contrast, strains Se585 and ISO1 possess P[6] type specificity [Griffin et al., 2002; Das et al., 2003], and strain Se585 has been shown to be a reassortant between L26 and strain US1205 or a strain closely related to those in the DS-1 genogroup [Griffin et al., 2002].

This is the first report on the detection of the G12 rotavirus in Japan. The two G12 strains detected in Japan are considered to be almost the same T152 strain in Thailand. These Japanese G12 strains were obtained at the same time from an infant and an adult, respectively, who live in neighboring cities and had not been abroad recently. It seems that they were infected with the G12 rotavirus in Japan. The G12 rotavirus probably had been transmitted from Thailand.

The detection of G12 strains in several countries implies the recent expansion of the distribution area of the G12 rotavirus. Since 1995, G9 rotavirus, although it was previously an uncommon G serotype, has been identified at a high frequency in various parts of the world [Gentsch et al., 1996; Unicomb et al., 1999; Griffin et al., 2000] and has become a common G serotype. There is a possibility that the G12 rotavirus will become prevalent as the G9 rotavirus in the future. Continued surveillance of G and P types of rotavirus is significant for developing effective vaccine and for analyzing rotavirus ecology.

Nucleotide Sequence Accession Numbers

Nucleotide sequence data described in this report have been submitted to the GenBank database and have been assigned the accession numbers AB125852 (CP727, VP7), AB125853 (CP1030, VP7), AB125854 (CP727, VP4), and AB125855 (CP1030, VP4).

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Characterizations of Adenovirus Type 41 Isolates from Children with Acute Gastroenteritis in Japan, Vietnam, and Korea

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Genetic and antigenic characterizations of 70 strains of adenovirus type 41 (Ad41), isolated between 1998 and 2001 from children in Japan, Vietnam, and Korea, were done by DNA restriction enzyme (RE) analysis, sequencing analysis, and monoclonal antibody (MAb)-based enzyme-linked immunosorbent assay (ELISA). Eight genome types were observed in the present study, among which D25, D26, D27, and D28 were novel genome types. These eight genome types were divided into two genome-type clusters (GTCs) based on phylogenetic analysis of the hypervariable regions (HVRs) of the hexon. GTC1 includes D1, D25, D26, D27, and D28, and the GTC2 contains D4, D12, and D22. The amino acid homologies among the members within a GTC were 97 to 100%, whereas between the members of different GTCs the homologies were 92 to 94%. The specificity of the GTC classification was confirmed by ELISA with MAb 1F, which was selected by the Ad41 prototype Tak strain. It was found that only the isolates of GTC1 but not of GTC2 reacted with MAb 1F. These results suggest that Ad41 isolates from the three countries should be classified into two subtypes. The accumulation of amino acid mutations located in HVRs of hexon are indicative for the classification of Ad41 subtype.

Adenoviruses are responsible for a wide range of disease symptoms. To date, there are 51 recognized serotypes of human adenovirus that are classified into six subgenera (A to F) based on several antigenic, morphological and molecular criteria. Among them, subgenus F, represented by the two serotypes adenovirus type 40 (Ad40) and Ad41, is associated with diarrhea in children; it is found in 1 to 20% of fecal specimens from children with acute gastroenteritis (3, 9, 12, 15, 22, 35). The two serotypes (Ad40 and Ad41) of subgenus F are termed enteric adenovirus (EAd) because of their tropism for the gastrointestinal tract (10, 32). The subgenus F adenoviruses grow poorly in most cell culture systems, in contrast to other cultivable "nonenteric" adenoviruses, and are therefore also termed "noncultivable" or "fastidious" adenovirus (7, 16).

Earlier surveys had shown that the occurrences of Ad40 and Ad41 are approximately equal (6, 11). However, several studies have recently reported a decrease in the rate of isolation of Ad40 and a concomitant increase in the rate of isolation of Ad41 (5, 8, 25, 34). After 1986, Ad41 infection became dominant over Ad40. Our previous studies confirmed that Ad41 was the prevailing serotype of adenovirus associated with acute diarrhea among children also in Japan, Vietnam, and Korea (19). The change in prevalence of Ad41 might have been caused by an antigenic drift, thus increasing the incidence of infection in susceptible individuals (3). Therefore, it is important to characterize these Ad41 strains. In the last decade, there were relatively few comprehensive epidemiological studies of subgenus F adenoviruses from children with diarrhea in Asian countries. The objectives of the present study were (i) to describe the genome types of Ad41 isolates by DNA restriction endonucleases (REs) analysis in Japan, Vietnam, and Korea and (ii) to describe the genetic and antigenic characterization of different genome types of Ad41 isolates in these three countries.

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MATERIALS AND METHODS

Fecal specimens. A total of 3,577 fecal specimens were collected from children between 1 month to 15 years of age with acute diarrhea from Japan, Vietnam, and Korea. These specimens included 1,991 specimens from Tokyo, Osaka, Saga, Maizuru, and Sapporo in Japan collected between July 1998 and June 2001; 1,355 specimens from Ho Chi Minh City in Vietnam collected between December 1999 and November 2000; and 231 specimens from Seoul in Korea collected between January 1998 and July 1999. By using polyclonal antibody-based enzyme-linked immunosorbent assay (ELISA) (24) and the combination of PCR and restriction fragment length polymorphism methods (31), adenoviruses were detected and serotyped. A total of 158 adenovirus-positive specimens were identified. Of these specimens, 101 specimens were typed as Ad41, the most common serotype, followed by Ad40 (12 isolates), Ad8 (12 isolates), Ad2 (10 isolates), Ad3 (10 isolates), Ad5 (8 isolates), and Ad31 (1 isolate). Four specimens remained untypeable. These Ad41 specimens were used for analysis in the present study.

DNA RE analysis. (i) Virus isolation. Ad41 prototype strain Tak was obtained from the National Institute of Infectious Diseases of Japan. Ad41 specimens were isolated and propagated in Caco-2 cells (human colonic carcinoma cells). The inoculation was performed as previously described (26). The cultured fluids were harvested after infection at the time of maximal cytopathic effect (CPE).

(ii) Extraction of viral DNA. A total of 8 ml of the cultured fluids were centrifuged at 1,353 × *g* for 30 min, and the cells were pelleted. In order to avoid mixing of cellular nucleic acid in the subsequent extraction of viral DNA, ultracentrifugation of the cultured supernatant was performed (40,700 × *g* for 3 h).

TABLE 1. Oligonucleotide primers for PCR sequencing

Primer	Polarity	Sequence (5'-3')	Position[a]
HX5-3	+	cac atc gcc gga cag gat gct tcg gag ta	40–68
HX3-4	−	gtg ttg tga gcc atg ggg aag aag gtg gc	1819–1847
S-29	+	gcc agc acr twc ttt gac at	289–308
S-52	−	ccc atg ttg cca gtg ctg ttg tar tac a	986–1013
S-51	+	ccc aac aga ccc aay tac	937–956
S-53	−	aag ggg ttg acg ttg tcc at	1555–1574

[a] The sequence positions are based on the Ad3 hexon region.

After centrifugation, the supernatant was removed, and the viral pellet was obtained. Subsequent extraction was conducted by a modified Hirt method (44).

(iii) Digestion of DNA by RE. Approximately 1 μg of viral DNA was digested with 10 U of the REs BamHI, BglI, BstEII, EcoRI, HindIII, KpnI, PstI, SacI, SmaI, and XhoI (TaKaRa Biotechnology Co., Ltd.) according to the manufacturer's instructions. After digestion, reaction products were loaded onto 1.0% agarose gel containing ethidium bromide (0.5 μg/ml) and run at 50 V for 5 h in Tris-acetate (TAE) buffer. The gel was photographed under UV light. The genome typing system proposed by Adrian et al. (1) was used as the basis for our study. By comparing the resulting patterns with the published restriction profiles of prototype strains, genome type identifications could be achieved.

PCR sequencing analysis of HVRs. In order to understand the genetic relationships between different genome types of Ad41 strains isolated from the three countries in the present study, eight Ad41 representing strains were randomly selected from eight genome types, and their nucleotide sequences of hypervariable regions (HVRs) were determined.

Viral DNA diluted 1:100 was used as a template DNA for PCR. The primers were designed according to the method of Takeuchi et al. (37). In the first PCR, a pair of primers, HX5-3 and HX3-4, was used. The PCR product of 1,800 bp contained all seven HVRs. The second PCR was conducted by using the first PCR product as a template with a combination of the following two pairs of primers: S-29 and S-52 or S-51 and S-53. The primer pairs used are listed in Table 1. The cycling conditions of the PCR were modified in 35 cycles (94°C for 1 min, annealing at 45°C for 2 min, and primer extension at 72°C for 3 min, with a final product extension at 72°C for 7 min). The second PCR products were extracted from 1% agarose gel by using phenol and chloroform. The purified DNA was used to perform enzymatic extension reaction for DNA sequencing with a DNA sequencing kit (BigDye Terminate v3.0 cycle sequencing ready reaction; ABI Prism; Applied Biosystems, Warrington, Great Britain). After the reaction products were cleaned, samples were analyzed by autosequencer (ABI Prism 310 genetic analyzer). Evaluation of all sequence data and analysis of identity were conducted by using DNASIS software (version 1994; Hitachi Software Engineering Co., Ltd., Tokyo, Japan). The sequence alignments of nucleotide and deduced amino acid were carried out by DDBJ (http://www.ddbj.nig.ac.jp) by using the CLUSTAL W program. The phylogenetic tree was constructed with MEGA version 2.1 (18) by using the neighbor-joining method and the bootstrap test (http://www.megasoftware.net/).

Nucleotide sequence accession numbers. Sequence data from the present study were entered in the GenBank/EMBL/DDBJ database under the following accession numbers: Ad41-D1-VN47 (AB103341), Ad41-D4-VN28 (AB103342), Ad41-D12-JP3171 (AB103343), Ad41-D22-Km079 (AB103344), Ad41-D26-JP3106 (AB103345), Ad41-D27-JP2149 (AB103346), Ad41-D25-Ks35 (AB103347), and Ad41-D28-VN1020 (AB103348).

ELISA based on MAb. ELISA based on the use of monoclonal antibodies (MAbs) was utilized to examine the antigen specificity of Ad41 strains. ELISA was performed as described by Nishio et al. (24). Anti-Ad40 rabbit serum was used as capture antibody. The MAbs 15D (adenovirus group-specific), 12D (Ad40 type specific), and 1F (Ad41 type specific) were used as detector antibodies. In each test, cell culture supernatants of Ad41 prototype strain were included as positive controls, and phosphate-buffered saline was used as a negative control. If the optical density value was >0.2, as well as 2-fold greater than the negative control well, the specimen was considered positive.

RESULTS

Genome types of Ad41 isolates from three countries. Seventy Ad41 fecal specimens were isolated successfully, and RE analyses were performed with 10 different REs: BamHI, BglI, BstEII, EcoRI, HindIII, KpnI, PstI, SacI, SmaI, and XhoI. To confirm the genome type, restriction patterns were compared to that of the Ad41 prototype Tak strain. The results of enzyme code and genome type determinations obtained in the present study are shown in Table 2. New restriction patterns were observed in cleavage with enzyme EcoRI, KpnI, PstI, SacI, and SmaI. According to the nomenclature system, the new patterns were named as follows: code 7 of EcoRI, code 4 of KpnI, code 4 of PstI, code 3 of SacI, and code 9 of SmaI (Fig. 1a to e).

