An ATP-free packaging of T4 DNA

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Abstract

Packaging of viral DNA into a capsid with the liquid crystalline density is a crucial step of the viral reproduction. The DNA packaging with the ATP fueled molecular motor is an established packaging system of viral DNA. However, the velocity and the efficiency are not well match with the 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 ambient concentration of phosphate decreases to the extra-cell concentration in the coexistence with divalent cations, Ca2+ and Mg2+. 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. The conformational changes of DNA between compact forms and coil forms were differentiated by the fluorescent microscopy. Identification between packaged- or unpackaged-DNA was confirmed by enzymatically. The infectious ability of the re-generated virions was confirmed by pfu. The concentration of ATP in the packaging process was measured by 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 conventional and new packaging processes may cooperate to accomplish the DNA packaging process of T4. 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 [1]. 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 [2] requires molecular motor using energy [3,4]. In the metabolism of T4, no net packaging of DNA into the capsid was observed at the concentration of ATP under 25 μM [5]. The energy is stored as highly pressurized DNA, which is used as the force for ejection of the DNA into the host [2,6]. Treatment with chelate agents, like EDTA, induces the ejection of DNA from the head of the bacteriophage [7,8], and phages so treated did not recover their activity [7–9]. Among the ions which create ion potential on the cell membranes essential for the activity for life [10], calcium ion (Ca^{2+}) and inorganic orthophosphate ion (Pi) form the steepest gradients [11]. 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 [12]. 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 [13,14] 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 10 g, glucose 1 g, NaCl 3 g, 0.1M CaCl2 1 ml, 0.1M MgCl2 10 ml, 0.1M KH2PO4 3.2 ml, in 1 l 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 [15]. 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 [15; Beckman XPN-90, SW32 rotor, 4 ℃, 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 against Milli-Q water 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 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. Inoculated plates were incubated at 36℃ 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 the 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 50 mM 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 40 mM 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 epifluorescent microscope, equipped with N.A. 1.35 UPlan Apo x100 objective lens, N.A. 0.4 UPlan Apo x10 objective lens and U-MWBV dichroic mirror unit. At the beginning of enumeration, the even distribution of the specimens on a filter was confirmed by a general view of the whole filter. Enumerations were carried out at randomly selected more than 20 fields along a diameter from one periphery to another periphery. Total numbers of the counted objects were more than 200, except for the cases where were almost no object.

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; 15 mM Na^{+}, 140 mM K^{+}, 0.1 mM Mg^{2+}, 10 mM Cl^{-}, 10 mM HCO^{3-}, 35 mM $HPO4^{2-}$ [17,18]. 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 10–105 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 ES, 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. Suspensions of virions are 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 [19] in EL, on the filter to degrade naked DNA. This process removes the original solvent to replace DNase I solution. The DNase treatments were incubated for 0.5–1 hour.

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) [20]. 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℃ for 15 min, was used as the control of the proteinase K treatment.

ATP concentration:

The concentration of ATP was measured with triplicate specimens. 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.). Hereinafter, the total amount of ATP, ADP and AMP is indicated as ATP concentration

3. Results

3.1. Ejection of DNA

The ejection of DNA from the head of a bacteriophage was induced by the increment of the ambient concentration of phosphate (Pi), a chelate agent [7–9]. 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). 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. All the DNA molecules of T4 virions immersed in 30 mM Pi were completely degraded by the on-filter DNase I treatment and no intact virion was left (Fig. 1D).

In order to ascertain the conformations of naked T4 DNA in the low Pi concentration coexistence with divalent cations, the capsid proteins of T4 virions were degraded with proteinase K in EL. The naked 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 in 10^2-fold to 10^5-fold. Following this, the suspension was diluted with EL in 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 tightly compacted globule forms outside of capsids or packaged into capsids to form virion heads [27]. 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 Sect. 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 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 connection, the packaging process undergoes on 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 undergoes on 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 [26,28].

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 at 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 [5]. 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

In order to elucidate the acts of DNase I in the individual conditions used in this study, in situ degradations and on filter degradations of DNA molecules in EL, 30 mM Pi and TE were examined (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 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 of 30 mM Pi to EL, 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, the 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 were remained after DNase I degradation in TE (Fig. 4H), while these globular DNA showed no infectious ability. On-filter treatment of DNase I degrades almost all DNA of T4 in TE (Fig. 4I).

