On the Mechanism of Integration of Transforming Deoxyribonucleate

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ABSTRACT The characteristics of the intermediates in the reaction, between DNA and pneumococcus, that results in genetic transformation are described in so far as they have been characterized. Transformation with DNA isolated from bacteria carrying in addition to genetic markers ^{32}P as a radioactive label and ^{2}H and ^{15}N as density labels has permitted the characterization of the product of recombination between the newly introduced DNA and the DNA of a recipient bacterium. The evidence for a single strand displacement mechanism producing a hybrid structure in the DNA of the recipient bacteria is presented. Progeny of single transformants have been examined. The results of these segregation studies permit the further characterization of this hybrid product of transformation as a genetically heterozygous structure.

The process whereby bacterial transforming deoxyribonucleate (DNA) is utilized by transformable bacteria to effect a genetic transformation has been examined in three organisms, pneumococcus (1), *B. subtilis* (2, 3), and *H. influenzae* (4, 5). For each organism there is now evidence that transforming DNA is physically integrated into the genomes of the recipient bacteria, but it is certainly clear that the mechanisms differ, at least, in detail.

Since other participants in this symposium will be discussing the other two systems, I will limit my discussion to what is known about the integration process as it occurs in pneumococcus with only limited reference to other organisms.

Formally the over-all reaction between bacteria and transforming DNA can be described in terms of two steps. The first is a rapidly equilibrating reversible reaction, whose rate is probably diffusion-limited, to form a complex. The second is a slow irreversible process, whereby the DNA is fixed in the sense that it cannot be removed by exhaustive washing nor is it any longer sensitive to deoxyribonuclease (DNAase) added to the medium (6).

The stoichiometry of the over-all reaction has been determined (7, 8). There is a strict linear correspondence between the number ot bacteria transformed with respect to a single marker and the quantity of DNA fixed by

the treated competent population. With the most active DNA preparations, the number of bacterial equivalents of donor DNA fixed, per bacterium transformed, is less than two and may approach one for some single markers. Since the population fixes fragments of the transforming DNA at random (9), and only occasionally does the fragment fixed carry the marker under examination, the process appears to be remarkably efficient.

Little is known about the state of the DNA that is presumed to be reversibly bound in the form of the complex. It might be bound to the bacterial surface but this is by no means the only possibility. The high efficiency of the over-all process suggests an alternative explanation. Material in the form of the complex may include elements of DNA that have reversibly penetrated various permeability barriers and are therefore able to "search," by diffusion, for homologous regions of the recipient genome. "Finding" the homologous region, perhaps by complementary pairing, might initiate the irreversible reaction and thus account for the remarkable efficiency of the process. This hypothesis is further suggested by the long time constant (about 180 min) (1) for the fixation step and by the rapidity and certainity with which the succeeding steps occur (10). In addition, this proposal is consistent with the failure to observe, in recently transformed pneumococcus, any DNAase-resistant donor DNA retaining its original double-stranded integrity.

In contrast to what is observed in pneumococcus, recently transformed populations of *H. influenzae* have, in fact, been shown to contain intact double-stranded donor DNA which cannot be removed by washing or by treatment with DNAase. It is likely that the complex that has been described includes a sequence of intermediate steps. All these intermediates must be considered to be reversible in the case of pneumococcus. In the case of *H. influenzae*, however, the passage of DNA into one of these intermediate states might be irreversible. On further incubation the donor DNA that has been fixed, intact, might then pass slowly through the various further steps that are involved in integration. Such a proposal could perhaps resolve the differences between the observations describing the integration processes in these two organisms (1, 4, 5, 11).

We return to the case of pneumococcus, and the DNA that has been irreversibly fixed. Total DNA, extracted immediately following a short exposure of bacteria to transforming DNA, manifests very little of the transforming activity of the newly fixed DNA present. During further incubation at 37°C the transforming activity, as measured in extracts, of the newly fixed DNA is recovered with a half-time of 3 min (10). Following this recovery and during subsequent growth of the transformed population the extracted DNA contains a constant ratio of donor to recipient transforming activities. It can be therefore concluded that following escape from eclipse the newly introduced transforming DNA multiplies in synchrony with the DNA of the recipient bacteria (12).

