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Topical Review

Transformation in *Haemophilus:* A Problem in Membrane Biology

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Introduction

Bacteria exchange genetic material by three basic mechanisms: conjugation, transduction, and transformation (Fox, 1978). Conjugation is a plasmid-mediated transfer of DNA from donor to recipient cells through a connecting cellular bridge. Transduction is mediated by bacterial viruses, which package donor cell DNA fragments at low frequency and pass them to recipient cells by infection. Transformation differs from the above two mechanisms in that recipient cells are able to take up donor DNA molecules (released by cell lysis) directly from the medium. Thus a unique feature of transformation is the presence of an efficient membrane transport system for large DNA molecules.

Transformation does not appear to be a common mechanism in bacteria. However, several species have developed highly efficient, genetically determined systems. Among these, the gram-positive organisms *Bacillus subtilis* and *Streptococcus pneumoniae,* and the gram-negative organisms *Haemophilus influenzae, H. parainfluenzae,* and *Neisseria gonorrheae* have been extensively studied in the laboratory. These naturally transformable bacteria should be clearly differentiated from several bacterial species (e.g. *Escherichia coli*), which can be made artificially competent for transformation by treatment with lysozyme (Chang & Cohen, 1979; Smith, Danner & Deich, 1981) or calcium ions (Mandel & Higa, 1970), but which do not appear to have specific genetic mechanisms for developing natural competence (ability to be transformed).

The overall process of transformation in naturally transformable organisms can be divided into three major steps: (1) development of competence, (2) uptake of donor DNA, and (3) integration of donor DNA into the recipient cell chromosome to form genetic transformants. Both gram-positive and gram-negative organisms follow these basic steps, but, possibly due to differences in their cell envelope structures, they have evolved somewhat different mechanisms.

Typical gram-positive bacteria have a cytoplasmic membrane surrounded by a thick peptidoglycan cell wall. As the cells become competent, there are significant changes in this cell envelope (Tomasz, 1972); DNA-binding proteins and nucleases specific for the transformation process make their appearance and are responsible for binding, cleavage, and processing of donor DNA molecules into single-stranded fragments during uptake (Lacks, 1977). Donor strands, once inside the cell, become tightly complexed with a newly synthesized protein which protects them from cellular nucleases (Morrison, 1977, 1978; Morrison & Baker, 1979). The segments of single-stranded donor DNA are then rapidly integrated into homologous regions of the chromosome, the whole process taking only a few minutes (Dubnau & Davidoff-Abelson, 1971).

Gram-negative bacteria have a three-layered cell envelope consisting of a cytoplasmic membrane, a thin peptidoglycan layer, and an outer membrane of unique composition. Despite extensive work, it remains puzzling as to how DNA penetrates these layers. However, two recent findings have provided insights into the mechanism. First, DNA uptake in *Haemophilus* (and *Neisseria*) is homospecific, i.e., the cells recognize and show preference for uptake of *Haemophilus* DNA (Scocca, Poland & Zoon, 1974). This specificity depends on interaction of short, frequently repeated sequences in the DNA with putative cell surface receptor molecules (Sisco & Smith, 1979; Danner, Deich, Sisco & Smith, 1980; Deich & Smith, 1980). Secondly,

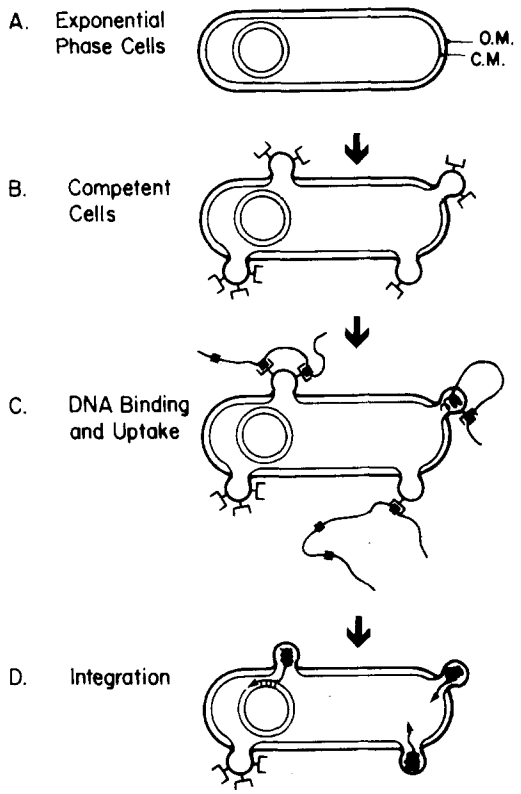

Fig. 1. A model for *H. influenzae* transformation. (*A*): Exponential cells are bounded by an outer membrane (*O.M.*) and an inner membrane (*C.M.*). The chromosome is shown as a duplex circular structure. (*B*): At the completion of competence development, 8 to 12 outer membrane vesicles (transformasomes) containing specific receptor proteins appear on the surface. The vesicles are thought to be located at points of attachment of inner and outer membranes (Bayer's foci). (*C*): Double-stranded *Haemophilus* DNA contains specific uptake sequences that interact with the vesicle receptors and initiate uptake. It is not known how the DNA enters the vesicles, although entry through pores is thought likely. (*D*): Once inside the transformasome structure, DNA is protected from nucleases in the external medium and from internal restriction systems. It is thought that the DNA exits the transformasome as a single strand, the other strand being degraded. Only a single strand becomes integrated into the recipient chromosome

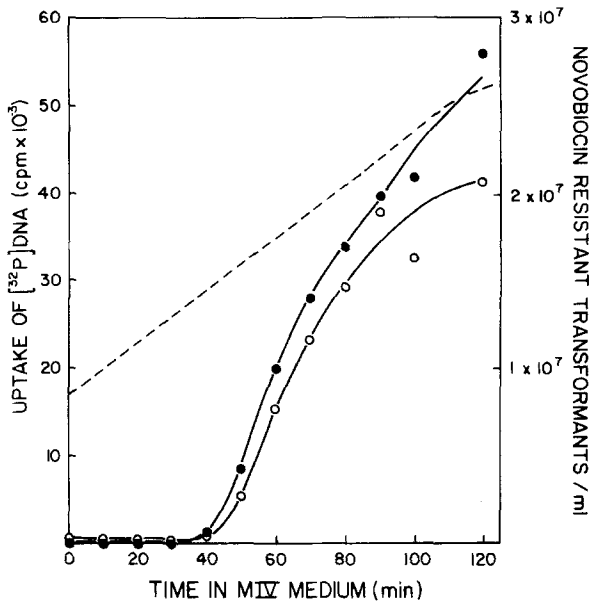

Fig. 2. Competence development of *H. influenzae* Rd in MIV medium. Cells were grown to about 8×10^8 colony forming units (cfu)/ml in heart infusion medium and then transferred to MIV medium at zero time. Incubation was carried out as 37°C with shaking, and samples were removed for assay. For DNA uptake, 0.5 ml of cells were incubated with 10 ng of nick-translated, ^{32}P-labeled pKS17 DNA (Sisco & Smith, 1979), 1×10^7 cpm/μg for 5 min. The cells were then DNase treated, washed with MIV medium containing 0.5 M NaCl and counted by Cerenkov radiation. For transformation, 0.01 ml of cells was incubated with 0.01 ml of novobiocin-resistant marker DNA (1 μg/ml) for 5 min and then plated for transformants. ○, [^{32}P]DNA uptake; ●, Nov^R transformants; ---, relative increase in cell particle concentration as determined by counting in a Petroff-Hauser chamber. Final viable cell concentration at 120 min was 2.6×10^9 cfu/ml

specialized outer membrane structures (transformasomes) appear on the surface of competent *Haemophilus* cells and play a role in taking up and holding donor DNA in a protected state until it can be subsequently moved into the cell for integration (Kahn, Maul & Goodgal, 1982; Kahn, Barany & Smith, 1983). Thus an elaborate membrane mechanism for DNA transport comes into play during *Haemophilus* transformation which differs in many respects from the mechanisms utilized for transport of smaller molecules.

Figure 1 presents our current conception of the overall mechanism for *H. influenzae* transformation based on our interpretations of recent experiments. In the first part of our review we will give a physiological description of transformation, and in the second part we will focus on the cell envelope and its role in the process. We have not attempted a comprehensive or critical treatment of the subject. Our aim is rather to acquaint the reader with current ideas in the field and to stimulate interest in this intriguing area of membrane biology. For other focal and critical reviews on transformation see Notani and Setlow, 1974; Dubnau, 1976; Lacks, 1977; Smith et al., 1981; Goodgal, 1982*a*.