In order to estimate the infective abilities of the virions, if any, resistant to DNase I degradation in 30 mM Pi, two steps deionization of the viral suspensions were proceeded. First, viral suspensions were deionized with dialysis against Milli-Q water for one week. Virions did not eject DNA at this step (data not shown), while they have lost the infective ability. The abundance of virions resistant to deionization of dialysis and maintaining infectious ability was less than one percent (Table 3). The immersion of these dialyzed virions in ES recovered the infective ability to 76%. The next deionization was the immersion of the dialyzed virions in 30 mM Pi. The virions totally ejected DNA at this deionization (data not shown). The dilution of 30 mM Pi suspension reproduced compact DNA covered by capsids (data not shown) and recovered the infective ability to 139% (Table 3).

4. Discussions

4.1. Ejection of DNA

High pressure inside of capsids is thought to be used for the ejection of DNA from capsids [21,22]. The stress inside the phage capsid decreases by polyvalent cations [23,24]. The full capsid has a slightly smaller radius than the empty capsid [25]. These imply that the internal pressure of the mature head is not higher than the external pressure. The naked T4 DNA produced by degradation of capsid proteins with proteinase K show globules (Fig. 2), not self-repulsive strings like the ejected DNA in 30 mM Pi (Fig. 1C). DNA molecule itself takes a globular 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 [7,8,21,22,26], 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 E. coli.

4.2. Packaging of DNA

Prior to discuss packaging, it should be appropriate to examine that there were no or few, if any, intact virions in 30 mM Pi suspension in where most of the virions ejected their DNA. The FLM observations clearly indicated nearly complete ejection of DNA and almost no intact virion in 30 mM Pi (Figs. 1C, 4D). However, pfu values of the same specimens were equivalent to the original specimens (Figs. 4A, 4D). This apparent contradiction can be attributed to the concentration of Pi. The process of plating decreases the high concentrations of Pi of the suspensions, where virions ejected their DNA, into the low Pi concentration, which triggers the regeneration of virions in agar plate. Even though that is the case, some of the virions may not eject their DNA in 30 mM Pi and maintain the infectivity. In order to find the abundances of virion particles survived in 30 mM Pi condition, we tried to digest the naked DNA in 30 mM Pi suspension with DNase I (Fig. 4E). 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, the acts of DNase I in situ and on-filter to the suspensions of T4 virions in EL, 30 mM Pi and TE were examined (Fig. 4). As described at Materials and Methods section, in “in situ treatment”, the DNA molecules were degraded in the original solvents, i.e. in EL, 30 mM Pi and TE, and “on-filter treatment”, the suspensions were once filtered and collected DNA molecules on 0.02 μm Anodisc to remove the original solvents. Afterwards, the collected DNA was degraded on-filter with DNase I in EL.

In the T4 suspensions in EL, DNase I treatment did not change the FLM abundances and the pfu counts among the original specimen, in situ DNase I treated specimen and on-filter DNase I treated specimen (Fig. 4A, B, C). Intact virions are not digested with DNase I. The ejected DNA was not digested with DNase I treatment in 30 mM Pi (Fig. 4E). During the DNase I treatment, abundant globular DNA molecules were formed. 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 [14]. 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 of 30 mM Pi to EL, digested almost all coil and globular formed DNA molecules (Fig. 4E). This indicates the globular DNA observed in situ DNase treatments (Fig. 4F) were naked DNA and almost no intact virion survived in 30 mM Pi. The pfu counts show no difference between the original (Fig. 4A) and after 30 mM Pi DNase treatments (Fig. 4E). These results indicate DNase I does not degrade ejected DNA of T4 in 30 mM Pi, regardless of coil form or globular form. Contrary to the ejected DNA in 30 mM Pi, the eject DNA in TE (Fig. 4G) was degraded with DNase I in situ (Fig. 4H). Few globules of DNA were remained after DNase I treatment in TE (Fig. 4H), while both the initial population in TE and the globular DNA showed no infectious ability. All the DNA of T4 in TE was degraded with the on-filter 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 and in EL, but not in 30 mM Pi, the intracell mimic concentration of Pi. Modification of DNA protects T4 DNA from the nuclease digestion [29]. The protection of T4 DNA against DNase I works when the DNA is in intracell mimic concentration of Pi, but not when it is in TE and EL. The behaviors of ejected DNA in 30 mM Pi and in TE were also somewhat different. The 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.

