GENETIC MAPPING OF DNA: INFLUENCE OF THE MUTATED
CONFIGURATION ON THE FREQUENCY OF RECOMBINATION
ALONG THE LENGTH OF THE MOLECULE

A. W. RAVIN AND V. N. IYER

Department of Biology, University of Rochester, Rochester, New York

Received May 18, 1962

A NECESSARY assumption for the conventional genetic mapping of chromosomes is that the frequency of recombination between two sites is independent of the allelic condition of sites adjacent to the recombining ones. Without such an assumption, genetic mapping would be extremely difficult, if at all possible. Fortunately, when one deals with intergenic recombinations, that is, with recombinations between sites at distances of one Morgan unit or greater, this assumption is generally proved valid (Morgan 1926).

In recent years a large body of evidence has been accumulating to show that genes, defined as functional units, are complex structures, and that mutation and recombination can occur within their limits (PonTECORVO 1952; BenzE 1955). Indeed, within the limits of a gene the quantitative properties of recombination appear to differ from those of recombination at the intergenic level. For example, while the frequency of multiple recombinational exchanges between two sites at the intergenic level is positively related to the distance between them (positive interference), this frequency is inversely related to the distance between two sites at the intragenic level (negative interference; Pritchard 1955; Chase and Doermann 1958). A unitary hypothesis of recombination has been invoked to account for this phenomenon (Pritchard 1960), which postulates the existence of "effective pairing regions" within the limits of the gene. Hypotheses have also been invoked which propose different mechanisms of recombination at what may be termed the genic and chromosomal levels of organization (Roman and Jacob 1958).

One of the advantages of the study of genetic transformation induced by DNA is the possibility provided to analyze recombination at the intramolecular level. In transformation a host bacterium is infected by a molecule (or molecular fragment) of DNA extracted from a donor bacterium of different genetic constitution. Some interaction, probably preceded by an intimate homologous association (pairing or synapsis), occurs between the donor and the host molecules such that a segment of the donor molecule is integrated into the recipient molecule by

1 The research described in this article was supported by a grant awarded by the National Institute for Allergy and Infectious Disease.

2 Present address: Microbiology Research Institute, Canada Department of Agriculture, Ottawa, Canada.

Genetics 47: 1369-1384 October 1962.
replacement of a homologous region (EPHRUSI-TAYLOR 1958; Fox 1960; Voll and Goodgal 1961). A theoretical model has been developed to account for the intramolecular recombination that occurs in transformation (Balassa and Prévost, personal communication; see also EPHRUSI-TAYLOR 1961, for a descriptive statement of the model). This model proposes a number of plausible parameters affecting the frequency of integration of a given segment of the donor molecule, but it is based on the assumption that pairing between donor and host molecules is largely constant all along their respective lengths. At least in certain situations, this assumption is known not to obtain (Schaeffer 1958; Green 1959). In the present paper we wish to provide evidence that the frequency of recombination in a given region of the interacting molecules depends, in a significant way, upon the particular mutated configurations being confronted. This finding was made during an investigation in pneumococcus of recombination between a number of erythromycin-resistance mutations known to be carried by the same molecule of DNA (Ravin and Iyer 1961; Iyer and Ravin 1962).

MATERIALS AND METHODS

The strains, mutations, and method of transformation have been described in preceding papers (Ravin and Iyer 1961; Iyer and Ravin 1962). A great advantage of the group of erythromycin-resistance mutations is the fact that one can discriminate phenotypically not only among them, but also among the various combinations of the mutations that can be brought together by recombination. Thus, by the use of selective media containing a varying concentration of erythromycin, the different classes of recombinations produced in a given “cross” can usually be determined. Occasionally, the use of other macrolide antibiotics (such as tylosin or oleandomycin, which are analogs of erythromycin) can aid in the differentiation of recombinant classes that may be difficult to distinguish by the use of erythromycin alone (Iyer, Ache and Ravin, 1963). We are grateful to Dr. R. L. Hamill of Eli Lilly and Co. for supplying us with tylosin.

The transformations reported in this paper were all carried out according to the following manner: frozen aliquots of a competent batch of recipient cells were thawed to 32°C and treated briefly (15 minutes) with DNA bearing the described markers; exposure to DNA was terminated by addition of DNase; growth was allowed to occur at 37°C, and after two hours, samples were plated on selective and nonselective agar media. Whenever it was necessary to compare the action of several DNAs bearing different markers on a particular recipient strain, aliquots from the same frozen batch of competent pneumococci were used. Although saturating concentrations of DNA were used as a general practice, it was known from experiments performed with some DNA preparations that the distribution of recombinant types was independent of DNA concentration.

In all of the experiments the str-rl marker (which confers a high level of resistance to streptomycin) was included in the donor DNAs. This marker is unlinked to the group of ery-r mutations, and serves as a useful reference marker.
for normalizing the effects of competence or DNA quality, which may vary in different experiments (RAVIN 1960; RAVIN and IYER 1961).

