmids; (**C**) pU3DP$^{-}_{Tac}$, 106 plasmids; (**D**) pU3DGP$^{+}_{L}$, 103 plasmids; (**E**) pU3DP$^{+}_{Tac}$ in the presence of LacI, 60 plasmids.

directed deletions (seven plasmids derived from pU3DP$^{+}_{Tac}$ and nine from pU3DP$^{+}_{L}$, Figure 3A and B) revealed little or no homology between the M13 replication origin and the recombining regions (Figure 3C). Similarly, there was little homology between different end-points, although C and T were frequently present 4 and 3 bp, respectively, upstream of the deletion end-point and C was found in a majority of deletions 7 bp downstream from the deletion end-point (Figure 3D). The significance of this observation is not clear. Sequence-independent end-points are generally observed for deletions forming at the M13 replication origin (Michel and Ehrlich, 1986; Michel *et al.*, 1989).

A)	1158	C G A G G C G G C A T A A A T C G C C G T G A C
	1201	G G C G G C G C C T A C A A T C C A T G C C A A
	2271	A C A A T C T G C T C T G A T G C C G C A T A G
	2345	A A G A G C G C C T G A T G C G G T A T T T T C
	980	C C A T C C A G C C T C G C G T C G C G A A C G
	2446	C C C T G A T T C T G T G G A T A A C C G T A T
	1315	C T C C A A T T C T T G G A G T G G T G A A T C
	2424	A T T A C C G C C T T T G A G T G A G C T G A T
	2143	G G C A T C C G C T T A C A G A C A A G C T G T
B)	1074	C C C T G A T G G T C G T C A T C T A C C T G C
	1246	G C T C C A T G C A C C G C G A C G C A A C G C
	2472	C A C A T G T T C T T T C C T G C G T T A T C C
	2472	C A C A T G T T C T T T C C T G C G T T A T C C
	35371*	T T G G T G G T G T G T G G C A G T T G T A G T
	2274	A G T A C A A T C T G C T C T G A T G C C G C A
	2097	T G T C A G A G G T T T T C A C C G T C A T C A
C)	5769	T C C A C G T T C T T T **A A T A G T G G A C T C**
D)		**X X X X X C T X X** X X X X C X X X X

Fig. 3. Sequence analysis of deletion end-points. The sequences are presented in the 5′−3′ orientation, the numbers refer to pBR322 or λ (*) co-ordinates and indicate the nucleotide linked to the M13 replication origin. The sequences in light print are lost in the molecule and those in bold print are maintained. (**A**) Deletions formed in pU3DP$^{+}_{Tac}$; (**B**) in pU3DP$^{+}_{L}$; (**C**) DNA sequence of the M13 origin between nucleotides 5769 and 5792. No homology between the origin and the sequence flanking the deletion end-point was found for 7/16 molecules, homologies from 1 to 4 bp were detected in eight plasmids and a plasmid with 7 bp of homology (deletion at nucleotide 2472 of pBR322) was obtained twice. (**D**) Comparison of the 16 deletion end-points. X is any nucleotide. C and T were found four and three nucleotides, respectively, 5′ from the deletion end-point in 13/16 (81%) of cases, and C was found five nucleotides 3′ from the end-point in 9/16 (56%) of cases.

An operator-bound repressor can be a deletion hot spot

The results presented above indicate that properly oriented transcription directs deletion end-points to a region downstream of a promoter. Repression of transcriptional activity should therefore abolish the clustering of deletion end-points. To test this prediction we introduced plasmids carrying the promoters P_{Tac} or P_L into cells containing repressors LacI or C_I, respectively. Distribution of deletion end-points in pU3DP$^{+}_{Tac}$ is shown in Figure 2E. Two major differences were observed relative to the distribution in cells where P$^{+}_{Tac}$ promoter was not repressed (compare Figure 2B and E). First, the end-points were no longer constrained to a region downstream from the promoter. This confirms the prediction that repression of promoter activity abolishes clustering of deletion end-points. Second, a deletion hot spot was observed at the repressor binding site, where ~25% of the second deletion end-points were localized. Eight such deletion end-points were analysed by sequencing and all were found to be located within or just next to the P_{Tac} operator (Figure 4A). These results indicate that a repressor bound to the cognate operator can be a deletion hot spot. Orientation did not affect deletion hot spot activity of the bound Lac repressor, since 20% of the deletions ended very close to or within the P_{Tac} operator when plasmid pU3DP$^{-}_{Tac}$ was introduced in LacI-containing cells (not shown). Sequence analysis of four deletion end-points is summarized in Figure 4B.