Morphologically, 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). The infective abilities of these DNase I resistant DNA particles in 30 mM Pi were estimated by two steps deionization of the viral suspensions. After the first deionization by dialysis against Milli-Q water, the abundance of infectious virions resistant to this deionization step was less than one percent (Table 3). DNA molecules were not ejected at this step. The recovering of the infective ability to 76% by immersion of these dialysis virions in ES indicated the loss of infective ability was caused by the deficiency of ions in viral particles, probably divalent Ca^{2+} and Mg^{2+}. The next deionization with immersion of the dialysis virions in 30 mM Pi totally ejected DNA from virions (data not shown). One hundred times dilution of the suspension in 30 mM Pi reproduced compact DNA covered by capsids (data not shown) and recovered the infective ability to 139% (Table 3). Because the pfu values of the virion populations resistant to the first step deionization were decreased to less than one percent prior to the second deionization with Pi (Table 3), the initial pfu values of the second deionization steps were less than 1% and more than 99% of infective virions were reproduced by the dilution of the population suspended in 30 mM Pi.

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 [3,30–32]. This type of ejection-packaging system is hereinafter called ‘motor-ATP system’. Accordingly, the motor-ATP system cannot work without ATP [5].

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 [5]. 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 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 [32, 33], simply decreasing of the ambient

concentration of Pi induced the fluid-to-solid conformational change of DNA. This compaction of DNA is occurred inside of a capsid and the DNA is packaged into a capsid, 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 [34]. 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 [3,30], 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 [35]. 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 [36], while the efficiencies in the latter were > 90% in both FLM and pfu counts (Fig. 1). Besides these differences, 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 [7–9]. Indeed, if the packaging process is interrupted by the addition of ATPγS during the transportation process, the packaged DNA runs out from the capsid [37]. This indicates that at 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) [13,14], 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 [31] or by the ATP-motor system [3,30,31], 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 [30,38]. 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 ends 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 [36] 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 [39]. 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

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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References

1. Birge EA. *Bacterial and bacteriophage genetics*. Springer Science-Business Media, Inc: New York, USA, 2010.
2. Gelbart WM, Knobler CM. Pressurized Viruses. *Science* 2009; **323**: 1682-1683.
3. Casjens S. The DNA-packaging nanomotor of tailed bacteriophages. *Nature Rev Microbiol* 2011; **9**: 647-657.
4. Zhang H, Schwartz C, De Donatis GM, Guo P. "Push Through One-Way Valve" Mechanism of Viral DNA Packaging. In Łobocka M, Szybalski WT (eds) *Bacteriophages*. Adv Vir Res 2012; **83**: 415-465.
5. Kottadiel VI, Rao VB, Chemla YR. The dynamic pause-unpackaging state, an off-translocation recovery state of a DNA packaging motor from bacteriophage T4. *Proc Nat Acad Sci* 2012; **109**: 20000–20005.
6. Molineux IJ, Panja D. Popping the cork: mechanisms of phage genome ejection. *Nature Rev Microbiol* 2013; **11**:194-204.
7. Chow TY, Lin YT, Kuo TT. Stability of Phage Xp12. *Bot Bull Academia Sinica* 1971; **XII**: 57-65.
8. Kuo TT, Huang TC, Wu RY, Chen CP. Specific Dissociation of Phage Xpl2 by Sodium Citrate. *J gen Virol* 1971; **10**: I99-202.
9. Shafia F, Thompson TL. Calcium Ion Requirement for proliferation of bacteriophage φμ-4. *J Bacteriol* 1964; **88**: 293-296.
10. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. *Molecular biology of the cell*, 4th edn. Garland Pub, Inc New York, USA, 2002.
11. Clapham DE. Calcium Signaling. *Cell* 2007; **131**: 1047-1058.
12. Ukuku DO, Sapers GM, Fett WF. ATP Bioluminescence Assay for Estimation of Microbial Populations of Fresh-Cut Melon. J Food Protect. 2005; **68**: 2427–2432.
13. Gosule LC, Schellman JA. Compact form of DNA induced by spermidine. *Nature* 1976; **259**: 333-335.
14. Yoshikawa K, Matsuzawa Y. Discrete phase transition of giant DNA dynamics of globule formation from a single molecular chain. *Physica D* 1995; **84**: 220-227.
15. Sambrook J, Russell DW. 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
16. Christi K, Elliman J, Owens L. A Synthesis of the divalent cation requirements for efficient adsorption of bacteriophage on to bacterial cells. In: Harrington D (ed). *Bacteriophages: an overview and synthesis of a re-emerging field*. *Bacteriology Research Developments*. Nova Science Publishers Inc, New York, USA, 2017, pp. 43-69.
17. Mason PW, Carbone DP, Cushman RA, Waggoner AS. The Importance of Inorganic Phosphate in Regulation of Energy Metabolism of Streptococcus lactis. *J Biol Chem* 1981; **256**: l861-1866.
18. Milo R, Phillips R. *Cell Biology by the Numbers*. Garland Science: New York, USA, 2015.
19. Anderson S. Shotgun DNA sequencing using cloned DNase I-generated fragments. *Nucleic Acids Res* 1981; **9**: 3015-3027.

