Inhibition of DNA Repair by Neighbouring Mismatched Bases in *Streptococcus pneumoniae*

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(Received 31 May 1988; revised 25 July 1988)

A set of pneumococcal strains containing immediately adjacent or nearby double mutations at the *amiA* locus, conferring resistance to amethopterin, has been isolated by oligonucleotide site-specific mutagenesis. Repair of these double mutations has been measured by transformation of wild-type strains with DNA extracted from these strains. In several transformations we have observed an inhibition of repair by neighbouring mismatches. This inhibition ranges from mild to severe depending upon the interfering mismatch. Unrepaired mismatches can strongly inhibit repair of an adjacent repairable mutation. This suggests that the repair-complex proteins attach not only to repairable mismatches but also to some mismatches known to escape the repair system.

INTRODUCTION

A central role for DNA polymerase in the fidelity of DNA replication has been documented in numerous studies (Drake *et al.*, 1969; Ripley, 1981; Ripley & Shoemaker, 1983). *In vitro*, using well-characterized multi-enzyme complexes, the errors in DNA replication are several orders of magnitude more frequent than spontaneous mutations observed *in vivo*. It has been suggested that a post-replication repair mechanism efficiently contributes to fidelity (Hibner & Alberts, 1980) and that the most frequent errors of replication would be specifically eliminated by a correction process (Radman & Wagner, 1986). The first evidence for such a correcting system was obtained in studies of spontaneous mutants at the *amiA* locus in *Streptococcus pneumoniae*. These mutants occur at high frequency because the locus is quite long and it contains several successive open reading frames (Gasc, 1987). The spontaneous mutation rate was found to be higher in a strain unable to repair mismatched bases at the *amiA* locus (Tiraby & Sicard, 1973) as well as in other genes (Tiraby & Fox, 1973). The spontaneous mutants which are efficiently eliminated by the wild-type repair system were eventually sequenced. The mutations were shown to be either transitions, or one- or two-base additions or deletions (Claverys *et al.*, 1981*b*, 1983; Lacks *et al.*, 1982; Gasc & Sicard, 1986; Gasc *et al.*, 1987). As the frequency of transitions and transversions remaining after repair is similar, it is likely that the repair system corrects the most common errors in DNA replication, i.e. transitions.

The mismatch repair system that eliminates some errors of replication also acts at the level of heteroduplex DNA during recombination, where it is more easily studied. Recombination between markers during transformation in *S. pneumoniae* depends strongly on the marker scored (Ephrussi-Taylor *et al.*, 1965; Lacks, 1966). It is possible to correlate mutational changes in the DNA of each marker with its ability to be repaired (reviewed by Claverys & Lacks, 1986): both G/T and C/A mismatches resulting from transitions are most prone to repair, as well as G/G which results from a transversion; most C/T mismatches are repaired; A/A and T/T are partially repaired; A/G is poorly or not at all repaired; C/C has never been found to be repaired.

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One- or two-base additions or deletions are most frequently repaired whereas longer heterologies (more than five base-pairs) completely escape this repair system (Gasc *et al.*, 1987).

The repair mechanism excises several kilobases of DNA to eliminate a single mismatched base (Mejean & Claverys, 1984). This accounts for the observation that a mismatch prone to repair can provoke the repair of a non-repairable mismatch according to the distance between them (Lacks, 1966; Sicard & Ephrussi-Taylor, 1966; Sicard *et al.*, 1985). However, when two mismatches are separated by very few base-pairs, we have observed some cases in which repair of both can be depressed (Claverys *et al.*, 1983). Studies of this latter effect require construction of very closely linked markers that is quite difficult to achieve by classical genetic means but is feasible by site-directed mutagenesis.

In this paper we describe mutants obtained by this method. They contain nearby or adjacent double-base substitutions. We demonstrate that the double mutations create interferences in the process of excision and repair of mismatched bases, suggesting that the repair protein complex can attach to a non-repairable mismatch without effecting its repair.

