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Replication of chicken anemia virus (CAV) requires apoptin and is complemented by VP3 of human torque teno virus (TTV)

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ABSTRACT

To test requirement for apoptin in the replication of chicken anemia virus (CAV), an apoptin-knockout clone, pCAV/Ap(−), was constructed. DNA replication was completely abolished in cells transfected with replicative form of CAV/Ap(−). A reverse mutant competent in apoptin production regained the full level of DNA replication. DNA replication and virus-like particle (VLP) production of CAV/Ap(−) was fully complemented by supplementation of the wild-type apoptin. The virus yield of a point mutant, CAV/ApT^{108}I, was 1/40 that of the wild type, even though its DNA replication level was full. The infectious titer of CAV was fully complemented by supplementing apoptin. Progeny virus was free from reverse mutation for T^{108}I. To localize the domain within apoptin molecule inevitable for CAV replication, apoptin-mutant expressing plasmids, pAp1, pAp2, pAp3, and pAp4, were constructed by deleting amino acids 10–36, 31–59, 59–88 and 80–112, respectively. While Ap1 and Ap2 were preferentially localized in nuclei, Ap3 and Ap4 were mainly present in cytoplasm. Although complementation capacity of Ap3 and Ap4 was 1/10 of the wild type, neither of them completely lost its activity. VP3 of TTV did fully complement the DNA replication and VLP of CAV/Ap(−). These data suggest that apoptin is inevitable not only for DNA replication but also VLP of CAV. The common feature of apoptin and TTV-VP3 presented another evidence for close relatedness of CAV and TTV.

Chicken anemia virus (CAV) was first isolated in Japan (Yuasa et al., 1979). CAV causes anemia by apoptosis of hemocytoblasts in bone marrow (Taniguchi et al., 1983). Chicken thymocytes and lymphoblastoid cells can also be infected with CAV and undergo apoptosis (Jeurissen et al., 1992). The virus is immunosuppressive in newborn chickens, and induces generalized lymphoid atrophy, severe anemia and increased mortality (Adair, 2000).

CAV is a small non-enveloped virus with a single-stranded circular DNA genome in the size of approximately 2.3 kb (Noteborn et al., 1991; Todd et al., 1990). CAV is the only member of genus *Gyrovirus* within family *Circoviridae*. CAV has open reading frames (ORFs) only on the antigenomic strand, in contrast to the other members of circoviruses that have ambisense genome (Bendinelli et al., 2001; Miyata et al., 1999; Noteborn et al., 1991). The major structural protein (VP1) of circoviruses is coded for by the genomic strand, while at least two other proteins (VP2 and VP3) by the antigenomic strand.

On the other hand, the arrangement of ORFs of CAV is quite similar to that of torque teno virus (TTV) (Bendinelli et al., 2001; Hino and Miyata, 2007; Miyata et al., 1999; Mushahwar et al., 1999). These two viruses have a >80% similarity in a 36-nt stretch near the replication origin, but nucleotide sequences of the remaining genome show no apparent similarities (Hino and Miyata, 2007; Miyata et al., 1999). Their transcription patterns to produce three or more spliced mRNAs are also similar to each other (Kamada et al., 2006; Kamahora et al., 2000; Qiu et al., 2005). TTV is currently classified into genus *Anellovirus* in family *Circoviridae* (Bendinelli et al., 2001; Hino and Miyata, 2007; ICTVdB, 2006). However, because of enormous diversity of TTVs, a new family *Anelloviridae* will probably set up in the near future (Anonymous, 2007). In contrast, arguments to relocate CAV outside *Circoviridae* seem to be less extensive.

