GENETIC RECOMBINATION IN DNA-INDUCED TRANSFORMATION OF PNEUMOCOCCUS. V. THE ABSENCE OF INTERFERENCE, AND EVIDENCE FOR THE SELECTIVE ELIMINATION OF CERTAIN DONOR SITES FROM THE FINAL RECOMBINANTS[1]

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HIGH negative interference or positive correlation of recombination events, especially over short segments of a genome, is a phenomenon that has been reported to obtain during genetic recombination in several organisms (PRITCHARD 1955; CHASE and DOERMANN 1958; DE SERRES 1958). That high negative interference is a general phenomenon (DEMEREC 1928; STURTEVANT 1951; PRITCHARD 1955; ST. LAWRENCE 1956; ST. LAWRENCE and BONNER 1957) was suggested by CHASE and DOERMANN (1958). Whether high negative interference is also observed when transforming DNA recombines with a bacterial chromosome is the principal question that prompted the investigations reported in this paper.

PRITCHARD (1955) has suggested that recombination is very intense in localized areas that represent effectively paired segments of the chromosome, and that recombination is virtually nonexistent along other segments. CHASE and DOERMANN (1958) further suggested that recombinational events are, indeed, clustered within the "switch areas," which occur randomly throughout the genome of phage T4, these switch areas constituting only a small part of the genetic structure. These proposals have been advanced to explain high negative interference. In phage, the "switch areas" have now been identified with regions of heterozygosity (DOERMANN 1965) which may be internal, or the result of terminal redundancies in a permutated linear chromosome structure (STREISINGER, EDGAR and DENHARDT 1964). Thus, DOERMANN (1965) concludes that in order to explain high negative interference in phage there is no need to assume that the occurrence of one exchange has an effect on adjacent exchanges.

Three-point crosses such as those to be reported in this paper afford quantitative measurements of both the intensity and independence (or dependence) of multiple recombinational events in DNA-induced transformation, which is in many respects the simplest of all genetic recombination systems (EPHRUSSI-TAYLOR 1960). The presence of interference (positive or negative) in a transforming system would indicate that genetic exchanges are not random in even this simplest

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of genetic systems, and that the biochemical events underlying recombination at the molecular level also are nonrandom. The absence of interference would indicate randomness of exchanges at the molecular level and randomness of these biochemical processes, a conclusion which would agree with that of DOERMANN.

The relative linear order of 54 *amiA-r* mutations in *D. pneumoniae* has been established by means of two-point transformation crosses with the *ami-r* mutations in the *trans* configuration, and the efficacy of this system in genetic fine-structure analysis has been demonstrated (SICARD and EPHRUSSI-TAYLOR 1965). In the course of this present work, the positions of additional *amiA-r* mutations have been determined in similar experiments. *amiA-r* mutations fall into two nonoverlapping efficiency classes; high efficiency (HE) mutations are those which are recombined into (or out of) a (recipient) genome with approximately equal frequency as the standard unlinked reference marker gene *str-r41*, while low efficiency (LE) mutations are integrated with only one-tenth the frequency of the reference marker (EPHRUSSI-TAYLOR, SICARD and KAMEN 1965).

It has been demonstrated that recombination frequencies are roughly additive up to values approaching 30% (SICARD and EPHRUSSI-TAYLOR 1965). One less favorable feature of transformation, insofar as fine-structure studies are concerned, is that recombination frequency is apparently influenced by the physiological state of the recipient population (RAVIN and IYER 1961; SICARD and EPHRUSSI-TAYLOR 1965); it is not unusual to observe as much as a twofold difference in the recombination frequency between two sites when two independently prepared cultures of the same recipient strain are transformed by the same donor DNA preparation. This inherent variability of recombination frequency must be taken into account in making comparisons of sets of data obtained with different recipients, or different preparations of the same recipient culture. It sets the limit to measurements of interference in that observed and predicted frequencies of recombinants must differ by more than four to eightfold, depending on the crosses, before it may be securely inferred that interference is present, predicted frequencies being based on two independent two-point crosses in many instances.

The central portion of the *amiA-r* locus is especially well suited to the analysis of interference by means of three- and four-factor transformation experiments because the recombining *amiA-r* mutations in this well mapped segment are quite closely linked (SICARD and EPHRUSSI-TAYLOR 1965). Recombination frequencies between neighboring marker mutations, of both high and low efficiency classes, are generally of the order of 10% or less. Furthermore, through a refinement of the selective system developed by SICARD (1964) it has proven possible to measure quantitatively the occurrence of very rare recombinational events.

It will be shown in this report that significant positive and negative interference are absent from recombination in the *amiA* locus of *D. pneumoniae*. Furthermore, the preparation of strains bearing two mutations in the *amiA* locus, necessary for effecting three- and four-factor crosses, permitted the examination of the consequencies of having an HE and an LE mutation present in *cis* position during transformation, in two-point crosses of double mutant cells by wild-type DNA,

or the reverse cross. It is found that the LE mutation in *cis* position relative to the HE mutation suppresses the high transforming efficiency of the HE site, in agreement with observations recently reported by LACKS (1966). A model of events during recombination which explains low efficiency, as well as the results of all types of genetic crosses based on the present data, has already been presented (EPHRUSSI-TAYLOR and GRAY 1966). We are publishing here for the first time the data which inspired this model. It is shown how this model explains observations of workers in other laboratories on other genetic systems as well as our own. The features of our data which are incompatible with an alternate model proposed by LACKS will also be briefly discussed.

MATERIALS AND METHODS

Strains: Most of the strains used here, and their derivations, have been described previously (SICARD 1964; EPHRUSSI-TAYLOR, SICARD and KAMEN 1965; SICARD and EPHRUSSI-TAYLOR 1965). Clone 3, the standard wild-type strain used in this laboratory, is descended from strain R36A of AVERY and is sensitive to 0.1×10^{-5}M aminopterin. Many of the *amiA-r* mutants derived from Clone 3 are of spontaneous origin, while others were induced by mutagens such as nitrous acid and ethyl methanesulfonate. In general, *amiA-r* strains are resistant to $1–2 \times 10^{-5}$M aminopterin. However, four strains, *amiA-r31*, *r34*, *r39* and *r60*, show a maximum resistance level of 0.5×10^{-5} M. *amiAr34* and *r60* are nonrecombining mutations of independent origin.

Several strains bearing two *amiA-r* mutations have been prepared for these investigations, using the four *amiA-r* mutants which have only a moderate level of resistance to aminopterin. In principle, among the transformed progeny arising from a cross of one *amiA* mutant by DNA of another, three classes should be present: transformants like the DNA donor, and two recombinant classes, one bearing the two mutated sites, the other bearing the two corresponding wild-type sites. When a strain showing moderate resistance to aminopterin is crossed by DNA of a strain showing higher resistance, and samples plated in concentrations of aminopterin varying from 0.1 to 3.5×10^{-5}M, characteristic decreases in the number of colonies on the plates are observed at 0.75×10^{-5}M, and at 1.5×10^{-5}M. The first decrease results from inhibition of growth of untransformed cells, and the second from inhibition of growth of transformants of donor type. Recombinants bearing two *amiA-r* mutations are slightly more resistant than either parent, and are the predominant colonies on plates containing from 1.5 to 3.5×10^{-5}M aminopterin. Clones were established by picking colonies from plates at the higher concentrations of aminopterin. The identity of each double mutant strain was established by "backcrossing" the suspected double mutant with DNA from each of the parents, these DNAs bearing also the standard reference marker *str-r41*. When no *amiA-s* recombinants were observed for several hundred thousand *str-r41* transformants scored, using either parental DNA, the recipient strain was considered to be a double mutant bearing the parental mutations. Strains prepared in this manner are enumerated in Figure 1. The recipient strain employed in the preparation of a double mutant strain appears first, followed by the site in the donor DNA fragment. Some of these strains carry two LE mutations, while others bear both an LE and an HE mutant site in the chromosome; in some strains the HE site is located to the left of the LE site and in other strains the right-left relationship is reversed. A double mutant strain bearing two HE *amiA-r* mutations was not isolated because of technical difficulties arising from the fact that all of our HE mutants show maximal resistance to aminopterin.

Experimental procedures: The salient features of two-point transformation experiments have been reported (SICARD and EPHRUSSI-TAYLOR 1965); three- and four-point transformation crosses are performed in essentially the same manner. SICARD (1964) reported the details of the selective system utilized in this laboratory for scoring aminopterin sensitive (wild-type) transformants in crosses of *amiA-r* mutants by DNA of other *amiA-r* mutants. One important modification of the plating procedure has been introduced. It has been found that if a small amount of complete

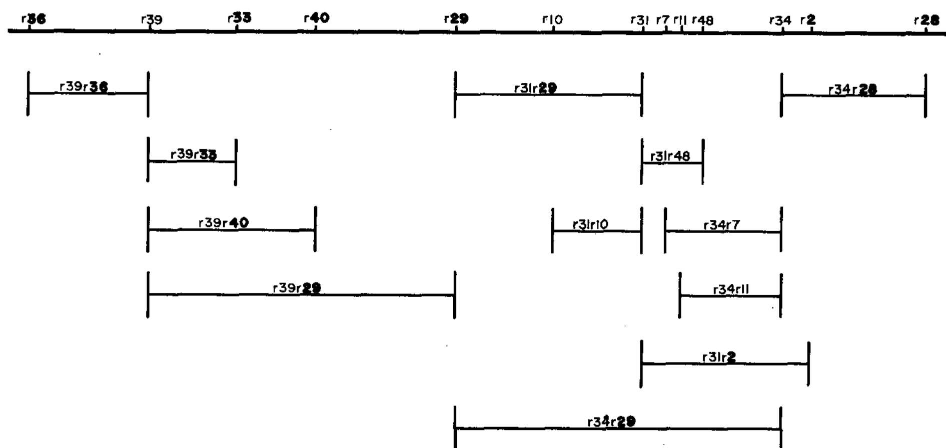

FIGURE 1.—Diagram of the 12 double-mutant strains employed in the crossing experiments. The segment extending from *amiA-r36* to *r28* comprises approximately 75% of the entire aminopterin region. High efficiency sites are indicated by bold-face numerals, while normal numerals indicate sites of low efficiency. For nomenclature of the various double mutant strains, the following convention has been adopted: the recipient strain employed in the preparation of the double mutant strain appears first, followed by the site in the donor DNA fragment (see text for full explanation).

