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Functional complementation of a conserved non protein-coding element (CNE) of *Autographa californica* multiple nucleopolyhedrovirus by heterologous CNE originating from *Malacosoma neustria* nucleopolyhedrovirus

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Aim. To assess the functional conservation of a baculovirus CNE. **Methods.** CNE deletion from the baculovirus genome and its replacement with a heterologous CNE was performed by using the bacmid-based technology. Transfection-infection assays were used to investigate cell-to-cell spread of the recombinant virus. **Results.** The recombinant *Autographa californica* multiple nucleopolyhedrovirus genome carrying the *Malacosoma neustria* nucleopolyhedrovirus CNE in place of the innate CNE was constructed. The recombinant virus was able to transmit the infection in a cell culture. **Conclusions.** The CNE essential function was found to be conserved between the representatives of I and II groups of the genus *Alphabaculovirus*.

Key words: Baculovirus, baculovirus CNE, functional complementation

Introduction

Baculoviridae is the name of a family of enveloped, double-stranded DNA viruses infecting invertebrates. The family is specific in that its representatives form the inclusion bodies in infected cells. The shape of inclusion bodies (polyhedra or granules) formed by different viruses determines a predetermined baculovirus division onto nucleopolyhedroviruses (NPV) and granuloviruses (GV) respectively [1]. The family members are widely studied for various applications: besides the traditional recognition of baculoviruses as safe insecticides, the genetically modified representatives of the family are considered to be efficient expression vectors, vaccine producers and prospective gene therapy vectors [2]. Large and complex circular baculovirus genomes comprise up to 180 genes as well as non-coding functional elements, from which the transcriptional regulators and origins of replication of different kinds have been well studied [3]. The phylo-

genetic relationships established among the family representatives have revealed the synchronous development of virus and host lineages over large evolutionary timespans [4]. This tendency is reflected in the modern baculovirus taxonomy according to which the family is divided into four genera, *Alpha*-, *Beta*-, *Gama*- and *Deltabaculovirus* comprising lepidopteran NPV, lepidopteran GV, hymenopteran NPV and dipteran NPV respectively [5]. A phylogenetic analysis also illustrates subdivision of the genus *Alphabaculovirus* onto group I and group II [6]. The representatives of two groups differ in an envelope protein that provides the fusion of a virus envelope with host membrane: gp64 serves as an identifier of group I, whereas F protein appears to be a marker of group II [7]. To date, more than 70 baculovirus genome sequences have been determined. A homology search approach applied for the analysis of the content of diverse baculovirus genomes has allowed the interpretation of each genome element as that mediating

ubiquitous, lineage-specific, or specie-specific function. Recently, by using this approach a conserved non protein-coding element (CNE) 154-156 bp in length has been identified in all alphabaculovirus genomes sequenced to date [8]. This alphabaculovirus-specific element was shown to be indispensable for the life cycle of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), the prototype of *Alphabaculovirus* genus. The CNE essential function was shown not to be related either to CNE coding capacity (CNE overlaps short ORF and several non protein-coding RNA genes in AcMNPV genome), or to its activity as a transcriptional activator of the adjacent gene *ie2*. CNE is characterized by an extreme level of nucleotide conservation. This makes it different from other baculovirus non-coding elements because the latter evolve much faster during virus evolution. The CNE essential function has not yet been defined as well as the nature of CNE extreme conservation remains unknown. As noted above, CNE appears to be polyfunctional element and its conservation within the genus *Alphabaculovirus* points to the fact that at least some of CNE function(s) should be common for the genus members. Accordingly, the below-described experiment was designed to test whether as yet unknown essential function is shared by representatives of the genus. The replacing of the AcMNPV CNE with its *Malacosoma neustria* nuclear polyhedrosis virus (ManeNPV) equivalent revealed functional conservation of this element despite the relatively high evolutionary distances between two viruses (AcMNPV and ManeNPV belong to alphabaculovirus group I and group II respectively [9]). These results provide the support for the suggestion that the CNE essential function specifies the genus *Alphabaculovirus*.

Materials and Methods

Bacmids, viruses, cell lines

Recombinant AcMNPV genomes, derivatives of the original bacmid bMON14272 [10] were maintained in *E. coli* cells DH10B.

The Sf9 cell line originated from the AcMNPV natural host, fall armyworm, *Spodoptera frugiperda*

was used in the transfection-infection experiments. The MCA cell line derived from the Chinese tasar moth *Antheraea pernyi* was used for the propagation of ManeMNPV. Cells were cultured in TC100 medium (Sigma Aldrich) supplemented with 10 % fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin (2.5 µg/ml) at 27 °C.