Certain pairs of markers are said to be genetically linked since they are frequently carried on single fragments of transforming DNA (9). Linkage provides a biological criterion for examing the establishment of molecular associations that occur as a consequence of the recombination events in transformed bacteria. DNA extracted from transformed bacterial populations can exhibit activity characteristic of newly created linkage pairs, one member of which has been provided by the donor DNA and the other by the DNA recipient bacterium.

The formation of this new association and hence the recombination process itself occurs with a half-time of about 6 min at 37°C. This reaction has been shown to be distinct from that involved in the recovery from eclipse (10). Both these reactions can be completed at their maximum rates even under conditions wherein there has been a less than 5% and probably less than 1% net increase in DNA in the transformed bacterial population (13).

Incorporation into transforming DNA of radioactive phosphorous (^{32}P) at high specific activities leads to destruction of the transforming activity as a primary consequence of the ^{32}P harbored in individual molecules (14). This observation has been used to examine the degree to which the integrity of the newly introduced DNA is retained through the process of fixation, recovery from eclipse, and establishment of linkage (15). Disintegration of ^{32}P destroys the biological activity with respect to a single marker present in the heavily labeled transforming DNA at a rate consistent with a minimum sensitive target of about 600,000 daltons (14).

Heavily labeled DNA that was about to lose transforming activity as a consequence of ^{32}P disintegration was used to transform cold bacteria. DNA was isolated from the treated bacteria after allowing sufficient time for the newly introduced DNA to escape from eclipse and nearly complete the establishment of linkage. The transforming activity of a marker present in the donor DNA was determined after allowing various amounts of ^{32}P disintegration to occur. The biological activity of the newly introduced marker decayed at the same rate as did the same marker in the original transforming DNA. It follows therefore, that the elements of DNA manifesting a newly introduced biological activity, have the same arrangement of ^{32}P atoms and must have retained their integrity over a molecular region of at least 600,000 daltons.

Given the demonstration that elements of transforming DNA are themselves genetically integrated into the genomes of the recipient bacteria, it now becomes possible to inquire into the physical structure of the integrated product. This inquiry has been accomplished by the use of isotopic density labels coupled with density gradient centrifugation. The technique permits the separation and identification of different species of DNA molecules by virtue of their different densities.

The use of such methods has made it possible to demonstrate that the prod-

uct of the reaction between transforming DNA and the DNA of the recipient bacterium is physically a hybrid. The hybrid, composed of one strand of donor and one strand of host DNA, extends over a region of about 2 or 3 thousand nucleotide pairs. Furthermore, the terminals of the newly intro-