In the genetic terminology employed in this paper, the symbol s indicates the wild-type, or sensitivity-conferring allele of the corresponding r, or resistance-conferring mutation. Thus, ery-s6 is the wild-type homologue of ery-r6.

RESULTS

Relative distance between ery-r6 and ery-r2 and between ery-r6 and ery-r3: A single batch of competent ery-r6 cells was used as a recipient population for the following DNAs: ery-r2 str-r1 and ery-r3 str-r1. Aliquots from this batch of ery-r6 cells were treated with the DNAs simultaneously under identical conditions. Because of the lower level of resistance of ery-r6 cells, it is possible in this type of experiment to detect two types of recombinations produced by transformation. One type is the replacement of ery-r6 by the wild-type allele of ery-6 in addition to the integration of the donor marker (ery-r2 or ery-r3); this type results in a tenfold higher level of resistance. The other type is the integration of the donor marker (ery-r2 or ery-r3) without the simultaneous substitution of ery-r6 by its wild-type allele in the donor molecule; this type may be said to require a recombination between the ery-r6 site and the ery-r2 or ery-r3 site, and it produces a large increase in the level of erythromycin-resistance (25-fold in the case of the ery-r2-r6 combination, 75-fold in the case of ery-r3-r6). The results are indicated in Table 1.

It is clear that the ery-r6 mutation in the host molecule is replaced by the donor mutation (ery-r2 or ery-r3) more frequently than it recombines with the donor mutation (to form either the ery-r2-r6 or ery-r3-r6 recombinant type). This finding corroborates the previous finding (IYER and RAVIN 1962) that double marker integration (in the present case, ery-r3-s6 or ery-r2-s6) may occur more frequently than single marker integration (in the present case, r3 or r2 without the wild-type allele of r6).

<table>
<thead>
<tr>
<th>Mutations in donor DNA</th>
<th>Frequency ($\times 10^{-4}$) of str-r1 transformations</th>
<th>Frequency ($\times 10^{-4}$) of all ery-r transformations</th>
<th>Frequency ($\times 10^{-4}$) of ery-r transformations involving recombination between r6 and r2</th>
</tr>
</thead>
<tbody>
<tr>
<td>ery-r2 str-r1</td>
<td>10.0</td>
<td>5.2</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>5.4</td>
<td>4.5</td>
<td>1.2</td>
</tr>
<tr>
<td>avg. 7.7</td>
<td>avg. 4.8</td>
<td>avg. 1.7</td>
<td></td>
</tr>
<tr>
<td>ery-r3 str-r1</td>
<td>4.8</td>
<td>3.1</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>4.7</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>3.1</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>avg. 4.9</td>
<td>avg. 3.6</td>
<td></td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>

All aliquots from a single batch of ery-r6 cells were used for various DNAs. Treatment of cells with DNA was done in duplicate or triplicate, under identical conditions.
When \textit{ery-r2} is the donor mutation, about one third of the \textit{ery-r} transformations result in the formation of the \textit{ery-r2-r6} recombinant type. When, however, \textit{ery-r3} is the donor mutation, less than one in a hundred of the \textit{ery-r} transformations leads to a recombination between \textit{r6} and \textit{r3}.

Since some parameter other than size of the region to be integrated is limiting the frequency of integration, it is suggested that the distance between the mutations confronting each other is contributing to the relative frequency of single marker integration. On this assumption, the distance between the mutations \textit{r6} and \textit{r3} is much closer than that between \textit{r6} and \textit{r2}.

\textit{Order of \textit{ery-r2}, \textit{ery-r6}, and \textit{ery-r3} sites:} A single batch of competent \textit{ery-r2} cells was used as a recipient population for the following DNAs: \textit{ery-r3 str-r1} and \textit{ery-r6 str-r1}. Aliquots from this batch were treated with the DNAs simultaneously under identical conditions. This experiment was repeated for several different batches of \textit{ery-r2} cells in order to determine the effect of the physiological state of the recipient cells on the relative recombination frequencies. In this experiment, only one type of recombination can be detected, namely, the recombination occurring between the \textit{ery-r2} host site and the \textit{ery-r6} or \textit{ery-r3} donor site. Only this recombinant type has a higher level of erythromycin-resistance than the recipient \textit{ery-r2} cell (fourfold higher in the case of the \textit{ery-r2-r6} recombinant, 15-fold higher in the case of the \textit{ery-r3-r6} recombinant). The results are recorded in Table 2.

