An ATP-free packaging of T4 DNA

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Research

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Abstract

Packaging of viral DNA into a capsid with the liquid crystalline density is a crucial step in viral reproduction. The DNA packaging with the ATP fueled molecular motor is an established viral DNA packaging system. However, the velocity and the efficiency are not well matched with in vivo processes. On the other hand, DNA compacts with the conformational change by multivalent cations exclusively of the valences higher than three. The conformational change of DNA was not considered as the vehicle of DNA packaging of viruses. Here, T4 DNA, ejected from a capsid in the ambient concentration of phosphate corresponding to the intra-cell concentration, is packaged into the capsid when the phosphate concentration decreases to the extra-cell concentration in the coexistence with divalent cations, Ca$^{2+}$ and Mg$^{2+}$. The compaction and packaging process coincide with the conformational change of DNA. Divalent cations can compact T4 DNA when the counter anion is phosphate. The DNA-packaged and re-generated virions showed equivalent infective ability with the original populations. Fluorescent microscopy distinguished the conformational changes of DNA between compact forms and coil forms. Packaged- or unpackaged-DNA were confirmed enzymatically. Pfu was used as the measure of infectious ability of virions. The concentration of ATP was measured by the luminometric method. The packaging is proceeded in pM or lower concentration of ATP. This is a new packaging process of viral DNA practically free from ATP into which conformational change of DNA is incorporated. The results may provide new interpretations of the life cycle of T4.

1. Background

The life cycle of bacteriophage T4 is one of the most intensively studied virus–host systems [Birge 2010]. It is generally agreed that packaging of a long and self-repulsive string of the phage DNA into a small empty capsid as a liquid crystalline DNA [Gelbart, Knobler 2009] requires a molecular motor using energy [Casjens 2011,Zhang et al. 2012]. In the metabolism of T4, no net packaging of DNA into the capsid was observed concentrations of ATP under 25 μM [Kottadiel et al. 2012]. The energy is stored as highly pressurized DNA, which is used as the force for ejection of the DNA into the host [Gelbart, Knobler 2009,Molineux Panja 2013]. Treatment with chelate agents, like EDTA, induces the ejection of DNA from the head of the bacteriophage [Chow et al. 1971,Kuo et al. 1971], and phages so treated did not recover their activity [Chow et al. 1971, Kuo et al. 1971, Shafia, Thompson 1964]. Among the ions which create ion potential on the cell membranes essential for the activity for life [Alberts et al. 2002], calcium ion (Ca$^{2+}$) and inorganic orthophosphate ion (Pi) form the steepest gradients [Clapham 2007]. We hypothesized that Ca$^{2+}$ and Pi can be critical ions for the phage - host cell relationship. Here, a system of reversibly ejecting and packaging DNA induced by adjusting the ambient phosphate (chelate) and Ca$^{2+}$ (divalent cation) concentrations mimicking the inside and outside of a cell, respectively, will be introduced. As a result of ultracentrifugation, dialysis and dilution, the concentrations of ATP, when the packaging DNA proceeded, were at pM orders or lower [Ukuku et al. 2005]. Accordingly, the mechanism of packaging DNA described here is not relying on ATP or is an ATP-free packaging system. Virions that produced by this system are actively infectious equivalent to the original population. The conformational
change of DNA molecules [Gosule, Schellman 1976, Yoshikawa, Matsuzawa 1995] may be the vehicle of these ejection-packaging processes.

2. Materials And Methods

Strains:
The bacteriophage studied was T4 (ATCC 11303-B4), and its host bacteria were *Escherichia coli* (ATCC 11303).

The preparation of T4 virions:

Peptone broth (peptone 10g, glucose 1g, NaCl 3g, 0.1M CaCl₂ 1ml, 0.1M MgCl₂ 10ml, 0.1M KH₂PO₄ 3.2ml, in 1l solution, pH 7.2) was used for culturing the host bacteria, *E. coli*. T4 suspension was obtained by the plate lysate method and the small-scale liquid culture [Sambrook J, Russell DW 2001]. In the plate lysate method, the virions were extracted by adding 2-3 ml of an electrolyte solution (EL) including 1.8 mM NaCl, 0.12 mM MgSO₄, 0.12 mM MgCl₂, 0.034 mM CaCl₂ and 0.05 mM KCl, incubated several hours to elute virions and finally filtrate the eluent with 0.2 μm filter (Advantec AS020) to remove bacteria and bacterial debris. To exclude the effects of anonymous ions and ATP in the packaging experiments, the suspensions of T4 virions were purified with ultracentrifugation and dialysis. For ultracentrifugation, crude bacteriophage particles were purified by isopycnic centrifugation through CsCl gradients [Sambrook J, Russell DW 2001; Beckman XPN-90, SW32 rotor, 4 °C, 24 h]. Following to ultracentrifugation, T4 suspensions were dialysed with Nuclepore polycarbonate membrane filters (0.015 μm in pore size, Whatman Inc.) against 0.5 mM CaCl₂ for one week replacing the outside 0.5 mM CaCl₂ five times. In the case to remove ions in viral particles, the viral suspensions were dialyzed with Spectro/Por 6 dialysis membrane, MWCO 1000, against T-buffer including 100 mM NaCl, 2 mM MgSO₄, 0.5 mM phosphate buffer, pH 7.5, for one week replacing outside water twice a day. The chemicals used were special grade products from Wako Pure Chemical Ind. Ltd.