Analysis of 70 Ad41 isolates from three countries revealed the existence of eight genome types in the present study. Of these genome types, four (D1, D4, D12, and D22) were previously reported by van der Avoort et al. (43). In addition, four novel genome types D25, D26, D27, and D28 are reported here for the first time. The photographs of representative strains of these novel genome types cleaved by the panel endonucleases are shown in Fig. 2. They accounted for 61% (43 of 70) of all Ad41 isolates. The novel genome type D26 had enzyme code 2212141341 and was closely related to another novel genome type D25, differing only in cleavage with BamHI.

In Japan, the novel genome type D26 predominates with 26 isolates, followed by D12 and D27 with 17 and 4 isolates, respectively. In 3 years, D26 and D12 were observed every

TABLE 2. Genome types of Ad41 isolates in Japan, Vietnam, and Korea

Country	Genome type[b]	Enzyme code[a]									Total no. of isolates
		BamHI	BglI	BstEII	EcoRI	HindIII	KpnI	PstI	SacI	SmaI	XhoI	
Japan	**D26**	2	2	1	2	1	**4**	1	**3**	4	2	26
	D12	2	1	2	1	5	1	1	1	2	1	17
	D27	2	2	1	**7**	1	1	1	1	4	2	4
Vietnam	**D28**	2	1	4	4	4	2	**4**	1	**9**	1	10
	D4	3	1	1	1	3	1	1	1	4	1	4
	D1	1	1	1	1	1	1	1	1	1	1	2
Korea	D22	2	1	2	1	5	1	3	1	2	1	4
	D25	1	2	1	2	1	**4**	1	**3**	4	2	1
	D26	2	2	1	2	1	**4**	1	**3**	4	2	2

[a] The boldface numbers indicate a novel pattern.
[b] Novel genome types are indicated in boldface.

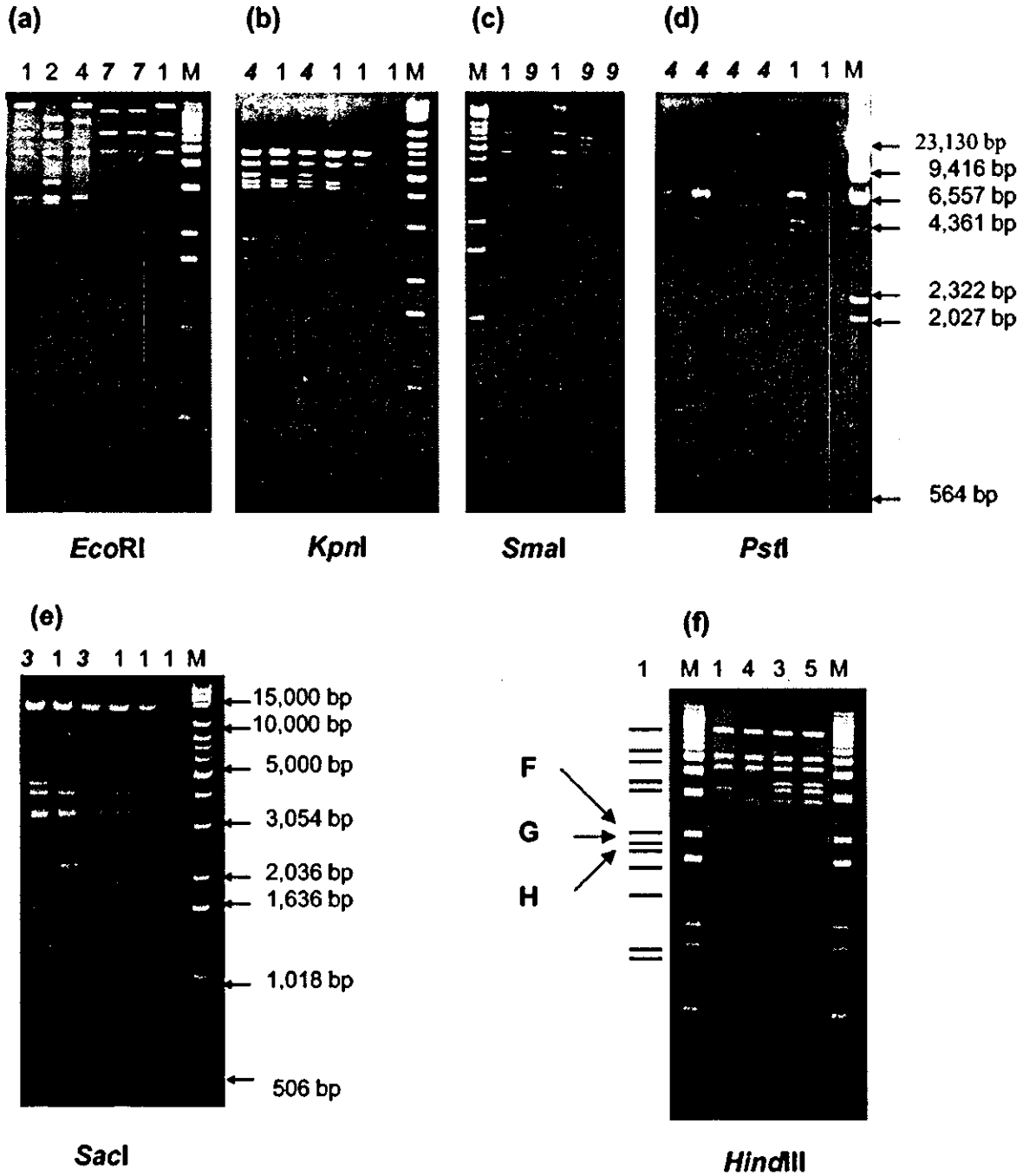

FIG. 1. Novel restriction patterns with EcoRI (a), KpnI (b), PstI (d), SacI (e), and SmaI (c). The numbers above the lanes are the enzyme code; lane M contains molecular weight standards (1-kb DNA extension ladder; Life Technologies), except for lane M of PstI. Lane M of PstI contains molecular weight standards (Marker II, λDNA HindIII digested; TaKaRa). Enzyme code "1" refers to the pattern of the prototype. The boldface italic numbers mark the novel patterns. (f) Restriction patterns with HindIII observed in the present study. The numbers above the lanes are the enzyme code; lane M contains molecular weight standards. The marker lines on the left give the model pattern of enzyme code 1. The arrowed letters F, G, and H refer to the names of the fragments according to the physical map given by Takiff et al. (40). The patterns of codes 3 and 5 lack fragment F.

year. From July 1999 to June 2000, the new genome type D27 appeared, but it was not found the next year. In Vietnam, D28, represented by 10 isolates, was the most common. D4 and D1 were observed in 4 and 2 isolates, respectively. In Korea, D22 predominated with four isolates. Interestingly, the novel genome type D26 was observed in Korea as well. It was present in two isolates, whereas only one isolate of novel genome type D25 was detected.

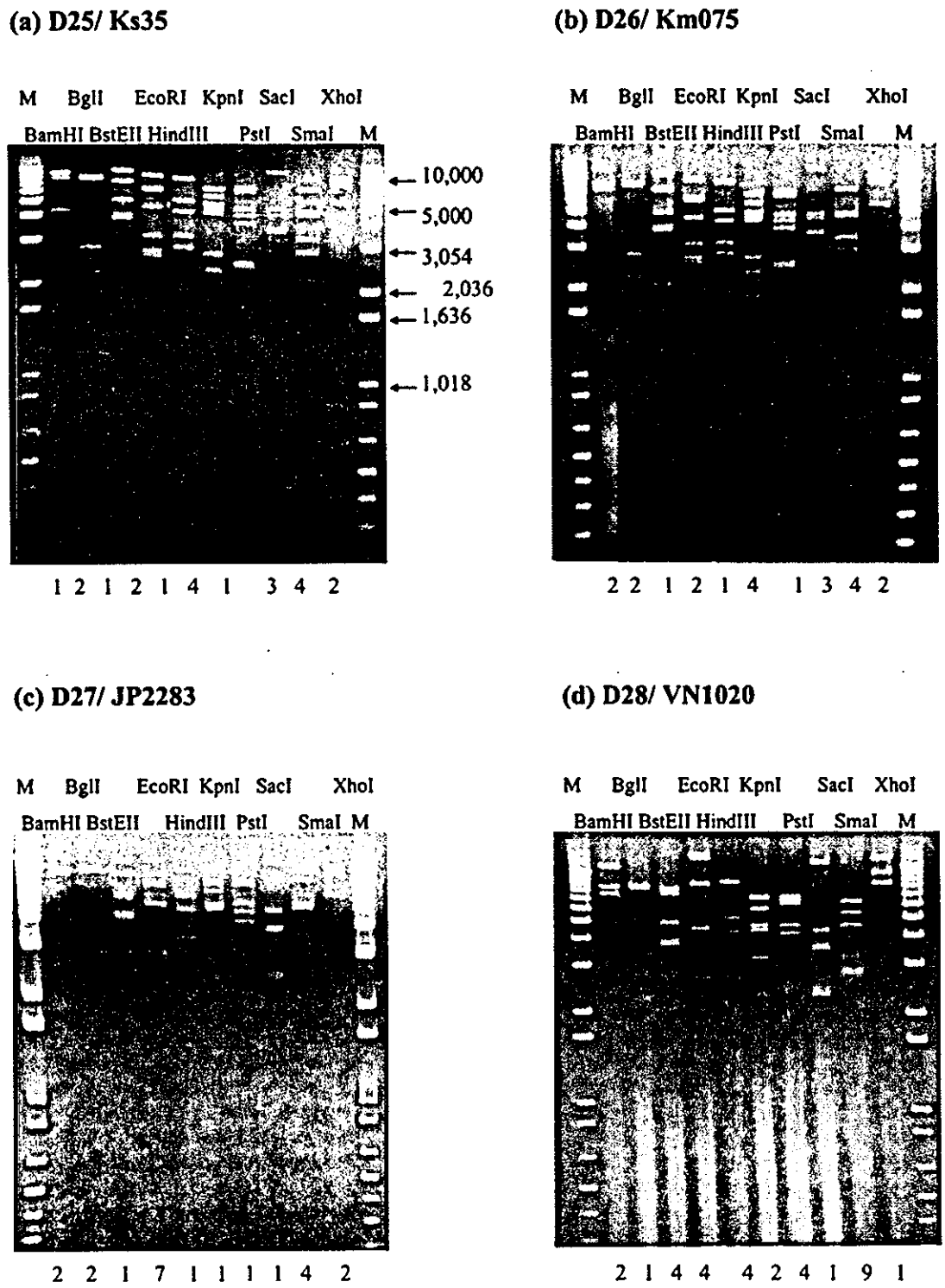

FIG. 2. (a to d) Restriction profiles of novel Ad41 genome type strains isolated from Japan, Vietnam, and Korea, obtained in the present study. (a) D25/Ks35; (b) D26/Km075; (c) D27/JP2283; (d) D28/VN1020. The numbers under the lanes are the enzyme code; lane M contains molecular weight standards.

