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Mismatch repair in *Streptococcus pneumoniae*: Relationship between base mismatches and transformation efficiencies

(marker effect/Hex system/DNA sequence analysis/bacterial transformation)

JEAN-PIERRE CLAVERYS, VINCENT MÉJEAN, ANNE-MARIE GASC, AND A. MICHEL SICARD

Centre de Recherche de Biochimie et de Génétique Cellulaires du Centre National de la Recherche Scientifique, 118 route de Narbonne 31062 Toulouse-Cedex, France

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ABSTRACT **Genetic transformation in *Streptococcus pneumoniae* involves the insertion of single-stranded pieces of donor DNA into a recipient genome. Efficiencies of transformation strongly depend on the mutations (markers) carried by donor DNA. Markers are classified according to their transforming efficiencies into very high, high, intermediate, and low efficiency. The last is approximately 1/20th as efficient as the first. This marker effect is under the control of the Hex system, which is thought to correct mismatches at the donor–recipient heteroduplex stage in transformation. To investigate this effect, wild type, mutant, and revertant DNA sequences at five genetic sites within the *amiA* locus were determined. The results show that low-efficiency markers arise from transitional changes A·T to G·C. The transversion A·T to T·A corresponds to an intermediate-efficiency marker. Transversions G·C to T·A and G·C to C·G lead to high-efficiency markers. Among the eight possible mismatches that could exist transiently at the heteroduplex stage in transformation, only two—namely, A/G and C/C—are not corrected by the Hex system. It is noteworthy that the four possible base pairs (A·T, T·A, G·C, and C·G) have been encountered at the very same site (*amiA6* site), which constitutes a good illustration of the marker effect. DNA sequence analysis also reveals that short deletions (33 or 34 bases long) are integrated with very high efficiencies. These results confirm that the Hex system corrects point mismatches harbored in donor–recipient heteroduplexes thousands of bases long. The correction pattern of the Hex system toward multiple-base mismatches has also been investigated. Its behavior toward double-base mismatches is complex, suggesting that neighboring sequences may affect the detection of mispaired bases.**

Genetic transformation in *Streptococcus pneumoniae* involves the insertion of single-stranded fragments of donor DNA into the chromosome of recipient bacteria (1–3). The frequency depends strongly on the marker scored (4, 5). Discrete classes of transformation efficiency were described for markers belonging to the same gene: very high (VHE), high (HE), intermediate (IE), and low (LE) efficiency, in the relative ratio of 2.0:1.0:0.4:0.15 (4–6). VHE markers yield transformants approaching one per genome equivalent of donor DNA taken up by the cells. Excision and correction of donor DNA induced by mismatched base pairs of donor–recipient heteroduplexes was proposed to account for the low efficiency of some markers (7). The existence of such a cellular mechanism was substantiated by the identification of mutant strains, denoted Hex$^-$, that are transformed with very high efficiency by single-site markers (8, 9). Resolution of the complementary strands and *in vitro* preparation of heteroduplex molecules led to measurement of the efficiency of individual strands (10). For LE markers, light (L) and heavy (H) strands are equally low in efficiency (11); both are high in efficiency for VHE markers (11, 12). For HE markers, one strand is low and the other is high in efficiency; this strand-preference property is also controlled by the Hex system (12). Within the same gene, the high-efficiency strand can be either the L or the H strand depending on the HE marker (11). This is interpreted as correction of one of the two reciprocal mismatched base pairs. Additional evidence for correction of mismatched base pairs at the donor–recipient heteroduplex level comes from studies of the kinetics of destruction of LE markers: these are consistent with elimination of the marker after heteroduplex DNA has been formed (13). To understand the relationship between base mismatches and transformation efficiencies, we have used DNA sequence analysis to define changes at five mutant sites within the *amiA* locus. Mutations within this locus confer resistance to aminopterin and have been widely used as markers in transformation studies. The ability to select both aminopterin-resistant (amiA$^-$) and aminopterin-sensitive (amiA$^+$) phenotypes (14) was used to construct a genetic map (15), to correlate it with a restriction map (16), and to clone *amiA* fragments (17, 18). We report DNA sequence analyses of mutational changes within this locus and their correlation with transformation efficiencies. A preliminary report of these results was given at the International Conference on Streptococcal Genetics, November 1981, Sarasota, FL (19).

