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Molecular Basis for the Transformation Defects in Mutants of *Haemophilus influenzae*

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To determine the molecular basis of transformation defects in *Haemophilus influenzae*, the fate of genetically marked, ^{32}P-labeled, heavy deoxyribonucleic acid (DNA) was examined in three mutant strains (*rec*$_1^-$, *rec*$_2^-$, and KB6) and in wild type having ^{3}H-labeled DNA and a second genetic marker. Transforming cells upon lysis with digitonin followed by low-speed centrifugation are separable into the supernatant fraction, containing mainly the unintegrated donor DNA, and the pellet, containing most of the resident DNA along with integrated donor DNA. Electron micrographs of digitonin-treated cells also indicate that the resident DNA is trapped inside a cellular structure but that cytoplasmic elements such as ribosomes are extensively released. DNA synthesis in digitonin-treated cells is immediately blocked, as is any further integration of donor DNA into the resident genome. Isopycnic and sedimentation analysis of supernatant fluids and pellets revealed that in strains *rec*$_2^-$ and KB6 there is little or no association between donor and resident DNA, and thus there is negligible transfer of donor DNA genetic information. In these strains, the donor DNA is not broken into pieces of lower molecular weight as it is in strain *rec*$_1^-$ and in the wild type, both of which show association between donor and recipient DNA. In strain *rec*$_1^-$, although some donor DNA atoms become covalently linked to resident DNA, the incorporated material does not have the donor DNA transforming activity.

Transfer of genetic information from donor deoxyribonucleic acid (DNA) to the resident genome during bacterial transformation is accompanied by the incorporation of discrete single-strand segments of the input DNA (2, 6, 17). Although the ultimate fate and distribution of the donor atoms is fairly well understood, very little is known about the various steps that lead to physical and genetic fixation of the information contained in the donor DNA. It is presumed that physical integration of the donor DNA and covalent linkage between the donor and resident DNA species are preceded by a process of recognition between homologous segments of the two DNA species and that the recognition between two homologs occurs by complementary base-pairing. If that were so, the generation of single-stranded stretches of donor and resident DNA prior to recognition might be expected. During transformation of pneumococcus, donor DNA is recoverable immediately after uptake only in denatured (single-stranded) or degraded form (9). Evidence has been presented that implicates single-stranded DNA as the precursor of genetically integrated DNA in pneumococcus (10). Recently, a denatured form of donor DNA in *Bacillus subtilis* transformation has been reported (18). On the other hand, in *Haemophilus influenzae* the bulk of the input DNA immediately after uptake is recoverable in biologically active, double-stranded form. In wild-type cells, most of this DNA is then progressively incorporated into the resident DNA. It has been shown that a major portion of the integration is single-stranded and that the molecular weight of the integrated, single-stranded segments is about 6×10^6 (17). An amount approximately equivalent to the integrated segment of donor DNA was considered to be degraded and resynthesized into the recipient genome (17). An intracellular donor

DNA species (species II) with lower molecular weight than that of input DNA has been observed, but it has the characteristics of a by-product rather than an intermediate in transformation (16). From the foregoing studies it appeared that a search for intermediates in recombination might be more rewarding in recombination-defective mutants.

The strains selected for this study were the transformation-defective mutants KB6 (1), the ultraviolet-sensitive (UV^s) DB117 (19), the ultraviolet-resistant (UV^r) Rd(DB117)$^{rec-}$ (1), and Rd(DB117)$^{UV^s}$ (Setlow, Boling, Beattie, and Kimball, J. Mol. Biol. *in press*). The latter two strains were derived by transformation of wild-type cells with strain DB117 DNA. Strain DB117 has been shown to be a double mutant, rec_1^- mex^-, whereas strain Rd(DB117)$^{UV^s}$ is apparently altered only at the rec_1 locus. The two rec_1^- strains, DB117 and Rd(DB117)$^{UV^s}$, gave similar results in the types of experiments reported in this paper and are referred to here as rec_1^-. It has been postulated that the UV^r strain Rd(DB117)$^{rec-}$ is mex^- rec_2^p, containing one mutant gene from strain DB117 and one derived from the selection technique, and that these genes together cause the recombination defect (Setlow, Boling, Beattie and Kimball, J. Mol. Biol. *in press*). This strain is referred to in this paper as rec_2^-.

The present study reports the fate of donor DNA in these transformation-defective mutants. Evidence will be presented to show that even though the mutant strains KB6 and rec_2^- have independent origins, and strain rec_2^- but not strain KB6 is defective in promoting phage recombination, both appear to be blocked at an early step in transformation, and there is virtually no transfer of atoms from donor DNA to resident DNA. The irreversibly bound donor DNA remains unassociated with resident DNA for over 30 min. On the other hand, in the rec_1^- mutants there is transfer of donor atoms to resident DNA, although this process is slower than in the wild type. It is probable that some macromolecular association between donor and recipient DNA is established, but this association does not culminate in the formation of stable recombinants, since there is no accompanying transfer of genetic information.

MATERIALS AND METHODS

Bacterial strains. *H. influenzae* wild-type strain Rd and mutants derived from the wild type, the rec_1^- mutants DB117 and Rd(DB117)$^{UV^s}$, the rec_2^- mutant Rd(DB117)$^{rec-}$, and mutant KB6 have been described (1, 19; Setlow, Boling, Beattie, and Kimball, J. Mol. Biol. *in press*). The residual transformations in strain KB6, the rec_1^- strains, and rec_2^- relative to that in the wild type are 10^{-4}, 10^{-6}, and 10^{-7}, respectively.

