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Characterization of the nuclear localization signals of duck circovirus replication proteins

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Summary. – Duck circovirus (DuCV) possess a circular, single-stranded DNA genome that requires the replication protein (Rep) for its replication. Based on the viral genotype, there are two categories of Rep proteins: Rep1 and Rep2. To characterize the nuclear localization signals (NLSs) conferring the nuclear localization of the Rep proteins, defined coding regions of the rep gene of two genotypes of DuCV were cloned and co-expressed with the red fluorescent protein DsRed2. The results showed that deleting the putative N-terminal NLS located at amino acid residues 10–37 of Rep1 and Rep2 abrogated nuclear translocation, while deleting the putative C-terminal NLS located at residues 244–274 of Rep1 did not significantly alter its subcellular localization, confirming that only the NLS located at residues 10–37 in the N-termini of the Rep proteins had nuclear targeting activity.

Keywords: duck circovirus; genotype; Rep protein; nuclear localization signal

Duck circovirus (DuCV) is a member of the genus *Circovirus* of the *Circoviridae* family (Hattermann *et al*., 2003). Infection with DuCV is characterized by feathering disorders, poor body condition, and low body weight (Hattermann *et al*., 2003), and it results in multiple local lesions in the spleen, thymus, and the bursa of Fabricius in ducks (Soike *et al*., 2004; Liu *et al*., 2010a). Our previous studies provided evidence of possible horizontal and vertical transmission of DuCV and the simultaneous transmission of DuCV-1 and DuCV-2 from breeder ducks to ducklings (Liu *et al*., 2010a, 2014). Surveys showed that there was a high prevalence of DuCV in duck flocks of different species (Hattermann *et al*., 2003; Soike *et al*., 2004; Jiang *et al*., 2008; Zhang *et al*., 2009; Liu *et al*., 2010a).

With a single-stranded circular genomic DNA of about 1.99 kb, DuCV was classified into two genotypes, DuCV-1 and DuCV-2 (Wang *et al*., 2011; Zhang *et al*., 2012). Both DuCV-1 and DuCV-2 have similar genomic organizations, with three major ambisense open reading frames (ORFs) (Zhang *et al*., 2012). ORF1 (*rep*) is located on the viral plus-strand, and it encodes the viral replication-associated protein (Rep) (Hattermann *et al*., 2003). Located on the complementary strand, ORF2 (*cap*) encodes the immunogenic capsid protein, while ORF3 encodes the ORF3 protein, which has pro-apoptotic activity (Hattermann *et al*., 2003; Xiang *et al*., 2012).

Although circoviruses lack an autonomous DNA polymerase and are dependent on the replication machinery of the host cell for *de novo* DNA synthesis, Rep is required to initialize viral replication (Tischer *et al*., 1987; Meerts *et al*., 2005). In the case of porcine circovirus (PCV), ORF1 encodes, via

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Abbreviations: CP = capsid protein; DuCV = duck circovirus; NLS(s) = nuclear localization signal(s); ORF(s) = open reading frame(s); PCV = porcine circovirus; Rep = replication protein

differential splicing, two viral replication-associated proteins, Rep and Rep', (Mankertz and Hillenbrand, 2001; Mankertz *et al*., 2003; Cheung, 2003, 2004). Rep and Rep' are responsible for viral replication, and the replication of the PCV genome requires interactions between the Rep complex (Rep-Rep') and the cis-acting element at the origin of replication (Ori) that is present in the minimum-binding-site recognition region (Mankertz and Hillenbrand, 2001; Steinfeldt *et al*., 2001, 2006; Cheung, 2004, 2006, 2007).

Previous studies have revealed that the nuclear accumulation of the PCV1 and PCV2 capsid proteins is mediated by their distinct nuclear localization signal (NLS) motifs (Liu *et al*., 2001; Shuai *et al*., 2008). For PCV1, of the three potential NLSs that are located in the identical N-termini of Rep and Rep', NLS1 and NLS2 are required to mediate nuclear import, whereas NLS3 enhances the nuclear accumulation of the Rep proteins (Finsterbusch *et al*., 2005). The nuclear localization of the capsid protein (CP) of the beak feather disease virus (BFDV) was shown to be directed by three partially overlapping, bipartite NLSs, whereas Rep that was expressed on its own in insect cells was restricted to the cytoplasm. When co-expressed with the CP, Rep localized to the nucleus, strongly suggesting that an interaction with the CP facilitates the translocation of Rep into the nucleus (Heath *et al*., 2006).