Genetic analyses of HVRs for eight genome types of Ad41. The HVRs of eight representative strains from eight genome types obtained in the present study were sequenced. The DNA sequences, containing seven hexon HVRs, with a total of 1,232 to 1,235 nucleotides, from the eight strains were determined. Alignments of the predicted amino acid sequences were performed. A phylogenetic tree based on the deduced amino acid sequences of eight representing genome types was subse-

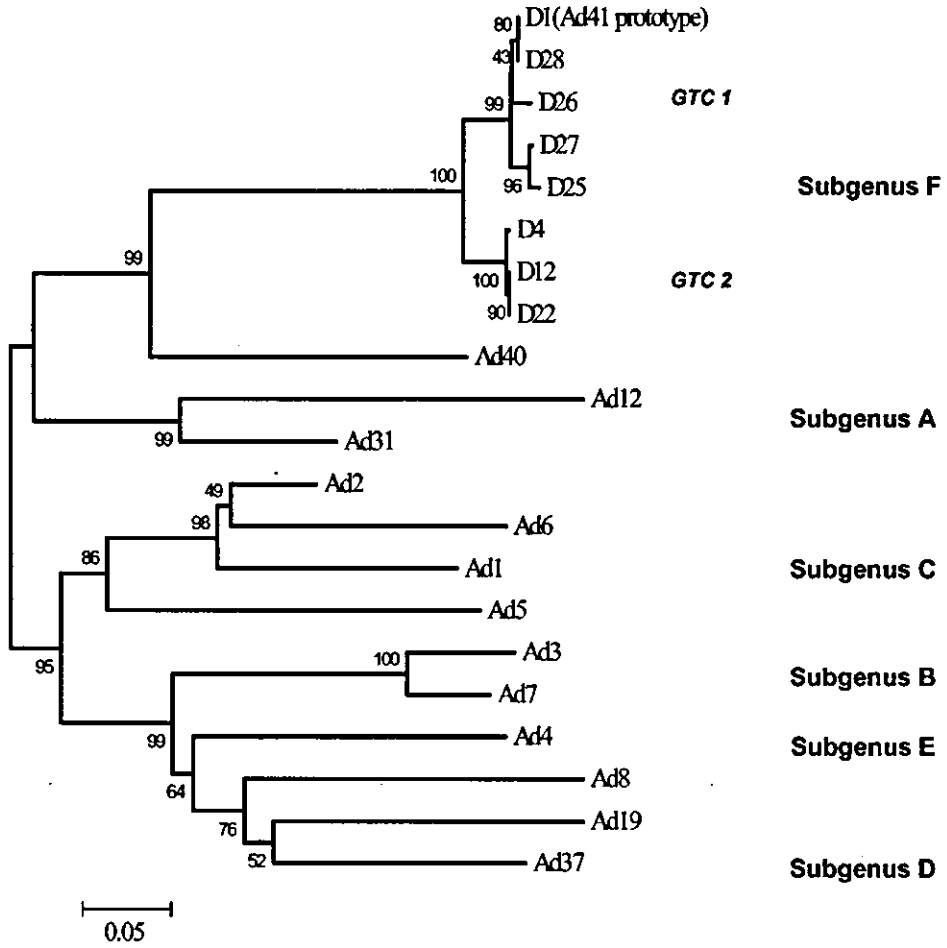

FIG. 3. Phylogenetic tree based on the deduced amino acid sequences of HVRs of Ad reference strains and Ad41 strains isolated from Japan, Vietnam, and Korea. Two GTCs, GTC1 and GTC2, are shown. The sequences of reference strains previously reported are listed in GenBank under the indicated accession numbers: Ad12 (X73487) (36), Ad31 (74661) (29), Ad1 (X67709) (28), Ad2 (J01917) (2), Ad5 (M73260) (17), Ad6 (X67710) (28), Ad3 (X76549) (30), Ad7 (Z48571) (21), Ad4 (X84646) (30), Ad8 (X74663) (29), Ad19 (X98359) (43), Ad37 (X98360) (43), Ad40 (X51782) (42), and Ad41 (X51783) (41).

quently constructed (Fig. 3). It revealed that in the eight genome types representing strains, two genome type clusters (GTCs) are present. GTC1 includes D1 (Ad41 prototype Tak strain), D28, D26, D27, and D25, and GTC2 consists of D4, D12, and D22. The amino acid homology rates in all seven HVRs among the eight representative strains are shown in Table 3. The highest amino acid homologies were observed within a GTC (97 to 100%). Between the members of different GTCs, the homologies were 92 to 94%. The alignments of HVRs predicted amino acid sequences are shown in Fig. 4. The same substitutions in amino acid (aa) 54 (HVR1), aa 74 (HVR2), aa 100 to 101 (HVR3), aa 133 (HVR4), aa 155 to 156, aa 160 (HVR5), aa 190 (HVR6), aa 314 to 316, and aa 323 (HVR7) were presented between GTC1 and GTC2 strains. The members of GTC2 have a common deletion in aa 73 and an insertion in aa 320.

Antigen specificity of Ad41 isolates. MAbs were used to examine the antigen specificity in all Ad41 isolates belonging to eight different genome types. All of these isolates reacted with MAb 15D; none of them reacted with MAb 12D. On the other hand, two antigenic subtypes among eight genome types were distinguished on the basis of the specificity of the MAb 1F selected by Ad41 prototype Tak strain. The isolates that belonged to genome types D1, D28, D26, D27, and D25, were recognized by MAb 1F, but the mem-

TABLE 3. Homology comparison of HVRs between Ad41 isolates and prototype strains Ad40 and Ad41

HVR	% Homology[a] with HVR:								
	Ad40	D1	D26	D27	D28	D25	D4	D12	D22
Ad40 (prototype)		72	72	72	72	72	72	72	72
D1 (Ad41 prototype)	68		98	98	100	98	94	95	95
D26 (JP3106)	68	98		98	98	98	94	94	94
D27 (JP2149)	67	98	99		98	98	94	94	94
D28 (VN1020)	68	100	98	98		98	94	95	95
D25 (Ks35)	67	98	97	99	98		94	94	94
D4 (VN28)	66	94	93	93	94	92		99	99
D12 (JP3171)	66	94	93	93	94	93	99		99
D22 (Km079)	66	94	93	93	94	93	99	100	

[a] The upper right data are the percent homologies of the nucleotide sequences; the lower left data are the percent homologies of the amino acid sequences.

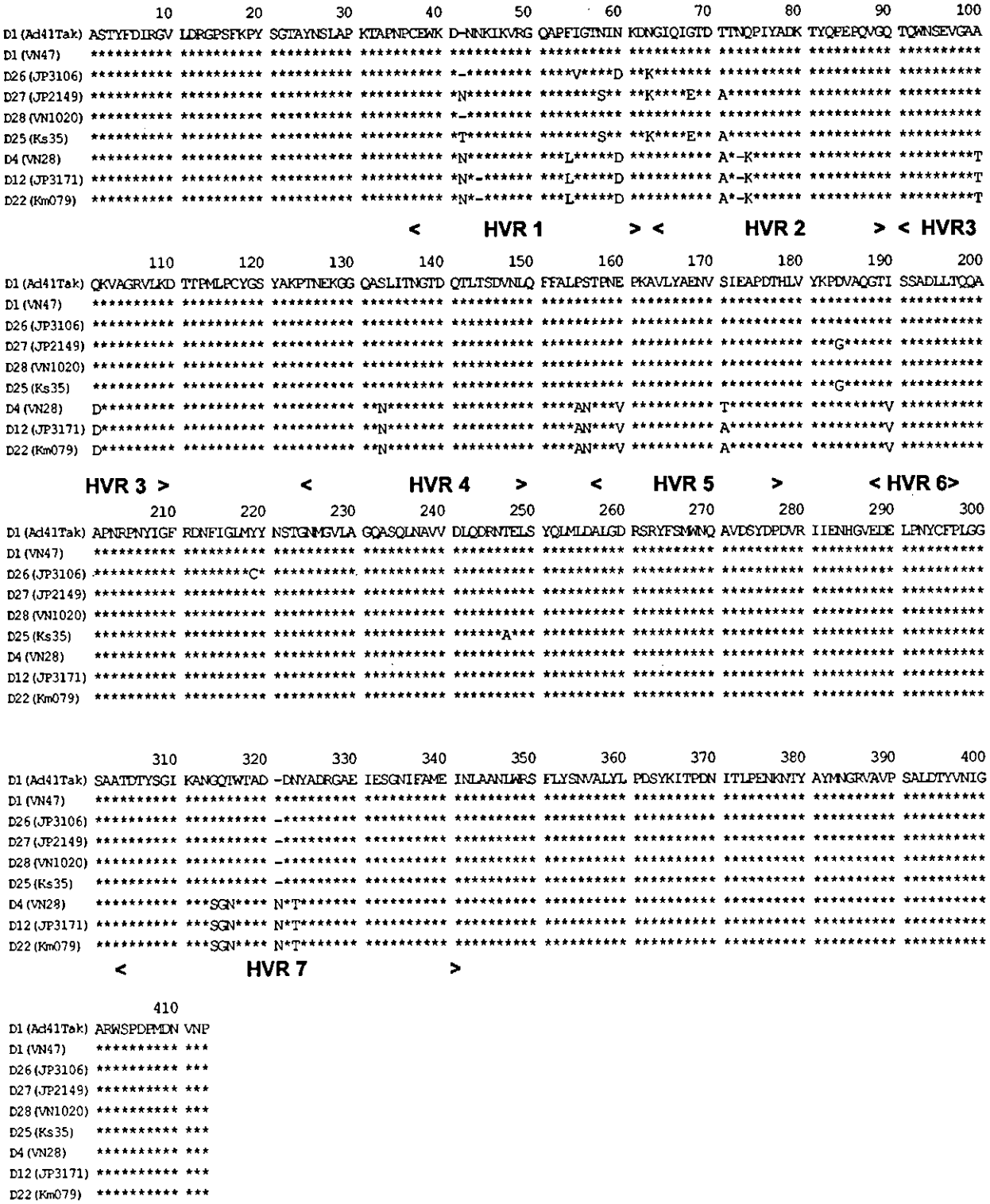

FIG. 4. Alignments of the deduced amino acid sequences of hexon, including seven HVRs from eight Ad41 genome type strains. The regions of seven HVRs are given. The borders of the seven HVRs are in accordance with the literature (37). Asterisks indicate the identical residues to the sequence of D1.

bers of D4, D12, and D22 could not be distinguished with the same MAb 1F.

DISCUSSION

In this study, the genome types of Ad41 strains isolated from fecal specimens in Japan, Vietnam, and Korea were determined. In addition, the genetic and antigenic characterizations are given.

EAd are known as fastidious adenoviruses since they do not grow well in conventional cell lines. There are varied reports in the literature concerning the ability of EAd to replicate in different cell lines, such as Chang conjunctival cells, Hep-2 cells, 293 cells, and PLC/PRF/5 cells. The 293 cells, an Ad5-transformed HEK cell line that contains and expresses the Ad5 early region E1A and E1B, were considered useful for the growth of many Ad41 strains (40), but infection yields are still low. Recently, Pinto et al. (27) have reported the use of Caco-2 cell line in isolating laboratory strains of human enteric viruses, including the group A rotavirus type 3, astrovirus serotype 1, and Ad5, Ad40, and Ad41. In their study, for EAd, apparent CPE was observed with laboratory strains, whereas wild-type Ad40 and Ad41 strains failed to induce CPE in Caco-2 cells, though the amplification of viral nucleic acid was confirmed by dot blot hybridization. In our study, Caco-2 cells were used to propagate EAd from fecal specimens. Seventy Ad41 and ten Ad40 (unpublished data) strains from clinical specimens were successfully isolated. The present study confirmed that Caco-2 cells are a useful cell line for research on diarrheal viruses.

