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DNA sequences required to induce localized conversion in *Streptococcus pneumoniae* transformation

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Summary. In pneumococcal transformation a particular point mutation belonging to the *amiA* locus is able markedly to enhance recombination frequency when crossed with any other markers of this gene. This results from a polarized conversion of the mutation towards the wild-type sequence. In this report, by site-directed oligonucleotide mutagenesis, we have generated a series of mutants showing various degrees of conversion. We have found that the substitution 5′-ATTCAT→5′-ATTAAT is a sufficient signal for localized conversion. Changing individual bases within this sequence results in decreased conversion frequencies to levels that depend on the mutation, suggesting that there is a family to related sequences which may act as a substrate for a conversion system. Moreover, the length over which this conversion occurs has been estimated to be 12 base pairs on the average.

Key words: Oligomer mutagenesis – Mismatch repair – Pneumococcus – Recombination

Introduction

In general, genetic recombination involves a set of processes resulting in exchanges between homologous DNA segments and plays a central role in evolution by gene diversification as well as by gene homogenization. Indeed, recent observations on repeated mammalian genes have shown small clusters of base substitutions which appear to be products of a recombination event, probably a gene conversion, that is, an asymmetric transfer of genetic information (Powers and Smithies 1986). It is therefore of interest to understand the mechanisms of gene conversion at the molecular level. Since conversion cannot be experimentally induced in higher eucaryotes, it has been intensively studied during DNA transformation in *Streptococcus pneumoniae* where such limitations do not exist (for review see Sicard et al. 1985a; Claverys and Lacks 1986; Sicard 1987). At least two types of conversions have been described:

1. Long-patch gene conversions in which several thousand bases are excised and DNA rapidly becomes homoduplex by a repair process specific for mismatched bases.
2. Localized conversion in which fewer than 27 bp are involved (Sicard et al. 1985b).

It is likely that this latter conversion is related to the gene conversion found in repeated genes in higher eucaryotes because the clusters of base substitutions are often confined to small numbers of bases (Bentley and Rabitts 1983; Powers and Smithies 1986). For this reason our interest has focused on localized conversion in pneumococcus.

The original observation that led to the concept of localized conversion was the description of a particular mutation (*amiA*36) belonging to the *amiA* locus which confers resistance to 10^{-5} M amethopterin. This mutation markedly enhances recombination rates in genetic transformation with all closely linked markers (Lefèvre et al. 1984). This hyper-recombination does not modify the recombination frequencies between outside markers, which suggests that a conversion towards wild-type recombinants occurs frequently at this site. The mutation results from a C/G to A/T transversion in the sequence 5′-ATTCAT-3′→ 5′-ATTAAT-3′ (Lefèvre et al. 1984). We have reported that it is a polarized process because only one of the two artificial heteroduplexes, 5′-ATTAAT-3′/3′-TAAGTA-5′, is specifically converted (Sicard et al. 1985b). Moreover, only one mutant is converted to wild type i.e. 5′-A-3′ is converted to 5′-C-3′ (Mostachfi and Sicard 1987).

A peculiarity of the *amiA*36 marker is that this mutation generates a palindrome of 6 A.T base pairs. This palindrome could be a coincidence or it could be a structure required for the localized conversion process. Conversion enzymes might specifically interact with such structures in a way reminiscent of some restriction or methylation enzymes. Conversion could then be explained by methylation of an adenine in this palindrome that would be hemimethylated at the heteroduplex stage during recombination and thus sensitive to nucleolytic action. Although there is no evidence for such methylation in pneumococcus, this possibility is not excluded.

If this hypothesis was correct, as methylating enzymes are specific for palindromes, conversions should not be possible without palindromic structures. In this report we describe the use of oligonucleotide-directed mutagenesis to change individual bases of this palindrome. This leads to decreased conversion frequencies whose levels depend on the particular mutations. Therefore localized conversion does not require a palindrome but instead, a family of related structures may serve as substrates for conversion. This result furthermore suggests that methylating process is not involved in localized conversion.