Based on the phylogenetic analysis of complete amino acid sequences, the Rep protein of DuCV has been classified into two types: Rep1 and Rep2, respectively (Zhang *et al*., 2012). Our previous study showed that two independent, bipartite NLSs were located in the N-terminus of the CP at amino acid residues 1–17 and 18–36 (Xiang *et al*., 2013). Therefore, in this study, we aimed to determine whether the putative NLSs in Rep1 and Rep2 are required for their nuclear localization.

Using the online computer program cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi), the Rep of DuCV-1 (Rep1) and the Rep of DuCV-2 (Rep2) were analyzed, and two potential NLSs were found at residues 10–37 and 244–274 of Rep1, whereas only a single NLS was found at residues 10–37 of Rep2 (Fig. 1). To test whether these potential NLSs were functional, we constructed a series of plasmids that expressed truncated Reps, which lacked the putative NLSs, fused with the red fluorescent protein DsRed2.

Two plasmids, pDuCV-1, which includes the complete genomic DNA of the DuCV-1 strain FJ0601 (EF370476) (Jiang *et al*., 2008), and pDuCV-2, which includes the complete genomic DNA of the DuCV-2 strain WF0701 (EU022375) (Zhang *et al*., 2012), were constructed and stored in our laboratories. Using plasmid pDuCV-1 as template, the complete *rep1* gene was amplified by PCR with primers Rep1-F and Rep1-R (Table 1). The entire *rep2* gene was amplified with primers Rep2-F and Rep2-R using plasmid pDuCV-2 as a template. The PCR products were digested with *Eco*RI and *Bam*HI (TaKaRa, Dalian, China) and inserted into the corresponding region of pDsRed2-N1 (Clontech, Mountain View, CA, USA). The recombinant plasmids, named pDs-Rep1 and pDs-Rep2, were used to express Rep fusion proteins tagged with the red fluorescent protein DsRed2.

Using pDs-Rep1 and pDs-Rep2 plasmid DNA as a template, four pairs of mutagenic primers were designed to construct the recombinant plasmids by the site directed mutagenesis method (Table 1). PCR was carried out in a 50 µl volume with 100 ng template plasmid DNA, 0.2 mmol/l (each) mutagenic primer, 200 µmol/l (each) deoxynucleoside triphosphates, 5 µl of 10×*Pfu* PCR buffer (containing Mg2+), and 2.5 U of cloned *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA). The amplification parameters consisted of an initial 5 min denaturation at 95°C, followed by 18 cycles of 1 min at 95°C, 1 min at 60°C, and an extension step at 68°C, where the extension time in minutes was twice the length of the plasmid in kilobases, and a final 15 min extension step at 72°C. After checking the DNA quality by agarose gel electrophoresis, the PCR products were digested with ten units of DpnI (TaKaRa) at 37°C for 1–2 hr. Then, 1–2 µl of the DpnI-treated DNA was used to transform *Escherichia coli* DH5α competent cells (Li *et al*., 2008). A series of recombinant plasmids, pDs-Rep1Δ10–37, pDs-Rep1Δ244–274, pDs-Rep1Δ10–37Δ244–274, and pDs-Rep2Δ10–37 were constructed and verified by sequence analysis.

The human non-small-cell lung carcinoma cell line H1299 (ATCC CRL-5803) was obtained from the American Type Culture Collection (Rockville, MD, USA). In a six-well culture plate, H1299 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (GIBCO BRL), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were maintained at 37°C in a humidified 5% CO_2 incubator. When the cells reached approximately 70% confluence, they were washed twice with phosphate-buffered saline (8.1 mmol/l Na_2HPO_4, 1.5 mmol/l KH_2PO_4, 140 mmol/l NaC1, and 3.0 mmol/l KC1, pH 7.2) and transfected with 2 µg of purified recombinant plasmids (pDs-Rep1, pDs-Rep1ΔN10–37, pDs-Rep1ΔN244–274, pDs-Rep1ΔN10–37ΔN244–274, pDs-Rep2, or pDs-Rep2ΔN10–37) per well using the Xfect transfection reagent (Clontech) according to the manufacturer's protocol; H1299 cells transfected with an equal amount of the pDsRed2-N1 vector were used as negative controls.