Competence Development

Haemophilus transforms poorly when growing exponentially; only about one cell in ten thousand is competent. However, under conditions of slowed

growth, competence is induced in the entire cell population. *H. parainfluenzae* attains high levels of competence by overnight broth culture in a stationary petri dish (Gromkova & Goodgal, 1979). *H. influenzae* is also induced to competence by a shift from an aerobic culture to an anaerobic standing culture (Goodgal & Herriott, 1961); however, a high level of competence is most reproducibly obtained by use of a specially designed synthetic (MIV) medium (Herriott, Meyer & Vogt, 1970) that contains nutrients and amino acid supplements but is lacking several ingredients necessary for sustained growth. Essentially all of the cells become competent during a 100-min aerobic incubation at 37°C in this medium. DNA uptake ability and transformability begin to appear abruptly at 45 min and approach a plateau at 100–120 min (Fig. 2). The viable cell and particle concentrations increase by about a factor of two during the incubation, and there is a corresponding increase of DNA synthesis.

Several factors have been empirically determined to influence competence development. Lactate and inosine have a stimulatory effect during the first phase of growth in heart infusion medium (Ranhand & Lichstein, 1969), while valine, nicotinamide adenine dinucleotide (NAD), and inhibitors of protein synthesis inhibit competence development during the MIV incubation (Spencer & Herriott, 1965; Herriott et al., 1970). Competence is again lost after one or two generations of growth in broth (Scocca & Haberstat, 1978). An interesting additional observation is that cAMP induces partial competence when added to broth cultures, suggesting a catabolite repression effect (Wise, Alexander & Powers, 1973).

The genetic pathway controlling the induction of competence has not been determined. However, a number of competence-deficient mutants have been isolated (Caster, Postel & Goodgal, 1970; Beattie & Setlow, 1971; Setlow, Boling, Beattie & Kimball, 1972). Some of these are deficient in DNA uptake, while others are impaired in their ability to integrate donor DNA into the chromosome. It is possible that all of the competence genes (perhaps 10 or 20 in number) might constitute a single operon under coordinate control; however, evidence for this is currently lacking. Since *Haemophilus* is not well suited for classical genetic analysis, recent efforts have been directed toward isolating the genes by cloning.

DNA Uptake

Once cells become competent, they are capable of binding relatively large amounts of DNA in a specific and irreversible fashion (Barnhart & Herriott, 1963). Neither high salt washes (0.5 M NaCl) nor extensive DNase treatment is able to remove the adsorbed DNA, and it is presumed to have been taken up into the cell. Recent work (to be described in later sections) indicates that the DNA becomes sequestered in the outer membrane transformasomes where it persists until incorporated (Kahn et al., 1983). Noncompetent cells are also capable of binding DNA in medium of low ionic strength; however, the absorbed DNA is entirely removed by high salt washes or DNase treatment (Barnhart & Herriott, 1963). The DNA in this case seems to be only adsorbed to the outer surface by reversible ionic attractions.

A number of factors influence DNA uptake. Optimal uptake is achieved in a neutral medium containing 1 mM Ca^{2+} and 100 mM NaCl (or KCl) at 35 to 37°C. Uptake has been reported to be decreased by metabolic inhibitors such as dinitrophenol (Barnhart & Herriott, 1963; Stuy, 1965), suggesting that it is an active process. Single-stranded DNA is poorly taken up. However, Postel and Goodgal (1966, 1967) have described single-stranded DNA uptake in competent *H. influenzae* cells under conditions of low pH (4.5–4.8) and 1 mM EDTA. This uptake is biologically relevant since the efficiency of transformation with single-stranded DNA approaches that for duplex DNA.

The kinetics of DNA uptake in *H. influenzae* are illustrated in Fig. 3. The rapidity of the process is quite phenomenal, taking only one or two minutes for completion. This uptake could represent either a few complete molecules, or portions of a large number of molecules taken up per cell. Evidence strongly supports the former. Circular or linear 14-kb plasmid (Barany et al., 1983) or 32-kb HPIcI phage DNA molecules (H. Smith, *unpublished*) can be recovered intact from cells (by lysis and phenol extraction) within 1 min after uptake. These recovered molecules are fully within the cell since DNase-treatment and washing do not remove the donor DNA.

Another important property of the uptake mechanism is that it tends to saturate according to the number of molecules taken up rather than the total mass of DNA (Deich & Smith, 1980). For example, in the experiment of Fig. 3, the saturation value of 70 ng per 5×10^8 cells corresponds to about four 30-kb molecules taken up per cell. If the DNA is sheared to a 3-kb size, approximately one tenth as much DNA is taken up, but the number of molecules is still about four per cell (Deich & Smith, 1980). This finding has led to the conjecture that only a limited number of entry sites exist on the competent cell which are used only once. While this

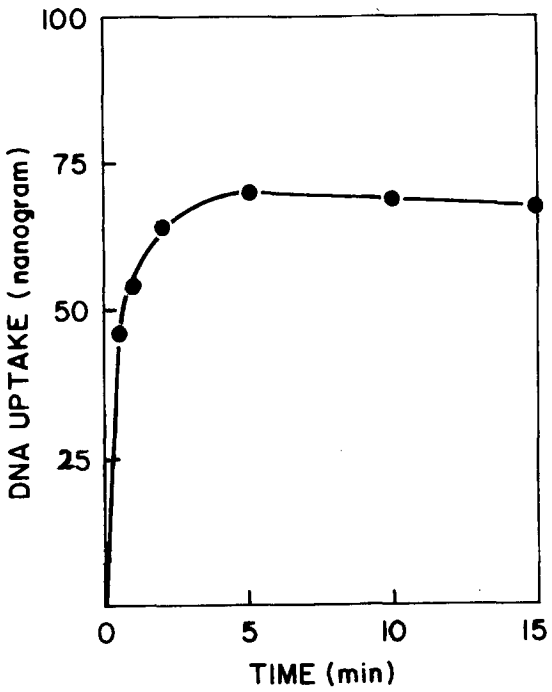

Fig. 3. Kinetics of DNA uptake by competent *H. influenzae* cells. *H. influenzae* [^{32}P]DNA was added at a concentration of 1 μg/ml to a suspension of cells at 5×10^8 cfu/ml in MIV medium at 37°C. At intervals, 1-ml aliquots were removed and pipetted into 1 ml of ice-cold 10 mM Tris (pH 7.5), 5 mM $MgCl_2$, and 50 μg/ml DNaseI. After 20 min, cells were washed and counted by Cevenkov radiation to determine uptake of ^{32}P

view is perhaps oversimplified, the transformasome mechanism discussed in later sections (*see also* Fig. 1) provides a rationale for the observations; i.e., one could easily imagine the number limitation to be determined by the packaging of a few individual DNA molecules into each of a limited number of surface transformasomes.

Homospecificity of Uptake and its Dependence on Specific Uptake Sequences in DNA

A particularly novel feature of *Haemophilus* transformation is the specificity of the uptake process. Several investigators have noted that competent *H. influenzae* cells have a strong preference for taking up *Haemophilus* DNA as compared to foreign DNAs (Schaeffer, Edgar & Rolfe, 1960; Scocca et al., 1974). Scocca et al. (1974) were the first to clearly document this effect and to focus on the possible mechanisms for the apparent ability of the cells to recognize DNA at the cell surface. They suggested that *H. influenzae* and *H. parainfluenzae* DNA carried either a specific modification pattern or a specific repeated sequence as the basis for recognition.

To discriminate between these two possibilities, Sisco and Smith (1979) cloned segments of *H. parainfluenzae* DNA in *E. coli* so that *Haemophilus*-specific modification would be lost. They demonstrated that the cloned *H. parainfluenzae* DNA fragments were still preferentially taken up; hence, recognition presumably depended on specific sequences (sites) within the DNA. Four small *Haemophilus* DNA fragments (ranging from about 90 to 260 bp) with high preferential uptake were isolated from restriction digests of two of the clones (pKS11 and pKS17) and sequenced (Danner et al., 1980). Three of the fragments contained one copy of an eleven-basepair sequence, 5′-AAGTGCG GTCA, and the fourth fragment contained two copies of this sequence (Fig. 4A). Since there were no other comparable sequence homologies between the four fragments, it was inferred that this sequence was necessary for preferential uptake.