With different batches of competent \textit{ery-r2} cells there is a change in the relative ability of the \textit{ery-r3} or \textit{ery-r6} marker to combine with \textit{ery-r2} (i.e., in the relative ability of \textit{ery-r3} or \textit{ery-r6} to be integrated without the wild-type allele of the \textit{ery-r2} marker). This fact is indicated in the columns marked “a” and “b” of Table 2, in which the frequency of occurrence of such \textit{ery-r} combinations is compared to the frequency of \textit{str-r1} transformations. There is over a threefold variation in the relative frequency with which recombinations occur in the region between the \textit{ery-r2} marker in the host molecule and the \textit{ery-r3} or \textit{ery-r6} marker in the donor molecule. Despite this variation, the ease with which \textit{ery-r2} and \textit{ery-r3} combine \textit{relative} to the ease with which \textit{ery-r2} and \textit{ery-r6} combine is

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Mutations in donor DNA} & \textbf{Batch of competent \textit{ery-r2} cells} & \textbf{Average no. of transformants in 0.1 ml capable of growing in 2.5 \mu g ery/ml, 100 \mu g strep/ml} & \textbf{Ratio ($\times 10^{-3}$)} & \\
& & & \textit{ery-r2-r3} & \textit{ery-r2-r6} \\
\hline
\textit{ery-r3 str-r1} & I & 11 & 726 & 1.51 & 0.58 & 2.6 \\
\textit{ery-r6 str-r1} & I & 5 & 858 & \ldots & \ldots & \ldots \\
\textit{ery-r3 str-r1} & II & 30 & 882 & 3.40 & \ldots & \ldots \\
\textit{ery-r6 str-r1} & II & 11 & 1204 & \ldots & 0.91 & 3.7 \\
\textit{ery-r3 str-r1} & III & 27 & 318 & 8.4 & \ldots & \ldots \\
\textit{ery-r6 str-r1} & III & 14 & 611 & \ldots & 2.2 & 3.6 \\
\hline
\end{tabular}
\caption{Relative frequency of integration of single \textit{ery-r} markers into \textit{ery-r2} recipient cells}
\end{table}
fairly constant in the different experiments. This fact is indicated in the column in which the value of “a” is divided by the value of “b”.

Since ery-r2 and ery-r3 combine more frequently (in such crosses in which ery-r2 is the recipient) than do ery-r2 and ery-r6, and assuming that this difference is due primarily to the relative distances between the mutated sites in question, it is concluded that the mutation ery-r6 is closer to ery-r2 than ery-r3 is to ery-r2. In view of the previous finding that ery-r6 is more closely linked to ery-r3 than it is to ery-r2, we conclude that ery-r6 is located between the sites of ery-r2 and ery-r3. The order of the mutations is, therefore, r2-r6-r3, with the r6-r3 distance probably much smaller than the r2-r6 distance.

Relative frequency of double marker integration and verification of the order of the ery-r sites: A single batch of competent sensitive (ery-s) cells was used as a recipient population for the following DNAs: ery-r2-r6 str-r1, ery-r3-r6 str-r1, and ery-r2-r3 str-r1. In this experiment three classes of erythromycin-resistant recombinants can be detected, corresponding to the integration of each single ery-r marker from the donor DNA molecule as well as of both ery-r markers simultaneously. The total frequency of transformants capable of resisting erythromycin was determined by selecting originally at a concentration of erythromycin which all recombinants could withstand; the frequency of streptomycin-resistant transformants was determined in the usual way. By transferring a sample of the erythromycin-resistant colonies that were originally selected and subjecting them to further test, it was possible to measure the proportion of ery-r transformants that received both ery-r markers from the donor DNA. The results are recorded in Table 3.

The order of the ery-r sites hypothesized above (namely, r2—r6-r3) predicts that the highest frequency of double ery-r marker integration relative to str-r integration will be that of the r6-r3 pair and the lowest will be that of the r2-r3 pair. This prediction is verified by the results of the experiment just described.

It may be noted however, that the frequency of integration of the r2-r3 pair is not significantly lower than the frequency of integration of the r2-r6 pair. This quantitative result is not what would be expected from the previous results

<table>
<thead>
<tr>
<th>Mutations in donor DNA</th>
<th>Average no. of transformants in 0.1 ml capable of growing in 0.1μg ery/ml 100μg strep/ml</th>
<th>Proportion of total ery-resist. transformants that possess both ery-r markers</th>
<th>Ratio* double ery-r transf. str-r transf.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ery-r2-r6 str-r1</td>
<td>395</td>
<td>337</td>
<td>17/59</td>
</tr>
<tr>
<td>ery-r3-r6 str-r1</td>
<td>144</td>
<td>216</td>
<td>53/59</td>
</tr>
<tr>
<td>ery-r2-r3 str-r1</td>
<td>221</td>
<td>388</td>
<td>34/58</td>
</tr>
</tbody>
</table>

* Ratio given by column (4) \( \times \) column (2) / column (3).

