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

Afiono Agung Prasetyo [a,1], Toshio Kamahora [a], Ayumu Kuroishi [a,2], Kyoko Murakami [b], Shigeo Hino [a,*]

[a] Division of Virology, Faculty of Medicine, Tottori University, 86 Nishi, Yonago 683-8503, Japan
[b] Department of Virology II, National Institute of Infectious Diseases, Toyama, Shinjuku, Tokyo 162-8640, Japan

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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

* Corresponding author. 4-3-1F, Medical Scanning, Kanda-Surugadai, Chiyoda, Tokyo 101-0062, Japan.
E-mail address: shg.hino@gmail.com (S. Hino).
[1] On leave from Department of Microbiology, Faculty of Medicine, Sebelas Maret University, Jl. Ir. Sutami 36A, Surakarta 57126, Indonesia.
[2] Current address: Department of Viral Infections, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita-shi, 565-0871, Osaka, Japan.

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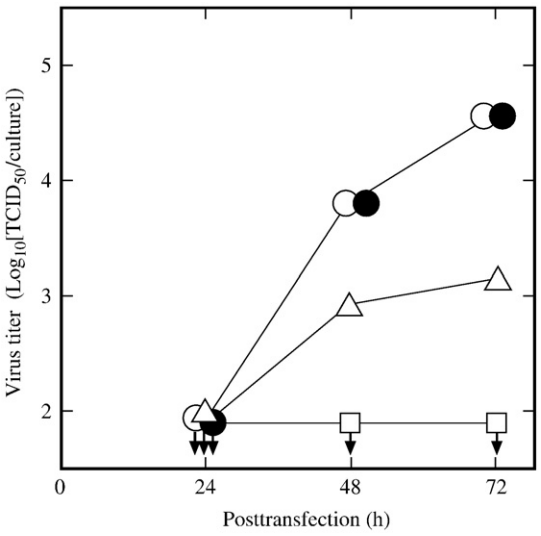

Fig. 1. Infectious CAV in the culture fluid after transfection with RF DNA. Culture fluids of MDCC-MSB1 cells transfected with CAV/WT (○), CAV/Ap(−) (□), CAV/ApRM (●) or CAV/ApT^{108}I (△) were tested for infectious CAV titer. Arrows indicate the titer less than the detection limit of the assay (1.9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

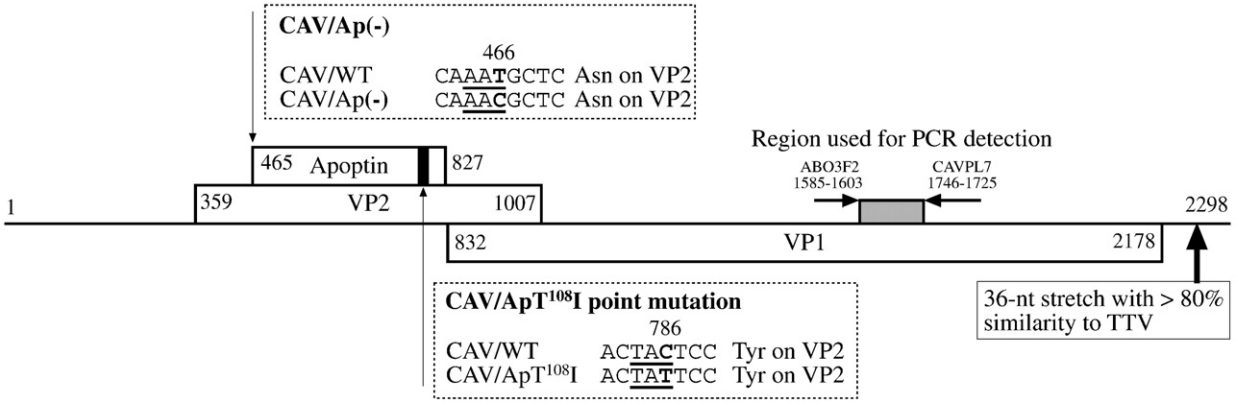

Fig. 2. Schematic diagram on genomic structure of CAV. An apoptin knockout clone, pCAV/Ap(−), was designed to point-mutate the initiation codon of apoptin without inducing an amino acid change in the overlapping VP2. pCAV/ApT^{108}I was obtained by a similar strategy. The region used for PCR detection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

Table 1
DNA replication and infectious virus of CAV within cells transfected with RF DNA of CAV and its mutants at 72 h posttransfection

Transfection with	Co-transfected with	CAV DNA Copies/culture (log_{10})	Virus titer $TCID_{50}$/culture (log_{10})
RF DNA of			
CAV/WT	None	8.2	5.6
	pHM6	8.2	5.6
CAV/Ap(−)	None	ND	ND
	pAp/WT	8.2	ND
	pHM6	ND	ND
CAV/ApRM	None	8.2	5.6
CAV/ApT^{108}I	None	8.2	4.0
	pAp/WT	8.2	5.6
	pHM6	8.2	4.0
pCAV/WT (plasmid form)	None	ND	ND

ND: not detectable.
CAV DNA and infectious virus were undetectable after transfection by RF DNA of CAV/WT without transfection reagent, FuGENE HD. Minimum detection level of CAV DNA was 6.2 log_{10} copies/culture and of infectious virus titer was 1.9 log_{10} $TCID_{50}$/culture. Three independent experiments gave essentially the same results.

than that of extracellular fluid up to 72 h posttransfection (Table 1 and Fig. 1).