Direct evidence that competent cells interact with this sequence was suggested by experiments analogous to those used by Gilbert and coworkers to identify contact points between RNA polymerase and promoter sites in DNA (Siebenlist, Simpson & Gilbert, 1980). A small fragment containing a single eleven-bp sequence was labeled with radioactive phosphorus at one 5′ terminus and treated with the alkylating agent ethylnitrosourea (Siebenlist & Gilbert, 1980). The fragment was then incubated with competent cells and absorbed fragments recovered, cleaved at the points of ethylation of phosphate groups on the backbone by alkali treatment, and analyzed by the sequencing gel technique. A number of ethylations clustered in the region of the eleven basepair sequence were found to inhibit uptake (Fig. 4*B*). These were interpreted as potential contacts between a putative competence-specific receptor protein and the DNA uptake sequence (Danner et al., 1980).

Further confirmation of the importance of the eleven-bp sequence for uptake has come from the demonstration that a synthetic copy of the sequence confers uptake activity when inserted into foreign DNA (Danner, Smith & Narang, 1982). Surprisingly, however, activity was found to vary considerably depending on location of the inserted sequence. While this effect is not yet understood, there appeared to be a strong correlation between activity of the synthetic site and the AT-richness of flanking sequences. The sequenced natural sites are also frequently situated in regions of high AT-content, but this is most consistent in the 3′ flanking region (Fig. 4*A*).

A.

DNA		SEQUENCE			ACTIVITY
pPUP3·	AAAAAAAAAAAAAAA	AAGTGCGGTCA	TTTT	TTTTTTTTTTT	high
pKS17	AAAATTAAAATTTAA	AAGTGCGGTCA	TTTT	GACCGAGATTT	high
pKS17	GGTTGTGCATTGTTG	AAGTGCGGTCA	AAAA	ATCGGAAATTT	high
pKS11	GTTCGCCCCAAAGGA	AAGTGCGGTCA	TTTT	ATAGGTTGGAT	NT
pKS11	TTACTTATAAAATAA	AAGTGCGGTCA	ATTT	CAAAACAGTTT	NT
pKS11	GTAATCCTAAACAGA	AAGTGCGGTCA	ATTT	TAAAACTGTTT	NT
pEUP1	TCGTCTTCAAGAATT	AAGTGCGGTCA	AATT	CTCATGTTTGA	medium
pRSUP1	CTTGGTTATGCCGGT	AAGTGCGGTCA	ACTG	CCGGGCCTCTT	medium
pBUP2	GCGTAGAGGATCCGG	AAGTGCGGTCA	CCGG	ATCCACAGGAC	low
HPICI(2309)	CAATAATAAACAAGC	AAGTGCGGTCA	TGTT	CCTAAATGTTT	high
HPICI(7877)	GTGGGATTATGTGTT	AAGTGCGGTCA	AATT	TCCCTTCATAA	high
HPICI(4508)	CAGTCAATACGGCGA	AAGTGCGGTGG	ATTT	TCTGCGATCTT	high
HPICI(7147)	ATCTATCGCAGGGGT	AAGTGCGGTAG	AAAA	TTTAGAAGATT	high
HPICI(3288)	ACTTTGTTTAAGTAA	AAGTGCGGTGG	TTTC	TGCTTGCTTTT	high
HPICI(354)	TCCGTTTTTTTAAAT	AAGTGCGGAAT	TCCT	TCTTGGTGTTC	low

B. pKS11

GTTCGCCCCAAAGGA AAGTGCGGTCA TTTT ATAGGTTGGAT
CAAGCGGGGTTTCCT TTCACGCCAGT AAAA TATCCAACCTA

Fig. 4. (*A*): Nucleotide sequence of the *Haemophilus* DNA uptake site. Plasmids pPUP3, pEUP1, pRSUP1, and pBUP2 are pBR322 derivatives into which a synthetic copy of the 11-bp sequence has been inserted at different positions and varying flanking sequence contexts (Danner et al., 1982). Plasmids pKS11 and pKS17 are *H. parainfluenzae* DNA clones which contain natural 11-bp sites (Sisco & Smith, 1979). The *Haemophilus* phage HP1*c*1 sequences are from an 8-kb region of the phage DNA; numbers in parentheses refer to nucleotide position in the sequenced region (Fitzmaurice, 1983). The HP1*c*1(354) sequence has low uptake activity (1%) compared to the other phage sites. (*B*): Position of phosphate groups which when ethylated lead to decreased (▽) or increased (▼) uptake

Frequency of Uptake Sequences in *Haemophilus* DNA

The number of copies of the uptake sequence per *H. influenzae* and *H. parainfluenzae* genome has been estimated at about 600 by competition experiments (Sisco & Smith, 1979). This corresponds to about one site per 4-kb of DNA on the average. More direct evidence for the presence of multiple copies of the sequence has been obtained by Southern hybridization analysis using the ^{32}P-labeled synthetic sequence as a probe (Danner & Smith, 1983). A large number of hybridizable sequences are found in *H. influenzae* DNA, while *E. coli* DNA gives no detectable hybridization (Fig. 5). Interestingly, one *Eco*RI fragment of phage lambda DNA hybridizes to the probe. A search of the lambda sequence (Daniels et al., 1983) reveals a perfect 10-bp match to the eleven-bp sequence in this fragment. Apparently, the stringency of the hybridization was such as to permit detection of contiguous 10-bp matches.

Uptake of Heterologous DNA

Several investigators have pointed out that *H. influenzae* will take up heterologous DNAs (Steinhart & Herriott, 1968; Newman & Stuy, 1971), and some of these do not have complete 11-bp sites (Deich & Smith, 1980; Goodgal, 1982*b*). This activity might be accounted for by partial (incomplete) sites present in the DNA. In competition experiments, *E. coli* DNA appears to have the equivalent of about 40 uptake sites per genome (Deich & Smith, 1980). However, statistically, no more than two complete sites would be predicted, and none are detected by direct hybridization (*see* Fig. 5). Thus it seems reasonable to ascribe this observed uptake to a larger number of incomplete sites whose total activities are equivalent to about 40 complete sites. Recent experiments by Goodgal (1982*b*) might also be interpreted in this way. When pBR322 DNA is cleaved into multiple fragments by a restriction enzyme, some of the fragments are poorly taken up, but a

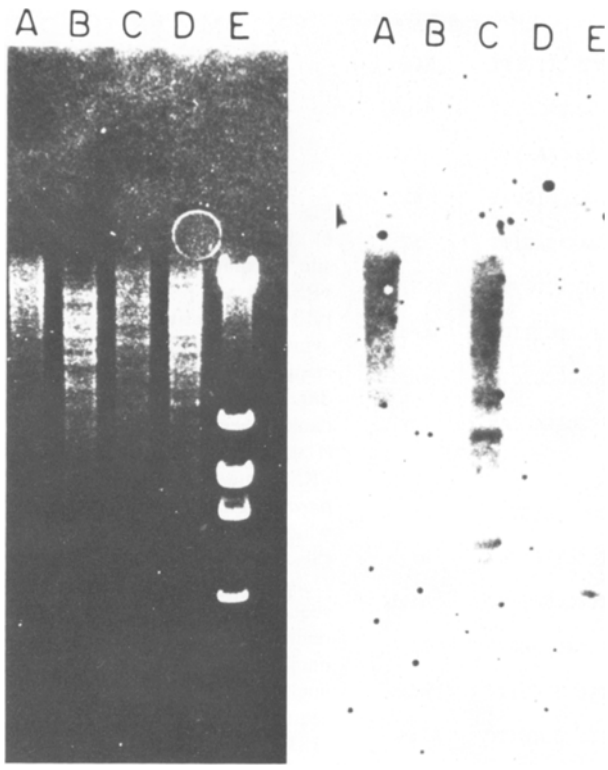

Fig. 5. Occurrence of sequences complementary to the 11-bp sequence in *H. influenzae, E. coli,* and phage lambda DNA as determined by Southern hybridization analysis. Lanes *A* to *E* (on left): Ethidium bromide stained agarose gel of *Pst*I-cut *H. influenzae* and *E. coli* DNA, *Eco*RI-cut *H. influenzae* and *E. coli* DNA, and *Eco*RI-cut phage lambda DNA, respectively. Lanes *A* to *E* (on right): Autoradiogram obtained when DNA was transferred to nitrocellulose and hybridized with ^{32}P-end-labeled synthetic 11-mer DNA at 14°C

few show significant uptake activity. These latter fragments contain one or more contiguous or hyphenated 8- to 9-bp matches to the 11-bp site, which may be responsible for the uptake. However, the actual sequences conferring the uptake have not been identified. An alternative possibility is that the sites may bear little sequence relationship to the 11-bp sequence and that some feature of the sites other than sequence (for example, a structural feature) might also provide a basis for recognition.

