Transfection in *Pneumococcus*: Single-Strand Intermediates in the Formation of Infective Centers

RONALD D. PORTER† AND WALTER R. GUILD*

Biochemistry Department, Duke University, Durham, North Carolina 27710

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Transfection has been found and characterized in pneumococcus. For replicating $\omega 3$ phage DNA extracted from infected cells, transfection was relatively efficient and rose linearly with DNA concentration and quadratically with time, according to $T(T - 3.5) \text{ min}^2$. For mature DNA extracted from phage particles, transfection was hardly detectable below $1 \mu\text{g/ml}$ but increased about as the cube of the DNA concentration up to $100 \mu\text{g/ml}$, and was still rising at concentrations over $200 \mu\text{g/ml}$. The kinetics suggest a dependence on a mixed cubic function of the time of exposure of cells to mature DNA. Cell and phage DNAs competed with each other for transformation and transfection. Transfection was reduced much more strongly than transformation in cells that were deficient in the membrane-bound endonuclease required for conversion of donor duplex DNA to intracellular single strands; these data agree with the kinetic data in implying that independent entry of segments of two strands is necessary for transfection by replicating $\omega 3$ phage DNA and entry of at least three strands is necessary for transfection by mature DNA. To reconcile differing DNA concentration dependences of transfection and transformation with a common entry path, it was necessary to reexamine data on transformation and to recognize that this process continued to rise slowly through the concentration region usually described as "plateau." These results and the transfection data reflect multiple binding and nicking events that occurred on the cell surface before entry. Our conclusion is that transfection in pneumococcus occurs by association inside the cell of segments of single strands of phage DNA that have entered independently, creating gapped structures that need repair synthesis to create infective centers. Physical recombination is therefore automatically a prerequisite to transfection.

Although there is substantial literature on transfection, the infection of transformable bacteria by phage DNA (8), many points of its mechanism have remained unknown, except that competence for uptake of cellular DNA is also required (39). For *Escherichia coli*, there is little evidence on whether or not the entry of DNA in transformation or transfection is similar to that in species that have evolved high levels of natural competence, nor is it clear that among the latter species the entry processes are the same. The majority of studies on transfection mechanism have been reported for *Bacillus subtilis*; these have often been interpreted in terms of entry of one or more pieces of phage DNA in duplex form (39). Reports (5, 31) that the major entry path leads to single strands of donor DNA inside the *B. subtilis* cells have raised questions about these interpretations.

Of particular interest in this respect are experiments on the fate of heteroduplexes pre-

pared in vitro from opposite strands of the DNAs of mutant phages. If the DNA enters as a duplex, then such studies might help characterize the process by which mismatched base sequences are corrected to homozygosity, a phenomenon known to occur with considerable specificity in pneumococcal transformation (11) and thought to be the mechanism of gene conversion in higher organisms (15, 25). Experimental results on *B. subtilis* transfection by heteroduplexes of SPP1 DNA have been interpreted as implying strand and/or allele specificities in the conversion process (37, 39). An initial recombination event is known to be mandatory for the establishment of an infective center, apparently serving to eliminate one or more inactivating events that accompany or follow entry (7, 9, 10, 30, 39).

The finding that transforming DNA appears to enter as single strands did not rule out entry of duplex DNA for transfection. The efficiency per unit of DNA in solution is much lower for transfection than for transformation, and the dependence of the two processes on DNA con-

† Present address: Radiobiology Laboratories, School of Medicine, Yale University, New Haven, CT 06510.

centration differs significantly, suggesting there could be a difference in entry pathways. Thus, duplex DNA might enter in small amounts, difficult to detect by physical methods in the presence of large amounts of single strands. The fact that the latter have been reduced in length by cuts inserted before entry (6, 26) is consistent with inability to yield transfection and leaves open the possibility of entry of duplex DNA.

The recent discovery of phages of pneumococcus (24, 38) allows transfection in this system to be examined. Two particular advantages of pneumococcus with respect to the above questions are: (i) it has a mismatch-correction system that has been proven to operate at the heteroduplex stage (36) and for which mutants (*hex*⁻) are available that do not attack heteroduplexes (18), and (ii) mutants exist for a membrane-bound endonuclease that is required for donor DNA to enter recipient cells (20), where it is found as segments of single strands (26, 27).

The experiments described here characterize transfection in pneumococcus by phage ω 3 DNA. By showing that transfection requires multiple entries by the endonuclease-dependent pathway, they imply that the intracellular intermediates are segments of single strands. Association of these in the cell to create an infective center automatically makes a primary recombination event mandatory. However, to reconcile the strongly differing dependences on DNA concentration, it has been necessary to reexamine prior models for binding and entry of transforming DNA, and the analysis below gives new insight into that process also.

A preliminary report of these results has been presented (33), and further details have been

reported by R. D. Porter (Ph.D. thesis, Duke University, Durham, N.C., 1976).

MATERIALS AND METHODS

Bacteria, phage, and general procedures. Table 1 lists the bacterial strains used here. Strain Rx1 and endonuclease-deficient derivatives of it (see below) were used as recipients for transfection and transformation. Phage ω 3 was received from J. G. Tiraby. Media, buffers, growth, purification, and characterization of ω 3, and plaque assay methods have been described previously (32).