The effect of repression on deletion formation was also tested using plasmid pU3DP$^{+}_{L}$ in *E.coli* cells expressing the λ repressor C_I (a JJC42-derived λ lysogen was used). There was no clustering of second deletion end-points in any of the derived plasmids, and the distribution profile resembled that of pU3D (not shown). This confirms that repression of transcription abolishes the clustering of deletions. However, in contrast to plasmids carrying P_{Tac},

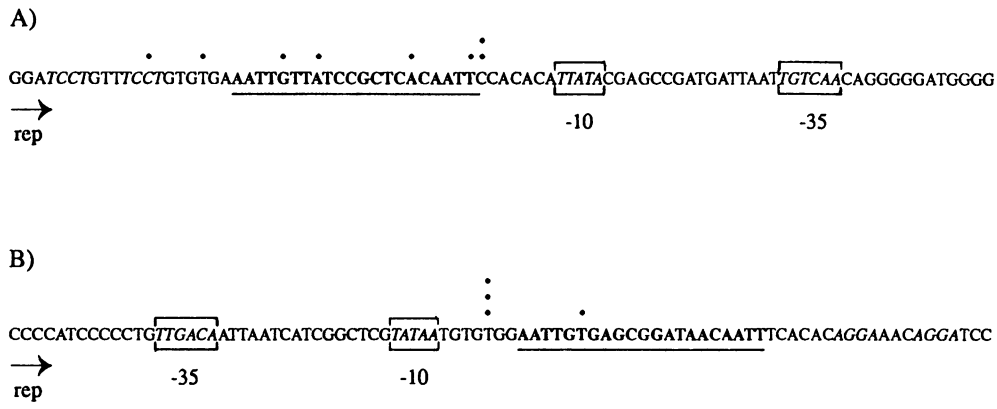

Fig. 4. Deletion end-points in (**A**) pU3DP$^{+}_{Tac}$ and (**B**) pU3DP$^{-}_{Tac}$ formed in the presence of LacI. The nucleotide sequence of the P_{Tac} promoter region is shown in the 5′−3′ orientation, leading strand synthesis initiated at pBR322 replication origin is coming from the left (rep, arrow). The P_{Tac} promoter −35 and −10 consensus sequences are boxed, the operator is underlined and is in bold print, the Shine−Dalgarno sequences are italicized. The asterisks indicate the positions of deletion end-points. Sequences 5′ from the end-points were joined to the M13 + strand at the origin nick site. For 4/8 pU3DP$^{+}_{Tac}$-derived plasmids, no homology was found at the junction and homologies from 1 to 3 bp were detected for the remaining molecules. One bp homology at the junction was found for the four pU3DP$^{-}_{Tac}$-derived plasmids.

repressor binding did not generate a deletion hot spot, since only 5% of deletion end-points mapped near the repressor binding site, irrespective of the orientation of the P_L promoter. These results indicate that different repressor−operator interactions have different capacities to direct deletion formation.

Effect of the orientation of pBR322 replication

Orientation of transcription units carried on pU3D affects the second deletion end-points (Figure 2), which suggests an interaction between the transcription within these units and another oriented process taking place on the plasmid. To test whether pBR322 replication, known to be unidirectional, might be this process, we inverted a fragment of pU3P$^{+}_{Tac}$ which carries the pBR322 replication origin. In the resulting plasmid, pU4P$^{+}_{Tac}$ (Figure 1), pBR322 replication and P_{Tac} transcription are co-directional. The overwhelming majority of plasmids derived from pU4P$^{+}_{Tac}$ (163 out of 165) resulted from deletions ending downstream from P_{Tac}. This result is similar to that observed with pU3P$^{+}_{Tac}$, which argues against interaction of transcription and pBR322 replication. To ensure that the deletions did not involve putative replication forks propagating in the opposite direction from the major pBR322 replication, we inserted the *E.coli* replication terminator *terB* (Hill *et al.*, 1988) upstream of the pBR322 origin (plasmid pU5P$^{+}_{Tac}$, Figure 1). A large majority of derived plasmids (80 out of 103) still resulted from deletions ending downstream of P_{Tac}, which confirms that pBR322 replication does not affect transcription-directed deletion end-points.

