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Hyperrecombination at a specific DNA sequence in pneumococcal transformation

(aminopterin resistance/cloning/mismatch repair/gene conversion)

J. C. LEFÈVRE, A. M. GASC, A. C. BURGER, P. MOSTACHFI AND A. M. SICARD*

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

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ABSTRACT **In pneumococcal transformation, recombination frequency between point mutations is usually proportional to physical distances. We have identified an aberrant marker belonging to the *amiA* locus that appeared to markedly enhance recombination frequency when crossed with any other markers of this gene. This mutation results from the C-to-A transversion in the sequence A-T-T-C-A-T → A-T-T-A-A-T. This effect is especially apparent for short distances as small as 27 base pairs. The hyperrecombination does not require the wild-type function of the pneumococcal gene for an ATP-dependent DNase (which is homologous to the product of the *Escherichia coli recBC* genes) or of the *hex* genes, which correct certain mismatched bases in transformation. The hyperrecombination is affected by the presence of nearby mismatched bases that trigger an excision–repair system. It is proposed that the mutation that shows hyperrecombination is sometimes converted to the wild-type allele at the heteroduplex stage of transformation.**

General recombination involves the exchange of genetic information between homologous DNA segments. The frequency of recombination has been postulated to be approximately constant throughout homologous regions. However, this assumption is not always true, as has been shown by genetic studies. Many examples of map distortions have been reported. In fungi as well as other eukaryotes, the frequency of exchange between genes can be strongly affected by mutations (1). The nature of the genetic factors controlling the recombination events has remained elusive. A major step in the molecular understanding of one such factor has been the studies by Stahl and his collaborators of sites (*Chi* elements) that promote recombination in bacteriophage λ (2). Such sites are also found in the *Escherichia coli* chromosome and may affect bacterial recombination (3). It was found that the enhancement of recombination due to *Chi* sites was asymmetric, and it required the wild-type activity of *recBC* genes. A specific octamer is present in all reported *Chi* elements (3). Recombination hotspots also have been observed in recombination between *E. coli* plasmids. These hotspots appear to be different from *Chi* elements in that they do not require the *recBC* pathway and they are active in either orientation (4). Moreover, Lieb identified mutations that enhance recombinations in bacteriophage crosses (5). Such mutations are particularly susceptible to mismatch correction by short patch repair and are quite different from the *Chi* elements. In studies of marker effects in bacteriophage T4 recombination, short patch repair has also been reported (6, 7). Therefore, it appears that there are a variety of factors that control recombination, which may be more or less specific to the organism under investigation. Moreover, the mechanisms of enhanced recombination could be better understood if genes that promote such an effect were known.

The subject of this report is the description of a mutation belonging to the *amiA* locus of *Streptococcus pneumoniae* that appears to enhance markedly recombination rates in genetic transformation with all closely linked markers. This mutation results from the transversion C → A in the sequence A-T-T-C-A-T → A-T-T-A-A-T. The hyperrecombination extends over very short distances on both sides of the mutation and does not require the wild-type function of the pneumococcal gene homologous to the *E. coli recBC* gene (8).

A striking feature of genetic studies of recombination in *Streptococcus pneumoniae* is the difference in the efficiency with which single-site mutations are integrated by transformation (9, 10). Discrete classes of transformation efficiency have been described: very high efficiency (VHE), high efficiency (HE), intermediate efficiency (IE), and low efficiency (LE) (10, 11). It has been proposed that excision and correction of donor DNA induced by some mismatched base pairs of donor–recipient heteroduplexes account for the low efficiency of some markers (12). This hypothesis was substantiated by the identification of a mutant strain, denoted *hex*$^-$, which is transformed with very high efficiency for all single-site markers (13). In this report we show that hyperrecombination is observed in *hex*$^-$ strains, whereas in *hex*$^+$ strains the excision–repair process induced by an LE mismatch base pair interferes with hyperrecombination. This suggests that the mechanism of hyperrecombination acts at the heteroduplex stage.