Simultaneous exposure of aliquots from a single batch of competent sensitive cells to DNAs indicated. Plated after two hours expression; colonies appearing on 0.1 μg erythromycin agar are transferred to sectors of antibiotic-free agar and then tested by velvet replication.
described, in which the formation of the r2-r6 combination (using ery-r2 cells as recipients) is three times easier to produce than the r2-r3 combination. If distance between the sites in question were the sole parameter affecting recombination frequency, one should expect that the r2-r3 combination would be three times easier to dissociate by recombination than the r2-r6 pair. Since the latter is not observed, we conclude that several factors contribute to the frequency of recombination between two mutations. One of these factors may very well be the distance between the sites of mutation, the greater the distance, the greater the probability of recombination. Another factor is probably the intrinsic efficiency of integration of each mutation, which differs for different ery-r markers (Iyer and Ravin 1962) and may be related to the size and/or composition of the mutation. A third factor will be described below as the “marker effect.”

Location of the ery-r5 mutation: When the ery-r6 strain is treated with DNA extracted from the ery-r5 strain, it is found that all of the transformants that are thereby enhanced in their level of resistance to erythromycin bear the ery-r5 mutation in place of the ery-r6 mutation. It is possible to perform this experiment because ery-r6 cells have a tenfold lower resistance to erythromycin than do ery-r5 cells. It was performed in the following way. Competent ery-r6 cells were treated with DNA ery-r5; transformants were selected on agar containing a concentration of erythromycin slightly higher (0.25 μg/ml) than that which the recipient cells are maximally capable of resisting (0.1 μg/ml). By velvet replication it was found that the several hundred transformants thus obtained were indistinguishable in phenotype from ery-r5 cells. In addition, one of the transformants originally selected in each of ten independent experiments was isolated and a DNA preparation obtained from it. These ten DNA preparations were tested on sensitive (ery-s) cells and transformants were selected at random on an erythromycin concentration (0.075 μg/ml) which ery-r6 cells can resist. Fifty to 60 such transformants obtained with each of the ten DNA preparations were isolated and tested for their maximum level of resistance. All had the phenotypic level of resistance of ery-r5 cells (1.0 μg/ml).

This evidence indicates that, when the mutation ery-r6 is confronted in a transformation reaction with the mutation ery-r5, there is generally a replacement of r6 by r5. Therefore, either r5 is allelic to r6 or it is very closely linked to it.

In regard to resistance to erythromycin, ery-r5 cells are indistinguishable from ery-r2 or ery-r3 cells, and it is not possible to detect any transformants enhanced in their level of resistance to erythromycin when ery-r2 (or ery-r3) cells are treated with DNA ery-r5 (or conversely, when ery-r5 cells are treated with DNA ery-r2 or ery-r3; Ravin and Iyer 1961). In certain crosses it was possible, however, to construct an ery-r2-r5-r3 recombinant and to show that the ery-r2-r5 and ery-r5-r3 recombinant types can exist (Ravin and Iyer 1961). Recently, it was possible to show that recombinations occur frequently between the sites of ery-r5 and ery-r2. This was done by taking advantage of the fact that the ery-r2 mutation confers a higher level of resistance than does the ery-r5 mutation to the erythromycin-analogue, tylosin. If ery-r5 cells are treated with DNA ery-r2
MUTATION ORDER 1375

and transformants selected on tylosin agar, all of the transformants thus produced are indistinguishable phenotypically from ery-r2 cells. However, upon treatment with DNA ery-r3, these transformants produce a class indistinguishable from ery-r2-r5-r3 (which resists only 15 µg erythromycin per ml) and different from ery-r2-r3 (which resists up to 40 µg erythromycin per ml).

These findings support the conclusion, therefore, that ery-r5 is medial in location to the sites of ery-r2 and ery-r3, and that it may be allelic to (i.e., overlap) ery-r6.

Location of the ery-r7 mutation: In earlier investigations (RAVIN and IYER 1961) it was found that the mutation ery-r7 could replace ery-r2. We now have the results of seven independent experiments in which ery-r2 recipient cells were treated with DNA ery-r7. It is possible to detect transformants because ery-r7 cells have a 15-fold higher level of resistance to erythromycin. In one of the seven experiments, the insertion of the wild-type allele of ery-r2 in addition to the integration of the ery-r7 mutation occurred most frequently. However, in the other six experiments recombination between ery-r2 and ery-r7 appeared to be the most frequent event. In some of these experiments the majority of the transformants had a higher level of resistance than ery-r2 but lower than ery-r7; in other experiments the majority of the transformants had a higher level of resistance than ery-r7. The level of resistance of the transformants isolated was a genetic property of the strain, since it persisted in a stable manner through clonal reproduction.

One possibility to account for these results is that ery-r7 is a large mutation, capable of being broken up by transformation, the different pieces of which can produce different levels of erythromycin-resistance in conjunction with the mutation ery-r2. In this regard, we know that only the single level of resistance characteristic of the donor strain is conferred on an erythromycin-sensitive strain treated with the DNA of any one of the ery-r mutants (RAVIN and IYER 1961). Moreover, we can detect phenotypically only a single transformant class when a sensitive recipient strain, after being treated with DNA ery-r7, is challenged with one of the macrolide antibiotics, say tylosin, and isolated transformants are then tested for level of resistance to other macrolide antibiotics, such as oleandomycin or erythromycin. Finally, it should be pointed out that variability in the resistance level of the recombinants, such as produced by the interaction of the mutations ery-r2 and ery-r7, have never been observed in the case of recombinations involving the mutations ery-r2, -r3 and -r6.