METHODS

Bacterial strains and genetic analyses. The pneumococcal strains used in this study were derivatives of the R6 strain. Amethopterin-resistant mutants at the *amiA* locus (Sicard & Ephrussi-Taylor, 1965) were isolated in strain R6 *hex*⁻ 801 (Lefevre *et al.*, 1979) and were resistant to a concentration of 10⁻⁵ M-amethopterin. Isolation and purification of DNA, and transformation procedures, were similar to those described by Claverys *et al.* (1981*a*). Efficiency of transformation was measured in strain R6 *hex*⁺ 800 (Lefevre *et al.*, 1979) as the ratio of amethopterin-resistant transformants to streptomycin-resistant (*str*-41) transformants.

Statistical analysis of efficiencies was performed by the method of Kimball (1961). Briefly, if *x* and *y* are the total number of colonies on each selective medium, and *Z* is the ratio *x/y*, then the variance of *Z* is

$$V_z = \frac{x}{y^3} (x + y)$$

This calculation assumes that *x* and *y* follow a Poisson distribution. This is a reasonable assumption since *x* and *y* are always larger than 100 and usually more than 500.

Induction of *amiA* mutants by oligonucleotide-directed mutagenesis. Wild-type fragments A1 and B2 belonging to the *amiA* locus (Gasc *et al.*, 1987) were cloned in phage M13 derivatives (Zoller & Smith, 1982). Single-stranded DNA was extracted and hybridized with synthetic oligonucleotides complementary to the wild-type pneumococcal sequence except for the bases to be substituted. The procedures described by Kramer *et al.* (1984) and by Norris *et al.* (1982) were used to obtain these mutants in *Escherichia coli*. They were sequenced and the M13 replicating form was transformed into strain R6 *hex*⁻ 801, deficient in mismatch repair. It was possible to induce these mutants directly in pneumococcus by another procedure, as follows. M13 DNA containing the cloned A1 or B2 fragments was hybridized to the required oligonucleotide, and second-strand polymerization and ligation was carried out as described by Kramer *et al.* (1984). After linearization by *Bgl*II, samples were used to transform the wild-type pneumococcal strain 801 and amethopterin-resistant transformants were selected. Up to 2 × 10⁵ mutants ml⁻¹ were obtained. Unstable strains that resulted from rare insertion-duplication events were discarded. Stable mutants were intercrossed to ascertain their homoallelism. One transformant for each site-directed mutagenesis was sequenced.

RESULTS

Six double mutants in the A1 fragment and one in the B2 fragment of the *amiA* locus have been obtained by site-directed mutagenesis. All of them contain a non-sense mutation yielding the highest level of resistance to amethopterin. They consist either of adjacent base substitutions or of substitutions separated by one, two or three base-pairs. The base changes involve either transitions associated with several classes of transversions, similar transversions or a transversion with an A/T deletion (Table 1). Such a variety of double mismatched bases enables us to address the question: Is a repairable or an unrepairable mismatch able to interfere with the repair of a second and neighbouring mismatch?

Repair can be measured by transformation efficiency since the respective values are inversely related (Gray & Ephrussi-Taylor, 1967). The results of these experiments are reported in Table 1. The transformation efficiency of strain *amiA*-135 is 0.15, the same as that found for fully

Table 1. Transformation efficiency of double mismatches

Wild-type bacteria were transformed by DNA carrying a double mutation and the reference marker *str-41*. Transformation efficiency is the ratio of amethopterin transformants to streptomycin transformants. Without interference, the predicted transformation efficiency is the efficiency of the best-repaired single-site mutation: when both heteroduplexes are repaired this value is 0.15 on the average; when only one heteroduplex DNA is repaired this value is 1.20. Repair is inversely related to transformation efficiency. Sequences are written in the order 5'→3' corresponding to the mRNA strand. A1, wild-type sequence in the A1 fragment of the *amiA* locus in the *amiA*-36 region; B2, wild-type sequence in the B2 fragment of the *amiA* locus in the *amiA*-128 region (Gasc *et al.*, 1987). Statistical fluctuations were calculated as described in Methods.