DuCV-1-positive sera and DuCV-2-positive sera, which were the mixtures of serum samples from ten DuCV-1-positive ducks and ten DuCV-2-positive ducks (Liu *et al*., 2010b), were stored in our laboratories. The H1299 cells were harvested 48 hr post-transfection and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blotting as previously described (Xiang *et al*.,

2013). DuCV-positive sera were used at a 1:100 dilution; anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mouse monoclonal antibody (ProteinTech Group, Chicago, IL, USA) was used at a 1:2,000 dilution; anti-DsRed2 mouse monoclonal antibody (Santa Cruz Biotechnology, Dallas, TX, USA) was used at a 1:200 dilution; and horseradish peroxidase (HRP)-labeled goat anti-duck antibody (KPL, Gaithersburg, MD, USA) and HRP-labeled goat-anti-mouse IgG (Santa Cruz Biotechnology) were used at a 1:10,000 dilution. All constructs expressed proteins that corresponded to their expected molecular sizes, and no protein was detected in H1299 cells transfected with pDsRed2-N1 using the DuCV-positive sera (Fig. 2a,b).

The H1299 cells were washed, mounted, and analyzed by fluorescence microscopy (AF6000, Leica, Wetzlar, Germany) at 48 hr after transfection. The results showed that DsRed2-tagged Reps (Rep1-DsRed2 and Rep2-DsRed2) were observed exclusively in the nuclei of transfected H1299 cells,

Table 1. The primers used in this study

Primers	Oligonucleotide sequence in 5'–3'direction
Rep1-F	CCAGAATTCGCAGGATGGCTAAGAGTGG
Rep1-R	CCGGGATCCTGGTAGTTTATTGGAAAGGGGAG
Rep2-F	CCAGAATTCGCAGGATGGCGAAGAGCGGCAA
Rep2-R	CCGGGATCCTGGTAGTTTATTGGGAACGGGAGGGTA
Rep1-Δ10-37 F	TAAGAGTGGCAACTACTCCTACTTCGCAATCGTCGGA
Rep1-Δ10-37 R	TCCGACGATTGCGAAGTAGGAGTAGTTGCCACTCTTA
Rep1-Δ244-274 F	GTTGCTAAGACGTTGATCGTTGACAAGTATGAACCTGCCC
Rep1-Δ244-274 R	GGGCAGGTTCATACTTGTCAACGATCAACGTCTTAGCAAC
Rep2-ΔN10-37 F	GAAGAGCGGCAACTACTCATACTTTGCTATCGTCGGCGAG
Rep2-ΔN10-37 R	CTCGCCGACGATAGCAAAGTATGAGTAGTTGCCGCTCTTC

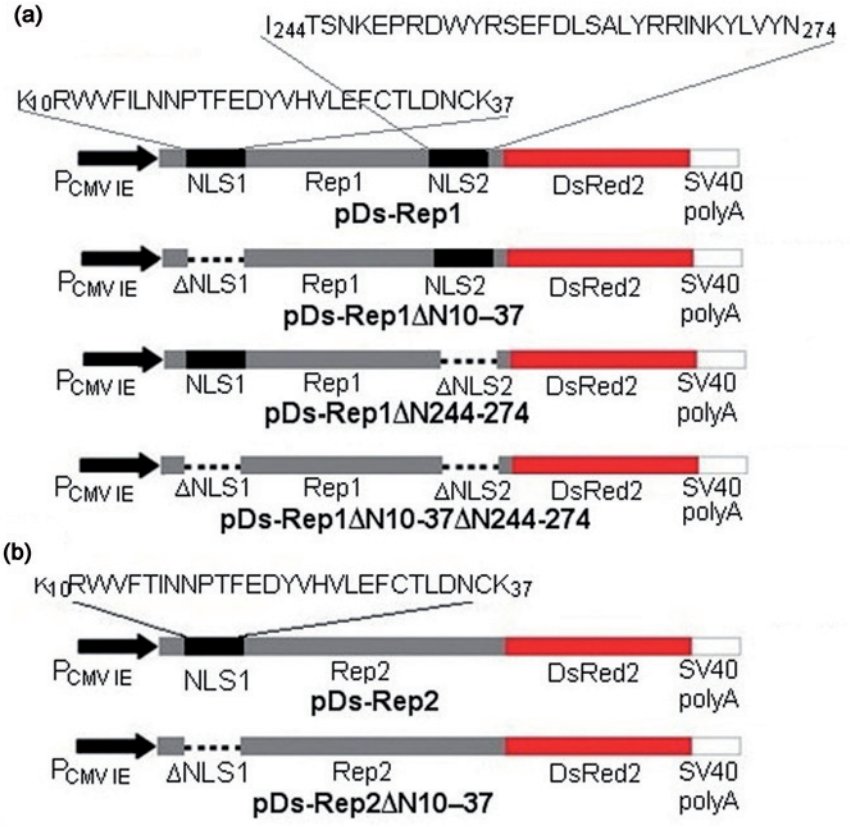

Fig. 1

A schematic representation of the replication protein (Rep) derivatives of DuCV used in this study

(a) Expression vector carrying the red fluorescent protein (DsRed2)-tagged truncated recombinant DuCV Rep1 proteins. (b) Expression vector carrying the DsRed2-tagged truncated recombinant DuCV Rep2 proteins. The human cytomegalovirus (CMV) immediate early promoter (black arrows) was fused to the N termini of the Reps (gray boxes). The regions of the Reps corresponding to their putative NLSs are indicated by thick black lines. Residues constituting the potential NLSs are indicated. Dashed black lines represent the deleted regions in each of the truncated derivatives. Thick red lines represent the DsRed2 protein fused to the C termini of the Reps.