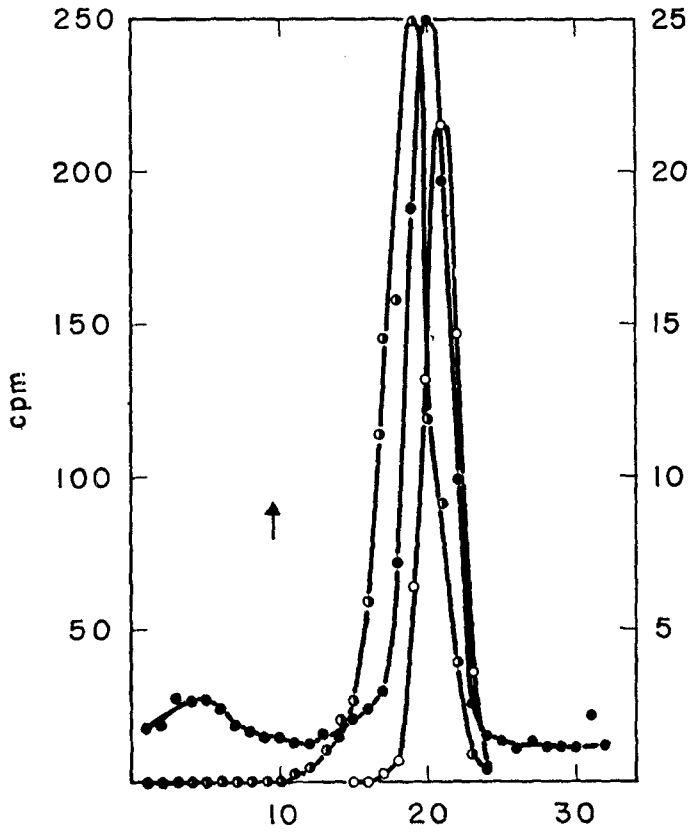

FIGURE 1. Equilibrium density gradient of DNA isolated from light cold bacteria that had been treated with triply labeled transforming DNA for 15 min at 30°C, followed by 3 min at 37°C. The arrow indicates the gradient position to be expected for heavy DNA. The distribution shows the radioactivity (●) and transforming activity (◑) of the donor DNA and the transforming activity of the recipient DNA (○). The scale on the right is in transformants per ml $\times$ 5 $\times$ 10^{-3} for the donor marker and $\times$ 10^{-5} for the recipient marker.

duced polynucleotide chain are covalently bound to elements of the host DNA.

This conclusion is reached by an examination of the DNA extracted from unlabeled bacteria that had been transformed with triply labeled DNA carrying the density labels ^{2}H and ^{15}N and the radioactive label, ^{32}P. DNA was isolated from recipient bacteria after a 15 min exposure to the transforming

DNA, at 30°C and 3 min at 37° to allow escape from eclipse. This DNA was allowed to come to equilibrium in a CsCl density gradient centrifuged at 140,000 × *g*. The distribution of the various components is described in Fig. 1.

All the biological activity of the newly introduced DNA and the bulk of its ^{32}P are found at a density position very near that of the host DNA. There is none to be found at the position characteristic of the donor DNA. The covalent linkage between the donor DNA and the recipient DNA is demon-

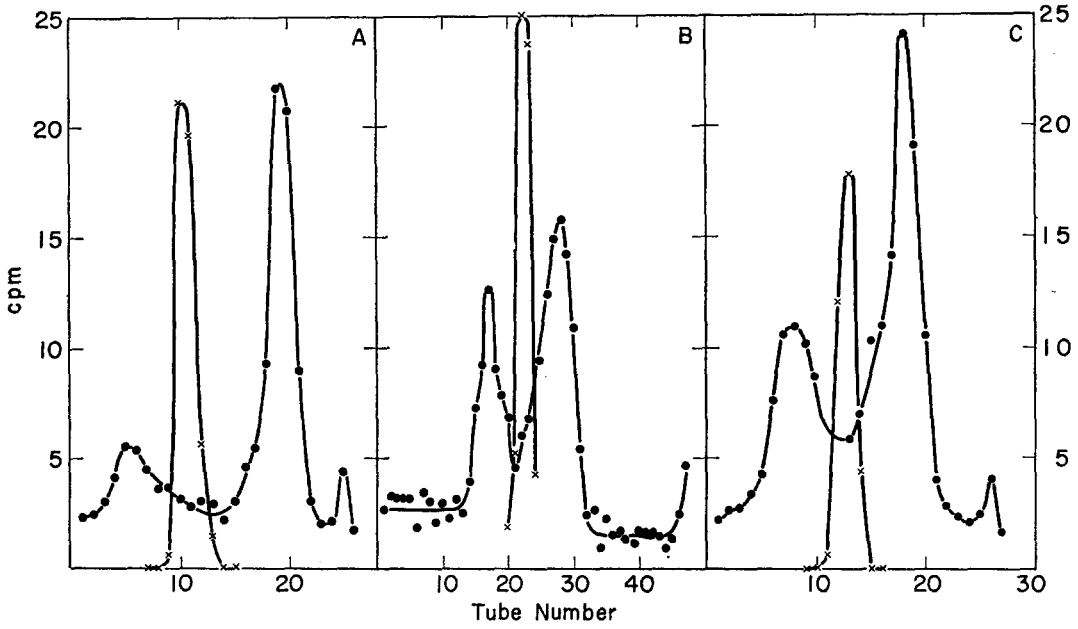

FIGURE 2. The extract from transformed bacteria showing the distribution of radioactivity (●) present in the donor DNA. The extract was (*A*) untreated, (*B*) heat-denatured, and (*C*) alkali-denatured. The biological activity of added heavy DNA (*X*) constitutes a position marker.

strated by the persistence of their association following either thermal or alkali denaturation, as indicated in Fig. 2.