Strains	Sequence	Mismatched heteroduplexes	10 ⁻⁵ × No. of <i>amiA</i> transformants ml ⁻¹	10 ⁻⁶ × No. of <i>str-2</i> transformants ml ⁻¹	Transformation efficiency
<i>amiA</i> ⁺ (A1)	CCAGATTTCAT				
<i>amiA</i> -135	-----AG-	$\frac{CA}{T}$ and $\frac{GT}{CAG}$	4.89 3.82	3.20 2.73	0.15 ± 0.02 0.14 ± 0.03
<i>amiA</i> -142	----G--A--	$\frac{A--C}{C--T}$ and $\frac{T--G}{G--A}$	1.75 1.46	7.11 5.20	0.25 ± 0.03 0.28 ± 0.04
<i>amiA</i> -148	-----C-A--	$\frac{T-C}{G-T}$ and $\frac{A-G}{C-A}$	2.02 1.32	7.33 5.51	0.27 ± 0.03 0.24 ± 0.03
<i>amiA</i> -145	-----A-C	$\frac{C-T}{T-G}$ and $\frac{G-A}{A-C}$	1.08 0.82	3.62 3.15	0.30 ± 0.04 0.26 ± 0.03
<i>amiA</i> -136	TG-----	$\frac{CC}{AC}$ and $\frac{GG}{TG}$	29.8 13.4	4.30 1.81	0.69 ± 0.09 0.74 ± 0.07
<i>amiA</i> -146	-----G-A--	$\frac{T-C}{C-}$ and $\frac{A-G}{TG-A}$	67.2 23.5	5.60 2.20	1.20 ± 0.12 1.07 ± 0.10
<i>amiA</i> ⁺ (B2)	CGTTTTAATG				
<i>amiA</i> -126	--TTTTT--T	$\frac{T---G}{AA---A}$ and $\frac{A---C}{TT---T}$	3.69 1.49	1.15 4.95	0.32 ± 0.04 0.30 ± 0.05

repaired single mismatches (Gasc & Sicard, 1986). One of the substitutions in this strain is a transition which is always repaired. Therefore the A/G mismatch which is not repairable does not interfere with the repair of adjacent G/T and C/T mismatches and does not modify the repair of A/C. This absence of interference between highly and poorly repairable markers when they are adjacent is similar to the same observation when they are far apart (Lacks, 1966; Gray & Ephrussi-Taylor, 1967; Sicard *et al.*, 1985).

An interference was found, however, when strain *amiA*-142 was used in transformation. In this strain, the same mismatches as in *amiA*-135 are separated by two base-pairs. The efficiency of transformation was twice the value expected if there was no interference. Therefore at least one of the three mismatches A/C, T/G, C/T, which are individually repairable, partially escapes correction due to the presence of another mismatch located two base-pairs away.

A similar interference was found for the two strains *amiA*-148 and *amiA*-145. The base substitutions here result from two transitions, one of them being the same transition, C/G → A/T, as in strain *amiA*-142. A similar result was also obtained when a fully repairable mutation (addition of A/T) was present three bases away from the mismatches A/G and C/T, in strain *amiA*-126.

The inhibition reported above was small but reproducible. It became much larger in strain *amiA*-136, where the efficiency of transformation was increased fourfold over that obtained with fully repaired mutations (Table 1). In this case, a mismatch derived from a transition is adjacent to a C/C or a G/G mismatch. At least one heteroduplex is poorly repaired. It was possible to determine which heteroduplex is repaired because the elimination of a mismatch extends over several kilobases and will depress transformation efficiency if the excluded strands are opposite. This depression is a function of the distance between the markers (Sicard & Ephrussi-Taylor, 1966). We have used strain *amiA*-9 *rev*-81, which results from a C/G → G/C transversion. In a

cross between this strain and wild-type DNA, the conserved mismatch is C/C whereas G/G is fully repaired. When *amiA-9 rev-81* was transformed by *amiA-136 str-41* DNA, the efficiency of transformation was 0.38. This is much lower than the level of non-repaired mismatches (1.2). This depression, which is not reduced to the basic level (0.15) due to recombination between the markers, cannot result from the elimination of the G/G mismatch at site *amiA-9*, which is anyway fully repaired. Therefore the conserved double mismatch is AC/CC in the pairing between wild-type and *amiA-136*. The mismatch C/C, which alone is unrepaired, strongly inhibits repair of the adjacent A/C mismatch. This is an agreement with a previous observation at another site (Claverys *et al.*, 1983).

