*ON THE MECHANISM OF DEOXYRIBONUCLEATE INTEGRATION IN PNEUMOCOCCAL TRANSFORMATION**

By Maurice S. Fox and Marcia K. Allen†

DEPARTMENT OF BIOLOGY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY

Communicated by S. E. Luria, June 22, 1964

In the transformation of *Diplococcus pneumonia*, fragments of the transforming deoxyribonucleate (DNA) are inserted into the genome of recipient transformable bacteria. This entire process requires little or no net DNA synthesis.[1, 2]

Immediately following its fixation, the transforming DNA can be reisolated from the transformed bacterial population and can be demonstrated to be without biological activity. The newly introduced DNA recovers its activity with a half time of about 3 min and thereafter replicates in synchrony with the bulk DNA of the recipient bacteria.[1, 3] Lacks[4] has examined the inactive transforming DNA extracted from transformed bacteria. Some of the material was degraded and the remainder was denatured. He concluded that the denatured DNA was single-stranded and proposed an integration mechanism on this basis.

We will present evidence demonstrating that the transforming DNA extracted, prior to its replication, from transformed bacteria is a hybrid, which is apparently formed with the DNA of the recipient bacteria and which extends over a region of about one or two million daltons. Furthermore, the newly introduced DNA appears to be covalently linked to the DNA of the recipient bacteria.

Experimental.—Transforming DNA carrying the density labels deuterium and nitrogen-15 was isolated from a streptomycin-resistant, p-nitrobenzoic acid-sensitive strain of pneumococcus,[5] RF_6S. The bacteria were allowed many generations of growth in a heavy-isotope-labeled medium containing P^{32} in the form of phosphate. The nutrients in the medium were in the form of sugar and amino acid extracts from algae that had been grown in a deuterium, nitrogen-15-substituted medium.[6] The algal extracts were generously provided by H. Crespi.

The bacteria were centrifuged, washed two times in 0.15 *M* NaCl, 0.01 *M* versene, pH 7.5, and then lysed with a mixture of sodium dodecyl sulphate and deoxycholate at a final concentration of 0.05% and 0.025%, respectively. The lysate was shaken with chloroform and isoamyl alcohol[2] and either (*a*) treated with boiled ribonuclease, alcohol precipitated, redissolved in *M*/40 phosphate buffered saline, pH 7.5, and filtered through an HA Millipore filter before using, or (*b*) DNA reisolated from a preparative equilibrium density gradient centrifugation in CsCl.

Equilibrium density gradient centrifugation was performed by adding DNA samples to a concentrated solution of CsCl (Trona) to give a final density of 1.70, a final volume of 1.5 ml, and a DNA concentration of less than 10 μg/ml. Samples were centrifuged at 36,000 rpm and 21°C for 42 hr. The centrifuge tubes were punctured with a needle, and 26–32 fractions of two drops each were collected. The drops were diluted with 5 vol of *M*/40 phosphate buffered saline, pH 7.5, and aliquots were scanned for radioactivity and assayed for biological activity. Both the donor marker, streptomycin resistance, and the recipient marker, p-nitrobenzoic acid resistance, were assayed on the test strain RF6 which is sensitive to both drugs. Under these conditions, native "heavy" DNA may be separated from native light DNA by 10 fractions as is shown in Figure 1. Denaturation in boiling water for 10 min, followed by rapid chilling, shifts the position of a given DNA preparation by 5 fractions. This shift is consistent with a density increase of 0.015 gm/cc. The heavy DNA, therefore, has a buoyant density in CsCl of about 0.03 gm/cc greater than that of native light DNA.