Interestingly, 80% of the transformants obtained with pU4P$^{+}_{Tac}$ contained, in addition to a plasmid carrying the β-lactamase gene, one or several plasmids resulting from deletions downstream of the β-lactamase promoter. In the smallest plasmids observed, the second deletion end-point must have been located just upstream of the RNAII promoter required for pBR322 replication. These plasmids do not confer Ap^r and are therefore counter-selected, which suggests that they are generated efficiently, possibly because of the transcription initiated at the β-lactamase promoter.

To test the effect of pBR322 replication on deletions at the operator-bound repressor, plasmids pU4P$^{+}_{Tac}$ and pU5P$^{+}_{Tac}$ were introduced in cells overproducing LacI repressor. Only two out of 40 and one out of 66 derived plasmids, respectively, could result from a deletion at the *lac* operator, as judged by size analysis. This is significantly lower than the 25% of deletion at this operator observed with pU3DP$^{+}_{Tac}$. This indicates that the deletions at the repressor-bound operator are due to replication of pBR322.

Discussion

In this work we investigated the influence of transcription on illegitimate recombination. The hybrid plasmids we used have several advantages. First, they undergo deletion with high frequency, since they transformed *rep*$^{-}$ *E.coli* with an efficency similar to that of a viable plasmid but the parental molecule, when present, was always accompanied by one or several derived plasmids. These had to be formed during the 30 generations of transformant selection and growth, at a deletion rate of at least 10^{-2} per cell generation. Secondly, one deletion end-point is highly specific, since in all characterized plasmids (a total of 202) it was localized at the M13 replication origin. This high specificity allowed us to map easily a large number of second deletion end-points (1310). To simplify the system the M13 rolling circle replication was inhibited by a *rep*$^{-}$ mutation.

We used two different promoters, P_{Tac} and P_L, and two different transcription terminators, λ t_0 and t_L, to show that a region downstream from an active promoter is rendered deletion prone in an orientation-dependent manner. The deletogenic region is not limited to the transcribed domain, which rules out any direct role of the transcription machinery in deletion formation. Dependence on orientation is not due to interaction of transcription and DNA replication, as shown by inverting the plasmid replication origin.

These results could be explained by postulating that plasmid superhelicity affects deletion formation. pU3D carries 14 transcription units, 13 of which are co-directional (counter-clockwise in Figure 1). Expression of a strong transcription unit (P_{Tac} or P_L) opposite to the main plasmid transcription could divide the molecule into two superhelical domains, positive (or relaxed) and negative, downstream and upstream from the cloned promoter, respectively (Liu and Wang,1987; Rahmouni and Wells, 1992). The former domain might be particularly deletion prone, by being more

Table I. Plasmids[a]

pHV727[b]	Replication origin of pC194 and Em^R gene of pE194 linked to pBR322
pHV750[b]	pHV727 deleted *in vitro* for the *Pvu*II−*Ava*I sequence (bp 2066−1425 of pBR322)
$pHV750P^+_{Tac}$	pHV750 carrying the P_{Tac} promoter and the λ t_0 transcription terminator
pU3D[b]	pHV750 linked to M13mp2 by *Eco*RI sites
$pU3DP_{Tac}$	pU3D carrying the P_{Tac} promoter and the λ t_0 transcription terminator
$pU3DP_L$	pU3D carrying the P_L promoter and the λ t_0 transcription terminator
$pU3DGP_L$	pU3D carrying the P_L promoter followed by the λ t_0 and the t_L transcription terminators
pHV770-P_{Tac}	pHV727 with the P_{Tac} promoter and λ t_0 terminator in the *Nru*I site in the + orientation and the *Pvu*II−*Aat*II fragment inverted
$pU3P^+_{Tac}$	as $pU3DP^+_{Tac}$ but with the entire pBR322 sequence
$pU4P^+_{Tac}$	pHV770-P_{Tac} linked to M13mp2 at *Eco*RI site
$pU5P^+_{Tac}$	*terB* cloned in $pU4P^+_{Tac}$ between nucleotides 3231 and 3152 of pBR322
$pACmLacI^q$	Replication region and Cm^R gene of pACYC184[c] linked to the $lacI^q$ gene of pSO1000[d]