Plaque forming unit (pfu):

Plaque forming units (pfu) were measured duplicately by plating aliquot of virions, adjusted to 10 ~ 500 plaques per plate as possible, on 1% agar peptone plates and 0.5% agar peptone top agar. For measuring the time course of pfu, Fig. 5, a single plate was prepared repeatedly during the time course. We have done the similar sets of time course experiments several times. All the results were homogenous. We chose the most complete set among them. Inoculated plates were incubated at 36°C for 12 hours before counting. The pfu values of T4 samples were enumerated frequently to confirm the activity of the T4 virion during the experiments.
Fluorescent light microscopic observation (FLM):

Virion particles and ejected DNA were distinguished with fluorescent microscopy. Aliquots of the suspensions of virions and DNA were collected on 0.02 μm Anodisc (ø25 mm), backed by a pre-moisturized filter (Millipore HA). Virions and DNA collected on the filters were stained with filtration of ca. 30 μl of 0.001 dilution of SYBR-Gold (Molecular Probes, Inc.) including 0.1 or 10 mM phosphate buffer (pH 8.0), 0.1 mM EDTA (pH 8.0) and 50mM dithiothreitol (Wako Pure Chemical Ind. Ltd. for molecular biology). Sample filters were mounted on a glass slide with a mounting medium composed with 50% glycerol (Wako Pure Chemical Ind. Ltd., Special grade), 0.1 or 10 mM phosphate buffer (pH 8.0) and 40mM dithiothreitol. The phosphate buffer concentration was selected in accordance with the viral specimen, i.e. 10 mM for ejected DNA and 0.1 mM for compact DNA. In addition, 10 mM MgCl2 were added for the specimen of compact DNA. Samples were observed with Olympus BX50 epiuorescent microscope, equipped with N.A. 1.35 UPlan Apo x100 objective lens, N.A. 0.4 UPlan Apo x10 objective lens and U-MWVB dichroic mirror unit. Samples were prepared at least two filters. At the beginning of enumeration, one sample filter which showed even distribution of the specimens on a filter confirmed by a general view of the whole filter was chosen. Using this sample filter, enumerations were carried out at randomly selected more than 20 fields along a diameter from one periphery to another periphery. Total numbers of counted objects were more than 200, except for the cases where there were almost no visible objects.

Ejection of T4 DNA:

The ejection of DNA from T4 virion was examined with biological (Pi, citrate and an electrolyte mimicking cell sap) and an artificial (EDTA, Tris-HCl and TE (Nippon Gene Co., LTD)) chelates (pH 8.0). The composition of an electrolyte mimicking cell sap is; 15mM Na+, 140mM K+, 0.1mM Mg2+, 10mM Cl−, 10mM HCO3−; 35mM HPO42− [Mason PW, Carbone DP, Cushman RA, Waggoner AS 1981,Milo R, Phillips R 2015]. The virions were exposed to combinations of counterion concentrations, i.e. phosphate buffer (pH 7.6) including 0.01 – 100 mM Pi vs. 0 – 1 mM CaCl2 or 0 – 1 mM Mg Cl2, for several minutes.

Packaging of T4 DNA:

The packaging of DNA into the capsids of T4 was induced by 10 – 10^5 times dilution of the ejected specimens. The solvent used for the dilution were 1 mM CaCl2 solution or EL. The diluted suspensions of T4 virions were incubated for ca. 10 min. The total number of reformed virions were measured visually by FLM and their infective ability was estimated by pfu counts of the plate method.
**DNase I treatment**

DNase I (recombinant DNase I, Takara) treatment was applied to discriminate the DNA covered with capsid proteins from the naked DNA without capsid coverings. Two types of DNase I digestions were proceeded. One was DNase I degradations *in situ*. DNase I was added to suspensions of T4 in EL, 10 mM or 30 mM Pi and TE with the concentrations of 1 U/40 μl plus 1 mM MnCl₂. The specimens were incubated for 2 hours.

The other treatments were the on-filter degradation. This process had two branches. One was on-filter-dry. In this branch, suspensions of virions and DNA molecules were filtrated on 0.02 μm Anodisc (ø25 mm), followed by mounting of DNase I solution, ca. 0.1 – 0.2 ml of 1 U/40 μl dilution plus 1 mM MnCl₂ [Anderson S 1981] in EL and in EL plus 10 mM or 30 mM Pi, on the filter to degrade naked DNA. Virions and DNA molecules were once exposed to air and adsorbed on the surface of a filter, and the original solvent was replaced by DNase I solution in this process. The other was on-filter-wet. In this process, the *in situ* suspension was mounted on the filter, slowly filtrated, and supplied DNase solution carefully when the suspension on the filter decreased. The DNA molecules and virions were filtrated on the surface of a filter but not exposed to air during filtration. The DNase treatments were incubated for 0.5 - 1 hour for on-filter-dry treatment and 1 - 2 hours for on-filter-wet treatment.

After incubation, specimens were filtrated and stained with SYBR-Gold.

**Digestion of capsid proteins by proteinase K**

To obtain naked DNA molecules of T4 free from capsids, the capsid proteins of viral suspension was digested with proteinase K (Takara, ≥ 600 mAnson U/ml) [Steward GF, Culley AI 2010]. Virions were suspended in EL, pH 8.0, with 1 % v/v of proteinase K, which was incubated at room temperature for one day. Tris, EDTA and SDS, which were normally included in the lysis buffer, were not included in the present mixture of proteinase K degradation in order to avoid any negative effects on pfu (This will be discussed in the text). Inactivated proteinase K, heated the enzyme solution at 95°C for 15 min, was used as the control of the proteinase K treatment.