In order to examine the fate of the heavy transforming DNA, transformation was carried out in the following manner. About 300 ml of a frozen transformable culture[7] of strain R6 (resistant to p-nitrobenzoic acid and streptomycin-sensitive) were incubated at 30° for 15 min, centrifuged in the cold, and resuspended in 10 ml of casein hydrolyzate medium[8] supplemented with 10^{-3} *M*

$CaCl_2$. The labeled DNA was added to the culture at 30°C. After 8 min, DNase (Worthington, IX crystallized) was added to give a final concentration of 10 μg/ml and the culture was transferred to 37°C. After 1, 4, and 15 min of incubation, samples were chilled to 0°C, centrifuged in the cold, and resuspended in an equal volume of casein hydrolyzate medium containing 10 μg/ml DNase. The samples were further washed three times by centrifuging and resuspending in 2 ml of 0.15 *M* NaCl, with 0.01 *M* versene, and 0.02% albumin. This washing was sufficient to remove residual traces of transforming DNA that had not been fixed by the treated bacteria. After final resuspension, the culture was lysed with 0.05% sodium dodecyl sulfate and 0.025% deoxycholate. The suspensions cleared in about 1 min at 37°C and were shaken for 15 min at room temperature with an equal volume of chloroform and 1/20 vol of isoamyl alcohol. The aqueous layers were recovered and examined by density gradient centrifugations. Small variations in the above procedure are described in the text.

The procedures that have been described permit the recovery in the density gradient of between 80 and 90% of the radioactivity added to the cesium chloride solution, and this in turn accounts for at least 90% of the radioactivity that has been irreversibly fixed by the transformable bacterial population.

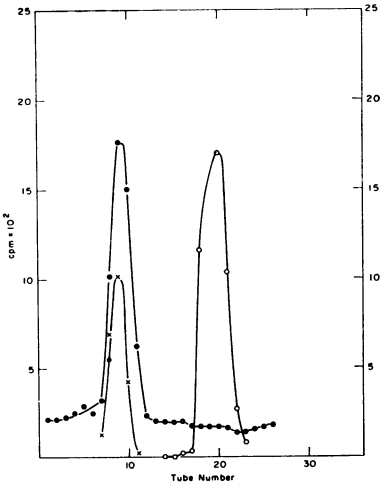

FIG. 1.—Distribution of pneumococcal transforming activities of light (O) and heavy (X) DNA after CsCl density gradient centrifugation. The heavy DNA is also labeled with P^{32} (solid dots). Transformants: X, heavy marker (in 10^{-3} ml); O, light marker (in 10^{-3} ml).

Results.—The density gradient patterns of DNA extracted from transformed bacteria that had been treated in the manner described above are illustrated in Figure 2. A small amount of cold heavy transforming DNA was added to the gradients as a position marker. At 1 min, only 21 per cent of the biological activity of newly introduced DNA has recovered from eclipse; by 4 min, about 60 per cent has recovered; and by 15 min, the newly introduced DNA has almost reached equilibrium. The distribution of radioactivity among the DNA fractions, heavy denatured, light nativelike, and soluble in 5 per cent trichloroacetic acid (TCA), is given in Table 1. It can be seen that there exists little or no material in the gradients

TABLE 1

DISTRIBUTION OF RADIOACTIVITY IN EXTRACTS OF TRANSFORMED BACTERIA

Incubation time following DNase addition (min)	% TCA-soluble	% Heavy denatured	% Resembling light native	% Recovery from eclipse
1	43	21	36	21
4	37	5	54	60
15	19	0	81	90

that physically resembles the original heavy transforming DNA. At a time when the input marker has recovered only 21 per cent of its biological activity, all of this activity and more than a third of the DNA radioactivity has been so altered as to band in a position very near that of the light recipient DNA. The remaining DNA radioactivity is distributed so that 20 per cent appears in a position to be expected of heavy denatured DNA and 40 per cent is in degraded TCA-soluble fragments. These latter components are lost on incubation, and their P^{32} becomes associated with light native DNA. Lacks[4] has similarly reported the denaturation and deg-