In any case, it now seems likely that ery-r7 is fairly distal to the cluster of ery-r2, -r3, -r5 and -r6 sites. On the basis of the results described up to this point, we would be inclined to represent the map of the erythromycin-resistance mutations as indicated in Figure 1. We have assumed a linear distribution of these mutations on the DNA molecule bearing them, and we have attempted to indicate the probable intersite distances based upon the recombination frequencies that have just been reported.

Three-factor crosses and the "marker effect": With regard to the ery-r2, -r3 and -r6 mutations, we have considered up to this point two-factor crosses only.

Downloaded from https://academic.oup.com/genetics/article/47/10/1369/6033899 by guest on 20 February 2022
In these the two ery- r mutations were either confronted in the \textit{trans} position (i.e.,
one mutation in the host molecule, the other in the donor molecule), or they were
present in the \textit{cis} position (i.e., both mutations in the donor molecule). The results
of these crosses were consistent with the order of the mutated sites shown in
Figure 1.

When three-factor crosses were begun, in which one mutation was in the host
molecule and the other two in the donor molecule, some unexpected results were
obtained. For example, when \textit{ery-r6} cells were treated with DNA \textit{ery-r2-r3}, the
frequency of \textit{ery-r3-r6} recombinants obtained was considerably higher than
anticipated on the basis of the close linkage of the \textit{-r3} and \textit{-r6} sites concluded from
the two-factor crosses.

Since the previous study (IYER and RAVIN 1962) showed the significance of
the physiological state of the recipient cells on the distribution of recombinant
types produced, it was necessary to control the state of the host. This was done
by testing aliquots from a single batch of competent \textit{ery-r6} cells separately, but
under simultaneous and identical conditions, with the following DNAs: \textit{ery-r2
\textit{str-r1}}, \textit{ery-r3 \textit{str-r1}}, and \textit{ery-r2-r3 \textit{str-r1}}. These three tests are referred to as
crosses (1), (2), and (3), respectively. The various recombinant types were
screened and tested in the usual manner. Moreover, in order to insure that we
were scoring the genotypes of the recombinants correctly, DNAs were prepared
from one or two transformants of each distinct phenotypic class. These DNAs
were then tested on sensitive cells, and the phenotypes of the transformants
determined by the usual procedure. In this way, we could verify the genotypic
behavior of each of the recombinant classes recovered in the original crosses.
Thus, for example, the DNA of an \textit{ery-r2-r6} recombinant produced three pheno-
typic classes, when tested on the sensitive strain: transformants that resist 0.1 \(\mu\)g
erythromycin per ml, transformants that resist one \(\mu\)g per ml, and those that
resist five \(\mu\)g per ml.

The results of such an experiment are shown in Table 4. Figure 2 is presented
to help visualize and interpret the results. At the top of the figure is a diagram
representing the possible results issuing from a confrontation of the host and
donor molecules. It is assumed that a recombination is produced by a “switch”
from the host to the donor molecule; this could occur as a result of either a
breakage-reunion or copy-choice mechanism. The recombinant molecule may
be considered to originate at point “0” of the host molecule and to contain parts
of the donor molecule integrated as a result of one or more “switches” to the
donor molecule. A final return to the host molecule is assumed to occur in the
The influence of the mutant configuration of the donor molecule on the frequency of recombination in various regions of the molecule. I. ery-r6 cells as recipients

<table>
<thead>
<tr>
<th>Cross number</th>
<th>Mutations in donor DNA</th>
<th>Frequency (×10⁻²) of transformants in treated population capable of growing in 100µg strep/ml 0.25µg ery/ml Tested</th>
<th>Number of ery-r transformants phenotypically and genotypically</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>ery-r2 str-r1</td>
<td>1.1 1.1 36 23 0 13 0 0 0</td>
<td></td>
</tr>
<tr>
<td>(2)</td>
<td>ery-r3 str-r1</td>
<td>1.1 1.0 2564 0 2564 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>(3)</td>
<td>ery-r2-r3 str-r1</td>
<td>1.8 1.3 90 2 21 27 36 0 4</td>
<td></td>
</tr>
</tbody>
</table>

Aliquots from same batch of competent cells used simultaneously and under identical conditions for various DNAs.

