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SHORT COMMUNICATIONS

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Intracellular Localization of Regulatory Proteins of the German Cockroach *Blattella germanica* Densovirus

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Abstract—The use of western blot analysis of nuclear and cytoplasmic extracts of BgDNV densovirus-infected German cockroach, *Blattella germanica*, the intracellular localization of the regulatory proteins of the corresponding densovirus was investigated in cell culture. It was demonstrated that two proteins, namely NS1 and NS3, were predominantly localized in the nucleus, whereas NS2 protein was equally distributed in the nuclei and the cytoplasm. The data obtained are important for understanding the potential functions of densovirus regulatory proteins. The intracellular localization of NS3 protein was determined for the first time for any densovirus.

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Densovirus of German cockroach *Blattella germanica* (BgDNV) belongs to the Densovirinae subfamily of the Parvoviridae family. Viruses of this subfamily are highly species-specific pathogens of insects and a number of crustaceans, causing fatal diseases. Densoviruses are characterized by an icosaedral capsid without lipoprotein envelope. The genomes of densoviruses are represented by linear single-stranded DNA, 4000–6000 nt in length. The distinguishing feature of the densovirus genome is noncoding terminal palindromic sequences that are crucial for virus genome replication.

The analysis of genomes of a number of densoviruses revealed that all of them contain two or three open reading frames (ORFs) encoding regulatory proteins [1]. Our understanding of the functions of corresponding proteins and their roles in densovirus life cycle at present is quite incomplete. It is known that one regulatory protein, namely NS1, which is present in all densovirus genomes, presumably takes part in the following processes of the densoviral life cycle: the initiation of genome replication, transcription regulation, and the encapsidation of viral DNA. NS1 protein possesses ATP-dependent helicase activity, site-specific endonuclease activity, and could bind specific regions of the viral genome [2, 3].

NS2 regulatory protein was also found in the genomes of all known densoviruses. According to Azarkh et al. [4], this viral protein is necessary for effective viral genome replication and the formation of virus progeny capable of productive infection. Nevertheless its precise function remains unclear.

NS3 protein can be found in the genomes of only a few densoviruses. The S3 amino acid sequence is characterized by the presence of a zinc finger motif, and it was shown previously that the expression of this protein is essential for the completion of a productive virus infection [5].

Unlike NS1 protein, there is slight homology between NS2 and NS3 proteins of different densoviruses, which could indicate that the functions of the corresponding proteins are specialized for each densovirus and, most likely, play adaptive roles [6].

Thus, it seems important to investigate the functions of densovirus regulatory proteins to further understand the mechanisms of viral pathogenesis, as well as of the regulation of viral infection by the host cell.

Densovirus of the German cockroach was described for the first time in 2000 [7]. It was shown that the BgDNV genome comprises three ORFs that encode regulatory proteins (ORF3, ORF4, and ORF5, Fig. 1). All three corresponding proteins, NS1 (ORF3, 60 kDa), NS2 (ORF4, 29 kDa), and NS3 (ORF5, 31 kDa), were shown to be synthesized during viral infection [8].

It is well known that all proteins, including the viral ones, fulfill their functions in certain cell compartments. Thus, the elucidation of the intracellular localization of each of the densoviral proteins would further our understanding of their role in the virus infection cycle. In the current work, we determined the intrac-

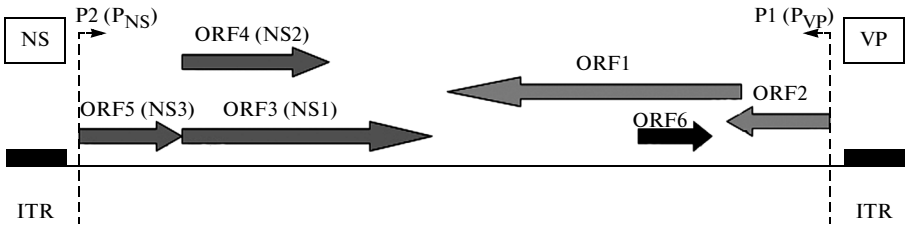

Fig. 1. German cockroach densovirus BgDNV genome organization. NS is portion of the genome encoding regulatory proteins, VP is portion of the genome encoding capsid proteins. Black rectangles indicate noncoding terminal inverted repeats. Arrows indicate open reading frames; the open reading frame names and the proteins encoded by them are given above the arrows. The open reading frames encoded by the opposite genome strands are indicated by the arrows in the opposite direction. Dotted arrows indicate promoters driving expression of capsid protein genes (P1) and regulatory protein genes (P2).