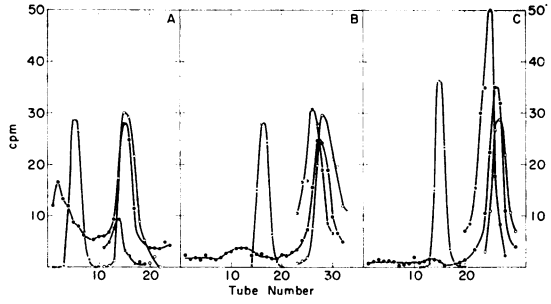

FIG. 2.—Density gradient distributions of extracts from transformed bacteria that had been allowed 8-min exposure to labeled DNA followed by (*A*) 1-min, (*B*) 4-min, and (*C*) 15-min incubation with 10 μg/ml DNase at 37°C. The distributions show the radioactivity (●) and biological activity (◐) of the donor DNA, the biological activity of the recipient DNA (○), and the biological activity of nonradioactive heavy DNA added as a position marker (X). Gradient (*A*) contains 30% more extract than the others. Transformants: ○, resident marker (in 4×10^{-4} ml); ◑, input marker (in 0.25 ml); X, heavy marker (in 4×10^{-2} ml).

radation of newly fixed transforming DNA. The quantitative differences that exist between Lacks' results and those reported here are probably the consequence of a difference in methods of DNA isolation.

In order to increase the amount of material available for study, the remaining experiments were carried out on extracts from bacteria that had been allowed 15 min exposure at 30°C to the transforming DNA, followed by 3 min at 37° with 10 μg/ml of DNase. Under these conditions, most of the newly introduced DNA has recovered from eclipse, and there has been no detectable multiplication of the DNA element responsible for the transformation.[2] The density gradient pattern of such an extract is shown in Fig. 3*A*, and the distribution of radioactivities is given in Table 2. All of the biological activity and most of the radioactivity of

TABLE 2

DISTRIBUTION OF RADIOACTIVITY IN GRADIENT FRACTIONS

Treatment of extract	% TCA-soluble	% Heavy denatured	% Light denatured	% Resembling native
None	36	17	...	46
Heat-denatured	39	19	42	...
Alkali-denatured	30	25	45	...
Sonicated	38	11	...	51
Sonicated and heat-denatured	37	39	23	...
Sonicated and alkali-denatured	34	39	27	...

the newly introduced DNA is physically associated with sufficiently large elements of light native DNA so as to mask about 90 per cent of the density label. A similar association between the transforming DNA and the DNA of the recipient bacteria has been observed in *B. subtilis* by Szybalski[9] and Bodmer and Ganesan.[10] In addition, it can be seen that there is no biological or physical material resembling the native heavy DNA with which these bacteria had been transformed. The irreversible fixation of transforming DNA must, therefore, require a reaction more complex than the mere passage across the bacterial membrane.

Zone sedimentation in a sucrose gradient demonstrates that the macromolecular P^{32} in the transformed bacterial extracts sediments at the same rate and with the same distribution as does freshly isolated H^3-labeled T7 DNA. Since T7 DNA has

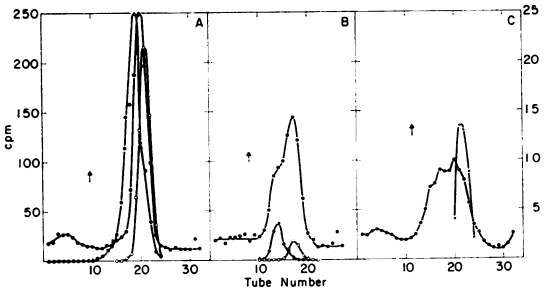

FIG. 3.—Density gradient distributions of extracts from transformed bacteria subjected to (*A*) no sonication, (*B*) 1-min sonication, and (*C*) 6-min sonication. The arrows indicate the gradient position to be expected for heavy DNA, and the biological activity (X) of added light DNA constitutes a position marker in gradient (*C*). The distributions show the radioactivity (●) and biological activity (◑) of the donor DNA, and the biological activity of the recipient DNA (○). Transformants: ○, resident marker (in 10^{-5} ml); ◑, input marker (in 5×10^{-3} ml); X, light marker (in 10^{-4}).

a molecular weight of 20 million,[11] the P^{32} must be associated with elements having approximately the same molecular weight. Sonication of the extract for 1 min and 6 min, in the manner described by Freifelder and Davison,[12] reduces the sedimentation coefficient from 30*S* to 13*S* and 11*S*, respectively (on the basis of linear distance moved in the gradient). These sedimentation coefficients correspond, according to Doty *et al.*,[13] to molecular weights of about two and one million, respectively.