The hybrid structure of the intermediate was demonstrated by shearing the DNA extracts prior to CsCl centrifugation. Shearing by sonication was used to reduce the molecular weight of the DNA in the extract from 20 million to about 1 to 2 million. The effect of this shearing is described in Fig. 3*A*. The reduction in molecular weight results in shifting of the buoyant density of the DNA carrying the newly introduced marker from a position near that of the light host DNA to a position nearly halfway between the buoyant densities of the heavy donor and light recipient DNA's. A substantial fraction of radioactivity, perhaps half, also assumes the hybrid density.

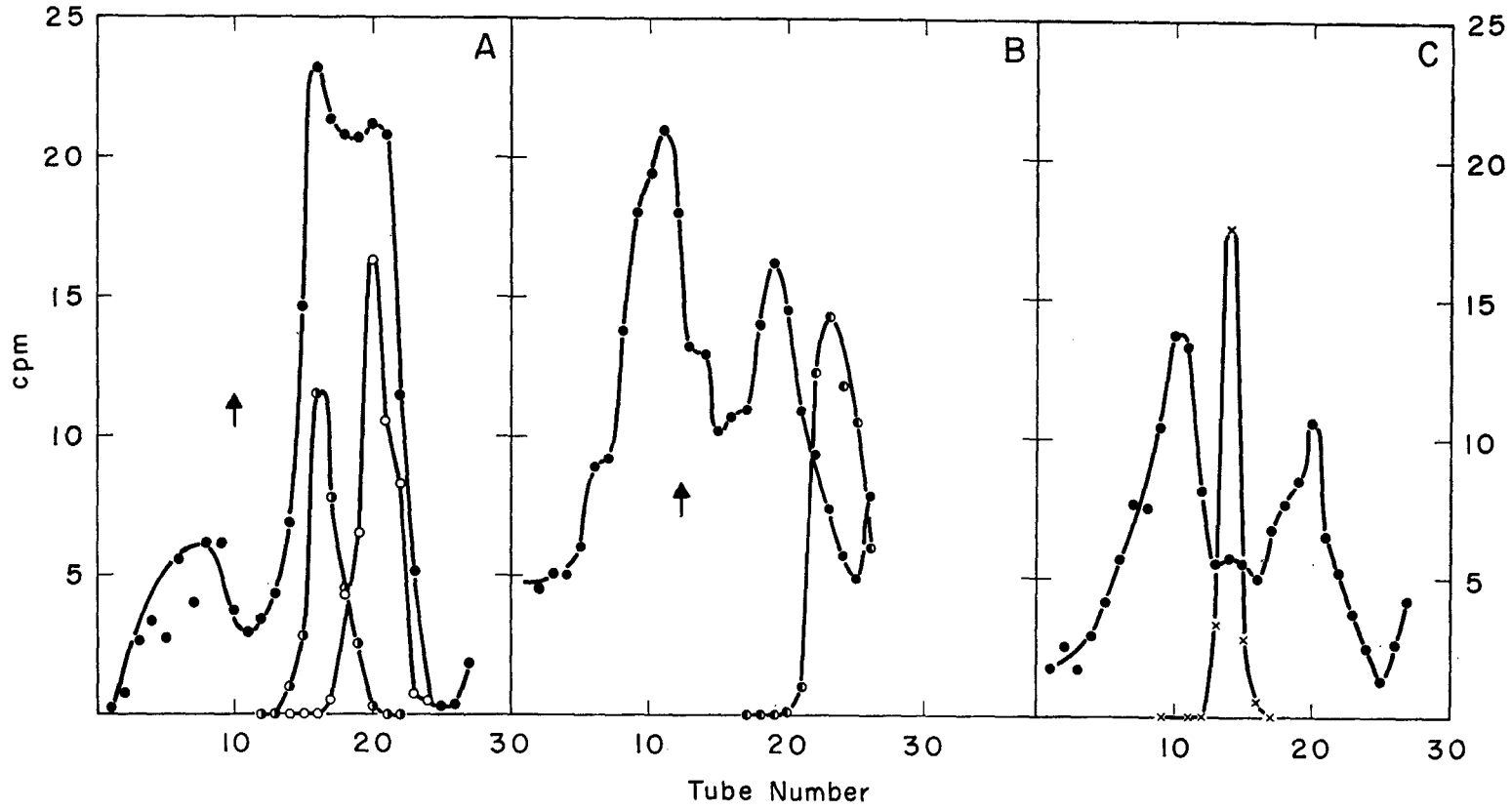

FIGURE 3. Density gradient distributions of an extract from transformed bacteria that had been sonicated (*A*), then (*B*) heat-denatured, or (*C*) alkali-denatured. The arrows indicate the position that native heavy DNA would be expected to assume. The distributions show the radioactivity (●) and biological activity (◑) of the donor DNA and the biological activity of the recipient DNA (○), as well as the biological activity of a light position marker (◑) in (*B*) and a heavy position marker (*X*) in (*C*). The scale on the right is in transformants per ml × 1.25 for the donor marker, × 10^{-3} for the resident marker, × 2 × 10^{-2} for the heavy position marker, and × 5 × 10^{-5} for the light position marker.

Further shearing fails to move either the biological activity or the radioactivity to a density position greater than that of half-heavy and half-light DNA.

In contrast to what is observed as a consequence of denaturation of the intact extract, denaturation of a sheared DNA isolate, described in Fig. 3*B* and *C*, results in the bulk of the ^{32}P banding in the position of heavy denatured DNA as if most of it had lost its association with light DNA. Thus the DNA which had become hybrid in density as a consequence of shearing was hybrid because it was heavy in one strand and light in the other.

The first intermediate carrying the transforming activity of the donor DNA can now be described in some detail. A segment of the DNA of the recipient bacterium has been displaced by a single strand of the donor DNA. A similar structure has been described as the product of the transformation of *B. subtilis* (2, 3). The demonstration of this intermediate confirms the displacement mechanism proposed by Lacks (16) and by Guild and Robison (17). In pneumococcus, this region is 2 to 3 thousand nucleotides in length, substantially shorter than the usual length of molecules of transforming DNA. Furthermore, the terminals of this single polynucleotide sequence are covalently coupled with the DNA of the host bacterium.