[a]Nucleotide coordinates used for pBR322 are from Sutcliffe (1978) and Backman and Boyer (1983), those for M13 from Van Wezenbeck *et al.* (1980), those for pC194 and pE194 from Horinouchi and Weissblum (1982a, b).
[b]Michel *et al.* (1989).
[c]Chang and Cohen (1978).
[d]Oehler *et al.* (1990).

accessible to enzymes capable of introducing DNA breaks. We have previously proposed that deletions form at the M13 replication origin by joining the nucleotide 5′-phosphate, generated by the action of the gene II protein, to a non-adjacent 3′-hydroxyl nucleotide, which could result from breaks in DNA (Bierne *et al.*, 1991; Michel and Ehrlich, 1986; Michel *et al.*, 1989). For example, *E.coli* DNA gyrase, which normally removes positive supercoils and has been shown to affect illegitimate recombination (Ikeda *et al.*, 1981; Miura-Masuda and Ikeda, 1990), might act preferentially on the positively supercoiled plasmid domain and possibly generate 3′-hydroxyl ends. However, two observations make a direct role of DNA gyrase unlikely. First, there is no homology between the gyrase consensus sequence and the deletion end-points (Lockson and Morris, 1985, Figure 3) and secondly, the pattern of deletions observed with various plasmids did not change in a *gyr*A−*top*I double mutant (not shown). Other enzymes (nucleases?) should therefore be postulated to provide the required ends.

Inversion of the pBR322 replication origin, which modifies the direction of plasmid replication, also modifies direction of Ap^r gene transcription, and should therefore constrain the domain of positive supercoiling to the region downstream of the β-lactamase promoter. 80% of transformants obtained with the modified plasmid harboured one or more plasmids generated by deletions ending in this region. Even more such plasmids might have been formed and then been counter-selected, since they did not confer Ap^r. The additional appearance of deletion end-points upstream of the β-lactamase and downstream of the P_{Tac} promoter may simply reflect the greatest strength of P_{Tac}.

Another type of deletion, forming at the operator-bound LacI repressor, may be analogous to deletions resulting from binding of the Tus protein at the replication terminator *terB* (Bierne *et al.*, 1991). Two hypotheses might be considered to account for these. The LacI−RNA polymerase−operator complex could locally increase the DNA susceptibility to breakage and the resulting DNA ends could be linked to the nick at the M13 replication origin. Alternatively, the complex could slow down the progression of the pBR322 replication fork, and the temporarily arrested fork would be the deletogenic structure (Bierne *et al.*, 1991). Since the frequency of deletions at the *lac* operator sharply decreased when the replication of pBR322 was inverted we consider the second hypothesis more likely and suggest that other protein−DNA complexes capable of arresting, or slowing down, DNA replication might also be deletogenic.

Deletions were not stimulated by λ repressor, which suggests that its binding to the O_L operator does not interfere with the progression of the pBR322 replication fork. The repression complex formed at the *lac* operator is associated with the RNA polymerase (Straney and Crothers, 1987; Lee and Goldfarb, 1991) and is therefore likely to be more stable and more bulky than the O_L-C_I complex.

Our observations imply that processes which control gene expression can greatly influence the likelihood that the gene undergoes a deletion. This was shown here for a model system, using constructed transcription units and studying a particular class of deletions, forming at a nicked replication origin. However, other transcription units are likely to have a similar effect, as suggested by deletions forming within the β-lactamase gene. Futher work should show how general these implications might be.

Materials and methods

Bacterial strains and plasmids

Escherichia coli strain JJC42, which is an *hsdR rep* derivative of AB1157 (*thi-1 his-4 argE3 lacY1 galK2 ara-14 xyl-15 mtl-1 tsx-33 rpsL31 supE44 hsdR rep3*) was used as recipient for unstable plasmids and *B.subtilis* strain HVS49 (*trpC2 hisA aroB2 tyrA1*) was used for plasmid construction (Bierne *et al.*, 1991). Plasmids are listed in Table I. Unstable plasmids were constructed in two steps. First, the transcription units were inserted in plasmid derivatives devoid of M13mp2 sequences. Next, the resulting plasmids, which could be maintained stably in *E.coli*, were joined to M13mp2 and introduced in *B.subtilis* HVS49 harbouring the plasmid pHV1020 (Gros *et al.*, 1987), which provides the replication protein required to activate pC194 replication origin.