The distribution of material in density gradients of extracts that had been sonicated for 1 and 6 min are shown in Figure 3*B* and *C*. As a consequence of the sonication, the biological activity of the input DNA shifts away from that of the resident DNA toward a position of higher density and approaches a density that is halfway between that of heavy and light native DNA. Moreover, the P^{32}-containing material, with increasing sonication, becomes spread over a broader and broader density region which ultimately appears to extend from the position of light DNA to the position where one might expect to find "hybrid" DNA. Fractions taken from various positions in the broad band of the sonicated sample retain their positions with respect to light DNA when centrifuged again in a density gradient. It is thus demonstrated that the DNA molecules produced by sonication are heterogeneous with respect to density. In spite of the very substantial reduction in molecular weight to one or two million daltons, little, if any, radioactivity appears in the density position of native fully heavy DNA.

Sonication of an artificial mixture of P^{32}-labeled heavy DNA and a large excess of light DNA produces no P^{32} in the position of light native DNA, nor does the P^{32}-band pattern manifest any heterogeneity.

The structure of the complex between the heavy transforming DNA and the light DNA of the recipient bacteria is further elucidated by examining its fate upon heat and alkali denaturation. The heat denaturation was accomplished by placing the DNA extract in boiling water for 7 min and chilling rapidly. Alkali denaturation was brought about by adding enough alkaline phosphate to raise the pH to 12.3 (Beckman combination electrode) incubating at 45°C for 5 min, chilling, and neutralizing with HCl. The density gradient pattern of the DNA extracted from transformed bacteria is shown in Figure 4, which compares the untreated extract with the heat-denatured and the alkali-denatured samples. On denaturation a

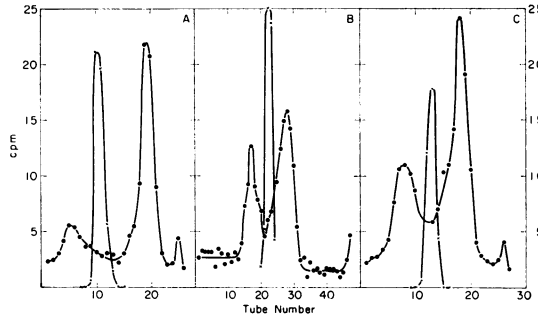

FIG. 4.—The extract from transformed bacteria showing the distribution of radioactivity (●) present in the donor DNA. The extract was (*A*) untreated, (*B*) heat-denatured, and (*C*) alkali-denatured. The biological activity of added heavy DNA (X) constitutes a position marker. Transformants: X, heavy marker (in 4×10^{-2} ml).

small additional amount of P^{32}-containing material moves to the position of heavy denatured DNA, but most of the P^{32} is still associated with large elements of light, now denatured, DNA.

The same DNA extract described in Figure 4 was sonicated for 4 min, and aliquots were heat- and alkali-denatured. The density gradients are shown in Figure 5. Following sonication and denaturation, a large portion of the macromolecular P^{32} behaves like heavy denatured DNA. Table 2 summarizes the distribution of radioactivity among the various fractions.

Sonication creates no fully heavy native DNA, only hybrid material which can, by denaturation, be demonstrated to be largely if not entirely an association of fully heavy and light subunits.