Purification of virus DNA, amplification of CNE-containing fragment

ManeNPV DNA was purified from the infected cells using a standard phenol-chloroform extraction procedure as described [11]. The amplification of the ManeNPV CNE from the ManeNPV DNA template was carried out with the primers derived from the regions surrounding the CNE in ManeNPV genome: MnD 5'-CGGAATTCAGGAATAGATAACGAAC-3' and MnR 5'-GCGGATCCCAAATGCGTCATGG AA-3'.

Recombinant virus construction

CNE knock out bacmid vAc^{CNE-Ko} obtained earlier [8] was used for the construction of the CNE repaired bacmid, where the genuine AcMNPV CNE was replaced by the CNE of ManeNPV. The insertion of ManeNPV CNE in the particular position of vAc^{CNE-Ko} was performed by using commercially available Bac-to-Bac system (Invitrogen) according to the manufacturer instruction. Briefly, the amplified ManeNPV CNE-containing fragment was cloned into BstI site of pFastBac-Dual-EGFP, the recombinant plasmid represented by pFastBac-Dual with green fluorescent protein gene inserted under the control of polyhedrin promoter. The EGFP-CNE-containing cassette was transposed into vAc^{CNE-Ko} propagated in DH10B through the cell transformation with resulting transfer vector pFastBac-Dual-CNE-EGFP followed by homologous recombination. The resulting recombinant bacmid vAc^{CNE-Ko}-RepMane-EGFP as well as bacmids used as controls were purified as described by Bac-to-Bac manufacturer.

Transfection, infection procedures

DOTAP transfection reagent (Sigma-Aldrich) was used to introduce bacmid DNA into Sf9 cells. 3 µl

DOTAP and 1 μ g bacmid DNA were mixed with 100 μ l and 150 μ l of HBS buffer respectively, after that two suspensions were combined. After the incubation for 30 min 350 μ l of TC100 medium without antibiotics were added to the DNA-DOTAP complex and the resulting mixture was layered onto the cell monolayer (1×10^6 cells per 30 mm dish).

After 96 hours the post transfection (hpt) supernatants were harvested. 100 μ l aliquots were used for the infection of fresh monolayers on 30 mm dishes.

The efficiency of transfection/infection was monitored by the fluorescent microscopy.

Results

Replacement of the original *AcMNPV* CNE by the heterologous CNE

Replacement of the genome element by its homolog originated from a heterologous genome followed by functional studies is a widely used method to test the functional conservation/diversification of these elements in the course of evolution. *AcMNPV* and

ManeNPV which appear to be the representatives of *Alphabaculovirus* group I and group II respectively were chosen as a subject of investigation aimed to test the functional conservation of CNE between two lineages of the genus. It is notable that *AcMNPV* efficiently disseminates the infection from cell to cell in the SF9 cell culture. In contrast, *ManeNPV* was shown not to be able to infect the Sf9 cells, that was an important factor enabling the design of functional complementation experiment.

The CNE knock out bacmid $vAc^{CNE-Ko-EGFP}$ as well as $vAc^{CNE-Ko-Rep-EGFP}$, the CNE repair bacmid which contains its own CNE in heterologous location (9 kb away from the original position) were obtained earlier [8]. It was shown that while $vAc^{CNE-Ko-EGFP}$ is not able to spread the infection from cell to cell, the CNE repaired virus efficiently does it, so these bacmids were used as controls in the experiment described below. The CNE deletion mutant vAc^{CNE-Ko} was used for the construction of the second CNE repaired bacmid which represents the analog of $vAc^{CNE-Ko-Rep-EGFP}$, where the genuine *AcMNPV* CNE was

