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MAIN FEATURES ON TAILED PHAGE, HOST RECOGNITION AND DNA UPTAKE

Lucienne Letellier, Pascale Boulanger, Laure Plançon, Pierre Jacquot, and Monica Santamaria

Institut de Biochimie et Biophysique Moléculaire et Cellulaire, UMR CNRS 8619, Université Paris Sud, Bât 430; 91405 Orsay cedex, France

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1. ABSTRACT

Phage nucleic acid transport is atypical among membrane transport and thus poses a fascinating problem: transport is unidirectional; it concerns a unique molecule the size of which may represent 50 times that of the bacterium. The rate of DNA transport can reach values as high as 3 to 4 thousands base pairs / sec. This raises many questions, which will be addressed in this review. Is there a single mechanism of transport for all types of phages? How does the phage genome overcome the hydrophobic barrier of the host envelope? Is DNA transported as a free molecule or in association with proteins? Is such transport dependent on phage and / or host cell components? What is the driving force for transport? Data will be presented for a few selected tailed phages, which are the most common type of phages and for which DNA transport has been most extensively studied. Part of the review is devoted to recent *in vitro* data which have allowed to partly decipher the mechanism of phage T5 DNA transport.

2. INTRODUCTION

Transport of nucleic acid across cell membranes is an essential natural process that occurs in all-living organisms. In bacteria nucleic acid transport takes place at the early stages of many processes: conjugation, genetic transformation, T-DNA transfer from bacteria to plant cells and bacteriophage infection. During the last few years many genes involved in DNA transport have been identified, sequenced and their gene products have been characterized. These studies have emphasized striking functional and structural similarities in the processes of conjugation, T-DNA transfer and transformation. In contrast, we still have a poor understanding of the transport mechanisms of phages genomes.

A common feature of almost all phages is that only their genome is transferred to the host cytoplasm while the phage capsid and tail remain bound to the cell surface. This situation is very different from that found in most eukaryotic viruses the envelope of which fuses with the host plasma membrane so that the genome is delivered without contacting directly the membrane. In the case of viruses infecting Gram-negative bacteria the naked hydrophilic DNA (or RNA) has to overcome two hydrophobic barriers: the outer and the inner membrane. Furthermore, it has to cross the periplasm, the space separating the two membranes that contains nucleases and the rigid polymeric sugar structure of the peptidoglycan. Phage nucleic acid transport poses a fascinating problem: transport is unidirectional; it concerns a unique polyanionic molecule the size of which

may represent 50 times that of the bacterium (50 micrometers of contour length for the double-stranded DNA of T4 phage). The rate of DNA transport, although varying from one phage to another, can reach values as high as 3 to 4 thousand base pairs / sec, a value significantly larger than that attained during the transport of DNA in conjugation and natural transformation (100 bases / sec). This raises many questions. Is there a single mechanism of transport for all types of phages? How does the phage genome overcome the hydrophobic barrier of the host envelope? Is DNA transported as a free molecule or in association with proteins? Is such transport dependent on phage and / or host cell components? What is the driving force for transport?

This review focuses predominantly on tailed phages for which DNA transport has been the most extensively studied. *Caudovirales* which represent the largest group of bacteriophages (96,4%) include about 5000 members occurring in over 100 genera of bacteria (1). It is our belief that despite the broad diversity of phages some general rules will emerge from these studies applying to the transport of genomes of other phages.

3. CHARACTERISTICS OF TAILED PHAGES

Tailed phages are formed of a proteinaceous head (capsid) and of a thin tube or tail. The size of the capsid which varies usually between 50 and 100 nm, contains the viral genome. Tail length differs greatly between phages types and can be up to 200 nm. The tail ends with a tail tip and fibers, which ensure the binding of the phage to the host. Almost all tailed phages contain a linear duplex DNA molecule. This DNA is highly condensed into the capsid. As a mater of example the DNA of phage T4 (172 kbp), occupies a volume of 4 x 10^9 nm^3 and has a radius of gyration of about 1000 nm when released in aqueous solution, whereas it occupies a volume of 5x 10^5 nm^3 when confined into a 50 nm capsid. Therefore the packing of the polyanion represents an increase of its density by a factor of 10^4. Although the length of the DNA strongly varies from one phage to the other -ranging from 4 kbp to 640 kbp (a mycobacteriophage)- (2) its concentration within the capsid remains relatively constant, about 450 mg/ml (3). The DNA adopts a quasi-crystalline structure within the capsid that can be visualized by cryo-electron microscopy. Images of isolated capsids from phage T7 (4) and phage T4 (5) showed that the DNA strands are organized in concentric rings structure as a spool. The packing of DNA into the capsid relies on a molecular machine, the portal or connector complex located at one vertex of the capsid. The portal complex ensures the connection between the capsid and the tail and forms the channel through which the DNA is packaged during morphogenesis and exits during infection (6).