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Markers. All pneumococcal and *Escherichia coli* strains used in this study were previously described (17). Ten recombinant plasmids carrying fragments of the *amiA* locus with various markers (see Fig. 1) were used for determination of nucleotide sequence: pR10 (*amiA9* and *amiA29*), pR15 (*9rev*), pR16 (*29rev*), pR21 (*6rev2*), pR22 (*6rev3*), pR23 (*6rev4*), pR32 (*amiA6*), pR33 (*amiA10*), pR34 (*amiA22*), and pR35 (*amiA141*) (18). *9rev* etc. refers to revertants to wild or pseudo wild type at these sites (see below).

Genetic Analysis. Culture medium, transformation procedure, and selection of transformants were similar to those described (17). Introduction of the *amiA144* mutation into *6rev2*, *6rev3*, or *6rev4* recipients was done by transformation of these recipients with DNA of the recombinant plasmid pR4 (pBR325::B*amiA144*; ref. 17), which carries the *Eco*RI B fragment of the *amiA* locus, covering the area to the right side of the *amiA6* site (see Fig. 1). Similarly, the *amiA150* mutation was introduced by transformation of *6rev2*, *6rev3*, and *6rev4* recipients with DNA from the recombinant phage λ::B*amiA150* (17). Construction of the pneumococcal strain carrying

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Abbreviations: H (heavy) and L (light) refer to the buoyant density of DNA strands complexed with poly(G,U) in CsCl density gradients; VHE, very high efficiency; HE, high efficiency; IE, intermediate efficiency; LE, low efficiency.

both *amiA9* and *amiA29* mutations has been described (20).

DNA. Plasmid DNA was prepared as described (17). Isolation of native pneumococcal DNA, resolution of complementary strands, and preparation of heteroduplex DNA were as described (11).

Chemicals and Enzymes. *Bam*HI, *Eco*RI, *Nco* I, and *Rsa* I restriction endonucleases were purchased from New England BioLabs and used as recommended by the manufacturer. Bacterial alkaline phosphatase was from Worthington, and polynucleotide kinase was from P-L Biochemicals. Chemicals used have been described (21).

Nucleotide Sequence Determination. Nucleotide sequence was determined by the method of Maxam and Gilbert (22). End labeling and sequence analyses were as described (21).

RESULTS

Choice of Markers. The *amiA22*, *amiA29*, *amiA9*, *amiA6*, *amiA10*, and *amiA141* mutations were selected for DNA sequence determination because they covered all efficiency classes. Their map positions are shown in Fig. 1. Because genetic results suggested that point mismatches could give all efficiency classes, we focused our attention on a few sites and tried to obtain reversions of some mutants, hoping to get all possible base-pair changes at the same site. Spontaneous revertants to wild phenotype occur (14) but frequently are not true revertants, because the integration efficiency of each mutation is changed when strains carrying their respective reversions are used as recipients (5, 23) (e.g., *29rev*, a reversion of *amiA29*; *9rev*, a reversion of *amiA9*; see ref. 23). We have isolated three different reversions of *amiA6* (*amiA10*), two mutations that turn out to be identical (see below).

Integration Efficiencies. In the wild type, *amiA22*, *amiA29*, and *amiA141* are HE markers with a preferred (more efficient) H strand (11). *amiA9*, *amiA6*, and *amiA10* are LE markers. In the *9rev* recipient, *amiA9* behaves as a HE marker with a preferred L strand (11). In the *29rev* recipient, *amiA29* becomes a VHE marker (6). Results obtained for *amiA6* (*amiA10*) in their three reversions are shown in Table 1. In both *6rev2* and *6rev4*, *amiA6* becomes a HE marker but with opposite strand preference: L strand preferred into *6rev2* and H strand preferred into *6rev4*. In the *6rev3* recipient, *amiA6* behaves as an IE marker.