Among these genome types observed in Japan, Vietnam, and Korea, four novel genome types (D25, D26, D27, and D28) were observed for the first time, and the other four genome types (D1, D4, D12 and D22) were reported in earlier studies (13, 14, 43). Genome type D12 was present in 17 isolates in Japan over a period of 3 years. This genome type was first isolated in 1982 from The Netherlands and was highly prevalent in the 1980s (43). In Sweden, an identical D12 was also reported, specimens of which were mainly obtained from an outbreak of gastroenteritis in a long-term pediatric ward at a hospital of Stockholm in 1988; it was then found sporadically for several years afterward (13). Another genome type D22 observed in Korea was first isolated during 1984 in The Netherlands, too (43). In the present study, genome type D4 identified in Ho Chi Minh City, Vietnam, was also first found in Malaysia in 1979 (14). These phenomena may indicate that genome types of Ad41 have a scattered global distribution. It is quite possible that the novel genome types observed in the present study are also present in Europe and other continents. Another possibility is that, because of limited research work these novel genome types were not detected.

In studying the profiles of Ad41 isolates obtained with 10 restriction enzymes in three countries, it was found that almost all restriction patterns that appeared in Korean strains were also present in Japanese strains and, in contrast, Vietnamese isolates always exhibited different patterns. For example, Japanese and Korean Ad41 isolates have the patterns 1 and 5 upon cleavage with enzyme HindIII, but Vietnamese strains showed patterns 3 and 4, along with pattern 1. In addition, novel genome type D26 was observed in both Japan and Korea. These data might represent a gradient of antigenic drift reflecting increased travel activities between the two countries.

The occurrences of novel genome types reflect the proceeding mutation of viral DNA. Hexons are important capsid proteins of adenovirus. It was reported that seven HVRs located in hexons take part in the neutralization reaction and contain the epitopes of serotype determination (4). It is interesting to estimate what role these regions might have in identical adenovirus serotypes. Previous studies revealed that hexon contains HVRs that are conserved in different genome types of Ad3 and Ad7 belonging to the subgenus B (20, 38). In the present study, the sequence analyses of HVRs of different genome types of Ad41 isolates from three countries also demonstrated a high level of conservation. The homologies ranged from 92 to 100% and the eight genome types of Ad41 were divided into two clusters according to the predicted amino acid sequences of HVRs. The classification of GTCs had a curious coincidence with the result of ELISA with MAb 1F. It has been confirmed that the MAb 1F may react with hexon protein of Ad41 (24). Therefore, the results presented here suggest that HVRs of hexons may contain epitopes that are associated with the Ad41 subtype.

In comparing the ten enzyme codes, one difference can be found in cleavage with HindIII between GTC1 and GTC2. The members of GTC1 have the HindIII code 1 and 4; the members of GTC2 have codes 3 and 5. Physical map of Ad41 was published by Takiff et al. (39). Tak strain HindIII fragments F and H were determined to lie between map units 51 and 60, which is within the confines of the hexon gene. van der Avoort et al. (43) revealed that D4, D12, and D22 could not react with MAbs 1-23, 3-10, 3-18, and 7-14 and lacked fragment F in common (Fig. 1f). However, in the present study, the nucleotide sequence analysis of hexon-containing HVRs confirmed that the guanine-for-adenine substitution in nucleotide 696, which leads to failure of HindIII-cut viral DNA of GTC2 isolates, was a conservative substitution.

Recently, ELISA with MAbs specifically reacting with Ad40 and Ad41 is widely used for the diagnosis of EAd in fecal specimens. It is a rapid, readily available means for identification of Ad40 and Ad41. MAb 1F was used successfully for screening Ad41 in clinical specimens (24). In the present study, the Ad41 isolates belonging to genome types D4, D12, and D22 did not react with MAb 1F. The application limitation of MAbs was reported in earlier studies (5, 23, 33, 43). Due to virus strain mutation, the MAb may be safely used for screening purposes only temporarily. The genetic variation within one type of adenovirus can evidently affect the diagnosis capability of highly specific MAbs. It has been reported that a commercial MAb-based ELISA could not demonstrate the presence of a highly prevalent genome type of Ad41 in Canada (33). In South Africa, the locally circulating adenoviruses were not detected with a commercial kit utilizing MAbs directed against type-specific epitopes on the adenovirus hexon (23). For Japanese specimens, MAb 1F distinguished all of the Ad41 strains isolated from 1982 to 1988 (24) but failed to react with some Ad41 isolates in 1998 to 2001. These results also confirmed that antigenic drift on the surface of adenovirus occurred frequently. Hexon and fiber compose most of the external capsid of adenovirus. It may be difficult to find other, better targets for reagent antibodies except the hexon and

fiber. The use of pooled MAbs to hexon may provide a practicable way to avoid omissions in detection such as the ones described above when a single MAb was used.

In conclusion, eight genome types of Ad41 were found in Japan, Vietnam, and Korea. Four novel genome types were investigated for the first time in the present study. The existence of two GTCs and the antigenic difference between them may reflect the accumulation of amino acid mutations located in HVRs of the hexon. These amino acid mutations may affect classification of the Ad41 subtype. These data contribute to our understanding of the molecular epidemiological characterization of adenoviruses in these countries. Further studies are needed to obtain additional established strains of the Ad41 genome types in order to clarify the genetic and antigenic relationships between them.

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Evaluation of a Bedside Immunochromatographic Test for Detection of Adenovirus in Respiratory Samples, by Comparison to Virus Isolation, PCR, and Real-Time PCR

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An immunochromatography (IC) kit for human adenovirus (HAdV) was evaluated with 138 patient nasopharyngeal samples. The samples were collected at a sentinel clinic in Japan from January through June 2003. Patients were diagnosed by clinical manifestation of pharyngoconjunctival fever (n = 38) or exudative tonsillitis (n = 100). The IC kit was positive for 84% (116 of 138) of patients diagnosed at bedside. The remaining extract solution of the IC kit test was transferred into maintenance medium and tested via laboratory diagnoses. The IC kit had 95% sensitivity (116 of 122 patients) with HAdV isolation (isolation) as the standard and 91% sensitivity (116 of 128 patients) with PCR as the standard. All of the IC kit-positive samples were isolation and PCR positive. Similarly, all the isolation-positive samples were PCR positive. Twenty-two IC kit-negative samples were evaluated by real-time PCR. Six samples were IC kit negative and isolation positive and contained 3.8×10^7 to 2.5×10^9 copies of the HAdV genome/ml. Five samples that were only PCR positive contained 3.0×10^4 to 3.8×10^5 copies of the HAdV genome/ml, but one sample was real-time PCR negative. We conclude that the IC kit is a useful bedside diagnostic tool for HAdV infections because it has 95% sensitivity (compared to isolation), but a negative result does not always rule out HAdV infection.

Human adenoviruses (HAdVs) are one of the most commonly isolated viruses and are a significant cause of diseases of the respiratory tract and eye. Pharyngoconjunctival fever (PCF) is a widely known adenoviral disease. HAdVs, particularly types 7 and 3, are also known to cause pneumonia (12, 13). Furthermore, HAdVs are increasingly being recognized as fatal pathogens in immunocompromised patients (1, 2, 5, 18).

Recently, four different kinds of diagnostic methods for HAdV infection became available. The virus isolation technique (isolation) is usually considered the "gold standard" but is time-consuming. PCR is a rapid and sensitive diagnostic technique that can be completed in a few hours, and sequencing the amplified products yields useful data. Real-time PCR for HAdVs has recently become available. Real-time PCR produces results quickly, and quantitative data can be obtained (5, 8, 18). In addition to these laboratory diagnostic techniques, immunochromatography (IC) kits have become available for HAdV diagnosis at the patient's bedside (6, 7, 16, 20, 21).

IC kits for HAdVs have been evaluated in comparison with an isolation technique (6, 20), an enzyme-linked immunosorbent assay kit (16), and PCR (7, 21). However, they have not been evaluated in comparison to the combination of isolation, PCR, and real-time PCR. Quantitative evaluation alone was insufficient; therefore, we evaluated an IC kit in comparison to these three methods. The sensitivity and specificity of the IC kit change according to which method is considered to be the gold standard (15).

The purpose of this study was to evaluate the IC kit, qualitatively and quantitatively, in comparison to multiple, sensitive laboratory diagnostic methods. The reliability and limitations of the IC kit were evaluated. The detection limit of the IC kit was evaluated with HAdV type 1, 2, 3, 5, 6, and 7 isolates.

We found that the IC kit had a higher sensitivity than reported previously, although it was less sensitive than isolation, which was less sensitive than PCR and real-time PCR. However, the IC kit was found to have 95% sensitivity compared to HAdV isolation, the diagnostic technique usually considered the gold standard.

MATERIALS AND METHODS

Patients and clinical samples. A total of 138 nasopharyngeal swabs were collected from patients suffering from pharyngoconjunctival fever (n = 38) or exudative tonsillitis (n = 100) during the period of January through June 2003. The mean age of the patients, who ranged in age from <1 to 10 years, was 3.6 ± 0.19 years (mean ± standard deviation [SD]), except for one adult patient who was 38 years old. There was no significant difference in the sex of the patients (72 males and 66 females). All of the patients had temperatures in the range of 38.0 to 40.7°C; the mean temperature was 39.4 ± 0.05°C (mean ± SD). The clinical picture of patients with typical HAdV-associated exudative tonsillitis includes a high fever (>38°C) which persists for about 5 days, and C-reactive protein is usually strongly positive. All clinical samples were collected at the Okafuji Pediatric Clinic, Himeji City, Japan. The protocol was reviewed and approved by the local institutional review board, and informed consent was obtained from all patients.

Clinical diagnosis at bedside. The IC kit used in this study was the SAS Adeno Test (SA Scientific, Inc., San Antonio, Tex.), which was sold under the name Check Ad (AZWELL, Osaka, Japan). The tonsils and posterior pharynx of each patient were vigorously rubbed with a cotton swab moistened in sterile physiological saline. The swabs were extracted with 500 μl of mucolytic agent provided

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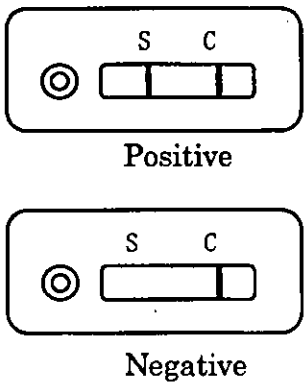

FIG. 1. Detection of adenovirus in a clinical sample by the IC kit. The test is positive if two colored lines appear in the sample (S) and control (C) areas. The test is negative when only one colored line appears in the control area.

by the manufacturer and rubbed in a soft tube. An aliquot of the extract (200 μl) was filtered and dropped into the IC kit device. Both the test tube and filter were provided by the manufacturer. The IC kit indicated an HAdV-positive result when two colored lines appeared in the device. When only one colored line appeared in the control area of the IC kit, the result was HAdV negative (Fig. 1). The remaining extract solution (about 200 μl) was transferred into a test tube containing 2 ml of Dulbecco's modified Eagles's medium (Sigma) and used as a clinical sample for a later laboratory diagnosis. The test tubes were kept at −80°C until use.

Virus isolation and serotyping. Clinical samples were inoculated onto 80% confluent monolayers of HEp-2 cells in duplicate wells of a 24-well plate (Nippon Becton Dickinson, Tokyo, Japan). For cultivation, Dulbecco's modified Eagle's medium (Sigma) supplemented with 2% heat-inactivated fetal calf serum and antibiotics was used as a maintenance medium. The HEp-2 cells were subpassaged three to eight times to allow time for the cytopathic effect (CPE) to develop. When the CPE became evident, the HAdV isolates were serotyped by a neutralization test (NT) by use of antisera purchased from Denkaseiken (Tokyo, Japan). HAdV type 7 (HAdV 7) used in the detection limit test was isolated and identified in 1998 by the same technique. Enteroviruses were isolated and identified in the same way. Isolates which could not be identified by NT using antiserum for HAdV 1 to 7 were identified by a combination of PCR and sequencing techniques.