4. PHAGE - HOST RECEPTOR RECOGNITION

4.1. General features of phage receptors

Infection of Gram-negative bacteria by tailed phages follows almost the same general scheme: the tail fibers make first contact with the cell surface. This binding is followed by specific and irreversible binding of one of the proteins located at the tip of the tail to an outer membrane component. Phage binding triggers conformational changes that are transmitted along the tail to the connector allowing its opening and the release of the viral genome from the capsid. The genome is then transferred via the tail, through the host envelope. Interestingly, for certain phages like T5 no component other than the phage and the host receptor are required for DNA release (see § 9). Almost all surface components including flagella, pili, capsules, lipopolysaccharides and proteins serve as receptor for phages. Most of the outer membrane phage receptors have been identified and their interactions with phage receptor-binding proteins demonstrated mainly by genetic approaches (for an exhaustive review see (7). On the other hand, few of the interactions have been investigated starting from the isolated purified proteins. The interaction between the *Escherichia coli* phage lambda and its receptor, the maltoporin LamB, is mediated by the tail protein gpJ. Specific interactions between the C-terminal portion of gpJ and purified LamB were demonstrated by dot blot immunoassays (8). In the case of the *Salmonella* phage P22, the tailspike protein binds reversibly to the O-antigen repeating units of the lipopolysaccharide. This allows the phage to move over the surface of the bacteria and to partially destroy the lipopolysaccharide by its endoglycosidase activity. Crystal structures of the tailspike protein in complex with oligosaccharides were determined at 1.8 Å resolution allowing the identification of the active-site topology (9). Phage PrD1 has very interesting features. It infects a broad range of Gram-negative bacteria harboring a conjugative plasmid such as IncP plasmid RP4. Its 15 kbp double-stranded DNA molecule is confined inside a membrane that is enclosed in a proteinaceous capsid. PrD1 is functionally and structurally related to adenovirus suggesting an evolutionary relationship between bacterial and animal virus (10). Its receptor binding protein, P2, is the structural equivalent of the adenovirus receptor binding spike knob (11). A functional form of P2 was purified that binds to its receptor with very high affinity (apparent K_d = 0.2 nM) (12). Low-resolution models of the P2 protein showed that the protein has an elongated shape (13). Further studies of the structural and functional differences between this prokaryotic virus and adenovirus might be of general interest to unravel the evolutionary relationship of viruses.

4.2. FhuA, an E.coli outer membrane iron transporter and phage receptor

FhuA is a 78 kDa *E.coli* outer membrane protein that transports iron chelated to the siderophore ferrichrome. Iron-ferrichrome transport across the outer membrane is coupled to the electrochemical gradient of protons in the cytoplasmic membrane *via* the cytoplasmic membrane-anchored protein complex TonB-ExbB-ExbD. Besides its physiological function FhuA is also the receptor for phage T1, T5, UC1 and phi80. Infection by phages T1, and phi80 – but not by T5-, is coupled to TonB and requires an energized cytoplasmic membrane (reviewed in (14). The recent determination of the three-dimensional structure at 2.5 Å resolution of FhuA (15);(16) has revealed an unexpected feature of this protein. FhuA is composed of a barrel domain consisting of 22 antiparallel beta-strands lodged in the membrane and of an amino-terminal globular domain that

folds inside the barrel and occludes it. This “plug” or “cork” domain, which spans most of the interior of the beta-barrel, consists of a four-stranded beta-sheet and four short helices which are connected to the beta-barrel and to hydrophilic loops facing the external medium. One of this loop (L4) serves as binding site for phages T1, T5 and phi80 (17). *In vitro* studies have allowed to characterize at a molecular level the interactions of FhuA with phage T5.

4.2.1. FhuA-phage T5 *in vitro* interactions

T5 is a phage with a flexible non-contractile tail, 160 nm in length and a double-stranded DNA of 121 kbp. Host recognition is initiated by reversible binding of the long tail fibers to the O-antigen of the lipopolysaccharide (18). Then the phage irreversibly binds to FhuA by means of pb5, a 67.8 kDa protein located at the distal end of the phage tail (19-21). This binding triggers conformational changes that are transmitted through the tail to the head-tail connector initiating its opening and the release of the DNA which is then transferred through the bacterial envelope (reviewed in (22)). An interesting feature of T5 is its ability to functionally interact with FhuA *in vitro* (7, 23). Regions of FhuA and pb5 were defined that are essential for phage binding and DNA ejection. Data from Killmann *et al.* suggested that a hexapeptide purified from the L4 loop of FhuA is sufficient to trigger DNA release from phage T5 particles (17). The receptor-binding domain of pb5 was shown to be confined to ca. 200 aa within the N-terminal half of pb5 (24, 25).

pb5 was overproduced in *E.coli* and purified (24, 26). The protein, which is monomeric, has a high beta sheet content (51 %), a property shared with the adsorption proteins of phage P22, PRD1 (9, 12) and of adenovirus (27) suggesting that this type of secondary structure could be relevant for the function of these proteins in phage infection. pb5 was functional *in vivo*. It impaired infection of *E.coli* cells by phage T5 and phi 80. pb5 also inhibited growth of bacteria on iron-ferrichrome suggesting that it either hindered the ferrichrome binding site or changed the conformation of FhuA so that ferrichrome binding or transport were impaired. pb5 was also functional *in vitro* since addition of equimolar concentration of pb5 to purified FhuA prevented *in vitro* DNA release from phage T5. Direct interaction of pb5 with FhuA was demonstrated by isolating an equimolecular complex of pb5 and FhuA using size exclusion chromatography. This complex was stable over a large range of pH between 4.0 and 8.8 and it was not dissociated by 2% of the detergent SDS even if the temperature was raised up to 70 °C. The formation of the complex was correlated with a strong stabilization of both pb5 and FhuA since thermal denaturation of the complex occurred at 86°C while pb5 and FhuA were denatured at 45°C and 74 °C respectively. The strength of the association between pb5 and FhuA is reminiscent of the irreversible nature of the phage adsorption step in the infectious process. No complex was formed when pb5 was added to FhuA delta021-128, a mutated FhuA of which almost all the cork had been removed. Removal of the cork domain of FhuA also impaired phage T5 binding and DNA release *in vitro* (28). Altogether this indicates that the binding affinity of phage T5 depends not only on the externally facing loop L4, but also on the global structure of the protein, the integrity of which is ensured by cork.