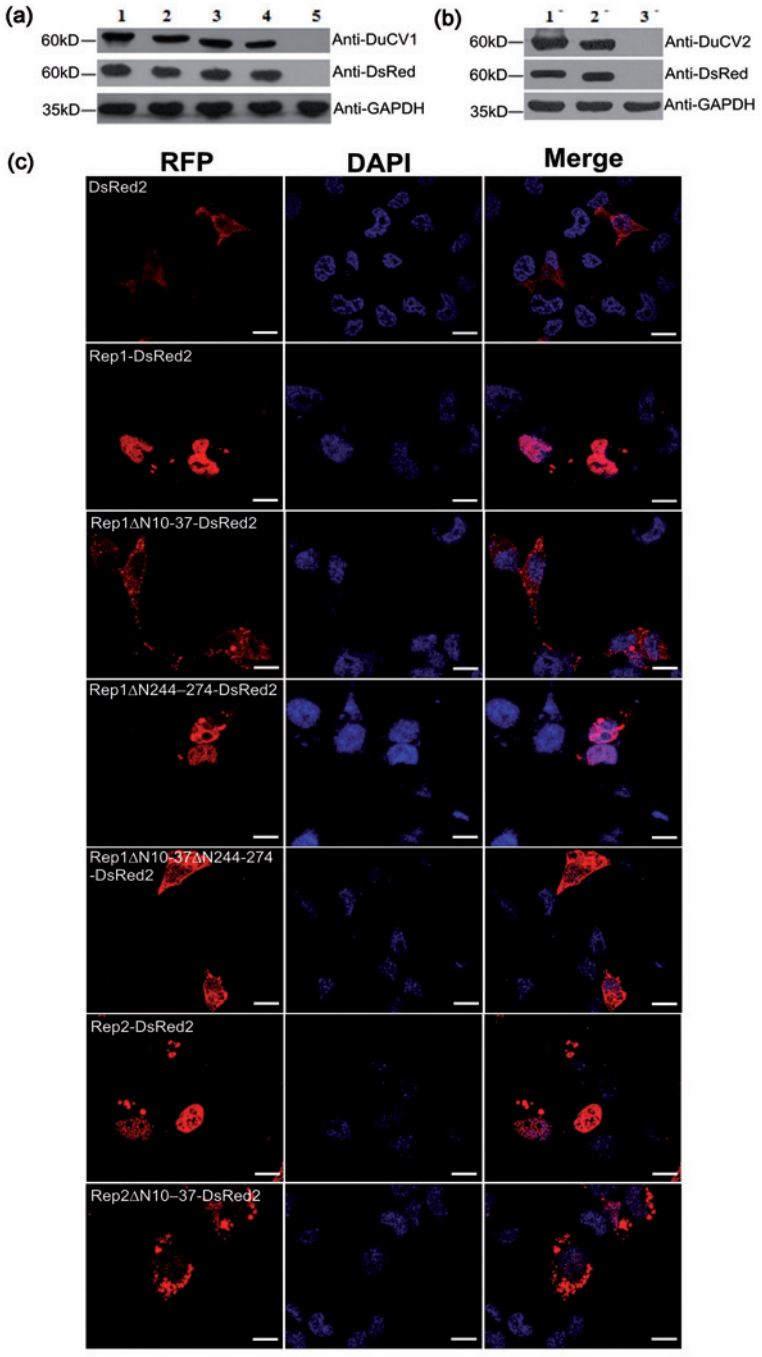

Fig. 2

Analysis of Rep expression in H1299 cells

(a) Analysis of truncated DuCV Rep1 proteins by Western blotting. Rep1-DsRed2 (lane 1), Rep1ΔN10–37-DsRed2 (lane 2), Rep1ΔN244–274-DsRed2 (lane 3), Rep1ΔN10–37ΔN244–274-DsRed2 (lane 4), and DsRed2 (lane 5). (b) Analysis of truncated DuCV Rep2 proteins by Western blotting. Rep2-DsRed2 (lane 1), Rep2ΔN10–37-DsRed2 (lane 2), and DsRed2 (lane 3). (c) Subcellular distribution of DuCV Rep1 and Rep2 proteins in H1299 cells. In the top row, DsRed2 expressed alone is distributed throughout the cytoplasm of infected cells. In the second and sixth rows, DsRed2-tagged Reps were observed exclusively in the nuclei of infected cells. In the third and seventh rows, deletion of the first putative NLS (N-terminal amino acid residues 10–37 of Rep1 and Rep2) completely abolished the translocation of the fusion proteins Rep1ΔN10–37-DsRed2 and Rep2ΔN10–37-DsRed2 into the nuclei of infected cells, resulting in their distribution throughout the cytoplasm, as occurred for DsRed2. In the fourth row, removal of the second putative NLS (residues 244–274) of Rep1 clearly did not impair the nuclear import of the Rep1-DsRed2 fusion protein. In the fifth row, deletion of residues 10–37 and 244–274 completely abolished the translocation of the Rep1ΔN10–37ΔN244–274-DsRed2 fusion protein into the nuclei of infected cells, resulting in its distribution throughout the cytoplasm, as occurred for DsRed2.

and fluorescence was generally distributed evenly throughout the nuclear region, while DsRed2 was distributed throughout the cytoplasm of transfected cells (Fig. 2c). Similarly to the Rep1-DsRed2 proteins, the Rep1ΔN244–274-DsRed2 fusion protein was distributed exclusively in the nuclei of transfected H1299 cells (Fig. 2c), thus the deletion of the putative NLS located at residues 244–274 of Rep1 did not significantly alter its subcellular localization. In contrast, the Rep fusion proteins Rep1ΔN10–37-DsRed2, Rep2ΔN10–37-DsRed2, and Rep1ΔN10–37ΔN244–274-DsRed2 were uniformly distributed throughout the cytoplasm of the transfected H1299 cells, as was DsRed2, which indicated that deleting N-terminal residues 10–37 of Rep1 and Rep2 completely abrogated the nuclear translocation of their respective fusion proteins (Fig. 2c). The results showed that only the NLS located at residues 10–37 of Rep1 and Rep2 had nuclear targeting activity.

