

Mutations produced by DNA polymerase III holoenzyme of *Escherichia coli* after *in vitro* synthesis in the absence of single-strand binding protein

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Summary

Single-stranded plasmid DNA, containing the *mnt* gene, was replicated *in vitro* with DNA polymerase III holoenzyme. *Escherichia coli mutH* bacteria, defective in mismatch repair, were transformed with the products of *in vitro* synthesis. Mutations in *mnt* were readily identified and 33 out of 65 isolates were single base changes including transition, transversion and frameshift mutations. The remaining 32 isolates were deletions of apparently random length and substitutions (deletion/insertions). The intergenic deletions as well as the transition and frameshift mutations were identical to those previously isolated from mismatch repair-defective cells *in vivo*.

Introduction

To clarify the mechanisms of spontaneous mutation in *Escherichia coli* we have determined mutation spectra in wild-type and various mutator (*mut*) strains using the *mnt* (maintenance) repressor gene of bacteriophage P22 as a target. These studies have shown that insertion sequence (IS) elements are the major cause of mutations in the wild type (Carraway *et al.*, 1987; Rewinsky and Marinus, 1987).

In contrast to the wild type, single base changes predominate in the spectra of *mut* bacteria. A *mutD5* strain predominantly yielded transversion mutations, suggesting that polymerase-associated proof-reading preferentially repairs potential transversion mispairs (Wu *et al.*, 1990). On the other hand, cells defective in Dam-directed mismatch repair produce mainly transition mutations (Carraway *et al.*, 1987; Rewinsky and Marinus, 1987; Wu *et al.*, 1990). These results suggest that the two repair systems act in a complementary fashion to correct replication errors. In addition to the repair systems

described above at least two others are known. These require the products of the *mutY* gene, which acts to repair G–A mispairs (Au *et al.*, 1988; 1989; Lu and Chang, 1988); the *mutT* product is required for repair of A–G mispairs (Akiyama *et al.*, 1989) and C–C mismatches are also repaired but the genes involved have not been identified (Radicella *et al.*, 1988). We know of no data which indicate that the VSP ('very short patch'; Lieb, 1983) system repairs T/G mispairs that arise from replicative errors.

To complement the *in vivo* data above, we have initiated studies in which the same target gene is replicated *in vitro* by DNA polymerase III holoenzyme (PolIII HE). This polymerase activity is primarily responsible for chromosome replication in *E. coli* (Kornberg, 1988). We wish to determine what kinds of mutations are produced in the *mnt* gene after *in vitro* synthesis and which repair system in *E. coli* prevents such mutations from being fixed. Eventually we should be able to catalogue, for each mutable base in the gene, the mispairs that can be formed at that position and their fate.

We have begun these studies by monitoring base mispairs that can result in transition mutations produced by PolIII HE. Replication was carried out on templates in the absence of single-strand binding protein (Ssb) in order to maximize mutation yield (Loeb *et al.*, 1980).

Results

Experimental system

We have determined mutation spectra in Dam-dependent mismatch repair-defective strains *in vivo* (including *mutH34*), using a pBR322 plasmid with an M13 *ori* and which had the *mnt* target gene cloned between the *EcoRI* and *HindIII* sites to produce an operon fusion (Carraway *et al.*, 1987; Rewinski and Marinus, 1987; Wu *et al.*, 1990). The wild-type *mnt* gene product represses transcription of the *tet* gene, while mutations inactivating Mnt repressor elicit transcription thereby imparting tetracycline resistance to cells (Fig. 1). In mismatch repair-deficient strains, AT to GC and GC to AT mutations in *mnt* predominate, suggesting that A–C and G–T mispairs are not corrected prior to replication.