The availability of the pb5/FhuA complex opens the way to crystallographic studies and to the determination of the three-dimensional structure of the complex. This should provide new insights into the conformational changes undergone by FhuA upon binding of pb5 in the absence or presence of ferrichrome.

4.2.2. Role of TonB in infectivity by phage phi80

The mechanism that supports iron transport across the outer membrane and phage infection seems a non-solved paradox. This transport is an energy-dependent process. However, energy sources are neither available within this membrane nor in the periplasm. Gram-negative bacteria have solved this dilemma by evolving a system whereby the energy derived from the electrochemical gradient of protons generated by the electron transfer chain in the cytoplasmic membrane is transduced across the periplasm to the outer membrane receptors. Energy is transduced by means of a protein complex consisting of three cytoplasmic membrane-anchored proteins TonB, ExbB and ExbD (14). TonB is the only protein of the complex required for infection by phages phi80 and T1.

TonB is a 26-kDa protein. Its N-terminus is in the cytoplasm and the protein is anchored in the inner membrane by its uncleaved N-terminal signal sequence. The central domain contains a large proline-rich sequence that is thought to confer to TonB a conformational rigidity and an extended shape allowing its C-terminal domain to contact the outer membrane receptor (29). The crystal structure of the C-terminal domain of TonB (residues 164 to 239) from *E. coli* was solved at 1.55 Å resolution (30). TonB forms a tightly intertwined antiparallel dimer that is cylinder-shaped with a length of 65 Å and a diameter of 25 Å. The relationship between this dimeric structure and the functioning of TonB remains speculative. Extensive studies have allowed the determination of the regions of TonB and of the receptors that physically interact (31). A short stretch of conserved amino acids in the N-terminal region of the outer membrane receptors known as TonB box is in part, responsible for the physical interaction with TonB. The periplasmic domain of TonB is sufficient for interaction with the receptors but TonB recognizes preferentially the ligand-loaded receptors both *in vivo* and *in vitro* (31, 32). How phage binding to FhuA depends on TonB is still badly understood. The TonB-dependent infectivity of T1 and phi80 is preserved upon removal of the cork domain of FhuA suggesting that TonB not only interacts with the cork (via the "TonB box") but also with the barrel (33, 34). This also implies that TonB generates large conformational changes in FhuA that spread from the periplasmic side to the surface loop L4.

TonB fragments that contained part or the entire periplasmic domain of the protein were expressed in *E.coli* (35). Overexpression of these fragments that were targeted to the periplasm, abolished sensitivity of cells to phage phi 80 suggesting that they can functionally interfere with wild-type TonB presumably by competing for binding sites at the

transporters or by forming non functional dimers with TonB. It is therefore likely that neither recognition nor binding to siderophore receptors is the energy-requiring step in the TonB-receptor interactions.

5. CROSSING THE PERIPLASM

One major obstacle to the traversal of the periplasm by phage DNA is the presence of nucleases. How do phages avoid the space separating the two membranes? On the basis of electron microscopy observations, Bayer proposed that infection by phages would take place at specific regions of the envelope where the inner and the outer membrane are in close contact (36). Thirty-five years later the origin of these contact sites is still a matter of debate: are they preexisting in non-infected cells or formed transiently during infection? Are they made up of lipids, proteins? The participation of phage proteins in forming these contact sites is suggested by fractionation of the envelope of T5-infected *E.coli* cells: a protein belonging to the phage tail, pb2, was recovered in a membrane fraction containing proteins of both the inner and the outer membrane (37). pb2 was only present in the envelope upon DNA transfer, in agreement with previous results suggesting that pb2 would form a "channel" for DNA transport (38). Arguments in favor of such hypothesis will be presented below.

6. CROSSING THE PEPTIDOGLYCAN

Another still unsolved and almost always-ignored problem in phage DNA transport is the traversal of the peptydoglycan. On the basis of data on the three dimensional structure of the peptidoglycan and of measurements of penetration of fluorescein-labeled dextrans, Koch and collaborators (39) concluded that the cut-off value for the passage of proteins through the peptidoglycan is about 50 kDa and that the peptidoglycan meshwork would contain a few holes with a diameter larger than 4 nm. The size of these holes might be sufficient to permit the passage of the proteins forming the tail tip of different phages (T5, T4, see below). Alternatively peptidoglycan hydrolases might play a role in breaking down the murein wall thus facilitating the passage of the viral genome. Such a proposal is suggested by a recent search in protein databases that revealed sequence similarities between catalytic domains of transglycosylases of bacteria and bacteriophages (40).

7. CROSSING MEMBRANES: IS THE DNA PUSHED OR PULLED?

The phage genome starts to be transferred to the host cytoplasm within a few seconds following binding to the receptor. Available evidence indicates that the DNA of tailed phage crosses the host membranes linearly nucleotide pair by nucleotide pair and in the direction opposite to that in which it was packaged. Two general hypothesis of DNA transport have been formulated. The first one proposed that the packaging machinery might simply introduce DNA under sufficient pressure in the capsid during morphogenesis to allow spontaneous ejection of the genome upon infection (41, 42). The second hypothesis proposed that the electrochemical gradient of protons across the host cytoplasmic membrane would be the driving force for DNA transport (43).