The three transcription units used were named P_{Tac}, P_L and GP_L. P_{Tac} consisted of the promoter P_{Tac} (de Boer *et al.*, 1983) followed by the phage λ transcription terminator t_0, P_L of the phage λ promoter P_L followed by a 5′-region of the λ N′ gene and t_0 and GP_L of P_L, the N′ gene inactivated by a mutation and two λ terminators, t_L and t_0 (Figure 1). The P_{Tac} promoter was purchased from Pharmacia as a 97 bp *Hin*dIII−*Bam*HI *tac* promoter gene block and P_L was cloned as a *Bam*HI−*Hpa*I fragment from the plasmid pPL-λ of Pharmacia. To obtain GP_L, a derivative of the pPL-λ vector was constructed in which the N′ gene was inactivated by insertion of an 8 bp oligonucleotide in the *Hpa*I site and the GP_L was excised as a *Bam*HI fragment. t_0 transcription terminator was cloned from plasmid pSS9 (Scholtissek and Grosse, 1987). The oligonucleotide used to insert *terB* (Hill *et al.*, 1988) in $pU5P^+_{Tac}$ was:

5′-CTGCAGAATAAGTATGTTGTAACTAAAGTAG-3′
3′-GACGTCTTATTCATACAACATTGATTTCATC-5′

This oligonucleotide was cloned in a *Sna*BI linker inserted between nucleotides 3232 and 3251 of pBR322 in pU4P$^+_{Tac}$, which gave plasmid pU5P$^+_{Tac}$.

Plasmid preparation and analysis, preparation and transformation of *B.subtilis* and *E.coli* competent cells have been described previously (Michel *et al.*, 1989). Restriction enzymes, ligase and polymerase were from commercial sources and were used according to the suppliers' recommendations. For overproduction of LacI in P_{Tac}-repressed experiments, we used the plasmid pACmLacI^q.

Deletion analysis

One of the deletion end-points is invariable and corresponds to the nick in the M13 replication origin, which allowed us to map the other deletion end-point to within 100 bp simply by measuring plasmid size. To determine this size, DNA of at least 20 independent transformants was extracted from 1.5 ml of overnight cultures as described by Birnboim and Doly (1979) and compared, intact or linearized by restriction enzyme cleavage, with standard size markers on agarose gels. The transformants generally contained several different plasmids and the proportion of plasmids in a given size range was expressed as a ratio of the number of plasmids within this range and the total number of detected plasmids, disregarding the relative plasmid amount in the transformant. To map deletions within 5–10 bp the plasmids of interest were digested with *Hin*fI and analysed on 4% Nusieve–agarose horizontal gels. The sequence of the deletion end-points was determined by the dideoxy sequencing method, using sequenase on double-stranded templates (Boehringer Mannheim sequencing kit was used).

Distribution of deletion end-points is not influenced by selection

Two types of plasmid were generated by deletions, preserving or losing the transcription unit carried by the parental plasmid. If the plasmids preserving the unit were less viable and therefore counter-selected in our experiment, the distribution of the deletion end-points would give a distorted image of the deletion frequency in different intervals. To test the viability of plasmids carrying a transcription unit we used pBR322 derivatives containing P_{Tac} in the + orientation, named pHV750P$^+_{Tac}$. This plasmid transformed *E.coli* cells as efficiently as the plasmids lacking P_{Tac}. The growth rates of two types of transformants were similar. In addition, pHV750P$^+_{Tac}$ derivatives were stably maintained in *E.coli* cells for 70 generations without selection (longer times were not tested) and the plasmid copy number did not vary during this period. We conclude that the plasmids carrying the P_{Tac} promoter were probably not counter-selected. This conclusion is corroborated by the observation that in 81% of the transformants obtained with pU3DP^+_L, the parental molecule was visible together with the plasmids, which suggests that plasmids retaining the P_L promoter would not have been counter-selected.

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