We have used the same plasmid in this *in vitro* study since it allows for the easy isolation of single-stranded

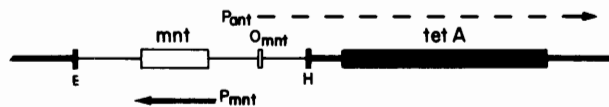


Fig. 1. The control of tetracycline resistance by Mnt repressor. Mnt repressor binds to O_{mnt} preventing rightward transcription of the *tet* gene from P_{ant} ; plasmids carrying this fusion confer a tetracycline-sensitive phenotype. Mutation in the *mnt* gene or O_{mnt} prevent repressor binding and permit transcription of the *tet* gene from P_{ant} . The thin line indicates the 500bp fragment of P22 DNA and the thick line indicates pBR322 sequences. E and H denote *EcoRI*- and *HindIII*-recognition sites, respectively.

template DNA and also because it allows us to compare results obtained *in vitro* and *in vivo*. We reasoned that after *in vitro* replication by PolIII, transition mismatches (A–C, G–T) in *mnt* would not be repaired in a *mutH* strain after transformation and should produce tetracycline-resistant mutations. Accordingly, an oligonucleotide primer was annealed to single-stranded circular plasmid DNA and extended with PolIII HE. After ligation, *mutH* cells were transformed to ampicillin resistance and tetracycline-resistant derivatives were identified by replica plating. Plasmid DNA was isolated and the *mnt* region sequenced to identify mutations. In the results presented below, we found only one mutation in *mnt* in each plasmid isolate.

There are two reasons why this technique was successful. First, the replica-plating screening for tetracycline-resistant derivatives among ampicillin-resistant cells has a low background level (less than 1 in 100 000). This low background is important because we assume that mutations are produced during *in vitro* synthesis and not after transformation in the host cell. We found that the mutation rate after transformation was about 10^{-5} . This value is higher than the spontaneous resistance to tetracycline in *mutH* cells transformed with either control (Mnt^+) double-stranded covalently closed circular plasmid DNA (10^{-6} – 10^{-7}) or after transfection of *mutH* cells with unreplicated single-stranded plasmid DNA (Mnt^+) which yielded tetracycline-resistant colonies at a frequency of about 10^{-7} .

A second reason for the success of the technique is that in *mutH* cells transformed with *mnt* heteroduplex plasmid DNA, colonies are formed which contain both tetracycline-resistant and -sensitive cells. The resistant clones can be detected by replica plating. In other words, the recovery of transition mutations is high. This high recovery rate holds for a variety of mismatched bases and for deletion/insertions of up to 18 bases (B. Parker and M. G. Marinus, unpublished data). The data presented below indicate that the actual recovery rate for deletion/insertions greater than 18 bases must also be high.

Single base changes

We have characterized 65 mutations in the *mnt* gene

obtained after *in vitro* synthesis. Of these, 33 were single base changes and these are listed in Table 1. Transitions, as expected, are the largest class (24/33), followed by transversions (6/33) and single base deletion/insertion (3/33). Since the sequence of the template is known, the actual mispairs formed during synthesis can be deduced. Both A–C and G–T mispairs were formed in both orientations (i.e. template-strand purine daughter strand pyrimidine and vice versa), but A–C mismatches outnumbered G–T by 21 to 3, respectively. Of the transversion mispairs, G–G, T–T and C–C were formed but not A–A, although this is probably not significant because of the small sample size.

The mutation spectrum in a *mutH* strain *in vivo* shows diagnostic 'hotspots' of AT to GC changes at nucleotides 41, 106 and 116 (Rewinski and Marinus, 1987). Since these are not apparent in Table 1 this indicates that the mutations cannot have arisen as replica-plating errors and were probably derived from *in vitro* replication mistakes. All the AT to GC and GC to AT changes as well as the frameshift mutation listed in Table 1 were also seen in mutation spectra obtained *in vivo* from mismatch repair-deficient strains.

Intergenic and intragenic deletions

Table 2 lists the deletions with one end in *mnt* and the other end in the gene coding for β -lactamase (*bla*). We define these as simple deletions in contrast to the more complex rearrangements shown in Table 4. Seven intergenic deletions were identified but this represents a lower

Table 1. Single base changes in the *mnt* gene.