Mutational Changes. The strategy for determination of nucleotide sequence is shown in Fig. 1*B*. All the mutations used are located on a 1,050-base-pair-long *Bam*HI/*Eco*RI fragment. The wild-type sequence for >300 bases from the *Bam*HI site has been published (21). The wild-type sequence from the *Eco*RI site is shown in Fig. 1*A*. In both regions, and in other places within the *amiA* locus (21), only one reading frame is open out of a possible six. The transcribed strand, identified as the H strand by phenotypic expression studies (11), appears to be the r chain, and the locus is transcribed from left to right. Mutational changes observed at the five genetic sites studied are shown in Fig. 2. With the exception of *amiA29*, all mutations are point mutations that introduce nonsense codons in the open reading frame: amber for *amiA22* and *amiA141*, ochre for *amiA9*, and opal for *amiA6* and *amiA10*, which appear to be the same mutation. The *amiA29* mutation is a complex change: the sequence A-T-G-G-A-T is mutated to A-T-T-T-G-C-T, which results in a frameshift of +1 base. This frameshift mutation leads to a stop codon a few bases downstream. Fig. 2 also shows results obtained for revertant sequences. Each reversion, *29rev* excepted, was a single base-pair change from a nonsense to a sense codon. The reversion *29rev* restores a normal reading frame by deletion of 34 bases that cover the *amiA29* mutation. At site 6,

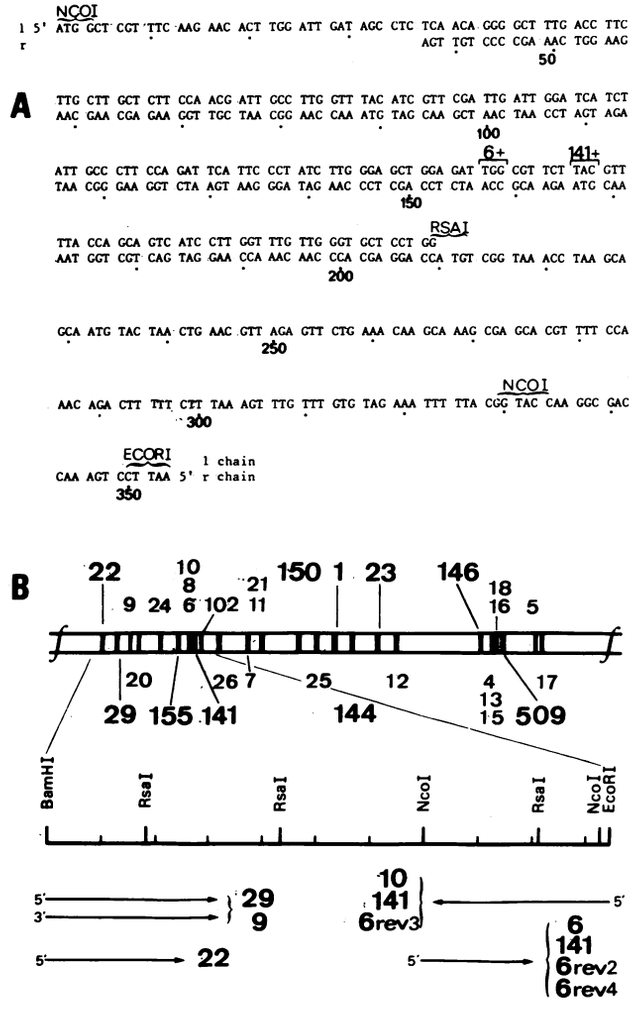

FIG. 1. Genetic map of the *amiA* locus, strategy for determination of nucleotide sequence, and partial sequence of the wild-type *amiA* fragment. (*A*) r and l, rightward and leftward transcribed chains. Nucleotides are numbered from the internal *Nco* I site toward the *Eco*RI site. According to this definition the site of the *amiA6* mutation is at position 159, and the site of *amiA141* is at position 168. As observed for the sequence from the *Bam*HI site, only one reading frame out of a possible six is open, and the *amiA* locus is transcribed from left to right (*B*) from the r chain. (*B*) *Upper*, markers are ordered according to map position established from genetic crosses (15). Large numbers correspond to HE and VHE markers; small numbers correspond to LE markers. *Lower*, arrows represent orientation and size of sequence determined by using the individual plasmid carrying the mutation. The *Bam*HI/*Eco*RI fragment is 1,050 base pairs long (21). A restriction map of whole region is shown in ref. 19.