Both extracellular and intracellular virus titers after transfection of CAV/ApT^{108}I were 1/40 of those after transfection of CAV/WT (Fig. 1 and Table 1). Nevertheless, DNA replication of CAV/ApT^{108}I was in the same level as that of CAV/WT (Table 1). The data suggest that the point mutation affects the process on virus particle production than viral DNA replication, and that the mechanism of apoptin action on CAV replication consists of at least these two different steps.

Apoptin complements DNA replication of CAV/Ap(−) and CAV/ApT^{108}I

To test if the CAV/Ap(−) replication could be complemented by apoptin, RF DNA of CAV/Ap(−) was co-transfected with an apoptin expressing plasmid, pApWT. As expected, infectious virus production was not detected after this complementation (Table 1). However, the DNA replication was fully complemented to the level of the wild type by supplying competent apoptin. The backbone plasmid, pHM6, could complement neither infectious virus production nor DNA replication of CAV/Ap(−) (Table 1).

Moreover, the infectious virus titer of CAV/ApT^{108}I (1.0×10^4 $TCID_{50}$/culture) was fully complemented with the wild-type apoptin to that of wild-type virus (4.0×10^5 $TCID_{50}$/culture) (Table 1). Sequencing of the resultant high titered virus revealed no reversed mutations at T^{108}I (data not shown). The CAV DNA level in cells co-transfected with CAV/ApT^{108}I RF DNA and pAp/WT was within that of the wild type (Table 1).

Complementation of CAV/Ap(−) DNA replication by apoptin mutants

In the literature, a bipartite nuclear localization signal (NLS) is responsible for apoptin nuclear translocation in tumor cells: NLS1 at aa 84–90 (PSKKRSC) and NLS2 at aa 112–118 (PRTARRR) (Oorschot et al., 2003). The disruption of these NLSs reduced nuclear transport and apoptosis induction in tumor cells. Apoptin also possesses a leucine-rich sequence (LRS) in its N-terminus (aa 33–44, IRIGIAGITITLSL), which is considered as nuclear export signal or cytoplasmic retention signal (CRS), because apoptin without LRS is essentially retained in the nucleus (Poon et al., 2005b).

To find out which region within apoptin molecule is responsible for the CAV replication, plasmids expressing mutant apoptins, pAp1, pAp2, pAp3, and pAp4, were constructed in which aa 10–36, 31–59, 59–88 and 80–112 of apoptin were deleted, respectively (Fig. 3A). Ap1 lacks just N-terminus three amino acids of LRS and Ap2 lacks whole LRS, while Ap3 and Ap4 lack NLS1. In addition, pApT^{108}I with T^{108}I mutation was also prepared.

Expression of these mutated apoptin with HA tag was first tested in floating MDCC-MSB1 cells under fluorescent microscopy. However, their intracellular distributions could not be visualized because of extremely low efficiency in transfection and of scanty cytoplasm in floating MDCC-MSB1 cells (data not shown). In COS-1 cells, >60% of cells expressing the wild-type apoptin showed a nuclear dominant staining. Nuclear dominant staining was observed in >80% of cells expressing Ap1 and Ap2, while in <30% of cells expressing Ap3 and Ap4 (Fig. 3B). Nuclear dominant staining was observed in approximately 40% of cells expressing ApT^{108}I, which value was closer to those of Ap3 and Ap4 (Fig. 3B). Interestingly, the replication of CAV/Ap(−) RF DNA was complemented fully with Ap1 and Ap2, and to the level of 1/10 with Ap3 and Ap4 (Table 2). The mutant, ApT^{108}I, also showed full capability of complementation (Table 2). As expected from the nature of complementation, none of these mutants recovered the infectious CAV production from CAV/Ap(−) (data not shown).