**ATP concentration:**

The concentration of ATP was measured with triplicate specimens. ATP in a specimen can be converted to ADP or AMP during processing of the specimen. The measured amount of ATP itself can be underestimation. The total concentration of ATP + ADP + AMP between 10 pM and 10 μM was measured by a reagent set, Lucipack A3 water, and Lumitester Smart (Kikkoman Biochemifa Co.). Here, ADP and
AMP are enzymatically converted to ATP, afterwards the amount of ATP is measured. Therefore, the amount of ATP measured by the present system is clearly overestimation. Hereinafter, the total amount of ATP, ADP and AMP is indicated as ATP concentration.

**Statistical processing:**

Statistical processing has been done with functions in Microsoft Excel 2016. Standard deviations were calculated as the arguments were a sample of population. T-test was carried out with two-tailed distribution and the two samples were assumed to have the equal variances.

### 3. Results

#### 3.1. Ejection of DNA

The ejection of DNA from the head of a bacteriophage was induced by incrementing the ambient concentration of phosphate (Pi), a chelate agent [Chow et al. 1971, Kuo et al. 1971, Shafia, Thompson 1964]. When the concentration of Pi and Ca$^{2+}$ were higher than 10 mM and lower than 0.1 mM, respectively, the virions instantly ejected their DNA (Table 1, Fig. 1C). The intramolecular Brownian motion of the Gaussian random coil DNA produced a blur ball images (Fig. 1C). Hydrodynamic flow of the mount medium stretched coil DNA and the stretched state was adhered on the filter surface by seeping mount medium including divalent cation from the backside (Fig. 1G). The random coil DNA was stretched to 10 – 20 μm long, but not totally extended (Zinchenko et al. 2003, Tang et al. 2011, Renner and Doyle 2015). Ca$^{2+}$ acts counteractively against Pi to suppress the ejection of DNA. Mg$^{2+}$ was also counteractive against Pi, while the strength of suppression of ejection was weaker than Ca$^{2+}$ (Table 1). Immersion in an electrolyte mimicking cell sap also induced the ejection of T4 DNA. The ejections of DNA molecules outside from the capsids were confirmed by the degradation of specimens with DNase I in EL or 30 mM Pi, according to the original suspensions. All the DNA molecules of T4 virions immersed in 30 mM Pi were completely degraded by the on-filter-dry DNase I treatment and no intact virion was left (Fig. 1D).

In order to ascertain the compact conformation of T4 DNA in the capsid in the low Pi concentration coexistence with divalent cations, the capsid proteins of T4 virions were degraded with proteinase K in EL. The T4 DNA showed globules (Fig. 2), not self-repulsive strings (Fig. 1C).

#### 3.2. Packaging of DNA

DNA molecules of virions were totally ejected by immersion in a solution of 10 - 30 mM Pi. In this process of ejection, suspensions of virions were diluted by 10$^2$-fold to 10$^5$-fold. Following this, the suspension was diluted with EL by 10$^2$-fold, which reduced the Pi concentration to 0.1 or 0.3 mM. The reduction of Pi
concentration instantly induced the condensation of DNA (Fig. 1D). At this point, it was not known whether the condensed DNA was in tightly compacted globule forms outside of capsids or packaged into capsids to form virion heads [Tongu et al. 2016]. If the DNA was naked, it would be degraded with DNase I. No significant differences in the FLM abundances were observed between the original and regenerated populations and between the pre- and the post-degraded populations (Fig. 1). This indicates, in the virion populations, almost all of them ejected DNA, packaged the ejected DNA into capsids by dilution of Pi concentration and protected from DNase degradation. The reproduction of virions by decrement of the ambient concentration of Pi also recovered the infectivity (pfu) nearly 100% (Fig. 1E). The pfu values of the viral population in 30 mM Pi is estimated in the section 3.4. Increasing the concentrations of Ca$^{2+}$ also stimulated the packaging of DNA (Table 1), while the increment of Ca$^{2+}$ concentration was not inevitable.

The loss of infectivity of condensed DNA without capsid is confirmed with proteinase K treatment of the virions. After the capsid protein of virions was degraded by proteinase K (Fig. 2B), following on-filter-dry DNase I treatment degraded all the virion-like particles (Fig. 2D), which confirmed these virion-like particles were naked DNA particles. The treatment of inactivated proteinase K also produced similar virion-like particles, which were resistant to DNase I degradation (Fig. 2C). The infectious ability, pfu, of the suspensions treated with proteinase K was less than 1/10,000 that of the T4 populations treated with inactivated proteinase K. Globular naked T4 DNA have no ability to infect *E. coli* cells. This also confirm the results that infectious bodies in the population regenerated by justifying the ambient Pi concentrations were not the naked DNA but newly produced intact virions.

If we assume the ejection separates the ejected DNAs and empty capsids, and the ejected DNAs and empty capsids need to meet for packaging, the process is carried out on the second order reaction. Consequently, the higher the initial densities of ejected DNA and capsids, the higher the densities of regenerated virions. On the contrary, if the ejected DNA and the capsid remained connected, the packaging process occurred within the first order reaction, and the initial density would not affect the final concentration of regenerated virions. The differences of the concentrations of DNA when the regeneration processes initiated did not affect the final densities of regenerated infectious virions (Table 2). Accordingly, it is reasonable to infer that the packaging process occurs within the first order reaction, and the ejected coil DNA and capsids are not separated totally but maintain the connection after the ejection until the moment of packaging. In phage λ, the ejected DNA remains attached to the capsid [Li et al. 2015,Grayson et al. 2007].