20. Steward GF, Culley AI. Extraction and purification of nucleic acids from viruses. *MAVE Chapter* 2010; **16**: 154–165.
21. Liu T, Sae-Uenga U, Lia D, Landerb GC, Zuoc X, Jönssond B et al. Solid-to-fluid–like DNA transition in viruses facilitates infection. *Proc Nat Acad Sci* 2014; **111**: 14675–14680.
22. Leforestier A, Livolant F. The bacteriophage genome undergoes a succession of intracapsid phase transitions upon DNA ejection. *J Mol Biol* 2010; **396**: 384–395.
23. Evilevitch A, Lavelle L, Knobler CM, Raspaud E, Gelbart WM. Osmotic pressure inhibition of DNA ejection from phage. *Proc Nat Acad Sci* 2008; **100**: 9292–9295.
24. Fuller DN, Rickgauer JP, Jardine PJ, Grimes S, Anderson DL, Smith DE. Ionic effects on viral DNA packaging and portal motor function in bacteriophage ø29. *Proc Nat Acad Sci* 2007; **104**: 11245–11250.
25. Effantin G, Boulanger P, Neumann E, Letellier L, Conway JF. Bacteriophage T5 Structure Reveals Similarities with HK97 and T4 Suggesting Evolutionary Relationships. *J Mol Biol* 2006; **361**: 993–1002.
26. Li D, Liu T, Zuo X, Li T, Qiu X, Evilevitch A. Ionic switch controls the DNA state in phage λ. *Nucleic Acids Res* 2015; **43**: 6348–6358.
27. Tongu C, Kenmotsu T, Yoshikawa Y, Zinchenko A, Chen N, Yoshikawa K. Divalent cation shrinks DNA but inhibits its compaction with trivalent cation. *J Chem Phys* 2016; **144**: 205101.
28. Grayson P, Han L, Winther T, Phillips R. Real-time observations of single bacteriophage λ DNA ejections in vitro. *Proc Nat Acad Sci* 2007; **104**: 14652–14657.
29. Bryson AL, Y Hwang, S Sherrill-Mix, GD Wu, JD Lewis, L Black, TA Clark, FD Bushman 2015 Covalent Modification of Bacteriophage T4 DNA Inhibits CRISPRCas9. mBio 6(3): e00648-15.
30. Aksyuk AA, Rossmann MG. Bacteriophage Assembly. *Viruses* 2011; **3**: 172-203.
31. Vafabakhsh R, Kondabagil K, Earnest T, Lee KS, Zhang Z, Dai L, et al. Single-molecule packaging initiation in real time by a viral DNA packaging machine from bacteriophage T4. *Proc Nat Acad Sci* 2014; **111**:15096–15101.
32. Zinchenko A. DNA conformational behavior and compaction in biomimetic systems: Toward better understanding of DNA packaging in cell. *Adv Col Interf Sci* 2016; **232**: 70–79.
33. Todd BA, Parsegian VA, Shirahata A, Thomas TJ, Rau DC. Attractive Forces between Cation Condensed DNA Double Helices. *Biophys J* 2008; **94**: 4775–4782.
34. Vranjes J, Kono Energy in density gradient. *Phys. Plasmas* 2015; **22:** 012105.
35. Black LW, Rao VB. Structure, Assembly, and DNA Packaging of the Bacteriophage T4 Head. *Adv Virus Res* 2012; **82**: 119–153.
36. Black LW, Peng G. Mechanistic Coupling of Bacteriophage T4 DNA Packaging to Components of the Replication-dependent Late Transcription Machinery. *J Biol Chem* 2006; **281**: 25635–25643.
37. Morita M, Fujisawa H. How do bacteriophages recognize and package their own genome DNA? *Prot Nucleic Acid Enzy* 1997; **42**: 609-618.

38. Arisaka F, Yap ML, Kanamaru S, Rossmann MG. Molecular assembly and structure of the bacteriophage T4 tail. *Biophys Rev* 2016; **8**: 385–396.
39. Kutter E, Kellenberger E, Carson K, Eddy S, Neitzel J, Messinger L, North J, and Guttman B. Effects of bacterial growth conditions and physiology on T4 infection. *In* JD Karam et al. ed. Molecular biology of Bacteriophage T4. ASM Press 1994.