Discussion.—In extracts from transformed bacteria, the heavy transforming DNA that has been fixed is associated with elements of recipient DNA sufficiently large to obscure most of its characteristic buoyant density. Since most of these elements remain associated with the light DNA after either thermal or alkali denaturation, they would appear to be covalently linked to the light DNA of the recipient bacteria. After sonication, to reduce the molecular weight of the DNA in the extracts from twenty million to about one million, the elements with which the biological activity of the heavy DNA are associated increase in density, approaching the density to be expected of hybrid DNA.

Extracts also contain components of the heavy transforming DNA that have been denatured and that have been degraded. These components are rapidly converted into material that resembles light native DNA.

When the DNA of a sonicated extract is denatured by heat or exposure to high pH, the bulk of the P^{32} is then found associated with heavy denatured DNA. A substantial amount is also found in association with light denatured DNA. This light denatured band is probably best explained as the product of new synthesis of DNA using predominantly light precursors but reutilizing the acid soluble P^{32}-containing products of that portion of the transforming DNA that has been degraded. The heavy denatured band represents material which could not have been more than hybrid in the native configuration and which becomes fully heavy on denaturation. The fraction of the native material that was hybrid must therefore have been half heavy because it was heavy in one strand and light in the other.

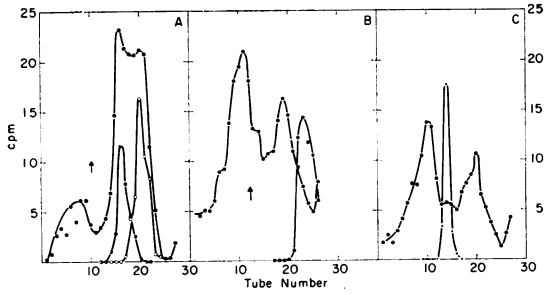

FIG. 5.—Density gradient distributions of an extract from transformed bacteria that had been sonicated for 4 min (*A*), then (*B*) heat-denatured or (*C*) alkali-denatured. The arrows indicate the position that native heavy DNA would be expected to assume. The distributions show the radioactivity (●) 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*). Transformants: ○, resident marker (in 10^{-3} ml); ●, input marker (in 1.25 ml); X, heavy marker (in 5×10^{-2} ml); ◐, light marker (in 5×10^{-5} ml).

It is with this hybrid that all of the donor-type biological activity of the reisolated DNA is associated. These findings demonstrate that the transforming DNA that has recovered from eclipse in transformed bacteria is in the form of a duplex that contains only a single strand of the physical material of the native donor transforming DNA. The single strand is present in elements whose molecular weight is of the order of one to two million daltons and is both chemically and genetically[1] coupled with the DNA of the recipient bacteria.

A number of hypotheses will be considered regarding the origin of the hybrid that has been described. One might assume that only a unique strand of the transforming DNA is used. If this unique strand were to form a hybrid with the complementary DNA of the recipient bacterium, then after one bacterial doubling there would have been no increase in the transforming activity of the newly introduced marker—a strand of DNA would have been synthesized that would not be active in transformation. This model can be excluded, since the transforming activity of the newly introduced DNA multiplies in synchrony with that of the recipient DNA.[2-4]

A second possibility is that either strand can be used in transformation and that the strand used forms a hybrid with a rapidly synthesized new complementary strand of itself. Evaluation of this hypothesis can be made by re-examining the results of experiments in which the sensitivity of transforming activity to the disintegration of incorporated P^{32} was measured in preparations of DNA heavily labeled with P^{32}.[2] The rate of loss of the biological activity of DNA that has been reisolated very soon after fixation by transformable bacteria is not distinguishable from that of the original DNA. This model can, therefore, also be excluded, since the newly synthesized strand would carry biological activity and would not contain lethal P^{32} atoms.