3.3. ATP concentrations

The original peptone broth contained ca. 1 μM concentration of ATP (Fig. 3). After the growth of host bacteria, *E. coli*, and lysis by T4, the extracted crude T4 suspension included ca. 30 nM of ATP. Dialysis of
the crude T4 suspension decreased the ATP concentration to ca. 30 pM ATP. In the other specimens; including Milli-Q water, phosphate buffers, and diluted dialyzed suspensions used in the experiments of ejection and packaging of DNA; the measured ATP concentrations were equivalent to the lower limit of the detection, ca. 10 pM, and no significant difference between each other. All these concentrations of ATP measured were significantly lower than 25 μM, the lower limit concentration of ATP for packaging DNA with the molecular motor [Kottadiel et al. 2012]. As previously described, DNA molecules of T4 virions were packaged into capsids at up to 10^7-fold dilution of dialyzed suspension including ca. 30 pM of ATP. This condition is practically ATP-free in the context of packaging of DNA.

3.4. pfu values of 30 mM Pi specimens and acts of DNase I in EL, 30 mM Pi and TE

The behaviors of DNase I in the conditions used in this study, *i.e.* in situ degradations and on filter degradations of DNA molecules vs. in EL, 10 - 30 mM Pi and TE, were elucidated (Fig. 4).

In the T4 suspensions in EL, the abundances of virion-like globular DNA and the pfu counts showed no difference among the original specimen, in situ and on-filter DNase I treated specimens (Fig. 4A, B, C). In situ and on-filter-dry DNase treatments do not affect the abundances of virions and their infectious abilities.

In 30 mM Pi, T4 virions eject their DNA (Fig. 4D). The pfu values of the populations in 30 mM Pi were equivalent with the populations in EL (Fig. 4D). After the naked DNA in 30 mM Pi suspension was treated with DNase I in situ, abundant coil and globular DNA molecules were observed and pfu counts did not decrease from the original (Figs. 1A, 4E). The globular DNA observed in 30 mM Pi (Fig. 4F) were apparently bigger than intact virions (Fig. 4A, B, C). On-filter DNase I treatment, which replaced the solvent to 10 mM or 30 mM Pi, digested almost all coil and globular formed DNA molecules (Fig. 4E).

T4 virions eject their DNA in TE (Fig. 4G) like the ejection of DNA in 30 mM Pi (Fig. 4D). However, clear differences were observed in infectious ability and DNase I sensitivity between the DNA-ejected virions in TE and in 30 mM Pi. In contrast to the ejected DNA in 30 mM Pi, the ejected DNA in TE showed no infectious ability (Fig. 4G) and was degraded with DNase I in situ (Fig. 4H). Few virion-like globular DNA remained after in situ DNase I degradation in TE (Fig. 4H), while these globular DNA showed no infectious ability. On-filter-dry treatment of DNase I degrades almost all DNA of T4 in TE (Fig. 4I).

To confirm whether virions ejected their DNA in 30 mM Pi solution can recover their infectivity during the plating process, which dilutes the concentration of phosphate and triggers the re-production of infective virions, the recovering rates of virions were monitored as follows. In a set of experiments, the ultra-centrifuged T4 virions were dialysed against T-buffer and stored in T-buffer. These T-buffer suspensions were diluted twice. First dilution with Milli-Q water 100-fold and acclimated to this condition for five minutes. At this point, the ionic concentrations of the viral suspensions were 0.01 T-buffer. Second, the Milli-Q water dilutions were diluted again 100-fold with the test solutions. The final concentrations of
original T-buffer in the test solutions were 0.0001 T-buffer. The time courses of pfu in the original T-buffer indicates stable activity through 60 min. (Fig. 5, Tb). In 0.01 T-buffer, pfu was gradually decreased, and after the acclimation period, 5 min., more than 95% of virions maintained their infectivity (Fig. 5, 0.01Tb). When the second dilution was inoculated in Milli-Q water, 0.0001 T-buffer, the infectivity was lost instantly (Fig. 5, 0.0001Tb). The second dilution was inoculation into 30 mM Pi, which included 30 mM phosphate ion and 60 mM Na\textsuperscript{+}, the pfu values were equivalent or higher than the original x1 T-buffer suspension (Fig. 5, 30 mM Pi, Tb). As 30 mM Pi includes 60 mM Na\textsuperscript{+}, the first dilution was inoculated into 60 mM Na\textsuperscript{+} solution. The time course of this inoculation was equivalent with 0.01 T-buffer suspension (Fig. 5, 60Na, 0.01Tb). Cation Na\textsuperscript{+} just maintains the infectivity of the pre-inoculation suspension, but no more than that. On the contrary, the inoculation into 30 mM Pi produces higher pfu than the pre-inoculation suspension or the inoculation into 60 mM NaCl throughout the time course (Fig. 5, 30 mM Pi, 0.01Tb, 60Na). The average rate of pfu of (30 mM Pi inoculation)/(60 mM Na\textsuperscript{+} inoculation) was 1.57±0.18 (av. ±SD).

