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Fate of Donor Deoxyribonucleic Acid in a Highly Transformation-Deficient Strain of *Haemophilus influenzae*

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A transformation-deficient strain of *Haemophilus influenzae* (efficiency of transformation 10^4-fold less than that of the wild type), designated TD24, was isolated by selection for sensitivity to mitomycin C. In its properties the mutant was equivalent to *recA* type mutants of *Escherichia coli*. The TD24 mutation was linked with the *str*-r marker (about 30%) and only weakly linked with the *nov*-r2.5 marker. The uptake of donor deoxyribonucleic acid (DNA) was normal in the TD24 strain, but no molecules with recombinant-type activity (molecules carrying both the donor and the resident marker) were formed. In the mutant the intracellular presynaptic fate of the donor DNA was the same as that in the transformation-proficient (wild-type) strain, and the radioactive label of the donor DNA associated covalently with the recipient chromosome in about the same quantity as in the wild type. However, many fewer donor atoms were associated with segments of the mutant's recipient chromosome as compared with segments of the wild-type chromosome. In the mutant the association was accompanied by complete loss of donor marker activity. The lack of donor marker activity of the donor-recipient complex of DNA isolated from the mutant was not due to lack of uptake of the complex by the second recipient and its inability to associate with the second recipient's chromosome. Because the number of donor-atom-carrying resident molecules was higher than could be accounted for by the lengths of presynaptic donor molecules, we favor the idea that the association of donor DNA atoms with the mutant chromosome results from local DNA synthesis rather than from dispersive integration of donor DNA by recombination.

Recombination-deficient (*rec*$^-$) strains of transformable bacteria are potentially useful in attempts to define the molecular events that result in genetic recombination. A number of such strains of various transformable species has been described (5, 6, 10, 11, 14, 20, 23, 24, 25, 27, 28, 32, 33, 35). In an attempt to obtain *rec*$^-$ strains of *Haemophilus influenzae*, we previously reported that by selection for sensitivity to mitomycin C (MC), poorly transformable strains can be isolated (17). Three of these mutants have been investigated in more detail, all of which turned out to be recombination proficient (17, 19). The poor transformability of these mutants resulted from a cell envelope deficiency, causing lack of phenotypic expression of the antibiotic-resistance donor markers (19).

In *Escherichia coli* most recombination-deficient mutants fall into two genetically and phenotypically distinguishable groups: *recA* and *recB-recC* types. *recA* mutants are almost completely recombination deficient (7) and highly sensitive to ultraviolet (UV) irradiation (9, 15, 16, 21), produce a very low level of bacteriophage λ both spontaneously and after UV irradiation (3, 7, 13), and degrade their deoxyribonucleic acid (DNA) to a high extent after UV irradiation (8, 16, 34). *recB-recC* mutants show a substantial level of residual recombining potential and are less sensitive to UV than are *recA* mutants (7, 34); they lack the adenosine triphosphate (ATP)-dependent exonuclease (2, 4, 22).

In further exploration of our transformation-deficient mutant collection, we characterized a highly recombination-deficient mutant showing properties that seem to justify its classification as a *recA* type of mutant. The present communication also deals with the biological and physicochemical fate of transforming DNA in this mutant.

MATERIALS AND METHODS

Strains. W used the Rd strain of *H. influenzae* (1), sensitive to 1 μg of novobiocin, 1 μg of erythromy-

cin, and 3 μg of streptomycin per ml. The *str*-r, *nov*-r2.5, and *ery*-r strains are resistant to at least 250 μg of streptomycin, 2.5 μg of novobiocin, and 6 μg of erythromycin per ml, respectively. The strains were obtained from J. H. Stuy in 1965. For phage HP1c1 plating, an RD 200 strain (30) was used as indicator. The transformation-deficient (TD24) strain was derived from the *str*-r strain by means of selection for sensitivity to MC and poor transformability as described (17). In all experiments TD24 strains were used in which the TD24 locus of the original MC-sensitive strain had been transferred to Rd (*nov*-s *str*-s, *nov*-r2.5 *str*-s, and *nov*-s *str*-r) strains by transformation and selection for MC-sensitive transformants.

Media, preparation of donor DNA and of [^{3}H]-thymine-labeled DNA, competence, transformation assays, and determination of sensitivity to MC and UV irradiation. All were essentially as described previously (17). Diluted medium denotes medium diluted 10-fold with buffered saline.

Preparation of ^{32}P-labeled DNA. ^{32}P-labeled DNA was prepared from bacteria grown in the chemically defined medium M, described by Stuy (29). The phosphate was replaced by 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.0), and 80 μg of carrier-free radioactive phosphate per ml was added. After the culture had reached the stationary phase, the cells were collected by centrifugation and the DNA was isolated as described previously (17). The specific activity of the ^{32}P-labeled DNA was about 10^6 counts per min per μg of DNA.

Preparation of heavy (^{15}N, ^{2}H) ^{32}P-labeled DNA. Heavy ^{32}P-labeled DNA was prepared as described by Goodgal and Postel (12), except that 0.2 ml of deuterated algal whole hydrolysate (Merck, Sharp and Dohme, Canada) was added per 10 ml of medium. The medium was inoculated with cells that had been adapted to growth in 99.9% D_2O by stepwise transfer of cultures grown in brain heart infusion medium prepared with 50 to 99.9% D_2O.

Counting of radioactivity. Samples (0.3 ml) of ^{3}H- and ^{32}P-containing solutions were added to 5 ml of toluene-Triton X-100 (100:30, vol/vol) scintillation fluid containing 0.5% (wt/vol) 2,5-diphenyloxazole and 0.005% (wt/vol) 1,4-bis-[2]-(5-phenyloxazolyl)-benzene in 20-ml scintillation vials. The radioactivity was counted in a Nuclear Chicago liquid scintillation counter for 10 min.

MMS sensitivity. Logarithmically growing cells (about 5×10^8/ml) were collected by centrifugation and resuspended in the original volume of 0.05 M Tris-maleate buffer, pH 6.9 (37 C). MMS (methylmethane sulfonate) was added to a final concentration of 7 mM, and the cells were further incubated at 37 C. At various times thereafter samples were removed, appropriately diluted, and plated in 10 ml of Levinthal agar.

X irradiation. Samples (0.55 ml) of logarithmically growing cultures in Levinthal broth (about 10^8 cells/ml) were irradiated in a 5-ml glass beaker and agitated at 50 Hz. The samples were irradiated with a Machletti OEG-60 tube fitted with a 1-mm beryllium window. The tube was operated at 50 kV and 30 mA. The dose rate was about 600 rad/s as measured by a Philips dosimeter.

Determination of spontaneous and UV-induced HP1c1 phage production in lysogenic cells. Cultures of wild type and strain TD24 were infected with phage HP1c1 and after appropriate dilution plated for infective centers. After incubation overnight at 37 C, lysogenic cells were picked from the center of phage plaques and cultured. Cultures of lysogenic cells (2 ml) were washed twice, and the cells were resuspended in 2 ml of brain heart infusion medium diluted 10-fold with buffered saline. Then 1 ml of the suspension was irradiated for 10 s with UV light (254 nm; dose rate 40 erg/mm^2·s) and plated for infective centers.