7.1. Pressure inside the capsid and DNA ejection

The confinement of DNA into the tiny volume of the capsid, the electrostatic repulsion between the highly charged DNA chain, its folding in dimensions comparable to its persistence length and the important reduction of entropy involved in this packaging are at the origin of the strong energy stored in the capsid. Several models proposed the ejection to be directly governed by the difference between the pressure inside and outside the capsid. The outside pressure is related, *in vivo*, to the high concentrations of proteins inside the bacterial cytoplasm and *in vitro*, to the osmotic pressure of the solution surrounding the phage. The pressure inside the capsid and the ejection forces were estimated theoretically (44). Gelbart and collaborators (45) calculate the forces required to package the DNA and to eject it from the capsid as a function of the length of DNA loaded. They conclude that the initial ejection is a passive process, being driven by the pressure gradient. They also conclude that the passive stage of DNA injection in the host should in general be incomplete given the osmotic pressure inside the host cell, which is believed to be on the order of a few atmospheres. Recent experimental data were obtained on phage phi29, a *Bacillus subtilis* tailed phage, that provided new insights into the dynamic process of nucleic acid packaging within the capsid during morphogenesis and allowed to estimate the internal capsid pressure. The connector of phage phi29 was visualized *in situ* by cryo electron microscopy (46) and its structure was determined at atomic resolution (47, 48). A model of DNA packaging was proposed on the basis of the structural data and of the analysis of cryo electron microscopy image reconstructions: ATP would drive the rotation of the connector step by step. Each rotation would translate two base pairs of DNA into the capsid. The reverse process is likely to proceed upon opening of the connector during DNA ejection but in that case the energy would not be provided by ATP (which is not present in the capsid) but by the internal capsid pressure. The possibility to package phage phi29 *in vitro* has allowed the group of Bustamante to measure the forces involved in the packaging of a single DNA molecule into an isolated capsid (49). Using laser tweezers they demonstrated that the portal complex is indeed an extremely powerful motor that couples rotation to DNA translocation using ATP as an energy source. They could measure the rate of packaging of the 19 kbp phi29 genome and the force generated by the motor throughout the entire packing process. At maximum the motor pulled with about 60 picoNewtons of force. The built force makes the connector of phi29 one of the strongest molecular motors reported to date. By dividing this force by the DNA hexagonal cell surface area they estimated the pressure inside the capsid to be roughly 6MPa (60 Atmospheres), which represents about ten times the pressure inside a bottle of champagne!

7.2. The chemiosmotic theory of DNA transport

On the basis of the chemiosmotic theory of Peter Mitchell (50), Grinius (43) postulated a “ universal ”

mechanism of DNA transport in which the electrochemical gradient of protons generated across the host cytoplasmic membrane by the electron transfer chain would be the driving force for DNA transport. The polyanionic DNA molecule would be co-transported with protons down their gradient. This attractive hypothesis was supported by experiments showing that phage T4 DNA transport took place only in energized membranes and above a threshold of membrane potential of -90 mV (51). However, further experiments revealed that the membrane potential was not required for transport of the DNA but rather for opening of a voltage-gated "DNA channel" (52). This hypothesis also failed to explain T5 DNA transport since it was shown that the DNA could cross the cytoplasmic membrane of de-energized cells (53). Obviously, the chemiosmotic theory did not provide a framework to understand phage DNA transport.

The complexity of phage DNA transport is attested by the fact that none of the phages (T4, T5 and T7) for which it was studied uses similar strategies. Yet, a common feature of these phages is that the transfer of their genome is accompanied by a transient efflux of cytoplasmic potassium down its gradient that strictly follows the timing of DNA transport. A quantitative analysis of this efflux allowed to define some general characteristics of this transport (reviewed in (22)).

8. CROSSING MEMBRANES: DIFFERENT PHAGES ADOPT DIFFERENT STRATEGIES

8.1. Phage T4

Binding of T4 phage to its receptor, the lipopolysaccharide, triggers tail contraction; the tip of the internal tube of the tail is then brought close to the cytoplasmic membrane and the 172 kbp of the DNA cross the membranes in about 30 sec at 37°C. This represents the highest rate (approximately 4000 bp / sec) observed for DNA transport. As a matter of comparison, the rate of the ATP-dependent DNA translocation through the connector during packaging is of the order of 140 bp / sec (48). The crystal structure of the center of the baseplate called by Rossmann and collaborators "the cell-puncturing device" was recently solved (54). It consists of two proteins, gp5 and gp27, that form a trimeric complex. The structure of the complex resembles a torch with a length of 19 nm. The gp27 trimer forms the head, a hollow cylinder with an internal diameter of 3 nm. The gp5 trimer, which forms the handle, consists of three domains: the carboxy-terminal domain is a triple-stranded long beta-helix, the middle is a lysozyme domain which serves to digest the peptidoglycan and the amino-terminal, an antiparallel beta-barrel domain that inserts into the cylinder formed by gp27. The diameter of gp27 is of a size that can accommodate a double-stranded DNA helix. The authors proposed that upon attachment of the base plate to the cell surface, the tail sheath contraction exerts a force onto the tail tube towards the cell membrane. This force would be transmitted through the gp27 cylinder and the N-terminal domain of gp5 to the beta-helix, allowing the later to act as a membrane-puncturing device. As the contraction of the tail sheath progresses, the beta helix would span the outer membrane allowing the lyzozyme domains to digest the peptydoglycan and the penetration of the tail tube through the inner membrane for injection of the DNA into the host cytoplasm. This splendid structure, which, in addition, fits into a cryo-electron microscopy map of the base plate-tail tube assembly, gives for the first time a mechanistic view of how phage tail proteins cross the bacterial envelope. We previously concluded that T4 DNA transport takes place through a phage protein forming a voltage-gated channel that opened above a threshold of membrane potential and remained opened only during DNA transport (52).We speculate that the membrane potential dictates the conformation of gp5 and its insertion in the membrane. What is then the "driving force" for T4 DNA transport? We are having no satisfying answer to that question. Gabashvili and Grosberg (55) proposed a theoretical model that described the dynamics of DNA release as a reptation process through the phage tail, the driving force being the decrease of the condensed DNA free energy. T4 DNA transport takes place within 30 sec, a too short delay to permit transcription of the phage genome so that it is unlikely that the DNA is mechanically pulled by its own polymerase. Alternatively, the DNA might be pulled and condensed while entering the cytoplasm by attachment of host histones like HU (56).