MATERIALS AND METHODS

Pneumococcal strains used in this study were derivatives of R6 (14, 15) in which *hex*$^-$ or aminopterin-resistant mutations (9), or both, were introduced by transformation. Deletion mutations were obtained by the procedure described earlier (15). Isolation, purification of DNA, and transformation procedures were similar to those as described (16). Wild-type transformants belonging to the *amiA* locus were scored by plating in synthetic medium containing an excess of isoleucine (9). To correct for fluctuations in competence, wild-type recombinants are measured as a ratio (the efficiency) to streptomycin-resistant (*str41*) transformants. To correct for this in a two-point cross, the recombination frequency is divided by the efficiency of the recipient marker (10) to give a "recombination index."

The mutation *amiA36* has been cloned by the rapid procedure previously described (18). The recombinant plasmid pR28 (18) carrying an erythromycin-resistance marker, was integrated inside the *amiA36* gene of the pneumococcal chromosome. It was extracted by *Pst* I restriction enzyme treatment, circularized by ligation, and used to transform *E. coli*. Nucleotide sequence was determined by the method of

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Abbreviations: HE, high-efficiency; LE, low-efficiency; bp, base pair(s).

*To whom reprint requests should be addressed.

Maxam and Gilbert (19). End labeling and sequencing-gel procedures were as described (20).

RESULTS

Genetic Mapping of the Mutation *amiA36*. Many mutants conferring resistance to 10 μM aminopterin have been isolated in our laboratory. Because they are sensitive to an imbalance in concentrations of branched-chain amino acids, wild-type recombinants can by easily scored (9). The analysis of hundreds of two-point crosses showed that these mutants map in a single locus, *amiA* (9). The recombination frequencies are additive and are proportional to physical distances, the frequency of recombination being estimated to be 0.03% per base pair (bp) separating two mutations (21). Although such recombination frequencies fluctuate somehow with physiological factors (9, 10), the genetic location of many mutants has been confirmed by restriction mapping of this locus (21, 22) when cloned fragments of this gene became available (16, 18) or by DNA sequence determination for some mutants (20, 23, 24). An aberrant marker, *amiA36*, showed a frequency of recombination exceeding 20% with all tested reference markers. The location of this marker was investigated by using cloned fragments of the *amiA* locus (J. P. Claverys, personal communication). The strain *amiA36* was transformed by several cloned DNAs. Wild-type recombinants were only obtained when the donor DNA contained a 1050-bp-long segment (Fig. 1, fragment A_1).

The mutation was further positioned with the help of deletions. We transformed the strain *amiA36* with DNA bearing the spontaneous mutation *amiA109*, which covers several sites in the middle of the A_1 fragment (22), and nine other induced deletions, which start at the junction between A_1 and B fragments and extend into A_1 (15). Wild-type recombinants were not recovered when deletions *amiA507*, *-513*, *-577*, *-502*, *-505*, and *-579* were used as donor DNA. Because the mutation *amiA36* recombined with the other deletions, it is assigned to the short interval between the left ends of *amiA502*, *-505*, and *-579* and *amiA590* (Fig. 1).

Table 1. Efficiency of the *amiA36* marker

Recipient strain	Donor DNA	Efficiency
hex$^+$ *amiA36*	*amiA*$^+$ *str41*	1.31 ± 0.14
hex$^+$ *amiA*$^+$	*amiA36 str41*	1.28 ± 0.24
hex$^-$ *amiA36*	*amiA*$^+$ *str41*	0.98 ± 0.07
hex$^-$ *amiA*$^+$	*amiA36 str41*	0.90 ± 0.08

The integration efficiency is the ratio of the number of transformants for *amiA*$^+$ or *amiA36* markers to the number of transformants for the reference marker *str41*. Statistics were as described by Kimball (17).