Spontaneous and UV-induced endogenous DNA degradation. Ten milliliters of logarithmically growing wild-type and strain TD24 cultures (approximately 5×10^8 cells/ml) in Levinthal broth supplemented with 3 μg of tritiated thymidine per ml (methyl-^{3}H, specific activity 5,000 mCi/mmol; Radiochemical Centre, Amersham) and 300 μg of deoxyadenosine per ml were washed four times with a medium consisting of 1 volume of Levinthal broth and 10 volumes of buffered saline, supplemented with 100 μg of non-radioactive thymidine per ml. Cells were resuspended in 50 ml of the same medium and the cultures were divided into two parts. One part was irradiated for 10 s with UV light (254 nm; dose rate 40 erg/mm^2·s) in a 10-cm petri dish. The cultures were incubated at 37 C with gentle shaking. After various intervals, 1-ml samples were removed, added to 1 ml of cold (0 C) 10% (wt/vol) trichloroacetic acid, and placed in an ice bath for at least 1 h. To determine the amount of solubilized radioactivity, the acid samples were centrifuged for 15 min at 12,000 × *g* and the radioactivity of the supernatants was counted.

ATP-dependent exonuclease assay. Cultures (2 liters) of wild-type and TD24 cells were grown in Levinthal broth to an absorbance of approximately 1.2 at 600 nm (1-cm light path) and centrifuged for 10 min at 10,000 × *g*. The cells were washed by centrifugation with 20 ml of 0.05 M Tris-hydrochloride buffer (pH 8.3) containing 2 mM mercaptoethanol, resuspended in 5 ml of the buffer, and sonicated for 5 min at 0 C in an MSE sonic oscillator.

The suspension was centrifuged for 10 min at 10,000 × *g*. The supernatant was preincubated for 2 h at 37 C and centrifuged for 10 min at 10,000 × *g*. After storage overnight (0 C) the extracts of wild type and TD24, containing 36 and 39 mg of protein per ml, respectively, were assayed for ATP-dependent exonuclease activity. Enzyme activities of 1, 3, 5, and 10 μliters of extracts diluted to 0.1 ml with 0.05 M Tris-hydrochloride buffer (pH 8.3) were measured in a solution consisting, in addition, of 0.2 ml of 0.2 M Tris-hydrochloride buffer (pH 8.3) containing 20 mM $MgSO_4$, 2 mM ATP, 2 mM mercaptoethanol, and 0.1 ml of ^{32}P-labeled native DNA. As controls, extracts were also incubated in the absence of ATP. The reaction was started by the addition of extract and allowed to continue for 1 h at 37 C. The reaction was stopped by addition of 0.2 ml of unlabeled salmon sperm carrier DNA (1.25 mg/ml) and 0.2 ml of 6% (vol/vol) per-

chloric acid. The mixtures were left for 15 min at 4 C. After centrifugation for 20 min (4,500 × *g*), the radioactivity in the supernatants was determined. These radioactivities were corrected for the radioactivity in the supernatant obtained from an incubation mixture without extract. The radioactivity in the supernatant of an incubation mixture untreated with perchloric acid was taken to be 100%.

Assay of uptake of donor DNA. A 20-ml amount of competent cultures of strains Rd *str*-r and TD24 *str*-r was incubated with 5×10^{-3} μg of [^{3}H]thymine-labeled *nov*-r2.5 donor DNA per ml for 10 min at 37 C (specific activity of the DNA, 3.5×10^5 counts per min per μg of DNA). Two minutes after the addition of deoxyribonuclease (DNase) (25 μg/ml), the cells were collected by centrifugation, washed four times with buffered saline, resuspended in 1 ml of SSC (0.15 M NaCl + 0.015 M sodium citrate), and lysed with 0.1% (wt/vol) sodium lauryl sulfate (SLS) at 45 C. The DNA was re-extracted and the radioactivity was measured as described.

Donor marker and recombinant-type activity at 37 C. A 300-ml amount of competent cultures of the strains Rd *str*-r and TD24 *str*-r was chilled to 17 C. The cultures were incubated in the presence of *nov*-r2.5 donor DNA (final concentration, 0.5 μg/ml) for 30 min and DNase was added (final concentration, 25 μg/ml); 5 min later the cultures were transferred to 37 C. After various intervals the DNA was re-extracted from 50-ml samples as described (19), and the resident marker (*str*-r), donor marker (*nov*-r2.5), and recombinant-type (*str*-r *nov*-r2.5) activities were assayed on a second recipient, sensitive to both novobiocin and streptomycin.

Fate of donor DNA at 17 C. An 88-ml amount of competent cultures of the wild-type (*str*-r) and the TD24 (*str*-r) strain was chilled to 17 C and incubated in the presence of *nov*-r2.5 [^{3}H]thymine-labeled donor DNA (final concentration, 7.5×10^{-3} μg/ml) for 20 min. Five minutes after the addition of DNase (final concentration, 25 μg/ml), the cells were washed four times with cold (4 C) diluted medium M and resuspended in 10 ml of the same medium. Two 2-ml portions of the cell suspension were incubated in the presence of non-radioactive thymidine (final concentration, 20 μg/ml [chasing conditions]) and two were incubated without thymidine at 17 C for 60 min. Then the cells were collected by centrifugation (4 C), the supernatant was membrane filtered (Millipore HAWG 02500), and the cells were resuspended in 2 ml of SSC and lysed with 0.1% (wt/vol) SLS. The radioactivity of the filtered supernatants and lysates was determined. The lysates were additionally assayed for donor marker (*nov*-r2.5) and resident marker (*str*-r) activity.

Analysis of association of donor DNA with resident DNA. A 320-ml amount of competent cultures of strains Rd *str*-r and TD24 *str*-r was incubated at 37 C in the presence of *nov*-r2.5 [^{3}H]thymine-labeled donor DNA (final concentration, 0.01 μg/ml) for 5 min. Two minutes after the addition of DNase (final concentration, 25 μg/ml), the cells were washed with four changes of 25 ml of cold (4 C) diluted medium and resuspended in 10 ml of the same medium. The suspension was divided into portions of 2.5 ml each, which were incubated in the absence or presence of non-radioactive thymidine (20 μg/ml) at 37 C for 0 or 60 min. After incubation the cells were collected by centrifugation (4 C), the supernatants were membrane filtered (Millipore HAWG 02500), the cells were resuspended in 1.25 ml of SSC and lysed with 0.1% (wt/vol) SLS, and the DNA was re-extracted as described (19). The radioactivity of the filtered supernatants, the lysates, and the DNA was counted and the donor marker, resident marker, and recombinant-type activities of the DNA were assayed on a second recipient. Samples (0.1 ml) of the re-extracted DNAs were layered onto a 4.5-ml sucrose gradient (5 to 20% in SSC) and centrifuged at 35,000 rpm in a Spinco SW50 rotor for 120 min at 20 C. Twenty fractions of equal volume were collected in scintillation vials. For determination of the donor marker, resident marker, and recombinant-type activities, 0.05 ml of the fractions was removed; then the fractions were diluted with SSC to 0.3 ml and the radioactivity was counted.