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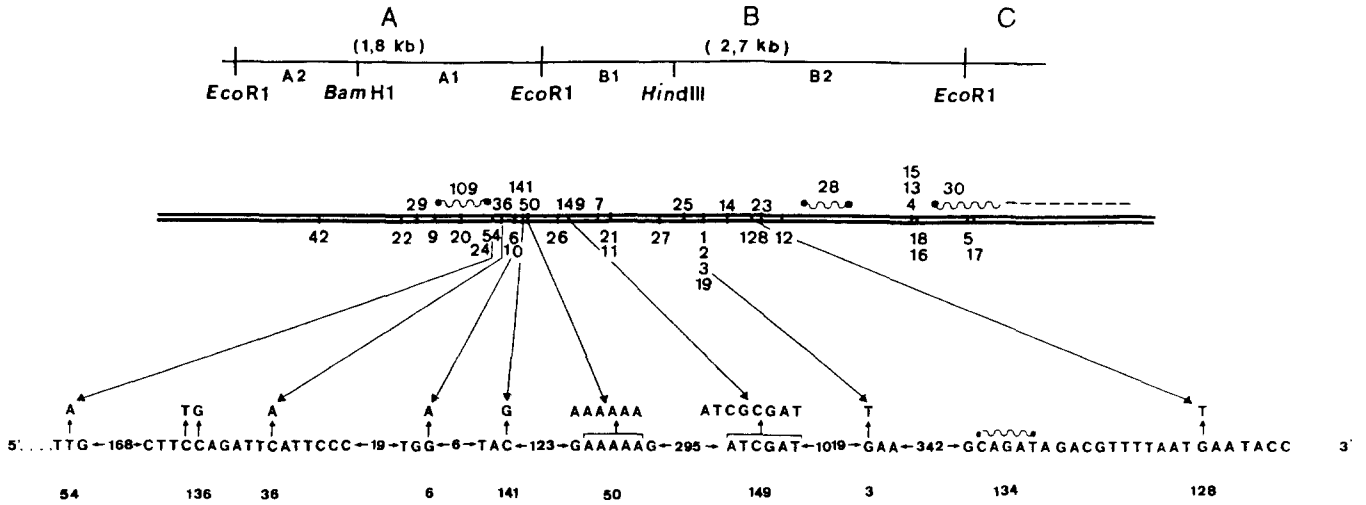

Fig. 1. Genetic and physical map of the *amiA* locus. Restriction fragments are shown on the *upper line*. The genetic map is marked by a number of alleles shown near the *double line*. *Wavy lines* refer to deletions. Wild-type and mutant sequences are indicated below

Materials and methods

Strains and transformation. Pneumococcal strains used in this study are derivatives of R6 (Lefèvre et al. 1979; Claverys et al. 1980a). Recipient strains are *hex*B$^-$, i.e. they transform all markers at the same high efficiency (Claverys et al. 1980b). The markers belong either to the *amiA* locus, conferring resistance to 10^{-5} M amethopterin (Sicard and Ephrussi-Taylor 1965) or to a locus that confers resistance to 2 mg/ml of streptomycin (the *str*41 marker). Isolation, purification of DNA, and transformation procedures were as described (Claverys et al. 1981). Wild-type transformants belonging to the *amiA* locus were scored by plating on synthetic medium containing an excess of isoleucine (Sicard and Ephrussi-Taylor 1965). To correct for fluctuations in competence, wild-type recombinants are measured as a ratio (the efficiency) to streptomycin resistant (*str*41) transformants. To correct for this in a two-point cross, the recombination frequency is divided by the efficiency of the recipient marker (Lacks 1966) to give a "recombination index".