Electron microscopy of competent digitonin-treated cells. Samples were fixed with osmium tetraoxide (8), dehydrated in ethanol, and embedded in Epon by the method of Luft (11). The polymerized Epon blocks were sectioned with a Porter-Blum MT-2 ultramicrotome with a diamond knife. Sections were stained for 1 hr in droplets of 2% uranyl acetate in water. After being rinsed in water, the grids were further stained for 5 min with lead citrate. A Siemens Elmiskop IA electron microscope was used.

Measurement of DNA synthesis in digitonin-treated cells. Exponentially growing wild-type cells were washed with 0.15 M NaCl plus 0.015 M trisodium citrate (SSC) and suspended at a concentration of about 10^9 cells/ml in SSC containing 25 μCi of [^{3}H]deoxythymidine (dThd) per ml, with or without 0.1% digitonin (Matheson, Coleman, and Bell). The two mixtures (0.1 ml of each) were incubated at 37 C. At intervals 10-μliter samples were pipetted onto paper disks, processed, and counted as previously described (13).

Preparation of ^{32}P-, ^{2}H-, and ^{15}N-labeled DNA. Streptomycin-resistant (Str) cells were adapted to growth in 99.9% D_2O by stepwise transfer of cultures grown in Brain Heart Infusion (BHI) medium (19) made with 50 to 99.9% D_2O. The adapted culture was then inoculated into a defined medium (7) containing 20 μCi of $^{32}PO_4$ per ml. After overnight growth, cells were washed three times with SSC and lysed at 37 C with sodium lauryl sulfate (final concentration 0.5%). NaCl was added to a final concentration of 2 M. The lysate was shaken with an equal volume of chloroform-amyl alcohol (24:1) for 1 hr by the method of Marmur (12). The mixture was then separated by low-speed centrifugation, and the water layer was treated at 37 C for 30 min with ribonuclease (heated to inactivate contaminating deoxyribonuclease) at 100 μg/ml. After a second cycle of deproteinization, the preparation was passed through a Sephadex G-100 column (bed volume 60 ml, hold-up volume ~20 ml) and eluted with SSC. A 4-ml fraction containing most of the DNA was used in the experiments. The specific activity of such DNA was ~1 to 2 $\times$ 10^5 counts per min per μg.

Preparation of ^{3}H-labeled, competent recipient cells. Cathomycin (novobiocin)-resistant (Nov) cells were grown in BHI medium containing 350 μg of adenosine per ml and ~1 μCi of [^{3}H]methyl thymidine per ml (specific activity ~20 Ci/mmole). The cells were made competent in M IV medium (20). In some experiments, the genetic marker in the cells was *str* and the donor DNA was *nov*. This reversal did not affect the results.

Digitonin lysis and preparation of DNA extracts. Digitonin lysates were prepared by a procedure previously developed (15). A mixture of 3 ml of ^{3}H-labeled competent cells and 0.3 ml of ^{32}P-labeled DNA (~0.5 μg) was incubated at 37 C for 5 min, and deoxyribonuclease was added to a final concentration of 30 μg/ml. After 3 min of further incubation, a

1-ml sample was withdrawn, washed three times with SSC, suspended at the same cell concentration in SSC containing 0.1% digitonin, and incubated at 37 C for a minimum of 3 hr. This was called the "0-min" sample. Other samples were incubated for additional times before being washed and treated with digitonin. After digitonin treatment, the samples were centrifuged for 10 min at 5,000 rev/min (3,000 $\times$ *g*), the supernatant fractions were removed, and the pellets were suspended in 1 ml of SSC. The supernatant fractions and pellets were then sheared on a Vortex, Jr. Mixer (Scientific Industries, Inc.) for approximately 1 min, and the DNA was extracted once by treating the solution with chloroform-amyl alcohol. In some experiments, the last step was omitted. In one set of experiments, pellet material was denatured by heating at 100 C for 7 min, followed by rapid cooling.

Velocity sedimentation. Samples of 0.2 to 0.3 ml were layered on 5 to 20% sucrose gradients containing 0.15 M NaCl. Tubes were centrifuged in an SW65 rotor of a Beckman centrifuge at 36,000 rev/min for 3 hr.

Density-gradient sedimentation. A rapid equilibrium method (4) was used, as suggested by G. Kellenberger-Gujer (*personal communication*). Two CsCl solutions were prepared with densities of 1.7415 and 1.8457, and 1.87 ml of the higher-density solution was placed in a 5-ml cellulose nitrate or polyallomer tube. A sample (0.7 ml) was mixed with 1.5 ml of the lower-density solution and gently layered on top. The tube was then filled with 1 ml of mineral oil and centrifuged at 32,000 rev/min in an SW50.1 rotor for a minimum of 16 hr, by which time equilibrium was already established.

Collection and assay of fractions. About 65 five-drop fractions were collected directly on paper strips, processed, and counted by the procedure of Carrier and Setlow (5); or about 32 ten-drop fractions were collected in test tubes, part of the sample was placed on paper for measurement of radioactivity, and part was used for biological assay. For the biological assay, fractions were diluted by a factor of eight to avoid the inhibiting effect of CsCl on transformation. Samples of 0.05 ml of the diluted fractions were mixed with 1 ml of competent wild-type cells and incubated with shaking for 30 min. Appropriate dilutions of the mixture were then plated and incubated for 1.5 to 2 hr, at which time the plates were overlaid with agar containing either 5 μg of cathomycin per ml, 500 μg of streptomycin per ml, or both.