Viral DNA preparation. HAdV genomes and enterovirus RNAs were prepared directly from the clinical samples with a QIAamp blood kit (QIAGEN) or a High Pure viral nucleic acid kit (Roche Diagnostics). DNAs from HAdV 1, 2, 3, 5, and 7 isolates were extracted with the High Pure kit.

PCR and sequencing. A single-tube multiplex PCR for HAdVs was carried out as reported previously (3). HAdVs were distinguished by the size of the amplified products. HAdVs which amplified to produce 188- and 301-bp DNA fragments were typed as HAdV 3 and non-subgroup B HAdVs, respectively.

Another primer pair for HAdV 3 (and HAdV 7) was designed and used for PCR and sequencing of the hexon region of HAdVs. The sequences of the primers were 5'-AGAATCATGGACTGATACTGATG-3' (sense) and 5'-AGC CTGTCATTGCCAGGCCAGC-3' (antisense). Amplification reactions were conducted in 50 μl of reaction mixture containing a 0.5 μM concentration of each of the primers, a 200 μM concentration of each dideoxynucleotide, 1.25 U of *Taq* polymerase (TaKaRa Shuzo, Shiga, Japan), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$. The reaction was carried out with a cycle of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min and was continued for 35 cycles. In the first cycle, the denaturation step continued for 5 min at 94°C, and in the last cycle, the extension step continued for 5 min at 72°C. The expected product size was 1,596 bp (from position 591 to 2186 based on the hexon sequence of HAdV3; GenBank accession number X76549).

Two RT-PCR methods for enteroviruses developed by Ishiko et al. (11) and Oberste et al. (14) were used for enterovirus identification. PCR-amplified products were sequenced directly as reported previously (3, 4, 17). PCR and RT-PCR were performed using a Thermal Cycler Dice (TaKaRa Shuzo). The nucleotide sequence was determined with a model 310 genetic analyzer (Applied Biosystems Japan [ABI]).

TABLE 1. IC kit and typing results of detected viruses (n = 138)

Virus type(s) identified[a]	No. (%) of patients	No. (%) of patients positive by:			
		IC kit	Virus isolation		Multiplex PCR
			HAdV	Enterovirus	
HAdV 1	11 (8.0)	9 (7.8)	11 (9.0)		11 (8.6)
HAdV 2	17 (12)	17 (15)	17 (14)		17 (13)
HAdV 3	86 (62)	80 (69)	84 (69)		86 (67)
HAdV 5	4 (2.9)	4 (3.4)	4 (3.3)		4 (3.1)
HAdV 6	2 (1.4)	2 (1.7)	2 (1.6)		2 (1.6)
HAdV 3 + 5	1 (0.7)	1 (0.9)	1 (0.8)		1 (0.8)
HAdV 3 + CB 2	2 (1.4)	2 (1.7)	2 (1.6)	2 (33)	2 (1.6)
HAdV 3 + CB 5	1 (0.7)	1 (0.9)	1 (0.8)	1 (17)	1 (0.8)
HAdV NI + CB 2	1 (0.7)			1 (17)	1 (0.8)
HAdV NI	3 (2.2)				3 (2.3)
CB 2	2 (1.4)			2 (33)	
Total	138[b]	116	122	6	128

[a] NI, not identified.
[b] Includes eight patients (5.8% of the total number) negative for all viruses.

Real-time PCR. Real-time PCR for a wide range of HAdVs was used in this study. Primers derived from the highly conserved HAdV hexon 3 and 4 genes by Echavarria et al. (1) were used. The sequences of the primers were 5'-GACAT GACTTTCGAGGTCGATCCCATGGA-3' (Hex3) and 5'-CCGGCTGAGAA GGGTGTGCGCAGGTA-3' (Hex4). The expected product size was 140 bp (from position 21589 to 21728 based on the complete sequence of HAdV2; GenBank accession number J01917). One microliter of template DNA was added to a final volume of 25 μl containing 1× SYBR green PCR master mix (ABI) and a 160 nM concentration of the primers Hex3 and Hex4. The real-time PCR was carried out with an ABI PRISM 7900 HT sequence detection system for a cycle of 94°C for 1 min, 57°C for 1 min, and 72°C for 1.5 min, which was continued for 40 cycles. During thermal cycling, the emissions from each sample were recorded and SDS (sequence detection system) software processed the raw fluorescence data to produce threshold cycle (C_T) values for each sample. The SDS software then computed a standard curve from the C_T value of the diluted standards and extrapolated absolute quantities for the unknown samples based on their C_T values. The DNA of the prototype HAdV 2 was amplified by the PCR system of Echavarria et al. (1) as described above. The product (140 bp) was cloned into a pCR2.1 vector and used as a standard.

Detection limit of the IC kit. HAdV 1, 2, 3, 5, and 6 isolated in this study and HAdV 7 isolated in 1998 were diluted in phosphate-buffered saline and tested with the IC kit. Diluted solutions showing slight but clear positivity by the IC kit were used to determine the detection limits of the IC kit. The detection limits were evaluated by determining the 50% tissue culture infective dose ($TCID_{50}$) per milliliter by use of HEp-2 cells (10) and real-time PCR. Additionally, repeated IC kit detection limit tests were performed with a twofold serially diluted HAdV 3 solution to check the reproducibility. At each concentration, 10 IC kits were tested.

RESULTS

Virus isolation and identification. All the IC kit-positive samples were isolation positive. Six IC kit-negative samples were isolation positive and typed HAdV 1 or 3. Enteroviruses were also isolated individually or as a mixture with HAdVs (Table 1). The IC kit was able to detect HAdVs in coinfected samples. Because enteroviruses grow faster than HAdVs in HEp-2 cells, the CPE of coinfected samples was indistinguishable from that of enterovirus-infected samples. However, the supernatant of coinfected samples was IC kit positive. The IC kit was also useful for confirming HAdV isolation when the HAdV CPE was not clear.

TABLE 2. Results of real-time PCR and typing of the IC kit-negative samples (n = 22)

Test results[a] (n)	Virus type identified in each sample[b]	Copies of genome/ml[c]
PCR pos.; isolation pos. (6)	HAdV 1	3.8×10^7
	HAdV 3	9.0×10^7
	HAdV 3	4.0×10^8
	HAdV 1	4.5×10^8
	HAdV 3	1.3×10^9
	HAdV 3	2.5×10^9
PCR pos.; isolation neg. (6)	HAdV 3	ND
	HAdV 3	3.0×10^4
	HAdV NI	7.1×10^4
	HAdV NI	1.1×10^5
	HAdV NI + CB 2[d]	2.1×10^5
	HAdV NI	3.8×10^5
PCR neg.; isolation neg. (10)	Neg. (n = 9)	ND (n = 9)
	Neg.[e] (n = 1)	3.3×10^5

[a] pos., positive; neg., negative.
[b] NI, not identified.
[c] ND, not detected (the multiplex PCR was positive [188 bp] but the band was weak).
[d] The isolate was neutralized by anti-CB 2 serum.
[e] This sample was recorded as PCR negative because only a very weak band (301 bp) was observed.

PCR, RT-PCR, and real-time PCR. All isolation-positive samples, including IC kit-positive samples, were positive by PCR. However, all HAdV PCR-positive and isolation-negative samples were IC kit negative (Table 1). Samples positive by isolation but IC kit negative had higher concentrations of HAdVs than PCR-positive but isolation-negative samples (Table 2). One isolation-positive sample was coinfected with HAdV (2.1×10^5 copies/ml), and only coxsackievirus type B2 (CB 2) was isolated. Two PCR-amplified products were not clear when stained with ethidium bromide. The two samples did not give concordant results with PCR and real-time PCR. A sample containing HAdV (3.3×10^5 copies/ml) was recorded as PCR negative because only a very weak signal was observed after multiplex PCR. Similarly, one real-time-PCR-negative sample was multiplex PCR positive, but the product band was weak.

IC kit for HAdV. The detection limit of the IC kit was $10^{1.6}$ to $10^{4.0}$ $TCID_{50}$/ml and $10^{4.6}$ to $10^{5.8}$ copies of the HAdV genome/ml. The IC kit was not less sensitive in detecting HAdV 3 and 7 than in detecting HAdV 1, 2, 5, and 6 (Table 3). In repeated IC kit tests, HAdV 3 was positive 10 of 10 times at detection limit concentrations, 3 of 10 times at a twofold serial dilution, and 0 of 10 times at a fourfold serial dilution. A high reproducibility for the IC kit was ascertained.

Five clinical samples that were IC kit and isolation negative but PCR positive contained 3.0×10^4 to 3.8×10^5 copies of the HAdV genome/ml. However, six clinical samples were IC kit negative in spite of the presence of 3.8×10^7 to 2.5×10^9 copies of the HAdV genome/ml. These samples were all isolation positive, and the IC kit had sensitivity to the isolates.

At the bedside, 40 patient samples were recorded as strongly positive, since in each case a positive colored line appeared before the control line appeared. Seventy-three samples were positive within 10 min. Only three samples required 20 min.

TABLE 3. Detection limit of IC kit

Virus type	Detection limit	
	$TCID_{50}$/ml (10^n)	Copies of HAdV genome/ml (10^n)
HAdV 1	2.9	4.6
HAdV 2	3.2	5.4
HAdV 3	1.9	5.8
HAdV 5	3.9	5.8
HAdV 6	4.0	5.3
HAdV 7[a]	1.6	5.6
Mean ± SD (CV)[b]	2.9 ± 1.0 (0.34)	5.4 ± 0.4 (0.08)

[a] This strain was isolated in 1998. Other serotypes were isolated in this study.
[b] CV, coefficient of variation.

DISCUSSION

The IC kit has proven to be a useful method for bedside diagnosis of HAdV infections. HAdV infection is difficult to diagnose by the presence of symptoms alone. The sensitivity of the IC kit was 95% in comparison to HAdV isolation in HEp-2 cells. This percentage is higher than that reported earlier by Tsutsumi et al. (20) (72.6%; 69/95). As stated in the manufacturer's guide for the IC kit, the test is highly dependent on the technique of sample collection. We took a great deal of care in sample collection, transportation, and preservation. Additionally, we had more than 10 years of experience in sampling HAdV for isolation before the IC kit became available, which may explain the high detection level. Hara (7) reported a sensitivity of 94.2% (97 of 103 samples) for the same kit in comparison to isolation and that the high sensitivity is dependent on the method of sample collection.

All IC kit-positive samples were isolation positive. Two enterovirus-infected samples were negative by the IC kit and other HAdV diagnoses; the specificity of the IC kit was 100%, as reported previously (6, 20).

In this study, we were able to identify many HAdV-infected patients within a short period of time because a large outbreak of HAdV occurred in Japan in 2003 (13). This outbreak was the largest of its kind in the previous 10 years. The main causative agent was HAdV 3, which accounted for 69% (84 of 122) of the patients diagnosed by NT. According to previous reports, the IC kit is faster than the enzyme-linked immunosorbent assay kit (which takes at least 40 min) and is more useful for detection of respiratory HAdVs (21). Therefore, the IC kit is the only practical bedside diagnostic tool for respiratory HAdV infections.

The IC kit was useful for detecting HAdV in samples coinfected with HAdV and enterovirus. There was no clinical difference between coinfected patients and other HAdV-infected patients. Therefore, HAdV seemed to be the main causative agent of infection in these patients. Coinfection of HAdV3 and HAdV5 could be identified by PCR-based sequencing (17) and use of the HAdV 3- and 7-specific primers designed in this study.