Four possible naive models can be distinguished that permit further definition of the structure of this intermediate. One pair of models assumes that the physical hybrid is genetically heterozygous: The light strand is genetically "host" and the heavy strand genetically donor. One member of this first pair of models further assumes that the heterozygous structure arises from the introduction of a particular one of the two donor strands. On the basis of this model, the product of the first semiconservative replication would be a double-stranded homozygous structure in which the newly synthesized strand was inactive in transformation. Since only the particular strand could transform, the first round of bacterial replication would yield no increase in the biological activity of the newly introduced DNA. Contrary to this expectation, following recovery from eclipse, the DNA carrying the activity of the newly introduced marker multiplies at the same rate as does the bulk host DNA, with no lag in its replication (12). Furthermore, the first model fails to account for the observed reduction of sensitivity to ^{32}P disintegration of heavily labeled DNA reisolated from transformed populations that had been allowed to undergo one doubling. We will reserve consideration of the second model in which either strand can participate in the formation of the heterozygous structure.

An additional pair of models assumes that the physical hybrid is in fact genetically homozygous. Rapid "conversion" of the complementary strand of the recipient DNA could perhaps occur as the consequence of new synthesis or some kind of process of excision and repair (18, 19). Such a genetically homozygous physically hybrid structure might occur as a consequence

of the introduction of either of the two strands of a fragment of donor DNA. This model holds that a heavily ^{32}P-labeled element of newly integrated DNA would rapidly create its "converted" complement. The complement would be biologically active, harbor no ^{32}P atoms, and therefore be much less sensitive or insensitive to subsequent ^{32}P disintegration. Contrary to this expectation, heavily labeled transforming DNA, reisolated from transformed bacteria manifests little, if any, loss in its sensitivity to subseqeunt ^{32}P disintegration (15).

The remaining possibility may be considered single strand displacement, with a particular one of the two complementary strands participating, followed by conversion. This model is to be contrasted with the more appealing model of single strand displacement, with either strand participating and resulting in the formation of a genetic heterozygote.

Recent studies in this laboratory by Guerrini (20) on the segregation of progeny of single transformed bacteria have ruled out simple models involving conversion. The conversion models predict that all the progeny of a single newly transformed bacterium be transformant. The heterozygote model predicts that a single bacterium transformed at a given locus will give rise to a mixed clone. Each clone would be expected to contain bacteria transformed at the indicated locus as well as bacteria that manifest the recipient genotype at that locus.