RESULTS

Effect of digitonin on the structure of cells and on DNA synthesis. Treatment of competent cells with digitonin (Fig. 1) results in a progressive degradation of the cell wall, leading to the formation of spheroplasts. These spheroplasts lyse, due either to fragility or to further action of digitonin on the cell membrane. Although lysis removes much of the cytoplasmic elements, such as ribosomes, the DNA fibrils appear to remain trapped within the cell fragment.

Table 1 shows that there is almost no DNA synthesis in digitonin-treated cells in SSC, even after only 10 min of treatment, whereas cells in SSC alone synthesize some DNA for about 20 min and then stop, presumably as a result of depletion of intracellular pools of precursors.

Distribution of label from donor DNA and resident DNA in digitonin lysates. The distributions of resident and donor DNA labels in digitonin supernatant fractions and pellets of strains Rd (wild type), KB6, rec_1^- (DB117), and rec_2^- cells are shown in Table 2. The digitonin lysis followed various times of incubation of the cells containing donor DNA. Between 92 and 97% of the resident DNA activity in all the strains is in the pellet. In the wild type, only about 35% of the donor label is in the pellet at 0 min, but the amount increases with time to about 70%, while there is a corresponding decrease in the counts in the supernatant fraction. The kinetics of appearance of label in the digitonin pellet are similar to those reported by Notani and Goodgal (17) for association of heavy donor DNA with light resident DNA, as determined by equilibrium centrifugation of DNA extracted from cells during transformation. Therefore, we conclude that the digitonin pellets contain complexes of donor and resident DNA, whereas the digitonin supernatant fractions contain mainly unintegrated donor DNA.

In contrast to the wild type, in strains KB6 and rec_2^- there is no increase in the ^{32}P-label from the donor DNA in the pellet with incubation time, and the ^{32}P-label in the supernatant fraction is also constant. In strain rec_1^-, there is an increase in donor ^{32}P counts in the pellet, but less than in the wild type in absolute counts as well as per cent. In all the strains, the total number of donor and recipient counts is approximately a constant as a function of incubation time, except that in rec_1^- cells there is about a 10 to 15% decrease.

Transforming activity of digitonin lysates. Table 3 shows the transforming activity of the two fractions obtained after digitonin treatment. With increasing incubation time between DNA uptake and digitonin lysis in wild-type cells, transformation by the donor DNA streptomycin marker (*str*) decreases in the supernatant fraction and increases in the pellet, while there is little or no change in the resident DNA cathomycin marker (*nov*). There is also an increase in the number of double (*str*,*nov*) transformants from the pellet, but