Genetic Nature of the *amiA36* Mutation. The behavior of the mutation *amiA36* suggests that it may not be a point mutation but might result from insertion or deletion. When deletion mutants are used as donor in transformation of *hex*$^-$ pneumococcus, there is an enhancement of ≈20% in the frequency of wild-type recombinants for close markers but no enhancement for the reciprocal cross (insertion into a deletion) (15). Reciprocal frequencies for *amiA36* were similar, so it is unlikely that it is a deletion. Transformation efficiencies for short deletions are high (10)—1.6 to 2.0 relative to *str41* (25)—and they decrease for longer deletions, falling below unity and becoming unequal in reciprocal crosses (10).

We measured the efficiency of the *amiA36* marker in reciprocal crosses in both *hex*$^+$ and *hex*$^-$ recipients (Table 1). The efficiency in the latter type of recipient was similar to that of a point mutation or of a short deletion. However in a *hex*$^+$ recipient, the efficiency of such a deletion should be close to 2, whereas that of *amiA36* was around 1.3. These results are consistent with the hypothesis that it is an HE point mutation. Additional evidence was provided by the isolation of spontaneous reversions of this mutation to wild type. Finally, electrophoretic studies of fragments obtained

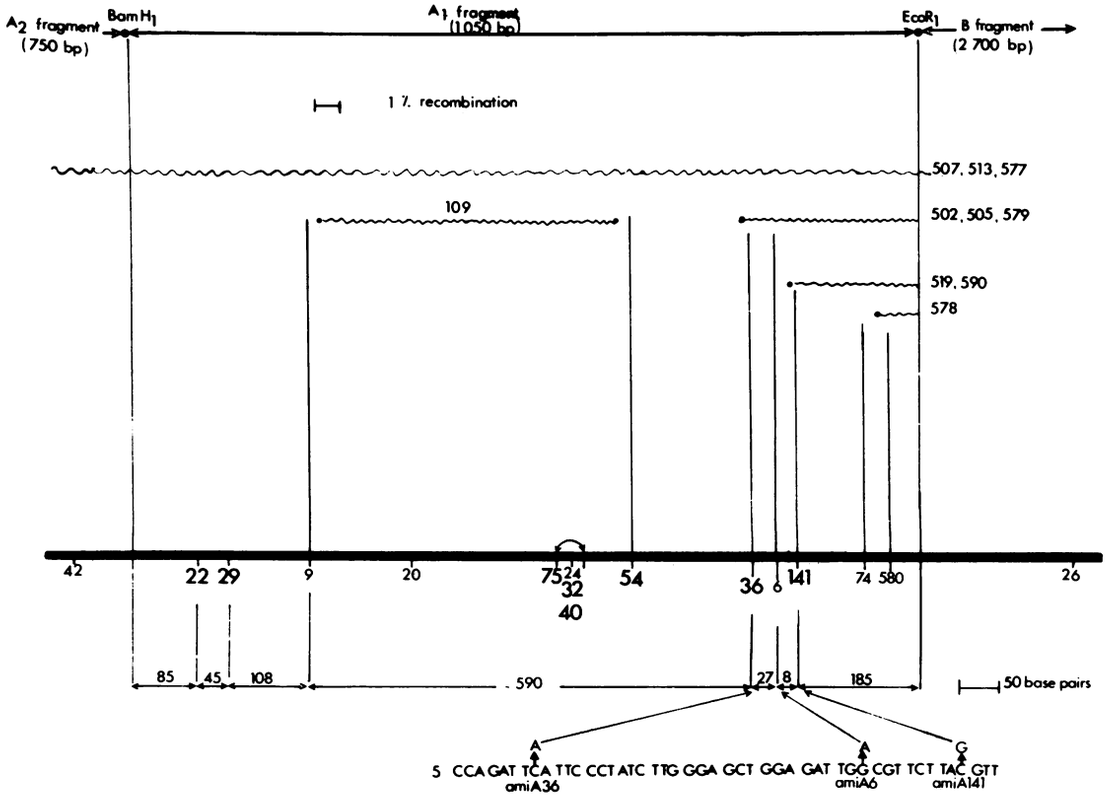

FIG. 1. Genetic map of the left side of the *amiA* locus and sequence of the *amiA36–amiA6–amiA141* region.

by restriction enzyme treatment showed that the 1050-bp A_1 fragment carrying the *amiA36* mutation migrated at the same rate as the fragment containing the wild-type sequence. Identical sizes for both types of fragment were obtained also with the smaller fragment generated by *Rsa* I.