To examine the presence or absence of covalent bonds between the donor and recipient moieties of DNA in the complex, competent cultures (132 ml) of wild type and strain TD24 were incubated in the presence of [^{3}H]thymine-labeled donor DNA (final concentration, 0.01 μg/ml) for 5 min at 37 C. At 0 and 30 min after the addition of DNase (final concentration, 25 μg/ml), the DNA was re-extracted from 60-ml samples as described (19). DNA (0.6 ml) was layered onto a 16-ml sucrose gradient (5 to 20% in SSC) and centrifuged at 23,000 rpm in a Spinco SW25.3 rotor for 8 h at 20 C. Thirty fractions of equal volume were collected and the radioactivity was counted. The fractions of unintegrated donor DNA (0-min reisolate) or of resident DNA containing donor atoms (30-min reisolate) were pooled, dialyzed against SSC, and then concentrated by dialysis against 20% (wt/vol) polyethyleneglycol 6000 (Merck, Darmstadt). A 0.2-ml amount of the concentrated samples of DNA was layered onto an alkaline sucrose gradient (5 to 20% in SSC; pH adjusted with 1 N NaOH to 12), and centrifuged at 35,000 rpm in a Spinco SW50 rotor for 2.5 h at 20 C. Twenty-four fractions of equal volume were collected and the radioactivity was counted.

To examine the nature of the association of donor atoms with resident DNA by isopycnic centrifugation, 240 ml of wild-type and TD24 cells grown to competence in the presence of [^{3}H]thymidine (specific activity, 5,000 mCi/mmol) was incubated with heavy (^{15}N, ^{2}H) ^{32}P-labeled donor DNA (final concentration, approximately 5×10^{-3} μg/ml) for 10 min at 37 C. After addition of DNase (final concentration, 25 μg/ml), the cells were incubated for 60 min at 37 C and then washed three times with SSC (4 C); the DNA was re-extracted and finally dissolved in 2 ml of SSC. The DNA solution was mixed with 20 ml of a cesium chloride (Suprapur; Merck, Darmstadt) solution (final refractive index [n^{25}] of 1.3993, determined by refractometry), and sheared with a Virtis 45 microhomogenizer to approximately 6×10^6 daltons. A 10.2-ml amount of the sheared DNA in cesium chloride solution was centrifuged at 29,000 rpm for 72

h at 20 C in a type 40 fixed-angle rotor of a Spinco L2 ultracentrifuge. The gradients were fractionated from the top in 0.3-ml fractions and the radioactivity was counted.

Analysis of the fate of the donor-recipient complex of DNA in a second recipient. To determine whether the second recipient is able to take up the donor-recipient complex of DNA isolated from the mutant, 5 μg of ^{32}P-labeled donor DNA per ml was added to 240 ml of competent [^{3}H]thymine-labeled wild-type or TD24 cells, and after 10 min of incubation at 37 C uptake was stopped with DNase (final concentration, 25 μg/ml). After the addition of DNase, the cells were incubated for 60 min at 37 C; then the cells were washed three times with SSC at 4 C and the DNA was isolated as described (17). The re-extracted DNA was added to 240 ml of a wild-type second recipient. DNase was added (final concentration, 25 μg/ml) after 10 min of incubation at 37 C. The cells were further incubated at 37 C for 60 min and washed three times with SSC (4 C), and the DNA was isolated as described (17). The DNA was finally dissolved in 0.5 ml of SSC and the uptake was determined by counting the radioactivity.

To determine whether the donor-recipient complex of DNA isolated from the transformation-deficient first recipient associates with the DNA of the transformation-proficient second recipient, this complex was separated from unintegrated DNA by layering onto a neutral sucrose gradient and centrifuging in a Spinco SW25.3 rotor as described; the fractions containing the donor-recipient complex of DNA were pooled and added to 240 ml of the wild-type second recipient. After incubation of the cells in the same way as before, DNA was re-extracted and dissolved in 0.5 ml of SSC, and 0.2-ml samples were analyzed by neutral sucrose gradient centrifugation in a Spinco SW50 rotor as described.

RESULTS

Characterization of the TD24 mutant. (i) Transformability. *nov*-r2.5 *ery*-r *str*-r donor DNA produced transformants in the mutant with a frequency approximately 10^4-fold less than in the transformation-proficient (wild-type) strain (Table 1). In addition, the frequency of residual transformants varied widely among the various markers examined.

TABLE 1. *Transformability of wild type and strain TD24 with nov-r2.5 ery-r str-r donor DNA*

Strain	Donor marker	Transformation frequency
Rd (*nov*-s *ery*-s *str*-s)	*nov*-r2.5	3.7×10^{-3}
	ery-r	3.2×10^{-3}
	str-r	2.9×10^{-3}
TD24 (*nov*-s *ery*-s *str*-s)	*nov*-r2.5	9.5×10^{-7}
	ery-r	2.5×10^{-7}
	str-r	4.7×10^{-7}

TABLE 2. *Sensitivity of wild type and strain TD24 to mitomycin C*

Strain	Lethal concn of MC (μg/ml)[a]
Rd *str*-r	0.02
TD24 *str*-r	0.01

[a] Defined as the concentration preventing the formation of visible colonies.

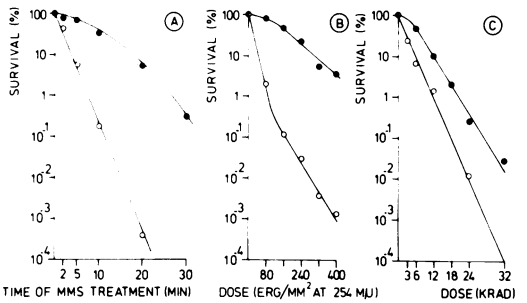

FIG. 1. *Cell survival after MMS (7 mM) treatment (A), UV irradiation (B), and X irradiation (C) of wild-type strain (●) and strain TD24 (○).*

(ii) Sensitivity to deleterious agents. All agents tested are more deleterious to the mutant than to the wild type (Table 2 and Fig. 1).

(iii) Phenotypic similarity to E. coli recA mutants. Apart from yielding very low frequencies of recombination and being sensitive to UV, *E. coli recA* mutants degrade their DNA continuously to a certain extent, and very severely after UV irradiation. In addition, *recA* mutants lysogenic for bacteriophage λ yield low numbers of bacteriophage, both spontaneously and after induction with UV. The TD24 mutant degraded the DNA continuously to a considerable extent, which became very extensive after irradiation with UV (Fig. 2). Upon irradiation with UV, the wild type also showed extensive degradation of its DNA; however, this stopped after a phase of rapid increase.

Lysogenic TD24 cells produced reduced numbers of free phage HP1c1 and were insignificantly inducible upon UV irradiation as compared with the wild type (Table 3). In conjunction with the other properties displayed by the TD24 mutant, these two additional properties suggest that the TD24 mutant is of a type comparable to the *recA* class of *rec* mutants of *E. coli*. The ATP-dependent exonuclease activity in the TD24 mutant was equal to that in the wild type (Fig. 3).