four different base pairs corresponding to wild type, mutant, *6rev2*, and *6rev3* sequences were found at the same position. Reversion to *6rev4* occurred on a different base of the same triplet.

Relationship Between Mismatches and Transformation Efficiencies. When donor and recipient DNA differ by one base change, four pairs of mismatches are expected. All four have been found in this study and related to transforming efficiency (Table 2). A transitional change results in a LE marker. The two mismatches A/C and G/T are equally strongly corrected, because there is no strand preference and the total efficiency of markers carried by these strands is low (0.15). The HE markers result from two transversions, A·T to C·G and C·G to G·C, leading to two pairs of mismatches (A/G and C/T, C/C and G/G). One member of each pair is corrected and the other is not. The

Table 1. Transforming efficiencies and relative strand efficiencies at the *amiA6* site

Hex$^+$ recipient	Relative marker efficiency*	Relative strand efficiency†
Wild type	0.15 ± 0.03	1.34 ± 0.16
6rev2	1.02 ± 0.10	9.3 ± 1.00
6rev3	0.47 ± 0.07	0.37 ± 0.08
6rev4	0.86 ± 0.15	0.13 ± 0.02

Results represent mean ± 2 SD.

* Ratio of transformants for this marker to those for the reference marker when native donor DNA is used. Standard reference marker is *str41*, a HE marker conferring resistance to streptomycin.

† Ratio of the transformation yield for heteroduplex DNA bearing this marker on the light strand (Lm/H+) to that for heteroduplex DNA bearing it on the heavy strand (L+/Hm).

last transversion, A·T to T·A, leads to A/A and T/T mismatches and results in an IE marker. This agrees with the proposal that some IE markers are point mutations (5). For intermediate efficiency, both strands must be corrected. The absolute transforming activity of each strand, using heteroduplex DNA as donor, suggests that A/A is as low as A/C and G/T for example, whereas T/T exhibits a higher transforming activity (data not shown). Sequence analysis of *29rev* reveals that a deletion of 34 base pairs behave as a VHE marker.

Mismatches Between Wild-Type and *rev* Sequences. The integration efficiency of mismatches between phenotypically wild-type sequences cannot be tested directly but can be deduced from their effect on the efficiency of HE or VHE markers on the same DNA fragment (exclusion effect; see refs. 5 and 23). We used HE markers of both types—either the L strand (*amiA144*) or the H strand (*amiA22*, *amiA141*, or *amiA150*) was preferred for integration—and compared them in various combinations with phenotypically silent mismatches. The results are given in Table 3.

Single-base mismatches. (*i*) Wild type and *6rev2*: Exclusion affects *amiA144* but not *amiA22*, *amiA141*, and *amiA150* (Table 3: part 1, wild-type recipient; parts 1 and 2, *6rev2* recipient). Because the mismatch between *6rev2* and wild type affects only the L strand, this confrontation is of the HE type. The strand preference is such that L+/H*6rev2* (G/G) is recognized and corrected by the Hex system. A similar conclusion can be drawn from transformation of the wild-type recipient with DNA that carries both *6rev2* and *amiA150* (part 2, wild-type recipient).