**Figure 2.** Analysis of recombination involving the *ery-r6* mutation in the host and the *ery-r2* and *ery-r3* mutations in the donor. Crosses refer to those listed in Table 4. The symbol “+” refers to the wild-type or sensitivity-conferring s allele at the indicated site.

region indicated by the dashed crossed lines, if a return to the host molecule has not already occurred prior to this region. Although likely *a priori*, this assumption is not necessary for the following argument. The “switch” regions of interest in the present case are those between point “0” and the *ery-r2* site, between the *ery-r2* site and the *ery-r6* site, and between the *ery-r6* site and the *ery-r3* site; these are indicated by the solid crossed lines labelled “a”, “b” and “c”, respectively. In cross (3), for example, a “switch” to the donor molecule at region “a” followed by a return in the region of the dashed crossed lines will result in the recombinant *r2*-s6-*r3* (or simply *r2-r3*); this type is referred to as an “a” type. Another example would be a switch at region “b” followed by a return in region
“c”; this will result in a s2-s6-s3 (or completely wild-type or sensitive) recombinant, and is referred to as a “(b,c)” type of recombinant. In the middle section of Figure 2 is a table listing, for crosses (1), (2) and (3), the recombinant types resulting from the various kinds of possible “switches.” A “0” indicates that the recombinant type produced cannot be detected by the method of screening employed in this experiment. In the lower part of Figure 2 the ratio, in terms of number of transformants produced, between the types of recombination giving rise to distinct classes of detectable transformants in cross (1) is compared with that same ratio obtained in cross (3). The results of cross (2) are likewise compared with those of cross (3). For example, r2-r6 cells are produced in cross (1) as a result of recombinations of type (a,b,c) and (a,b); r2 cells are produced as a result of recombinations of type a and (a,c). The ratio of the number produced by the former to the number produced by the latter is 13/23. In cross (3) the corresponding ratio is 4+27/0+2 or 31/2. It is clear that the ratio changes greatly in going from cross (1) to cross (3). A similar result is observed in going from cross (2) to cross (3). These results mean that the relative frequencies of the different kinds of recombinations change according to the particular confrontation of mutated sites involved in a given cross. Specifically, for example, switching between the r6 and r3 sites is increased as a consequence of the presence of the r2 marker. We refer to this phenomenon, therefore, as the “marker effect.”

Another example of the “marker effect” is given by the results of the following experiment. Aliquots from a single batch of competent ery-r2 cells were tested separately, but under simultaneous and identical conditions, with the following DNAs: ery-r3 str-rl, ery-r6 str-r1 and ery-r6-r3 str-r1. These three tests are referred to as crosses (1), (2) and (3), respectively. The various recombinant types were again determined in the usual fashion, and the scoring of genotypes verified as mentioned above. The results are recorded in Table 5, and their interpretation is aided by means of Figure 3. In cross (1) only one recombinant genotype can be detected, and it is produced by recombinations of type b and type c. Therefore, we can compare the number of transformants produced by these two types of recombination in cross (1) to the number of transformants produced by the same types of recombination (b + c) in cross 3. We must correct also for the differences in the relative activities of the DNA used in crosses (1) and (3), which are reflected in the different frequencies of str-r transformations observed in the two

### TABLE 5

The influence of the mutant configuration of the donor molecule on the frequency of recombination in various regions of the molecule. II. ery-r2 cells as recipients

<table>
<thead>
<tr>
<th>Cross number</th>
<th>Mutations in donor DNA</th>
<th>Number of transformants in 0.1 ml capable of growing in 100ug strep/ml 2.5ug ery/ml Tested</th>
<th>Number of transformants phenotypically and genotypically r2-r3 r2-r6 r6-r3 r2-r6-r3</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>ery-r3 str-r1</td>
<td>285 110 439 439 0 0 0</td>
<td>439 0 0 0</td>
</tr>
<tr>
<td>(2)</td>
<td>ery-r6 str-r1</td>
<td>388 40 159 0 159 0 0</td>
<td>0 159 0 0</td>
</tr>
<tr>
<td>(3)</td>
<td>ery-r6-r3 str-r1</td>
<td>395 165 71 0 5 5 61</td>
<td>71 0 5 5 61</td>
</tr>
</tbody>
</table>

Aliquots from same batch of competent cells used simultaneously and under identical conditions for various DNAs.
crosses. Similarly, a comparison is made between crosses (2) and (3) with respect to the numbers of transformants produced by the two types of recombination \([b + (b,c)]\) that give rise to the unique recombinant genotypes detectable in cross (2). These comparisons are shown in the lower part of Figure 3. The difference between the numbers obtained for crosses (2) and (3) is highly significant. We conclude again that a "marker effect" is operating.