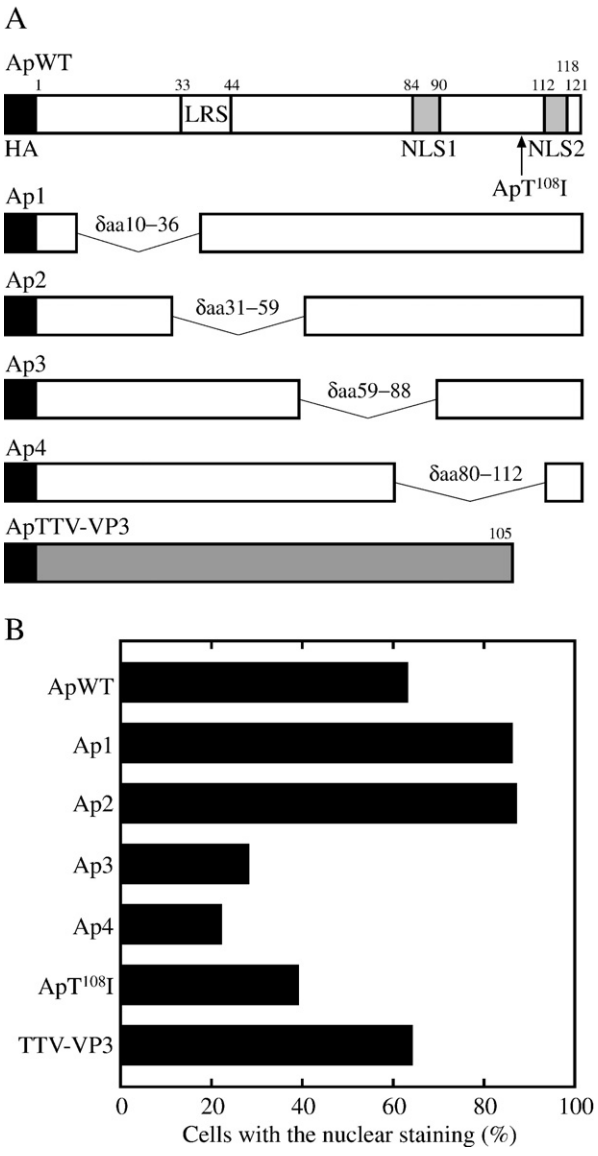

Fig. 3. Tagged apoptin and TTV-VP3 in expression vector, pHM6, and their subcellular localization in COS-1 cells. (A) Schematic diagrams of the wild-type apoptin and its four mutants, and of TTV-VP3. They were tagged with an HA-epitope at its N-terminus. Positions deleted in Ap1 through Ap4 are shown. (B) Proportion of cells with the nuclear staining (those with >50% signals in the nuclear region) after transfection with one of the expression plasmids. At least 100 transfection-positive cells were counted. The results are the average of three independent experiments.

Table 2
Complementation of CAV/Ap(−) DNA replication by apoptin and its mutants and by TTV-VP3

Transfection RF DNA	With apoptin	CAV DNA Copies/culture (log_{10})
CAV/Ap(−)	None	ND
	ApWT	8.2
	$ApT^{108}I$	7.2
	Ap1	8.2
	Ap2	8.2
	Ap3	7.2
	Ap4	7.2
	TTV-VP3	8.2

ND: not detectable.
See legend of Table 1.

Complementation of Ap(−) CAV DNA replication by TTV-VP3

On the third frame of TA278 (GenBank AB008394), one of the subgroup 1 TTVs, resides an open reading frame (ORF) for a 105-aa protein (VP3) spanning nt 372–686 (Miyata et al., 1999). Kooistra et al. (2004) reported that this protein had a higher apoptosis-inducing activity in human hepatocellular carcinoma (HCC) cells than in non-HCC cells, and named it TTV-derived apoptosis-inducing protein (TAIP).

Complementation by TTV-VP3 on the replication of CAV/Ap(−) was tested. MDCC-MSB1 cells were co-transfected with RF DNA of CAV/Ap(−) and pTTV-VP3. Surprisingly, the replication of CAV/Ap(−) was fully complemented by TTV-VP3 (Table 2). Intracellular localization of TTV-VP3 was also tested by HA-tagged protein in COS-1 cells. The level of cells with dominant nuclear staining with TTV-VP3 was approximately 60% nearly equal to that of the wild-type apoptin (Fig. 3B), which is consistent with the full complementation capacity of TTV-VP3 for CAV/Ap(−) DNA replication.

Southern blot analysis

Southern blots were performed to confirm these PCR data, because the background signal of input DNA could not be eliminated even after DpnI restriction. In the Southern blot, the double- and single-stranded DNA bands of CAV were observed after transfection of RF CAV/WT DNA (Fig. 4, lane 1). However, no appreciable signals were detected by mock transfections, either of CAV/WT DNA in the plasmid form (lane 12), or of RF CAV/WT DNA without the FuGENE HD transfection reagent (lane 13). These data suggest that the signal on the Southern blot reflects de novo synthesized CAV DNA.

In comparison to CAV/WT, CAV/$ApT^{108}I$ showed the same level of single and double stranded DNAs with or without complementation with ApWT (lanes 3 and 2, respectively). No replications of CAV DNA

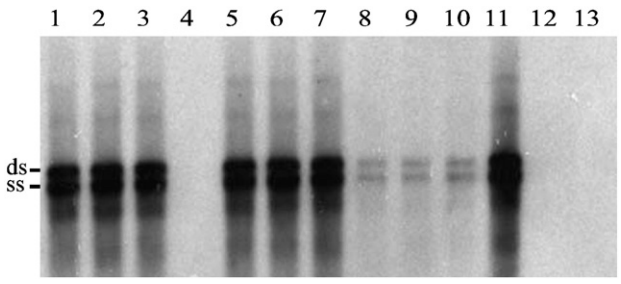

Fig. 4. Southern blot of the MDCC-MSB1 DNA transfected with RF DNA of CAVs. Lanes, 1: CAV/WT; 2: CAV/$ApT^{108}I$; 3: CAV/$ApT^{108}I$ co-transfected with pApWT; 4: CAV/Ap(−); 5 through 11: CAV/Ap(−) co-transfected with pApWT, pAp1, pAp2, pAp3, pAp4, pAp$T^{108}I$ and pTTV-VP3, respectively; 12: pCAV/WT (as circular plasmid) with FuGENE HD; 13: RF CAV/WT DNA without FuGENE HD. Bars on the left margin point to the CAV-DNA, ss: ssDNA; ds: dsDNA.