Fitzmaurice (1983), in a study of the *Haemophilus* phage HPIcl, has found that the phage DNA has a high content of active uptake sites. He sequenced several restriction fragments of the phage DNA, and also measured the uptake activity of the fragments. Those fragments with high uptake activity contained either a complete 11-bp site (two cases), or incomplete copies in which the first nine base-pairs were matched (three cases) (*see* Fig. 4*A*). He referred to these as [1-9]9-mers. A fragment with a [1-8]8-mer had low (1%) uptake activity, while several [3-11]9-mer-containing fragments gave essentially no uptake. It is evident from these observations that the determinants for uptake activity are as yet imperfectly understood and warrant further study.

Role of the Cell Envelope in DNA Uptake

It is appropriate at this juncture, having acquired some knowledge of the conditions favoring DNA uptake, to begin to think in terms of a specific mechanism by which DNA might penetrate the membrane barriers of the cell. So far, we know that the uptake process is rapid, that it saturates according to number of molecules rather than mass of DNA, and that it depends on recognition of specific sequences in the donor DNA. These facts fit best with the notion that DNA transport is a localized process rather than a diffuse property of the membrane. Thus we can imagine a few (perhaps eight or ten) localized structures on the competent cell surface involved in DNA uptake. These might, for example, be specialized pores or channels. Furthermore, receptors are clearly required for DNA recognition, and these receptors could well be outer membrane proteins situated on, or in proximity to, the localized uptake structures (*see* Fig. 1).

With these ideas in mind, investigators have recently begun to study the *Haemophilus* cell envelope, focusing on the appearance of new proteins or visible structures during competence induction. While these studies are still quite preliminary, it has become clear that a number of competence-specific changes in protein composition occur and that a discrete membranous structure makes its appearance on the surface of competent cells. In the next several sections, the normal cell envelope is described to serve as a baseline for comparison, and changes in composition and structure in competent cells are reviewed. Where possible, current thoughts as to the functions of the induced proteins and structures are discussed.

Structure and Composition of the Noncompetent *Haemophilus* Cell Envelope

The *Haemophilus* envelope appears similar to that of other gram-negative bacteria in electron micrographs (Fig. 6). Some clinical isolates possess, in addition, a thick (0.25 μm) external polysaccharide capsule. Of the six antigenic types (a-f) (Pittman, 1931), the b-type capsule is best characterized, and consists of a negatively charged, phosphodiester-linked, ribose-ribitol copolymer (Crisel, Baker & Dorman, 1975). Surprisingly, highly competent *H.*

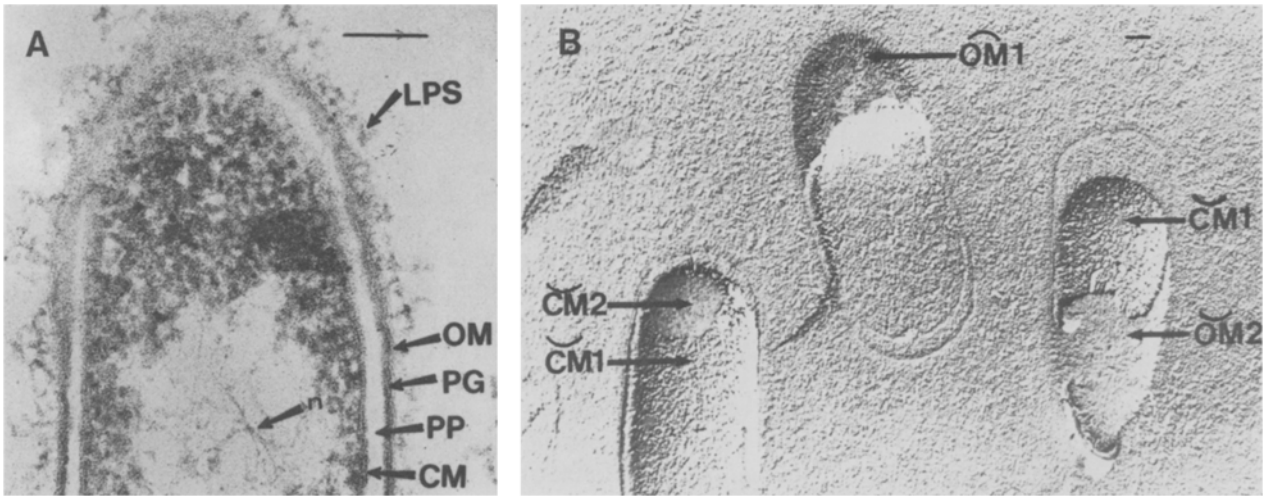

Fig. 6. Morphology of noncompetent *H. parainfluenzae* cells. (*A*): Longitudinal thin section demonstrating cell envelope structures. The outermost fibriller layer is lipopolysaccharide (*LPS*). Other structures in order are: outer membrane (*OM*), peptidoglycan (*PG*), the periplasmic (*PP*), cytoplasmic membrane (*CM*), and the cell nuclear material (*n*). (*B*): Freeze etching demonstrating unique morphology of membrane surfaces. *OM1*, convex view of outer membrane revealing the fibriller lipopolysaccharide layer. *OM2*, concave view of inner surface of the outer membrane; *CM1*, concave view of the outer surface of the cytoplasmic membrane, note patchy appearance; *CM2*, concave view of inner surface of the cytoplasmic membrane. Bar represents 100 nm

influenzae type b cells can be prepared (E.R. Moxon, *personal communication*).

The outer membrane is the first barrier encountered by DNA. Lipopolysaccharide is a major constituent of the outer leaflet of this membrane. It contains carbohydrate (Raichuarg, Brossard & Agnery, 1980) and lipid A (Raichuarg, Grenounov, Brossard & Agnery, 1981), but the structure is not fully characterized. Less than 5% of the carbohydrate is comprised of glucosamine and galactosamine (Raichuarg, Brossard & Agnery, 1979), but these charged residues may contribute to nonspecific DNA binding. The phospholipids of the *H. influenzae* and *H. parainfluenzae* membranes are phosphatidyl ethanolamine (85%) and phosphatidyl glycerol (15%) (Sutrina & Scocca, 1976). The majority of the fatty acids are C16:1 and C16 (White & Cox, 1967).

Seven major polypeptides (a-g) make up the bulk (80%) of the protein content of the *H. influenzae* outer membrane. The molecular weights are estimated by SDS-polyacrylamide gel electrophoresis to be 46, 38, 37, 34, 28, 26, and 15 kilodaltons, respectively (Loeb, Zachary & Smith, 1981). Although the outer membrane profiles from *Haemophilus* isolates vary, e, g, and a minor polypeptide, h, appear to remain constant (Loeb & Smith, 1982). Protein d is related immunologically to the OmpA protein of *E. coli* (van Alphen, Riemens, Poolman & Zanan, 1983). The others have not been characterized, but some are presumably the porins responsible for size-dependent transport of hydrophilic molecules across the outer membrane (Nakae, 1976*a,b*).

The thin peptidoglycan layer beneath the outer membrane (Fig. 6), while uncharacterized, is thought to be similar to that in other bacteria. It either represents no barrier to DNA, or possibly is discontinuous at the sites of uptake. A hydrophilic region, the periplasmic space, separates the inner and outer membranes (Fig. 6). About 7% of the cell's volume is contained in this space based on measurements of freeze-fractured cells (M.E. Kahn, *unpublished*). The inner membrane represents the final obstacle for the passage of donor DNA into the cell (Fig. 6). A complex pattern of polypeptides is observed when purified inner membranes are analyzed by electrophoresis on SDS-polyacrylamide gels (Loeb et al., 1981). The inner membrane may play a role in the active transport of DNA into the cell. Bayer's foci (Bayer, 1968) are also observable in thin sections. These zones of apparent adhesion between inner and outer membranes could possibly function as entry sites for DNA.

Envelope Changes Associated with Competence

There have been several recent efforts to detect biochemical components of the transformation mechanism. Attention has been focused primarily on the cell envelope, and the basic strategy has been to

look for specific changes in going from the noncompetent to the competent state.

The phospholipid content of the membranes does not appear to change during competence induction. Recently Reusch and Sadoff (1983) have noted a correlation between the presence of D-(−)-poly-β-hydroxybutyrate in the membrane and competence. In addition LPS has been reported to increase by 85% and there appears to be an increase in the neutral sugar content of LPS in competent cells (Zoon & Scocca, 1975).