The \(ery-r2-r6-r3\) recombinant: In the three-factor crosses just mentioned a recombinant class appeared which has not heretofore been described. This class is characterized by a high level of resistance to erythromycin. It resists over 50 \(\mu g\) per ml of erythromycin, which is higher than the level of resistance of the \(ery-r2-r3\) recombinant. However, the sure means of distinguishing the \(ery-r2-r6-r3\) cell from the \(ery-r2-r3\) cell is by comparing the action of DNAs prepared from each of them on erythromycin-sensitive cells. DNA from the former produces principally two classes of transformants: cells resisting one \(\mu g\) per ml of
erythromycin (corresponding in phenotype to \textit{ery-r2} or \textit{ery-r3} cells); and cells resisting 15 \(\mu\)g per ml of erythromycin (corresponding in phenotype to \textit{ery-r6-r3} cells). DNA from the latter produces two classes of transformants: cells resisting one \(\mu\)g per ml of erythromycin, and cells resisting 40 \(\mu\)g per ml of erythromycin (the latter corresponding to \textit{ery-r2-r3} cells). Thus, DNAs prepared from presumably \textit{ery-r2-r6-r3} cells and \textit{ery-r2-r3} cells are distinct in their actions on erythromycin-sensitive recipient cells.

**DISCUSSION**

It is worth emphasizing a feature of recombinations between erythromycin-resistance mutations in pneumococcus that makes possible a more probing analysis of intragenic recombination than has been generally possible in microbial genetics. This feature is the phenotypic recognizability of most of the recombinant types that issue from a given cross. With other types of mutations, such as plaque morphology in bacteriophage or growth factor independence in molds, one can screen for either mutant or wild-type, but not for the various combinations of mutant sites. The kind of analyses described in this paper should encourage the development of other systems in which the different mutant combinations give rise to distinct phenotypes. Mutations and recombinations affecting resistance to antibiotics may be particularly useful in this regard (Hotchkiss and Evans 1958).

On the basis of the two-factor crosses involving \textit{ery-r2}, \textit{-r6} and \textit{-r3} mutations, it has been possible to establish an order for these three mutations on the molecule of DNA that bears the information for erythromycin-resistance. While the hypothetical order of these mutant sites is compatible with all of the results obtained in the two-factor crosses, it is not possible to establish unequivocally the distances between the sites in terms of recombination frequencies. The frequency with which two mutations on the same molecule are dissociated by recombination appears not to depend exclusively on the distance between them. That the distance between the mutated sites does play a role, however, is indicated by the fact that double marker integration can occur more frequently than the integration of one of the markers in the combination. We interpret this finding to mean that the relative frequency of double marker integration increases when the distance between the markers is short relative to the size of the markers themselves. In the work of Lackss and Hotchkiss (1960) and in our previous paper (Iyer and Ravin 1962) evidence was furnished that different mutations have quite different intrinsic efficiencies of integration. This may be due to such properties of the individual mutation as relative size and/or nucleotide composition, which may affect its pairing or the probability of exchanges ("crossovers") on either side of it (Hotchkiss 1958; Hotchkiss and Evans 1958). We are inclined to give some credence to the idea that the composition of the specific mutation itself influences the probability of its integration. This view is supported by the discovery of the "marker effect," which reveals that the particular molecular configurations that are confronted during the pairing that precedes recombinat-
tion affect strongly the probabilities of exchanges at specific regions along the lengths of these molecules. In other words, it is conceivable that the different efficiencies of integration of the various individual markers have the same basis as the "marker effect".

It should be pointed out that, in discussing the "marker effect," we adopted for convenience the order of the -r2, -r6 and -r3 sites which was suggested by results of the two-factor crosses. The conclusion that the "marker effect" is operating in the three-factor crosses does not depend, however, on the correctness of this order. By proceeding in the manner illustrated in Figures 2 and 3, it can easily be shown that, on the basis of any order of these sites, the probability of exchanges in certain regions of the recombining molecules changes according to the particular mutated states adjacent to those regions.

We are not prepared to offer a physico-chemical explanation of the "marker effect," except to suggest, as has always seemed likely a priori, that recombining DNA molecules are physical continuums without "spacers" of specialized DNA (or non-DNA in nature) in which the actual exchanges or "switches" of recombination take place. (The work of Rubenstein, Thomas and Hershey [1961], and Davison, Freifelder and Levinthal [1961] demonstrates, in this regard, that there are no specific regions of the DNA molecule especially susceptible to rupture by shearing forces). Consequently, at the intragenic or molecular level, changes in nucleotide composition in any region of the molecule will alter not only the functional capacity of that region, but its pairing and recombinational capacities as well. Since the chromosome of animal and plant cells is a complex structure in which DNA molecules may be organized in a discontinuous array, and in which the non-DNA portions may have as much or more to do with intergenic recombination as the DNA portions, recombination between different genes, even if linked, may be expected to display entirely different properties. One of these properties would be that the frequency of recombination between genes would be largely independent of the allelic state of those genes. This kind of argument favors, therefore, separate mechanisms of recombination at the genic and chromosomal levels of organization.