Tables

Due to technical limitations, Tables 1-3 are provided in the Supplementary Files section.

Figures

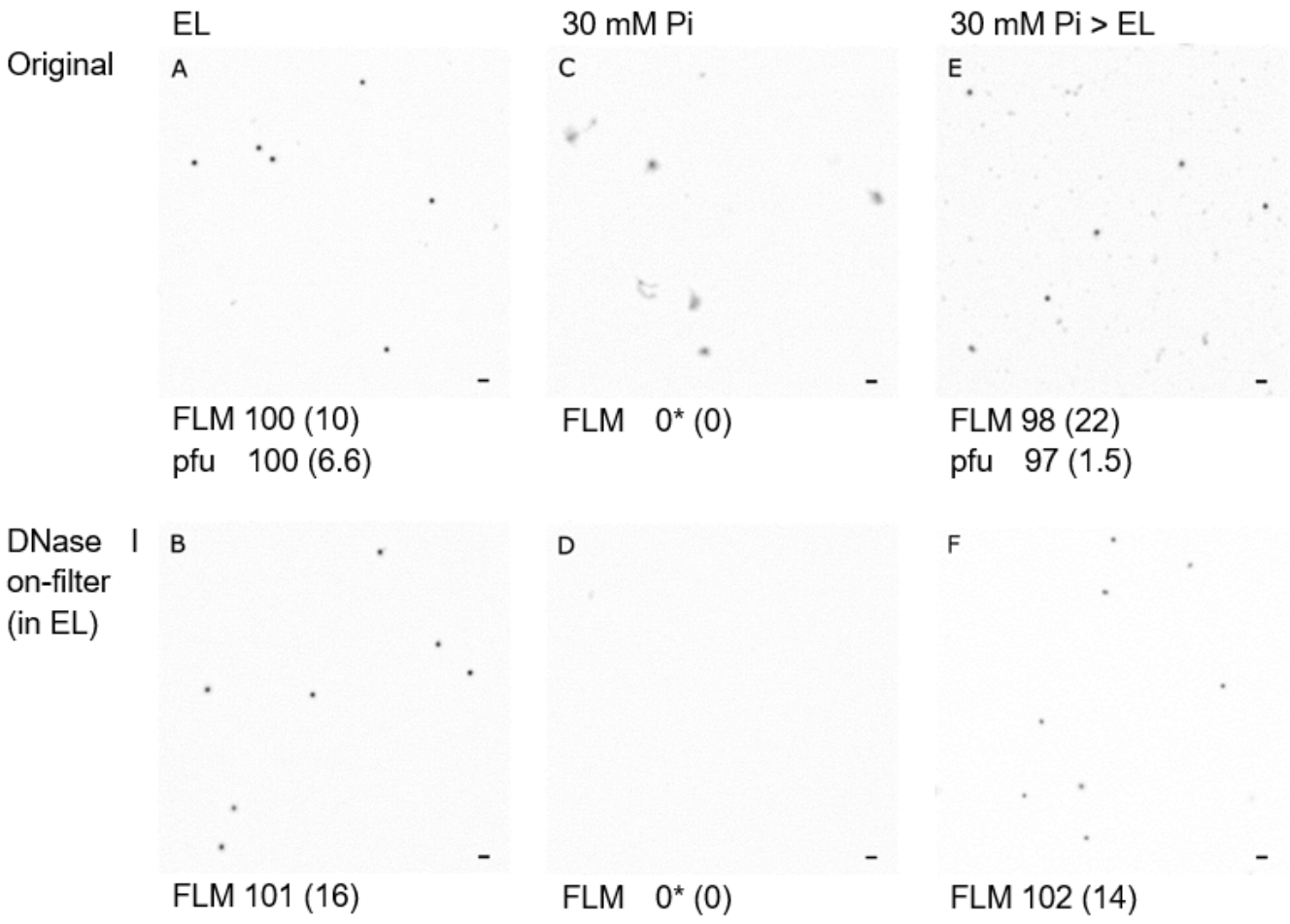

Figure 1

Fig. 1. On-filter DNase I treatment on UDT4 virions. Top: original virions, middle: virions immersed in 30 mM Pi, bottom: virions immersed in 30 mM Pi were diluted 100-fold by EL to 0.3 mM Pi Left: virions

without DNase I treatment, right: virions digested with 0.2 ml of 1 U/40 µl DNase I for one hour on the Anodisc, 0.02 µm, ⌀ 25 mm A. Original image of ultracentrifuged-dialyzed suspension (UDT4). B. The abundance of virion-like DNA particles was equivalent to the original population (A). C. Immersed in 30 mM Pi, UDT4 virions ejected their DNA molecules from the capsules. The Brownian motion of DNA molecules showed cloud-like appearances. No clear virion-like globular DNA was observed. D. After DNase I treatment, DNA molecules observed in (C) were disappeared and no virion-like particle left. E. The ejected DNA molecules were compacted. The abundance and the infectious ability of re-generated 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: 1µm av. % (SD %), * significantly different from the original no-DNase I treatments (n=10, both side, $p<0.01$)