medium is carried over with the cells when they are plated in synthetic medium, no time need be allowed for phenotypic expression of wild-type recombinants. Platings of undiluted transformed populations ("complexes") may thus be made directly into medium containing an excess of isoleucine, permitting detection of even very rare recombinants. When complexes must be diluted for plating, this is done in either preculture (P) medium or in transforming (NS) medium, rather than in a balanced salt solution containing 0.02% serum albumin as previously reported. From 0.1 to 0.6 ml of diluted or undiluted complexes are plated directly into 6 to 8 ml of synthetic-agar medium containing an excess of isoleucine. Generally, selection of wild-type recombinants is optimal in the presence of 1.3 to 2.6 mg/ml excess isoleucine. Selection is adequate when the excess isoleucine ranges from 0.65 to 6.5 mg/ml. In order to standardize the procedure, 2.6 mg/ml excess isoleucine was generally added to the synthetic medium.

Whenever practical, two-point transformation crosses, which serve as the basis for predicting the results of three-factor crosses, were performed simultaneously with comparable three- and four-point crosses. This procedure served to minimize variations in recombination frequencies that are often seen with different batches of competent cells and are presumably due to differences in environmental conditions which we have not yet explored. Thus, when a three-point cross of a recipient culture of *amiA-r31r2* by a series of donor DNAs was performed, competent cultures of both *amiA-r31* and *r2* were transformed by the same series of donor DNAs, in the same medium. However, this practice does not completely eliminate inherent variations because data on two recipient strains must be used in many of the experiments in order to obtain all of the recombination frequencies for calculation of expected double or triple exchanges.

Types of crosses performed: The types of crosses performed in these investigations are presented diagrammatically in Figure 2. A cross of *amiA-r* cells by DNA extracted from wild-type (aminopterin sensitive) cells constitutes a one-point cross (TYPE I) which defines the incidence of transformation at that particular site. The only requirement for obtaining a wild-type recombinant in such a cross is that one or any odd number of exchanges on either side of the site must occur. No restriction is placed upon the positions of the exchanges except that they must flank the mutant site. Presumably they could occur between the site and the adjacent base pair, or

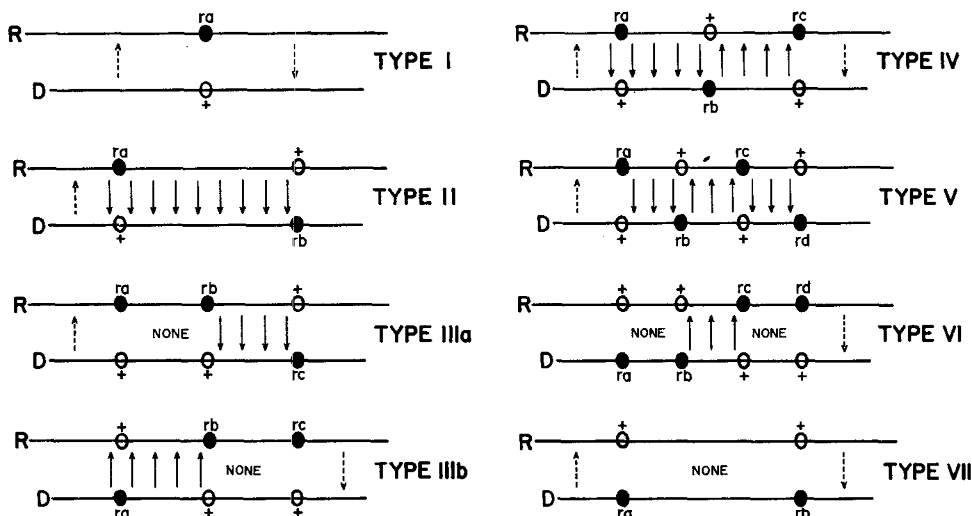

FIGURE 2.—Diagram of the seven kinds of transformation crosses. R = recipient nucleotide sequence; D = donor nucleotide sequence; ● = mutated site; O = ++ site; a solid arrow indicates location of a restricted exchange event and a dashed arrow indicates location of an unrestricted exchange event. TYPE I. One-factor cross. In order to recover a ++ recombinant, two unrestricted exchange events must occur, one on either side of the recipient site which must be eliminated from the recombinant chromosome. TYPE II. Two-factor *trans* cross. Recovery of a ++ recombinant is dependent upon the occurrence of a restricted exchange event in the interval between the recipient and donor sites, as well as the occurrence of an unrestricted exchange event outside this interval. TYPE III (a and b). Three-factor cross. Recovery of a ++ recombinant depends upon the occurrence of a restricted exchange event in one interval and the absence of an odd number of exchange events in a contiguous interval. The position of the single unrestricted exchange event is unspecified. TYPE IV. Three-factor cross. Two restricted exchange events, one in each of two adjacent intervals, and two unrestricted exchange events are required in order to recover a ++ recombinant. TYPE V. Four-factor cross. Recovery of a ++ recombinant is dependent upon the occurrence of a restricted exchange event in each of three contiguous intervals, as well as the occurrence of a single unrestricted exchange event. TYPE VI. Four-factor cross. One unrestricted exchange event whose position is largely unspecified and one restricted exchange event in the interval *rb* to *rc* are required in order to recover a ++ recombinant. TYPE VII. Two-factor *cis* cross. Recovery of double transformant (*ra rb*) is dependent upon the occurrence of two unrestricted exchange events, accompanied by no or an even number of exchange events in the interval separating the two recipient sites.

several hundred nucleotides away from the mutated site. Thus, a portion of the recipient sequence is replaced by the sequence in the donor fragment.

In a two-point cross of mutant cells by DNA derived from a different mutant (TYPE II), where the mutant sites are in *trans* position, a minimum of two exchanges are also required in order to produce a wild-type recombinant. In this case, however, one of the two exchange events must occur within the interval defined by the two sites; which of the two recombinational events is thus restricted depends upon whether the donor site lies to the left or right of the site in the recipient genome. In the restricted interval one or any odd number of exchange events must occur if the recipient site is to be excluded from the recombinant structure. Presumably, the farther removed the two mutant sites are from one another, the greater is the probability of the occurrence of an exchange in the restricted region. In all our transformation crosses, the frequency of recombination is expressed as the ratio of the *amiA-s* recombinants to transformants

TABLE 1

Frequencies of wild-type recombinants relative to str-r41 *transformants obtained in two-factor crosses of recipient cells bearing an* amiA-r *mutation with a series of donor DNAs, each bearing a different* amiA-r *mutation*

Site in donor DNA	Mutated site in recipient genome										
	r36	*r39*	*r33*	*r22*	*r29*	*r10*	*r31*	*r48*	*r34*	*r2*	*r28*
r36	....	0.316	0.093	....	....	....	....	....	....	....	....
r39	0.221	....	0.218	....	....	....	....	....	....	....	....
r33	0.169	0.173	....	....	....	....	....	....	....	....	....
r45	0.084	0.148	0.058	....	....	....	....	....	....	....	....
r74	0.081	0.123	0.054	....	....	....	....	....	....	....	....
r54	0.425	0.215	0.069	0.376	....	....	....	....	....	....	....
r75	0.108	0.256	0.094	0.235	0.125[a]	....	....	....	....	....	....
r40	0.469	0.281	0.168	0.230	0.202[a]	....	....	....	....	....	....
r49	0.212	0.314	0.160	....	....	....	....	....	....	....	....
r41	0.442	0.454	0.472	0.059	0.081[a]	....	....	....	....	....	....
r42	0.463	0.528	0.409	0.061	0.093[a]	....	....	....	....	....	....
r22	0.616	0.399	0.379	....	0.036	0.444	0.432	....	....	....	....
					0.032[a]						
r29	....	....	....	0.018	....	0.371	0.373	....	....	....	....
r9	....	....	....	0.043	0.041	0.357	0.405	....	....	....	....
					0.048[a]						
r20	....	....	....	0.159	0.072	0.271	0.296	....	....	....	....
r24	....	....	....	....	0.109	0.193	0.261	....	....	....	....
r6	....	....	....	....	0.243	0.0003	0.152	....	....	....	....
r8	....	....	....	....	0.213	0.0006	0.168	....	....	....	....
r10	....	....	....	....	0.203	....	0.118	....	....	....	....
r47	....	....	....	....	0.332	0.109	0.254	....	....	0.894	....
r26	....	....	....	....	0.313	0.215	0.004	....	0.262	0.578	....
									0.468[a]	0.615[a]	....
r31	....	....	....	....	0.294	0.219	....	0.285	0.159	0.600	....
									0.560[a]	0.739[a]	....
r43	....	....	....	....	....	0.390	0.176	0.113	0.307	0.437	....
										0.501[a]	....
r7	....	....	....	....	....	0.418	0.205	0.109	0.346	0.249	....
									0.353[a]	0.452[a]	....
r11	....	....	....	....	....	0.484	0.190	0.074	0.102	0.408	....
									0.455[a]	0.437[a]	....
r21	....	....	....	....	....	0.406	0.202	0.074	0.205	0.422	....
									0.283[a]	0.437[a]	....
r44	....	....	....	....	....	0.542	0.300	0.052	0.120	0.355	....
r48	....	....	....	....	....	0.669	0.389	....	0.101	0.301	....
									0.111[b]	0.170[a]	....
r25	....	....	....	....	....	0.626	0.379	0.935	0.057	0.155	....
									0.071[b]	....	....
r27	....	....	....	....	....	0.581	0.441	0.067	0.056	0.175	....
									0.078[a]	....	....
r34	....	....	....	....	....	....	0.407	0.077	....	0.057	....
r1	....	....	....	....	....	0.707	0.476	....	....	....	....
r2	....	....	....	....	....	0.723	0.406	....	0.041[c]	....	0.316
											0.104[a]
r3	....	....	....	....	....	0.652	0.492	....	....	....	....