The Rep protein is the viral replication-associated protein of DuCV (Hattermann *et al*., 2003). To date, we have very little knowledge about the functional role of this protein in the replication and maturation of DuCV. In this study, the proteins Rep1 and Rep2 of DuCV were successfully expressed in non-small cell lung cancer cells. A western blot assay showed that Rep1 and Rep2 were detected by DuCV-positive serum (Fig. 2a,b), which indicated that the Reps of DuCV are in fact expressed in ducks infected with DuCV.

Two potential NLSs are located at the N- and C-termini of DuCV Rep1, whereas only one potential NLS is located at the N-terminus of DuCV Rep2 (Fig. 1). To test whether these potential NLSs were functional, we constructed a series of plasmids by sequentially deleting the N-terminal and C-terminal regions between amino acid residues 10–37 and 244–274 of Rep1 and Rep2, respectively. The results indicated that deleting the N-terminal NLSs (residues 10–37) of Rep1 and Rep2 clearly abrogated nuclear translocation, while deleting the putative C-terminal NLS located at residues 244–274 of Rep1 did not significantly alter its subcellular localization, confirming that the karyophilic activity of these proteins was associated with their N-terminal NLSs. The data also suggested that the nuclear translocation mechanism of DuCV Rep1 might be the same as that of DuCV Rep2.

Many different viruses have been shown to either target nuclear proteins for use in virus infection, and viral proteins have been shown to localize to the nucleus. Although Rep is required to initialize viral DNA replication, the continuation of the process is dependent upon cellular enzymes expressed during the cell's S phase, and it commences only after the host cell has passed through mitosis (Tischer *et al*., 1987). The Rep protein of the beak feather disease virus, when expressed alone, is restricted to the cytoplasm of insect cells, whereas it localizes to the nucleus when co-expressed with the CP (Heath *et al*., 2006). In a stark contrast, there are three NLSs with nuclear targeting activity that are located at the identical N-termini of Rep and Rep' of PCV (Finsterbusch *et al*., 2005). In this study, the Rep proteins of two DuCV genotypes actively localized to the nucleus when they were expressed in non-small cell lung cancer cells (Fig. 2c). The data presented in this study and in our previous study (Xiang *et al*., 2013) showed that the CP and Reps each have one NLS at their N-terminus, which indicates that these proteins might independently translocate into the nucleus during viral replication.

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