Transformation and transfection. Competent cells previously prepared and stored frozen at -85°C in 10% glycerol were thawed and added to DNA at 37°C, and the exposure was terminated by addition of pancreatic DNase (10 μ g/ml), as described previously (11). The cultures were then diluted as necessary and plated either for transformants or for infective centers (before lysis) as in a standard plaque assay. The competent cultures typically have 3×10^7 to 5×10^7 cells per ml at the time of thawing and about twice as many at the time of plating; 0.15 ml makes a good lawn. This amount of uninfected culture was added to the plating tube when the transfected culture had been diluted. A limitation when few transfectants are expected is that 0.15 ml of undiluted culture is nearly the maximum that can be plated on a single plate for reproducible lawn formation, sometimes making multiple platings mandatory to accumulate statistically significant numbers of plaques.

Preparation of phage DNA. Phage were grown and concentrated, and DNA was extracted with phenol and dialyzed against SSC (0.15 M NaCl-0.015 M trisodium citrate) as described (32), except that the phage concentration before lysis was increased by banding on CsCl step gradients to titers as high as 5×10^{13} PFU/ml, containing over 2.6 mg of DNA per ml before dilution.

Preparation of RI DNA. To prepare replicative intermediate (RI) DNA, a drug-resistant strain (1003

TABLE 1. *Strains of pneumococcus*^a

Strain	Relevant properties	Origin	Reference/source
Rx1	<i>hex</i> ⁻	Ravin	36
Derivatives of Rx1			
1003	<i>str-r41 nov-r1 hex</i> ⁻	This work	
1219	<i>noz-19 (end)</i> ⁻	Rx1 transformed with DNA from 410 (Shoemaker)	Personal communication
1600	<i>hex</i> ⁺	Shoemaker	36
1602	<i>hex</i> ⁺ <i>str-r1 nov-r1 ery-r2</i>		
1648	<i>hex</i> ⁺ <i>ery-r2</i> Add	1600 <i>ery-r2</i> transformed with DNA from 608 (Shoemaker)	
1771-1776	<i>hex</i> ⁻ <i>end</i> ⁻	This work	
R6 strains			
410	<i>end-1 exo-2 noz-19</i>	Lacks	19
608	Add	Lacks	Personal communication

^a *Streptococcus pneumoniae*, until recently called *Diplococcus pneumoniae* in the United States.

or 1602) was grown to 3×10^8 to 4×10^8 cells per ml in K-CAT (32) at 37°C and infected with phage at a multiplicity near 3. At the desired time, the cells were diluted twofold into SSC at 0°C and were centrifuged for 7 min at $6,000 \times g$ to pellet the infected cells. The cell pellet was suspended in 0.1 volume of SSC containing 0.02% sodium dodecyl sulfate, and then was placed at 65°C for 60 min to lyse the cells and to inactivate any intact phage particles. The lysate was then cooled to room temperature, and SSC was added to bring the volume to that of the original culture. This material was then stored at 4°C.

***end⁻* mutants.** To produce endonuclease-deficient (*end⁻*) mutants, Rx1 cells were washed and then mutagenized by exposure to 1 M hydroxylamine (pH 6) at 37°C for 20 min, followed by 20-fold dilution to cold medium, further washing, resuspension, and division into six separate subcultures. The survival after hydroxylamine treatment was 10%. After several generations of growth to ensure segregation, the subcultures were diluted and plated on DNA-methyl green agar to select for small zone-formers by the method of Lacks (18). In each subculture, about 1 in 400 colonies formed small zones, and among these were found some that reproducibly gave properties relative to Rx1 of smaller zone, lower transformability, and lower levels of RNA-inhibited endonuclease assayed in extracts by the method of Lacks et al. (20). Lysates made from one such clone from each subculture were used to transform Rx1 cells, from which small zone-formers were selected again on DNA-methyl green, at frequencies consistent with transfer of a single marker. The transformants showed RNA-inhibited endonuclease activities and transformabilities similar to those of the mutant donors. The strains retained were numbered 1771 through 1776, each carrying an *end⁻* mutation from a separate subculture transferred back once to Rx1.

Table 2 gives the apparent endonuclease activity in extracts, measured as the RNase-induced stimulation of digestion of [³H]DNA (20), and the transformability of these strains, determined as the highest level ever observed in their competence cycle. The precise correlation of RNase stimulation and transformability

among the *end⁻* strains was not strong, but in each case the levels were low compared with wild-type Rx1, and the methyl green plate assay selected strains low in both endonuclease and transformation, similar to those described by Lacks et al. (20). Strain 1771 has been tested for DNA binding and uptake by D. A. Morrison (personal communication) and has been found to bind DNA externally to an extent comparable to Rx1, whereas the uptake to a DNase-resistant state is greatly reduced, as would be expected from its transformability. This behavior is like that described by Lacks et al. for their *end⁻* mutants (20). Strain 1219, an Rx1 derivative to which Lacks's *noz-19* mutation has been transferred (Table 1) by the same selection techniques, has very low endonuclease activity and transformability, as does the *noz-19* donor.

RESULTS

Initial attempts to transfect pneumococcus with mature $\omega 3$ phage DNA at concentrations in the range from 1 to 10 $\mu\text{g/ml}$ produced only a few infective centers (IC) per milliliter, for yields near 2 IC per 10^{10} phage equivalents in solution. At such concentrations, transformation in pneumococcus is near saturation, and transfection in *B. subtilis* is also near maximum (39). We then tried RI DNA extracted from infected cells, and this gave high levels of transfection. Later work showed that mature DNA also will give high levels of IC, but only at concentrations much greater than are needed for transformation by cell DNA or for transfection by RI DNA.

We have characterized both kinds of transfection as they depend on DNA concentration, on time of exposure of cells to DNA, on DNA-DNA competition, and, of most significance, on the level of the membrane-bound endonuclease.