4. Discussions

4.1. Ejection of DNA

High pressure inside of capsids is thought to be used for the ejection of DNA from capsids [Liu et al 2014, Leforestier A, Livolant F 2010]. The stress inside the phage capsid decreases by polyvalent cations [Evilevitch et al. 2008, Fuller et al. 2007]. The full capsid has a slightly smaller radius than the empty capsid [Effantin et al. 2006]. The latter two results imply that the internal pressure of the mature head is not higher than the external pressure. The T4 DNA produced by degradation of capsid proteins with proteinase K show compact forms (Fig. 2), not self-repulsive strings like the ejected DNA in 30 mM Pi (Fig. 1C, G). The DNA molecule itself takes a compact conformation within and without a capsid under the regular extracellular ambient concentrations of ions, pressure, and temperature without addition of any multivalent cations. The globule-to-coil phase transition of phage DNA, which is necessary for the transportation of phage DNA into the host bacteria [Chow et al. 1971, Kuo et al. 1971, Liu et al 2014, Leforestier A, Livolant F 2010, Li et al. 2015], can be induced solely by increment of ambient concentration of Pi to the concentration corresponding to cell electrolyte in T4 DNA (Fig 1). It is implicated that this process of DNA ejection may have contribution to the process of DNA ejection of T4 to the host cell of \textit{E. coli}.

The step-by-step mechanism of the ejection of DNA was not investigated in this study. However, the completed head and tail are joined spontaneously after DNA packaging is completed in the assembly of bacteriophage [Aksyuk, Rossmann 2011, Arisaka et al. 2016], we presume that in the ejection of DNA from a capsid the tail may detach from the connector first and the ejection of DNA from the connector follows according to the increment of the ambient phosphate concentration.
4.2. Packaging of DNA

Prior to discussing packaging, it would be appropriate to examine again that there were no or few, if any, intact virions in the 30 mM Pi suspension. The FLM observations clearly indicated nearly complete ejection of DNA and almost no intact virion in 30 mM Pi (Figs. 1C, 4D). After 25 mM phosphate treatment virions separate into DNA, empty heads, and tails [Chow et al. 1971]. Ultracentrifugation of 3 mM sodium-citrate treated virions indicates almost no intact virion remains after chelate treatment [Kuo et al. 1971]. It becomes appropriate to estimate few, if any, intact virions remain in 30 mM Pi suspension of T4.

However, pfu values of 30 mM Pi suspensions were equivalent to the original specimens (Figs. 4A, 4D). This apparent contradiction can be attributed to the change of the concentration of Pi during plating. The high concentrations of Pi of the T4 inocula, where virions ejected their DNA, decreases when the suspensions were inoculated into the peptone agar of low Pi concentration for plating, which triggers the regeneration of virions in the agar plate. To prove this process, T4 suspensions in T-buffer were inoculated into 30 mM Pi, 60 mM NaCl and Milli-Q water and the pfu values were monitored (Fig. 5). The solution of 60 mM Na\(^+\) was chosen as the equivalent cation concentration with 30 mM Pi. The infective ability was immediately lost when 0.01 T-buffer suspension was diluted 100-fold into Milli-Q water (Fig. 5, 0.0001Tb). The monovalent cation, 60 mM Na\(^+\), preserves but no more pfu than the infectious ability of the 0.01 T-buffer inocula throughout the time course (Figs. 5, 60Na, 0.01Tb). The infectivity recovery of the inoculation from 30 mM Pi, in contrast, was higher than the 0.01 T-buffer inocula, and moreover, it was equivalent or higher than the original 1 T-buffer suspension (Figs. 5, 30 mM Pi, Tb). Plating of virions ejected their DNA in 30 mM Pi on the normal peptone agar triggers DNA packaging and produce more infective virions than inocula, which indicates not only recovering of the infective ability but also reset some virions lost their infectivity to infective virions.

To support this, we tried to digest the naked DNA in 30 mM Pi suspension with DNase I. However, abundant coil and globular DNA molecules were observed after the DNase I treatments and no decrease of pfu counts was observed (Figs. 1A, 4E). Consequently, we compared the DNase I degradation in situ and on-filter vs. the suspensions of T4 virions in EL, 30 mM Pi and TE (Fig. 4). As described at Materials and Methods section, in “in situ treatment”, the DNA molecules in the original solvents were degraded, i.e. in EL, 30 mM Pi and TE, and “on-filter-dry treatment”, the suspensions were filtered through once and DNA molecules collected on 0.02 μm Anodisc to remove the original solvents. By this filtration, coil DNA molecules and virion particles are exposed to air and adsorbed on the filter surface. Afterwards, the collected DNA molecules and virion particles were degraded on-filter with DNase I in EL or 10 - 30 mM Pi, according to the original suspensions. In “on-filter-wet treatment”, the suspensions with DNase I were mounted on 0.02 μm Anodisc and filterate slowly. When the sample suspension decreased, additional DNase solution was supplied. By this filtration, coil DNA molecules and virion particles are filtrated on the
filter surface but not exposed to air during DNase degradation. In the T4 suspensions in EL, DNase I treatment did not change the FLM abundances and the pfu counts among the original specimens, *in situ* and on-filter-dry DNase I treated specimens (Fig. 4A, B, C). On-filter-wet DNase I treatment also did not change the FLM abundance (data not shown). Intact virions are not digested with DNase I. The ejected DNA in 30 mM Pi was not digested with *in situ* DNase I treatment (Fig. 4E). During the DNase I treatment parts of DNA molecules became globular (Fig. 4E) which were bigger than intact virions (Fig. 4A, B, C), and it is known that the globular conformation is more stable than coil conformation and DNA in the latter conformation naturally transformed to the former conformation [Yoshikawa, Matsuzawa 1995]. On-filter-dry DNase I in 10 mM and 30 mM Pi treatments digested almost all DNA molecules (Fig. 4F). While on-filter-wet DNase I in 10 mM and 30 mM Pi treatments did not digest coil or globular DNA molecules (data not shown). This indicates following facts; first, the DNase I in 10 mM and 30 mM Pi maintained the ability of the degradation of DNA, and second, almost no intact virion survived in 30 mM Pi. The abundances of DNase I resistant DNA particles in 30 mM Pi, determined by FLM, were ca. 1% or less of the original viral abundances (Figs. 1, 4). Contrary, few globules of DNA remained after DNase I treatment in TE (Fig. 4H), while both the initial population in TE and DNase I treated suspensions showed no infectious ability. All the DNA of T4 in TE was degraded with the on-filter-dry DNase I treatment (Fig. 4I). It becomes clear, DNase I, an endonuclease from bovine pancreas that digests single- and double-stranded DNA, digests T4 DNA in TE, but not the coil DNA in 30 mM Pi, the intracell mimic concentration of Pi. After two days of the above preparations, the on-filter-dry preparation of T4 virions in EL decreased their size dramatically, while the on-filter-wet preparations of T4 virions in EL and coil or globular DNA molecules in 30 mM Pi did not change the FLM condition (data not shown). The compact DNA molecules in the capsids were gradually digested by DNase I after the virions were exposed to air. Probably the virion heads were deformed by the exposure to air and a trace of DNase I might seeped into the heads to digest packaged DNA molecules. Modification of DNA protects T4 DNA from the nuclease digestion [Bryson et al. 2015]. The protection of T4 DNA against DNase I works when the DNA is in coil form in intracell mimic concentration of Pi, but not when it is attached on a filter or in the compact conformation. The inactivity of DNase I in 30 mM Pi also can be explained by the lack of enough concentration of metal ions, e.g. Mg$^{2+}$, Ca$^{2+}$ and Mn$^{2+}$ which are necessary for DNase activity [Price 1972, Price 1975], because of the chelation of metal ions by Pi. While DNase I in 10 – 30 mM Pi digests coil DNA attached on a filter, the concentrations of metal ions in 10 – 30 mM Pi are high enough for the activity of DNase I. The behaviors of ejected DNA in 30 mM Pi and in TE were also somewhat different. The coil DNA molecules in 30 mM Pi showed active Brownian motion during the microscopic observation, while the ejected DNA molecules in TE were more likely to stick on the surface of the filter.