8.2. Phage T7

Phage T7, in contrast to T4 does not contain a contractile, penetrating tail. It has a small genome (40 kbp). T7 is one of the best-characterized genetic systems and the phage genome can easily be modified for specific purposes. Phage T7 is the only one for which it has been clearly demonstrated that DNA internalization is coupled to transcription (reviewed in (57)). About 850 bp of the left end of the genome are passively transferred into the host. Promoters located on this short sequence allow the host RNA polymerase to initiate transcription and to pull about 20% of the genome into the cell. T7 RNA polymerase is then synthesized and the remaining DNA is transcripted by the phage enzyme. The internalization of the genome takes about 10 min at 30°C and is associated with an efflux in two steps of cytoplasmic K^+ that correlates with the two transcription events (58). This slow rate suggests that the limiting step in transport could be the rate of transcription. *In vitro* studies using laser tweezers (59) have elegantly demonstrated that a transcribing RNA polymerase is a strong and efficient molecular motor. The characteristics of this motor would permit the phage genome to be mechanically pulled into the cytoplasm by the polymerase. However, this proposal failed to explain how T7 mutants that did not require transcription internalize their genome. In such mutants, the rate of DNA transport is constant over the entire genome. Such observation is not consistent with the proposal of Kindt *et al.* (45) that the gradient of pressure between the inside and the outside of the capsid governs the ejection of the DNA. Mutations that uncoupled DNA transport from transcription were all located in gp16, a protein that together with gp14 and gp15, forms the internal core of T7. Since all three proteins were found in membranes of infected cells, this led the authors to propose that they would form the DNA channel across the host envelope. How these proteins contribute to pull the DNA remains speculative.

8.3. Phage T5

A unique feature of phage T5 is that its genome (121 kbp) is transferred in two steps (60) : 8% of the DNA (First Step Transfer or FST DNA) enters first the cytoplasm. Then, there is a pause of about 4 minutes (at

37°C) during which proteins encoded by this fragment are synthesized. Two of these proteins (A1 and A2) are required for the transfer of the remaining DNA (92%) or second step transfer (SST) DNA. A1 (M_r = 64 kDa) is implicated in at least three functions: the degradation of host DNA, the shutoff of pre-early transcription and together with the product of gene A2 in completion of phage DNA transfer. A2 (M_r = 15 kDa) is a homodimeric DNA-binding polypeptide that appears to form an oligomeric structure with A1 (61, 62) .If one prevents synthesis of A1 and A2, then the SST DNA still connected to the phage head and attached to the injected FST fragment, crosses the outer and inner membranes without being degraded suggesting that the DNA is protected from periplasmic nucleases during its transport. The reason why phage T5 transfers its DNA in two steps remains unclear. The arrest of transfer at the FST stage is not due to newly synthesized phage proteins encoded by the FST DNA since it takes place in the presence of inhibitors of protein synthesis. Cloning and sequencing of the terminal region of the FST DNA fragment revealed that it contained several large inverted repeats forming potential stem-and-loop structures that could jam the DNA during its transfer (63). Data from Davison & Brunel (64, 65) suggest that T5 specifies a restriction protection function, encoded by the FST DNA that may protect the viral DNA against several bacterial restriction systems (EcoRI, EcoKI, EcoPI). We believe that host factors are not involved in the transfer in two-steps *in vivo* since phage T5 DNA ejection also proceeds in two steps when triggered *in vitro* by interaction with its receptor (see below). Filali Maltouf and Labedan (53) previously demonstrated that both the FST and SST DNA could be transferred in cells deprived of metabolic energy sources (electrochemical gradient of protons and ATP). This again raises the question of the force driving DNA transport. Relief of the capsid internal pressure is likely to explain the passive transfer of the FST DNA but not that of the SST DNA. Indeed, the capsid and tail of phage T5 can be sheared off the bacterial cell surface once the FST DNA has been transported into the cytoplasm. Under these conditions the SST DNA (approximately 27 micrometer in length) is floating in the surrounding medium, but is still attached to the FST DNA and crossing the envelope. If one provides the energy for the synthesis of A1 and A2, then the free DNA is transported through the membranes. It is therefore obvious that the DNA has to be pulled inside the cytoplasm. One reasonable hypothesis is that bacterial histones and/or the DNA binding protein A2 encoded by the FST DNA, contribute to this transport. The possibility to mimic T5 DNA transport into liposomes (see below) should help to validate this hypothesis.

The transfer of phage T5 DNA is correlated with a transient depolarization of the cytoplasmic membrane and an efflux in two steps of cytoplasmic K^+ that strictly follows the timing of DNA penetration (66). We previously showed that this efflux was the consequence of the opening in the cytoplasmic membrane of a channel, the function of which was to transfer the DNA: this channel opened upon FST DNA transfer then closed during the synthesis of the FST-encoded proteins and reopened transiently during the transfer of the SST DNA. The participation of the tail fiber phage protein pb2 in forming the DNA channel was first proposed on the basis of electrophysiological studies (38) and further supported by fractionation of the envelope of T5-infected E.coli cells (37). Arguments in favor of this proposal are given below.