Of potentially greater interest are the changes in the outer membrane proteins. Zoon and Scocca (1975) examined stained polyacrylamide gel electrophoretic profiles of crude envelope fractions and observed the appearance of three polypeptides, 120, 95, and 70 kilodaltons in size, during competence induction. By incorporating radioactive amino acids during competence induction, they detected several additional apparently competence-related polypeptides of sizes 95, 80, 78, 64, 58.5, and 40.5 kilodaltons (Zoon, Habersat & Scocca, 1976). Using surface-specific iodination methods, Concino and Goodgal (1981) observed a competence-specific 29-kilodalton polypeptide. Several other proteins of sizes 43, 46, 71, and 76 kilodaltons were also observed to label more prominently in competent as compared to noncompetent cells. In addition, several polypeptides of sizes 88, 83, 44, 42, 36, 33, 29, 27, and 23 kilodaltons were observed to decrease in labeling after addition of DNA to the competent cells, implying that they are either complexed to DNA, or participate in the DNA uptake process in some way.

While interesting, these observations have been difficult to interpret. First of all, in comparing one method to another, there is little agreement among the species of proteins identified. Many of the changes may simply reflect the altered physiological state of competent cells, rather than indicating specific components of the transformation mechanism. It is also possible that some of the transformation-specific proteins are in small amounts and escape detection. Finally, no assays are available to confirm the role of the various proteins in transformation.

One approach to overcome these difficulties is to analyze competence-deficient mutants. Concino and Goodgal (1981) observed the absence of a 29-kilodalton polypeptide in several mutants using the ^{125}I surface-labeling method. Sutrina and Scocca (1979) isolated a DNA-binding protein from cells by osmotic shock, which is also decreased or absent in several competence mutants.

Another approach has been to use the known ability of competent cells to bind preferentially to *Haemophilus* DNA as a method for purifying the receptor for DNA sites. R.A. Deich (*unpublished*) prepared detergent-solubilized ^{35}S-methionine-labeled membrane proteins from competent cells and chromatographed the extracts on *Haemophilus* DNA-agarose. He was able to purify two polypeptides of approximately 52 and 28.5 kilodaltons. The 28.5-kilodalton protein is perhaps the best candidate for the receptor since it may correspond to the 29 kilodalton polypeptide described by Concino and Goodgal (1981) and to the 29.5-kilodalton polypeptide present in transformasomes (M.E. Kahn et al., *unpublished*). Progress in this area will depend heavily on advances in genetic analysis of the system, and the correlation of specific genes with particular membrane proteins.

Membrane Vesicles Associated with Competence

The early idea that competence involves a general change in permeability of the cell envelope to large molecules has given way in recent years to the concept of a few discrete portals of entry. With this change in thinking, investigators have attempted to correlate visible structures on the cell surface with competence. In several organisms, transformability is strongly correlated with piliated strains (Ottow, 1975), but such a correlation is not found in *Haemophilus* (Kahn & Gromkova, 1981). However, Kahn et al. (1982) have recently described bleb-like structures, about 10 to 12 in number, on the surface of competent *H. parainfluenzae* cells. These are clearly seen in electron micrographs of thin sections of competent cells, and are rarely observed in noncompetent cells (Fig. 7*A*).

The significance of this finding could have easily been overlooked if not for the observation that the addition of donor DNA to competent *H. parainfluenzae* cells result in the disappearance of the surface vesicles (blebs) and the concomitant appearance of approximately ten periplasmic and cytoplasmic vesicles (Fig. 7*B*). Donor DNA can be recovered from disrupted cells as a DNase-resistant membrane-DNA complex of high density, suggesting that donor DNA enters the cell in association with the vesicles. Competent *H. influenzae* cells also have vesicles on their surfaces, while noncompetent cells do not (Kahn et al., 1982). However, the addition of donor DNA to competent *H. influenzae* cells does not result in the loss of the vesicles from the cell surface, possibly implying a basic difference in the transport mechanism between the two strains (Kahn et al., 1982).

Vesicles from both species have similar morphology. They average 20 nm in diameter and extend an average of 40 nm from the cell surface.

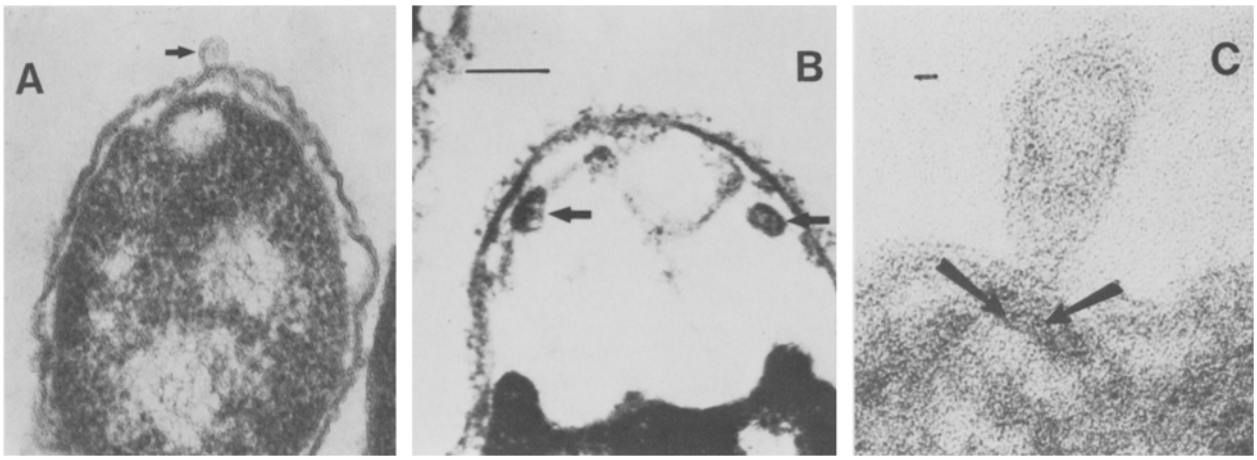

Fig. 7. Unique morphological features of competent *Haemophilus* cells. (*A*): Thin section of a competent *H. parainfluenzae* cell demonstrating a "bleb"-like structure (transformasome) on the cell surface (arrow). (*B*): Thin section of a competent *H. parainfluenzae* cell that had been incubated with transforming DNA for 3 min. Note the appearance of periplasmic vesicles (arrows). Bar represents 100 nm. (*C*): Under higher magnification, a pore structure can be observed (arrow) at the base of the transformasome. Bar represents 10 nm

Higher magnification of the vesicles reveals a pore structure with an opening of approximately 5 nm, at the point of attachment of the vesicle to the cell membrane (Fig. 7*C*) (Kahn, 1981; Kahn et al., 1983). In *H. influenzae* this point of attachment often appears to be localized at fusion points of the outer and inner membrane. Additional pore-like structures are sometimes observed on the body of the vesicle (M.E. Kahn, *unpublished*).

Vesicle-Shedding Mutants

A unique transformation deficient mutant of *H. parainfluenzae, com*$^-$10, has been isolated which sheds vesicles during competence induction rather than accumulating them on its surface (Kahn et al., 1979). The vesicles are readily isolated from cell-free supernatants and they possess DNA binding activity. In fact, they bind far more DNA than the cells from which they arise. An analogous vesicle shedding mutant of *H. influenzae, com*$^-$51, has also been identified (Kahn, Concino, Gromkova & Goodgal, 1979; Concino & Goodgal, 1982). Electron micrographs of thin sections of the mutant cells presented in Fig. 8 show both free vesicles and budding vesicles.

Concino and Goodgal (1982) have analyzed the polypeptide composition of vesicles shed from *com*$^-$51 cells using the cell surface iodination method. A 29-kilodalton polypeptide, possibly identical to the 29-kilodalton competence-specific polypeptide described in the previous section, was present in the vesicles, but was lacking in the cell membrane extracts. The authors also noted an enrichment for 33 and 42 kilodalton polypeptides, and to a lesser extent for 27 and 36 kilodalton polypeptides in the vesicle preparations. These polypeptides are similar in size to some of the polypeptides that they had characterized as being competence related, on the surface of wild type competent cells (*see* previous section). It would be useful to compare the results of ^{125}I-surface-labeled polypeptides to total polypeptides contained in *com*$^-$51 vesicles since some proteins of importance to the transformation process may not be exposed to the surface. However, these results are in basic agreement with recent results obtained in our laboratory (M.E. Kahn, F. Barany, and H.O. Smith, *unpublished*), analyzing structurally intact vesicle-DNA complexes released by extraction of competent cells with organic solvents (*see* below).