No. of isolates	Base no. ^a	Base pair change	Actual mispair ^b
1	–34, –35	–T	
1	–33	GC to AT	G–T
2	–11	AT to GC	A–C
1	–7	AT to GC	T–G
1	25	–G	
2	40	GC to AT	C–A
1	40	GC to CG	C–C
1	41	AT to GC	A–C
2	52	GC to AT	C–A
1	53	GC to CG	G–G
1	56	AT to TA	T–T
1	71–73	+G	
1	110	GC to CG	C–C
5	110	GC to AT	C–A
1	115	AT to GC	A–C
3	116	AT to GC	A–C
1	121	GC to AT	G–T
1	122	AT to GC	A–C
1	125	AT to TA	T–T
1	130	GC to AT	C–A
3	139	GC to AT	C–A
1	152	GC to CG	C–C

a. Base numbering is in relation to the start site of transcription.

b. The first base in this column is in the template strand.

Table 2. Deletions with endpoints in *mnt* and pBR322 vector sequences.

No. of isolates	Base no.	Size of deletion	Sequence at fusion ^a
1	31-3305	1329	<div style="text-align: right; margin-right: 20px;">30</div> T G G C T A G A G A T G A T C C G C A C T C A C T G A T T A A G C A T T G G T A 3306
1	66-4191	410	<div style="text-align: right; margin-right: 20px;">65</div> T G C C T A T G G A A G T C A G G G A G A C A A T A A C C C T G A T A A A T G C 4192
1	92-4318	256	<div style="text-align: right; margin-right: 20px;">91</div> A A A T T C A G G G C G G A G G C G A A T T T T A T A G G T T A A T G T C A T 4319
1	114-4248	307	<div style="text-align: right; margin-right: 20px;">113</div> G G A G A T C A A T G A A C T C C G A G C G G A A C C C C T A T T T G T T T A T 4249
1	124-4248	317	<div style="text-align: right; margin-right: 20px;">123</div> G A A C T C C G A G T T G T T A C A A A C G G A A C C C C T A T T T G T T T A T 4249
1	235-4308	123	<div style="text-align: right; margin-right: 20px;">234</div> G A A G A T G G T G T T T G A T A C G C T T A A T G T C A T G A T A A T A A T G 4309
1	244-4178	245	<div style="text-align: right; margin-right: 20px;">243</div> G T T T G A T A C G C T G A A G G A T T T A A A T G C T T C A A T A A T A T T G 4179

a. For each deletion, the top and bottom lines are sequences within the *mnt* and the *bla* genes, respectively. The space in each line represents the deletion borders. The sequences in bold type remain in the deleted derivatives. The *bla* numbering is that for pBR322. Deletion 31-3305 confers ampicillin-sensitivity to cells bearing this plasmid.

limit because those sensitive to ampicillin are selected against in the isolation procedure. In the two instances in which cells containing ampicillin-sensitive mutated plasmids were isolated they arose on plates each containing several hundred ampicillin-resistant colonies. Presumably the crowded conditions led to a decrease in effective ampicillin concentration and allowed some sensitive cells to grow.

Table 3 lists simple deletions within the *mnt* gene. Note that the endpoints of the mutations in Tables 2 and 3 are variable with respect to DNA sequence, i.e. no short DNA sequence repeats were detected. The lengths of the deletions appeared to be variable. Computer-aided analysis of the DNA sequence in the fusion regions did not yield folded structures capable of generating these deletions, or any significant sequence homology within 20 base pairs on either side of the deletion endpoints.

Complex deletions/insertions

Twelve *mnt* mutations were complex rearrangements involving deletion and concomitant insertion of DNA bases

(Table 4). These occurred as both intergenic (Table 4A) and intragenic (Table 4B) events. Most of the intergenic events involve the *mnt* and *bla* genes, except for the first mutation (nucleotides -37 to 61) which has its endpoints in the *Mnt* operator and the *tet* gene, respectively. This results in an operon fusion which is constitutive for tetracycline resistance.