CAV replicates via a circular double-stranded replicative form (RF) (Noteborn et al., 1991). The major transcript of CAV is an unspliced polycistronic mRNA which possesses three overlapping ORFs encoding VP1 (52 kDa), VP2 (24 kDa) and VP3 (14 kDa) (Claessens et al., 1991; Noteborn et al., 1991). VP1 is the major viral capsid protein. VP2 is a non-structural protein with phosphatase activity of dual specificities and has been shown to interact with VP1 (Noteborn et al., 1998; Peters et al., 2002). Both VP1 and VP2 are indispensable for CAV replication (Peters et al., 2006; Yamaguchi et al., 2001). VP3, also named apoptin, is a non-structural protein made of 121 amino acids

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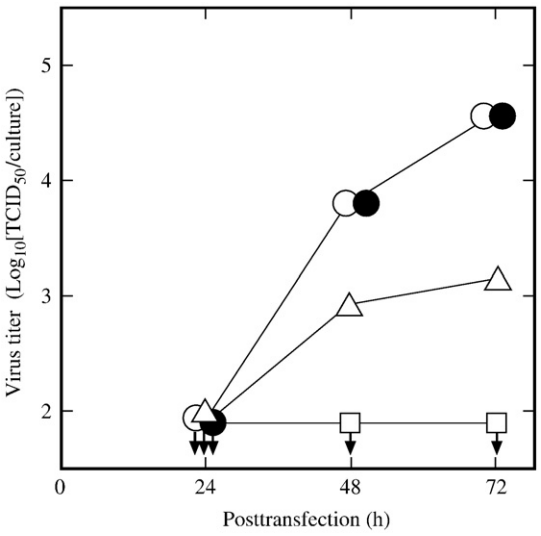

Fig. 1. Infectious CAV in the culture fluid after transfection with RF DNA. Culture fluids of MDCC-MSB1 cells transfected with CAV/WT (○), CAV/Ap(−) (□), CAV/ApRM (●) or CAV/ApT^{108}I (△) were tested for infectious CAV titer. Arrows indicate the titer less than the detection limit of the assay (1.9 $\log_{10}$ [$TCID_{50}$/culture]). Three independent experiments gave essentially the same results.

(aa). Although additional proteins can be coded for by spliced mRNAs of CAV, their biological meanings have not been elucidated (Kamada et al., 2006).

Apoptin has a number of proline, serine and threonine residues, and positively charged at C-terminus (Noteborn, 2004). Apoptin induces apoptosis selectively in transformed cells but not in non-transformed cells (Maddika et al., 2006; Oorschot et al., 1997). Maintenance of the apoptin gene in CAV implies the significance of apoptin in life cycle of CAV (Noteborn et al., 1994); however, no direct evidences for the necessity of apoptin in CAV replication have been ever given to the best of our knowledge. Interestingly, Kooistra et al. (2004) reported the apoptotic activity of an ORF-3 protein (105 aa) of TTV, with a limited similarity in their sequence to apoptin. Because of similarity in the gene localization of this protein and that of apoptin, starting at near 5′-terminus of VP1 and residing on the frame 3 (Miyata et al., 1999), we call this protein as TTV-VP3 in this report. The present study provided the first evidence of the necessity of apoptin in DNA replication and virus particle production of CAV, and ability of TTV-VP3 to complement apoptin.

Results

Infectious virus after transfection with RF DNA of CAV

Following transfection with RF DNA of the wild-type CAV, CAV/WT, into transformed chicken MDCC-MSB1 cells, infectious viruses in the culture fluid were titrated at 24, 48 and 72 h. Infectious viruses were first detected at 48 h posttransfection in the titer of 3.8×10^3 $TCID_{50}$/culture, which increased significantly at 72 h to the titer of 4.6×10^4 $TCID_{50}$/culture (Fig. 1).

In order to test the requirement of apoptin in the replication of CAV, pCAV/Ap(−) was constructed by disrupting the initiation codon of apoptin in pCAV/WT (Fig. 2). No infectious viruses were detected at all even at 72 h after transfection with RF DNA of CAV/Ap(−) (Fig. 1). A reverse mutant with the reorganized apoptin gene obtained from CAV/Ap(−), CAV/ApRM, regained full recovery in the infectious virus production (Fig. 1). The data suggest that apoptin is indispensable for infectious virus production of CAV.