Concentration dependence. Figure 1 shows log-log plots of transfectants from RI DNA and from mature phage DNA, and transformants from the cellular DNA in the RI preparation,

TABLE 2. *Endonuclease levels in crude extracts and transformability of mutants*

Strain	Endonuclease assay ^a			Maximum transformability ^b
	-RNase	+RNase	Difference ^c	
Rx1	6,776	56,415	49,639(100)	2×10^6 (100)
1771	6,243	16,918	10,675(22)	7.3×10^4 (3)
1772	11,406	20,564	9,158(18)	5.8×10^5 (29)
1773	7,232	17,262	10,030(20)	3.1×10^5 (15)
1774	6,552	15,479	8,927(18)	3.7×10^5 (18)
1775	7,532	20,289	12,857(26)	4.4×10^5 (22)
1776	7,477	20,285	12,808(26)	2.2×10^5 (11)
1219 ^d	8,565	8,856	291(0.6)	1×10^4 (0.5)

^a Trichloroacetic acid-soluble counts per 10 min produced in 30 min at 30°C by the method of Lacks et al. (20).

^b Transformants per ml, observed at the peak of competence for each strain, for addition of 1 μg of DNA per ml for 20 min at 37°C. Some batches of Rx1 showed higher levels than 2×10^6 , and some lower, but this number was a typical result. Numbers in parentheses indicate percentages.

^c Numbers in parentheses indicate percentages.

^d An Rx1 derivative carrying Lacks's *noz-19* mutation (see Table 1).

FIG. 1. Dependence of transfection on concentration of RI and of mature phage DNA from $\omega 3$. *Rx1* cells were exposed for 30 min at 37°C to the indicated concentrations of an RI DNA preparation prepared 70 min after infection of an *str-r nov-r* host DP1003 (data on left: Δ , transfectants per ml; $\circ$, *str-r* transformants per ml), or to DNA extracted from the mature phage (data on right: $\bullet$, $\square$, transfectants per ml). For the experiment represented by $\square$, the data were: for 1 $\mu\text{g/ml}$, 151 plaques in 8.85 ml of culture plated; for 2 $\mu\text{g/ml}$, 215 plaques in 3.45 ml plated; for 4 $\mu\text{g/ml}$, 145 plaques in 0.90 ml plated.

as functions of DNA concentration in the assay tube. The slope for transformants and RI transfectants is 1.0, whereas the line drawn through the transformants from mature DNA has a slope of 3.0. Closer examination shows the latter data actually fit an S-shaped curve, varying from a slope of 1.5 in the 1- to 4- $\mu\text{g/ml}$ region (note the statistically significant numbers of plaques counted, as given in the figure legend) to about 3.5 in the region from 16 to 66 $\mu\text{g/ml}$, and starting to level off above 100 $\mu\text{g/ml}$. A number of experiments have each shown the S shape, and the deviations from a slope of 3.0 are statistically very significant.

For RI DNA, the slope has varied from 1.0, as here, to 1.25 in several experiments. The physical properties of this preparation, to be described below, show that it is a large, rapidly sedimenting complex containing DNA strands much longer than a single phage genome. Further, the cell DNA remains at a high molecular weight after infection and gives excellent transforming activity that is useful as an internal control. Banding in CsCl in the analytical ultracentrifuge showed that this preparation was 2/3 phage DNA, 1/3 cell DNA. Though transformation was starting to saturate at the highest concentrations, transfection by the RI DNA showed no sign of saturation.

Such results are comparable to those seen in

some *B. subtilis* systems, except that 10 μg of mature DNA per ml is usually sufficient to saturate them (39), whereas here the PFU still increased between 132 and 264 μg of DNA per ml. The yield of IC per phage equivalent in solution rises to about 1×10^{-6} at the shoulder of the mature DNA curve, and is approximately 4×10^{-5} for the RI DNA. It is worth noting that at 264 $\mu\text{g/ml}$ there are 4.7×10^{12} phage DNA molecules per ml, equal to 10^5 per cell present and to about 2 per volume of a single cell of diameter 10^{-4} cm. The culture is distinctly viscous.

Dependence on time of exposure of cells to DNA. The kinetics of appearance of transfectants or transformants serve to indicate the number of independent entry events required, as distinguished from the collision events measured by concentration response. Kent and Hotchkiss (17) first used this approach to study linked and unlinked double transformation, and transfection has also been studied in this manner (7, 39).

Transfection and transformation as functions of time of exposure of cells to RI DNA are shown in Fig. 2. Single transformants for *str-r* and *nov-r*, which are not linked, increased linearly at early times, whereas double transformants and transfectants started up more slowly. The latter data are plotted against T^2 in Fig. 3. In both cases, the data fall on a straight line for the first 9 to 10 min. However, the double transformants extrapolate to $T = 0$, whereas the transfectants extrapolate to about 12 min². An

FIG. 2. Dependence of transformation and transfection on time allowed for uptake of RI DNA. Competent *Rx1* cells were exposed at 37°C to RI DNA prepared 40 min after infection of a drug-resistant host, DP1602. Samples were diluted into DNase at the indicated times and scored for single and double transformants and for transfectants. $\bullet$, *str-r*; $\circ$, *nov-r*; $\square$, *str-r nov-r* double transformants; Δ , transfectants.

FIG. 3. Quadratic time dependence for RI transfectants and for independent double transfectants. The data of Fig. 2 are plotted versus T^2 . $\square$, *str-r nov-r* double transfectants; Δ , transfectants.

intercept of 9 to 13 min² has been found in each of several such experiments, as though there is a minimum time of 3 to 3.5 min of exposure before any transfectants can appear.