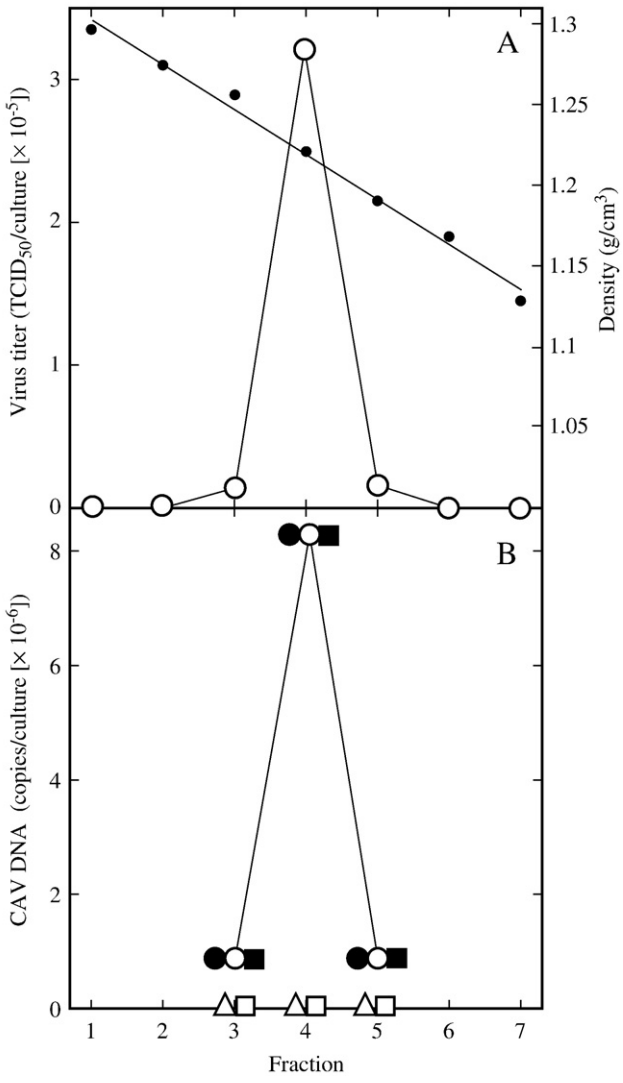

Fig. 5. CAV virus-like particle (VLP) production after complementation of CAV/Ap(−) by co-transfection with pApWT or pTTV-VP3. (A) Titer of CAV/WT in each fraction after a 30–60% sucrose density gradient centrifugation and density of each fraction. (B) Co-migration of CAV DNA with the VLP fraction. VLPs in each fraction were pelleted by ultracentrifugation and the resuspended pellet was treated with DNase. Thereafter, DNA was extracted, treated with DpnI to digest out the input RF DNA, and used as a template for CAV DNA titration by PCR. CAV/WT (○), CAV/Ap(−) complemented with pApWT (■) or with pTTV-VP3 (●). Negative controls: CAV/Ap(−) (□) or RF CAV/WT only (DNA without the transfection reagent, FuGENE HD) (△).

were detectable by the transfection of RF CAV/Ap(−) (lane 4), which was fully complemented by ApWT (lane 5) and by TTV-VP3 (lane 11). While CAV/Ap(−) was completely rescued by two deletion mutants at the N-terminal region, Ap1 and Ap2, it was only partially rescued by the other three deletion mutants at the C-terminal region, Ap3, Ap4 and $ApT^{108}I$ (lanes 6–10).

Virus-like particle production after complementation with apoptin and TTV-VP3

Both CAV-apoptin and TTV-VP3 were found to complement the replication of CAV/Ap(−) DNA. Next obvious question is if the complementation by these proteins is associated with the virus-like particle (VLP) production. VLP was partially purified by a sucrose density centrifugation. The peak of infectious virus particle was observed at the fraction 4 by titration of infectious viruses (Fig. 5A). CAV DNA in the cell extract transfected with CAV/WT revealed that the viral DNA was co-fractionated with the infectious virus particles

(Fig. 5B). In cells transfected with RF DNA of CAV/Ap(−), the CAV-specific DNA did co-migrate to the same fraction as VLP after complementation with ApWT, and also with TTV-VP3, (Fig. 5B). Neither cells transfected with RF DNA of CAV/Ap(−) alone, nor those sham transfected with RF DNA of CAV/WT in the absence of the transfection reagent, FuGENE HD, did not show VLP peak in the same fraction.

Discussion

Apoptin of CAV induces apoptosis in chicken thymocytes and lymphoblastoid cells. Apoptosis of human cancer cells by apoptin has been studied extensively (Maddika et al., 2006; Noteborn et al., 1994; Oorschot et al., 1997); however, the requirement apoptin for CAV replication has not been elucidated. This study provides the first direct evidence that apoptin is indispensable for CAV replication. Not only virus particle production but also DNA replication was completely knocked out in CAV/Ap(−). The defect of CAV/Ap(−) was fully recovered by the reverse mutation. The DNA replication of CAV/Ap(−) is restored by substitution of apoptin, but the production of infectious virus particles could not be rescued at all, indicating the mechanism of recovery in the DNA replication is complementation, not recombination.

Threonine^{108} of apoptin is phosphorylated in transformed cells, and the loss of hydroxyl group for phosphorylation on T^{108} significantly reduces its apoptotic function (Lee et al., 2007; Maddika et al., 2007; Poon et al., 2005a; Rohn et al., 2002). Poon et al. (2005a) claimed that this phosphorylation is directly associated with its nuclear export using apoptin mutants. An apoptin lacking in aa 1–73 remained in cytoplasm, while an apoptin devoid of aa 74–121 stayed in the nucleus. For the induction of apoptosis, nuclear localization is required but not sufficient (Zhang et al., 2004).

While the CAV DNA replication of CAV/ApT^{108}I was as high as that of the wild type, the infectious CAV/ApT^{108}I production was 1/40 that of CAV/WT. These data demonstrate that the T^{108}I mutation does affect the CAV virus particle production but not the DNA replication of CAV. When cells were supplemented with the wild-type apoptin by co-transfection of pAp/WT, the virus titer of infectious CAV/ApT^{108}I recovered to the level of the wild type without increasing the replication of viral DNA. Virus particles produced after the complementation retained the T^{108}I mutation, suggesting that the increased virus particle production is directly associated with the function of the wild-type apoptin.