Role of Transformasomes in DNA Uptake

Evidence for the primary role of surface vesicles, or transformasomes as they are now frequently referred to, in DNA uptake can be summarized as follows: isolated transformasomes from both *H. influenzae com*$^-$51 and *H. parainfluenzae com*$^-$10 possess DNA binding ability, and they are enriched for several competence-related membrane proteins. The average number of transformasomes per competent cell (10 to 12) correlates well with the number of DNA molecules taken up per cell (4 to 20 in

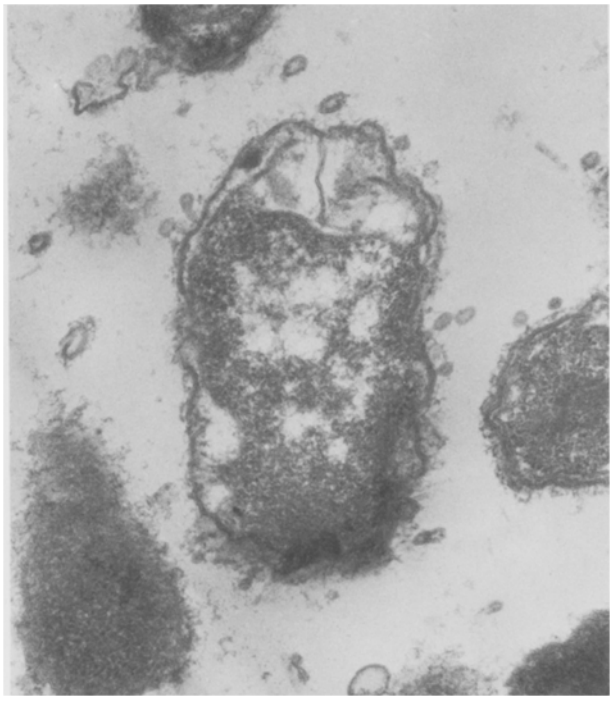

Fig. 8. Thin section of the *H. parainfluenzae* competence-deficient mutant *com*$^-$10. Note the shedding of vesicles somewhat larger in size than wild-type transformasomes. *Com*$^-$10 vesicles are composed of both outer membrane and competence-specific polypeptides, and bind DNA

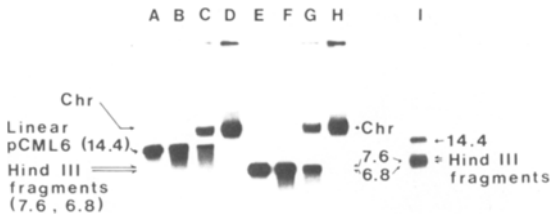

Fig. 9. Uptake and incorporation of radioactive donor DNA into competent *H. influenzae* cells. Nick-translated linear plasmid DNA was added to competent cells and DNA taken up by the cells was reisolated after 2, 15 and 40 min for electrophoretic analysis on agarose gels. Lane *A*: input linear DNA; Lanes *B* to *D*: reisolated donor DNA after 2, 15, and 40 min, respectively. Lane *E*: input DNA digested with *Hind*III restriction endonuclease. Lanes *F* to *H*: material from lanes *B* to *D*, respectively, digested with *Hind*III restriction endonuclease *in vitro*. *Chr* is chromosomal DNA. Numbers represent DNA sizes in kilobase-pairs

various studies). Transformasomes disappear from the surface of *H. parainfluenzae* cells within 3 min after addition of donor DNA, and a similar number of vesicles appear within the cell. Furthermore, the absorbed DNA can be reisolated as a DNase resistant, membrane-DNA complex. These observations fit well with the hypothesis that in *H. parainfluenzae* DNA is first packaged into the transformasomes and then transported into the cell by endocytosis.

However, transport by endocytosis does not seem to occur in *H. influenzae*. Instead, packaged DNA appears to be held temporarily on the surface in the transformasomes. DNA molecules then exit linearly from the transformasomes as transformation proceeds toward integration. The overall process of DNA *entry* can thus be described as a two-step process involving first, the *uptake* of DNA into the transformasome, and second, the *transport* of DNA out of the transformasome into the cytoplasm where integration occurs (Kahn et al., 1983; Barany, Kahn & Smith, 1983).

The two steps can be separated experimentally as shown in Fig. 9 (Kahn et al., 1983). Competent *H. influenzae* cells were incubated with linear molecules of ^{32}P-labeled plasmid DNA carrying a cloned homologous insert, and samples were taken at various times for analysis by agarose gel electrophoresis to determine the fate of the DNA. In the first two to three minutes, the majority of the donor DNA could be recovered intact from the cells, i.e., it was taken up as DNaseI-resistant material and had undergone no physical alteration in the cell (Fig. 9, lane *B*). Furthermore, it was still unmodified by the cellular *Hind*III methylase since it could be cleaved by *Hind*III restriction enzyme *in vitro* (Fig. 9, lane *F*). Thus a cytoplasmic location for the DNA seemed unlikely; it was in a "protected state." At subsequent time points, donor DNA radioactivity progressively moved into the chromosomal fraction (Fig. 9, lanes *C* and *D*) which is modified and hence resistant to *in vitro* cleavage by *Hind*III (Fig. 9, lanes *G* and *H*). By 40 min, no DNA remained in the protected state.

Further evidence for a two-step process comes from examining the topological requirements for DNA entry as illustrated by the experiment in Fig. 10 (Kahn et al., 1983). A mixture of linear, open-circular, and closed circular molecules was added to competent cells. All were efficiently taken up, but the majority of the closed circular species could be recovered intact after one hour (*compare* lanes *A* and *B* of Fig. 10). Radioactivity associated with the chromosome was derived predominantly from the linear and open-circular species. The closed circular species remained unmodified, arguing against a cytoplasmic location, (Fig. 10, lane *C*). Similar results are obtained with linear DNA molecules sealed at their ends by hairpin loops and by molecules with protein covalently bound to the 5′ termini (Barany et al., 1983). Thus all topological forms appear to be readily taken up into a DNase-

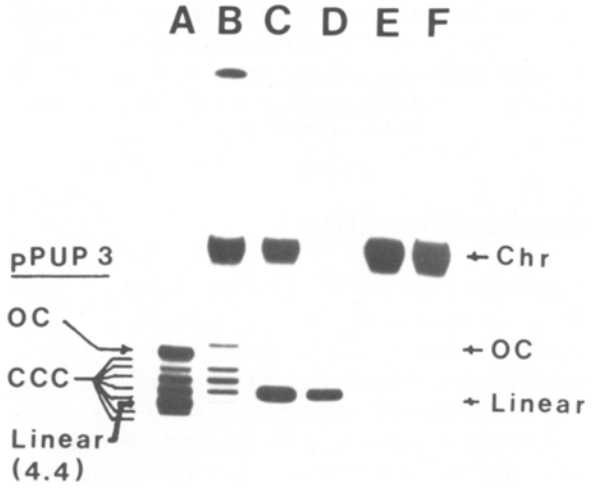

Fig. 10. Autoradiogram showing the fate of CCC, OC, and linear DNA after uptake. Nick-translated pPUP3 DNA (Kahn et al., 1983) in various conformations was added to competent *H. influenzae* cells, reisolated after a 60-min incubation, and analyzed on agarose gels. Lane *A*: input mixture of linear, open circular, and closed circular forms of pPUP3. Lane *B*: reisolated after a 60-min incubation; note the presence of open circular and closed circular forms in the protected state. Lane *C*: digestion of material in Lane *B* with *Hind*III *in vitro*. Lane *D*: input linear pPUP3 DNA; Lane *E*, reisolated after 60 min, and Lane *F*, digested *in vitro* with *Hind*III. Note that all of the donor DNA label was incorporated into the chromosome and resistant to *Hind*III