To see to what extent this is an absolute-minimum time, a higher-activity RI preparation was tested. There were 9 transfectants per ml at 2 min, 558 at 3 min, and 5,160 at 4 min, after which the activity rose 100-fold further, as in Fig. 2, to 5×10^5 /ml at 30 min, extrapolating to an apparent intercept at about 13 min². Therefore, though over 99% of the events can be described in terms of a function of the form $T(T - T_{\min})$, a small number can occur more rapidly.

Figure 4 shows the dependence of transfection by mature phage DNA on time of exposure, along with single transformants and RI transfectants assayed at the same time on the same tube of competent cells. The single transformants again increased linearly at early times, although with this culture they showed a lag of about 1 min. Transfectants from the mature phage DNA showed much more lag than those from the RI DNA, and had clearly different kinetics. Further discussion will be deferred, other than to note that the curve for mature phage DNA reasonably approximates a cubic relationship with time.

Dependence on endonuclease. Competent cells that contain adequate amounts of the major endonuclease of pneumococcus, a membrane-bound enzyme (21), bind homologous or heterologous native DNA to their surface, insert double-strand cuts into it (26), and proceed to convert the bound DNA to two forms: acid-soluble oligonucleotides that are released to the medium

FIG. 4. Time dependence for mature DNA compared with RI DNA. Portions of the same tube of competent Rx1 were added to RI DNA and to mature phage DNA at the same time. Samples from each tube were diluted into DNase to terminate DNA uptake at the indicated times. *str-r* transfectants were scored from the cellular DNA in the RI DNA preparation.

(20, 26) and single strands, resistant to added DNase and thereby defined as intracellular, of the same length distribution as those bound outside the cell in a DNase-sensitive form (26). The weight median size of the strands is 2.2×10^6 to 2.5×10^6 daltons, even when the initial size of the DNA is much larger, and all intracellular strands have been cut at least once (26). No native donor DNA can be detected in a DNase-resistant form even very soon after exposure of cells to DNA, though substantial amounts of single strands have become DNase resistant (27). In *end⁻* mutant cells, only single-strand cuts appear in the bound DNA (19), and the conversion of donor to oligonucleotides and DNase-resistant strands is reduced strongly, to the same extent as is transformation (20). Though the complete reaction sequence is not yet defined, the results at hand show that the membrane-bound endonuclease is essential for the conversion of extracellular duplex DNA to shorter segments of intracellular single strands.

A critical question, then, was how the level of endonuclease would affect transfection. If there were a separate entry path for duplex DNA, reduction of endonuclease might reduce cutting of the phage DNA and thereby increase the efficiency of transfection. Alternatively, if both DNAs use the endonuclease-dependent pathway, transfection should be decreased in *end⁻* mutants.

Several Rx strains that were sufficiently deficient in the endonuclease to reduce transformation were constructed (Table 2). Table 3 sum-

marizes a series of experiments in which various RI and mature phage DNA preparations were exposed to an *end*⁻ mutant and to wild-type Rx1 at essentially the same time. In each case, the mutants were at or near the maximum of their competence cycle. The relative numbers of transformants and transfectants in the mutants and in the Rx1 controls of each experiment are tabulated. Defining the ratio for transformation as f , the observed ratios of mutant to wild-type transfectants are compared with f^2 for RI transfectants and with f^3 for mature phage DNA transfectants. The clear result is that a reduction in the endonuclease levels reduces RI transfection much more than it reduces transformation, roughly by f^2 , and transfection by mature DNA is even more drastically reduced, by about f^3 . The conclusion is that the same membrane-bound endonuclease required for the uptake of transforming DNA is also required for the uptake of transfecting DNA in pneumococcus. This strongly implies that transfecting DNA passes through a single-stranded state before formation of IC.

Competition. When DNA concentration is in excess, transformation by a particular marked DNA can be reduced by competition by another DNA species; it is well established that this results from competition for binding and/or entry into the cell rather than at a later step (22). RI transfection did not show saturation at a concentration where transformation was saturating, and mature phage DNA transfection showed no sign of saturating at DNA concentrations 100-fold greater than were required for the

saturation of transformation (Fig. 1). Such a result implies that the DNA uptake pathway is not the same for the two processes, in contrast to the conclusion drawn from the endonuclease results.

To examine whether saturation of RI transfection could be achieved, greater DNA concentrations were needed, and this was not technically feasible with RI DNA alone. Therefore, cell DNA was added to increase the total DNA concentration. The RI data from Fig. 1 have been replotted in Fig. 5, along with an experiment in which the same RI DNA was mixed with a 200-fold excess of cellular DNA carrying *ery-r*. The two experiments were done on consecutive days with two different tubes of the same batch of competent cells. The presence of a 200-fold excess of competitor DNA reduced the RI DNA transformants and transfectants by 20- to 30-fold, implying some competition for entry. However, the RI transfectants still showed only minimal signs of saturation at these high DNA concentrations, which were comparable to those used for mature phage DNA alone. The data for the transformants, both from the RI preparation and from the competitor DNA, do not show true saturation either, and this is an important observation that will be discussed later.

In other experiments (not shown), mature phage DNA competed for entry of transforming DNA about as expected (addition of 10 μ g of phage DNA to 2 μ g of cell DNA per ml reduced transformation fivefold). Because in these circumstances every cell should have taken up

TABLE 3. Transformation and transfection in endonuclease-deficient cells^a

Strain ^b	Relative transformation (f)	Relative transfection			
		RI DNA		Mature phage DNA	
		Observed	f^2	Observed	f^3
Rx1	1	1	1	1	1
1771	0.031	0.0006	0.0010	<0.0001	0.00003
	0.087	0.009	0.008		
	0.018	0.00016	0.00032	0.000009	0.0000056
1775	0.38	0.010	0.14		
	0.077	0.0037	0.0060	0.00015	0.00046
1776	0.10	0.011	0.010	0.0023	0.001
	0.14	0.0036	0.020	0.0017	0.0028
	0.31	0.049	0.096		
	0.088	0.004	0.008	0.00032	0.0007
1219	0.0045	≤0.0003	0.00002		

^a Each line represents an experiment with a particular batch of cells near its maximum level of competence, with transfection and transformation done simultaneously, and compared to Rx1 cells done at the same time.