In these experiments, we could not specify the region within an apoptin molecule indispensable for the CAV replication, because Ap1 and Ap2 had full complementation capacity as the wild-type apoptin. However, the DNA replication levels of CAV/Ap(−) complemented by Ap3, Ap4 and ApT^{108}I were approximately 1/10 that of by the wild-type virus. The results suggested that C-terminal region of apoptin molecule is more responsible for the DNA replication of CAV. The results of PCR and Southern blot experiments were consistent each other. In the literature, apoptin deleted of 11 codons at its 3′-terminus retained apoptotic ability (Oorschot et al., 1997; Zhuang et al., 1995). Also, a truncated apoptin consisting of aa 1–59 was sufficient to bind Hippi, a protein interactor of Huntingtin-interacting protein 1 (Cheng et al., 2003). On the other hand, another apoptin mutant, truncated of the C-terminus and without the phosphorylation site, kept the nuclear translocation and the apoptotic capacity (Guelen et al., 2004). The N-terminus (aa 1–69) and C-terminus (aa 80–121) domains of apoptin induce apoptosis independently, although the apoptotic potency of these proteins were less than that of the wild-type apoptin (Oorschot et al., 2003). These previously reported data suggest that the functional domains of apoptin are not restricted in a single region (Rohn et al., 2005). They may be consistent with the current observation that none of four apoptin mutants completely lost the complementation capacity for the DNA replication of CAV/Ap(−).

At present, the action mechanism of apoptin on VLP production has not been elucidated. Association of nuclear localization found between apoptin and VLP production in this study seems rational, because both DNA replication and VLP formation of CAV should be intranuclear processes considering that CAV is a small DNA virus. VP1 on the frame 1 has been suggested to act as the capsid protein, and the VP2 on the frame 2 has been claimed to be scavenger protein for the virus particle formation (Koch et al., 1995; Noteborn et al., 1998; Todd et al., 1990). These proteins may be required by the virus for replicative or transcriptional functions, since VP2 and VP3 are produced very early in the infectious cycle (12 h postinfection) and deposited in large amounts within the nucleus of infected cells (Douglas et al., 1995). The data presented here clearly demonstrated the requirement of apoptin in CAV DNA replication and its virus particle formation. The functions of other putative proteins dependent on the spliced mRNAs (Kamada et al., 2006) await further studies.

CAV has a similarity to TTV in its genomic organization (Miyata et al., 1999; Mushahwar et al., 1999). By splicing, not only TTV but also CAV produces at least three different classes of mRNAs with the same 5′- and 3′-termini, using common TATA-box and polyA-site (Kamada et al., 2006; Kamahora et al., 2000). The 36-nt stretches in the genome of these two viruses have an >80% sequence similarity (Miyata et al., 1999). Although the nucleotide sequence similarity of these viruses is not observed in other parts of the genome (Miyata et al., 1999), the N-termini of both CAV-VP1 and TTV-VP1 have a short amino acid motif common for proteins associated with DNA replication by the rolling-circle mechanism (Bendinelli et al., 2001). Both CAV-VP2 and TTV-VP2 are the protein phosphatase of dual specificities (Peters et al., 2002). Furthermore, both viral DNA replication and virus particle formation of apoptin-defective CAV/Ap(−) were complemented either by apoptin itself or by TTV-VP3. Thus, all three major proteins of CAV and TTV have common features, suggesting the close relation between these two viruses. Lines of evidence provided by this report added further evidence for the functional similarity in VP3 s of CAV and TTV.

However, even though Kooistra et al. (2004) pointed out the similarity of these proteins by apoptotic activity, proline-rich nature and hydrophobic amino acid stretch, similarity of their amino sequences does not seem to be substantial. Although an N-mylistoylation site was observed on CAV apoptin [located at aa 39–44 (GITITL) and aa 48–53 (GCANAR)] and on TTV VP3 aa 57–62 (GCRLSR), it is not likely the key factor for this complementation, because AP2 completely lacking this domain still showed the significant capacity of complementation. To date, no conformational similarities in these proteins are available. Reason for the comparable capacity for complementing CAV/Ap(−) DNA replication remains a target in further studies. Complementation of apoptin deficiency by TTV-VP3 cannot be common, because ORF of VP3 is not open in all TTV genotypes. Furthermore, the expression of this ORF has not been confirmed in a full length TTV clone.

Materials and methods

Cell culture

Chicken T cells transformed by Marek's disease virus, MDCC-MSB1, were used to replicate CAV (Akiyama and Kato, 1974). Cells were subcultured twice weekly at 39 °C in RPMI1640 medium (Sigma, Stenheim, Germany) supplemented with 10% fetal bovine serum (FBS, Immuno-Biological Laboratories, Fujioka, Japan), 2 mM glutamine, and 0.01% each of penicillin and streptomycin. African green monkey kidney cell expressing SV40 T antigen, COS-1, was used to visualize intracellular localization of apoptin and its mutants. Cells were subcultured twice weekly at 37 °C in Dulbecco's modified Eagle medium supplemented with 10% FBS, 2 mM glutamine and 0.01% each of penicillin and streptomycin.