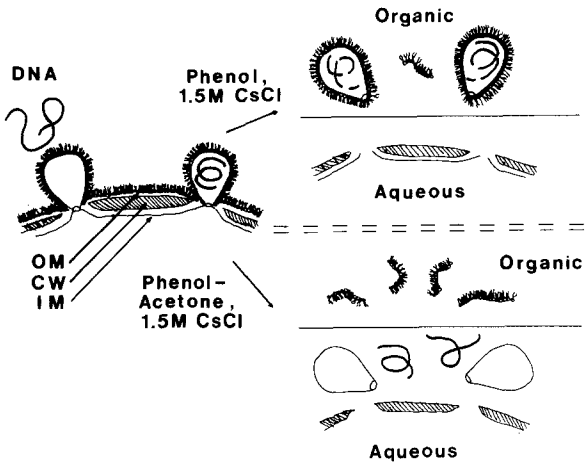

Fig. 11. Schematic summary of the removal of transformasome-associated DNA from competent cells by extraction with organic solvents. When competent cells (in 1.5 M CsCl) are extracted with an equal volume of phenol, structurally intact transformasomes containing donor DNA can be recovered from the phenol phase. The preferential solubility of transformasomes in the phenol phase is thought to be due to the coating of LPS on the surface of these structures. After dialysis of the phenol fraction, donor DNA appears to have undergone some degradation (probably an artifact of the isolation procedure). In order to isolate undegraded donor DNA, competent cells in 1.5 M CsCl were extracted with an equal volume of phenol/acetone (1 : 1). Under these conditions, transformasome-associated DNA could be recovered intact from the aqueous phase. *OM* is outer membrane; *CW* is cell wall; *IM* is inner membrane

resistant form (Step 1), but subsequent movement into the cytoplasm appears to require a free, unblocked terminus (Step 2).

Additional evidence that protected-state (Step 1) DNA exists in a separate compartment (the transformasome) has been obtained in phase extraction experiments (Kahn et al., 1983). When cells are gently extracted with phenol in the presence of 1.5 M cesium chloride, chromosomal DNA remains in the cellular fraction and is recovered at the interphase by centrifugation, while the majority of the protected-state DNA partitions into the phenol phase. After dialysis of the phenol phase, much of the recovered DNA is unable to penetrate a 0.4% agarose gel during electrophoresis, and is resistant to DNaseI and *Eco*RI digestion. Electron microscopy of the material reveals 85 nm diameter membrane vesicles and some cellular debris. Treatment with 1% SDS at 65°C, plus additional treatment with organic solvents such as chloroform, is required to release DNA from the vesicles. Protected-state DNA obtained by this method is partially degraded; however, degradation seems to be the result of the isolation procedure since a similar extraction of competent cells in 1.5 M CsCl with phenol/acetone (1 : 1), liberates intact protected-state DNA into the aqueous phase. This DNA is no longer resistant to DNaseI. A schematic summary of these results is shown in Fig. 11.

A sedimentation analysis of the recovered phenol phase DNA has been carried out in sucrose step-gradients (Kahn et al., 1983). The majority of radioactive label is localized as a broad band corresponding to free, partially degraded donor DNA. However, about 15% pellets to the bottom in association with membrane vesicles. The polypeptide components of this sucrose pellet material have been compared with the specific sets of proteins extractable into the phenol phase from competent and noncompetent cells (in the presence of 1.5 M CsCl). Polypeptides of 29.5, 32.5, 36.9, 50.6 and 67 kilodaltons are found in the phenol phase proteins from competent cells but are not evident in the noncompetent cell extracts. Several of these are similar in size to those reported by Concino and Goodgal (1981). The sucrose pellet is enriched for the 29.5, 32.5, 50, and 67 kilodalton competence-related polypeptides. Polypeptides of 42.5, 43.5, 55, and 64 kilodaltons are present in all three extracts and ap-

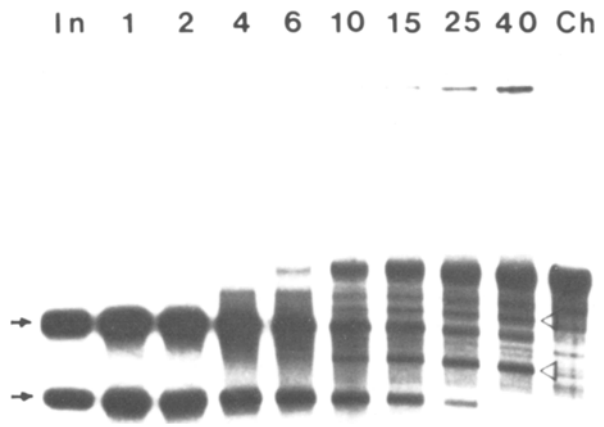

Fig. 12. Autoradiogram demonstrating the kinetics of homologous integration of linear pCML6 (Barany et al., 1983) donor DNA into the chromosome. Competent cells were incubated with nick-translated linear pCML6 DNA, and after various indicated times DNA was reisolated, digested with *Bst*EII, and electrophoresed on an agarose gel. *Bst*EII cleaves the linear input (*In*) DNA molecules once, yielding fragments of 9.6 kb and 4.8 kb (indicated by arrows). However, after incorporation, radioactivity corresponding to homologously integrated DNA is contained in chromosomal junction fragments of 12.5 kb and 7 kb (open triangles). *Ch* represents a *Bst*EII digest of DNA reisolated from cells which had taken up a nonhomologous 14-kb fragment

pear to represent normal protein constituents of outer membranes.

Although the effect of phenol extraction on cells is poorly understood, the following points can be made: (1) under high cesium salt conditions, phenol removes components rich in LPS from the outer membrane along with a subset of outer membrane proteins; (2) when phenol extracts of competent and noncompetent cells are compared, the release of competence-specific polypeptides, including a 29-kilodalton polypeptide observed by Concino and Goodgal (1981, 1982), is observed; (3) material in the phenol pellet is greatly enriched for some of the competence-related polypeptides and contains a subset of outer membrane proteins; and (4) protected-state DNA preferentially enters the phenol phase and appears to be contained within discrete membrane structures.

The above experiments lend strong support to the hypothesis that DNA is packaged into transformasomes. Transformasomes contain a 29-kilodalton competence-specific protein which may be the DNA receptor. This receptor, by binding tightly to specific sites in DNA, could hold the DNA long enough for packaging to be initiated. But the unanswered question relates to how the DNA actually enters the transformasome. The process may be analogous to phage DNA packaging. Both lambda phage and *Salmonella* phage P22 initiate packaging from specific DNA sites into preformed head structures (Earnshaw & Casjens, 1980). Perhaps an evolutionary relationship exists between the two processes.

Integration of Donor DNA

To complete the transformation process, donor DNA must recombine with the cell chromosome. This final step appears to be considerably more time consuming than the initial rapid uptake of DNA into the transformasome. Genetic experiments show that recombination reaches a maximal level about 60 min after the addition of donor DNA, although some recombination is observed as early as 5 min (Voll & Goodgal, 1961). Experiments with radioactive donor DNA give similar results (Barany et al., 1983; Kahn et al., 1983). By reisolating total DNA from competent cells at various times after the addition of radioactive donor DNA, and digesting this material with restriction enzymes, junction fragments representative of homologous integration are observed as early as 6 to 10 min, but it takes 40 min for maximal incorporation (Fig. 12). The lag between uptake and integration may represent the time it takes for donor DNA to successfully search for its homologous region in the chromosome.

Donor DNA molecules with free ends do not appear to remain dormant within transformasomes for any substantial time. They begin to exit almost immediately after uptake, and movement into the cell is accompanied by considerable degradation (Stuy, 1965; Notani, 1971; Barany et al., 1983; Kahn et al., 1983). This degradation is not unexpected since Notani and Goodgal (1966) have demonstrated by density labeling that only a single strand of donor DNA integrates into the chromosome. Thus 50% of each donor DNA molecule is not used. However, recent studies using uniformly-labeled cloned (10 kb) donor DNA show greater than 50% degradation (Barany et al., 1983; Kahn et al., 1983). In fact, only about 10 to 15% of the donor label can be localized by restriction analysis in junction fragments indicative of homologous recombination. Approximately 85 to 90% of the donor DNA is degraded and most of the label is randomly reincorporated into the chromosome.