T5 infective process requires the presence of at least 0.1 mM calcium. Below this threshold FST-encoded proteins are not produced and infected cells enter a dormant state. We observed that calcium regulated the membrane permeability changes occurring upon the transfer of the DNA. Decreasing calcium below 0.1 mM depleted the bacteria of K^+, caused a complete membrane depolarization and a decrease of cytoplasmic ATP. To account for these observations we proposed that calcium controls the conformation of the protein forming the DNA channel and that below 0.1 mM the channel remained opened. This would create an energetic state of the host unfavorable to the synthesis of phage components and lead to abortion of the infectious process (67).

9. THE MECHANISM OF PHAGE T5 DNA EJECTION CAN BE REPRODUCED *IN VITRO*

9.1. binding of phage T5 to purified FhuA triggers DNA ejection and channel opening

An attractive feature of T5 is that DNA release from the phage can be triggered *in vitro* simply by interaction of the virus with purified FhuA solubilized in detergent (23). DNA release was demonstrated spectroscopically using a fluorescent DNA intercalant YO-PRO-1 (68) the fluorescence of which is increased in proportion to the amount of DNA freed from the capsid. Phages bound to FhuA ejected virtually all their DNA in the surrounding medium in less than a few seconds. We recently followed the dynamics of the ejection of single T5 DNA molecules by fluorescence microscopy. Interestingly, DNA ejection proceeded very fast (within a few hundred msec) and in two steps. This indicates that DNA release in two steps is due to an intrinsic property of the phage or of the FhuA receptor (Mangenot *et al.*, in preparation).

Binding of phage T5 to FhuA incorporated into a planar lipid bilayer triggered the opening of a large channel (single channel conductance: 4 nS in 1.5 M KCl (69). The origin of the channel is still not totally clear. We considered two main hypotheses. One possibility is that binding of phage T5 to FhuA unmasks an inner channel in the FhuA protein that would be used by DNA to cross the outer membrane. This hypothesis is consistent with the finding that deletion in the FhuA protein of the loop which constitutes the binding site for T5, converts FhuA into a high conductance channel (70). Yet, given the crystal structure of FhuA (15, 16), it remains an open question whether such drastic conformational changes can take place. Indeed, within the beta barrel is a channel the entrance of which is constricted by three surface-exposed hydrophilic loops on one side and totally obstructed on the opposite side by the plug domain. The passage of DNA would require the unplugging of the channel and disruption of the many interactions between the plug, the β barrel and the loops. The second possibility is that the observed channel is part of

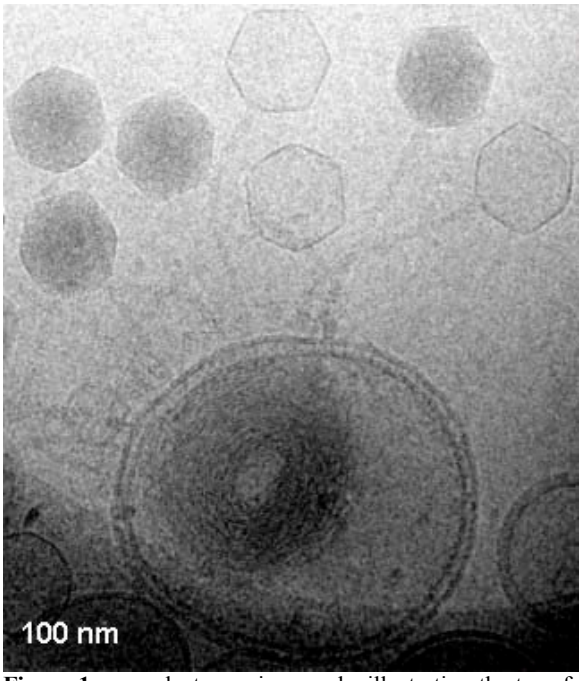

Figure 1. cryo-electron micrographs illustrating the transfer of phage T5 DNA into a double lipid layer proteoliposome loaded with 50 mM spermine : the DNA of several phages has crossed the two lipid layers and is condensed into a unique toroid occupying a volume smaller than the liposome (Bar = 100 nm). Taken from reference 82.

phage T5. Since T5 by itself did not induce channel activity, this activity would be triggered upon anchoring of a phage protein in the lipid bilayer as a result of its interaction with FhuA. The interaction of FhuA with the purified phage receptor protein pb5 was not sufficient to trigger channel opening suggesting that channel activity either necessitates another conformation of pb5 or other phage tail proteins that participate in the DNA ejection process (26). Feucht *et al.* (38) showed that isolated pb2, the protein forming the straight fiber (see § 9.5), has a porin-like activity in planar lipid bilayer. The possibility that pb2 would be responsible for the channel activity we observed remains an open question.

The above experiments show that there is no other requirement than the phage and its purified receptor FhuA, for T5 DNA to be fully ejected from the capsid. Such efficiency in DNA ejection has not been found in other systems. High conductance channels opened as a result of the interaction of phage lambda with its receptor, the maltoporin LamB from *Shigella sonnei* but this interaction was not sufficient to permit significant DNA release (71).The *E.coli* LamB receptor can trigger phage lambda DNA ejection but only when treated with chloroform of ethanol (72). Phage phi 80 binds to FhuA *in vitro* but does not release its DNA. This corroborates former data showing that phi80 infectivity requires not only FhuA but also an energized cytoplasmic membrane and the protein TonB (73). We recently expressed and purified the TonB protein carrying the amino terminal transmembrane domain (Santamaria *et al.* to be published). We observed a significant increase of YO-PRO 1 fluorescence when phage phi 80 was added to FhuA preincubated with purified TonB suggesting that some phages had ejected their DNA. This implies that purified TonB can interact with FhuA *in vitro.* We speculate that purified TonB shuttles between different conformations as proposed *in vivo* by Larsen *et al.* (74) and that those TonB proteins in an "energized conformation" (forming dimers?) (30) participate in functional interactions with FhuA. FhuA would then adopt a conformation that permits phage phi80 to bind and eject its DNA. Alternatively, we cannot exclude that ejection of Φ80 DNA is triggered as a consequence of the interaction of the phage with both FhuA and TonB.