Sequence of the Mutation *amiA36*. The zone of the *amiA* locus bearing the *amiA36* mutation has been cloned (18), and the wild-type sequence has been determined (23, 24). In order to determine the sequence for the mutation, we cloned it in the BR325 derivative containing an erythromycin gene that is expressed either in *E. coli* or pneumococcus (18). The A_1 fragment contains a 330-bp segment between two *Nco* I sites. Genetic studies show that *amiA36* should be in this fragment. Therefore, the hybrid vector was cut by restriction enzyme *Nco* I and end-labeled. As there is a single *Rsa* I site in this *Nco* I fragment (24), we cut this fragment by enzyme *Rsa* I and determined its sequence.

The autoradiogram of sequencing gels shows that *amiA36* resulted from a transversion of C to A in the sequence 5'...A-T-T-C-A-T to 5'...A-T-T-A-A-T (Fig. 1). Therefore, this mutation is 27 bp to the left of *amiA6*, a result consistent with previous genetic analysis. Thus, it appears that the *amiA36* mutation introduces a six-base palindrome. No other substitutions were detected 70 bp on the right and 131 bp on the left of this transversion (Fig. 1).

Hyperrecombination with *amiA36*. The mutation *amiA36* was transferred into the strain 801 (*hex*$^-$), which is unable to correct mismatched bases (14). All point markers are equally efficient when this strain is used as recipient. In this genetic background, possible interference by the *hex* system should be avoided. Two-point crosses were performed between the mutation *amiA36* and a set of closely linked markers. Transformation was done in both directions, with the recipient strains always carrying the *hex*$^-$ mutation. The results of these reciprocal crosses are summarized in Table 2. The recombination index (i.e., the percentage frequency of recombination) between *amiA36* and any other marker was >19%, even for the closest markers such as *amiA54*, *amiA6*, and *amiA141*. In contrast, the recombination between these latter three markers was much lower—for example, the recombination index for *amiA6* and *amiA54* was 6.6% and that for *amiA141* and *amiA54* was similar (8.1%). As *amiA6* and *amiA141* are only 8 bp apart and recombined at a rate of 0.5%, the average distance between *amiA54* and *amiA6* or *amiA141* was about 7.3%. With *amiA36* at 27 bp from *amiA6*, the sum of the recombination frequency with *amiA6* (or *amiA141*) and *amiA54* should not exceed 8%, whereas they totaled 43–50%. There was an enhanced recombination (hyperrecombination) with neighboring sites. It appeared that both sides of the *amiA36* mutation were equally affected. Recombination frequencies tended to be higher when *amiA36* was crossed with a more distant marker such as *amiA22*. The observed frequency of recombination may be the sum of a minimum frequency (20%) plus the component related to the distance between *amiA36* and the marker.

Table 2. Recombination indices involving the *amiA36* site

amiA36 donor DNA		*amiA36* recipient strain	
Recipient strain	Recombination index, %	Donor DNA	Recombination index, %
amiA42	56	*amiA42*	36
amiA22	50	*amiA22*	47
amiA75	49	*amiA75*	27
amiA40	19	*amiA40*	28
amiA24	28	*amiA24*	33
amiA54	24	*amiA54*	24
amiA6	24	*amiA6*	26
amiA141	19	*amiA141*	22
amiA74	34	*amiA74*	32
amiA580	36	*amiA580*	20

Recipient strains are *rec*$^+$*hex*$^-$, and all DNA carries the *str41* reference marker. Recombination indices are determined in two-point crosses by dividing the recombinant frequency by the efficiency of the site in the recipient cells, the latter being measured in a cross of the same recipient by wild-type DNA. Markers are ordered according to their map position (Fig. 1).