We are aware that, before attempting to resolve the differences, if any, between chromosomal and intramolecular recombination, further work is called for to determine the generality of the "marker effect." Is it peculiar to the group of erythromycin-resistance mutations in pneumococcus, or can it be observed at other functional loci and in other organisms? While definitive answers to these questions await the development of other appropriate systems of measuring intragenic recombination, as discussed above, there is already some evidence that the "marker effect" is not restricted to the erythromycin-resistance locus of pneumococcus. Demerec, Goldman and Lahr (1958) provided evidence suggesting that, in some cases, Salmonella transduced for multiple linked markers tended to select the wild-type allele from either the donor or recipient preferentially to the mutant alleles. Hotchkiss and Evans (1958) discussed several cases of recombination in pneumococcus, including their own analysis of the sulfanilamide-resistance locus, in which there appeared to be specific "marker" effects on the
frequency of recombination. HELINSKI and YANOFSKY (1962) have recently reported in *E. coli* "aberrantly" high frequencies of recombination of a mutation (A-23) with other very closely-linked mutations that affect the same amino acid in the A protein of tryptophane synthetase. KAPLAN, SUYAMA and BONNER (1962) have also shown in *Neurospora crassa* that the presence of a second *td* mutation adjacent to the *td 201* mutation affects the latter's mapping characteristics. The "marker effect" accounts, moreover, for certain anomalous situations that had been described in an earlier report of the erythromycin-resistance mutations (RAVIN and IYER 1961). We pointed out two cases, other than those mentioned in this paper, in which the frequency of recombination between given sites on the host and donor DNA molecules varied according to the genetic composition of the recombining molecules. For example, "...when *ery-r3* is crossed with *ery-r6*, the recombinant *ery-r3-r6* is rare. On the other hand, when *ery-s3-s6* treated with DNA *ery-r3-r6*, the frequency of the recombinant *ery-s3-r6* is relatively high. Yet in all respects, except for the specific nature of the sites confronted in these crosses, the recombinations involved are similar." The "marker effect" may even account for the different recombination frequencies often observed in reciprocal crosses (DEMEREC, GOLDMAN and LAHR 1958; LACKS and HOTCHKISS 1960), although the interpretation given by EPHRUSSI-TAYLOR (1961) based on different sizes of the recombining mutations is sufficient to explain such results.

In the meanwhile, if we are permitted to assume a generality of the "marker effect," a certain caution seems necessary in the interpretation of recombination data at the intragenic level. The notion of genetic distances is more illusory than ever, since the frequencies of recombination between mutational sites, on which distance determinations are based, vary according to the state of those sites; genetic distances are not fixed, but are rather elastic, however constant the physical distances may be. This fact obviously complicates considerably the entire problem of mapping DNA molecules.

Moreover, it does not seem likely that the effectiveness of pairing is constant all along the length of the recombining DNA molecules. Nor is it likely that a "switch" or "attack" from the host to the donor molecule is equally probable all along their lengths. For the "marker effect" shows that the frequency of a certain type of exchange relative to that of some other type of exchange varies considerably according to the mutant composition of the donor molecule. This means that models, such as those of BALASSA and PRÉVOST (personal communication, and described in EPHRUSSI-TAYLOR 1961), which assume constancy of pairing and "switching" efficiency along the length of the DNA molecule, must be modified.

Finally, the "marker effect" has some significance for the quantitative estimation of negative interference. Our experiments in themselves do not allow us to determine whether negative interference is operating in recombinations within the erythromycin-resistance locus. However, they do show that estimates of the degree of negative interference which are based on the assumption that genetic distances are fixed may be misleading. In the experiments of CHASE and DOERMANN (1958), for example, the observed frequency of wild-type phage issuing
from a three-factor cross of mutant phage is compared with the frequency expected on the basis of distances determined from two-factor crosses. The deviation of observed from expected is taken as a measure of the intensity of negative interference. This measure is unreliable, however, to the extent that the distance between two mutated sites is altered by the presence of a third mutated site on one of the parental phage DNA molecules. Future quantitative studies of negative interference should involve multifactor crosses from which all of the recombinant types can be recovered.

SUMMARY

For certain erythromycin-resistance mutations (ery-r2, -r3, and -r6) a series of crosses was carried out involving combinations of two of the mutations in either the cis or trans confrontation. On the basis of the results an order could be established for these mutations on the molecule of DNA bearing them. Other crosses made it possible to locate two additional mutations (ery-r5 and -r7) in relation to the first group.

Several factors appear to determine the frequency of recombination between sites of erythromycin-resistance mutations. These include the intrinsic nature of the markers themselves, as well as the distance between the recombining sites relative to the size of the mutations involved. Three-factor crosses revealed an additional factor, namely, that the particular mutant configurations that are confronted prior to recombination strongly influence the probabilities of exchanges at various specific regions along the lengths of the recombining molecules.

ACKNOWLEDGMENTS

The authors are deeply grateful to the helpful discussions we have had with Dr. Allan M. Campbell and to the dependable technical assistance of Miss Ruth Mary Hohman.

LITERATURE CITED


Fox, M. S., 1960 Fate of transforming desoxyribonucleate following fixation by transformable bacteria. II. Nature 187: 1004-1006.
1384

A. W. RAVIN AND V. N. IYER