9.2. Delivering a phage genome into liposomes

In an attempt to decipher the mechanism of phage T5 DNA transport we reconstituted FhuA into large unilamellar liposomes (100 nm in diameter) in which the fluorescent DNA probe YO-PRO-1 was entrapped. Phage T5 bound to the proteoliposomes and triggered an increase in fluorescence consistent with the transport of part of the DNA into the vesicles (75, 76). The high resolution of cryo-electron microscopy enabled the visualization of the phage-proteoliposome interactions and the unequivocal demonstration that the phage genome was delivered into the liposome (77). Surprisingly the DNA did not disturbed the morphology of the liposomes although its concentration within the vesicles reached values as high as 130 mg/ml. The amount of entrapped DNA decreased with the volume of the vesicle. This is consistent with a passive transport mechanism: DNA transfer should stop upon equilibrium of the osmotic pressure in the phage capsid and in the liposome.

Polyamines like spermine are polycations of physiological relevance that modify electrostatic interactions between DNA segments through neutralization of the charges along the DNA molecules (78). Their addition to aqueous solution of DNA led to the formation of toroidal structures (79) resembling those found in phage capsids. We raised the question whether spermine, by condensing the DNA would permit to raise the amount of entrapped DNA (80). Figure 1 shows a cryo electron micrograph of phages T5 bound to a FhuA-containing liposome made of two bilayers and loaded with 50 mM spermine. T5 phages are bound to the proteoliposome by the tip of their tails. Some of the capsids are filled with a dark gray striated material corresponding to DNA. Others are totally empty and the genome of these empty phages is visible inside the vesicle. The most striking feature is that the phage tail tip can cross not only one bilayer as shown previously (77) but two lipid bilayers upon delivery of the phage genome inside the vesicle. While no ordering of the entrapped DNA was visible in the absence of polycations (77) we observed that spermine modified the morphology of the packaged DNA which formed large ordered toroidal structures that showed no contact with the liposome. Remarkably, spermine allowed many DNA strands to be transferred in a liposome (up to 6 to 10). In all cases they formed only one toroidal structure indicating that a single toroid can collect many DNA strands that are delivered either independently or in a synchronized manner.

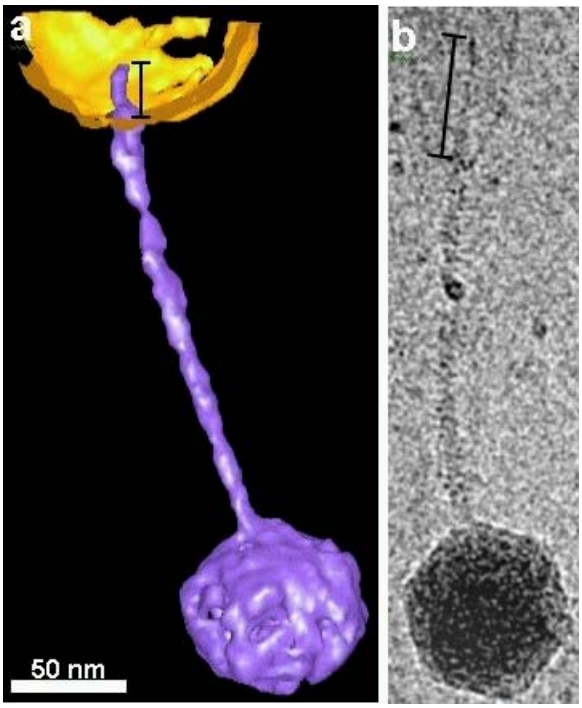

Figure 2. A: 3D reconstruction of cryo electron tomography images of phage T5 bound to a proteoliposome before DNA release. The vesicle is displayed in gold, the phage in blue. The tip of the tail is visible inside the vesicle. B: 2D cryo electron micrograph of a free T5 phage. Taken from reference 82.

Apart from understanding the mechanism of DNA transport and condensation these results might be of interest for gene therapy applications. These DNA-containing liposomes might serve as alternative vehicles to transfer foreign genes into eukaryotic cells. Vectorisation of foreign DNA is currently achieved by using cationic lipids, which form complexes with DNA through charge interactions (81). The DNA-containing liposomes described here are conceptually very different from the DNA-cationic lipids complexes since the DNA is truly entrapped in the liposomes. Furthermore, since the DNA does not interact with the lipids, this allows the lipid composition of the vesicles to be varied to contain different ratios of neutral to charged lipids, glycosylated lipids, or even cholesterol. The reconstitution procedure also allows other proteins / molecules to be co-reconstituted with the phage receptor to favour gene delivery or to be entrapped within the liposomes as shown above for spermine to protect the DNA from degradation. Finally, the origin of the DNA allows its manipulation by means of phage molecular biology strategies. The currently sequencing of the phage T5 genome (Boulanger *et al.*, to be published) should contribute in defining regions of interest to introduce foreign genes.

Cryo-electron tomography associated with images reconstructions improved the resolution of 2D electron micrographs and allowed to visualize the phage-proteoliposome interactions at a molecular level (82). Figure 2a depicts the 3D reconstruction of a T5 phage bound to a proteoliposome before the release of its genome. pb2, the protein forming the straight tail fiber is visible inside the vesicle. Interestingly, the tail fiber is shorter and larger when inserted in the liposome (23 nm in length and 4 nm in diameter) than on free phages (50 nm in length and 2 nm in diameter) (Figure 2b). Such shortening of the tail fiber is also observed when phages have released their DNA inside the liposome (not shown). To improve the significance of these observations we further characterized pb2.