Guerrini used a pair of allelic markers concerned with sulfanilamide resistance (d, d+) that have been described by Hotchkiss and Evans (21). Either allelic form of the pair may be detected by a selective procedure. Mutant bacteria, carrying the genotype d, are sensitive to *p*-nitrobenzoic acid, but are able to grow in the presence of sulfanilamide, whereas wild type bacteria, d+, are sensitive to sulfanilamide and may be identified by their capacity to grow in the presence of *p*-nitrobenzoic acid.

Guerrini has distributed newly transformed populations in an agar growth medium containing a small amount of sulfanilamide which limits the growth of wild type bacteria to small colonies, while permitting normal growth for transformants. Total colonies of transformants were picked, resuspended, and grown in a nonselective medium. Each clone was tested for the presence of bacteria carrying the d marker and also for bacteria carrying the d+ marker.

The colony-forming units in a transformable population of pneumococcus are chains containing on the average about four bacteria. As a consequence the clones isolated in the manner described above would all be expected to be mixed and among about 200 clones that have been examined all are indeed mixed. To reduce the number of bacteria that participate in the formation of a colony, the newly transformed bacteria were heat-killed to the point where only about 5% of the colony formers survived. Assuming that thermal

inactivation occurs at random, these colony-forming units would only rarely be expected to include more than one bacterium. Clones of transformed bacteria were isolated after the treatment and characterized with regard to their composition. Twenty-seven of the twenty-eight clones analyzed were mixed. In these mixed clones the bacteria carrying the recipient genotype occurred with frequencies between 0.1 and 10%.

To confirm this observation, freshly transformed populations were exposed to ultrasonic agitation, so as to allow only about 10^{-3} to 10^{-4} survivors, prior to plating on low levels of sulfanilamide. Here again random inactivation would be expected to yield colony formers containing more than one bacterium only rarely. Among the thirty-five colonies that have been examined, again all were mixed.

The following corollary experiment justifies the assumption that the clones that were examined indeed arose from single bacteria and confirms the heterozygote model. A transformed population was allowed to increase 40% in the number of bacteria as observed by counting in a Petroff-Hauser chamber. The culture was then sonicated and plated as above in the presence of a low concentration of sulfanilamide. Of 44 clones examined, 36 were still mixed. The remaining 8 clones contained only transformants. Of 78 clones isolated as above from a transformed population that had been allowed one doubling (Petroff-Hauser count) before sonication and plating, 71 clones proved to be pure transformant and only 7 were still mixed. The segregation of almost all the transformed bacteria after one doubling justifies the assumption that the colony-forming units after sonication are single bacteria. Even more interesting, this observation excludes most models involving heterozygosity arising as the consequence of multiple nuclei.

Guerrini's results mean that transformants are genetically heterozygous, and that segregation is practically complete by the first bacterial doubling. This observation excludes conversion models and, barring some rather unlikely assumptions, itself suggests single strand displacement as the mechanism of integration of transforming DNA.

We can summarize the major conclusions:

1. Recently integrated transforming DNA exists as a physical hybrid with its complementary segment in the recipient bacterium.
2. The fragment simultaneously carries the genetic information of both participating DNA's.
3. Neither conversion nor double strand events are involved in a significant fraction of transformation events.

A speculative cartoon describing the local structure of the donor and recipient DNA molecules is shown as structure I in Fig. 4. We know that the molecules of transforming DNA are substantially larger than the average element integrated, and that multiple exchanges can occur between a single

donor molecule and the recipient genome (22, 23). These concepts are incorporated in structures II or III in Fig. 4. A single transformation event might have as its consequence any of the three possibilities.

The cartoons significantly avoid indicating strand-switching events in which first a length of one strand of donor DNA is incorporated and then a