The high percentage of degradation can be interpreted in two ways. Either 70% of the donor molecules are completely degraded and a single strand from each of the remaining 30% of the molecules is efficiently integrated into the chromosome, or on average, each molecule undergoes limited degradation, and a single strand of each partially degraded

molecule efficiently integrates. Experimental evidence suggests that the latter interpretation is more correct. Barany et al. (1983) examined the kinetics of uptake and integration of radioactive donor DNA and noted a lower molecular weight intermediate (*see* Fig. 9) that was most prevalent 6 to 10 min after uptake and seemed to chase into the chromosome at later times. This species is associated with the transformasome because it remains sensitive to *in vitro Hind*III digestion and can be observed by electron microscopy of phenol-acetone extracts of competent cells (which selectively removes protected state DNA (Kahn et al., 1983)). It appears to be generated by progressive degradation of the DNA terminus entering into the cell. Four lines of evidence support this conclusion: (1) Gel analysis and electron microscopy show that the transformasome-associated DNA molecules are double-stranded but have short single-stranded regions at their ends (M.E. Kahn, *unpublished*), (2) covalently-closed circular DNA molecules and molecules with covalently-closed termini remain in the transformasome and are not degraded, (3) a significantly greater percentage of donor label enters junction fragments if donor molecules are internally labeled rather than uniformly labeled, and (4) the relative amount of donor label entering junction fragments increases as donor DNA length increases (Barany et al., 1983; Kahn et al., 1983).

These findings fit best with the hypothesis that one strand undergoes obligatory degradation during exit from the transformasome, while the other strand either becomes integrated or undergoes further degradation. The internalizing strand can be imagined to search the chromosome for a region of homology while at the same time undergoing continuous degradation. As a consequence of such a competitive process, each entering DNA molecule could be degraded by some average amount before successful homologous pairing occurs. Thus short molecules would suffer a greater percentage of degradation than larger molecules; and, conversely, the fraction of a small molecule integrated into the chromosome would be less than for a large molecule (Barany et al., 1983; Kahn et al., 1983). Preliminary experiments with cloned *Haemophilus* markers on different lengths of DNA support this conclusion.

When no homology exists, complete degradation should occur, and this should be at the same initial rate observed with homologous DNA. This is indeed found when heterologous donor DNA is used (Stuy, 1974; Kahn et al., 1983). Another consequence of the competing degradative process is that donor DNA would not accumulate in the cytoplasm as a free single strand (and none has been observed experimentally). The only observed intermediate is that associated with the transformasome. Since only a short length of single-stranded DNA is imagined to protrude from the transformasome into the cell interior, it seems likely that recombination takes place in close proximity to the transformasome.

A final question concerns whether it is the incoming 5′ or 3′ strand which integrates. This has been answered by constructing donor DNA molecules with one terminus covalently closed, thus forcing exit from the transformasome by the free end. To make unambiguous analysis possible, the molecules were internally labeled at a unique *Bst*EII restriction site. Therefore, integration of the 3′ strand could be distinguished from integration of the 5′ strand by determining which *Bst*EII-generated junction fragment became labeled. A strong preference for integration of the 3′ strand was observed (Fig. 13; Barany et al., 1983).

Efficiency of Integration

Although integration is not a rapid process and is associated with significant degradation of donor DNA, numerous lines of evidence suggest that it is quite efficient. Under saturating conditions for donor DNA, *H. influenzae* cells can be transformed at frequencies of from 0.5 to 5% for a single chromosomal marker. Several laboratories have determined that 10^9 competent cells will take up about 100 ng of DNA (Barnhart & Herriott, 1963; Deich & Smith, 1980), averaging 15×10^6 daltons (Marmur, 1961), or about four molecules per cell. Estimates of the *Haemophilus* genome size range from approximately 0.8×10^9 (Berns & Thomas, 1965; Michalka & Goodgal, 1969) to 1.4×10^9 (MacHattie, Berns & Thomas, 1965) daltons, so four molecules should represent from 4 to 8% of the bacterial genome. Therefore, observed frequencies are within a factor of 2 to 3 of theoretical. A similar conclusion is reached from calculations of the specific biological activity of serially diluted donor DNA (R.M. Herriott, *personal communication*).

Therefore, one would predict that transformation frequencies of 50 to 100% should be achievable using cloned DNA possessing selectable markers. However, recent studies with cloned antibiotic markers yield lower than expected frequencies (less than 10%) (M. Pifer, *personal communication*). This paradoxical result is presently not understood, but donor DNA length may possibly be an important factor in determining maximal transformation frequency. There may also be spatial effects in which the proximity of the entering donor DNA to

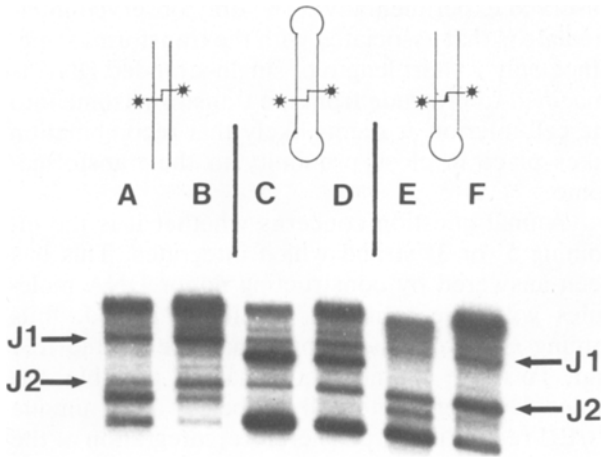

Fig. 13. Evidence for the preferential integration of the entering 3′ strand of donor DNA. Plasmid pCML6 DNA molecules were labeled at the *Bst*EII site by 5′-end labeling and then religated. These molecules were then cleaved opposite to the labeled site to produce linear molecules with internal label. After incorporation, reisolation, and recleavage with *Bst*EII, junction fragments of 12.5 kb (*J1*), and 7 kb (*J2*) representing homologously integrated material were obtained. Lanes *A* and *B* are DNA reisolated after 20 and 40 min incubation. Note that both junction fragments show approximately equal intensity on the autoradiogram of the dried electrophoretic gel. In lanes *C* and *D* the same DNA was used, but with the free ends capped with poly(dG-dC) hairpins (Barany et al., 1983). Since a free end is required for exit of donor DNA from the protected state, only those molecules with nicks can exit. Therefore, after a 20 min (*C*) or 40 min (*D*) incubation, much of the donor DNA remains unincorporated. Of the molecules that did exit and become incorporated, there is approximately equal labeling of the junction fragments. When one covalently closed end was removed by restriction to allow oriented exit, radioactive label entering junction fragment *J1* resulted from the integration of the 5′ strand, while label incorporated into junction fragment *J2* resulted from integration of the 3′ strand. Since more label is incorporated into junction fragment *J2* than *J1* after both a 20 min (*E*), and 40 min (*F*) incubation, there appears to be preferential integration of the 3′ strand

its homologous region in the chromosome determines the probability of successful integration.

Recombination Deficient Mutants

A series of *rec*-1 mutants defective in transformation have been described (Notani & Setlow, 1974). Several of those showing transformation efficiencies of less than 10^{-4} of wild type completely degrade the donor DNA (Setlow et al., 1972; Kooistra & Venema, 1974; Kooistra & Setlow, 1976). In another class of *rec*-1 mutants, *ird* (Kooistra & Venema, 1980), recombination is only partially impaired. A third class of *rec*-1 mutants is characterized by inability to replicate donor-recipient complexes (Kooistra, Boxel & Venema, 1983). Since the *rec*-1 gene is phenotypically similar to the *rec*A gene of *E. coli*, it is possible that pairing of donor and recipient DNA during transformation takes place by a *rec*A-type mechanism (Radding, 1982).

A second gene involved in recombination during transformation is *rec*-2. *Rec*-2 mutants transform 10^{-7} less well than wild type and are unable to degrade donor DNA (Notani & Setlow, 1974). It would be of interest to follow the biochemical fate of donor DNA in these cells in light of the recently proposed transformasome theory.

Epilogue

DNA entry in *H. influenzae* transformation is operationally divisible into two steps. In the first step, DNA traverses the outer membrane and becomes sequestered in specialized outer membrane structures, the transformasomes. This step corresponds to classically described DNA uptake, in which DNA becomes irreversibly bound and DNase-resistant. In the second step, the sequestered DNA linearly traverses the cytoplasmic membrane by a mechanism reminiscent of DNA entry in *Streptococcus* and other gram-positive organisms.

To unravel the membrane transport mechanism, a first step will be to isolate the relevant genes and determine their products. It will be difficult to accomplish this by standard genetic analysis, but cloning provides a new approach. To understand the mechanism at a biochemical level it will be important to develop an *in vitro* system. Isolated vesicles, preferably from wild type cells, should provide a good starting point. Ultimately, as protein components of the mechanism are isolated, it may be possible to construct a reconstituted system capable of specific binding and DNA transport.

We will have achieved our objective in writing this review if we are successful in attracting even one membrane biologist into this intriguing field, as progress can no longer be expected purely from traditional genetics, but will depend more and more on a combined molecular and biochemical approach.

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