Pleiotropism and location of the TD24 mutation. One hundred of *ery*-r transformants obtained by transformation of the TD24 strain with *ery*-r donor DNA were tested for MC,

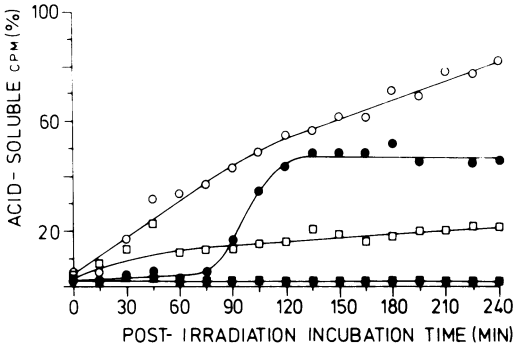

FIG. 2. *DNA degradation in nonirradiated and UV-irradiated wild-type and TD24 cells. Symbols:* ■, *Wild type, unirradiated;* ●, *wild type, UV irradiated (400 erg/mm²);* □, *TD24, unirradiated;* ○, *TD24, UV irradiated (400 erg/mm²).*

TABLE 3. *Spontaneous and UV-induced phage HP1c1 production in lysogenic wild-type and TD24 cells*

Lysogenic strain	Phage/ml	
	Unirradiated	UV-irradiated
Rd	8.6×10^6	1.4×10^8
TD24	5.9×10^4	2.5×10^5

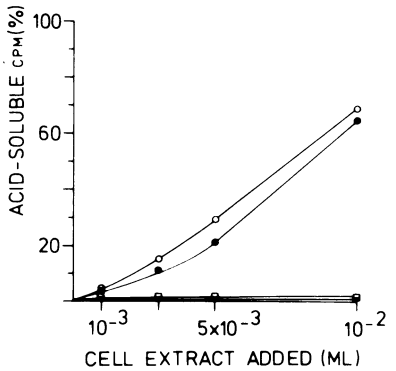

FIG. 3. *Degradation of DNA by ATP-dependent exonuclease in extracts of wild-type and TD24 cells. Symbols:* ●, *Wild type, + ATP;* ■, *wild type, − ATP;* ○, *TD24, + ATP;* □, *TD24, − ATP.*

MMS, and UV sensitivity and for transformability with *nov*-r2.5 donor DNA. Only one of the *ery*-r transformants yielded the wild-type level of transformation and was found to be resistant to MC, MMS, and UV. The other *ery*-r transformants were transformation deficient and continued to be sensitive to MC, MMS, and UV. These results suggest that the same mutation causes both sensitivity to UV, MC, and MMS, and poor transformability.

In transformation of an Rd (*nov*-s *str*-s *ery*-s) population with DNA of a *nov*-r2.5 *str*-r *ery*-r TD24 strain, sensitivity to MMS showed 30% cotransformation with the *str*-r marker (Table 4). Since the TD24 mutation confers sensitivity to MMS, the TD24 mutation is linked to the *str*-r marker. Since the linkage between the TD24 mutation and the *nov*r2.5 marker is very weak (about 4%) and *nov*-r2.5 and *str*-r are linked, the position of the TD24 mutation with respect to *str*-r and *nov*-r2.5 must be TD24 *str*-r *nov*-r2.5.

In the reciprocal transformation (Table 4), the linkage between the TD24 mutation and *str*-r marker (27%) corresponded with that determined in the previous cross. Again a very weak linkage was present between the *nov*-r2.5 and TD24 mutation. In view of the fact that the TD24 recipient yields varying transformation frequencies for different markers (Table 4), resulting in apparently decreased linkage between the *nov*-r2.5 and *str*-r loci, more reliable linkage data are obtained by using the wild-type recipient.

Uptake of donor DNA in the TD24 recipients. The amount of radioactive DNA taken up by the TD24 strain was of the same order of magnitude as in the wild type (Table 5). Nevertheless, in the TD24 strain the number of transformants relative to the amount of DNA taken up was reduced by more than 10^4-fold compared with the wild type.

Fate of donor DNA and absence of recombinant-type activity in TD24 recipients. To analyze the cause of the TD24 strain's extremely low frequency of transformation, we followed both the donor marker activity and formation of recombinant-type activity as a function of time of incubation after uptake, and studied the physicochemical fate of the donor DNA after uptake. The level of donor marker activity in the re-extracted DNA from the mutant was initially not very much different from that of the wild type (Fig. 4). However, contrary to the wild type, in which after the initial decrease the donor marker activity was not further reduced, the donor marker activity in the mutant continued to decrease steadily.

As a function of time of incubation, the recombinant-type activity increased in the wild type, in contrast to the mutant, in which the low recombinant-type activity initially present decreased even further. The low level of recombinant-type activity found in transformation of DNA re-extracted from the mutant did not represent the activity of molecules jointly carrying a donor and a resident marker. It was caused by independent uptake of separate re-extracted donor molecules and resident DNA molecules because this level of apparent recombinant-type

TABLE 4. *Transformation frequency and cotransformation of donor markers in transformation of wild type and strain TD24 with donor DNA from strains Rd nov-r2.5 ery-r str-r and TD24 nov-r2.5 ery-r str-r*

Recipient (*nov*-s *ery*-s *str*-s)	Donor DNA	No. of transformants/total number of viable centers	Ratio of no. of transformants	Cotransformation (%)
Rd	Rd (*nov*-r2.5 *ery*-r *str*-r)	*nov*-r2.5: 0.98×10^{-2} *str*-r: 0.94×10^{-2} *ery*-r: 0.95×10^{-2} *nov*-r2.5 *str*-r: 0.51×10^{-2}	*nov*-r2.5/*ery*-r: 1.03 *str*-r/*nov*-r2.5: 0.96 *str*-r/*ery*-r: 0.99 *nov*-r2.5 *str*-r/*str*-r: 0.52	*nov*-r2.5 and *str*-r (relative to *str*-r): 52
Rd	TD24 (*nov*-r2.5 *ery*-r *str*-r)	*nov*-r2.5: 1.15×10^{-2} *str*-r: 1.08×10^{-2} *ery*-r: 1.07×10^{-2} *nov*-r2.5 *str*-r: 0.66×10^{-2} *nov*-r2.5 (*mms*-r)[a]: 1.10×10^{-2} *str*-r (*mms*-r)[a]: 0.76×10^{-2} *ery*-r (*mms*-r)[a]: 1.07×10^{-2}	*nov*-r2.5/*ery*-r: 1.07 *str*-r/*nov*-r2.5: 0.94 *str*-r/*ery*-r: 1.00 *nov*-r2.5 *str*-r/*str*-r: 0.61 *nov*-r2.5 (*mms*-r)/*nov*-r2.5: 0.96 *str*-r (*mms*-r)/*str*-r: 0.70 *ery*-r (*mms*-r)/*ery*-r: 1.00	*nov*-r2.5 and *str*-r (relative to *str*-r): 61 *mms*-s and *nov*-r2.5 (relative to *nov*-r2.5): 4 *mms*-s and *str*-r (relative to *str*-r): 30 *mms*-s and *ery*-r (relative to *ery*-r): 0
TD24	Rd (*nov*-r2.5 *ery*-r *str*-r)	*nov*-r2.5: 2.40×10^{-6} *str*-r: 1.46×10^{-6} *ery*-r: 0.73×10^{-6} *mms*-r: 1.17×10^{-6} *nov*-r2.5 *str*-r: 0.57×10^{-6} *mms*-r *nov*-r2.5: 0.03×10^{-6} *mms*-r *str*-r: 0.39×10^{-6} *mms*-r *ery*-r: 0	*nov*-r2.5/*ery*-r: 3.31 *str*-r/*nov*-r2.5: 0.61 *str*-r/*ery*-r: 2.02 *mms*-r/*str*-r: 0.80 *nov*-r2.5 *str*-r/*str*-r: 0.39 *mms*-r *nov*-r2.5/*nov*-r2.5: 0.01 *mms*-r *str*-r/*str*-r: 0.27 *mms*-r *ery*-r/*ery*-r: 0	*nov*-r2.5 and *str*-r (relative to *str*-r): 39 *mms*-r and *nov*-r2.5 (relative to *nov*-r2.5): 1 *mms*-r and *str*-r (relative to *str*-r): 27 *mms*-r and *ery*-r (relative to *ery*-r): 0