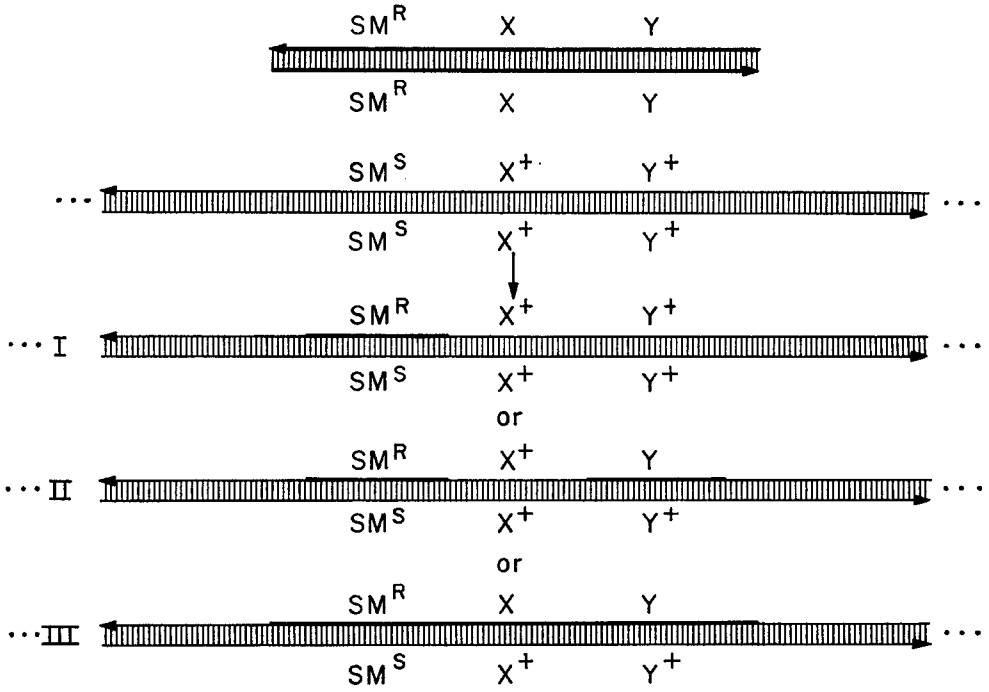

FIGURE 4. Cartoon describing several kinds of products of a single integration event. Light horizontal lines represent the strands of the host DNA and heavy horizontal lines represent the strands of donor DNA. As an example SM^R might represent the region of the DNA responsible for resistance to streptomycin and SM^S the homologous region in sensitive cells. The X^+ and Y^+ are meant to designate the recipient genotype and the X and Y other adjacent genetic elements present in the donor DNA and linked to SM^R.

length of the other. The reason for their omission is that such events occur only at the low frequency to be expected for random participation of two independent elements of transforming DNA (3).

The cartoons and the accompanying discussion are largely speculative. In this realm it seems worthwhile to discuss the possible mechanism of formation of the hybrid heterozygous structures. Some of the newly introduced DNA can, on reisolation, be characterized as denatured (16, 1, 3). Although this denatured DNA has been proposed as an intermediate in the integration process (16), it might equally well be a by-product of the reaction.

Still in the realm of cartoons, Fig. 5 illustrates possible intermediates in the formation of the recombinant structure. Since integration must involve breakage of the recipient genome and reunion with the donor DNA, a start can be made by breaking at position *A*. The process of trading strand partners would release one strand of the transforming DNA, as denatured material. A second break at position *B* would yield the structure illustrated as pathway I in Fig.