Of the *mnt-bla* intergenic mutations, those with ends at nucleotides 3275, 3294 and 3678 (pBR322 co-ordinates) are ampicillin-sensitive. Position 105 is represented five times out of six as an endpoint and the insertion sequence TCGGT (or some derivative of it) is seen in all cases.

In contrast, the deletion ends for the intergenic events appear more random. The deletion from -44 to -146 is the only other operator-constitutive mutation we have identified in this study. Since the operator region is only 1/25th that of *mnt*, only two to three mutations in this region are expected in this collection of 65 mutations.

For mutations 136-148 and 161-164, the insertion is the exact complementary sequence of regions 242-249 and 216-226, respectively. Furthermore, extensive base pairing flanking the complementary regions is predicted by

computer modelling. This suggests that these mutations could have arisen by base pairing of the flanking regions, followed by repair synthesis (Fig. 2). Figure 2 also shows that short direct repeats flanked, or were part of, the substitution. We were unable to detect similar sequence homology for the other mutations listed in Table 4, and consequently their origin remains obscure.

Discussion

The experimental system described here has allowed for

the identification of transition, transversion, frameshift, deletion and substitution mutations after *in vitro* synthesis by PolIII HE. This is a low estimate of the types and quantity of mutations since we have used only a *mutH* strain in this initial study. We plan to use other *E. coli* strains defective in recombination and repair to detect a greater variety of mutations as well as to determine the mechanism by which they originate.

The wide array of mutations obtained after *in vitro* synthesis by PolIII HE exceeds that of any *in vivo* spectrum of mutator strains (*mutD*, *mutH*, *mutL* and *dam*) we have

Table 3. Deletions in the *mnt* gene.

No. of isolates	Base no.	Size of deletion	Sequence at fusion ^a
1	21-233	213	<p style="text-align: right;">20</p> <p>G G A G T G A T G G C A T G G C T A G A A G A A G A T G G T G T T T G A T A C G 234</p>
1	25-175	151	<p style="text-align: right;">24</p> <p>T G A T G G C A T G G C T A G A G A T G C T G T G A C T G G A T A T C G C A A C 176</p>
1	31-37	7	<p style="text-align: right;">30</p> <p>C A T G G C T A G A G A T G A T C C G C A G A G A T G A T C C G C A C T T T A A 38</p>
2	33-223	191	<p style="text-align: right;">32</p> <p>T G G C T A G A G A T G A T C C G C A C G A G C T T G T T A A G A A G A T G G T 224</p>
2	45-138	94	<p style="text-align: right;">44</p> <p>A T C C G C A C T T T A A C T T C C G T A C A A A T C G T C C A A G A T G C T C 139</p>
1	121-158	38	<p style="text-align: right;">120</p> <p>A A T G A A C T C C G A G T T G T T A C T A T C A A A A C C A T C G C C T G T G 159</p>
1	136-163	28	<p style="text-align: right;">135</p> <p>G T T A C A A A T C G T C C A A G A T G A A A C C A T C G C C T G T G A C T G G 164</p>
1	136-217	82	<p style="text-align: right;">135</p> <p>G T T A C A A A T C G T C C A A G A T G C A G T C A G A G C T T G T T A A G A A 218</p>
1	145-158	14	<p style="text-align: right;">144</p> <p>C G T C C A A G A T G C T C T A T C A A T A T C A A A A C C A T C G C C T G T G 159</p>
1	158-170	13	<p style="text-align: right;">157</p> <p>C T A T C A A A A C C A T C G C C T G T C G C C T G T G A C T G G A T A T C G C 171</p>
1	159-279	121	<p style="text-align: right;">158</p> <p>T A T C A A A A C C A T C G C C T G T G C T G A C G G C G G G T T A A T T T T 280</p>

a. For each deletion, the top and bottom lines are sequences within the *mnt* gene. The space in each line represents the deletion borders. Sequences in bold are retained in the deletion derivatives.

those of Fersht and colleagues may stem from a variety of factors including the substrates, the holoenzyme preparations, the assay conditions, and the repair status of the host cells. Until some of these can be evaluated, a comparison is not meaningful.