Oligonucleotide-directed mutagenesis. The *amiA* locus has been subdivided into several fragments defined by restriction sites (Fig. 1). The A1 and B2 fragments were cloned in phage M13mp9. Oligonucleotides complementary to the cloned sequence but containing one or two base substitutions were synthesized and purified by preparative gel electrophoresis. The oligomer that was used to generate mutation *amiA*128 was a gift from D. Shire (Elf. Bio-Recherches). All other oligomers were synthesized using an Applied Biosystems 381 A DNA synthetiser. Oligonucleotide-directed mutagenesis was carried out using M13-derived vectors (Zoller and Smith 1982) following two methods to obtain the mutants: (1) the gapped duplex approach (Kramer et al. 1984); and (2) ligation with double primer and dot-blot hybridization (Norris et al. 1982).

A modification of the latter method has been devised to isolate some mutants directly in pneumococcus: a synthetic oligomer containing a single base change from wild type was annealed to a single-stranded wild-type genome, yielding a partial duplex containing the mismatched bases. In vitro enzymatic reactions with DNA polymerase (Klenow fragment) and DNA ligase formed a complete duplex. After linearization by *Bgl*II, aliquots were used to transform a wild-type pneumococcus strain unable to repair mismatched bases. Amethopterin resistant transformants were recovered at high frequency (2×10^5/ml). These could result from point mutations induced by the oligomer or from insertion of the circular duplex phage DNA inside the *amiA* locus by homologous recombination owing to pairing between B2 sequences in the chromosome and in the phage DNA (Méjean et al. 1981). This latter possibility should yield unstable clones. A set of amethopterin resistant transformants was grown on solid synthetic medium containing an excess of isoleucine to test their stability. Wild-type pneumococcus will yield colonies whereas amethopterin resistant strains will not grow (Sicard and Ephrussi-Taylor 1965). An average of 60% of the strains (29/48) was stable and was kept for further genetic analysis. Eight of the strains were intercrossed; they did not recombine and it was therefore assumed that they carry a mutation at the same site. Whatever the method used, all putative mutants were sequenced by the dideoxynucleotide method (Sanger et al. 1977).

Results

Localized conversion at the nucleotide level

To determine which bases in the palindrome in the *amiA*36 region are required to provoke conversion, we changed each base and tested the recombination indices with nearby markers that had already been sequenced. The criteria for choosing the base changes to be made were the following: they must create a nonsense codon so that they are amethopterin resistant and they must carry the A/G mismatch from the original *amiA*36 mutant. We have obtained a set of single and double mutants. Two-point crosses between these new mutants and the *amiA*6, *amiA*141 and *amiA*54 mutants, which are respectively -27, -35 and 180 bp apart, gave recombination indices which are higher than expected (Table 1). The expected values are calculated from an average of 1% per 27 bp (Lefèvre et al. 1984). The experimental indices were not, however, as high as those found in the

Table 1. Recombination indices in transformation between the constructed mutants and other closely linked markers

Recipient strain	Donor DNA			
	*amiA*6	*amiA*141	*amiA*54	*amiA*36
*amiA*148 (A C T A A T)	12	17	22	0
*amiA*142 (G T T A A T)	7	8	21	0
*amiA*137 (A T T G A T)	5	6	14	0
*amiA*135 (A T T A G T)	7	8	12	0
*amiA*146 (A G T A A T)	6	7	8	0
*amiA*145 (A T T A A C)	1.6	1.6	2.5	0
*amiA*136	5	7	9	0.4

Expected recombination index values would be 1, 1.2 and 6.6 for transformation by *amiA*6, *amiA*141 and *amiA*54 DNA respectively, in the absence of conversion. Underlined bases indicate a change from the sequence of *amiA*36 (ATTAAT). *amiA*136 is mutated 6 and 7 bases on the left of *amiA*36 resulting from the double substitution: 5′-CTT CCA GAT → 5′-CTT TGA GAT

Table 2. Recombination indices involving the *amiA*36 and *amiA*128 sites

*amiA*36 recipient strain			*amiA*128 recipient strain		
Donor DNA	Experimental	Expected	Donor DNA	Experimental	Expected
*amiA*54	24	6	*amiA*3	34	14
*amiA*141	22	1.2	*amiA*14	30	6
*amiA*6	26	1	*amiA*23	28	3