>NS1
MNYGRLTDFWSRFGVTMGDDAGRDSVSSSDMVEAVGGEPSGGPVQGQAEVTSSSVDQKLQELVDRFVSRLEEKNWKD
SGYYISDVYACESSERANALARRLEQRAESFGRGFIGIFIHNNHVHTIHACPYTSRTCRCQFKNFPEAKEDIRRLLR
KPPAIETFTRRDWENITKYFCTSGRRATFFKIFGHLQRLPLEITALSDSTISGQDGGGPDSGVENCNDPLEFHSGPE
VGDIPARPRANRRRKRRDQIVVGGDGGIGGATGIILDL/LSKCAVCPLTEIVFTKEYLQDPIVACKRLDSKEVKDAI
DTRASVINTWEREDFVAFYNNPNTILIWSARSLNAFDSYYFNYEESFNVVTELLTFQMGENLVQFCRNLVDTLECNI
PKRNCFVVCSPPSAGKNFFFDGVKDYYLNSGQMNNPNKYNQFAYQDCHNRRIIIWNEPNYEPREMENLKMLFAGDNL
SANVKCKPQANVKRTPVIVLTNSLPNFCQQTAFNDRVITYHWTQATFLKDYNKKPRPDACVDVLYSLLQ

>NS2
MAVSPTFGAALESLWEMMPDEIASHPATWWKLLEESPLEDRFKDKLKSLLVRWTKNYKNWLIGSFPALKKKIGKTVD
TILAMYMPANHLNELMHWLDDWSKELNLSEEDLSEYLSTIITSIQSTHAPTQAGRAGASSRTSLKRKKTLDDCFESL
QPSKRSHDETGKISQSIFVRQGDEQRSLKSLDTYKDYLLKLQLYPTLQYQAKMEEDRTQAWRTAMIRLNFTVDQKSG
IFQRVLELTDAVKDEIKLLLAETEESEELQE

Fig. 2. Localization of bioinformatically predicted putative nuclear transport signals in BgDNV NS1 and NS2 proteins. Single underlining indicates nuclear localization signals, double underlining indicates nuclear export signal.

ellular distribution of BgDNV regulatory proteins in the course of viral infection.

Bioinformatic analysis of amino acid sequences of BgDNV NS1, NS2, and NS3 proteins using Wolf PSORT (http://www.psort.org) and NetNES 1.1 software, designed for nuclear localization and nuclear export signal prediction, respectively, revealed the presence of a number of amino acid motifs, which can influence the intracellular distribution of the proteins. These are the nuclear localization signal-like sequences in NS1 protein, including PRANRR (239–245 aa), RRRKRR (243–248 aa), and KKPR (514–516 aa); nuclear localization-like sequences in NS2 protein: PALKKKI (66–72 aa) and KRKK (142–145 aa); and nuclear export signal-like sequence LPLEITAL (193–200 aa) in NS1 protein (Fig. 2). Amino acid residue numbers are given according to BgDNV protein amino acid sequences in GenBank (AY189948). No amino acid patterns similar to signals controlling nuclear transport were found in NS3 protein.

The most up-to-day technique of investigating intracellular distribution of proteins is immunocytochemistry, which uses specific primary antibodies and fluorescent-labeled secondary antibodies to demonstrate the localization of protein in an intact cell. Using bioinformatics tools we have previously predicted optimal epitopes in BgDNV NS1, NS2, and NS3 proteins. We obtained polyclonal rabbit antibodies (Genemed Synthesis Company, United States) against short (20–22 aa) polypeptide sequences that correspond to one of the predicted epitopes in each regulatory protein [8]. Utilizing immunocytochemistry, we showed that NS1 protein was localized in the nuclei of infected cells [8]. However, we were not able to determine the intracellular localization of NS2 and NS3 proteins because of the low specificity of the corresponding antibodies. Therefore, in the present study, we assessed the presence of these proteins in nuclear and cytoplasmic extracts of infected cells by immunoblotting, which is more sensitive that immunochemistry.

The study was performed using German cockroach cell culture BGE2. Cells were infected with the virus by adding a suspension containing densovirus viral particles into the culture medium. The virus-containing suspension was obtained by lysing a sample of previously infected cells.

Virus-infected and control noninfected cells were pelleted by centrifugation for 5 min at 4000 rpm; washed three times with cold phosphate-buffered saline (PBS); and lysed for 30 min at 4°C in 10 mM HEPES buffer (pH 7.9) containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1% Triton X-100, and protease inhibitor cocktail (Sigma). Then, nuclei were pelleted by centrifugation for 15 min at 6500 rpm at

4°C and the resulting supernatant was transferred to a clean Eppendorf tube. The pellet was washed with cold PBS several times to remove residual cytoplasm. The nuclei were subsequently lysed for 30 min at 4°C in 20 mM HEPES buffer (pH 7.9), containing 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 10 mM β-glycerophosphate, and protease inhibitor cocktail. Debris was removed by centrifugation for 5 min at 13000 rpm at 4°C.