A surprising feature of the transition mutations was that most arose from A–C rather than G–T mispairs. It is not immediately apparent as to why PolIII HE should produce the former more frequently than the latter. Perhaps residual repair activity in the *mutH* strain (if such is present) might preferentially remove G–Ts since they are corrected most efficiently (Su *et al.*, 1988). Alternatively, a repair system other than the Dam-dependent one may be removing mispairs. The VSP ('very short patch') system (Lieb, 1983), which removes G–T mismatches in specific sequences, is a possibility. VSP mismatches could occur at nucleotide positions –54, 87, 166, 172 and 272. We suspect that VSP repair is an unlikely explanation because mutations have never been recovered at these positions even in cells (*mutL*) defective in VSP repair (Wu *et al.*, 1990).

The transition mutation hotspots at nucleotides 41, 106 and 116, which are diagnostic for mismatch repair-deficient strains *in vivo*, were not seen *in vitro*. This indicates that such mutations are not produced at high frequency, relative to other transitions, by leading-strand replication. It is possible, however, that lagging-strand replication is responsible for their formation and we are currently testing this possibility. Alternatively, these mutations may be selected for during growth of the cells; perhaps they are dominant mutations analogous to *lacI*^{-d} alleles (Muller-Hill *et al.*, 1968).

The results of this study lead us to conclude that transition mismatches subject to repair by the Dam-dependent system are indeed errors of the replication process. This conclusion is based on the finding that identical mutations occur both *in vivo* and *in vitro* in *mutH* bacteria. Our results complement those of Schaaper and Dunn (1987), who proposed from *in vivo* data that mutations in *mutH*, *L*, and *S* strains reflect spontaneous replication errors. In addition to the transition mismatches, a frameshift mutation at a run of three Gs is seen in both cases and presumably results from 'slippage' of the polymerase on the template (Streisinger *et al.*, 1966). The data in this paper complement previous studies, using artificially constructed heteroduplexes, showing that the substrates for Dam-dependent mismatch repair are principally transition mispairs and frameshift-generating alterations (Claverys and Lacks, 1986; Radman and Wagner, 1986; Modrich, 1987; Meselson, 1988).

The intergenic deletions between *mnt* and *bla* have been found both *in vivo* (Wu *et al.*, 1990) and *in vitro*. It is unclear whether these and intragenic deletions are formed during *in vitro* replication or whether aberrant structures

are formed during *in vitro* replication which are subsequently processed in the *mutH* strain. An example of such processing could be illegitimate recombination by DNA gyrase action (Ikeda *et al.*, 1980) on relaxed circular substrates after entry of such molecules into the cell. The deletions we have isolated do not appear to have short repeated sequences at or near the deletion junctions and therefore probably do not arise by slipped mispairing (Streisinger *et al.*, 1966; Albertini *et al.*, 1982). Deletions have also been found after *in vitro* replication by other polymerases, for example *E. coli* DNA polymerase I (Papanicolaou and Ripley, 1989) and yeast DNA polymerase I (Kunkel *et al.*, 1989). Various models for the generation of deletions are discussed by these authors.

These models may also apply to the complex rearrangements listed in Table 4. It is also possible in this case that these mutations are artefactual since they have not been isolated *in vivo* and may be due to the absence of single-stranded binding protein in the reaction mixture. Alternatively, it is possible that there are systems in the cell that prevent their formation or repair the damage.

Experimental procedures

Bacterial strains, plasmid and phage

E. coli strain GM3856 is a *mutH::Tn5* derivative of MM294 (Meselson and Yuan, 1968). AB1874 is F-42 (*F-lac/lacY19*) and was obtained from Dr E. A. Adelberg (Yale University). GM2621 is pPY98/F-42/*recF143*, *thr-1*, *ara-14*, *leuB6*, *proA2*, *lacY1*, *glnV44*, *galK2*, *hisG4*, *rpsL31*, *xyl-5*, *mtl-1*, *argE3*, *thi-1*, *tsx-33*. Plasmid pPY98 has been described by Lucchesi *et al.* (1986). It is a derivative of pBR322 containing an M13 origin of replication in addition to the *immI* region of bacteriophage P22. Bacteriophage R408 was a gift from Dr M. Russel (Rockefeller University) and high-titre stocks were prepared by infection of strain GM2621. In agreement with Russel *et al.* (1986), we found this phage to be superior to IR-1 in its ability to package single-stranded plasmid DNA relative to phage DNA. Phage titres and transduction to ampicillin resistance were determined on strain AB1874.