Expected indices are computed from two-point crosses with the assumption that the expected recombination index between *amiA*36 and *amiA*6 is 1% (27 bp). Recombination indices in transformation of *amiA*36 as recipient were reported previously (Lefèvre et al. 1984)

original observation (21%) for the palindromic sequence ATTAAT (*amiA*36). The highest recombination index is observed in transformation with *amiA*148 which yielded an average of 14% in excess of the expected value. Therefore the 5′-T to 5′-C transition in the second position from the left of the palindrome reduced conversion from 21% to 14%, i.e. to 2/3. A stronger reduction of conversion was observed for the group of mutants *amiA*135, -137, -142, -146 which, on average, yielded an excess of 6.1% wild-type transformants. For these markers, conversion is reduced to 29% of the maximum value. The substitutions from the original palindrome consist of either a 5′-A to G transition in the first position, the same transitions in the fourth and fifth positions, or a 5′-T to G transversion in the second position. An increase of wild-type transformants was not found in crosses with *amiA*145 which results from a 5′-T to C transition in the sixth position. These results suggest that all six bases are involved in inducing conversion, the most significant being at the sixth position.

Another example of an increase in wild-type transformants was found for the *amiA*136 mutation which is five bp to the left of *amiA*36 (see next section). This mutation results from a two base substitution 5′-CTT CCA GAT to 5′-CTT TGA GAT. In two-point crosses with the three linked markers *amiA*6, -141 and -54, the recombination indices are, respectively, 5%, 7% and 9%, that is an average of 4% excess wild-type recombinants over the expected values. Thus aberrant recombination may be induced by some base configurations other than those of the original palindrome.

Among the other sequenced substitution mutants in the *amiA* locus, we have not observed any significant anomalies of recombination, except *amiA*9 which consistently yields twice as many recombinants with the closest sequenced marker (*amiA*29) as expected from the physical distance. Indeed *amiA*9 is a 5′-GTGGTCAATTCT to 5′-GTGGTTAATTCT transition which we have also found in the *amiA*142 mutation where it induced a conversion.

Since a palindrome was originally found to trigger conversion, a question that one might ask is whether any palindrome *per se* would induce conversion at the heteroduplex stage of recombination. A frameshift mutation has been induced in vitro by filling-in with DNA polymerase a *Cla*I site of the *amiA* locus, AT CGAT, yielding ATCGCGAT. The DNA fragment obtained creates a double frameshift (Claverys et al. 1983). When crossed with *amiA*50 located 303 bp away the recombination index is 8.5% and not statistically different from that expected (11.2%). Therefore a heteroduplex containing a palindrome *per se* appears not to be sufficient to induce conversion.

Minimum length of the signal sequence for localized conversion

To determine whether the neighbouring sequences of the original palindrome have an effect on conversion, we have created an identical palindrome in another region of the locus. Computer analysis of the sequenced segments of this locus indicated that a single nucleotide substitution could generate the ATTAAT/TAAGTA configuration at a site in the B2 fragment segment in *amiA*128.

The new *amiA*128 mutation is a G/C → T/A transversion which yields a nonsense codon. The resulting mismatches will be 3′-C(+)/5′-T(m) and 5′-G(+)/3′-A(m) very similar to the mismatches at the *amiA*36 site: 5′-C(+)/3′-T(m) and 3′-G(+)/5′-A(m), where it has been demonstrated that the only heteroduplex converted to wild type is the latter one. We have carried out transformations between *amiA*128 and three other *amiA* mutants: *amiA*3 which has been sequenced and is located 363 bp away from *amiA*128 (Fig. 1) and *amiA*14 and *amiA*23 which have been mapped in this same region (Sicard and Ephrussi-Taylor 1965). The results obtained (Table 2) show a high frequency of conversion (21%), quite similar to that observed with *amiA*36 (23%). This indicates that the 6 bp configuration 5′-ATTAAT-3′/ 3′- TAAGTA-5′ is a sufficient signal for localized conversion.