(*ii*) Wild type and *6rev3*: Exclusion promoted by the confrontation between wild type and *6rev3* does not affect *amiA144*

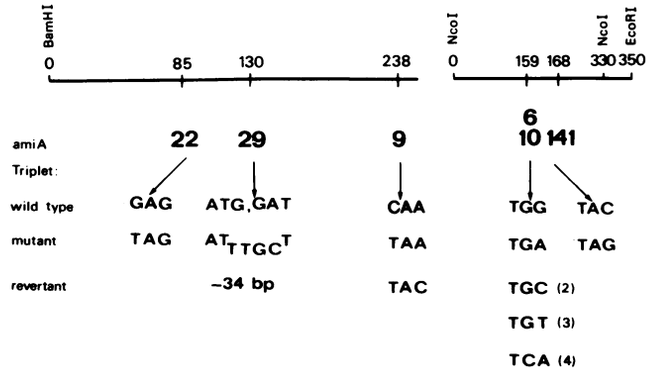

FIG. 2. Mutational changes at various *amiA* sites. Positions of each site are numbered taking as the 0 positions the *Bam*HI site for the left part and the internal *Nco* I site for the right part. For each site, bases are arrayed by triplet corresponding to the mRNA strand (L strand).

Table 2. Base specificity of mismatch repair

Cross	Cases, no.*	Mismatched base pairs		Efficiency class
A/T ⇌ × G/C ⇌	3 (9/+, 6/+, *6rev2*/*6rev3*)	A/C corrected	G/T corrected	LE
A/T ⇌ × C/G ⇌	4 (9/*9rev*, 6/*6rev2*, +/*6rev3*, 22/+)	A/G not corrected	C/T corrected	HE
C/G ⇌ × G/C ⇌	3 (141/+, 6/*6rev4*, +/*6rev2*)	C/C not corrected	G/G corrected	HE
A/T ⇌ × T/A ⇌	1 (6/*6rev3*)	A/A corrected	T/T corrected	IE

* Crosses that give these mismatches are listed in parentheses.

but affects *amiA22*, *amiA141*, and *amiA150* (Table 3: part 1, wild-type and *6rev3* recipients). The lower efficiency of *amiA141* compared with *amiA22* and *amiA150* is explained by the map location of these markers. *amiA22* and *amiA150* are expected to sometimes escape exclusion by integrating with independent DNA fragments. The confrontation between wild type and *6rev3* appears to be of the HE type. The strand preference is such that L*6rev3*/H+ (T/C) is corrected. This also results from the confrontation between wild-type recipient and DNA carrying both *6rev3* and *amiA144* (part 3, wild-type recipient).

(*iii*) *6rev2* and *6rev3*: This confrontation involves a single base-pair change (transition) and affects both *amiA144* and *amiA150* (Table 3: part 2, *6rev3* recipient; part 3, *6rev2* recipient). We conclude that it is of the LE type.

Confrontations involving double base mismatches. (*i*) *6rev4* and wild type: This confrontation involves two base-pair changes and affects both types of HE markers: *amiA144* and *amiA22*, *amiA150* (Table 3, parts 1 and 4, wild-type recipient; part 1, *6rev4* recipient). Thus, both G-G/G-T and C-A/C-C are recognized as LE mismatches. However, a striking result is observed with *amiA141* (part 1). Because *amiA141* is very close to the *6rev4* site (Fig. 1*B*), this marker should exhibit low efficiency, as seen in the *6rev3* recipient, instead of the intermediate efficiency observed. This result suggests that the confrontation between *6rev4* and wild type, normally corrected as a LE marker, is not recognized as efficiently when there is a second mismatch (between wild type and *amiA141*) separated by eight normal base pairs.

(*ii*) *6rev4* and *6rev2* or *6rev3*: Efficiencies of both *amiA144* and *amiA150* are affected by mismatches between *6rev4* and the two other reversions (Table 3: parts 2 and 3, *6rev4* recipient; part 4, *6rev2* and *6rev3* recipients), suggesting that these confrontations are of the LE type. However, the efficiencies are higher than for other LE mismatches, in the range 0.55–0.59 for C-A/C-G (L*6rev4*/H*6rev2*) and C-A/C-A (L*6rev4*/H*6rev3*) confrontations. This result suggests that these mismatches are corrected less frequently by the Hex system than are G-C/G-T, G-T/G-T, or single LE mismatches.