[a] *nov*-r2.5, *str*-r, and *ery*-r transformants, respectively, carrying the Rd MMS-resistance allele.

TABLE 5. *Transformation efficiency (ratio of number of transformants and amount of DNA incorporated) of strains Rd str-r and TD24 str-r*

Strain	Counts per min per ml of recipient culture	Transformants (*nov*-r2.5)/ml of recipient culture	Transformants per counts per min	Transformants per counts per min relative to Rd *str*-r
Rd *str*-r	120	2.3×10^5	1.9×10^3	1
TD24 *str*-r	210	13	6.2×10^{-2}	3.24×10^{-5}

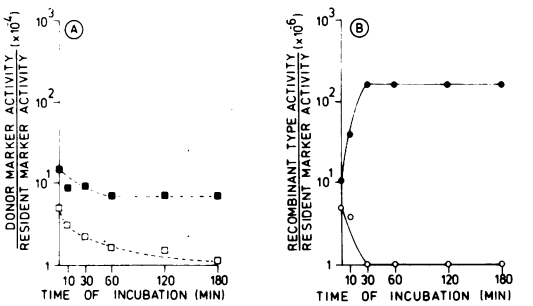

FIG. 4. *Donor marker and recombinant-type activity as a function of time of incubation at 37 C in wild-type (Rd str-r) and TD24 (str-r) cells. The donor DNA was isolated from an Rd nov-r2.5 strain. (A) ■, Donor marker activity, wild type; □, donor marker activity, TD24. (B) ●, Recombinant-type activity, wild type; ○, recombinant-type activity, TD24.*

activity is also present in DNA re-extracted from recipient cells incubated at 17 C, when recombination does not occur (18, 31).

There are two possible explanations for the continuing decrease of donor marker activity in the mutant and its failure to produce recombinant-type activity: irreversibly bound donor DNA is (i) degraded presynaptically or (ii) associates with the recipient chromosome, but the complex is biologically inactive. To test whether the donor DNA in the TD24 strain is broken down presynaptically we made use of the fact that *H. influenzae* is not able to integrate transforming DNA at 17 C (18, 31). After uptake of tritiated donor DNA, recipient cells of the mutant and the wild type were incubated at 17 C either in the presence or absence of non-radioactive thymidine (20 μg/ml), and, in addition to the donor marker

activity, the amount of breakdown products of donor DNA excreted into the medium was measured. Non-radioactive thymidine was added to chase exonucleolytic breakdown products from the cells and to prevent reutilization for replicative synthesis of recipient DNA (18). Under these conditions both the loss of donor marker activity and excretion of breakdown products were almost equal in the wild type and the mutant, indicating that presynaptically the fate of donor DNA in the mutant is equal to that in the wild type (Table 6). To determine whether the donor DNA in the TD24 strain associates with the resident DNA at 37 C, re-extracted DNA obtained at intervals after uptake of tritium-labeled donor DNA from cells incubated either in the presence or absence of non-radioactive thymidine was analyzed by sucrose gradient centrifugation.

Fig. 5 shows the results obtained with DNA re-extracted from cells in the presence of non-radioactive thymidine. Initially (0-min reisolate) the vast majority of the irreversibly absorbed donor DNA extracted from both the wild-type and the mutant strain (measured both biologically and radioactively) sedimented slower than the resident DNA (assayed biologically); only little radioactivity cosedimented with the faster-sedimenting resident DNA. In the 60-min re-extracted sample of DNA from the wild type, a substantial amount of both donor marker activity and donor radioactivity cosedimented with the resident DNA, and recombinant-type molecules have been produced, also cosedimenting with the resident DNA. In the 60-min reisolate obtained from the mutant, neither donor nor recombinant-type activity was present in the resident DNA moiety, although transition of slow to fast sedimenting radioactivity similar to that in the wild type had occurred. In conjunction with the observation that presynaptically the fate of donor DNA is the same in the wild type and the mutant, this indicates that the mutant is capable of

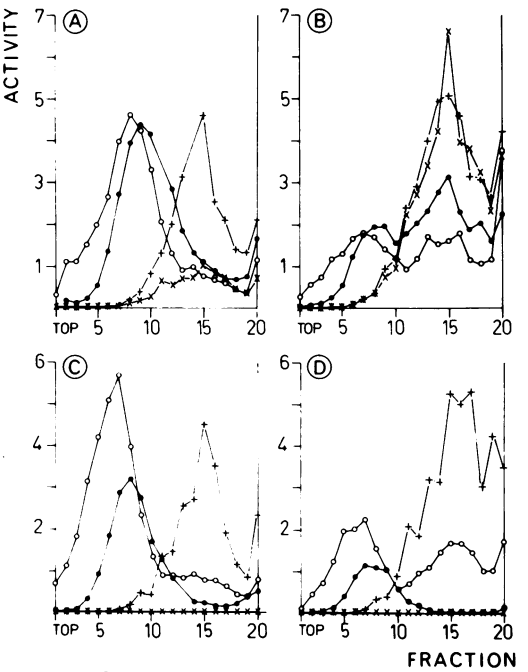

FIG. 5. *Sucrose gradient sedimentation patterns of [³H]thymine-labeled DNA re-extracted from wild-type (Rd str-r) and TD24 (str-r) cells. The donor DNA was isolated from an Rd nov-r2.5 strain. (A) Wild type, 0-min reisolate; (B) wild type, 60-min reisolate; (C) TD24, 0-min reisolate; (D) TD24, 60-min reisolate. Symbols: +, resident marker transformation (all graphs, 1.0 = 10⁶ str-r transformants/ml); ●, donor marker transformation (all graphs, 1.0 = 10³ nov-r2.5 transformants/ml); ×, recombinant-type transformation (all graphs, 1.0 = 2.10² nov-r2.5 str-r transformants/ml); O, radioactivity (³H) (all graphs, 1.0 = 10² counts/min).*