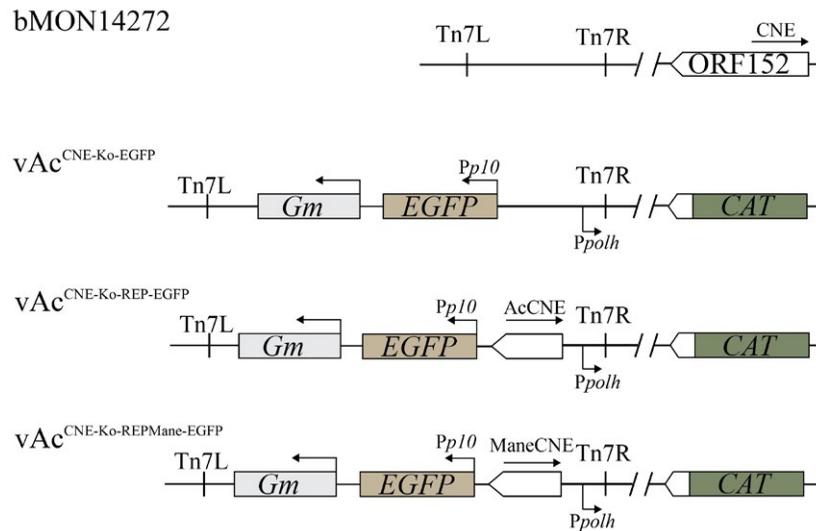


Fig. 1. Comparative presentation of the organization of analogous CNE-containing genome regions of the parental bacmid bMON14272 and its derivatives, CNE-knockout and CNE-repaired bacmids.

Recombinant genomes vAc^{CNE-Ko} and $vAc^{CNE-Ko-REP-EGFP}$ were derived earlier [8]. Bacmid vAc^{CNE-Ko} was used for generation of the CNE-repaired mutant $vAc^{CNE-Ko-REPMane-EGFP}$ carrying heterologous CNE originated from *ManeNPV* genome. $vAc^{CNE-Ko-REPMane-EGFP}$ was a result of the insertion of gentamicin-resistance gene (*Gm*)- *EGFP* gene - *ManeNPV* CNE cassette into the polyhedrin (*polh*) locus of vAc^{CNE-Ko} via transposon-mediated recombination using Bac-to-Bac system (Invitrogen Life Technologies). Tn7L and Tn7R indicate the left and right transposon arms, respectively. Arrows labeled Pp10 and Ppolh, denote the *polh* and *p10* promoters, respectively, and the unlabeled arrows denote native gene promoters. The arrowed white boxes indicate the complete and truncated Orf152, the black arrow above the boxes specifies CNE location within Orf152.

replaced by the CNE of ManeNPV (Fig. 1). The resulting recombinant $vAc^{CNE-Ko-RepMane-EGFP}$ also contained the *EGFP* gene which was inserted into the genome to simplify the detection of an infected cell using fluorescent microscopy. All construction manipulations were made using Bac-to-Bac system (see details in Material and Methods). $vAc^{CNE-Ko-RepMane-EGFP}$ together with $vAc^{CNE-Ko-EGFP}$ (negative control) and $vAc^{CNE-Ko-Rep-EGFP}$ (positive control) were used for the subsequent transfection of the Sf9 cells.

Transfection-infection assays

To test the ability of AcMNPV carrying the ManeNPV CNE to be propagated in the Sf9 cells the transfection-infection assays were applied as de-

scribed earlier [12]. The Sf9 cell monolayers formed by 1×10^6 cells per 30 mm cell culture dish were subjected to transfection by the $vAc^{CNE-Ko-RepMane-EGFP}$, $vAc^{CNE-Ko-EGFP}$ and $vAc^{CNE-Ko-Rep-EGFP}$. After 40 hours of incubation all cell monolayers were inspected by the fluorescent microscopy to estimate the transfection efficiency. The transfection efficiency was revealed to be quite comparable for each of transfected monolayers: nearly 10 % of cells in each monolayer illuminated with ultraviolet light emitted fluorescence. At 96 hpt the same amount of cells emitting fluorescence was observed in the $vAc^{CNE/Ko-E}$ – transfected sample but not in the samples transfected with either $vAc^{CNE-Ko-RepMane-EGFP}$ or $vAc^{CNE-Ko-Rep-EGFP}$ where amount of cells emitting green light increased