Site in donor DNA	Mutated site in recipient genome										
	r36	*r39*	*r33*	*r22*	*r29*	*r10*	*r31*	*r48*	*r34*	*r2*	*r28*
r19	....	....	....	....	....	0.623	0.464	....	....	....	....
r52	....	....	....	....	....	0.656	0.497	....	0.093[b]	0.054	0.293
										0.043[a]	0.137[a]
r63	....	....	....	....	....	....	....	....	0.134[c]	....	0.131
											0.078[a]
r50	....	....	....	....	...	0.652	0.516	....	0.053[c]	0.054	....
r51	....	....	....	....	....	0.686	0.449	....	0.082[b]	0.047	0.278
										0.049[a]	0.168[a]
r72	....	....	....	....	....	....	....	....	0.134[c]	....	0.020
											0.017[a]
r14	....	....	....	....	....	....	0.537	....	0.048[c]	0.084	0.084
										0.085[a]	....
r23	....	....	....	....	....	....	0.513	....	0.094[c]	0.251	0.182
										0.278[a]	0.066[a]
r71	....	....	....	....	....	....	....	....	0.038[c]	....	0.015
											0.097[a]
r12	....	....	....	....	....	....	0.443	....	0.098[c]	....	0.011[a]
r38	....	....	....	....	....	....	0.791	....	0.449[b]	0.574[a]	0.00005
r28	....	....	....	....	....	....	0.562	....	0.334[b]	0.467	....
										0.364[a]	....
r16	....	....	....	....	....	....	....	....	0.507[b]	....	0.216
r15	....	....	....	....	....	....	....	....	0.519[b]	....	0.214
r53	....	....	....	....	....	....	....	....	....	....	0.448
++	1.43	0.124	1.14	1.19	1.55	0.108	0.111	0.181	0.097	1.40	0.611
					1.02[a]	....	....	....	0.077[a]	1.39[a]	0.936[a]
									0.099[b]	....	....
									0.151[c]	....	....

A correction imposed by inherent physiological differences among the several recipient strains has been applied to the data. The figures represent the ratio of wild-type recombinants to *str-r41* transformants divided by the efficiency of integration of the marker in the recipient strain (as determined in a one-factor cross of mutant cells bearing the *amiA* mutation in question by wild-type donor DNA).

[a,b,c] represent recombination values obtained when different batches of recipient cells were transformed by the same donor DNAs.

Here, and in subsequent tables, unless very rare recombination events are involved, ratios are determined from colony counts of 2,000 or more transformants.

for the *str-r41* reference marker. The smaller this ratio, the greater is the linkage between the two sites.

Three-point crosses are of two types. One requires one restricted and one unrestricted exchange event to yield wild-type recombinants (TYPE IIIa and IIIb), while the other requires two restricted and two unrestricted exchange events (TYPE IV). In crosses of TYPE IIIa or IIIb, wild-type recombinants are recovered only if a single or any odd number of restricted recombinational events occur within the interval *rb* to *rc* or the interval *ra* to *rb*, depending upon the relative position of the mutated site in the donor fragment with respect to the mutant sites in the recipient genome. At the same time, no or an even number of recombinational events are permitted in the adjacent intervals, defined as *ra* to *rb* or *rb* to *rc*, respectively. Again, one outside exchange event whose position need not be defined is necessary for recovery of wild-type recombinants.

Crosses of TYPE IV, in which the mutated site in the donor fragment is flanked by the two mutant sites of the recipient chromosome, require a recombinational event in each of two adjacent intervals, the latter defined by the relative distances from *ra* to *rb* and *rb* to *rc*. Of course, two unrestricted events, one to the left of *ra* and one to the right of *rc*, are also required to yield wild-type recombinants.

The four-factor crosses reported in this paper are of two types, although other configurations are possible. In a cross of TYPE V, wild-type recombinants are recovered only if three restricted exchange events occur in the contiguous intervals *ra* to *rb*, *rb* to *rc* and *rc* to *rd*. The position of the single nonrestricted exchange need not be specified. In a four-point cross of TYPE VI, wild-type recombinants are recovered if a single restricted exchange occurs in the interval *rb* to *rc*; however, an odd number of exchanges within the intervals *ra* to *rb* and *rc* to *rd* is not permitted. Again, the position of the single unrestricted exchange event need not be specified.

The last type of cross (TYPE VII) performed in these experiments is a two-factor cross in which the *amiA-r* sites (*ra* and *rb*) are in the *cis* position in the donor fragment, and the recipient cells are wild-type. Among the progeny arising from such a cross are the recombinant types *ra sb* and *sa rb*, as well as the double transformant *ra rb*. The ratio of *amiA-r* transformants of all three classes to *str-r41* transformants scored affords a measurement of the overall efficiency of integration of *amiA-r* markers, both singly and simultaneously. Also, by establishing the genotypes of recombinants recovered from such a cross through transformation by DNAs bearing *ra* or *rb* singly, one can determine the frequency of segregation of the *amiA-r* marker mutations and the efficiency of integration of each. In this cross, therefore, the genotypes as well as the phenotypes of the recombinants are verified experimentally.

EXPERIMENTAL RESULTS

Of principal interest in these investigations is the segment of the *amiA* region extending from *amiA-r22* on the left to *amiA-r53* on the right. Comprising 39 mutated sites, of both high and low efficiency classes, this segment represents about half of the entire *amiA* region. The mutated sites are more densely distributed in this region than are the markers in either the right or left terminal portions: hence its usefulness in the present study. The recombination frequencies observed in two-factor crosses, and presented in Table 1, are those employed to calculate the recombination frequencies expected in three- and four-factor crossing experiments. In the table, sites are listed in left to right map order. A correction factor, imposed by the effect of differences in efficiency of the various recipient strains on measured recombination values, has been applied (LACKS and HOTCHKISS 1960; SICARD and EPHRUSSI-TAYLOR 1965). The data correspond to the observed ratio of *amiA-s* recombinants to *str-r41* transformants in the two-factor crossing experiments, divided by the efficiency of transformation of the recipient by DNA which is wild-type at the *amiA* locus.

Use of the term "exchange" in the following discussions will not imply an endorsement of either a copy choice or a breakage and reunion mechanism of genetic recombination. We shall mean by exchange unitary events leading to the assorting of donor and recipient patterns of base sequence in the recombinant structure.

Two-factor cis *crosses:* With rare exceptions, the efficiency of an *amiA-r* mutation is equal in both directions of one-factor crossing; that is to say, whether the mutated site is in the recipient chromosome or in the donor DNA fragment, it will be recombined into or out of the recombinant structure with the same probability. Experiments have now been performed to measure the frequency of double transformation in two-factor crosses with two *amiA* mutations in *cis* position (TYPE VII, Figure 2). Reciprocal crosses were done using (*a*) the standard wild-type strain and DNA extracted from double mutant strains (each bearing the standard

reference marker *str-r41* as well), and (*b*) the double mutant strains and DNA extracted from a strain wild-type for the *amiA* region, (but bearing also the *str-r41* marker). Results are presented in Table 2.

In cross (*b*), of doubly mutant cells by DNA of the wild-type strain, only a single recombinant class is scored, namely, that which includes both wild-type *amiA* alleles in the recombinant. These arise if no or an even number of exchanges occurred between the markers of the cross. An odd number of exchanges in the interval between the two mutated sites in the recipient chromosome leads to the formation of an aminopterin resistant recombinant bearing either one or the other *amiA-r* markers of the recipient, and such recombinants do not grow in the selecting medium (containing isoleucine in excess). Thus, the observed recombinant frequency in this direction of crossing represents the frequency with which both mutant *amiA-r* sites are simultaneously recombined out of the chromosome. In the reciprocal cross (*a*) of wild-type cells by DNA of a double mutant strain, transformation to aminopterin resistance is scored by plating at a level permitting growth of the least resistant recombinant, and, therefore, three recombinant classes are represented among the progeny. There are those which have integrated both *amiA-r* markers of the donor DNA fragment, as well as those which have integrated only one or the other of the donor sites. The recombinants can only be identified by "backcrosses" with two DNAs, each bearing one of the mutant sites in question. These "backcrosses" were made. When the frequency of recombining both mutant sites out of the recipient chromosome (cross b) is compared with that of integrating both mutant sites into the primary recombinant structure (cross a), very close agreement is observed (Table 2). We conclude that the frequency of integration of two mutant *amiA* sites in *cis* position is the same in both directions of crossing.

Of the double mutant strains tested for transformation to wild-type, some carry an LE and an HE mutation, while others bear two LE mutations (Table 2). In those crosses in which the double mutant recipient comprises markers of both efficiency classes, the frequency of wild-type recombinants is in the range of 0.080 to 0.11, irrespective of which pairs of markers were employed. We are, of course, dealing with fairly closely linked sites in *cis* position: were the efficiency of the HE site to dominate during recombination, we would expect wild-type transformant frequency to be equal to the probability of inclusion of the HE site, multiplied by the probability of the two wild-type sites in the donor DNA becoming separated by recombination. Conversely, were the efficiency of the LE site to dominate during recombination, we would expect wild-type recombinants to appear at a frequency equal to the transforming efficiency of the LE site multiplied by the dissociation frequency. The observed frequency of wild-type recombinants fits best dominance of the efficiency of the LE site, since it is equal to or slightly less than the efficiency of the LE marker of the pair. Thus, the probability of incorporation of the HE marker is clearly greatly reduced when it is in *cis* position relative to an LE marker.