We found that when the SYBR green real-time PCR method was used with isolated HAdV strains, the detection limit of the IC kit was $10^{4.6}$ to $10^{5.8}$ virus genome copies/ml for HAdV 1, 2, 3, 5, 6, and 7. However, there were clinical samples from which we isolated HAdV 3 (four samples) and HAdV 1 (two samples) that were negative by the IC kit despite concen-

trations of 9.0×10^7 to 2.5×10^9 virus genome copies/ml for HAdV 3 and 3.8×10^7 to 4.5×10^8 virus genome copies/ml for HAdV 1. These clinical samples had virus concentrations within the sensitivity limits of the IC kit; the detection limits for HAdV 3 and HAdV 1 were $10^{5.8}$ and $10^{4.6}$, respectively. According to Hierholzer (9), the bulk of newly synthesized HAdV product is not continuously released but remains cell associated. Only the products that are released into the extraction solution contribute to a positive result by the IC kit. This may explain the 5% false-negative rate.

The IC kit used in this study utilizes a monoclonal antibody made with the HAdV 2 hexon protein. Uchio et al. (21) reported that the IC kit had a lower sensitivity to HAdV 3 and 7 than to HAdV 4, when using serotypes 3, 4, 7, 8, 11, and 37, although these researchers used conjunctival swabs as samples. They reported that the minimum amounts of HAdV 3 and HAdV 8 detected by the IC kit were 4×10^3 and 4×10^4 PFU, respectively. Shimizu et al. (16) reported that the detection limit of an IC kit was $10^{4.45}$ (2.8×10^4) $TCID_{50}$/ml for HAdV 3 and that the sensitivities of other IC kits were similar. In our results, the IC kit was sensitive to $10^{1.9}$ to $10^{4.0}$ $TCID_{50}$/ml for HAdV 1, 2, 3, 5, and 7. Detection limits for the six serotypes were 2.9 ± 1.0 log $TCID_{50}$/ml and 5.4 ± 0.4 log copies of HAdV genome/ml (mean ± SD). The coefficients of variation were 0.34 and 0.08, respectively. Based on the real-time PCR results, the detection limit of the IC kit was approximately $10^{5.4}$ copies of HAdV genome/ml. In repeated IC kit tests, the high reproducibility of the IC kit was confirmed.

In this study period, no clinical samples containing HAdV 7 were obtained. HAdV 7 is known to cause fatal pneumonic infections in children, including nosocomial infections. We confirmed that the detection limits for HAdV 7 were $10^{5.6}$ copies of the HAdV 7 genome/ml and $10^{1.6}$ $TCID_{50}$ of HAdV 7/ml. The sensitivity of the IC kit was not lower for HAdV 7 than for the other serotypes in this study. According to Uchio et al. (21), the detection rate of the IC kit is 31% (8 of 26 patients) for HAdV 3 and 60% (3 of 5 patients) for HAdV 7 compared to that of PCR. We were able to detect the presence of HAdV 3 in respiratory samples with the IC kit 93% of the time (in 80 of 86 samples) in comparison to PCR.

The five samples that were IC kit and isolation negative but multiplex PCR positive contained 3.0×10^4 to 3.8×10^5 copies of the HAdV genome/ml. As isolation requires viable virus, PCR was more sensitive than isolation. It is known that many HAdV particles are not infectious, perhaps because they have genomic defects or lack fiber or some other protein (19).

In conclusion, the IC kit is a useful method for diagnosing HAdV diseases at the bedside because it has 95% sensitivity relative to that of isolation. To prevent nosocomial infections, rapid diagnosis at the bedside is necessary. Furthermore, the IC kit provides pediatricians with information about the prognosis of the respiratory diseases at the patient's first visit. However, because the IC kit test had a 5% false-negative rate compared to isolation, careful interpretation is required in cases where IC kit-negative results are obtained and HAdV infection is suspected.

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Poly(A)- and Primer-Independent RNA Polymerase of *Norovirus*

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Replication of positive-strand caliciviruses is mediated by a virus-encoded RNA-dependent RNA polymerase (RdRp). To study the replication of *Norovirus* (NV), a member of the family *Caliciviridae*, we used a recombinant baculovirus system to express an enzymatically active RdRp protein from the 3D region of the NV genome and defined conditions for optimum enzymatic activity. Using an RNA template from the NV 3′ genomic region, we observed similar levels of enzymatic activity in assays with and without a poly(A) tail. RdRp activity was not significantly affected by the addition of an RNA primer to the reaction mixture. Thus, the NV RdRp exhibited primer- and poly(A)-independent RNA polymerase activity. While the RdRp inhibitor phosphonoacetic acid inhibited NV RdRp activity, another gliotoxin did not. The active recombinant NV RdRp will be of benefit to studies of NV replication and will facilitate the development of specific inhibitors of NV proliferation.

Norovirus (NV), a member of the family *Caliciviridae*, is the leading cause of epidemic acute, nonbacterial gastroenteritis. NV infection causes nausea, vomiting, low-grade fever, and diarrhea, which can be severe in infants and young children. For these reasons, it is a major public health concern (7). An effective vaccine or other therapeutic agent would be valuable for preventing the significant morbidity and potential mortality associated with NV infections. However, the lack of an efficient culture system has hampered the biochemical characterization of the NV proteins, and thus far, molecular biological techniques have been the most useful tools for the study of NV.

The NV virion contains a polyadenylated plus-strand RNA genome of ~7.7 kb (11, 15). The structures of the full-length genome, phylogenetic trees, and genetic recombination among distinct genogroups have been analyzed in detail (13). Based on sequence similarities with other single-stranded RNA viruses, the NV open reading frame 1 (ORF1) is predicted to encode a large polyprotein that is cleaved into several viral proteins, including NTPase, proteinase, and RNA-dependent RNA polymerase (RdRp) (11, 15).

The RdRp encoded by the 3D region has a conserved amino acid motif, glycine-aspartic acid-aspartic acid (GDD), which is found in the active site of many viral RdRps (14), and thus might have an important role in NV replication. As in other positive-strand RNA viruses (2), NV genomic RNA likely acts as a template for the synthesis of minus-strand RNA. The minus-strand RNA then, in turn, serves as a template for the synthesis of progeny genomic plus-strand RNA molecules. Thus, RdRp is central to the synthesis of both plus- and minus-strand RNA molecules. Among the *Caliciviridae*, *Rabbit hemorrhagic disease virus* (RHDV) and *Feline calicivirus* (FCV) express an enzymatically active RdRp protein (27, 28). However, caliciviruses that infect humans have not been examined for RdRp activity.

The aims of this study were to develop a cell-free system that permits the identification of RdRp activity in vitro and to characterize the biochemical properties of RdRp in NV replication. We expressed the RdRp protein from the 3D region of the NV genome with a baculovirus vector and then tested the activity of the purified protein.

MATERIALS AND METHODS

Construction of plasmids. The primer sequences used for plasmid construction are listed in Table 1.

To obtain a cDNA fragment corresponding to the full-length genome of the NV genogroup 2 strain U201, we used primers U201LV1S-BglII and TX30SXN to amplify viral RNA extracted from a stool specimen (U201) (13). RNA extraction, reverse transcription, and PCR were performed as previously described (13). The 3D region was amplified from the full-length cDNA of strain U201 with the primers EagI-3D and 3D-EcoRI, digested with EagI and EcoRI, and cloned into the EagI-EcoRI site of the pVL1392 vector (BD Bioscience, San Jose, Calif.) to obtain pVLwt3D (Fig. 1).

The plasmid pVLwt3D was used as a template for mutagenesis, and DNA oligonucleotide primers 3D-GAA-F, 3D-GAA-R, 3D-GAD-F, and 3D-GAD-R were used to introduce amino acid substitutions at the conserved GDD motif. To obtain a mutant RdRp containing an amino acid change of the GDD motif to GAA, we amplified the 5′ and 3′ halves of the mutant 3D region by using primer set 3D-GAA-R and EagI-3D or 3D-GAA-F and 3D-EcoRI, respectively. Since the resulting two cDNA fragments partially overlapped each other, they were mixed, denatured, annealed, and filled in with Tbr EXT DNA polymerase (Fynzyme, Espoo, Finland) to obtain the full-length construct with a mutant 3D region. The resulting products were digested with EagI and EcoRI and ligated into the pVL1392 vector to form the plasmid pVLm3D(GAA) (Fig. 1).

We amplified two additional cDNA fragments. To obtain another mutant RdRp that changes the GDD motif to GAD, we amplified the 5′ or 3′ half of the mutant 3D region by using primer set 3D-GAD-R and EagI-3D or 3D-GAD-F and 3D-EcoRI with the same strategy as that used for the construction of plasmid pVLm3D(GAA). To obtain the full-length 3D construct with a GAD mutant, the resulting two cDNA fragments were mixed, denatured, annealed, and filled in with Tbr EXT DNA polymerase. The resulting products were digested with EagI and EcoRI and ligated into the pVL1392 vector to form the plasmid pVLm3D(GAD) (Fig. 1). Finally, the 3B region of the NV genome was

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TABLE 1. Sequences of DNA primers used for plasmid construction

Primer	Polarity	Sequence[a] (5'→3')
U201LV1S-BglII	+	GAagatctTCGTGAATGAAGATGGCGTCTAACGAC
TX30SXN	−	GACTAGTTCTAGATCGCGAGCGGCCGCCCTTTTTTTTTTTTTTTTTTTTTTTTTTT
EagI-3D	+	TTTcggccgATGGGAGGTGACGACAAGGGCACCTAT
3D-EcoRI	−	AAAgaattcTTATTATTCGACGCCATCTTCATTCACAAA
3D-GAA-F	+	TTCTATGGTG CTGCTGAGATTGTA
3D-GAA-R	−	CTCA GCAGCACCATAGAAAGAGTA
3D-GAD-F	+	TTCTATGGTG CTGATGAGATTGTA
3D-GAD-R	−	CTCATCA GCACCATAGAAAGAGTA
BamH-3B	+	CGCggatccATGGGAAAGAAAGGCAAGAACAAGTCC
3B-Xba	−	TGCtctagaTTATTATTCAAAATCTATCCTCTCATTGTA
KpnT7ORF3	+	CGGggtaccTAATACGACTCACTATAGGGAAGGATTCAATAATGGCTGGAG
201pAMS	−	TACGGCATGCacgcgtTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAAAAGATTCTAAATCAAATT
T7S608	+	TTATAATACGACTCACTATAGGACAGCTTTTGTCACTCCACCATC
201ASdelA-Xba	−	GtctagaAAAGATTCTAAATCAAATTTAGG

[a] Restriction sites used for cloning are represented by lowercase letters. The underlined nucleotides were changed to introduce amino acid substitutions at the conserved GDD motif.

amplified, using primers BamH-3B and 3B-Xba. The resulting cDNA was digested with BamHI and XbaI and cloned into the BamHI-XbaI site of the pVL1393 vector (BD Bioscience) to obtain pVL3B (Fig. 1).