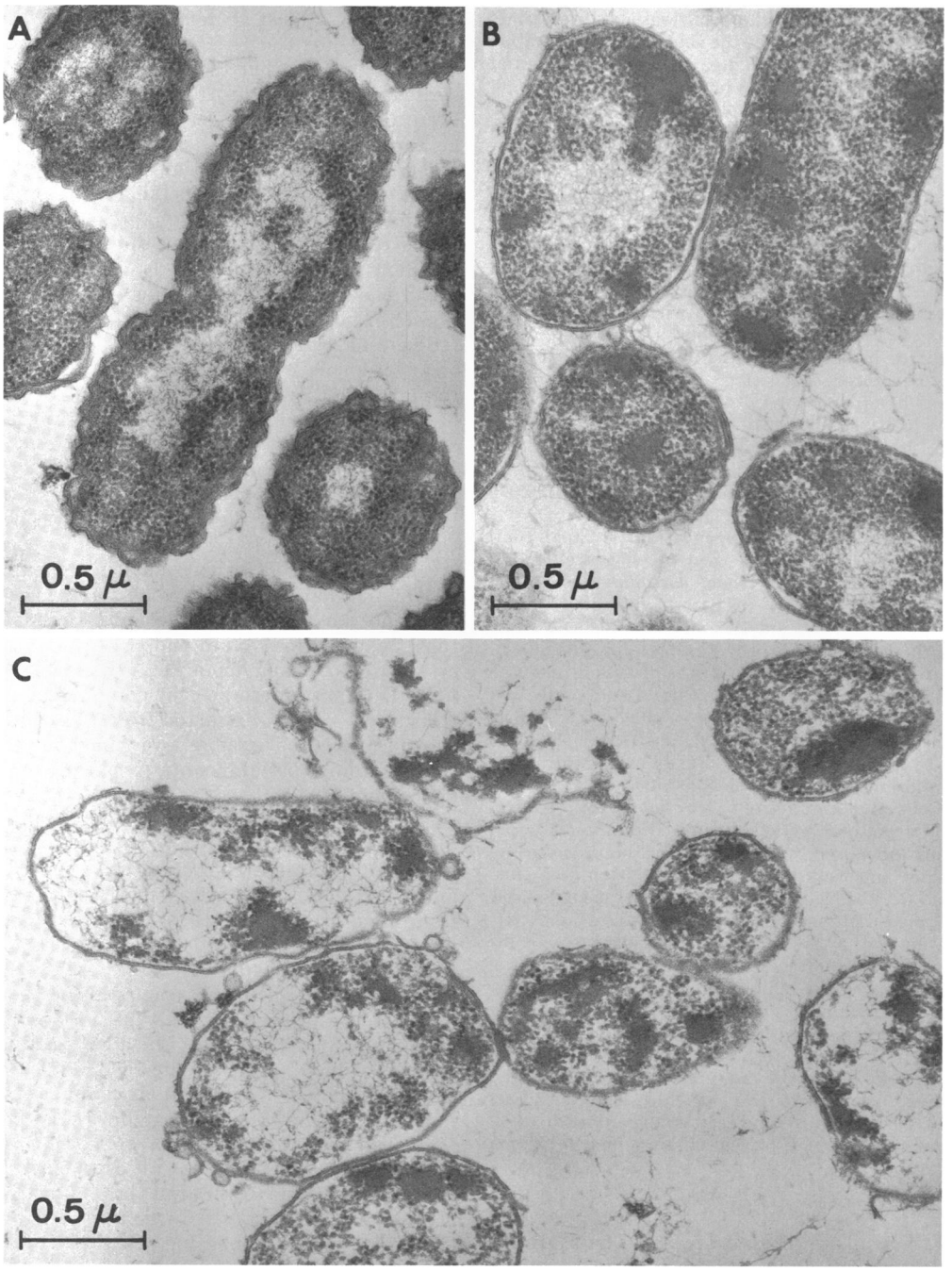

FIG. 1. *Thin-section electron micrographs illustrating the effect of digitonin on competent H. influenzae cells. (A) Untreated competent cells are characterized by a close association of the intact cell wall and membrane, ribosomes are clearly seen within the cytoplasm, and DNA fibrils can be distinguished within lightly stained nuclear areas. (B) One hour after treatment with digitonin a marked effect on the cell wall can be observed. Some cells have completely lost their cell wall, whereas others have wall fragments clinging to the cell membrane. (C) Three hours after digitonin treatment there is evidence of extensive lysis. The cytoplasmic material (ribosomes) appears diluted, and although the nuclear areas are dispersed the DNA fibrils can be seen within the cell.*

the number of such double transformants from the supernatant fraction remains negligible. These data provide additional evidence that the digitonin treatment separates the donor DNA into an unassociated portion (in the supernatant fraction) and an associated portion (in the pellet) containing molecules that bear genetic information from both donor and recipient and can give rise to double transformants. In contrast to the transformation by digitonin lysates of wild-type cells, donor marker activity in strain rec_1^- decreases with incubation time in both supernatant fraction and pellet. In spite of the association of donor and recipient radioactive label seen in the pellets of strain rec_1^- cells (Table 2), there is no evidence of formation of genetically recombinant molecules effective in transformation, as judged by the lack of *str,nov* double transformants from rec_1^- pellets.

TABLE 1. *Effect of digitonin on DNA synthesis in H. influenzae strain Rd ([^{3}H]thymidine counts incorporated into trichloroacetic acid-insoluble material)*

Time of exposure (min)	Digitonin-treated culture (net counts/min incorporated)	Control (without digitonin)
0	0	0
10	12	610
20	13	1,160
40	14	1,510
88	33	1,130

Specific biological activities of donor DNA in supernatant fractions and pellets are given in Table 4. In the wild type, the specific activity of the supernatant fraction decreases slightly with time of incubation and increases slightly in the pellet. In strain rec_1^- (DB117) and rec_2^-, there is little change in the supernatant fractions, but there is a substantial decrease in the pellet in strain rec_1^- and a small decrease in strain rec_2^-. It is noteworthy that the specific activity of strain rec_2^- supernatant fractions and pellets is much higher than that of either wild type or strain rec_1^-.

Sedimentation velocity of digitonin supernatants. The unassociated DNA in wild type

TABLE 2. *Donor DNA (^{32}P-labeled Rd DNA) and resident DNA (^{3}H-labeled from strains Rd, rec_1^-, rec_2^- and KB6 competent cells) in supernatant fraction and pellet of digitonin lysates as percentages of total ^{32}P or ^{3}H counts*

Interval between DNA uptake and digitonin lysis (min)	Rd		rec_1^-		rec_2^-		KB6	
	Donor	Resident	Donor	Resident	Donor	Resident	Donor	Resident
Supernatant fractions								
0	65	7	84	5	84	7	88	6
10	39	8	78	4	82	5	84	3
30	28	8	57	3	85	4	87	3
Pellets								
0	35	93	16	95	16	93	12	94
10	61	92	22	96	18	95	16	97
30	72	92	43	97	15	96	13	97

TABLE 3. *Transforming activity of supernatant fractions and pellets of digitonin lysates of strains Rd, rec_1^- and rec_2^- exposed to Rd DNA, as number of transformations per milliliter resulting from a mixture of 2 ml of Rd cells and 0.02 ml of supernatant fraction or pellet*[a]

Interval between DNA uptake and digitonin lysis (min)	Rd			rec_1^-			rec_2^-		
	nov	*str*	*nov,str*[b]	*nov*	*str*	*nov,str*[b]	*nov*	*str*	*nov,str*[b]
Supernatant fractions									
0	4.0×10^5	6.1×10^3	2.5	3.4×10^4	7.8×10^3	1	2.5×10^4	2.3×10^4	2.5
10	3.4×10^5	2.8×10^3	6	5.2×10^4	6.0×10^3	0	1.8×10^4	1.9×10^4	3
30	2.4×10^5	1.7×10^3	9	5.3×10^4	3.6×10^3	0	1.9×10^4	2.4×10^4	1
Pellets									
0	1.4×10^7	2.2×10^3	310	1.2×10^6	4.9×10^2	0	8.2×10^6	2.4×10^3	5
10	1.1×10^7	3.4×10^3	615	0.5×10^6	4.0×10^2	1	6.8×10^6	2.9×10^3	5
30	1.2×10^7	4.5×10^3	1015	0.7×10^6	2.4×10^2	0	6.6×10^6	1.8×10^3	0

[a] The cells were cathomycin-resistant (*nov*), and the Rd donor DNA carried the streptomycin resistance (*str*) marker.