In concordance with the results obtained with HE-LE double mutants, the LE-LE double mutants crossed by wild-type DNA show that it is the efficiency of the

TABLE 2

Frequency of double transformants obtained in two-factor cis *crosses of doubly mutant cells treated with ++ DNA (Column 4) or wild-type cells treated with donor DNAs, each bearing two* amiA-r *mutations (Column 5)*

1	2	3		4	5	
					Wild-type cells by double mutant DNA	
Mutated sites in recipient	Map distance between sites (%)	Efficiencies of sites in one-factor crosses		Doubly mutant cells by ++ DNA	Total *amiA-r* transformants	*rarb* transformants
		ra	*rb*			
r31r2	46.86	0.111	1.40	0.085	0.652	0.136
r31r29	57.30	0.111	1.16	0.080	0.509	0.061
r39r33	24.50	0.124	1.14	0.085	0.339	0.076
r39r36	33.00	0.124	1.43	0.084	0.181	0.067
r39r29	90.00*	0.124	1.16	0.109	....	....
r39r40	45.90	0.124	1.43	0.097	....	....
r34r28	42.60	0.099	0.936	0.085	....	....
r34r29	96.56*	0.099	1.16	0.081	....	....
r31r10	25.00	0.111	0.108	0.073	....	....
r31r48	16.36	0.111	0.181	0.085	....	....
r34r7	34.40	0.099	0.160	0.047	....	....
r34r11	30.40	0.099	0.100	0.056	....	....

* These distances were not measured directly in two-factor *trans* crosses because these sites generally show no, or only very weak, linkage. A reasonable estimate of the distance separating these sites can be obtained by treating a recipient strain bearing a site internal to the sites in question with DNA of each of the two sites; these values are then summed. (For example, the distance can be approximated by treating *amiA-r31* (an internal marker) with DNA of *r29* and *r34* and summing these values.)

marker having the lower probability of inclusion which dominates in determining the results: the frequency of bacteria transformed at both sites is lower than the efficiency of the least efficient of the two LE mutants. There is no compounding effect of low efficiencies.

The absence of compounding effects of efficiencies in these two-point *cis* crosses is a crucial result insofar as constructing a model of recombination in pneumococcus is concerned, as will be apparent below. Suffice it to say here that the absence of a compounding effect when two LE mutations are in the recipient chromosome indicates that whatever mechanism determines efficiency, it is most likely "triggered" by the nature of the LE site. Once the mechanism has been "triggered" by a single LE site, additional mutations of either high or low efficiency along the molecule do not serve as additional "triggering" sites. LACKS (1966) was able to detect similar effects with mutated sites in *cis* positions in the amylomaltase locus of pneumococcus.

The consequences of the presence of an LE site in *cis* position relative to an HE site are even more clearly demonstrated in the *cis* crosses of type *a* (wild-type recipient by double mutant DNA).

Four strains, each bearing an HE and an LE marker, were employed in these crosses. In two strains, *amiA-r31r2* and *r39r33,* the HE site lies to the right of the LE site, whereas in the others, strains *r31r29* and *r39r36,* the HE site is to the left of the LE marker. Wild-type cells (Clone 3) were transformed by DNA isolated from each of these strains, each of which also bears the standard reference

TABLE 3

Results of two-factor crosses in which the amiA-r *markers are in* cis *position in donor DNA*

Donor DNA 1	Recombinant genotype 2	Frequency relative to *str-r41* Observed 3	Expected 4	Recovery of marker (%) 5
r31r2	*r2*	0.462	0.657 + 0.472	40.9
(46.9)	*r31*	0.054	0.0521	103.7
	r31r2	0.136	0.0589	230.9
		(0.652)	(1.24)	
r31r29	*r29*	0.366	0.665 + 0.380	35.9
(57.3)	*r31*	0.081	0.0636	127.4
	r31r29	0.061	0.0474	128.3
		(0.508)	(1.156)	
r39r33	*r33*	0.221	0.279 + 0.661	23.5
(24.5)	*r39*	0.042	0.0304	138.2
	r39r33	0.076	0.0936	81.2
		(0.339)	(1.064)	
r39r36	*r36*	0.077	0.472 + 0.587	7.3
(33.0)	*r39*	0.039	0.0409	95.4
	r39r36	0.067	0.0831	80.6
		(0.183)	(1.183)	

Wild-type cells were transformed with four donor DNAs, each bearing two mutated sites, one of each efficiency class (as well as the standard reference marker *str-r41*). *amiA-r31* and *r39* are low efficiency sites, and *r2* and *r29* are high efficiency markers. Bracketed figures in Column 1 represent overall map distances between the markers in *cis* position obtained by summing the map distances between adjacent internal markers; those in Column 3 and 4 represent the total observed and expected frequencies of all three classes of *ami-r* transformants relative to *str-r* transformants.

marker *str-r41*. Samples of the transformed populations were plated in agar containing aminopterin at a final concentration of 0.25×10^{-5} M, low enough to allow growth of the least resistant transformant. After 18 hours incubation at 37°C, well isolated colonies were picked at random from the plates, and clones were established. The genotype of each clone was established by making the appropriate transformation "backcrosses." Table 3 presents the results of these experiments.

It is quite obvious that in all four type-*a* crosses it is the HE class of transformants that is deficient, regardless of the position of the HE site relative to the LE site in the donor DNA fragment. Both the LE and the double mutant HE-LE classes occur among the recombinants with near predicted frequency. The results clearly indicate that the efficiency of integration of the LE sites is unaltered in these crosses. They can be interpreted only if one postulates that the effect engendered by the presence of the LE site is extensive, and that it proceeds in both directions along the molecule, from the LE site.

In the crosses of Table 3, the severity of the effect of the LE sites upon the frequency of integration of the HE sites is inversely related to the map distance separating the two markers. In the donor DNAs of strains *r31r2* and *r39r33*, where the HE site is to the right of the LE site, the map distance between sites *r31* and *r2* is 47%, while the map distance between sites *r39* and *r33* is 25%. In these crosses, the *r2* site is integrated with a frequency of 41% relative to the ex-

pected frequency, whereas the frequency of integration of *r33* is only 24% relative to the expected frequency. Thus, the greater the distance separating the two markers, the greater is the frequency of integration of the HE site in the two crosses in which the HE site is to the right of the LE site. Clearly, however, the exclusion of an HE site is a phenomenon that is operative at sites relatively far removed from the LE site.

In the crosses where the HE site is to the left of the LE marker, the same relationships are observed; the map distance between *r31* and *r29* is 57% and the frequency of recovery of *r29* is 35% of the expected number, while the map distance between *r39* and *r36* is 33%, but the recovery of the *r36* is only 7% of that expected. These calculations show that the severity of depression of the recovery of recombinants bearing the HE donor site appears to be polarized. That is, for a given map distance separating the sites, when the HE marker lies to the left of the LE site with which it is associated in the donor DNA, the frequency of single HE recombinants among the segregants is lower than it is when the HE site lies to the right.

In summary, one can conclude that the severity of the exclusion effect diminishes as the relative distance between the markers increases and that it is more severe to the left of the LE site than to its right.

The characteristic efficiency of HE *amiA-r* markers is restored after they have segregated from an LE marker through recombination: *amiA-r2*, *r29*, *r33*, and *r36* segregants are transformed by wild-type DNA with high efficiency (and *r31* and *r39* segregants with low efficiency). In fact, the genotypes of segregants from individual two-point crosses can be reliably determined solely on the basis of the efficiency with which they can be transformed to wild type by wild-type DNA. The unalterability of efficiency observed in these experiments lends further support to the concept of site specificity of efficiency (EPHRUSSI-TAYLOR, SICARD and KAMEN 1965).

It is thus apparent from the above that the presence of a single LE site affects the frequency of integration of additional markers associated with it in *cis* position. We have tested the hypothesis that this effect is caused by a special configuration of donor and recipient base sequences during recombination; namely, that a particular kind of mutational *difference* between donor and recipient is responsible for exclusion of donor information from the final recombinant. It should be possible to free an HE site from the depressing effect of an associated LE marker in the following way: the difference between donor and recipient, at the level of the LE site, is eliminated if an HE-LE double mutant DNA is transformed into a recipient which already bears the LE site of the donor. Here, at the level of the LE mutation, donor and recipient are identical. If it is a *particular difference* between donor and recipient which gives rise to exclusion, then in this particular cross the HE site should regain its normal transforming efficiency. *amiA-r39*, a low efficiency recipient of low aminopterin resistance, was transformed by DNA of *amia-r39r36*; duplicate samples of the transformed population were plated in agar containing aminopterin at a level which kills *amiA-39* cells, but not cells bearing the *r36* mutation, and also in streptomycin. The ratio of *ami-r36* to

str-r cells in the population was thus determined. Marker *amiA-r36* is integrated with near normal efficiency in a cross of this type as opposed to its low efficiency of integration when the wild-type allele of *r39* is present in the recipient chromosome. Clearly, it is a particular kind of difference between donor and recipient which causes the low efficiency of integration of an LE marker, as well as the HE marker in *cis* position relative to the LE site. However, since mutations at the same site are always of the same efficiency class, one must assume further either that base composition in the immediate vicinity of a mutation also plays a role in determining efficiency, or that a given site tends strongly to mutate in only one way.