Wild-type CAV plasmid and apoptin mutants

A molecular clone of the wild-type CAV (CAV/WT), pCAV-A2C15 (pCAV/WT) (GenBank AB031296), was kindly supplied by Dr. Yamaguchi, National Institute of Animal Health, Japan (Yamaguchi et al., 2001). To construct a CAV clone knocked out of apoptin, an point mutation, A^{465}TG to ACG, was introduced to the initiation codon of apoptin gene on the frame 3 (Fig. 2). The mutation was designed to be synonymous in VP2 on the frame 2. Briefly, a reversed long PCR was performed by QuickChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA) using pCAV/WT as a template and a pair of primers, mApM-s (5′-GCA AGT AAT TTC AAA CGA A^{465}*C*G CTC TCC AAG-3′) and mApM-as (5′-CTT GGA GAG C*G*T^{465} TCG TTT GAA ATT ACT TGC-3′). The PCR was performed for 16 cycles with denaturing at 94 °C for 1 min, annealing at 55 °C for 1 min, and elongation at 72 °C for 11 min in each cycle. The mutant plasmid was named as pCAV/Ap(−). A reverse mutant, pCAV/ApRM, was prepared by a similar long PCR using pCAV/Ap(−) as a template and a pair of primers ApRM-s (5′-GCA AGT AAT TTC AAA CGA A^{465}*T*G CTC TCC AAG-3′) and ApRM-as (5′-CTT GGA GAG C*AT*^{465} TCG TTT GAA ATT ACT TGC-3′).

Poor performance of apoptin with a point mutation of threonine^{108} to isoleucine has been reported (Rohn et al., 2002). pCAV/ApT^{108}I was constructed by the similar reversed long PCR using two primers, mApT^{108}-s (5′-GC TTG ATT ACC ACT A^{786}*TT* CCC AGC CG-3′) and mApT^{108}-as (5′-CG GCT GGG *AAT*^{786} AGT GGT AAT CAA GC-3′). This point mutation was selected to induce a silent mutation in the ORF2 of CAV/WT (Fig. 2). The PCR condition was the same as above. All molecular clones in this report were sequenced by dideoxy-sequencing method (Sanger et al., 1977) with a SequiTherm Excel II sequencing kit (Epicentre Biotechnologies, Madison, WS). The nucleotide sequence was analyzed by Genetyx version 7.0 (Genetyx, Tokyo, Japan).

Tagged apoptin and its mutants in an expression vector (pHM6)

Molecular clones to complement apoptin-defective CAV, CAV/Ap(−), were constructed. A complete open reading frame of the apoptin gene of CAV/WT was amplified by PCR using primers 475-s (5′-CG*G GTA CCA*^{468} ACG CTC TCC AAG AAG ATA CT^{488}-3′) and 830-as (5′-CC*G AAT TC*T^{830} TAC AGT CTT ATA CAC CTT CT^{810}-3′). For cloning, each primer was attached with a KpnI or EcoRI recognition site (indicated in italics). The PCR condition was the same as above. The PCR product digested with KpnI/EcoRI was subcloned into the respective sites of pHM6 vector (Roche Diagnostics, Mannheim, Germany). The clone thus obtained, pApWT, expressed a wild-type apoptin tagged with HA-epitope at its N-terminus. Using this clone as a template, pApT^{108}I was constructed using same primers and PCR condition as in pCAV/ApT^{108}I construction.

To obtain deletion mutants of apoptin, Ap1, Ap2, Ap3 and Ap4, molecular clones, pAp1, pAp2, pAp3, and pAp4, were constructed (Fig. 3A). Briefly, pAp1 was constructed as follows. Restriction of pApWT (5796 bp) with HindIII gave 5481 bp and 315 bp fragments. The longer arm piece was dephosphorylated with calf intestinal alkaline phosphatase (New England BioLabs, Ipswich, MA) and was purified using QIAquick nucleotide removal kit (Qiagen, Hilden, Germany). The shorter piece was self-ligated using T4 DNA ligase (Promega, Madison, Wisconsin) and served in a reverse PCR with phosphorylated primers, ApM1L (5′-G^{971}GT GGA GTA TCT TCT TGG AGA GCG^{948}-3′) and ApM1U (5′-C^{1053}GC TGG AAT TAC AAT CAC TCT ATC G^{1077}-3′). The product was purified by electrophoresis in a 3% agarose S gel (Takara, Kyoto, Japan), recovered with the gel extraction kit (Qiagen), circularized by ligation, digested with HindIII, and then ligated back into the longer arm piece using T4 DNA ligase (Promega). Similar strategies were used to obtain pAp2, utilizing ApM2L (5′-C^{1034}TG CAG TGA GGG GTT TTC CAA C^{1014}-3′) and ApM2U (5′-T^{1125}GC GGA CAA TTC AGA AAG CAC TGG^{1148}-3′) primers.

To obtain pAp3, pApWT was digested with PstI into 4138 bp and 1658 bp fragments. The longer arm piece was dephosphorylated and purified. The shorter piece was recircularized and served for a reverse PCR with phosphorylated primers, ApM3L (5′-G^{1118}AT CTT AGC GTG GGA GCG CGA^{1098}-3′) and ApM3U (5′-C^{1209}TG CGA CCC CTC CGA GTA CA^{1228}-3′). PCR product was purified, circularized, digested with PstI, and then ligated back into the arm piece. To obtain pAp4, a pair of phosphorylated primers, ApM4L (5′-T^{1186}GG GTT GAT CGG TCC TCA A^{1168}-3′) and ApM4U (5′-C^{1283}CG CAA GAA GGT GTA TAA GAC TG^{1305}-3′), were used. The reversed PCRs were performed for 30 cycles with denaturing at 94 °C for 1 min, annealing at 53 °C (ApM1U/ApM1L), 53 °C (ApM2U/ApM2L), 57 °C (ApM3U/ApM3L), or 55,5 °C (ApM4U/ApM4L) for 1 min, and elongation at 72 °C for 2 min in each cycle.