TABLE 6. *Transforming activity and excretion of breakdown products of unintegrated DNA in the absence and presence of an excess of non-radioactive thymidine*

Strain	Time (min) of reisolation	Thymidine (20 μg/ml) added (+) or not added (−)	Ratio of donor marker activity to resident marker activity (*nov*-r2.5/*str*-r)	Ratio of *nov*-r2.5/*str*-r to that in 0-min reisolate	Transformation frequency	Percentage of radioactivity excreted into the medium
Rd *str*-r	0	+			0.52×10^{-3}	0
	0	−	0.77×10^{-3}	1		0
	60	+	0.53×10^{-3}	0.69		16
	60	−	0.52×10^{-3}	0.68		14
TD24 *str*-r	0	+			0.43×10^{-7}	0
	0	−	1.24×10^{-3}	1		0
	60	+	0.78×10^{-3}	0.63		16
	60	−	0.82×10^{-3}	0.66		11

associating donor DNA, or donor atoms, with resident DNA, and that as a consequence of the association donor DNA loses its biological activity. The results obtained with re-extracted DNA from cells incubated in the absence of non-radioactive thymidine are essentially the same as demonstrated for re-extracted DNA obtained from cells incubated in the presence of non-radioactive thymidine.

No effect of non-radioactive thymidine during incubation of transforming wild-type cells on the fraction of radioactivity cosedimenting with the resident DNA was observed (Table 7), suggesting that at least the wild type does not reutilize breakdown products under the experimental conditions used and that in the wild type, transition from slow to fast sedimenting material is the result of complex formation between the two species of DNA. Since the amount of breakdown products generated from donor DNA excreted into the medium and the fraction of radioactivity cosedimenting with the resident DNA were the same in the wild type and the mutant (Table 7), we conclude that the level of incorporation of donor atoms is determined by a step in the processing of donor DNA in which the mutant does not differ from the wild type.

It may be noticed (Fig. 5) that the ratio of transforming activity to radioactivity in the uncomplexed moiety of re-extracted donor DNA in the 0-min reisolate obtained from the wild type was higher than that of the mutant strain. Also, the presynaptic DNA from the wild type sedimented slightly faster than that of the mutant. These differences suggest that the molecular weight of the re-extracted DNA obtained from the mutant is slightly less than that isolated from the wild type. It is doubtful, however, that this difference originates from the nature of the mutant. Although the re-extraction experiments were performed with the same preparation of ^{3}H-labeled DNA, an interval of 21 days elapsed between the experiments carried out with the wild type and the mutant strain. Since ^{3}H disintegrations produce single- and double-strand breaks (26), it is likely that these must be held responsible for the differences in presynaptic DNA observed.

Properties of the donor-recipient complex of DNA. One of the possibilities that may explain the lack of donor marker and recombinant-type activity in the mutant's donor-recipient complex of DNA is the presence of discontinuities in the physical structure between its donor and recipient moieties. To test this, wild-type and mutant complexes collected from sucrose gradients of re-extracted DNA obtained 30 min after stopping uptake were analyzed on alkaline sucrose gradients. The results (Fig. 6) show that the distributions of the wild-type and mutant complexes over the alkaline gradients are practically the same and that the position of the donor atoms in the TD24 complex is very different from that of the presynaptic DNA re-extracted from the mutant. If the donor moiety of the complex was bound to the resident DNA by hydrogen bonds only, the radioactivity in the alkaline gradient should have shifted to either the position of presynaptic DNA in such gradients or beyond that position if shorter segments of DNA than those present in the presynaptic species were incorporated into the complex. The results indicate that the donor contribution to the TD24 complex is covalently linked to the resident DNA. (The insets of Fig. 6

TABLE 7. *Excretion of breakdown products of donor DNA, donor marker activity, and association of donor atoms with resident DNA at 37 C after 60 min of incubation in strains Rd str-r and TD24 str-r*

Strain	Time (min) of reisolation	Thymidine (20 μg/ml) added (+) or not added (−)	Percentage of radioactivity excreted into the medium	Ratio of donor marker activity to resident marker activity (*nov*-r2.5/*str*-r) relative to 0-min reisolate	Radioactivity[a]	
					Percentage in uncomplexed DNA	Percentage in donor-recipient complex of DNA
Rd *str*-r	0	+	2	1	86	14
	0	−	2	1	88	12
	60	+	44	0.64	47	53
	60	−	28	0.63	48	52
TD24 *str*-r	0	+	1	1	82	18
	0	−	1	1	83	17
	60	+	42	0.15	48	52
	60	−	22	0.16	56	44

[a] Percentage of radioactivity in uncomplexed and donor-recipient complex of DNA was calculated from the profiles presented in Fig. 5.

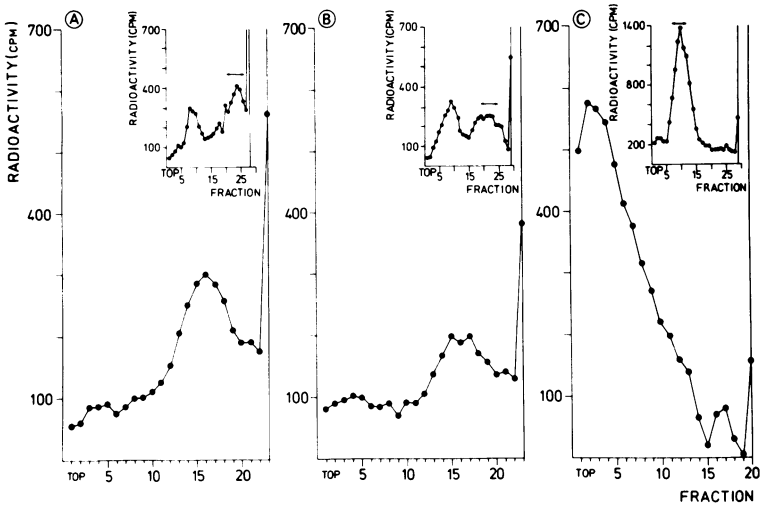

FIG. 6. *Alkaline sucrose gradient sedimentation patterns of the donor-recipient complex of DNA from the wild-type and TD24 strain. [³H]thymine-labeled DNA was re-extracted from the wild-type and TD24 strain 30 min after addition of DNase and first sedimented through neutral sucrose gradients in the Spinco SW25 rotor (insets). The fractions indicated by bars were pooled and recentrifuged on alkaline sucrose gradients in the Spinco SW50 rotor. (A) Wild-type donor-recipient complex of DNA; (B) TD24 donor-recipient complex of DNA; (C) presynaptic TD24 donor DNA. Inset: Neutral sucrose gradient centrifugation (Spinco SW25.3 rotor) of DNA re-extracted 0 min after addition of DNase.*

may suggest that the position of the donor-recipient DNA complex in Fig. 6B is, on the average, somewhat different from that of complex in Fig. 6A. This difference may be due to a slight inequality of the sucrose gradients used. The purpose of these sucrose gradient runs was to separate the donor-recipient complexes of DNA from unintegrated donor DNA. On the average, the size of the donor-recipient DNA complexes isolated from the mutant was the same as that of recombinant DNA molecules from the wild type. This follows from the observation that their position is the same in the sedimentation patterns depictured in Fig. 5 and 6.)