A question that one might ask is whether the close association of two identical mismatches known to be poorly repaired would induce some repair. Pairing between *amiA-146* and wild-type involves the A-G/G-A double mismatches which are not repaired when alone. Results indicate that this structure is not repaired because the transformation efficiency of *amiA-146* by wild-type DNA is 1.20, i.e. the same efficiency as observed when only one of these mismatches is present (Table 1). Moreover, using the same methodology as for strain *amiA-136*, we have found that the only repaired mismatches are T-C/C-T. For this double mutant there was no interference.

DISCUSSION

The main results reported in this work show that some unreparable mismatches are able to inhibit repair at a nearby site. This effect can be small or much larger depending upon the mutations. It thus seems possible that in double mutants not only the nature of the mismatches but the neighbouring sequences may affect repair, as has been reported for some single-site mutations (see Claverys & Lacks, 1986, for review). Moreover, as for single-site mutations (Modrich, 1987), it is impossible to attribute any physico-chemical state of the DNA to account for these interferences.

Although the concept and the first evidence for excision and repair of mismatched bases were reported long ago in pneumococcus by Ephrussi-Taylor & Gray (1966) to account for the elimination of single-site mutations during recombination, the mechanism of repair is only partially understood. From the facts that long deletions are not repaired by this system (Ephrussi-Taylor *et al.*, 1965; Lacks, 1966) and that the most systematically repaired mismatches are transition mutations, it was obvious that this process can accommodate only a very limited distortion of the DNA helix. This concept was further substantiated by the demonstration that the efficiency of repair is inversely proportional to the size of the heterology, as if looped-out structures could occur that escape recognition by the repair system (Gasc & Sicard, 1986). In fact, nuclear magnetic resonance studies of oligonucleotides have shown that a transition mismatch is more often intra-helical than an unrepaired transversion mismatch (Fazakerley *et al.*, 1986), supporting the role of DNA structure for repair specificity of mismatched bases. Extra-helical structures are more likely to occur when more than two mutations are clustered. This could explain why *amiA-29*, a spontaneous mutant, is not repaired (Ephrussi-Taylor *et al.*, 1965). This strain contains three adjacent mutations, one of them being the addition of a base-pair (Claverys *et al.*, 1981*b*) that by itself would be repaired.

However, DNA structures alone cannot explain some differences in repair efficiency between pneumococcus and *E. coli* or other organisms like yeast. In pneumococcus, C/T is repaired most of the time, and the complementary mismatch A/G is not repaired, whereas in *E. coli* these two mismatches are both repaired at the same level. A difference between pneumococcus and yeast is that in the latter organism A/A and T/T are repaired at the same level as transitions, which in turn are less repaired than single-base insertions (White *et al.*, 1985; Jones *et al.*, 1987). It would appear, therefore, that proteins recognizing repairable mismatches differ slightly in their specificity from one organism to another. Their affinity for some mismatched bases must depend upon neighbouring sequences in order to account for differences in repair efficiency between identical transversions located at several sites in the same organism.

To explain the reported inhibition of repair by an unreparable nearby mismatch we propose that one or several proteins of the repair complex are able to bind not only to the repairable

mismatches but also to others, especially C/C. This binding would inhibit attachment of this complex to nearby mismatches that are individually repairable. This model is supported by the demonstration that purified *mutS* protein, the *E. coli* homologue to the pneumococcal *hexA* protein of the repair complex (Claverys & Lacks, 1986), binds not only to repairable mismatches but equally well to unrepairable ones (Su & Modrich, 1986). The repaired mismatches are presumably further processed by an exonuclease activity on donor DNA by the *hex* system. This complex would travel along the DNA molecule and would degrade the free ends of donor DNA before its ligation to the chromosomal DNA as proposed by Guild & Shoemaker (1976). Such processing would not occur for unrepairable single-site mismatches like C/C that may hold onto the protein complex. One could expect then that competition for the repair complex occurs between neighbouring mismatches. However, there is no direct biochemical evidence in support of this hypothesis since the products of the pneumococcal *hex* genes have not been purified. The specificity of the systems that repair mismatched bases at the replication or the recombination levels would result from the dual effect of DNA secondary structure and the recognition by the protein complex at repairable mismatches.

We thank B. Stevens for help in preparing the manuscript. P. Garcia was supported by a fellowship from the Fundacion Juan March.

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