DISCUSSION

The marker effect in pneumococcal transformation results from the nature of the mutation and the Hex (mismatch repair) phenotype of the recipient. If the mutation is a short deletion (or addition), the relative transforming efficiency is maximal. The

Table 3. Effect of various confrontations at the *amiA6* site on transforming efficiency of neighboring markers

Part	Donor DNA	Recipient			
		Wild type	*6rev2*	*6rev3*	*6rev4*
1	*amiA22*	1.05 ± 0.19	0.91 ± 0.12	0.35 ± 0.03	0.26 ± 0.04
	amiA141	0.88 ± 0.09	0.91 ± 0.14	0.19 ± 0.03	0.46 ± 0.05
	amiA144	0.88 ± 0.18	0.42 ± 0.06	0.78 ± 0.11	0.37 ± 0.04
	amiA150	0.83 ± 0.16	0.94 ± 0.14	0.28 ± 0.04	0.39 ± 0.04
2	*amiA144-6rev2*	0.87 ± 0.13	0.84 ± 0.11	0.43 ± 0.05	0.42 ± 0.04
	amiA150-6rev2	0.35 ± 0.05	0.90 ± 0.12	0.31 ± 0.03	0.57 ± 0.07
3	*amiA144-6rev3*	0.43 ± 0.09	0.33 ± 0.07	0.82 ± 0.14	0.42 ± 0.04
	amiA150-6rev3	0.86 ± 0.13	0.38 ± 0.07	1.02 ± 0.13	0.58 ± 0.07
4	*amiA144-6rev4*	0.32 ± 0.04	0.59 ± 0.08	0.55 ± 0.10	0.87 ± 0.10
	amiA150-6rev4	0.33 ± 0.04	0.33 ± 0.05	0.32 ± 0.03	1.02 ± 0.13

Results (mean ± 2 SD) are expressed as relative transforming efficiencies (defined as in Table 1) of the *amiA* markers carried by donor DNA.

longer the deletion, the lower the efficiency whatever the genotype of the recipient Hex^+ or Hex^- (5, 8).

Point Mismatches in Hex^+ Cells. Data on wild-type, mutant, and revertant sequences at five genetic sites within the *amiA* locus have allowed us to correlate point mismatches with transformation efficiencies. Interestingly, all possible mismatches were encountered at the same site (*amiA6*) and correlated with low, intermediate, and high efficiency of transformation. Clearly, and this is our main conclusion, the Hex system can correct point mismatches present in donor–recipient heteroduplexes that are several thousand base pairs long (24). However, the behavior of the system toward single-site substitutions is complex. When a transitional change distinguishes donor DNA from recipient (three occurrences at two sites; see Table 2), the system corrects donor information quite efficiently, because transformation is decreased 1/10th to 1/20th compared to short deletions. A/C and G/T appear to be corrected equally, because no strand preference is observed for LE markers. This explains why the mutagens used—nitrous acid, hydroxylamine, ethyl ethanesulfonate (5, 15, 25), which are known to induce transitions preferentially—yielded only LE mutations. When a transversion distinguishes donor and recipient sequences, two types of markers have been observed: IE, associated with A·T to T·A change, and HE, associated with A·T to C·G and with G·C to C·G change. In the first case, A/A may be corrected more efficiently than T/T. In the second case, C/T for the first transversion (four occurrences at three sites) and G/G for the second transversion (three occurrences at two sites) are corrected efficiently, whereas the complementary mismatches, A/G and C/C, are not. To summarize the present results, five out of eight possible mismatched base pairs were corrected to similar extents by the Hex system: A/C, G/T, A/A, C/T, and G/G. One base pair, T/T, may be corrected less efficiently, and the other two, A/G and C/C, were not at a detectable frequency. That a similar level of correction may be achieved for A/C, G/T, C/T, and G/G mismatches is suggested by the result observed with *amiA141*. This marker goes down from HE to LE (transforming efficiency 0.19) when the G/T mismatch (wild type confronting *6rev3*) is present separated by eight normal base pairs. This suggests that C/T and G/G are corrected as efficiently as A/C and G/T, the two complementary LE mismatches. So far, among mismatches of the same type found at different sites, all have the same transformation efficiency. However, only four different sites have been studied so far, and more examples are required to analyze the importance of neighboring sequences. The recent report by Lacks *et al.* (26) of the same G·C to T·A change associated with two types of markers, IE and VHE, argues that, at least sometimes, recognition of the mismatch depends also on neighboring sequences.