[b] Each count was based on two plates containing either 0.2 or 0.5 ml of the transformation mixture, except with Rd pellets, which were based on 0.1- and 0.2-ml platings.

TABLE 4. *Specific biological activity of donor DNA in supernatant fractions and pellets of digitonin lysates of strains Rd, rec_1^- and rec_2^- competent cells exposed to ^{32}P-labeled, str DNA from Rd (ratio of transformations to ^{32}P counts)*

Interval between DNA uptake and digitonin lysis (min)	Rd	rec_1^-	rec_2^-
Supernatant fractions			
0	2.3	2.6	5.3
10	2.0	2.7	4.2
30	1.7	2.4	5.2
Pellets			
0	1.5	0.9	3.0
10	1.5	0.6	2.9
30	1.8	0.2	2.2

consists of at least two species: species I, which is similar to the input DNA in its sedimentation and biological properties, and species II, with a lower molecular weight and relatively low transforming activity (16). It was earlier suggested on the basis of kinetic data that species II molecules arise as a consequence of donor-recipient association (16). Thus we would expect association-defective strains to be unable to generate species II molecules. Figure 2 shows that this is indeed the case. The sedimentation profiles of digitonin supernatant fractions of wild type and of mutant KB6 are clearly different. In the wild type, but not in strain KB6, there is a large decrease in length of the DNA relative to the length of the original transforming DNA. The mutant KB6 is similar to strain rec_2^- in that very little donor label is found associated with recipient DNA (Table 2). Thus the relatively higher specific activity of strain rec_2^- supernatant fractions (Table 4) is presumed to result from the absence of species II molecules. On the other hand, similar experiments suggest that species II molecules are present in strain rec_1^- lysates (not shown), although possibly not to the same extent as in wild type, which explains at least in part the lower specific biological activity of digitonin supernatant fractions from these cells (Table 4).

Equilibrium sedimentation of transforming DNA and DNA from digitonin lysates. Figure 3 shows the isopycnic equilibrium sedimentation pattern of heavy (^{32}P-labeled) DNA as compared with light (^{3}H-labeled) DNA. It is seen that the peak of the heavy DNA is separated from that of the light DNA by seven fractions. The density difference is about 0.03 g/cm^3.

Supernatant fractions and pellets from digitonin lysates of transforming cells can be directly banded in CsCl. In the present experiments, DNA was extracted before banding. The distribution of radioactivity and transforming activity of wild-type supernatant fractions and pellets after CsCl equilibrium sedimentation is shown in Fig. 4 and 5. The donor label (^{32}P) in the supernatant fractions (Fig. 4) is not associated with the light resident DNA containing the ^{3}H-label. On the other hand, most of the ^{32}P-label in the pellets (Fig. 5) is distributed with the resident DNA. The donor transforming activity (*str*) of the supernatant and pellet fractions from wild-type cells in general corresponds to the distribution of donor radioactivity. *str* Transformants and the double transformants (*str,nov* recombinants) from the pellet are highest in the position where the donor and recipient label are associated. However, the donor transforming activity and radioactive label of the pellets are slightly displaced toward heavy density.

In strain rec_1^- cells, the supernatant fraction and pellet distribution of radioactive labels

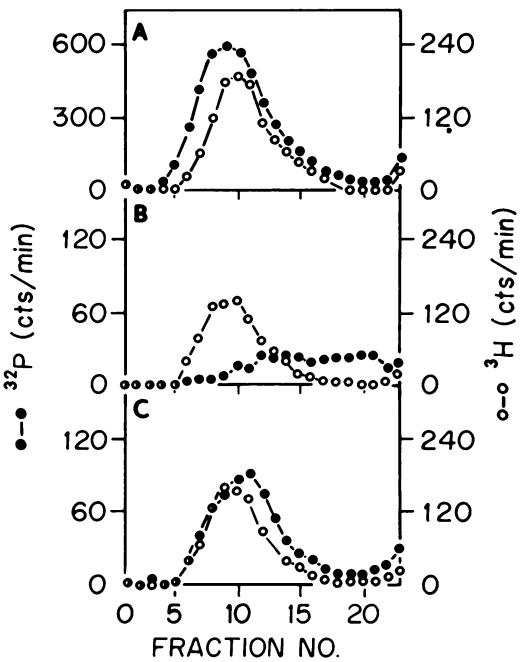

FIG. 2. *Sedimentation in 5 to 20% sucrose gradients of ^{32}P-labeled transforming DNA from strain Rd (A) and of the same DNA irreversibly bound but unintegrated at 30 min during transformation of wild type (B) and transformation-defective strain KB6 (C). ^{3}H-Labeled transforming DNA was added as a reference. Sedimentation was from right to left.*