A series of plasmids (i.e., pMT-ORF3pA, pUC608-pA, and pUC608delA) was used for in vitro transcription to prepare the RNA templates or RNA size markers for the analysis of RdRp products. These were constructed by PCR with the oligonucleotide primers described below. For the plasmid pMT-ORF3pA, primers KpnT7ORF3 and 201pAMS were used with the full-length cDNA template of U201 to amplify a cDNA fragment from ORF3 to the poly(A) region under the T7 promoter sequence. The resulting product was digested with KpnI and MluI and then cloned into the KpnI and MluI sites of the pMT1 vector (kindly provided by M. Tatsumi, National Institute of Infectious Diseases, Tokyo, Japan). Primers T7S608, which contained the T7 promoter, and 201pAMS were used with template plasmid pMT-ORF3pA to amplify a cDNA fragment corresponding to the 3'-terminal 232 nucleotides of the NV genome. The amplified product was ligated to the pT7Blue vector (Takara Bio, Shiga, Japan) to obtain pT7608-pA. The HindIII-EcoRI fragment of the cDNA was inserted into the HindIII-EcoRI site of the pUC119 vector and the plasmid was designated pUC608-pA. Primers T7S608 and 201AsdelA-Xba were used with template plasmid pMT-ORF3pA to amplify a cDNA fragment that corresponded to plasmid pUC608-pA except for a deletion of the poly(A) sequence. The PCR product was inserted into the pT7Blue vector to obtain pT7608delA. By the same strategy, the HindIII-EcoRI fragment of the cDNA was cloned into the pUC119 vector to obtain pUC608delA.

Preparation of RNA. The oligo$(U)_{15}$ RNA primer was purchased from Takara Bio. The RNA dimer UpU was purchased from Sigma (Tokyo, Japan). ORF3-pA, 608-pA, and 608-delA RNAs were transcribed in vitro by T7 RNA polymerase (Promega, Tokyo, Japan) from the templates MluI-digested pMT-ORF3pA, MluI-digested pUC608-pA, and XbaI-digested pUC608delA, respectively. The RNAs were purified, and their integrity was verified as described previously (6).

Expression and purification of RdRp protein. The procedures for generating recombinant baculoviruses and for subsequent large-scale production of the proteins were adapted from the work of Li et al. (16). Briefly, transfer plasmid pVLwt3D, pVLm3D (GAA), pVLm3D (GAD), or pVL3B was cotransfected into insect SF9 cells with linearized BaculoGold DNA (BD Bioscience) to obtain recombinant baculovirus BVwt3D, BVm3D(GAA), BVm3D(GAD), or BV3B, respectively. Insect Tn5 cells were infected with these recombinant baculoviruses for the large-scale expression of recombinant RdRp proteins. After 4 days of incubation at 27°C, cells were harvested, collected by centrifugation, and washed twice with phosphate-buffered saline.

The cell pellet was resuspended in ice-cold cell lysis buffer (20 mM Tris-HCl [pH 7.5], 1.0 mM EDTA, 10 mM dithiothreitol [DTT], 2% Triton X-100, 500 mM NaCl, 10 mM $MgCl_2$, 50% glycerol, and EDTA-free protease inhibitors)

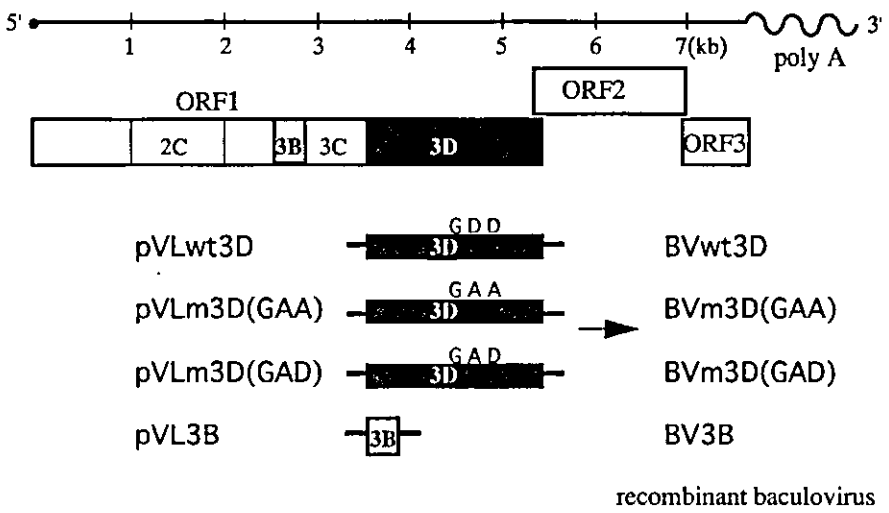

FIG. 1. Expression constructs for RdRp proteins. The genome structure of NV is shown to scale. The 3D region with the wild-type GDD motif (wt3D) and with amino acid substitutions of GDD to GAA [m3D(GAA)] or GAD [m3D(GAD)] and the 3B region of the NV cDNA were inserted into baculovirus transfer vectors to construct recombinant baculoviruses.

(Roche Diagnostics, Tokyo, Japan). The extract was sonicated, cleared of debris by centrifugation at 15,000 × *g* for 30 min, dialyzed against SP buffer (25 mM morpholineethanesulfonic acid [pH 6.4], 1.0 mM EDTA, and 1% Triton X-100), and applied to a HiTrap SP column (Amersham Biosciences) that was pre-equilibrated with SP buffer. Bound proteins were eluted by a linear gradient of 0 to 1.0 M NaCl in SP buffer and were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Coomassie blue staining. The RdRp protein was eluted with 320 to 550 mM NaCl, dialyzed against RdRp sample buffer (20 mM Tris-HCl [pH 7.7], 1.0 mM EDTA, 100 mM NaCl, 10 mM DTT, 2% Triton X-100, and 50% glycerol), and tested for RdRp activity. The fractions containing active RdRp protein were combined, dialyzed against Q buffer (20 mM Tris-HCl [pH 7.7], 1.0 mM EDTA, and 1% Triton X-100), and applied to a HiTrap Q column (Amersham Biosciences) that was pre-equilibrated with Q buffer. Bound proteins were eluted with a linear gradient of 0 to 1.0 M NaCl in Q buffer and were analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie blue staining. RdRp fractions eluted at ~520 mM NaCl. They were combined, dialyzed against RdRp sample buffer, and examined for RdRp activity.

RdRp and terminal nucleotidyl transferase (TNTase) assays. The RdRp reaction was performed in a 15-μl volume with 375 ng of RdRp protein and 5.0 pmol of in vitro-transcribed ORF3-pA RNA, 608-polyA RNA, or 608-delA RNA in a reaction buffer containing, unless otherwise specified, 20 mM Tris-HCl (pH 6.8), 2.0 mM $MnCl_2$, 100 mM NaCl, 20 mM DTT, 20 U of RNase inhibitor (Promega), 50 μg of actinomycin D/ml, 250 μM GTP, 125 μM ATP, 125 μM CTP, 5.0 μM UTP, and 4.0 μCi of [^{33}P]UTP (>2,500 Ci/mmol) (Amersham Biosciences).

Nuclease digestion was performed as described by Ishii et al. (10). TNTase assays were performed in the same buffer with specified nucleoside triphosphates. RdRp and TNTase reactions were done at 30°C for 90 min and stopped by the addition of 60 μl of a stop solution (10 mM Tris-HCl [pH 7.5], 10 mM EDTA, 100 mM NaCl). The RNA products were extracted with TRISOL LS reagent (Invitrogen, Tokyo, Japan) and precipitated with isopropanol. Products were dissolved with RNA sample buffer containing 80% formamide, 1 mM EDTA, and 0.1% bromophenol blue. After heat denaturation, the RNA products were separated in 6.0 or 10.0% polyacrylamide gels in 8.0 M urea. Radiolabeled RNA products were analyzed with the BAS 1000 system (Fuji Film, Tokyo, Japan).

For the examination of polymerase inhibitors, phosphonoacetic acid (PAA) and gliotoxin were purchased from Sigma. These materials were dissolved in H_2O, and then increasing amounts (25, 100, and 250 μM) of the inhibitors were mixed with the RdRp reaction buffer.

RESULTS

Expression and purification of enzymatically active RdRp proteins. In FCV, a member of the family *Caliciviridae*, the proteinase-polymerase precursor (3CD) rather than the 3D region itself is highly active, which may be attributed to inefficient proteolytic processing of the linker domain of 3CD (25, 28). Since efficient proteolytic processing of the NV polyprotein into 3D is observed in both *Escherichia coli* and mammalian cell expression systems (17, 24), we cloned and expressed the 3D region itself to study the biochemical properties of NV RdRp (Fig. 1). A recombinant baculovirus system was used to express NV RdRp. Soluble lysates of infected cells were separated by cation-exchange chromatography (Fig. 2A), and fractions were examined for RdRp activity.

Several investigators have shown that viral RdRps have primer-dependent RNA polymerase activities with homopolymeric RNA templates (5, 10, 28). To clarify the molecular mechanism of NV replication, it is more important to test the activity with the viral RNA template than with the homopolymeric RNA template. In this study, we examined the NV RdRp activity with synthetic RNA templates that corresponded to the 3′-terminal region of the NV genome. We believe that our strategy for measuring NV RdRp activity better replicates the production of minus-strand RNA from plus-strand genomic RNA.

The template ORF3-pA RNA (Fig. 2B) was incubated with RdRp fractions in the presence of [^{33}P]UTP and cold ribonucleotide triphosphates. Radiolabeled reaction products of RdRp assays were analyzed in denaturing polyacrylamide gels. [^{33}P]UTP was incorporated in fractions from BVwt3D-infected cell lysates that eluted at 320 to 550 mM NaCl (Fig. 2C).

We next examined whether the nucleotide length of the RNA product obtained by the RdRp assay was the same as that obtained with the template ORF3-pA RNA. As a size marker, we prepared T7 RNA polymerase-labeled RNA transcribed from MluI-digested plasmid pMT-ORF3pA (Fig. 2C, lane 1). The template RNA for the RdRp assays was also transcribed from the same starting material by T7 RNA polymerase (see Materials and Methods). The lengths of the RNA products from the RdRp assays were identical to that of the template, indicating that the RNA products of the RdRp reaction were transcripts of the template RNA. On the other hand, incorporation was at background levels in the lysates of negative control cells infected with BVm3D(GAA) and BV3Dm(GAD), which carried amino acid substitutions at a GDD motif (Fig. 2C).

To analyze the activity of the wild-type RdRp protein in detail, we collected the active RdRp fractions from cation-exchange chromatography and subsequent anion-exchange chromatography steps (Fig. 2A). Purified RdRp protein was used for further analyses.

Characterization of RdRp activity. We next examined the effects of pH and divalent cation concentrations, including $MgCl_2$, $MnCl_2$, and $CaCl_2$, on NV RdRp activity. ORF3-pA RNA synthesized in vitro was used as a template. Since the requirements of divalent cations are different for primer-dependent and primer-independent synthesis (21), the oligonucleotide primer was not used in the reaction. RdRp activity was markedly increased in the presence of 2.0 mM Mn^{2+}. A slight increase was detected with increased concentrations of Mg^{2+}, although to a much lesser extent than with Mn^{2+}. No increase was seen with Ca^{2+} (Fig. 3A). The RdRp activity was found to be optimal at pHs 6.8 to 7.5 (data not shown). Based on these results, we used pH 6.8 and 2.0 mM Mn^{2+} for further studies of NV RdRp activity.

We also examined the possibility that the synthesis was due to a DNA-dependent RNA polymerase in the extracts of insect Tn5 cells. To do this, we added actinomycin D to the reaction mixtures. Actinomycin D forms a complex with DNA and interferes with RNA synthesis. The production of labeled RNA with RdRp assays was not inhibited in the presence of various amounts of the inhibitor (Fig. 3B), indicating that the labeled RNA had not originated from the action of a DNA-dependent RNA polymerase.