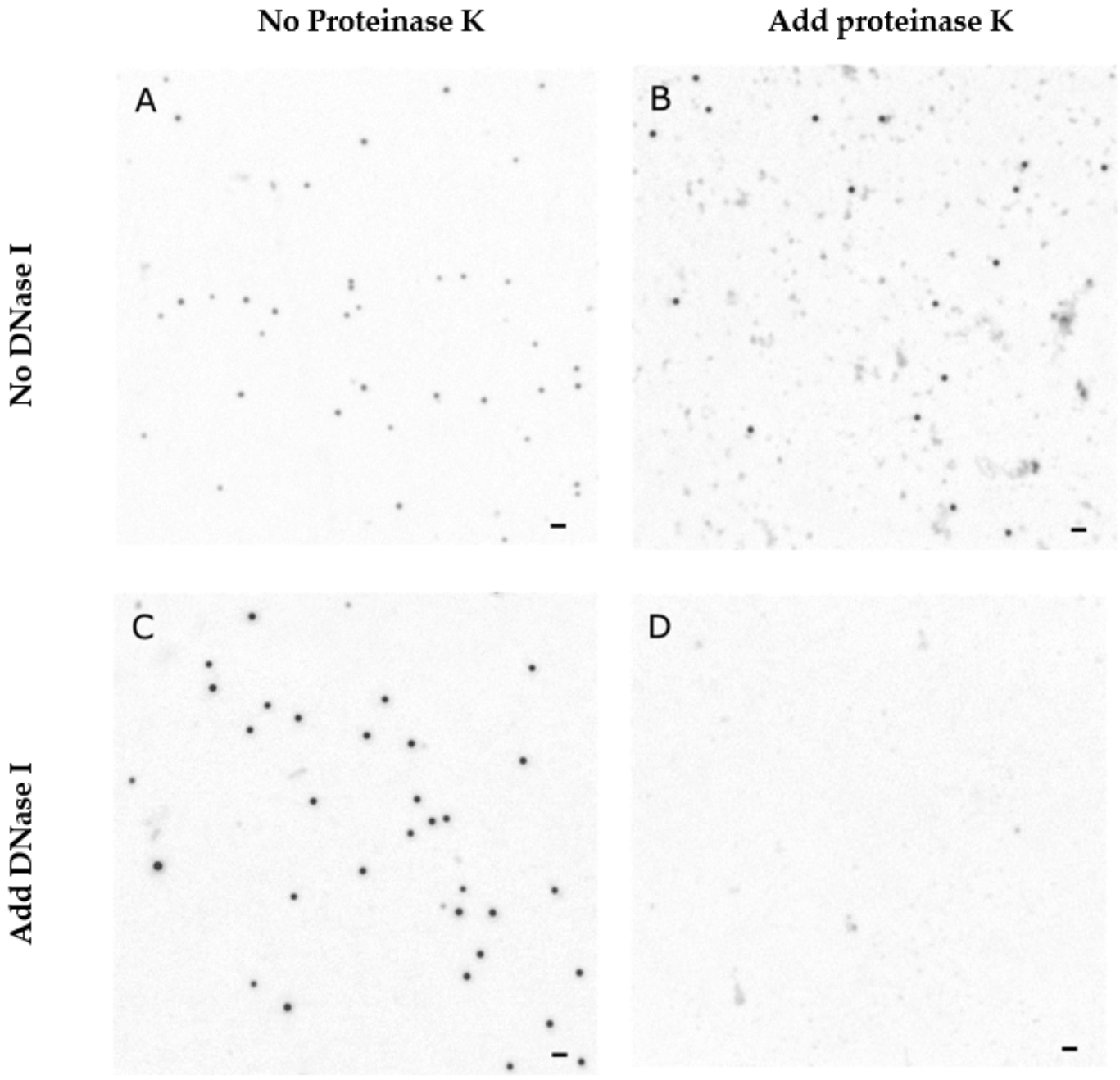

Figure 2

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 DNase I degradation. D. Proteinase K degradation following DNase I degradation removed the virion-like globular DNA in (B), which confirmed the globular DNA had no proteinous covering of a capsid. Scales: 1μm