Expression plasmid for TTV-VP3

To supply TTV-VP3 in cells, pTTV-VP3 was constructed. A complete open reading frame of the TTV-VP3 gene was amplified by PCR using pBKVT416(1.3G)CMV(−) (GenBank AB041007) (Kamahora et al., 2000) as the template, and a pair of primers VP3-Not (5′-TA*G CGG CCG CC*^{689}T AGC AGG TCT GCG TCT TCG^{670}-3′) and VP3-KspI (5–A*GC CGC GGT*^{373} GAT CAA CAC TAC CTT AAC TGG^{394}–3). Each primer contained NotI or SacII (KspI) site, for cloning purpose (indicated in italics). PCR was performed for 30 cycles with denaturing at 94 °C for 1 min, annealing at 55 °C for 1 min (for the first 15 cycles) and at 60 °C for 1 min (for an additional 15 cycles), and elongation at 72 °C for 1 min in each cycle. The PCR products digested with SacII and NotI were inserted into SacII and NotI site of pHM6, respectively.

Preparation of replicative form (RF) DNA

Each molecular clone of CAV in an amount of 5.0 μg was digested with XbaI. After electrophoresis on a 1% agarose, viral DNA was extracted by the gel extraction kit, and 200 ng of the DNA preparation was circularized by the T4 DNA ligase. DNA was extracted with phenol-chloroform (1:1) and precipitated with ethanol. The pellet was washed with 70% ethanol, dried, and resolved in 10 μL of filtered TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA).

DNA transfection

MDCC-MSB1 cells (1×10^6 cells/2 mL) in RPMI medium free from antibiotics were delivered to the 6-well plate. The RF DNA (200 ng) in 10 μL was transfected with FuGENE HD (Roche Diagnostics) according to manufacturer's instruction. For complementation experiments, the RF DNA was co-transfected with 1.0 μg of a plasmid expressing apoptin or its mutant protein. Every 24 h, cells were washed with dication-free phosphate buffered saline [PBS(−)] for three times, and resuspended with fresh complete RPMI medium to minimize the amount of DNA contamination.

Harvest

The culture at 72 h posttransfection, unless otherwise stated, was harvested for titration of infectious virus and DNA. The 2-mL supernatant was used for extracellular virus and DNA preparation after centrifugation at 3000 *g* for 5 min and filtration through a 0.45 mm Sartorius membrane filter. The cells were washed twice and resuspended in 2 mL of PBS(−). One-mL aliquot was centrifuged and the cell pellet was used for DNA extraction. Another aliquot was freeze-thawed three times, filtered through the membrane filter, and tested for infectious virus titer. Aliquots of the infectious virus and DNA samples were freeze-stocked at −20 °C.

DNA extraction

DNA was extracted using methods described previously (Laird et al., 1991). Briefly, 500 μL of lysis buffer (100 mM Tris–HCl, pH 8.5, 5 mM EDTA, 0.2% sodium dodecyl sulfate [SDS], 200 mM NaCl, 100 μg/mL proteinase K) was added into the sample, and incubated at 37 °C with agitation for 3 h. One volume of isopropanol (500 μL) was added to the lysate and the samples were mixed until precipitation is complete. The DNA precipitate was then recovered and dissolved in 100 μL of TE buffer. The concentration of DNA was measured using NanoDrop (NanoDrop Technologies, San Diego, CA).

DNA preparation for digestion of prokaryotic DNA by Dpn I

Sample obtained either from culture fluid or cell extract after transfection was heavily contaminated with prokaryotic input DNA. The maximum efficiency of the Dpn I digestion was approximately 99.9% (data not shown). To keep the undigested prokaryotic DNA undetectable after Dpn I digestion, the DNA sample was first titrated by a seminested PCR (Kamada et al., 2006), and adjusted the DNA concentration to $<4\times10^8$ copies CAV DNA/mL. Thereafter, the sample was digested with Dpn I at 37 °C for 16 h in NEBuffer 4 (New England BioLabs).

Titration of CAV DNA

CAV-DNA was titrated semiquantitatively using serial ten-fold dilutions and six tubes parallel in each dilution. Briefly, DNA sample (10 μL) was served for a single-step PCR using a pair of primers, ABO3F2 (5′-A^{1585}AA GGC GAA CAA CCG ATG A^{1603}-3′) and CAVPU7 (5′-^{1746}CCA CCA AGC TTG CGT GCT ATT C^{1725}-3), and 1.25 U of BIOTAQ polymerase (Bioline, London, UK) in 50-μL reaction volume. Following an initial 3-min denaturation step at 94 °C, 30 cycles of PCR were carried out comprising of 1 min at 94 °C, 1 min at 60 °C, and 2 min at 72 °C (10 min in the last cycle). After electrophoresis on a 3% agarose gel, the gel was stained with ethidium bromide. The sensitivity of this PCR assay was confirmed as 10^4 copies per reaction by quantifying pCAV/WT DNA as a template using the seminested PCR (Kamada et al., 2006). Because of contamination of prokaryotic DNA even after the optimized Dpn I digestion, this reduced sensitivity of the assay was selected. The titer was calculated using the Behrens–Karber method (Karber, 1931). Because the target region of this PCR is indifferent from apoptin gene, co-transfection of the apoptin expressing plasmid did not disturb the PCR. The experiments were repeated at least three times, and each experiment resulted in essentially the same results.