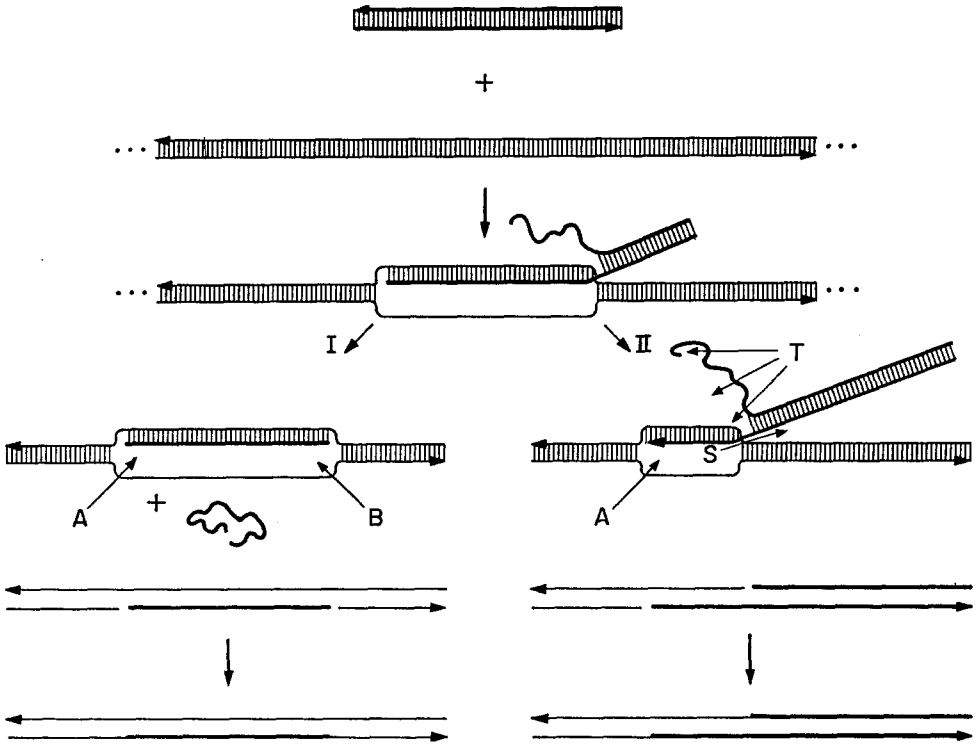

FIGURE 5. Cartoon describing a possible intermediate in the production of the fina integrated structure. Arrows are meant to designate points of breakage of polynucleotide chains. Light lines again represent strands of the host DNA and heavy lines, strands of the donor DNA.

5. Repair processes complete the reaction. Breaks of the kind illustrated in pathway II at *A* and *S* with hydrolysis of the single-stranded region at *T* are apparently excluded in pneumococcus. The product, however, is identical with that proposed to describe bacteriophage T2 heterozygotes (24) and bacteriophage λ recombinants (25).

The principal argument against the product of pathway I as a frequent occurrence in bacteriophage T2 or T4 recombination is the demonstration, by Levinthal (24), that most T2 heterozygotes are recombinant for outside markers. We now know that about one-third of the heterozygotes were prob-

ably of the terminally redundant type and would therefore be expected to be frequently recombinant (26). If the remaining heterozygotes were of the overlap variety a substantial fraction of them could be of the type described as the product of pathway I. This consideration could still yield the result that most T2 heterozygotes were recombinant for the outside markers.

Heterozygotes that are parental for outside markers have been reported in λ (27) as well as in T4 (28, 29), the latter occurring in substantial frequency. In both cases the heterozygotes have been accounted for by structures of the kind proposed as the products of pathway I (27, 29).

In three point crosses involving closely linked bacteriophage markers double cross-overs occur with an unexpectedly high frequency (high negative interference) (30, 31). Hotchkiss has suggested that this observation might be accounted for by assuming that a substantial fraction of recombination events were double events (32). The product of pathway I could be such a double event and in moderate frequency could account for high negative interference.

Both pathways may be considered possible as alternative products of a single recombination event in bacteriophage. Most recombinants for markers distant with respect to the average length of the heterozygous overlap, would therefore be expected to be products of pathway II. On the other hand, recombination events between closely linked markers might be produced frequently by either pathway.

It is obvious that the cartoon is incomplete, and that the completed product might be unrecognizable in these speculations. The fact is, a single strand of the donor transforming DNA displaces a segment of the DNA of the recipient bacterium to create a hybrid, heterozygous product.

The interesting questions that now arise are concerned with how such a product is created, and to what extent it might occur as a product of recombination in other organisms.

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Discussion

Dr. Walter R. Guild: Are there any strong positive arguments against the participation of a single-stranded intermediate?

Dr. Fox: Since it is clear that the transformation process involves the integration of only one strand of DNA, it becomes necessary in the case of transformation by double-stranded DNA to discard the complementary strand. Although this does not really constitute an argument, I think that there are no compelling reasons for excluding either possibility, a single strand as an intermediate or a single strand as a product, in the normal reaction. Even the clear demonstration of transforming activity of single-stranded DNA might not necessarily distinguish between these possibilities.

Dr. Guild: I could comment simply that we can fractionate strands far better than anything reported. Materials with density differences as large as 0.011 g/cm^3, which do not renature appreciably, unless mixed, have been separated from each other. It is quite clear that in this case material single-stranded in the region of the marker itself can constitute an intermediate. Now, whether it does, of course, in the normal case is another matter.