The high pfu abundances of DNase I treated 30 mM Pi suspensions are not derived from the intact virions in 30 mM Pi suspensions which are resistant to DNase I, but originated from DNA-ejected virions in 30 mM Pi which convert into infective virions during the plating processes which dilutes the concentration of phosphate and re-produce infective virions. The ejected DNA molecules from virions *in
situ are resistant from the degradation by DNase I (Figs. 4 D, E) and maintain their intact physiological activity (Fig. 5). The re-production of infective virions during the plating of DNA-ejected T4 suspension on peptone agar plate not only recovers the infectivity but also converts some non-infective virions into infective virions.

The currently accepted theory of DNA packaging of most dsDNA bacteriophages initiates when a terminase creates an end of the concatemeric DNA, which attaches to the portal vertex of a capsid and packages the self-repulsive DNA string into the empty capsid with the molecular motor, fueled by the energy of ATP hydrolysis [Casjens 2011, Aksyuk, Rossmann 2011, Vafabakhsh et al. 2014, Zinchenko 2016]. This type of ejection-packaging system is hereinafter called 'motor-ATP system'. Accordingly, the motor-ATP system cannot work without ATP [Kottadiel et al. 2012].

In our experiments, the initial concentration of ATP molecules in dialysed virion suspension prior to packaging was ca. 20 pM (Fig. 3), which is more than 1000 times lower than the minimum ATP concentration for packaging DNA in motor-ATP system, 25 μM ATP [Kottadiel et al. 2012]. The packaging of DNA molecules into capsids occurred at the condition up to 10^7-fold dilution from this concentration of ATP; 10^2-fold dilution for ejection of DNA in 30 mM Pi solution, following 10^3-fold dilution with 30 mM solution plus 10^2-fold dilution with EL at the compaction process (column IV of Table 2). Practically there was no ATP at the compaction/packaging process of DNA. This indicates the packaging system of DNA into a capsid introduced here is not ATP dependent nor a motor-ATP system. The sole agent causing the ejection and packaging of DNA in our protocol was the change of the ambient Pi concentrations. The DNA molecules in the capsids of T4 were first ejected by increasing the ambient concentration of Pi. Without addition of multivalent cations of the valences higher than three [Zinchenko 2016, Todd et al. 2008], simply decreasing of the ambient concentration of Pi induced the fluid-to-solid conformational change of DNA. This compaction of DNA occurs inside of a capsid and the DNA is packaged into capsids, which is confirmed by the resistance of the compacted DNA particles against DNase I and the ability of infection (Fig. 1). The efficiency of packaging to form the infective virion is nearly 100% (Fig. 1). This process does not require additional ATP for packaging of DNA. The concentration of the ambient ATP indicates the process is an ATP-free system. However, the process itself includes the change of the ambient concentrations of Pi, which implies the differences of the concentrations of ambient ions or the density gradient energy can be the source of the energy for the ejection and packaging processes [Vranjes, Kono 2015]. This type of packaging system is hereinafter called ‘conformational change system’.