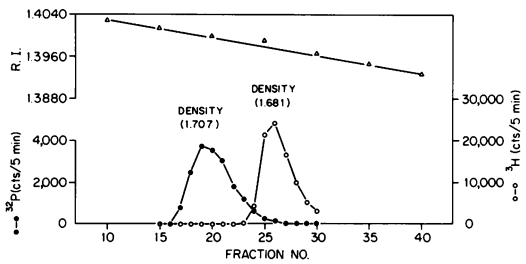

FIG. 3. *Equilibrium sedimentation profiles of ^{3}H-labeled light and ^{32}P-labeled heavy transforming DNA. The densities were calculated from the refractive indices (RI). Five drops per fraction were collected.*

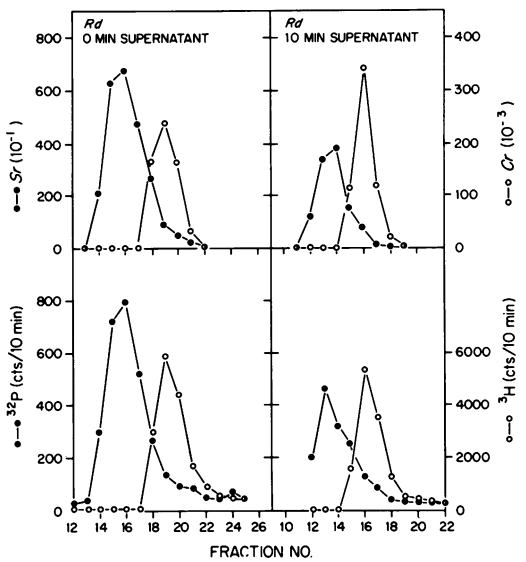

FIG. 4. *Distribution of radioactivity and transforming activity of DNA species from supernatant fractions of digitonin lysates of ^{3}H-labeled, nov, wild-type cells exposed to heavy, ^{32}P-labeled, str, transforming DNA centrifuged in CsCl gradients. Ten drops per fraction were collected. Sr: str; Cr: nov.*

(Fig. 6 and 7) is similar to that of the wild type, as is the transforming activity from supernatant fractions. However, while there is some association of donor and recipient label in the pellets, there is not a corresponding association of donor and recipient transforming markers.

There is some slight indication of a denatured form of DNA present in wild-type and strain *rec*$_1^-$ supernatant fractions, since the maximum of biological activity is sometimes in a slightly faster sedimenting fraction than is the maximum of donor radioactivity (Fig. 4, 10-min supernatant fraction; Fig. 6, 0- and 30-min supernatants). However, it is clear that most of the unintegrated DNA is native.

In strain *rec*$_2^-$ cells, there is neither association of radioactive label nor association of genetic-marker label in the pellet material (Fig. 8), even though these pellets contain around 20% of the donor label. The distribution of radioactivity and transforming activity in strain *rec*$_2^-$ supernatant fractions is similar to those of wild type and strain *rec*$_1^-$.

Figure 9 shows a comparison of the distribution of radioactive label from strain *rec*$_1^-$ pellets centrifuged in CsCl before and after denaturation. The presence of donor label associated with resident label in denatured pellet DNA as well as in native pellet DNA indicates that there is covalent bonding between resident and donor DNA.

DISCUSSION

The distribution of donor and resident DNA label in the supernatant fraction and pellet of digitonin lysates suggests that the resident DNA, either because of its large size or be-

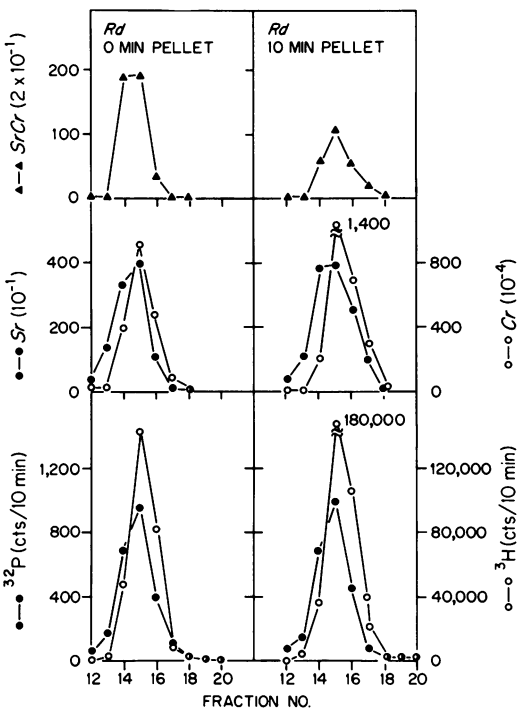

FIG. 5. *Distribution of radioactivity and transforming activity of DNA species from pellets of digitonin lysates of ^{3}H-labeled, nov, wild-type cells exposed to heavy, ^{32}P-labeled, str, transforming DNA, centrifuged in CsCl gradients. Ten drops per fraction were collected. Sr: str; Cr: nov.*