In vitro synthesis

Single-stranded DNA, extracted from R408 particles after infection of GM2621, was mixed with the 15-mer *EcoRI* site primer (New England Biolabs), heated to 70°C for 15 min and allowed to cool to room temperature over a 15 min period. A 26 µl volume contained 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 200 µg ml⁻¹ bovine serum albumin, 0.5 µg single-stranded DNA (wild-type *mnt* gene), 1 µM primer, 5 mM dithiothreitol, 50 µM deoxyribonucleoside triphosphates (Pharmacia) and 100 Units of DNA PolIII HE (a gift of Dr Charles McHenry, University of Colorado). The reaction was started by the addition of PolIII and incubated for 60 min at 37°C (the absence of Ssb protein slows processivity). Under these conditions, at least 50% of the single-stranded DNA was converted into a double-stranded nicked circular form which was stable for at least 60 min. Control experiments showed that

heating the DNA (either single or double-stranded) at 70°C did not detectably alter the mutation frequency.

Ligation and transformation procedure

EDTA (1 mM), ATP (1 mM) and 1 µl (400 Units) of DNA ligase (New England Biolabs) were added and the mixture incubated overnight at 12°C. Portions were diluted in PCM buffer (10 mM PIPES buffer (Calbiochem), pH 6.8, 10 mM CaCl₂ and 10 mM MgSO₄) to a final volume of 50 µl and mixed with twice the volume of ice-cold competent cells and incubated for 5 min on ice. The mixture was transferred to 37°C for 5 min and 350 µl of pre-warmed L-broth was added. After incubation for 60 min, portions were spread on Brain Heart Infusion (BHI) broth (20 g l⁻¹; Difco) solidified with 1.6% agar and supplemented with 40 µg ampicillin per ml. The plates were incubated overnight at 37°C.

Competent cells of strain GM3856 were generally prepared freshly each day and transformed with plasmid DNA as described by Beckingham and White (1980). Cells from an overnight BHI culture were diluted a 100-fold and grown, at 37°C with aeration, to a Klett reading of 85. The cells were collected by centrifugation and resuspended in ice-cold 50 mM CaCl₂-10 mM PIPES buffer, pH 6.8. After 20 min of incubation on ice, the cells were collected by centrifugation and resuspended in fresh buffer. These cells could be stored at 4°C for two days without significant loss of transforming ability.

Identification and analysis of mutations in mnt

Ampicillin-resistant transformant colonies were replicated onto BHI agar containing 10 µg of tetracycline per ml and incubated overnight at 37°C. Tetracycline-resistant colonies were purified by streaking twice on the same medium. The cells were inoculated into 5 ml of BHI supplemented with ampicillin and grown to saturation. Plasmid DNA was extracted by the method of Birnboim and Doly (1979) except that two phenol extractions were included. Supercoiled plasmid DNA was sequenced by the methods of Chen and Seeburg (1985) and Zagursky *et al.* (1985) using DNA polymerase I, Klenow fragment (New England Biolabs) as described by Sanger *et al.* (1977).

DNA sequence analysis

The programs of Conrad and Mount (1982) and Mount and Conrad (1984), version 3.8, were used for DNA sequence analysis. These programs were obtained from Dr D. W. Mount, University of Arizona, Tucson, AZ 85721, USA. The program PCFOLD, version 3.0, was used to determine the ability of single-stranded DNA to fold. It was obtained from Dr M. Zuker, National Research Council of Canada, Ottawa K1A 0R6, Canada.

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