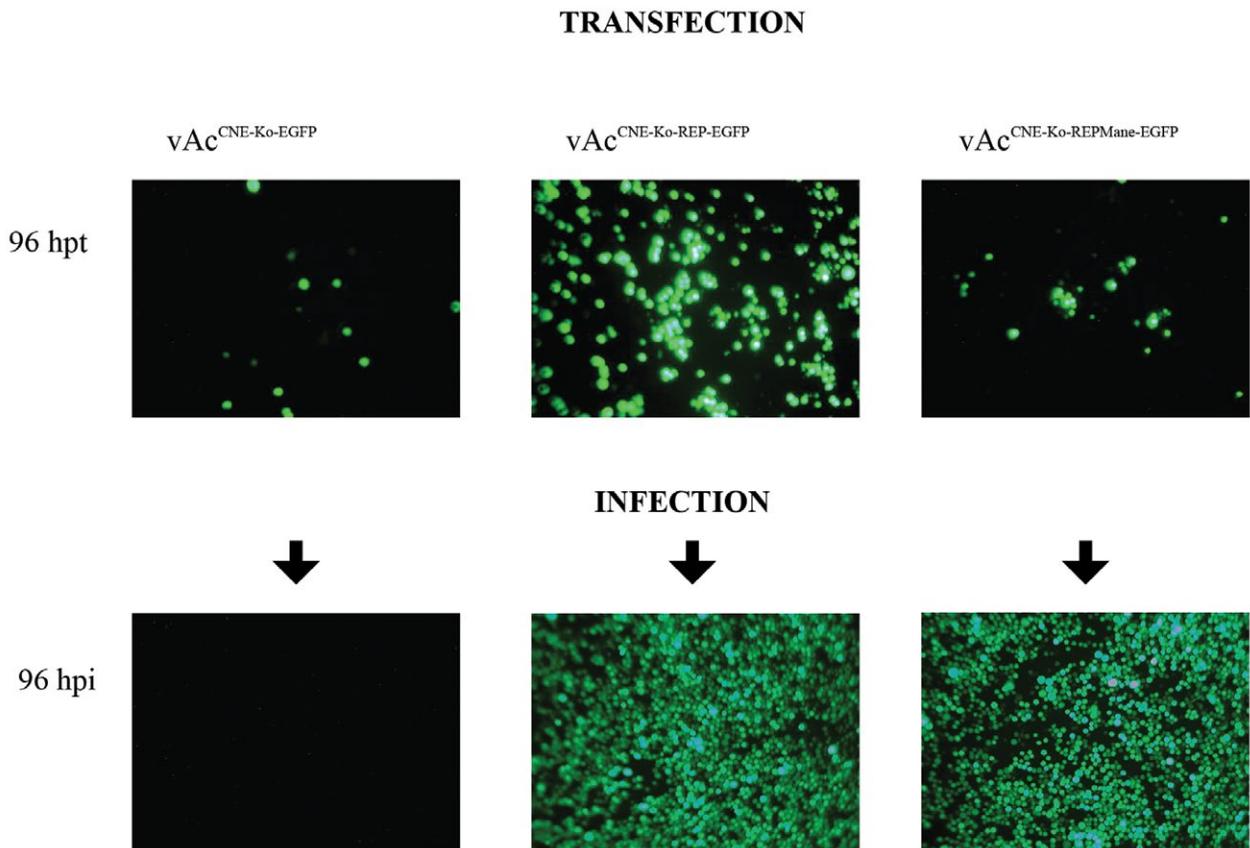


Fig. 2. Analysis of the infectivity of $vAc^{CNE-KO-EGFP}$, $vAc^{CNE-KO-REP-EGFP}$, $vAc^{CNE-KO-REPMane-EGFP}$. Cells transfected with DNA from indicated constructs (the top row of the image) and cells inoculated with the supernatants harvested from transfected cells (the bottom row of the image). Images were captured at 96 hpt and 96 hpi.

significantly (Figure 2). It is notable, however, that the vAc^{CNE-Ko-Rep-EGFP} and vAc^{CNE-Ko-RepMane-EGFP}-transfected monolayers were different in respect of the amount of green cells detected by the fluorescent microscopy: while the great majority of cells emitted fluorescence in the former, the discrete green islands formed by the cells surrounding the primarily transfected cell were visible in the latter. These data suggest that the ManeNPV CNE can rescue the lethal phenotype of AcMNPV carrying the CNE disruption, although the reduced amount of vAc^{CNE-Ko-RepMane-EGFP} - infected cells in comparison with the amount of vAc^{CNE-Ko-Rep-EGFP} - infected cells points that the ManeNPV CNE functions less efficiently than the original CNE. To further support these data the supernatants from transfected cells were harvested and used subsequently for the infection of fresh monolayers. At 96 hpi nearly 100% of vAc^{CNE/Ko-RepMane-EGFP} - and vAc^{CNE-Ko-Rep-EGFP}- infected cells exhibited green emission. No fluorescence emission was observed in the cell monolayers inoculated by the vAc^{CNE-Ko-EGFP} supernatant. These data were in accordance with the above suggestion about the conservation of CNE essential function between viruses.