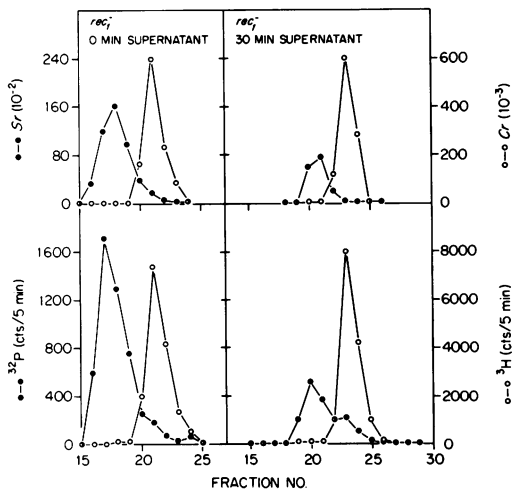

FIG. 6. *Distribution of radioactivity and transforming activity of DNA species from supernatant fractions of digitonin lysates of ^{3}H-labeled, nov, rec$_1^-$ cells exposed to ^{32}P-labeled, str, transforming DNA, centrifuged in CsCl gradients. Ten drops per fraction were collected. Sr: str; Cr: nov.*

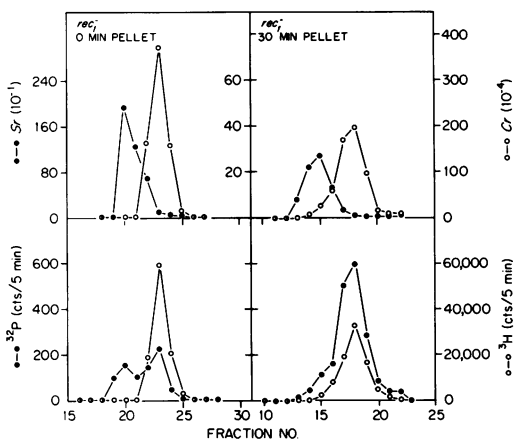

FIG. 7. *Distribution of radioactivity and transforming activity of DNA species from pellets of digitonin lysates of ^{3}H-labeled, nov, rec$_1^-$ cells exposed to heavy, ^{32}P-labeled, str, transforming DNA, centrifuged in CsCl gradients. Ten drops per fraction were collected. Sr: str; Cr: nov.*

cause of membrane association, remains trapped inside a cellular structure and thus precipitates on low-speed centrifugation. Electron micrographs of digitonin-treated competent cells also indicate that much of the resident DNA remains contained, even though ribosomal material has moved out. These observations suggest that the separation of the smaller, unintegrated but deoxyribonuclease-resistant donor DNA from resident DNA is possible because of small "holes" made in the

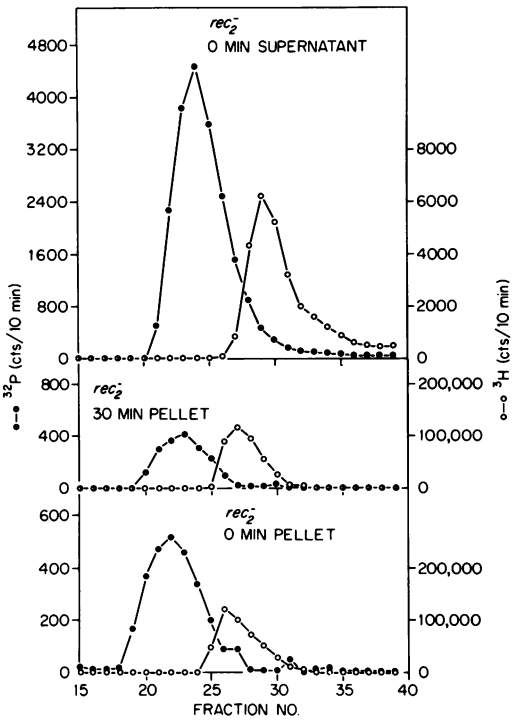

FIG. 8. *Distribution of radioactivity of DNA species from supernatant fractions and pellets of digitonin lysates of ^{3}H-labeled, nov, rec$_2^-$ cells exposed to heavy, ^{32}P-labeled, str, transforming DNA, centrifuged in CsCl gradients. Five drops per fraction were collected.*

cell envelope by digitonin. We have also shown that digitonin almost instantly blocks DNA synthesis, possibly because the integrity of the cell membrane may be necessary for this process.

While the separation of donor label is explicable in terms of associated and unassociated donor DNA, the presence of about 10% of recipient DNA label in the supernatant fraction is puzzling. One explanation is that a fraction of the cells are fully lysed by digitonin. However, the specific biological activity of resident DNA in the supernatant fraction is about three times lower than that of resident DNA in the pellet. Furthermore, the lack of genetic recombinant DNA in the supernatant fraction argues against complete lysis of some cells. It is possible that *H. influenzae* contains some episomal DNA, which would be expected to be released by digitonin treatment.

Strains KB6, rec$_1^-$, and rec$_2^-$ are transformed with very low efficiencies, although irreversible uptake of donor DNA by competent cultures of these strains is as high as in wild-type cells (1). Therefore, the transformation