9.3. Role of pb2, the T5 straight fiber protein, in DNA transport

The straight fiber consists of a unique protein, pb2 (M_r = 123 kDa) present in 5 to 6 copies in the phage structure (83). Fractionation of the envelope of infected cells previously showed that pb2 is the sole phage protein found in the membranes upon DNA transfer (37). Feucht *et al* also proposed that pb2 would play a role in channeling DNA (38). Secondary structure predictions deduced from the sequence (Genbank Accession Number AY303686 and Boulanger *et al.*, to be published) suggest pb2 to be mainly organized as a coiled-coil structure except for a short stretch of hydrophobic amino acids (30 residues) in the C-terminal part. Interestingly, the amino acid composition of the C-terminal hydrophobic stretch resembles that of viral fusion peptides (84) again suggesting an evolutionary relationship between bacterial and animal virus. We recently expressed in *E.coli* a truncated form of pb2 missing 80% of the N-terminal sequence but including this hydrophobic stretch. The protein, which is oligomeric in solution, shows multiple interesting features. pb2 induces instantaneous aggregation of bacteria and changes in their morphology particularly at division sites. It is a membrane active polypeptide as demonstrated by permeabilization assays. pb2 is also active *in vitro*. It inserts spontaneously into liposomes, and permeabilized them to solutes. Light scattering and fluorescence microscopy experiments showed an important increase in the liposomes size upon addition of pb2 suggesting that the peptide triggers the fusion of the vesicles (Jacquot *et al.*, to be published). What is the function of such a fusogenic sequence? It is noteworthy that pb2 changes conformation upon binding of the phage to FhuA and to membranes (it becomes sensitive to proteases (38) and its morphology is modified -see above-). It is tempting to propose that this change of conformation renders the peptide accessible to the host envelope and allows its hydrophobic domain to insert in the outer membrane and to fuse the outer and cytoplasmic membrane. The fusogenic domain of pb2 would have two functions: ensuring the contact between the two membranes so that to protect the DNA from periplasmic nucleases and by spanning both membranes it would form the DNA channel.

pb2 shares striking common characteristics with gpH (M_r = 92 kDa), the tape measure protein that determines the length of phage lambda tail (7, 85, 86). Both proteins are present at the same number of copies per phage particle and lose about a hundred amino acids by proteolytic cleavage at their C-terminal end during phage assembly. Secondary structure predictions suggest gpH to be mainly organized as a coiled-coil structure except for a short stretch of hydrophobic amino acids in the C-terminal part that also resembles that of viral fusion peptides (84). gpH is not only

involved in determining the length of the phage tail but also in DNA transfer into the host cell (87, 88). gpH like pb2 is resistant to detergent treatment in phage particles but not in phage ghosts or purified tails. On the basis of the analogy between pb2 and gpH, we speculate that the pb2 oligomer forms a five or six-stranded, partially coiled coil structure that spans the tail of phage T5, its N-terminal domain contacting the head-tail connector and its C-terminal domain forming the straight fiber. Upon interaction with the host the protein would undergo large conformational changes (38).

It is tempting to speculate that other phages share such fusion mechanism with T5. Indeed, the tail spike of phage p22 and gp3, a protein encoded by the filamentous phage fd also have fusogenic peptide sequences. Infection by phage fd is initiated by the binding of the N-terminus of gp3 to the tip of the F pilus. Binding is followed by large conformational changes of gp3 which then interacts with the cytoplasmic membrane-anchored protein TolA. These interactions lead to DNA transfer into the host (89). Interestingly, the oligomeric form of purified gp3 forms large pores when reconstituted into a lipid bilayer, the estimated diameter of which are large enough to allow the passage of the single-stranded DNA (90).

10. CONCLUDING REMARKS

We are far from understanding the mechanism by which a viral genome can be delivered into a bacterial cell. *In vitro* studies have been particularly stimulating allowing to approach, at a molecular level, the functioning of receptors as well as the mechanism of DNA transport and condensation. On the other hand, *in vivo* studies have been rather poorly informative. Genetics of phage has indeed contributed to identify and characterize phage receptors but similar approaches are lacking to analyse DNA transport since only few mutations affecting this specific step have until now been characterized. The sequencing of numerous genomes of phage infecting bacteria covering large phylogenetic domains with the perspective to identify new phage proteins that would share sequence homologies will certainly contribute to define some general rules of DNA transport. Progresses are also expected due to the increasing interest of scientists from different disciplines for phages. It is our belief that some of the conclusions might not be restricted to *E. coli* phages but rather being of broader significance. Indeed *Archaea* are also the targets of phages and some of these phages are of the same head and tail types as T phages and lambdoid phages. This is exemplified in the case of the haloarcheophage ϕH which resembles *E.coli* phage P1 in terms of structure and replication of its DNA and lysogeny (91). These observations strongly suggest that the common ancestor of bacteria and archaebacteria possessed viruses. Studies on virus might therefore be rich in information on evolution.

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Send correspondence to: Dr Lucienne Letellier, Institut de Biochimie et Biophysique Moléculaire et Cellulaire, UMR CNRS 8619, Université Paris Sud, Bât 430 ; 91405 Orsay cedex, France, Tel : (33) 1 69 15 64 29, Fax : (33) 1 69 15 47 27, E-mail : lucienne.letellier@biomemb.u-psud.fr