***recBC* Independence of *amiA36* Effects.** Investigation of the hyperrecombination mechanism would be aided by identification of genes that are needed for its action. In *E. coli* the fact that stimulation of recombination by *Chi* elements was specific for the *recBC* pathway lent support to a hypothesis on the mechanism of the enhanced recombination (2). This prompted the question of whether any *rec* genes were required to enhance the recombination frequencies with *amiA36*. The only well-characterized pneumococcal *rec* gene is homologous to the *E. coli recBC* genes. The pneumococcal *recBC*$^-$ is slightly UV- and γ-sensitive, lacks the ATP-dependent exonuclease activity, has a reduced viable count per culture density unit, and transforms 80% less than the wild type (8). As it had been studied in a *hex*$^+$ background, we first introduced the *amiA36* mutation into the *rec* strain. In order to make this strain *hex*$^-$ (to compare results with our previous experiments), we transformed it with DNA from recombinant plasmids carrying an internal fragment of genes *hexA* or *hexB* and an erythromycin-resistance gene that is expressed in pneumococcus. As these plasmids cannot replicate autonomously in these bacteria, the erythromycin-resistant transformants result from insertion of these plasmids in the region of homology (i.e., the *hex* genes), thereby inactivating them (J. P. Claverys, personal communication). The *hex*$^-$ *rec*$^-$ *amiA36* strains thus obtained were transformed by the DNA carrying closely linked mutations. Comparison of results in Tables 1 and 3 shows that the enhancement of recombination was not affected by the *rec*$^-$ mutation. It is also clear that, as far as hyperrecombination is concerned, the mutations *hexA*$^-$ and *hexB*$^-$ behaved similarly.

Interference of the *hex* Mismatch-Correcting System with Hyperrecombination. The results presented above indicate that the recombination induced by the *amiA36* marker does not require the action of the *hex* system. A question that we might ask is whether the hyperrecombination is affected by the presence of an LE marker that will trigger the excision–repair system in an *hex*$^+$ recipient. To answer this question, the marker *amiA36* was introduced in a *hex*$^+$ recipient and crossed to a set of neighboring markers (Table 4). When HE markers are in the donor DNA, hyperrecombination with *amiA36* is at the usual high level (20–30%). When LE markers were transformed into *amiA36*, the recombination frequencies were close to the low theoretical values. The contrasting behavior of LE and HE markers is especially obvious in comparing the crosses of *amiA6* (LE) and *amiA141* (HE), which are two well-defined nearby point mutations (see their sequence in Fig. 1). However, it appears that, in

Table 3. Recombination indices in transformation between *rec*$^-$ *hex*$^-$ *amiA36* strains, with other strains bearing closely linked markers

	Recombination index, %	
Donor DNA	*rec*$^-$ *hexA*$^-$ *amiA36*	*rec*$^-$ *hexB*$^-$ *amiA36*
amiA24	30	43
amiA54	30	26
amiA6	30	15
amiA141	23	29
amiA74	36	26

Definition of recombination index is in Table 2.

Table 4. Recombination indices in hex^+ transformation between *amiA36* strain with other strains bearing closely linked markers

Markers	Recombination index, %			
	amiA36 as recipient		*amiA36* as donor	Expected
	rec^+ hex^+	rec^- hex^+		
amiA24	9.1	9.0	15	9
amiA54	21.8	19.4	23.5	6.5
amiA6	2.7	2.1	8.3	1
amiA141	31.9	38.8	31	1.5
amiA74	7	6.9	11	5.4
amiA580	8	—	—	7

For donor DNA, boldface numerals designate HE markers, and light-face numerals designate LE markers. Expected indices are computed from two-point crosses with the assumption that expected recombination index between *amiA36* and *amiA6* is 1% (27 bp).

the cross with *amiA6*, there was not complete restoration to the expected value. The expectation was calculated from an average 0.03% per bp, which was found over long distances; possibly it should be higher for this short interval of 27 bp. It is also possible that a small fraction of hyperrecombination escapes the blocking effect of the LE marker. Similar results were obtained when the hex^+ recipient strain was also rec^- (Table 4).