The resultant nuclear and cytoplasmic extract samples were fractionated by electrophoresis in 10% PAGE [10]. In each well, 54 μg of total protein was applied. The protein content in the samples was estimated spectrophotometrically by the absorption rate at a wavelength of 280 nm. After fractionation proteins were transferred onto an Amersham Hybond-LFP (Amersham) PVDF membrane using semi-dry blotting technique in a buffer containing 47.9 mM Tris-HCl, 38.6 mM glycine, and 10% methanol (pH 8.3) for 2 h with constant current strength. Commercially available Precision Plus Protein Standards, Dual Color (Bio-Rad) was used as protein molecular weight markers, and the relative molecular weight of the proteins under study were estimated based on a calibration curve. For protein detection, after western blotting, we used a ProtoBlot II AP System with stabilized substrate (Promega) and primary antibodies against NS1, NS2, and NS3 proteins; all procedures were performed according to the manufacturer's recommendations.

The results obtained in our study are presented on Fig. 3. NS1 protein is localized mainly in the nuclei of virus-infected BGE2 cells, which is in agreement with the previously obtained data [8]. The intracellular distribution of NS1 corresponds well with its presumptive role in genome replication, which takes place in the nuclei of densovirus-infected cells, and transcription regulation, and is in agreement with the data obtained earlier for another densovirus PfDNV [11]. However, some NS1 was also observed in the cytoplasm. This fraction could possibly represent newly synthesized protein not yet transported into the nucleus. It is reasonable to suppose that the maintenance of some NS1 in the cytoplasm is caused by the presence of a nuclear export-like signal and, therefore, may serve some thus far unknown functions. It is possible that the presence of both nuclear localization and nuclear export signals is associated with some shuttle function of NS1 during the life cycle of BgDNV.

NS2 protein was found to be equally distributed between the nucleus and cytoplasm (Fig. 3). The similar intracellular localization was shown previously for NS2 protein of the yellow-fever mosquito densovirus (AeDNV) [4], as well as for homologous proteins of viruses from another parvovirus subfamily, infecting vertebrates [12].

Intracellular distribution of NS3 protein is also given in Fig. 3. As mentioned above, antibodies against NS3 appeared to have low specificity and to bind not

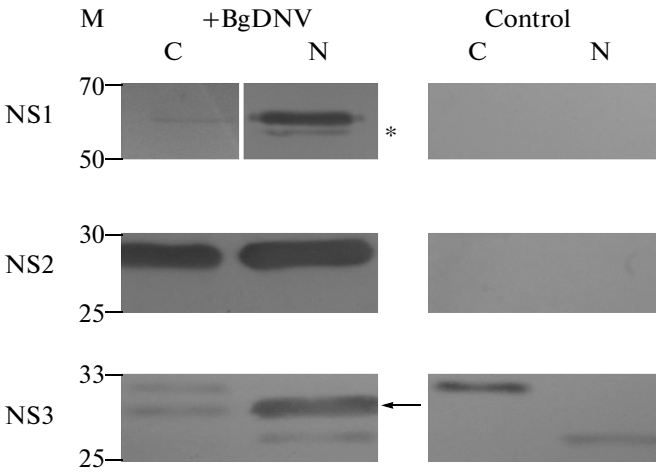

Fig. 3. Localization of NS1, NS2, and NS3 viral proteins in BGE2 cells infected by BgDNV virus detected using western blotting. Extracts of cells not infected by the virus were used as control. C indicates cytoplasmic extracts; N indicates nuclear extracts; M indicates protein molecular mass marker. * presumable nonmodified form of NS1 or the product of its partial proteolysis. An arrow indicates the band corresponding to NS3 specific signal.

only the viral protein, but also some cellular proteins, which are present in infected, as well as in control cells. Nevertheless, it can be seen in Fig. 3 that the band with molecular weight of 31 kDa (indicated with an arrow), which corresponds to NS3 protein, is only present in the infected cell sample. Thus, based on this result, it can be concluded that BgDNV NS3 protein is characterized by predominantly nuclear localization.

NS3 protein can also be found in the cytoplasm of infected cells, where it either fulfills some particular function, or accumulates because its transport into the nucleus is hindered. As mentioned earlier, the NS3 amino acid sequence contains no canonical nuclear localization signals; therefore the mechanisms providing nuclear localization of this protein remain unclear. However, it should be noted that, in western blot analysis, the molecular weight of NS3 (31 kDa) slightly exceeded the predicted molecular weight (26 kDa); this may be indirect evidence of posttranslational modifications of the protein, which are commonly known to be capable of regulating the intracellular localization of proteins [13].

In the current work, data on the intracellular distribution of BgDNV-encoded regulatory proteins were obtained. It should be noted that very few investigations have been conducted on the localization and functions of densovirus regulatory proteins, though this work is important for understanding the mechanisms of viral replication and different phases of the viral infectious cycle. Data on intracellular distribution of NS1 and NS2 proteins have so far only been obtained for two densoviruses PfDNV [11] and

AeDNV [4]. The data on NS3 protein localization were obtained for densoviruses for the first time.

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