A polarity effect in two-factor trans *crosses*. Experimental evidence suggesting a polarized and long-range effect of LE sites on the integration of donor DNA sites was, in fact, obtained some years ago in two-factor crossing experiments, but was not reported because no interpretation could be offered at the time. SICARD and EPHRUSSI-TAYLOR (unpublished) studied the manner in which wild-type recombinant frequency rises as the distance between the mutant site in the donor and that in the recipient increases. This was done by selecting two pairs of strains as recipients, one pair bearing mutations near the right end of the map, the other pair mutations near the left end of the map. In one strain of each pair the mutation in the recipient was HE, and in the other strain, LE. These recipients were crossed with donor DNAs bearing mutations farther and farther removed from the mutated site in the recipient. It was noted then that a right terminal LE mutation in the recipient strain gives fewer wild-type recombinants than does a right terminal HE mutation in the recipient, when crossed with left terminal donor mutations of either efficiency class. In formal genetic terms, we would say that an LE site at the right end of the map is more strongly linked to distant left-end sites than is an HE site at the extreme right end of the map. Recipients bearing left terminal sites of each class do not show this difference.

Table 4 presents some of the data of these crosses. Mutations *r36* and *r39* are left terminal mutations which are HE and LE respectively. LE mutation *r39* recombines with HE site *r36* with a frequency of 33%, and is thus more interior on the map. The average percent of wild-type recombinants recovered, in crosses with donor DNAs bearing extreme right marker mutations, was 74% for recipient *r36*, and 64% for recipient *r39*. This result is compatible with the relative positions of *r36* and *r39* on the map. In the cross with right terminal markers in the recipients, HE strain *r30* yielded an average value of 90% wild-type recombinants with left terminal donor sites (essentially freely recombining), while LE strains *r17*, *r53* and *r38* yielded values of 61, 48 and 46% respectively. Site *r17* is covered by site *r30*, while site *r53* is extremely close to the latter. One would expect, therefore, the same % recovery of wild-type recombinants with these three recipients. Yet sites *r17* and *r53* clearly are not freely recombining with left terminal sites of the map. The significance of these data is underlined if one considers the crossing results obtained with several HE recipients (Table 5) and the DNA of donor *r36*. Site *r1* lies one third of the total map distance in from the right end of the map, and is thus much closer to *r36* than are sites *r38*, *r53* and

TABLE 4

Relative degree of linkage of an HE as opposed to an LE site in recipient cells to very distant donor markers in the amiA *region*

Recipient strain	Donor DNA	Frequency of wild-type transformants	Percent of one-point cross by ++ DNA
r36	++	1.35	. .
E = 1.35	*r17*	1.05	78
	r35	0.93	69
	r53	1.01	75 Av. = 74
r39	++	0.207	. .
E = 0.207	*r4*	0.166	80
	r5	0.135	65
	r17	0.110	53
	r28	0.117	56
	r30	0.128	62
	r35	0.125	60
	r53	0.152	73 Av. = 64
r30	++	0.67	. .
E = 0.67	*r45*	0.48	72
	r40	0.62	93
	r33	0.71	106
	r39	0.68	100
	r36	0.53	79 Av. = 90
r17	++	0.109	. .
E = 0.109	*r45*	0.056	57
	r40	0.069	63
	r33	0.065	60
	r39	0.077	71 Av. = 61
r38	++	0.177	. .
E = 0.177	*r33*	0.088	50
	r36	0.096	54
	r39	0.059	33
	r45	0.084	47 Av. = 46
r53	++	0.101	. .
	r45	0.037	37
	r39	0.049	49
	r36	0.057	57 Av. = 48

E refers to efficiency of replacement of the recipient site in a one-factor cross with wild-type DNA. The right-hand column measures the degree of linkage of the donor site to the site in the recipient. See text for further explanation.

r17. Yet site *r1* is freely recombining with *r36*, while sites *r38*, *r53* and *r17* clearly are not.

These data reveal a polarized effect of the LE site in the recipient on the apparent degree of linkage of distant sites: the effect is observed when the LE site in the recipient is to the right of the mutant site in the donor DNA. Together the data obtained in two-factor crosses in which the mutated sites are in the *cis* and the *trans* positions demonstrate a long-range effect, of right-left polarity, of an LE

TABLE 5

Relative degree of linkage of an HE as opposed to an LE site in recipient cells to very distant genes in the amiA *region*

Recipient	DNA	Frequency of ++ transformants	Percent of one-point cross by ++ DNA
r1 (HE)	+	1.05	..
	r45	0.77	73
	r40	1.14	108
	r33	0.78	74
	r39	0.91	87
	r36	1.14	108 Av. = 90
r17 (LE)	+	0.109	..
	r45	0.056	51
	r40	0.069	63
	r33	0.065	60
	r39	0.077	71 Av. = 61
r30 (HE)	+	0.67	..
	r45	0.48	72
	r40	0.62	93
	r33	0.71	106
	r39	0.68	100
	r36	0.53	79 Av. = 90
r53 (LE)	+	0.101	..
	r45	0.037	37
	r39	0.049	49
	r36	0.057	57 Av. = 48
r38 (LE)	+	0.177	..
	r33	0.088	50
	r36	0.096	54
	r39	0.059	33
	r45	0.084	47 Av. = 46

Order of mutations, from right to left is: LE site *r17* right terminal; HE site *r30* right terminal multisite mutant covering *r17* and extending to left of latter. HE site *r1*, to right of map center, recombining with *r17* and *r30* about 35% of the time: HE gene *r22*, left of map center. To the left of *r22*, sites *r45*, *r40*, *r33*, *r39* and *r36*, are in the order cited. Although *r17* is well to the right of *r1*, it shows stronger linkage to left terminal sites than does *r1*.

site upon the integration of other markers. In the *cis* crosses, sites to the left of a point which, during recombination, is heterozygous for an LE mutation tend to be more severely excluded from the final recombinant than are sites to the right of such a point. In the *trans* crosses, wild-type sites in the donor DNA which are opposite LE mutations in the chromosome are more strongly associated with mutant sites to their left than with mutant sites to their right. We shall mention in the discussion what this may imply in terms of the hypothesis developed to explain exclusion of LE sites.

Three-factor crosses: It has been noted previously that three-point crosses are of two types: one (TYPE III) requires a single restricted exchange event in one specified interval as well as the absence of an odd number of exchanges within an adjacent specified interval, while the other (TYPE IV) requires two exchange

events, each within a specified interval. In the former type one unrestricted exchange event outside the marked zone is required, while in the latter type two unrestricted exchanges flanking the marked zone must accompany the restricted ones. The frequency with which a given exchange event occurs within a specified interval is measured directly in two-point crossing experiments (TYPE II) with strains bearing the restricting mutations; that is, in two-factor crosses of mutant *rb* by donor DNAs of mutants *ra* on the one hand and *rc* on the other, the frequency of wild-type recombinants observed measures the frequency of exchange events in the contiguous intervals *ra* to *rb* and *rb* to *rc*. Inasmuch as the frequency of exchanges within a given segment can be measured directly, the absence of an odd number of exchanges within that segment can be determined indirectly (expressed as one minus the frequency of wild-type recombinants observed in the two-point crosses of *rb* with DNA of mutants *ra* and *rc*).

Three-point crosses of TYPE III are of two kinds, which differ only with respect to the right-left position of the *amiA-r* marker in the donor DNA fragment relative to the two markers in the recipient chromosome. In TYPE IIIa the donor marker lies to the right of both markers in the recipient genome, whereas in TYPE IIIb the donor site is to the left of both recipient markers. In order to recover wild-type recombinants from a cross of either type, an exchange event must occur within the interval between the mutated site in the donor fragment and either the right or left marker in the recipient genome, depending upon the relative position of the donor marker. Further, an odd number of exchange events within the interval delimited by the mutated sites in the recipient chromosome must not occur. An even number of exchange events within this interval of course remain undetected. The frequency with which wild-type recombinants are recovered in these crosses is a measurement of the simultaneous fulfillment of both imposed conditions, and is, therefore, a measurement of the intensity and independence of exchange events in adjacent intervals.

Both the intensity and independence of multiple exchange events in this type of cross can be assessed in several different ways, all of which yield similar results. One can determine, for example, the frequency with which an odd number of exchange events *does not* occur in the interval defined by the *amiA-r* mutations in the recipient chromosome (the "forbidden" region) in the three-factor cross, and one can compare it with the frequency with which an odd number of exchanges *does not* occur in the same interval when measured in a two-factor cross; the latter value is referred to as the "expected" value in a three-point cross, and the former is the "observed" value in the three-point cross. Conversely, one can measure directly the frequency of recombinational events in the restricted intervals of both two- and three-point crosses and can compare the latter with the former. Results of this second calculation, presented in Table 6, show that the frequency of the restricted recombinational event of a three-point cross deviates but little from that of the comparable two-point cross. In three-factor crosses of this type, the ratio of "observed" frequency to "expected" frequency is based upon two independent measurements, each of which can vary by a factor of two for reasons discussed earlier. In order for positive interference to be inferred from

TABLE 6

Three-factor crosses of TYPE III (a and b) in which recovery of ++ recombinants is dependent upon the occurrence of a restricted exchange event in one interval and the absence of a similar event in the adjacent interval