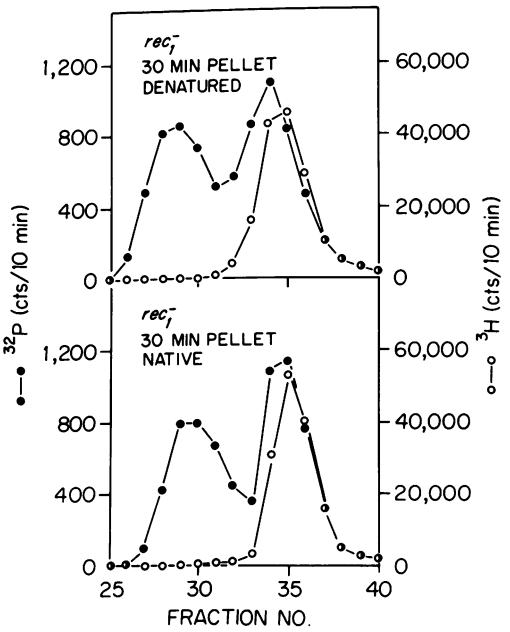

FIG. 9. *Distribution of radioactivity of DNA species from pellets of digitonin lysates of ^3H-labeled rec_1^- cells exposed to heavy, ^{32}P-labeled transforming DNA, centrifuged in CsCl before and after denaturation. Five drops per fraction were collected.*

defect in these strains must be due to a block in a step following uptake. Differences between wild-type and mutant strains in the transfer of donor DNA atoms to resident DNA have been noted from the observations with digitonin lysates separated into supernatant fractions and pellets. Strains KB6 and rec_2^- do not transfer any significant number of donor DNA atoms to resident DNA. Although this point is merely suggested by the data from digitonin lysates (Table 2), where there is some association indicated between resident and donor DNA in all strains, it is more clearly established by CsCl centrifugation of DNA from the pellets. In these gradients, the bulk of the donor DNA bands apart from the light resident DNA in strain rec_2^-. Thus there are obvious discrepancies between the extent of association between donor and recipient DNA species as measured by the digitonin method and as measured by the CsCl method. It is clear that by the digitonin method the extent of the association is overestimated. The overestimate could be due partly to residual supernatant material in the pellet, to imperfect release of donor DNA, or even to some real although noncovalent association that does not survive CsCl centrifugation.

The donor DNA in rec_2^- cells after uptake remains unassociated but stays biologically active. In fact, its specific biological activity is higher than that of the corresponding DNA from either wild-type or rec_1^- cells. It is presumed that the species II molecules, which are considered to be the by-products of transformation (16), are not generated in strain rec_2^-. This result is similar to that found for strain KB6. Thus a higher specific biological activity of donor DNA is observed in strain rec_2^-, apparently because there is little or no change in the structure or biological activity of this DNA.

It may be argued that strains KB6 and rec_2^- have an impermeable membrane for transforming DNA and that donor DNA after uptake remains between the cell wall and the membrane. This argument could be used for strain KB6, but probably not for strain rec_2^-, which, unlike strain KB6, is also defective in promoting phage recombination (Setlow, Boling, Beattie and Kimball, J. Mol. Biol. *in press*). Alternatively, KB6 and rec_2^- might be defective in a protein that brings about the initial interaction between donor and resident DNA species. There is no evidence to support this notion, and possibly there is no need to postulate a protein for a function which may be accomplished simply by diffusion. A third possibility might be that these strains are deficient in endonucleolytic nicking of the donor DNA, which may be required for recognition to occur by base-pairing between resident and donor DNA species. There is also no direct evidence for this possibility.

The pattern of transfer of donor DNA to resident DNA in strain rec_1^- is different from that in wild type. Transfer is slower in strain rec_1^-, but some transfer does occur. The distribution of donor atoms that are covalently linked to resident DNA has been shown to be bimodal in wild type (17). One mode, which was macromolecular and biologically active, was considered to be due to integration and to reflect genetic recombination. The second mode, devoid of biological activity, was postulated to be nonmacromolecular and was interpreted as arising from the degradation of one strand of input DNA and its resynthesis into resident DNA. If our observations on strain rec_1^- are to be interpreted in this way, it would appear that all the incorporation of donor label into resident DNA is nonmacromolecular, since there is no input or recombinant biological activity corresponding to these molecules. When molecules in the strain rec_1^- pellet are denatured and banded in CsCl, donor atoms are still associated with resident DNA, indicating that they are covalently

linked, but they band about half a fraction heavier than light molecules (Fig. 9). These data could be interpreted as due to integration of very small fragments of donor DNA rather than due to complete degradation and resynthesis. It should be pointed out that in normal wild-type transformation resident DNA fragments are released into the medium and are quantitatively equivalent to the amount of donor DNA integrated (20). Thus if there is extensive degradation and resynthesis of part of the donor DNA in wild type, it is necessary to explain why the resident DNA that is displaced by donor DNA appears in degraded form in the medium, rather than undergoing a similar process of degradation and synthesis within the cell. If degradation products of donor and resident DNA were chemically similar and not spatially separated, both should be included in resynthesis.

A third explanation for the absence of biological activity of donor DNA associated with resident DNA in strain rec_1^- is that these cells accumulate some kind of branched molecules that are intermediates in the integration process. There is as yet no direct evidence for this, but branched molecules have been implicated as intermediates in T4 phage genetic recombination (3). The lack of donor transforming activity from these hypothetical hybrid molecules could result either from their inability to enter the cell or from a bias in transformation by such molecules in favor of integration of the recipient DNA part of the molecule rather than the donor DNA part.

Resident DNA fragments are not released as a result of exposure of strain rec_1^- cells to transforming DNA (14). This finding would suggest that the displacement of resident DNA is one of the last steps in integration, and thus strain rec_1^- cells must be blocked either at this step or at some preceding step that follows association of donor and recipient DNA. We have demonstrated some covalent linkage between donor and resident DNA atoms in strain rec_1^-. Whatever may be the nature of donor DNA atom transfer in strain rec_1^-, it is certain that there is no concomitant transfer of genetic information, except at a level too small (10^{-6}) to detect physically.

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