Characteristic differences between the motor-ATP system and the conformational change system are: 1) ATP dependent in the former [Casjens 2011, Aksyuk, Rossmann 2011], while independent from the extra
ATP in the latter, 2) the packaging of DNA in the former is one-by-one mode and takes several minutes to package one DNA into a capsid [Black, Rao 2012]. In the latter, a group of packaging happens in parallel and in the ensemble average, ca. 80% of packaging might have been done within three minutes (data not shown), 3) the ratios of the regenerated virions were ca. 10% in the former [Black, Peng 2006], while the efficiencies in the latter were >90% in both FLM and pfu counts (Fig. 1). Besides these differences, the motor-ATP system has a limitation for working as the sole packaging system of DNA in a cell. As the globular DNA cannot be packaged into capsids, packaging of DNA should be initiated when they are in a coiled state under the intracellular high Pi concentration. On the other hand, packaged DNA flows out of a capsid in the high Pi condition [Chow et al. 1971, Kuo et al. 1971, Shafia, Thompson 1964]. Indeed, if the packaging process is interrupted by the addition of ATPγS during the transportation process, the packaged DNA runs out from the capsid [Morita, Fujisawa 1997]. This indicates that during the packaging process, once the pumping activity of the motor stops, the DNA molecule will automatically run out from the capsid, and complete virions cannot stay in a cell. Stabilization of the complete virions needs a lower concentration of Pi, which induces DNA coils into the globular conformational change (Figs. 1, 2, 4) [Gosule, Schellman 1976, Yoshikawa, Matsuzawa 1995], and disables the packaging by the ATP-motor system. This can be an obstacle if the ATP-motor system is the only packaging process. On the other hand, in the conformational change system, once the free ends of DNA molecules are connected, or partially packaged into capsids spontaneously [Vafabakhsh et al. 2014] or by the ATP-motor system [Casjens 2011, Aksyuk, Rossmann 2011, Vafabakhsh et al. 2014], the decrease of the chelate (Pi) concentration induces the packaging of DNA (this study), which is followed by the spontaneous joining of head and tail to form infectious virions [Aksyuk, Rossmann 2011, Arisaka et al. 2016]. Accordingly, the sole motor-ATP system or sole conformational change system may not be able to accomplish the packaging of DNA in a cell, but they need each other to accomplish DNA packaging. When one end of DNA attached or packaged into capsids by the motor-ATP system and the ambient Pi concentration is reduced at the end of viral reproduction [Black, Peng 2006] or even after the burst of the host cell (pfu of Figs. 4D, E), the packaging of DNA will be completed by the conformational change system. Because the process of the conformational change system does not require extra ATP and proceeds automatically, it has been ‘invisible’ and might be overlooked in previous observations.

5. Conclusions

It becomes clear, there are two genome packaging pathways, one is the motor-ATP system and the other is the conformational change system. Even it is yet unclear, these two pathways may fill different roles and cooperate in the process of DNA packaging. Conventionally, cations were regarded as the major effects of the intracellular ionic conditions for viral infection. Anions, especially phosphate, may receive more attention to this context [Kutter et al. 1994]. Further research is necessary to elucidate the actual roles of these systems in the viral life cycle.

Abbreviations
ATPγS: Adenosine 5'-O-(3-thio)triphosphate

EL: electrolyte solution

FLM: Fluorescent light microscopic observation

pfu: Plaque forming units

Pi: inorganic orthophosphate ion

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Tables

Table 1. Effects of phosphate and Ca\(^{2+}\) ions on ejections of DNAs from virions of T4.
The higher the concentration of Pi and the lower the concentration of Ca\(^{2+}\), the more DNA molecules were ejected from T4 heads. Pi and Ca\(^{2+}\) acted antagonistically on the ejection of DNA in vitro. More than 80% of virions ejected their DNA when the ambient concentration of Pi was higher than 10 mM and that of Ca\(^{2+}\) was lower than 0.1 mM. Mg\(^{2+}\) ion worked as a counteractive ion to Pi in the DNA ejection, while the effects were weaker than Ca\(^{2+}\).

| Ca\(^{2+}\) (mM) | Mg\(^{2+}\) (mM) |
|----------------|----------------|
| 0 | 0.1 | 1 |
| 100 | +++ | +++ | +++ |
| 10 | +++ | +++ | + |
| 1 | ++ | ++ | - |
| 0.1 | + | + | - |
| 0.01 | - | - | - |

Rates of ejections: +++: >80%, ++: 20 – 80%, +: 5 – 20%, -: <5%
Table 2. The effects of the differences of the initial densities on the regeneration rates of infectious virions.

Numbers, except for pfu, are the dilution rate, e.g. \(-n\) indicates \(10^n\)-fold dilution and 0 means no dilution. In all the cases, packaging of viral DNA, and the regeneration of virions, happened at the 100-fold dilution from 30 mM Pi to 0.3 mM Pi at the 2\(^{\text{nd}}\) dilution. The densities of the original specimens were ca. \(10^7\) ml\(^{-1}\). The case I packaging was started 1000 times higher density of ejected DNAs than the case IV. After packaging, the content densities of the specimens were adjusted to the same densities at the 3\(^{\text{rd}}\) dilution. The three orders of magnitude difference of the substrate densities at the 2\(^{\text{nd}}\) dilution induced less than three times difference on the regeneration rates of the infectious virions.

|          | I    | II   | III  | IV   |
|----------|------|------|------|------|
| original | 30 mM Pi | 0    | 0    | 0    | 0    |
| 1st dil. | 30 mM Pi | 0    | -1   | -2   | -3   |
| 2nd dil. | 0.3 mM Pi | -2   | -2   | -2   | -2   |
| 3rd dil. | 0.3 mM Pi | -3   | -2   | -1   | 0    |
| pfu av. (SD) | 100.0 (15.3) | 73.6 (1.0) | 124.3 (11.5) | 184.5 (18.2) |

Table 3. Estimation of the abundance of infectious virions in 30 mM Pi.