A third possibility is that a unique strand of the transforming DNA forms a hybrid with a complementary, rapidly synthesized new strand. This model can only be assessed by examining the sensitivity to P^{32} disintegrations of transforming DNA that has been reisolated after one replication in the transformed bacteria. At this time, the transforming activity should be equally distributed between stable

and fully sensitive elements. After infinite decay of the P^{32}, DNA isolated from such a population would be expected to retain at least half of its original transforming activity. Since it has been observed[2] that substantially more than half of the replicated products are in fact subject to inactivation by P^{32} disintegration, this possibility seems to be excluded.

Since it is clear that, as Lacks suggested,[4] only a single strand of the transforming DNA is being used intact, the P^{32} target must be a single strand. The survival, after infinite P^{32} decay, of less than half of the transforming activity, reisolated after one doubling, must be the consequence of a disintegration in one strand inactivating the complementary strand. The data that have been presented[2] are consistent with a cross-strand inactivation at a level of one in every five or six inactivating disintegrations. The absence of such a cross-strand effect to be expected for DNA reisolated soon after recovery from eclipse would reduce its sensitivity to P^{32} disintegrations by 17–20 per cent. Such a reduction was observed but was assumed to be the consequence of a small amount of DNA replication.

The remaining and most appealing model is that in which the hybrid is formed by either strand of the transforming DNA with a complementary region in the DNA of the recipient bacterial genome. This hybrid element would be half as active in transformation as an element which carries the marker on both strands. Observations on annealed mixtures of DNA are consistent with such an assumption.[14]

Further support is lent such a model by the observations of Guild and Robison[15] which suggest that for at least one transformable marker, there exist, in alkaline-denatured DNA, two density species, apparently complementary strands of about equal activity. The authors concluded that either strand could carry transforming activity and suggested a similar single-strand displacement mechanism. Furthermore, observations reported by Hotchkiss[16] demonstrate the existence of phenotypically transformed bacteria capable of yielding on replication both transformed and untransformed progeny, with respect to a single selective marker. This would indicate that the transformants must have been heterozygous with respect to the selective property. It would therefore appear that the hybrid that has been described is a hybrid between the newly introduced strand of DNA and its bacterial complement. This hybrid has been formed without a substantial amount of DNA synthesis and at a time when there has been little or no detectable replication of the DNA entity responsible for the transformation.[2]

It may be that fixation of elements of transforming DNA is initiated by one of its strands pairing with an open unpaired region of its bacterial complement. As this pairing proceeds, bacterial exonucleases would hydrolyze the unused strand of the transforming DNA as it is released. This would explain the absence of native heavy DNA in extracts of transformed bacteria as well as the presence of TCA-soluble fragments and what appears to be heavy denatured DNA. Upon completion of this pairing, the enzyme or enzymes responsible for repairing various DNA damages might excise that region of the recipient DNA whose complementary region is now occupied by a strand of the transforming DNA. This step might possibly be the step responsible for recovery from eclipse. Following this recovery, the establishment of chemical continuity between the recipient and the donor DNA could occur. This step might reasonably be considered identical with that in which genetic linkage is established.[1] Reactions such as those that have been de-

scribed are not substantially different from those that have been observed as being responsible for the dark reactivation of bacteria that have been irradiated with ultraviolet light.[17, 18]

In order to explain recombination within the region of the incorporated element of transforming DNA, it would be necessary to assume that the pairing process might be interrupted, perhaps by a break in the strand of donor DNA that is being incorporated. This might result in a switch, in which the other strand of donor DNA begins to pair with its complement in the host genome (later to be excised), and then a switch back to incorporate the remainder of the element of transforming DNA.

Whatever the final description of the mechanism of recombination, it is clear that, at least in the case of DNA-mediated transformation of *D. pneumonia*, the genetic exchange occurs by the physical insertion of a single strand of transforming DNA into the genetic material of the recipient bacterium.

The authors wish to thank Dr. P. F. Davison for his advice and suggestions, and Mr. J. Shamoun for his technical assistance.

* This work was supported by grants from the National Science Foundation (GB-644), and the National Institutes of Health (AI-05388).

† National Institutes of Health postdoctoral fellow.

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