Recipient strain	Donor marker	Relative position of donor marker	Frequency of restricted exchange event		Freq. 3-point/ Freq. 2-point
			3-point cross	2-point cross	
r31r2	*r6*	Left of	0.110	0.152	0.724
	r10	interval *r31r2*	0.105	0.118	0.890
	r24		0.196	0.261	0.751
	r20		0.258	0.296	0.872
	r9		0.271	0.405	0.669
	r29		0.343	0.373	0.920
	r22		0.246	0.432	0.569
	r52	Right of	0.020	0.043	0.465
	r50	interval *r31r2*	0.021	0.055	0.396
	r51		0.019	0.049	0.387
	r12		0.111	0.079	1.41
	r38		0.204	0.574	0.355
	r28		0.284	0.364	0.780
r31r10	*r24*	Left of	0.112	0.193	0.580
	r20	interval *r31r10*	0.130	0.271	0.480
	r9		0.209	0.357	0.585
	r29		0.177	0.371	0.477
	r22		0.210	0.444	0.473
	r43	Right of	0.123	0.176	0.699
	r7	interval *r31r10*	0.125	0.205	0.610
	r11		0.132	0.190	0.695
	r21		0.158	0.202	0.782
	r25		0.207	0.379	0.546
	r27		0.291	0.441	0.660
	r34		0.235	0.407	0.577
	r1		0.380	0.476	0.798
	r3		0.368	0.492	0.748
	r19		0.294	0.464	0.634
	r52		0.335	0.497	0.674
	r50		0.303	0.516	0.587
	r51		0.295	0.449	0.657
	r12		0.410	0.443	0.926
	r38		0.495	0.791	0.626
	r28		0.372	0.562	0.662
r31r29	*r22*	Left of interval *r31r29*	0.018	0.036	0.500
	r43	Right of	0.146	0.176	0.830
	r7	interval *r31r29*	0.167	0.205	0.815
	r11		0.196	0.190	1.03
	r25		0.365	0.379	0.963
	r27		0.320	0.441	0.726
	r34		0.309	0.407	0.759
	r1		0.460	0.476	0.966
	r2		0.451	0.406	1.11

TABLE 6—Continued

Recipient strain	Donor marker	Relative position of donor marker	Frequency of restricted exchange event: 3-point cross	Frequency of restricted exchange event: 2-point cross	Freq. 3-point/ Freq. 2-point
	r3		0.388	0.492	0.789
	r19		0.498	0.464	1.07
	r52		0.380	0.497	0.765
	r50		0.421	0.516	0.816
	r51		0.451	0.449	1.004
	r12		0.465	0.443	1.05
	r38		0.451	0.791	0.570
r31r48	*r10*	Left of	0.127	0.118	1.08
	r24	interval *r31r48*	0.268	0.261	1.03
	r25	Right of	0.050	0.035	1.43
	r27	interval *r31r48*	0.058	0.067	0.866
	r34		0.099	0.077	1.29
r34r28	*r27*	Left of	0.023	0.078	0.295
	r25	interval *r34r28*	0.048	0.071	0.676
	r48		0.081	0.111	0.730
	r44		0.100	0.120	0.833
	r11		0.124	0.102	1.22
	r16	Right of	0.074	0.216	0.343
	r15	interval *r34r28*	0.068	0.214	0.318
	r53		0.110	0.448	0.246
r34r29	*r22*	Left of	0.014	0.036	0.389
	r42	interval *r34r29*	0.033	0.093	0.355
	r2	Right of	0.031	0.041	0.756
	r52	interval *r34r29*	0.043	0.093	0.462
	r50		0.040	0.053	0.755
r39r33	*r36*	Left of interval *r39r33*	0.115	0.316	0.364
	r40	Right of	0.152	0.168	0.905
	r49	interval *r34r33*	0.137	0.160	0.856
	r41		0.269	0.472	0.570
	r42		0.298	0.409	0.729
	r22		0.290	0.379	0.765
r39r36	*r45*	Right of	0.002	0.148	0.014
	r33	interval *r39r36*	0.030	0.173	0.173
	r40		0.315	0.281	1.12
	r49		0.252	0.314	0.803
	r41		0.435	0.454	0.958
	r42		0.476	0.528	0.002
	r22		0.424	0.399	1.06

Recipient cells bearing two *amiA-r* mutations were crossed by a series of donor DNAs bearing a third *amiA-r* mutations, which lies either to the right or the left of the interval defined by the mutations in the recipient chromosome, and the standard reference marker *str-r41*. The frequency of occurrence of the restricted exchange event measured in the three-factor cross is compared directly with the frequency with which the same event occurs in the corresponding two-factor cross. In the three-factor crosses, the frequency of the restricted exchange event is expressed as the ratio of ++ recombinants to *str-r41* transformants divided by the frequency of simultaneous integration of the ++ alleles of both markers in the recipient chromosome.

the data this ratio must be consistently less than 0.5. On the other hand, this ratio must be consistently greater than 2.0 for one to infer that negative interference is present.

In three-factor crosses of TYPE IV, which requires a restricted exchange event in each of two adjacent intervals, the predicted frequency of occurrence of *both* recombinational events is the product of the frequency with which each required exchange event occurs independently of the other (as measured in two-point crosses). From our three-point crosses of this type the frequency of wild-type recombinants is a measurement of the frequency with which *both* exchange events do occur. Checking for interference in this type of cross consists in determining the ratio of observed to calculated frequencies of wild-type recombinants which, in this instance, is equal to the frequency of wild-type recombinants observed in the three-point cross divided by the product of the measured frequencies in the appropriate two-point crosses. Each of these three independent measurements can vary by a factor of two, as mentioned above. Therefore, any deviation in this ratio from unity must consistently exceed 2.0 in order to infer negative interference, and it must be consistently less than 0.25 before positive interference can be inferred. As was done with data from two-point crosses, a correction factor imposed by slight differences in efficiency of the double mutant strains was applied to the data obtained in three-point crosses. The result of these crosses are presented in Table 7.

An initial series of three-factor crosses of TYPE IV was performed, using as recipient double mutant strains bearing mutations *r31* and *r2*, *r31* and *r29* and *r31* and *r48*. A preliminary report on the results of these crosses has been published (EPHRUSSI-TAYLOR and GRAY 1966), and a curious pattern of positive interference was noted in one of the crosses. In the cross of *amiA-r31r2* by various donor DNAs, when the mutation in the donor lies close to either of the flanking mutations in the recipient, interference is absent or less severe than when the mutation in the donor DNA lies near the center of the segment delimited by the flanking mutations in the recipient. This pattern of positive interference seemed so peculiar a genetic phenomenon that we sought at the time to explain it as a possible further manifestation of the special properties of LE (low efficiency) markers. Indeed, all of the donor mutations employed in these three-factor crosses were LE transforming factors. Subsequent minor revision of this map segment and further refinement of the data resulted in a more uniform pattern of positive interference in this cross. Nevertheless, interference is less severe when the donor site lies relatively close to the flanking mutations in the recipient. A second series of similar crosses was established to test whether a peculiar pattern of interference is observed with LE donor sites, taking advantage of the fact that in the map segment *r34* – – – *r28*, mutations of both efficiency classes have been located. In these crosses, the striking observation is the almost total absence of either positive or negative interference, irrespective of the efficiency of the donor markers.

This has led us to alternative explanation of the aberrant pattern of positive interference of the cross involving strain *r31r2*. Doubly mutant strains are built making use of "leaky" *amiA-r* mutations, which resist only one-half the amount

TABLE 7

Three-factor crosses of TYPE IV in which the recovery of ++ recombinants is dependent upon the occurrence of a restricted exchange event in each of two contiguous intervals

Recipient strain	Donor marker	Position of donor site relative to recipient markers	Frequency of double exchanges		Observed/ Expected
			Observed	Expected	
r31r2	*r43*	Near *r31*	0.0039	0.0769	0.0507
	r7		0.0028	0.0510	0.0549
	r11	Medial	0.0029	0.0775	0.0374
	r21		0.0032	0.0852	0.0376
	r25		0.0034	0.0587	0.0579
	r27		0.0032	0.0772	0.0415
	r32	Near *r2*	0.0020	0.0232	0.0862
r31r29	*r26*	Near *r31*	0.0003	0.0013	0.231
	r6		0.0038	0.0369	0.103
	r8	Medial	0.0043	0.0358	0.120
	r24		0.0039	0.0284	0.137
	r20		0.0033	0.0213	0.155
	r9	Near *r29*	0.0026	0.0166	0.157
r31r10	*r26*	Near *r31*	0.0004	0.0009	0.444
r31r48	*r43*	Near *r31*	0.0032	0.0199	0.161
	r7		0.0023	0.0223	0.126
	r11	Medial	0.0026	0.0141	0.184
	r21		0.0026	0.0149	0.175
	r44	Near *r48*	0.0025	0.0156	0.160
r34r28	*r2*	Near *r34*	0.0020	0.0130	0.154
	r52		0.0077	0.0272	0.238
	r71		0.0042	0.0037	1.135
	r51		0.0044	0.0278	0.158
	r63	Medial	0.0256	0.0176	1.45
	r23		0.0195	0.0169	1.15
	r14		0.0029	0.0040	0.725
	r72		0.0023	0.0023	1.00
	r12	Near *r28*	0.0020	0.0011	1.82
r39r33	*r45*	Near *r33*	0.0025	0.0086	0.291
r34r29	*r9*	Near *r29*	0.0016	0.0305	0.0525
	r20		0.0035	0.0517	0.0677
	r24		0.0041	0.0724	0.0566
	r10		0.0065	0.0950	0.0684
	r26	Medial	0.0056	0.140	0.0401
	r31		0.0062	0.209	0.0297
	r7		0.0047	0.185	0.0255
	r11		0.0058	0.163	0.0356
	r25		0.0017	0.057	0.0298
	r27	Near *r34*	0.0016	0.138	0.0116

Recipient cells bearing two *amiA-r* mutations were crossed by a series of donor DNAs bearing a third *amiA-r* lying between those in the recipient, and the standard reference marker *str-r41*. The expected frequency of double exchanges is calculated as the product of the frequencies of each required single exchange as measured in two-factor *trans* crosses. The observed frequency of double exchanges is expressed as the ratio of ++ recombinants to *str-r41* transformants divided by the probability of simultaneous integration of the ++ alleles of both recipient markers of the recipient chromosome.

of aminopterin that most *amiA-r* mutations resist. Mutant strains *r31* and *r39*, both of the "leaky" type, are genetically unstable, readily acquiring secondary mutations in the *amiA* locus. Repeated cloning has been required to maintain them. Mutant strain *r31*, owing to its strategic location, was used in preparing all of the double mutants in the initial series of crosses of TYPE IV. It seemed quite likely that the peculiar positive interference observed in the crosses involving strain *r31r2*, as well as the rather severe positive interference observed in most crosses involving double mutants derived from *r31*, might be attributed to additional mutations having occurred in the delimited segment of the *r31* strain. Thus, in point of fact, we might have been performing crosses involving more than three mutational sites. Our expected frequencies of wild-type recombinants would be grossly exaggerated if this were the case.