Threonine^{108} of apoptin is phosphorylated specifically in tumor cells (Rohn et al., 2002). Apoptin with a point mutation at T^{108}A essentially stays in the cytoplasmic compartment, and has a reduced apoptotic activity (Poon et al., 2005a; Rohn et al., 2002). A point mutant with a silent mutation in the second frame, pCAV/ApT^{108}I, was constructed (Fig. 2). Infectious virus titer at 72 h posttransfection by transfection of CAV/ApT^{108}I, 1.0×10^4 $TCID_{50}$/culture, was 1/40 of that by transfection of CAV/WT, 4.0×10^5 $TCID_{50}$/culture (Fig. 1, Table 1). The data suggest that T^{108}I mutation of apoptin significantly reduces infectious virus titer of CAV.

CAV DNA replication in cells

Replication of CAV DNA was surveyed at 72 h posttransfection of CAV/WT RF DNA (Table 1). CAV DNA was detected at the level of 1.6×10^8 copies/culture. However, DNA replication was undetectable by transfection with the equal amount of pCAV/WT, nor by sham transfection in the absence of a transfection reagent, FuGENE HD. The level of DNA replication was not affected with or without co-transfection of the backbone vector plasmid used for apoptin supplement, pHM6. Moreover, DNA replication after transfection with RF DNA of CAV/Ap(−) was undetectable. This was fully rescued by supplementing apoptin, i.e. by co-transfection with pAp/WT (Table 1). However, co-transfection of backbone pHM6 could not. The level of DNA replication by transfection of CAV/ApRM was the same as that of CAV/WT. Thus, the apoptin itself was found inevitable for the DNA replication of CAV. Infectious virus titer in the cellular extract was approximately 10 times higher

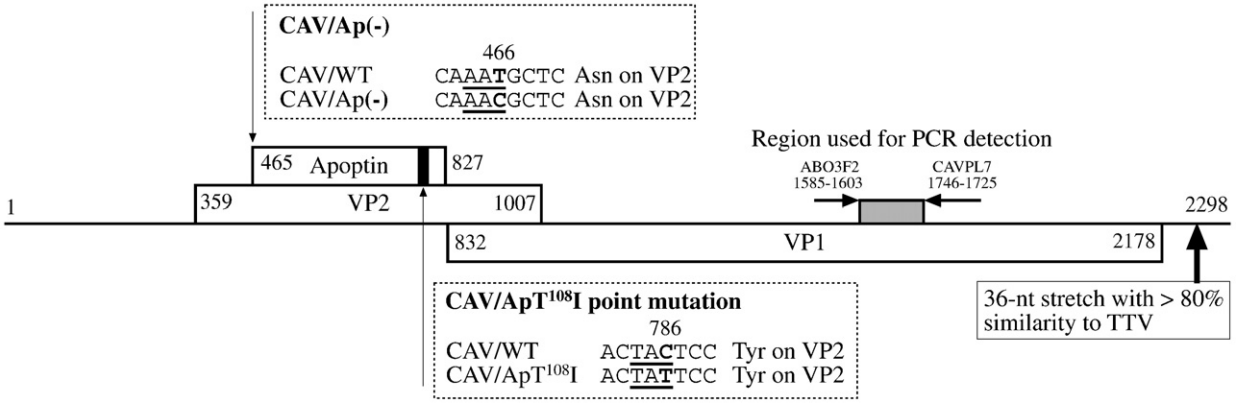

Fig. 2. Schematic diagram on genomic structure of CAV. An apoptin knockout clone, pCAV/Ap(−), was designed to point-mutate the initiation codon of apoptin without inducing an amino acid change in the overlapping VP2. pCAV/ApT^{108}I was obtained by a similar strategy. The region used for PCR detection of CAV DNA, nt 1585–1746, is located in the down stream.