As a possible cause of the absence of donor marker activity of the mutant donor-recipient complex of DNA, we envisaged the possibility that the complex is not incorporated into the second recipients, on which the presence or absence of biological activity is ultimately determined. To examine this we re-extracted ^{32}P-labeled donor DNA from ^{3}H-labeled recipients, added the re-extracted DNA to the second recipient, and re-extracted this DNA again. If the second recipient failed to incorporate the mutant complex, we would expect to observe a drastic decrease in the ^{32}P-to-^{3}H ratio in the DNA re-extracted from the second recipient as compared with the ratio in DNA obtained from the first recipient. The results (Table 8) show that the ratio of ^{32}P-to-^{3}H in DNA re-extracted from the second recipient is not much affected

TABLE 8. *Ratio of ^{32}P (donor) to ^{3}H (resident) in DNA re-extracted from a first and second recipient*

Experiment	First recipient	Ratio of ^{32}P dpm to ^{3}H dpm in DNA re-extracted from the first recipient	Ratio of ^{32}P dpm to ^{3}H dpm in DNA re-extracted from the second (wild-type) recipient	Ratio of ^{32}P dpm to ^{3}H dpm in DNA from second recipient relative to that in DNA from first recipient
1	Wild type	0.079	0.084	1.06
	TD24	0.082	0.096	1.20
2	Wild type	0.124	0.091	0.73
	TD24	0.114	0.090	0.79
3	Wild type	0.154	0.130	0.84
	TD24	0.116	0.091	0.78

by passage of the DNA through the second recipient. If the small decrease is real, it applies both to the wild type and mutant complex. These results suggest that the fate of the biological inactive complex re-extracted from the mutant in the second recipient is the same as that of the wild-type complex. In support of this are the results of sucrose gradient analysis of the donor-recipient complex of DNA from the mutant after its being passed through the second recipient. The position of the ^{32}P donor label coincided with that of the ^{3}H resident label (Fig. 7), indicating that the mutant complex can associate with the genome of the second

recipient. The lack of donor marker activity in the complex is therefore difficult to reconcile with the second recipient's presumed incapacity to incorporate and integrate the mutant complex of DNA.

Next we considered the possibility that the donor marker activity in the mutant is lost by disperse incorporation of donor atoms into the recipient genome. This possibility was investigated by re-extraction of heavy (^2H, ^{15}N) radioactive (^{32}P) donor DNA from ^3H-labeled mutant and wild-type cells and subsequent analysis of the re-extracted DNA by CsCl density gradient centrifugation. Before centrifugation the re-extracted DNA was sheared to a molecular weight of about 6×10^6. In the wild type, radioactivity banded at a position of substantially higher density than that of the resident DNA (Fig. 8), indicating that fairly long segments of donor DNA are incorporated into the recipient genome. The displacement of the ^{32}P position with respect to that of the ^3H radioactivity observed in the mutant was substantially less than that of the wild type. This suggests that shorter segments of DNA carrying donor atoms reside in mutant resident DNA as compared with wild-type resident DNA. Since the amount of donor atoms transferred to the resident DNA in the wild type is equal to that in the mutant, we conclude that the donor DNA is dispersively distributed over the mutant's genome. It may be noticed that, with respect to the amount of uncomplexed DNA, a large discrepancy exists between the results of the experiments given in Table 7 and Fig. 8. In all probability this difference is caused by different conditions to which the recipient cells have been exposed after uptake of DNA. The experiment summarized in Table 7 involves washing in the cold of the recipient cells and their resuspension in a medium unfavorable to growth. Presumably the resulting inhibition of metabolism interferes with the formation of donor-recipient complexes of DNA.

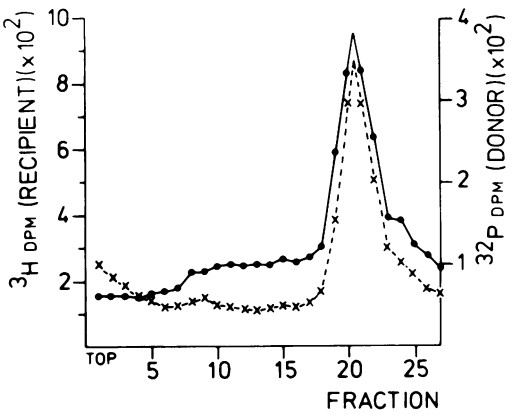

FIG. 7. *Sucrose gradient sedimentation pattern of DNA re-extracted from wild-type cells 60 min after uptake of donor (^{32}P)-recipient (^3H) complex of DNA produced by the TD24 strain. Symbols: ×, ^{32}P (donor); ●, ^3H (recipient).*

DISCUSSION

The ATP-dependent exonuclease-proficient mutant TD24 of *H. influenzae* described here resembles strongly *recA* type mutants of *E. coli* in all relevant aspects: strongly reduced recombining ability (7), sensitivity to UV (9, 15, 16, 21), X rays (15, 16), MC, and MMS (15), spontaneous endogenous degradation of chromosomal DNA and extensive degradation of the

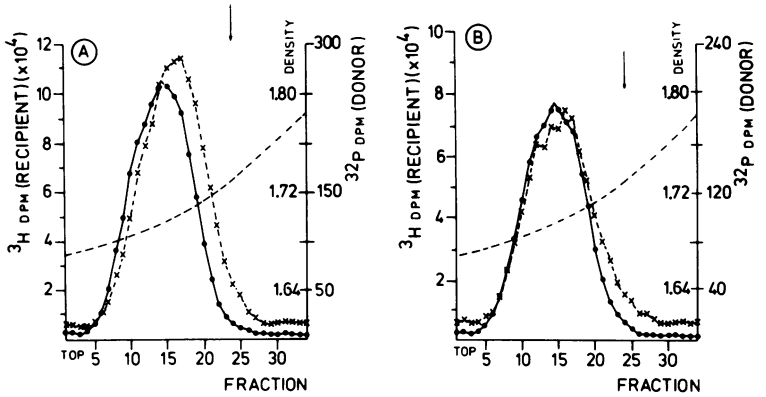

FIG. 8. *CsCl density gradient centrifugation of DNA re-extracted from wild-type and TD24 strains 60 min after uptake of heavy (^{15}N, ^2H) ^{32}P-labeled donor DNA by light ^3H-labeled recipient cells. Before centrifugation the re-extracted DNA was sheared to approximately 6×10^6 daltons. (A) Wild type; (B) TD24; ×, ^{32}P (donor); ●, ^3H (recipient); -----, density. The arrows mark the position of heavy native donor DNA.*

chromosomes after UV irradiation (8, 16, 34), and decreased production of both spontaneous and induced temperate bacteriophage by lysogens (3, 7, 13).