Results of crosses obtained using the newly prepared strain *r34r28* show that positive or negative interference is virtually nonexistent in this segment; therefore it is unlikely that any undetected anomalies in the *r31r2* strain are near *r2*. Further, in the three-point cross of *r31r10* by donor *r26*, interference is absent or only weakly positive, indicating that any undetected mutations must lie to the right of *r31*. A new double mutant strain was prepared using *r34* as the "leaky" mutant and introducing *r29* into it. Strain *r34* has proven thus far to be stable. The segment *r34* – – – *r29* includes most of the segment *r31* – – – *r2*, i.e., the segment in which very strong, peculiarly situated positive interference was observed. Results of three-factor crosses performed with strain *r34r29* are shown in Table 7. Interference again is quite strongly positive, with the most severe interference occurring to the right of *r31*, in the vicinity of *r7* and *r11*. These results support the idea that the strong positive interference observed in the three-point crosses involving strains *31r2* and *r34r29* may be the result of additional *amiA-r* mutations to the right of *r31*. We have, however, not yet succeeded in obtaining an *r31* isolate free of this anomalous behavior.

In conclusion, the majority of these experiments indicate that three-factor crosses of TYPE IV show little or no interference. Two exceptional sets of crosses did show strongly positive interference, and this can be tentatively attributed to the occurrence of undetected genetic alterations in the segments delimited by the two mutations introduced into the recipient strains.

Four-factor crosses: Three general configurations are possible in four-factor crosses of double mutant cells with doubly marked donor DNA. *Both* markers in the donor DNA may be either to the left or to the right of the segment delimited by the mutated sites in the recipient; *both* sites in the donor DNA may be included within the segment defined by the markers in the recipient; or one site in the donor fragment may be included in the segment delimited by marker sites in the recipient with the other site lying either to the right or the left of the segment. Recovery of wild-type recombinants depends upon one restricted exchange in the first configuration, two restricted events in the second and three restricted exchange events in the third.

Four-factor crosses of the first and third configuration were performed using the doubly mutant strain *r31r48* as recipient in each cross. (Table 8 shows the

TABLE 8

Results of four-factor crossses in which the doubly mutant strain r31r48 *was used as recipient in each cross*

Recipient strain	Markers in donor DNA	Frequency of ++ recombinants		Observed/Calculated
		Observed	Calculated	
r31r48	*r34r11*	0.00014	0.0011	0.127
	r39r29	0	0.00014	....
	r39r33	0	0.0046	....

result of these crosses.) There is no interference observed when *r31r48* is crossed with DNA of the strain *r34r11*; the observed to calculated ratio of wild-type recombinants is 0.127, within the limits of variability imposed by the system. (Four independent measurements of recombination are involved, one for the numerator, three for the denominator. Limits equal 0.125 to 2.)

In the crosses of *r31r48* with DNAs of *r39r29* no wild-type recombinants were observed. However, this is not surprising in view of the fact that the predicted frequency of wild-type recombinants is near the limits of resolution of the crossing system. The significient aspect of these data is the absence of negative interference in this type of cross. A twofold excess in the frequency of wild-type recombinants would have been detected.

Thus, on the basis of a limited number of four-factor crosses, which are a sensitive measurement of whether or not interference is occurring, we conclude that interference, both positive and negative, is absent in these crosses.

DISCUSSION

The experiments reported here in detail for the first time provided the information on the basis of which EPHRUSSI-TAYLOR and GRAY (1966) proposed a theory to explain efficiency differences in transformation. In order to facilitate the ensuing discussion, the essential features of this theory will be briefly presented.

In any type of transformation cross, regardless of the number of genetic markers on a molecule, or of their efficiencies, a primary heterozygous structure is presumably formed between the bacterial chromosome and the donor DNA molecule. Genetic exchanges then occur in an essentially random fashion, i.e., irrespective of the number of genetic markers involved, or their efficiencies. It is necessary to suppose *randomness* of these exchanges for two reasons. (a) The relative distances between genetic markers is not influenced by the efficiency of transformation of the site, or sites, in the recipient chromosome which must be replaced by donor sites (although absolute values of recombination frequency are; SICARD and EPHRUSSI-TAYLOR 1965). Thus, one basic exchange process must be operative in all crosses. (b) There is little or no evidence of interference, as demonstrated in the three- and four-factor crosses reported here. Following the establishment of a pattern of genetic exchanges, and before reduction of the partially hybrid chromosome to establish the definitive recombinant chromosome,

a correcting process is postulated to intervene. The correcting process must have the following properties in order to account for all of the special features of genetic recombination in pneumococcal transformation: (1) The correcting process must recognize specifically the heterozygous structure formed by an LE mutant site and its wild-type allele. (2) It must preferentially destroy or eliminate the genetic material originating from the DNA donor. (3) The destruction or elimination process must extend to the right and left of the heterozygous LE site, and be arrested at any point at which a genetic exchange has already occurred between donor and recipient. (4) Although donor genetic material is destroyed preferentially, about one tenth of the time it is, instead, the recipient genetic material which is destroyed.

The simplest molecular model which one can make of this process would be the localized formation of a heteroduplex region, with one DNA strand derived from the DNA donor, and the other from the chromosome of the recipient cells. The "correction" would consist of excision of one of the two single DNA strands at the level of the mutant site and its allele, excision extending along the condemned strand. The excision process would be arrested by a preexisting genetic exchange. Repair would then reestablish a double-stranded structure, copying from the persisting single strand. Evidence in favor of this particular molecular model has been advanced by EPHRUSSI-TAYLOR (1966), while others (FOX and ALLEN 1964) have advanced evidence suggesting that recombination in pneumococcal transformation involves at some relatively early stage pairing between single-standed homologous DNA molecules, the one of the donor and the other of recipient origin.

First, let us consider the bearing of the excision model on integration efficiency, in transformation of sites in the *amiA* region. The sites examined in the present study fall into two nonoverlapping efficiency classes: those which are integrated at the same frequency as the standard reference marker (HE, or high efficiency sites) and those integrated with one-tenth that frequency (LE, or low efficiency sites). According to our excision model, both types of sites are incorporated into a primary recombinant structure by a single kind of genetic exchange mechanism. Following the establishment of a pattern of exchanges, the correcting mechanism postulated above would "correct out" an LE donor site 90% of the time. The LE site would be permanently established in the chromosome the remaining 10% of the time, when it is the chromosomal strand that is eliminated. An HE site is presumed not to trigger the correcting mechanism. Accordingly, its transforming efficiency is high. If we assume in the latter instance random choice of donor or recipient homologous DNA in the genetic exchange process, the absolute frequency of integration of HE sites would be equal to 50% of the molecules which were fixed in a population of competent cells, and which bear such a site.

The possibility exists, however, that mutations showing an efficiency of 1 as transforming factors are subject to some excision, since mutations showing an efficiency of 2 are known (cf. LACKS' reference gene *Sd*. A few mutant sites have now been observed also in our *amiA* locus). Alternatively, however, this may

simply mean that excision is occurring in transformations with these "super efficient" mutations, but with a bias in the opposite direction of that found for LE sites: namely, it is the chromosome segment which is perferentially excised, and the donor DNA which is preferentially conserved and copied. Only very few such mutations have been found by us, and we cannot speak at present of having a third efficiency class. Rather, these mutations appear to be extremes in the HE distribution which is centered about an efficiency of 1.

On the basis of the excision model one can predict (a) that LE sites would preferentially destroyed subsequent to DNA uptake, and (b) that LE markers would be transmitted to sister cells one division earlier than HE sites. These features of transformation of LE sites have been demonstrated (EPHRUSSI-TAYLOR 1966). If excision and repair operate in transformation by HE sites showing an efficiency of 2, as suggested above, these markers also would show early transmission, but they would show no intracellular destruction. It is interesting to note that FOX (1966) finds some evidence of a transmission pattern of the *Sd* gene (efficiency of 2 on our scale) similar to that of our LE transforming factors. Only further experiments will reveal the significance of this. Clearly, however, one cannot deduce from transmission pattern alone how many donor strands are involved in recombination: correction processes could completely modify the outcome of recombination, and yield a modified transmission pattern.