Discussion

The nature of extended conservation of the CNE sequence across the genus *Alphabaculovirus* remains unknown, herewith, two non-mutually exclusive possibilities may be considered. A slow evolutionary rate of the CNE sequence may be caused by the overlap of several functional elements in the CNE locus, as well as may be the reflection of the intrinsic feature of some peculiar functional unit attributed to the CNE (Discussed in [8]). To clarify this issue, it is of great importance not only to establish so far unknown CNE function(s), but also to determine whether the essential or/and non-essential CNE functions remain conserved across the genus *Alphabaculovirus*. The fact of vital importance of the former for AcMNPV suggests a high but not absolute probability of its conservation among alphabaculoviruses, as can be deduced by analogy with the polyfunctional proteins: for instance, the F protein gene is found in the genomes of repre-

sentatives of all baculovirus genera, however F protein is expected to play an essential role of a fusion protein only in group II alpha-, beta- and deltabaculoviruses. In group I viruses, it appears to be inactive as a fusion protein and have been replaced by gp64 ([13] and references herein). Proceeding from it, the complementation experiment was designed to test the conservation of the CNE essential function between the relatively distant alphabaculoviruses, AcMNPV and ManeNPV, the representatives of alphabaculovirus group I and group II respectively. It was shown that the ManeNPV CNE can functionally substitute for the AcMNPV CNE that is in accordance with the assumption about the CNE essential function specifying the alphabaculovirus genus. Herewith, a decrease in the infectivity of vAc^{CNE-Ko-RepMane-EGFP} observed in the experiment points that the CNE activity is the characteristic of a particular virus species in respect of its efficiency which depends on the specific conditions provided by the virus or/and host cell. It is worth emphasizing that the replacement of the CNE in heterologous position in both vAc^{CNE-Ko-Rep-EGFP} and vAc^{CNE-Ko-RepMane-EGFP} genomes should lead to the loss of the CNE non-essential function, cis-regulation of the expression of an adjacent gene as well as its coding capacity of short peptide and ncRNAs, therefore the above conclusion is assigned precisely to the CNE essential activity.

An earlier comparative analysis of the alphabaculovirus genomes have demonstrated that the peptide-coding capacity of CNE is not universal and the vast majority of the alphabaculovirus CNEs do not overlap with any ORFs [8]. Conservation of the CNE essential function between relatively distant alphabaculoviruses has been demonstrated by the present study. Conservation/diversification of both CNE cis-regulatory activity and CNE capacity to encode ncRNA remains to be established in order to infer the nature of the extraordinary conservation of CNE.

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Функциональна комплементация консервативного некодирующего элемента (CNE) вирусу ядерного полиедроза *Autographa californica* гетерологічним CNE з вірусу ядерного поліедроза *Malacosoma neustria*

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Мета. Оцінити функціональну консервативність бакуловірусного CNE (conserved non protein-coding element).

Вивчити функціональне заміщення консервативного некодирующего элемента (CNE) вірусу ядерного поліедроза *Autographa californica* на CNE вірусу ядерного поліедроза *Malacosoma neustria*. **Методи.** Видалення CNE з вірусного генома і його заміщення гетерологічним CNE було виконано з використанням бакмідної технології. Трансфекційно-інфекційний аналіз був застосований для дослідження поширення рекомбінантного вірусу від клітини до клітини. **Результати.** Було сконструйовано рекомбінантний геном вірусу ядерного поліедроза *Autographa californica*, що несе в своєму складі CNE вірусу ядерного поліедроза *Malacosoma neustria* замість власного CNE. Показано, що рекомбінантний вірус здатний передавати інфекцію в культурі клітин. **Висновки.** Абсолютно обов'язкова функція CNE є консервативною для представників I і II груп роду *Alphabaculovirus*.

Ключові слова: бакуловірус, бакуловірусний CNE, функціональна комплементация

Функциональная комплементация консервативного некодирующего элемента (CNE) вируса ядерного полиедроза *Autographa californica* гетерологичным CNE из вируса ядерного полиедроза *Malacosoma neustria*

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Цель. Оценить функциональную консервативность бакуловірусного CNE (conserved non protein-coding element). **Методы.** Удаление CNE из вирусного генома и его замещение гетерологичным CNE было выполнено с использованием бакмидной технологии. Трансфекционно-инфекционный анализ был применен для изучения распространения рекомбінантного вируса от клетки к клетке. **Результаты.** Был сконструирован рекомбінантний геном вірусу ядерного поліедроза *Autographa californica*, несущий в своем составе CNE вірусу ядерного поліедроза *Malacosoma neustria* вместо собственного CNE. Было показано, что рекомбінантний вірус способен передавать інфекцію в культуре клеток. **Выводы.** Абсолютно обязательная функция CNE является консервативной для представителей I и II групп рода *Alphabaculovirus*.

Ключевые слова: бакуловірус, бакуловірусний CNE, функціональна комплементация

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