We recently isolated a transformation-deficient *H. influenzae* mutant lacking an ATP-dependent exonuclease and transferred the mutation into the TD24 strain. We observed that in the double mutant the UV-induced chromosomal breakdown was strongly reduced (manuscript in preparation). This lends further support to our conclusion that the TD24 mutant is equivalent to *recA* mutants of *E. coli* (34).

In transformation of the wild-type strain with TD24 *str*-r DNA, the TD24 mutation and *str*-r marker cotransform with a frequency of about 30%. In the reciprocal cross, approximately the same degree of linkage (27%) was determined. Setlow et al. (27) have described a poorly transforming mutant of *H. influenzae*, designated DB117, in which the *rec* mutation is also linked to the *str*-r marker. In transformation of the DB117 *rec*⁻ strain with wild-type DNA, the *rec* mutation and *str*-r marker cotransformed between 48 and 71%; however, in transformation of the *rec*⁺ strain with *rec*⁻ *str*-r DNA, only 4% linkage was observed between the *rec*⁻ mutation and *str*-r marker. Setlow et al. (27) supposed that in the latter transformation the linkage is underestimated because of interference of the *rec*⁻ mutation with its own integration as well as with the integration of markers linked with it. Our results provide no reason to suppose that the TD24 mutation interferes with its own integration, because in transformation of the wild-type strain with TD24 DNA, the donor markers all transform with almost the same efficiency regardless of whether the donor marker is linked with the TD24 mutation or not. We believe that linkage data obtained with the wild type as recipient are more reliable than those acquired with the TD24 mutant, because the efficiency of transformation for different markers varies considerably when this strain is used as recipient.

In view of the ambiguity in establishing the true linkage relationship between *str*-r marker and DB117 *rec*⁻ mutation, the presence of both the TD24 and DB117 *rec*⁻ mutation on the segment of DNA carrying the *str*-r marker is compatible with the possibility that they are both mutations in the same gene. This is supported by the fact that, as far as tested, the DB117 *rec*⁻ phenotype (27) resembles strongly that of the TD24 strain, except that the transformation is considerably lower in strain DB117 than in strain TD24.

A wide variation in the efficiency of transformation of various donor markers has also been observed in the *com*⁻56 strain of *H. influenzae* isolated by Caster et al. (6), which is also impaired in transformation (5). However, the *com*⁻56 mutation differs from the TD24 mutation because the *com*⁻56 strain transforms with about 50 to 10^3 higher frequency, depending on the marker used, than the TD24 strain, and, in contrast to the TD24 strain, is as resistant to X and UV irradiation as the wild type (5).

The results obtained in the experiment to judge the uptake of donor DNA by the mutant strain (Table 5) indicate that the low level of transformation of the mutant is not caused by inadequate incorporation of DNA from the medium. This is in accordance with the finding that the donor marker activity in early re-extracted DNA from the mutant is almost the same as that obtained from the wild type and that, if integration is inhibited (17 C), the re-extracted DNA from the mutant and the wild type show approximately the same level of biological activity. This finding, and the observation that the extent of degradation of presynaptic donor DNA at 17 C is the same in the wild type and the TD24 mutant, strongly suggests that the presynaptic fate of donor DNA in the mutant and the wild type is the same.

The TD24 mutant is able to associate atoms from the donor DNA with high-molecular-weight resident DNA. However, as soon as the donor DNA associates with the resident DNA, donor marker activity is lost. Caster and Goodgal (5) have obtained similar results with the *com*⁻56 mutant of *H. influenzae*. According to Postel and Goodgal's (24) classification, strain TD24 should be classified as donor-association biologically defective (*dab*⁻); the mutant strain DB117, described by Setlow et al. (27), which, similar to strain TD24, is also capable of covalently associating donor atoms to the recipient chromosome (20), apparently also classifies as a *dab*⁻ strain. It is unlikely that the donor-recipient complex of mutant DNA is lacking biological activity because of the second recipient's inability to incorporate it from the medium and to associate the original donor atoms with its chromosome. The pertinent experiments (Table 8; Fig. 6) do not favor this possibility.

At first sight a discrepancy exists between the results of the sucrose gradient centrifugation, indicating that an equal number of donor atoms associates with the wild-type and mutant resident DNA and those obtained by isopycnic centrifugation, showing that per segment of DNA obtained by shear (molecular weight of approximately 6×10^6) many fewer donor atoms are incorporated into the resident seg-

ments from the mutant than in those from the wild type. This discrepancy can only be resolved if we assume that the mutant distributes the donor atoms over a substantially higher number of segments of chromosomal DNA than the wild type. The segments carrying donor atoms could then have either one of two possible configurations: (i) the donor atoms reside in a small but original fragment of donor DNA, or (ii) they are dispersed between the resident atoms. Possibility (i) envisages disperse integration by recombination, and possibility (ii) envisages the local incorporation into the recipient chromosome through localized DNA synthesis. From the positions of donor radioactivity extracted from the wild type and the mutant in the CsCl gradient, it can be calculated that the wild-type segments of sheared DNA carry donor atoms approximately 10-fold in excess of those extracted from the mutant TD24, implying that the donor atoms are distributed over approximately 10 times as many molecules of 6×10^6 daltons (molecular mass of the sheared preparation of DNA) in the mutant as compared with the wild type. It is doubtful whether this could be accounted for by dispersive integration by recombination, because the size of the intracellular presynaptic DNA sets an upper limit to the number of molecules that can obtain donor atoms by integration. We have calculated that the molecular weight of the intracellular presynaptic DNA amounts to approximately 7×10^6 on the average. If the entire length of such molecules were available for dispersive integration by recombination, no more than two donor-atom-carrying molecules of 6×10^6 daltons (molecular mass of the sheared preparation of DNA) could be produced from one presynaptic donor DNA molecule. Since in normal (nondispersive) integration the lowest possible number of donor-atom-carrying molecules of 6×10^6 daltons that can be produced from one presynaptic donor DNA molecule amounts to one, disperse integration by recombination can maximally account for a two-fold excess of donor-atom-carrying molecules in DNA re-extracted from the mutant as compared with that re-extracted from the wild type. Since the difference observed is much higher, it is unlikely that, in the mutant, donor atoms are incorporated into the recipient chromosome by integration through recombination.

Nevertheless the incorporation of donor atoms into the mutant chromosome is localized. This may relate to the mutant's property to continuously degrade, and presumably to resynthesize, its endogenous DNA.

It is conceivable that synapsis of donor DNA initiates its depolymerization and that these breakdown products, together with those generated endogenously from the recipient chromosome, are used for local DNA synthesis. This would explain the observably increased buoyant density of segments of mutant recipient DNA carrying heavy donor atoms.

Since the number of donor atoms incorporated into the resident chromosome is equal in that of the mutant and the wild type, a further implication of this hypothesis is that the reutilization for DNA synthesis of these breakdown products must be highly efficient and is not affectable by addition of excess thymidine (Table 7). The breakdown products that can be chased from the cell by addition of excess thymidine could conceivably be those that are generated from presynaptic DNA.

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