^b 1771, 1775, 1776, and 1219 are *end*⁻ derivatives of Rx1 (see Tables 1, 2).

FIG. 5. Effect of competitor DNA on RI transfection. The data on the left are the concentration dependence data of Fig. 1, replotted. For the data on the right, a 200-fold excess of purified cell DNA carrying *ery-r2* was added to the same RI preparation, and then transformation and transfection assays were run on dilutions of this mixture as before. The total DNA concentration in the assay tube is the abscissa in both cases. ●, RI transfectants; ○, *str-r* transformants from the RI DNA; □, *ery-r* transformants from the competitor DNA.

several fragments of phage DNA as well as some cell DNA, this result implies that the presence of intracellular phage DNA did not prevent integration and expression of the cell DNA markers. In the 25- to 230- μ g/ml range, cell and phage DNA competed with each other for both transformation and transfection, though the latter was not reduced by as much as the former. Transforming and transfecting DNA therefore compete for the same pathways in terms of material uptake, and their common dependence on the endonuclease suggests that the biologically effective molecules use the same pathway. However, their radically different saturation behaviors suggest that the biologically effective molecules use different pathways.

Structure of RI and mature DNA. Prior studies (32) showed that mature $\omega 3$ DNA is a linear duplex of about 33×10^6 daltons (50 kilobases), and that the buoyant density of RI DNA is the same as that of the mature DNA (1.706 g/ml, relative to *E. coli* DNA at 1.710 g/ml).

RI DNA first becomes detectable in analytical CsCl gradients between 20 and 30 min after infection, whereas the amount of cell DNA appears to stay constant (data not shown). Transforming activity of the cell DNA does not fall until well after lysis. On the assumption that the RI phage DNA probably contained linear concatemers of the phage genome, we tried to estimate their size by sedimentation velocity. A lysate prepared 70 min after infection of a *str-r* host was mixed with an *ery-r* reference DNA sheared to 19S. After centrifugation for 3 h at 25,000 rpm in a 5 to 20% sucrose gradient in an SW39 rotor, the transfecting activity was on the bottom of the tube, whereas the *str-r* activity ranged from 20 to 100S, peaking near 50S. Further experiments showed that treatment with proteinase K did not affect the sedimentation of either activity and that the majority of transfecting activity sedimented with $s_{20,w} \geq 230$. No linear DNA should sediment this rapidly at 25,000 rpm (23), so the implication is that the RI DNA is in a more compact structure, insensitive to proteinase K and perhaps like those described for coliphages T4 (4, 16) and T7 (34). Preliminary electron micrographs (not shown) showed large complexes, less aggregated than those of T4 or T7 but with DNA strands much longer than those of the 33×10^6 -dalton linear duplexes of mature $\omega 3$ DNA.

Insensitivity of transfection to protease. Transfection by some *B. subtilis* phage DNAs is sensitive to protease digestion of the DNA preparation (13, 14). Proteinase K treatment did not affect $\omega 3$ transfection by either mature phage DNA or RI DNA (data not shown).

Insensitivity of transfection to level of ATP-dependent DNase. In *E. coli*, the presence of *exoV*, the ATP-dependent nuclease coded for by the *recB* and *recC* genes, strongly reduces the level of both transformation and transfection (1); this has been interpreted as reflecting an active degradation of entering linear DNA, particularly double-stranded DNA. Single-strand DNA is less affected by *exoV*, and is a better transformer for the T4 system than is double-strand DNA (40).

An ATP-dependent DNase similar to *E. coli*'s *exoV* exists in pneumococcus (41), and mutants deficient in it (Add) have been found. Strain 1648 is Add, and transfection is not significantly different in it than in Rx1 (Table 4). There is certainly not a higher level of transfection in 1648, as would be predicted from the *E. coli* results. The lower level of transformation appears to be mostly a reflection of lower viability of 1648, not a decreased recombination proficiency (N. B. Shoemaker, personal communication).

TABLE 4. *Transfection in a strain (Add) deficient in ATP-dependent DNase^a*

Strain	Transformants/ml	Transfectants/ml	
		RI DNA	Mature phage DNA
Rx1 Add ⁺	1.20×10^6	1.64×10^5	1.0×10^4
1648 Add	1.88×10^5	1.51×10^5	6.2×10^3

^a Rx1 and 1648 were exposed for 30 min at 37°C to the same RI DNA preparation (~1 µg/ml) and to mature ω3 phage DNA (45 µg/ml) on the same day. The transformants (*str-r*) are from the RI DNA, prepared from strain 1602.

Other phages. Results similar to the above have been observed with both RI and mature phage DNA from ω8, and preliminary experiments have shown transfection with RI DNA of two other phages.