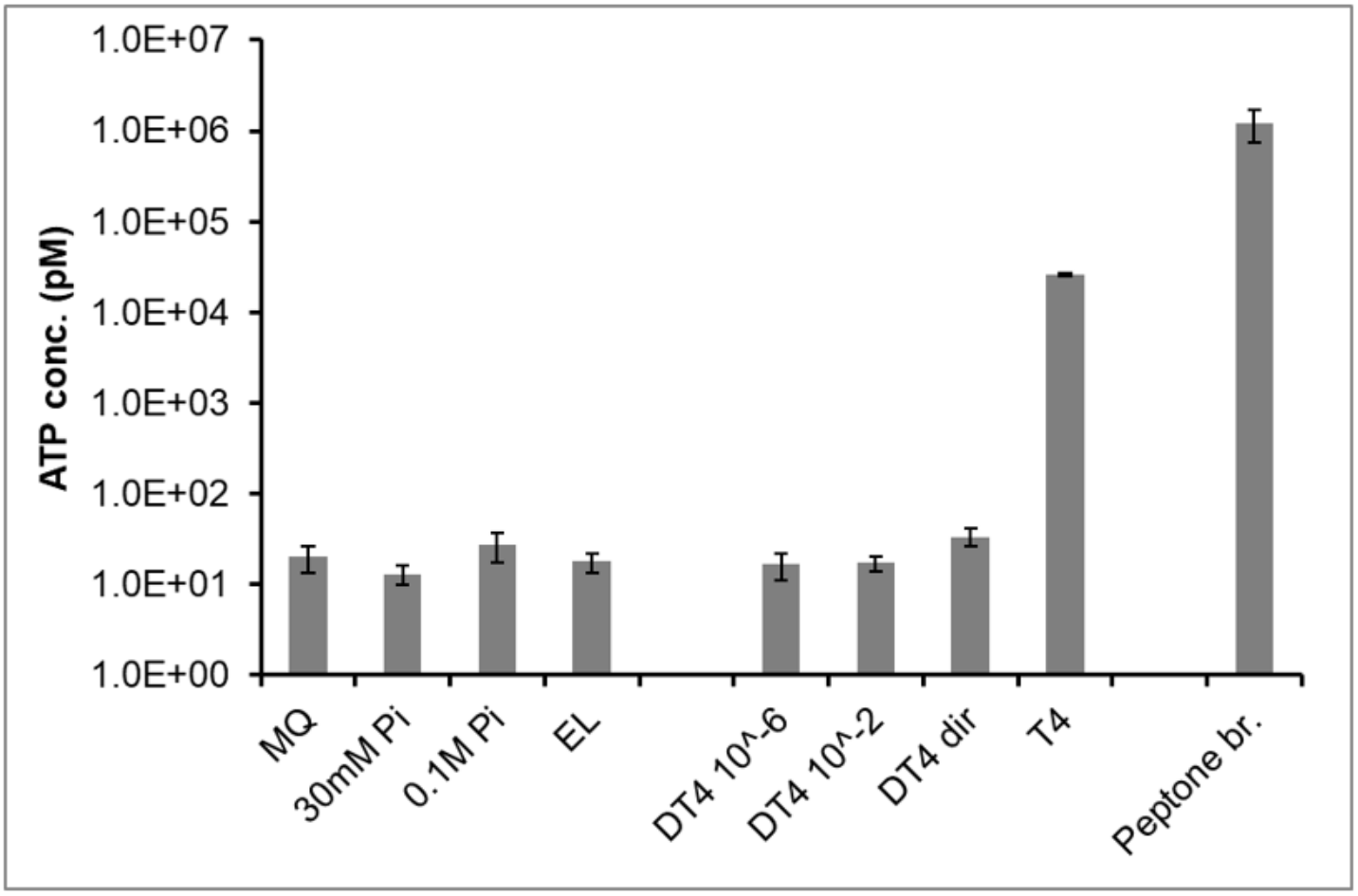

Figure 3

Concentrations of ATP. 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.