Titration of infectious CAV

CAV was titrated by a modification of the microtest method reported previously (Goryo et al., 1987). Serial ten-fold dilutions were performed and 25 μL of each dilution was inoculated into each of six wells containing 2×10^5 MDCC-MSB1 cells in 200 μL of the RPMI complete medium. After two days, a quarter of cells was subcultured in the complete medium. Two days after the subculture, cells were harvested. DNA was extracted and dissolved in 100 μL of TE, and a 10-μL aliquot of the DNA was used as template for the above-mentioned single PCR. This assay system was confirmed to have the same sensitivity as the previously described microtest method by Goryo et al. (1987), in which seven consecutive sub-cultures are required. Similar titration system has been reported by van Santen et al. (2004).

Southern blot analysis

MDDC-MSB1 cells were transfected with RF DNA of various forms of CAVs. The extracted DNA, 10 μg in each lane, was electrophoresed on 1% agarose gel, and blotted onto a Hybond-N+ filter (GE Healthcare, NJ) as described by Southern (1975). After UV cross linking, the DNA was prehybridized in DIG Easy Hyb solution (Roche) for 30 min at 49 °C and subsequently hybridized with DIG probe in the hybridization solution for 18 h at 49 °C. The DIG probe consisting of the genomic region used for PCR was generated using pCAV/WT as a template, and ABO3F2 and CAVPU7 as primers by a PCR DIG Probe Synthesis Kit (Roche). The blot was washed twice at the low-stringency condition for 5 min at 25 °C with 2×SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0) and 0.1% SDS, and twice at the high-stringency condition for 15 min at 68 °C with 0.1×SSC and 0.1% SDS. The DIG-labeled DNA was developed by a DIG Luminescent Detection Kit (Roche).

Purification of virus particles

Virus particles were partially purified by the previously described methods with minor modifications (Todd et al., 1990). The cells (2×10^6 cells/5 mL) in RPMI medium free of antibiotics were delivered to a 6-cm dish and transfected with 1 μg of RF DNA by FuGENE HD. For complementation experiments, the RF DNA of CAV/mAp(−) (200 ng) was co-transfected with 4.0 μg of pApWT or pTTV-VP3. After 72 h posttransfection, cells were washed three times with PBS(−), and resuspended in 1 mL of PBS(−). After three cycles of quick freezing-thawing, samples were treated with 0.5% SDS for 30 min at 37 °C. Cell debris was removed by centrifugation at 1.0×10^4 *g* for 30 min at 15 °C. The supernatant was collected, and a 500-μL portion of it was layered onto 10 mL of a continuous 30–60% sucrose gradient in TE, and centrifuged at 8×10^4 *g* for 17 h at 15 °C using a Beckman SW41Ti rotor. Fractions (1.5 mL each) were collected from the bottom. Parts of each fraction served for determination of sucrose density with a refractometer and for virus titration. Virus particles in each fraction were pelleted by centrifugation at 2×10^5 *g* for 30 min at 17 °C using a Beckman SW55Ti rotor, and resuspended in 125 μL of TE. The viral DNA was titrated by PCR described above.

Immunofluorescence

For analyzing the distribution of apoptin in cells, COS-1 cells were selected. The localization of apoptin was hardly visualized in MDCC-MSB1 cells, because transfection efficiency in MDCC-MSB1 cells was very low and small floating MDCC-MSB1 cells do not have much cytoplasmic area. One night prior to transfection, the cells were delivered into 8-well chamber slides. Cells were transfected with 1 μg of a expression plasmid with FuGENE HD, air-dried at 48 h posttransfection, and fixed with 4% paraformaldehyde in PBS(−) for 10 min at room temperature. After consecutive washings with PBS(−) and 0.1% Triton X-100 in PBS(−), the slides were blocked with 15 μL/well of 1.5% normal sheep serum in PBS(−) at 37 °C for 30 min. The cells were incubated with 1/100 dilution of anti-HA high-affinity rat monoclonal antibody (Roche Diagnostics), and then with 1/1000 Alexa Fluor 594 goat anti-rat IgG antibody (Invitrogen, Carlsbad, CA), each for 60 min at room temperature. The slides were mounted with 5 ng (5 μL)/well of Vectorshield with 4′-6-diamidino-2′-phenylindole dihydrochloride (DAPI, Vector Laboratories, Burligame, CA), and observed under a inverted fluorescent microscope (BX60: Olympus, Tokyo, Japan). The specimen was thoroughly washed with PBS(−) between each step unless otherwise stated.

To analyze the nuclear and cytoplasmic localization of apoptin and TTV VP3, three different methods were used because of the subjective nature of the analysis. First, cells double stained with anti-HA antibody and DAPI were examined manually under fluorescent microscope: cells with pink nuclei were scored as the nuclear staining. Second, double stained cells were scanned by “find edges function” of ImageJ, a public domain image analysis software (Abramoff, Magelhaes, and Ram, 2004). To eliminate background signals, the threshold range of apoptin color signal was determined using typical cells

stained for apoptin. Cells with >50% signal in nuclear region were scored as cells with the nuclear staining. Finally, merged image was re-processed using macro command written by Carmona et al. (2007). The results with these three different methods were almost identical (proportion of cells with dominant nuclear staining: ApWT, 60–75%; ApT^{108}I, 30–45%; Ap1, 80–95%; Ap2, 80–95%; Ap3, 5–30%; Ap4, 5–20%; TTV-VP3, 60–70%).

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