DISCUSSION

The responses of pneumococcal transfection to increases in DNA concentration and time of exposure are qualitatively much like those described for a number of *B. subtilis* systems, and they imply a requirement for multiple entry events followed by some sort of recombination process. The critical new experiments are those asking how transfection is affected by mutations in the endonuclease gene that reduce transformation. As reviewed above, the endonuclease is required in the pathway by which extracellular duplex DNA is converted to intracellular single strands that are shorter than those from which they were derived, and a reduction of its level might enhance transfection if the biologically effective DNA uses a separate, endonuclease-independent pathway. Instead, transfection is more dependent on the endonuclease than transformation is, to a degree that is quantitatively consistent with the kinetic data. That is, for RI DNA, two independent entry events are implied by the quadratic time dependence, and for mature DNA, an average of three events is implied by both the concentration and time dependence. Therefore, if the endonuclease is required for entry, when a deficiency of it reduces a single event by a factor f , transfection should be reduced by at least f^2 for RI DNA and f^3 for mature DNA. These are the observed results (Table 3), implying that transfecting DNA requires as much endonuclease per entry event as does a single entry of a segment of transforming DNA. This in turn implies that the biologically effective entry pathways are the same per event, and therefore strongly suggests that the intracellular intermediates in transfection are segments of single strands that have been cut at least once on the cell surface. The association of these segments into structures that can be regenerated

into complete genomes by repair synthesis automatically involves physical recombination.

However, before we can accept single-strand intermediates and consider models in more detail, it is essential to resolve an apparent paradox: transfection certainly requires entry of phage DNA, and entry of total DNA (as measured by radiolabel or by transformation) is largely saturated at 1 to 2 µg of DNA per ml; how then can transfection continue to rise rapidly at DNA concentrations 100-fold higher than this, if its DNA uses the same pathway of entry as does transforming DNA? The following analysis of this paradox gives important insights into transformation as well as transfection.

Entry of DNA. Physical entry of DNA into the cell is a limiting process for transformation, as shown directly by competition by excess unmarked homologous or heterologous DNA for entry of radiolabel and for transformation (22), and as implied by the shape of the response curve. This rises linearly at low DNA concentration and then starts to saturate, often following a curve which is like that for enzyme kinetics and is describable as an adsorption isotherm, $n/n_{\max} = KD/(1 + KD)$, where n is transformants or radiolabel, K is an apparent association constant, and D is DNA concentration (2). For transfection by mature DNA, one may ask whether the cube of this expression would generate the need for very high DNA concentration to reach maximum. For the same value of K , the cubed relation reaches half-maximum at only four times the DNA concentration for the first power relation, implying that transfection would be half-maximal at about 1 µg/ml. Because this is not the case for mature DNA, and because RI transfection is still rising at total DNA concentrations near 200 µg/ml (Fig. 1 and 5), there must exist a qualitative difference between the bulk of the entering DNA and that which is biologically effective for transfection. Although this qualitative difference could reflect a totally independent pathway of entry for a small fraction of the total DNA, and therefore possibly for double-stranded DNA, the endonuclease dependence argues against such a conclusion. The following considerations show how the results can arise naturally from the multiple binding and nicking that is observed with high-molecular-weight donor DNA (19, 26, 27).

Closer examination of transformation data for pneumococcus, both here and in other studies, shows that although results at low concentrations are often described well by an adsorption isotherm, the plateau at high concentrations is often not truly flat. Both transformants (Fig. 5) and uptake of radiolabel (R. Miao, Ph.D. thesis, Duke University, Durham, N.C., 1971) some-

times continue to rise slowly with DNA concentration in the region from 2 to 200 $\mu\text{g}/\text{ml}$, where the system has rarely been examined critically. Figure 6 shows the data of Fig. 5, replotted on linear scales. It is clear that the curve for transformation can be usefully resolved into the sum of two curves, I and II, where I is the adsorption isotherm, rising very rapidly at low concentrations, and II is a curve of initial slope about 1,000-fold less but still rising at 100 to 200 $\mu\text{g}/\text{ml}$. Most significantly, the transfection data follow curve II. This result implies that the path represented by I does not yield successful transfection; this is consistent with the fact that the bulk of donor DNA is cut on binding to segments of median length not greater than 6 to 8 kilobases (26), highly efficient for transformation but much smaller than the 50-kilobase phage genome.

Figure 7 shows two reaction schemes that are consistent with the above data. The first (A) can be thought of as free DNA in solution competing for entry sites with DNA that is previously bound, therefore nicked (19), and locally concentrated on the surface ready to interact with new sites. At sufficiently high concentrations in solution, the free DNA contributes significantly to the total DNA entering and should have few if any cuts due to binding at extraneous

FIG. 6. Concentration dependence of uptake of DNA as the sum of two curves. The data of Fig. 5 have been replotted on linear scales, using the *str-r* data (○) at low concentration and the *ery-r* data (●) at high concentration. Curves of similar overall shape are seen when a single DNA is scored over the entire range. The dashed curve (I) is that expected for an adsorption isotherm with $1/k = 0.2 \mu\text{g}/\text{ml}$ and $n_{\text{max}} = 2.6 \times 10^6$. The difference (□) between curve I and the observed *ery-r* transformants is labeled II, and the transfectants (●) follow curve II. The initial slope of I, per unit cell DNA, is about 1,000 times that of II in this experiment.

FIG. 7. Reaction schemes for DNA and cell surface sites that could generate results as in Fig. 6. In (A), it is permissible but not essential that initiation of entry is preceded by a binding step, even for path II. In (B), binding must precede entry, and the critical parameter affecting the length of intracellular strands is the number (n) of sites (S) with which each DNA reacts before completion of entry. An essential feature of each scheme is that new sites are continually appearing on the cell surface (see text).

sites. Binding and entry are separable processes, and it is conceivable that entry need not require the nicking associated with the first-step binding. However, it is plausible that the entry sites are also binding sites. Model B is an alternative description suggesting a more continuous transition from shorter entering segments when DNA is limiting to longer segments when most binding sites are full. Only in the latter case are some of the strands long enough to help create IC. RI DNA can give IC at low total DNA concentration (Fig. 1); this is almost certainly because a single large RI complex can saturate all the sites on a cell.