Multiple Mismatches. The sequences show that two base changes occurred between wild type and *9rev* (CAA and TAC, respectively). Their confrontation gives two mismatches, one normally LE and the other HE, separated by a normal base pair [C(A)A/A(T)G and T(A)C/G(T)T]. This double-base-pair change is still recognized as LE (21).

Change from wild type to *amiA29* involves replacement of three bases (G-G-A) by four bases (T-T-G-C). Interestingly, of the two complementary multiple mismatches between wild type and *amiA29*, G-G-A/A-A-C-G is not corrected by the Hex system. It involves A/G mismatches and a +1 base, which does not seem to be detected.

The confrontation between *6rev4*, on the one hand, and wild type, *6rev2*, or *6rev3*, on the other hand, involves two mismatched base pairs. (*i*) Between *6rev4* and wild type, C-A/C-C and G-G/G-T promote exclusion as LE markers. However, C-A/C-C is recognized less efficiently when a second mismatch (between wild type and *amiA141*) is present, separated by eight normal base pairs. (*ii*) Between *6rev4* and *6rev2*, C-A/C-G could be corrected less efficiently than G-C/G-T. It is worth noting that C-A/C-G (L*6rev4*/H*6rev2*) involves two mismatches that are not corrected when present singly. (*iii*) Between *6rev4* and *6rev2*, C-A/C-A also could be corrected less efficiently than G-T/G-T.

The fact that the correction of a pair of mismatches can be changed by placing a second mismatch separated by eight normal base pairs and that two mismatches, normally not corrected singly, are recognized when both are present suggests that local destabilization of the helix may influence the correction pattern. This comes back to the possible effect of neighboring sequences on Hex specificity. A simple explanation of the correction specificity built on the complementary base-pairing model proposed by Topal and Fresco (27) cannot account for our observations. It seems to be the structure between the mispaired bases that is recognized, rather than a given base *per se*, involved in a mismatch as we suggested previously (21).

Another open question concerns frameshift mutations. It has been shown that acridine-induced mutations were mainly of the LE type, and evidence has been presented that these were frameshift mutations (28). Our results suggest that the mis-

pairing between wild type and one frameshift mutation (*amiA29*) is not recognized by the Hex system. It is possible that the acridine-induced mutations studied were a complex of base substitution and frameshift mutations. This could explain the low efficiency of transformation of these mutations without need for recognition of frameshift *per se* by the Hex system. However, we have recently induced *in vitro* a frameshift mutation by filling in with DNA polymerase a *Cla* I site of the *amiA* locus (A-T ↓ C-G-A-T→A-T-C-G-C-G-A-T). The DNA fragment obtained carries a +C-G frameshift, as monitored from the appearance of a new *Nru* I site (T-C-G ↓ C-G-A). This +2 frameshift appears to be recognized as LE by the Hex system (unpublished data). Obviously, more results are needed to get a clear complete picture of the specificity of the Hex system toward frameshift mutations.

The only VHE confrontation observed involves *29rev* and *amiA29* or wild type; it corresponds to a short deletion (33 or 34 bases). As previous genetic work has shown that the Hex system does not correct deletions, it is likely that short deletions could account at least for some VHE markers.

In any case, it will be interesting to learn whether the specificity of mismatch repair we describe is general and applies to other organisms. The existence of an excision-repair system acting on mismatched base pairs has been postulated in *E. coli* to account for gene conversion of λ-heteroduplexes (29, 30). This system involves the products of the *mutH, mutL, mutS,* and *uvrE* genes (29, 31, 32), but so far its specificity remains unknown.

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