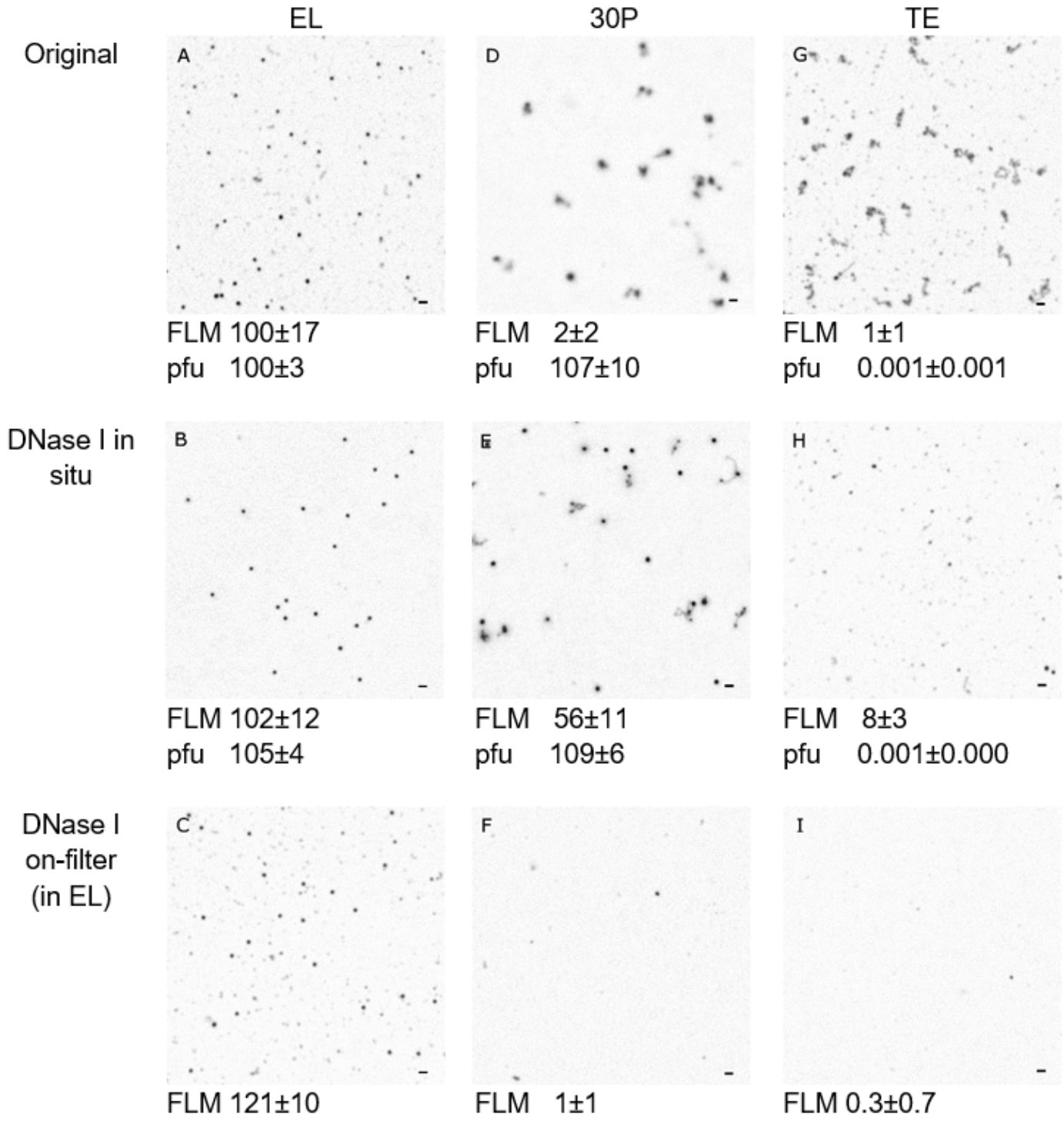

Figure 4

Acts of DNase I. DNA of T4 in EL, 30P and TE were degraded with DNase I in in situ solvent and on-filter conditions. At the on-filter degradation, DNase I was dissolved in EL. 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). Apparently, all the virions suspended in 30P ejected their DNA (D) which showed active Brownian motion. After two hours incubation with DNase I both coil form and globular DNA were observed (E). The diameter of the globular DNA was bigger than the intact

virions in EL (A, B, C). These coil form and globular DNA were totally degraded by on-filter degradation (F) which indicates the coil form and globular DNA were naked. The pfu values of T4 suspension in 30P with or without DNase degradation were equivalent with original pfu (A, D, E). DNase I did not degrade naked DNA in 30P condition. Regeneration of virions occurred after plating. T4 virions eject DNA in TE (G). In contrast to 30P, the ejected DNA was degraded with DNase I in TE (H). Almost no DNA was remained after on-filter 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).

Supplementary Files

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- Tables.docx