LACKS (1966), in a study of mutations in the amylomaltase locus, claims to discern four efficiency classes among mutations presumed to be point mutations, and a continuous spectrum of efficiencies among mutations known to be multisite. In his study, mutations were considered to be point mutations if there was no genetic evidence that they were multisite, and on the basis of their presumed origin from chemical mutagenesis. However, LACKS provided no proof that the mutagens he used to obtain his mutants were effective as mutagens in his experiments, and, indeed, in our hands, neither hydroxylamine nor EMS, two of the agents he employed, are mutagenic under the conditions he describes. One of us (GRAY, in preparation) has now examined the efficiencies of a large series of mutations in the *amiA* locus, induced by proflavine, hydroxylamine, and nitrosoguanidine, under conditions where less than 5% of the mutations could have been of spontaneous origin. It has been found that 38 proflavine induced mutations fall in a continuous spectrum of efficiencies, ranging from 0.03 to 2, while hydroxylamine and nitrosoguanidine (as HNO_2 was shown in the past to do; SICARD 1964), give rise only to LE mutations. Thus, proflavine, known to induce deletion mutations, is the only mutagen which has given mutations in the *amiA* locus whose efficiencies lie outside of our HE and LE classes. GRAY using a wider range of mutagens on the *amiA* locus than has been used in the past, has thus found no evidence of more than one class (LE) being formed by mutagens which generally give rise to transition and transversion mutations. These observations are certainly of importance in considering just what may constitute a recognition "signal" for exclusion of donor site from the final recombinant, and suggest that a single base-pair difference between donor and recipient constitutes such a "signal." Whether all such differences are signals, and to what extent "signals"

may be modified by adjacent base sequences cannot be decided at present. It should be added that we do not know whether the low efficiency of deletion-type mutations is due to the same mechanism as the low efficiency of most (if not all) point mutations, or to a different one.

While GRAY's observations with proflavine-induced mutations agree with LACKS' observations that deletion-type mutations show a broad spectrum of efficiencies, his experiments with hydroxylamine and nitrosoguanidine do not support LACKS' claim that point mutations give rise to four efficiency classes, nor LACKS' suggestion that transitions and transversions show different transforming efficiencies.

Considering the bearing of the excision model on the results of two-factor crosses, we note first that the observations which initially suggested an excision or "destruction-choice" model were those obtained in two-factor crosses with relatively closely linked *amiA-r* mutations in *cis* position in the donor DNA introduced into wild-type cells. When the genotypes of segregants were determined following crosses involving an HE-LE pair of mutations, it was found that the frequency of recombinants bearing the HE site was much less than expected on the basis of the efficiency of the latter. These results are readily interpreted by invoking an excision process which extends laterally from the LE "recognition" site: the presence of an LE site in *cis* position relative to the HE site would lead to excision of the latter, at a frequency inversely dependent on map distance. Dependence on map distance of the *cis* effect of LE sites is indeed observed. A left-right polarity in the severity of the effect of the LE mutations was also incidentally observed, in that when it lies to the right of the HE site, exclusion of the latter is more severe. According to our excision model, digestion of the condemned nucleotide chain proceeds more rapidly or farther, in the right to left sense of the map. One can note that a definite polarity in nonreciprocal recombination has been observed in certain Ascomycetes (LISSOUBA, MOUSSEAU, RIZET and ROSSIGNOL 1962; HOLLIDAY 1964). To the extent that our observations have any bearing on the mechanism of gene conversion, it is to suggest that polarity in nonreciprocal recombination may indeed arise from enzymatic processes operating at the molecular level, as suggested by WHITEHOUSE and HASTINGS (1965).

The excision model explains what has been a puzzling feature of two-factor *trans* crosses. Namely, it explains why the efficiency of the mutant site present in the donor DNA, is without influence on recombinant frequency. Since this site must be excluded by the basic genetic exchange process in order to obtain a wild-type recombinant, and since for independent reasons (cf. paragraph 2 of this discussion) these exchanges must be presumed to occur prior to the events determining efficiency, the efficiency of the mutant site in the donor DNA cannot play a role; it has already been eliminated from the primary recombinant structure, and cannot effectively "signal" to the excision process.

As shown above, a polarized effect of LE sites was also observed in two-factor *trans* crosses. Recombination frequencies between two distant points of the map tend to be low if the mutant site in the recipient is a right-end LE site. In such

a cross, the LE site of the recipient must be displaced by its wild-type allele derived from the DNA donor in order to obtain a wild-type recombinant. According to our model, it can only be displaced if the excision mechanism operates on the chromosome rather than on the donor DNA molecule. If excision proceeds more vigorously on the left side of the "signal" than it does on the right, as indicated from the *cis* crosses, and if the genetic exchange process also were to run in a right to left direction, even though the exchange process were to begin before excision, excision might tend to overtake the exchange events if the distance to run is long. This would lead to excision of the wild-type allele opposite the mutants site in the donor DNA, and lead to loss of wild-type recombinants.

The present crosses add further evidence that the essential feature responsible for low transforming efficiency is the difference between donor and recipient DNA molecules, and not the nature of the mutated base *per se*. This is clearly demonstrated in the cross of recipient *amiA-r39* by the doubly marked donor DNAs *r39r36* and *r39r33*, as opposed to the cross of these same DNAs into a wild-type recipient. In the first cross, donor and recipient have the same structure at the level of LE site *r39*, while in the second, they are different. In the first cross, integration of sites *r36* and *r33* is 1 relative to the reference gene, while in the second, their integration is very much decreased, owing to the "signal" provided by the difference at the level of the LE site.

A few remarks should be made concerning the results of three- and four-factor crosses presented in this paper, in particular with reference to the problem of high negative interference, usually observed in fine-structure work. The experiments presented here provide the first extensive test of interference in a transforming system. They show that, if present at all, interference is slightly positive. This result is consistent with PRITCHARD's (1960) proposal that chromosomal recombination is occurring in an intense fashion in relatively short regions of effective pairing. The approximate size of effective pairing regions, estimated by PRITCHARD from data on Aspergillus, is about that of a single molecule of pneumococcal DNA (0.4 map units, 4×10^4 base pairs per map unit; i.e. 16×10^4 base pairs in Aspergillus). A DNA molecule of 6 to 7×10^6 daltons contains about 2×10^4 base pairs.). Thus, our results are consistent with the idea that the entire donor molecule may be effectively paired with the bacterial chromosome, and that genetic exchanges are occurring relatively uniformly throughout the *amiA* locus, the latter distinctly smaller than the transforming molecule. This conclusion seems all the more inescapable in that our crosses covered more than two thirds of the map of the locus.

Some three-factor crosses did yield an aberrant pattern of positive interference. A preliminary report of the results of these crosses, involving doubly mutant strains *r39r2* and *r31r48*, has been presented (EPHRUSSI-TAYLOR and GRAY 1966). Interference was stronger at the center of the interval delimited by each pair of mutations than near the delimiting mutations, a result which would not be predicted by recombination theories. We believe this may be due to the presence of an additional, undetected genetic alteration in these strains lying in the interval

r31–r48. Some preliminary evidence was indeed obtained in favor of this interpretation.

The absence of strong interference of any kind in our crosses has a direct bearing on a model proposed by LACKS (1966) to explain low efficiency. This model advances the idea that a three-stranded structure is formed between a single donor strand and the double-stranded chromosome. A dynamic equilibrium is presumed to exist, allowing for regional displacements of the chromosomal polynucleotide chain which is homologous with the donor strand. Following this, through excision, the unpaired polynucleotides are eliminated. Poor efficiency is attributed to distortions in pairing, caused by the mutational differences between donor and recipient, leading to frequent exclusion of the donor strand. LACKS model, although much more detailed in concept than ours, differs from ours mainly semantically. There is, however, one crucial difference: LACKS supposed that the length of the pieces inserted into the recombinant chromosome is about 2000 base pairs. Whether this is a necessary postulate of his model, or a specification devised to accommodate the contention of FOX and ALLEN (1964), who, using density markers, believe to have shown that the inserted donor DNA has a molecular weight of 1×10^6 daltons, is not clear. In any event, a recombination mechanism which inserts pieces of such a size would lead to very strong positive interference. Genetic evidence is definitely against this postulate.

LACK's model calls for excision of unpaired regions of a three-stranded structure, but no repair is required. This is a second important difference between his model and ours. His model does not, therefore, account for the early transmission and phenotypic expression of LE transforming factors, as our model does.

Finally our model explains why, when two closely linked LE sites are present in *cis* position in transformation, their depressing effects on genetic integration are not compounded, while the model of LACKS does not explain this without further assumptions. Thus, although much of the difference between the two proposed models is semantic, it would seem at present that our model explains more observations with fewer suppositions.

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SUMMARY

Genetic data reported in this paper support the excision theory recently advanced by EPHRUSSI-TAYLOR and GRAY (1966) according to which low efficiency (LE) markers are integrated by a mechanism of excision and repair which leads to lower probability of integration and earlier phenotypic expression than high efficiency (HE) markers. Data obtained from crosses of ++ recipient cells by donor DNAs bearing an HE and an LE marker is *cis* position show that the probability of integration of the HE site is depressed by the presence of the associated LE site. Further, it has been shown that it is the particular difference between the base of the LE site and the unaltered base of the homologous strand that triggers the exclusion mechanism, rather than the nature of the LE muta-

tion *per se*. That integration efficiency is site specific was proposed by EPHRUSSI-TAYLOR and SICARD (1964) and is supported here by the observation that after the HE and LE sites are segregated from one another, the HE site regains its characteristic integration efficiency. A left to right polarity in the severity of exclusion from the recombinant structure of HE markers associated with LE sites in the donor DNA fragment has been observed. The probability that an HE site will be excluded is inversely related to the map distance separating the LE site from the HE site. In two-factor crosses in which the *amiA-r* markers are in *trans* position, a polarity effect is also observed; right-end LE sites in recipient cells are more strongly linked to distant markers than are HE sites of the right end. Recipients bearing left terminal markers of each efficiency class show, conversely, weak linkage to right-end markers. The results of three- and four-factor transformation crosses show an absence of interference in the *amiA* locus of pneumococcus. A degree of positive interference observed in two exceptional three-point crosses has been attributed to the presence of undetected mutations which do not alter the phenotype, bu which render ++ recombinants rarer than expected. Most important, negative interference was not observed in either three- or four-factor crosses. Therefore, recombinational events appear to occur randomly, frequently and independently of one another in the *amiA* locus. An alternative model proposed by LACKS (1966) to explain efficiency differences in transformation does not adequately explain the results obtained in experiments with *amiA-r* mutants.

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