In one sense, the transfection results can be thought of as resulting from competitive inhibition of extraneous nicking of the phage DNA at other binding sites. By itself, however, this interpretation would require the process to saturate at the same concentration as does binding, and it would not account for the curve II component of transformation and uptake of radio-labeled DNA. These and other results demand the inclusion of a steady supply of newly arising sites, either by de novo creation or by having completed the processing of another DNA segment. Overall, model A in Fig. 7 is amenable to formal analysis of the kinetics and leads to a reasonable description of the results (W. R. Guild, unpublished data). The formal analysis of model B is more complex. This model is, however, strongly suggested and reinforced by prior studies on the dependence of competition for transformation on the molecular weight of the DNAs (E. W. Green, M.A. thesis, Duke University, Durham, N.C., 1967) and by experiments using intragenic recombination to examine the length of integrated donor strand (J. E. Wagstaff, N. B. Shoemaker, and W. R. Guild,

in preparation). The quantitative features of the competition data described here are totally consistent with Green's results. Therefore, the difference in dependence of transformation and transfection on DNA concentration is not in conflict with both processes using the endonuclease for a late step in the entry process.

Mechanisms of forming IC. The above considerations argue strongly that transfection requires entry of DNA strands in segments of length much greater than that needed for transformation. Two further aspects of the results need to be considered, the much greater efficiency of RI than of mature DNA and the differences in their kinetics. In lysogenic systems of *Haemophilus influenzae* (29) and of *B. subtilis* (7), prophage DNA is also more efficient than mature DNA. This observation and the 10^5 - to 10^6 -fold greater efficiency of RI DNA at concentrations near $1 \mu\text{g/ml}$ (Fig. 1) both suggest that ends of the entering DNA are readily damaged or lost and that this inactivates mature DNA from a phage without a permuted genome. This interpretation has been proposed (7) for a model assuming double-strand entry of mature DNA. One expects RI DNA to contain concatemers and segments of it to be in effect a group of permuted genomes, no parts of which are preferentially at ends of the entering strands. The sedimentation and electron microscopic properties of $\omega 3$ RI DNA confirm that it must contain concatemers. We do not yet know whether mature $\omega 3$ DNA is unique sequence or permuted. All or almost all transfecting phage DNAs in other systems are reported to be unique sequence (7, 10, 12, 37).

Though the slope of 1 to 1.25 on the concentration response curve (Fig. 1) implies that a single RI DNA complex is usually sufficient, the endonuclease dependence (Table 3) and the time dependence (Fig. 2) show that two independent strand entry events are required. Further, however, the time dependence fits rather well to the relation, $\text{IC} = \text{constant} \times T(T - T_{\min})$, where $T_{\min}$ is between 3 and 3.5 min when uptake of single markers shows little or no lag. If this were the time needed for linear entry of one complete genome of 50 kilobases, it would imply a rate of about 15 kilobases/min, or 250 bases/s, which is consistent with data for the rate of entry of transforming DNA in pneumococcus (26).

As noted in the results, a detectable but very small fraction of IC can appear before 3 min. Most significantly, however, the data do not fit either T^2 or $(T - T_{\min})^2$. This suggests that what is needed for the most efficient production of an IC is one long strand and a shorter segment of a second strand. The few transfectants arising

earlier than 3 min could come from a fraction of the cells with more rapid uptake or from a less-efficient process where the right combination of shorter strands can suffice. Figure 8 illustrates the suggested process of transfection by RI DNA. Whether or not a circle is a necessary intermediate cannot be proven yet, but it should certainly be sufficient to initiate an infection.

Mature phage DNA. The process of creating IC from mature DNA is more complex than from RI. Three independent kinds of data are consistent with an average requirement for entry of three DNA strands. Although closer examination of the concentration curve (Fig. 1) shows that below about $10 \mu\text{g/ml}$ the slope is less than 3, the slope only indicates the number of collision events, which is a minimum for the number of entries, and it is reasonable to assume that some aggregates exist in the phage DNA preparation. At higher concentration, in the 16- to $66 \mu\text{g/ml}$ range for wild-type, the slope exceeds 3.0, showing that a four-particle interaction increases the efficiency of transfection.

The data on time dependence of transfection by mature DNA (Fig. 4) have been analyzed for fit to various plots of $T(T - T_{\min})^2$, $T^2(T - T_{\min})$, and so on. Such examination shows that there are too many adjustable parameters to allow firm conclusions about the best fit, other than to suggest a mixed function that must include cubic terms and could include a fourth power term also. There is also a suggestion that $T_{\min}$ here is less than the 3 to 3.5 min for RI.