Numbers are relative values of pfu. The original suspensions of T4 virions were deionized by two steps, dialysis against Milli-Q water for one week and immersion in 30 mM Pi. The first step deionization did not induce the ejection of DNA from virions but took infectivity away from the virions. The abundance of virions survived in this step was less than one percent. Immersion of the dialysis virion in EL recovered the infectivity to 76 %. The next deionization was immersion of dialysis virions in 30 mM Pi. The whole virions ejected their DNA at this deionization. Dilution of the suspension of virions in 30 mM Pi reproduced compact DNA covered by capsid and recovered the infectivity to 139%.

|          | H\(_2\)O | H\(_2\)O > 30 mM Pi | H\(_2\)O > 0.3 mM Pi | H\(_2\)O > EL |
|----------|----------|--------------------|----------------------|---------------|
| 100 (8.0) | 0.3 (0.2) | 139 (3.4)          | 76 (1.1)            |

pfu values: av. (SD)

Declarations

Ethics approval: Not applicable

Consent of publication: Not applicable

Availability of data and materials: Data and materials can be shared.
**Competing Interest:** The authors declare that they have no competing financial and non-financial interests.

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**Figures**

**Figure 1**

On-filter-dry DNase I treatment on ultracentrifuged-dialyzed suspension of T4.

Top: original virions without DNase I treatment, Middle: virions digested with 0.2 ml of 1 U/40 μl DNase I in EL or 30 mM Pi, according to the original suspensions, for one hour on the Anodisc, 0.02 μm, f 25 mm

Left: original virions, Middle: virions immersed in 30 mM Pi, Right: virions immersed in 30 mM Pi were diluted 100-fold by EL to 0.3 mM Pi

A. Original image of the suspension.

B. The abundance of virion-like DNA particles was equivalent to the original population (A).

C. Ejected DNA molecules from the capsids by immersion in 30 mM Pi. Elongated and folded forms of coil DNA molecules are shown.

D. After on-filter DNase I treatment, DNA molecules observed in (C) were no longer present and no virion-like particles remained.

E. The ejected DNA molecules were compacted and packaged into the capsids. The abundance of regenerated particles was equivalent to the original (A).

F. The abundance of DNase I resistant virion-like particles was equivalent to (B). The DNA of these virion-like particles were packaged into the capsids.

Scales: 5μm
av. % (SD %), * significantly different from the original no-DNase I treatments (n>20 fields, both side, p<0.01)

Figure 2

Degradations with proteinase K and DNase I.

A. Virions in the initial population.

B. Proteinous capsids were degraded with proteinase K. Naked DNA molecules showed the virion-like compact globules.

C. Majority of virions treated with the inactivated proteinase K were resistant to on-filter-dry DNase I degradation.

D. Proteinase K degradation following on-filter-dry DNase I degradation removed the virion-like globular DNA in (B), which confirmed the globular DNA had no proteinous covering of a capsid.

Scales: 5μm
Figure 3

In peptone broth, the concentration of ATP was ca. 1 μM. The extracted crude T4 suspension included several tenths nM of ATP. After dialysis, the ATP concentration in the T4 suspension decreased to several tenths pM of ATP, which was not significantly different from the concentration in Milli-Q water. The other specimens, the measured ATP concentrations were close to the lower limit, ca. 10 pM, of the detection and no significant difference each other. Abbreviations: MQ; Milli-Q water, 30mM Pi; 30 mM phosphate buffer, 0.1M Pi; 0.1M phosphate buffer, EL; electrolyte solution, DT4 10^{-6}; dialysed T4 suspension diluted to 10^{-6}, DT4 10^{-2}; dialysed T4 suspension diluted to 10^{-2}, DT4 dir; direct dialysed T4 suspension, T4; eluted crude T4 suspension, Peptone br.; peptone broth

Figure 4

DNA of T4 in EL, 30 mM Pi and TE were degraded with DNase I in *in situ* solvent and on-filter-dry conditions. At the on-filter-dry degradation, DNase I was dissolved in EL or 30 mM Pi, according to the original suspensions. Relative values of FLM and pfu are indicated under each photograph. In T4 virions suspended in EL the degradation of DNase I did not decrease the abundance and pfu of virions (A, B, C). All the virions suspended in 30 mM Pi ejected their DNA (D). After two hours incubation with *in situ* DNase I degradation DNA molecules were observed (E). These coil form and globular DNA were totally degraded by on-filter-dry degradation (F). The pfu values of T4 suspension in 30 mM Pi with or without DNase degradation were equivalent with original pfu (A, D, E). DNase I did not degrade naked DNA in 30 mM Pi conditions. Regeneration of virions occurred after plating. T4 virions eject DNA in TE (G). In contrast to 30 mM Pi, the ejected DNA was degraded with *in situ* DNase I in TE (H). Almost no DNA remained after on-filter-dry degradation (I). Few particles of DNA remained after DNase I degradation (H), while these particular DNA survived from DNase I degradation did not show infectivity (H).

Scales: 5μm
Figure 5

The time courses of pfu of T-buffer suspension (Tb), 100- and 10000-fold dilutions of T-buffer suspensions (0.01Tb, 0.0001Tb), 100-fold dilution of 0.01Tb in 30 mM phosphate buffer (pH=7.6, ca. 60 mM Na+) (30P), 100-fold dilution of 0.01 Tb in 60 mM NaCl (60Na)

Figure 6

A comparison between “on-filter-wet” and “on-filter-dry” DNase degradations. After two days of treatments, “on-filter-wet” treatment did not degrade virions (A, B), while “on-filter-dry” treatment digested almost all virion particles, but small blobs of DNA remained (B, C).

Scales: 5μm