The same set of markers was introduced into a hex^+ strain, and the resulting strains were transformed by *amiA36* DNA. Table 4 indicates that hyperrecombination was at the typical value (20–30%) when HE markers were concerned and was reduced when LE markers were transformed. However, the inhibitory effect of LE mismatches was only partial as compared to the reciprocal crosses.

DISCUSSION

This work demonstrates an enhancement of recombination when a spontaneous aminopterin-resistant mutant is crossed with closely linked markers belonging to the *amiA* locus. Several features of this hyperrecombination can be summarized. (*i*) Recombination is increased for distances as short as 27 bp to at least 20%, whereas the expected value for such a distance is 1%. (*ii*) The effect is obtained at similar levels in reciprocal crosses. (*iii*) No polarity can be detected. (*iv*) The pneumococcal equivalent of *recBC* genes is not required for this effect. (*v*) When an LE marker closely linked to the *amiA36* mutation is involved in a two-point cross with a hex^+ strain as recipient, the hyperrecombination effect is reduced. (*vi*) The mutation results from the transversion C → A in the sequence A-T-T-C-A-T → A-T-T-A-A-T. All these properties are quite different from the *Chi* activity described in λ prophages, and they suggest that a novel mode of enhanced recombination may act in genetic transformation.

When the *amiA36* mutation is carried by a hex^+-correcting system, which recognizes the LE mismatch and probably excises the donor DNA (25, 26), there is no enhancement of recombination. As it is known that the *hex* system acts at the heteroduplex step in DNA recombination (26–28), it is suggested that the hyperrecombination effect occurs at this stage also.

The hyperrecombination promoted by the *amiA36* mutation in its close neighborhood suggests that it is the result of a process limited to this site or its vicinity. This process could be a conversion with an equal probability—of 0.2—in both directions to account for the similar efficiencies of the *amiA36* marker in reciprocal transformation.

The inhibition of hyperrecombination by an LE mismatch can be explained by the mode of action of the *hex* system. When an LE mismatched base pair occurs at the heteroduplex stage, genetic results suggest that a long sequence (>1000 bp) of donor information is excluded (10, 29). The transformants are the results of a transfer of information to the chromosome (26–28). In a cross between *amiA36* and a LE *amiA* marker as donor, this process will lead to a homoduplex recipient DNA carrying the LE integrated marker and the $amiA^+$ allele as a result of cocorrection. The only wild-type transformants would result from exchanges or breaks between the two sites. This interpretation requires also that the elimination of the donor DNA is a faster process than the conversion directed by the *amiA36* mutation. Indeed, it has been shown that this elimination takes less than 2 min at 30°C (30). The interference of the *hex* system on hyperrecombination is smaller when the *amiA36* mutation is carried by the donor DNA. This may happen if the short-patch conversion of the *amiA36* marker to the wild type had occurred only partially before the *hex*-directed long-patch repair induced by LE mismatch. As it has been shown that the repair process is a fast process (31), it is likely that the proposed conversion at the *amiA36* site takes only a few minutes to occur.

Although the conversion hypothesis can well account for the observed hyperrecombination, conclusive proof, such as evidence of information transfer between DNA strands, is beyond the reach of the present analysis. Indeed when *amiA36* is paired with its wild-type allele, any event able to promote exchange of material from this location will explain this recombination. One or both heteroduplex structures could be specific recognition sites for the nicking of DNA by an endonuclease. This would provide a binding site for recombination enzymes such as recA protein or repair enzymes. Indeed, single-strand breaks are well documented as a source of recombination (32, 33). We can speculate as to the mode of recognition. If we suppose that the peculiar palindromic sequence (A-T-T-A-A-T) resulting from the mutation *amiA36* is not merely a coincidence, at the pairing step a hairpin may occur on DNA strands with a probability related to the observed hyperrecombination. Such intrastrand self-complementary structure could be the site of endonuclease action required for nicking DNA.

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