Figure 9 shows a number of ways that two,

FIG. 8. Mechanisms for creating IC from strands of concatemeric RI DNA. The data suggest that over 99% of IC arise from entry of one strand that is at least a full genome long, plus a segment of its complement. First-stage repair synthesis (-----), strand displacement, circularization, second-stage repair (—), trimmings of tails, and ligation lead to a closed circle that can begin a normal infection cycle. Rarely, two medium-length strands may suffice, as in model 2.

three, or four fragments of mature $\omega 3$ DNA could generate IC, all based on the assumption that the DNA is unique sequence and terminally redundant. The latter condition allows partial loss of ends, provided only that some terminal redundancy survives. Some of the models require circularization, with or without preceding repair synthesis, and others do not. Models A1 to A5 require only two strand fragments, but the results imply that they contribute at most a small fraction of the transfectants from mature DNA. Because it is difficult to see why the two partial strands of A4 and A5 would not yield an IC, it appears that strands longer than half a genome either enter only rarely or do not survive as such long enough to associate, and that therefore three or four segments are usually needed, as in models B and C (Fig. 9). A failure to survive might reflect a time-dependent intracellular inactivation of phage markers, like that described in *B. subtilis* by Green (10). He assayed them by their ability to transform or to be rescued by superinfecting mutant SP82 phage added later (a system sometimes called "helper phage transfection," but that has more parallels to transformation [40] than to transfection itself). We observed a similar postentry inactivation

in comparable assays in our system (data not shown); it had parallels to a loss of cell transforming markers observed when integration is delayed after DNA entry (3, 35). To determine whether or not this inactivation affects models A4 and A5, one must find out whether it occurs by partial (slow) or by complete (rapid when started) degradation of a given intracellular strand.

Alternatively, a failure of longer strands to enter would be consistent with the suggestion from the data that $T_{\min}$ may be shorter here than for RI DNA. This would imply that even when most binding sites are full, the typical mature DNA received at least one extra cut on the cell surface in addition to that needed to initiate entry.

Other systems. The results presented above apply directly, of course, only to $\omega 3$ transfection in pneumococcus. However, in *B. subtilis*, most of the data for a number of transfecting DNAs appear consistent with the interpretation presented here. For example, a specific question is how fragments shorter than half a genome can succeed (Fig. 9B and C) when both Green (9) and Havender and Trautner (12) found that half-molecules of SP82 or SP50 DNA, sheared once near their centers, had little or no transfecting activity. First, there would be preferential loss of strands to bridge the center. Second, the model implies that only the longest strands of the intracellular distribution cooperate to create IC, and we suggest that even these usually have suffered two cuts on the cell surface. Shear would specifically eliminate these longest strands even though the median of the total length distribution, controlled primarily by the surface cuts (6, 26), might be only slightly reduced. We predict a nonzero activity for sheared DNA, but with a dependence on perhaps the fifth or sixth power of DNA concentration, not readily detectable. In the absence of shear, the greater efficiency of transfection at lower concentrations of mature DNA in *B. subtilis* may reflect the wider spacing between surface cuts reported for this system (6).

B. Dishman (Ph.D. thesis, University of Texas, Houston, 1972) has shown transfection by denatured SPO2 DNA and a requirement for both complementary strands under conditions where fractionated single strands transform *B. subtilis*; and K. S. Loveday (Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, 1976) has presented strong if not compelling arguments for single-strand entry of the DNA of *B. subtilis* phage ϕ e. Transfection by SPO2 DNA is now reported to be sensitive to trypsin (14), but ϕ e is not. Entry may or may

FIG. 9. Mechanisms by which mature phage DNA could generate infective centers (—). The entering strand fragments, whose pairing is indicated; (→) 3'-OH termini; (---) repair synthesis on the primary recombinant structure. In some cases, this can generate an intact duplex genome directly. In others, circularization (Cir) with or without strand displacement (Dis) would be required, as in Fig. 8. The data imply that three or four strand fragments, as in (B) and (C), are usually needed for transfection by mature DNA (see text).

not be a different process for the protease-sensitive systems. Little can be said about entry of transfecting DNA in *H. influenzae*, where transforming DNA acquires DNase resistance while still double stranded (29).

Heteroduplex correction? The models in Fig. 9 show that apparent correction of mismatched phage DNA heteroduplexes prepared in vitro could arise from repair synthesis. However, there might also be some *hex*-type action on mismatches in the paired portions of the structures, and in principle one could examine this question by use of such heteroduplexes to transfect *hex*⁺ and *hex*⁻ cells. At this time, however, the results imply that heteroduplexes of mature DNA would have to be prepared at concentrations near 1 mg/ml, and this is likely to present major technical problems. It is also unlikely that useful heteroduplexes could be prepared from RI complexes of large and uncertain structure.

Single-strand entry? The evidence on entry of donor DNA in pneumococcus and its dependence on the endonuclease, as reviewed above, has led to the strong inference that the conversion of duplex to single strand precedes or accompanies entry, so that no duplex as such acquires DNase resistance. It was our first impression that the above interpretations of the transfection data explicitly depend on this inference. Further consideration shows that they are almost certainly independent of it. The argument is as follows.

Although no evidence supports it, one may entertain the hypothesis that conversion of duplex to single strands follows entry so rapidly that it has yet to be detected. Further, granting that entry itself requires the endonuclease and that it cuts DNase-sensitive donor DNA, one could argue that the actual conversion to single strands need not require it, if the evidence on oligonucleotide release to the medium were explained by alternate hypotheses. One could not, however, argue that intact duplex DNA survives as such long enough to participate in reactions with other duplexes that may enter 10 min or more later, unless one were also to postulate that, in the undetected short interval between entry and conversion to single strand, phage sequences could be transcribed and eventually translated into specific inhibitors of the conversion to single strands. In that case, donor phage DNA should begin to accumulate in the cells as duplexes. Loveday (Ph.D. thesis) has been unable to detect such material in *B. subtilis* exposed to ϕ e DNA. In the absence of such phage-specific inhibition, the products found immediately after entry would be those we describe as

the products of entry, and all further interpretations of the transfection process would be the same.

Therefore, to argue against single-strand intermediates requires invoking a series of ad hoc assumptions, each of which is unlikely to be true. We believe that the results presented and their interpretations as summarized in Fig. 7 to 9 provide a useful framework for further analysis of the processes of transfection and transformation in pneumococcus and perhaps in other systems.

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