Length of the conversion event

It has been shown that the conversion process involves at least 5 bp on the right of *amiA*36 (Sicard et al. 1985b) but no more than 27 bp (Lefèvre et al. 1984). To estimate more precisely the length over which this event occurs we have isolated two mutants by site-specific mutagenesis: *amiA*136 at 5 bp to the left of *amiA*36 and *amiA*134 at 12 bp to the left of *amiA*128. These mutants were crossed with their neighbours that induce conversion. In the transformation of the strain *amiA*36 by *amiA*136 DNA, the recombination

index was 0.4% (Table 1), slightly more than the expected value (0.2%) but much below 21%. We interpret this result as a co-conversion event which simultaneously created a wild type at the *amiA*36 site and the mutant at the *amiA*136 site. Conversion at the *amiA*36 site, therefore, appears to extend further than 5 bp on both sides of the *amiA*36 mutant site. In transformation between *amiA*128 and *amiA*134, the recombination index was 12%, that is about half of the maximum conversion frequency for the marker *amiA*128. Together, these data suggest that the conversion event involves an average of 10–12 bases i.e. a turn of the DNA helix probably on either side of the signal.

Independence of the localized conversion process and excision of UV-induced damage

Nothing is known about the molecular processes leading to localized conversion. It could be the result of the excision of a few bases close to a mutation. This model would be similar to the short-patch repair system of pyrimidine dimers after UV irradiation of DNA which also involves a 12 base excision (Sancar and Rupp 1983). We have transferred the *amiA*36 mutation into the strain 402 which is UV sensitive, transforms at a normal rate and does not repair UV-induced damage on a virulent phage DNA (Tiraby 1974). When crossed with *amiA*6, there is 26% wild-type transformation, similar to values obtained with *amiA*36 carried in non-UV sensitive strains. Therefore, localized conversion does not involve the repair step which is missing in this UV sensitive strain.

Discussion

In this report we have shown that in the *amiA* locus a short stretch of 6 bases is able to induce hyper-recombination when strains carrying the stretch are crossed with strains containing closely linked markers. Since two separate six base palindromes at different locations in the locus give similar conversion frequencies, the neighbouring sequences have a negligable effect. Moreover, all substitutions in the original palindrome sequence reduced the hyper-recombination to levels depending upon the mutation. Thus, non-palindromic sequences can be still quite accessible to the conversionogenic system. This result argues against the hypothesis that localized conversion acts in a manner similar to restriction modification enzymes. It is more likely that a family of related sequences are recognized in this system by one or more specific enzymes to produce localized conversion. If this is so, mutants deficient in this conversion should be found. Their selection at present, however, is precluded by the lack of an anticipated phenotype.

In lambda phage it has also been reported that some particular mutations recombine anomalously. The highest recombination frequency with nearby markers was observed when a C to T change occurs in the palindrome sequence 5′-CCAGG which is methylated by the product of gene *dcm*. Related sequences such as 5′-CAGG or 5′-CCAG which are not methylation sites likewise induce high recombination levels (Lieb et al. 1986). Thus the very short-patch process acting in λ phage crosses shares some properties with localized conversion in pneumococcus: the sequence signal is not unique but is a family of related sequences. It is very short and, moreover, the length over which the conversion process occurs is small. On the other hand, the signal sequences are different in the two organisms, and the very short-patch repair in lambda phage is depressed in *Escherichia coli* strains deficient in the methyl-directed excision repair system, while in pneumococcus deficient in a similar excision-repair process, localized conversion is not modified.

The function of localized conversion in pneumococcus is not known. It is able to correct the A/G mismatch which is not recognized by the long-patch repair system (Claverys et al. 1983), thus participating in the protection of the genome. It may have some other essential biological functions since we have found it in several pneumococcal strains of independent origins (unpublished results). Further studies are required to reveal the mechanism of localized conversion which will help to understand its function in pneumococcus.

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