Characterization of RdRp products. Hepatitis C virus (HCV) RdRp expressed in recombinant baculovirus systems shows TNTase activity (20). TNTase activity might confound the interpretation of the actual properties of RdRp. Since TNTase labels the 3′ ends of RNA, the RNA product in our RdRp assays could result from the addition of [^{33}P]UTP to the 3′ end of the template RNA by TNTase. To examine this possibility, we performed TNTase assays without cold ATP, CTP, and GTP. The template 608-pA RNA contained the NV

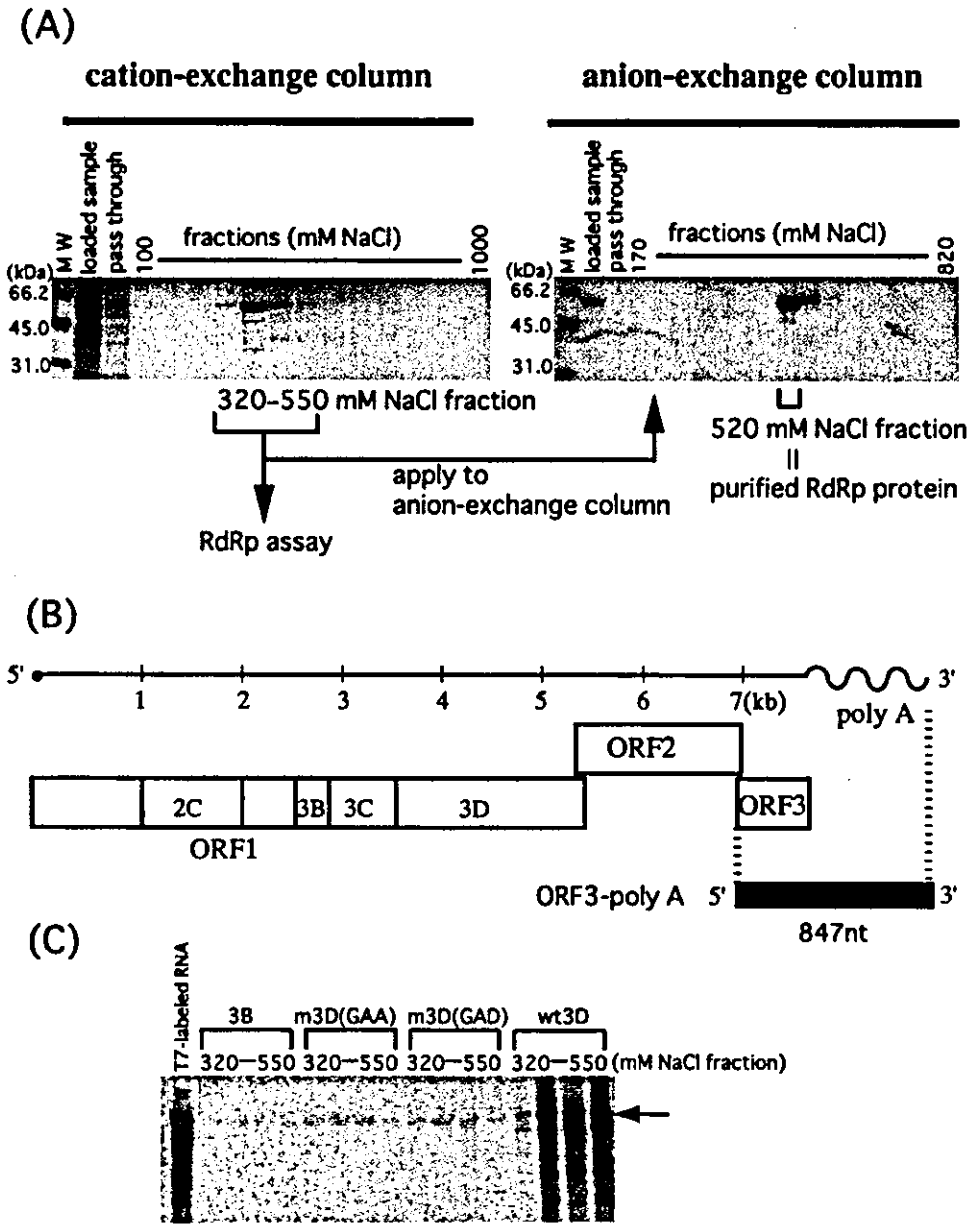

FIG. 2. Purification and enzymatic activity of NV RdRp. (A) Eluted proteins from a HiTrap SP (cation exchange) column (left) and a HiTrap Q (anion exchange) column (right) were separated by SDS-polyacrylamide gel electrophoresis and visualized by Coomassie blue staining. The portions of fractions containing RdRp protein eluted by 320 to 550 mM NaCl in SP buffer from the cation-exchange column were used to examine enzymatic activity. The fractions with active RdRp from the cation-exchange column were then subjected to further purification in the anion-exchange column. (B) Genome structure of NV shown to scale. The RNA template used for RdRp assays is represented by a black bar. (C) RdRp reaction with the fractions from a cation-exchange column. At the same time, m3D(GAA) and m3D(GAD), which had substitutions in the GDD motif to GAA and GAD, respectively, and 3B protein as a negative control were separated in cation-exchange columns, and the eluted fractions were subjected to an RdRp assay using the same strategy as that used for the wild-type protein (wt3D). RNA products were separated in a denaturing polyacrylamide gel, analyzed by the BAS1000 system, and presented together with [^{33}P]-incorporated molecular size markers transcribed by T7 RNA polymerase from MluI-digested pMT-ORF3pA (T7-labeled RNA).

3'-terminal region and 30 nucleotides of poly(A) sequence (Fig. 4). [^{33}P]-labeled RNA products were not detected in the TNTase assays in the presence of [^{33}P]UTP only or [^{33}P]UTP plus cold UTP, without any other NTPs (Fig. 4, lanes 2 and 3). The RdRp protein did not display any activity that added UTP to the 3' end of the template RNA. Therefore, the labeled RNA product was not the result of TNTase activity.

We next determined whether the products synthesized from the NV RNA template were single or double stranded. Double-stranded RNA would indicate that cRNA is synthesized

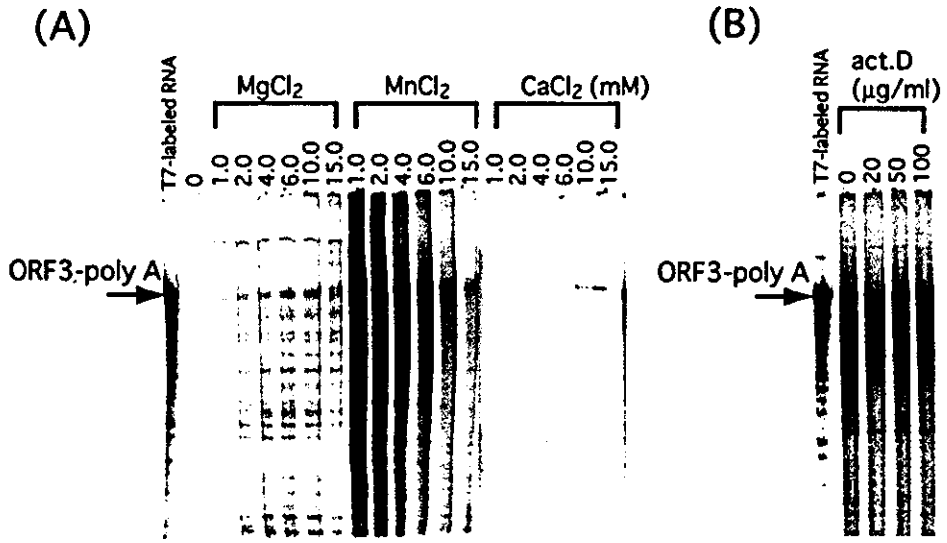

FIG. 3. Optimization of RdRp assay conditions and effects of actinomycin D on enzymatic activity. The RNA template used for RdRp assays is shown in Fig. 2B. (A) Divalent cation optimization. (B) Effects of increasing amounts of actinomycin D on enzymatic activity. RNA products were analyzed using methods similar to those described for Fig. 2.

from the template RNA by RdRp. Since S1 nuclease catalyzes the specific degradation of single-stranded RNA to mononucleotides, the RdRp product was incubated with S1 nuclease. The 608-pA RNA containing the 3′-terminal region of the NV genome and the poly(A) sequence was used as a template. The RNA product was resistant to S1 nuclease digestion (Fig. 4, lanes 5 and 6). However, S1 nuclease treatment after heat denaturation almost completely degraded the RNA (lane 7). This result indicated that the product formed by the RdRp reaction was double-stranded RNA.

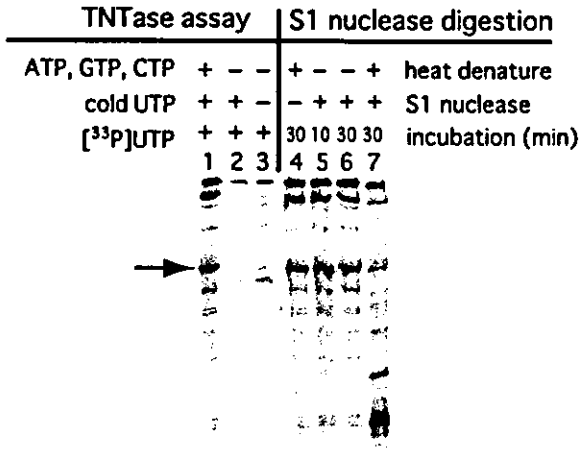

FIG. 4. TNTase assay and S1 nuclease digestion. An in vitro-transcribed RNA, 606-polyA, corresponding to the 3′-terminal 232 nucleotides of the NV genome, was used as a template for the RdRp reaction. For TNTase assays, reactions were performed without ATP, GTP, and CTP (lane 2) or without ATP, GTP, CTP, and cold UTP (lane 3). An RNA product from a standard reaction mixture is shown (lane 1) as a control. For the S1 nuclease digestion analyses, RdRp products were heat denatured at 95°C for 2 min (lanes 4 and 7) before treatment without (lane 4) or with (lanes 5, 6, and 7) S1 nuclease for the indicated times. The 232-nucleotide band, identified by an arrow, is missing from lanes 2, 3, and 7. RNA products were analyzed by methods similar to those described for Fig. 2.

Poly(A)- and primer-independent RdRp activity. The results described above indicated that NV RdRp was able to synthesize cRNA without the primer at the 3′ region of the NV genome. Since the NV genome contains a poly(A) sequence at the 3′ end (11, 15), we investigated whether the poly(A) sequence is required for the initiation of RNA synthesis by NV RdRp. RdRp activity was measured by using NV 3′ genomic RNA with or without a poly(A) tail. The 608-pA template RNA contained the 3′-terminal region of the NV genome and 30 nucleotides of poly(A) sequence; 608-delA RNA contained the same NV sequence but not the poly(A) sequence (Fig. 5A). [^{33}P]UTP incorporation was similar in both reactions. Furthermore, the labeled RNA size markers driven by T7 RNA polymerase confirmed that the RdRp product was the same size as the template RNA, irrespective of the presence of the poly(A) tail on the template (Fig. 5B). The results strongly indicated that the 3′ poly(A) sequence is not necessary for the initiation of synthesis or for synthesis to the exact length of the cRNA.

We next examined the influence on RdRp activity of the addition of an oligo(U)$_{15}$ primer or UpU dimer primer to the template RNA containing the poly(A) tail (Fig. 5C). When the oligo(U)$_{15}$ primer was added to the reaction, the amounts of RdRp product increased slightly with increasing amounts of primer. Also, a distinct RNA product was observed which migrated faster than the templates. The reason for the presence of this smaller RNA is not known. The presence of the oligo(U)$_{15}$ primer made little difference on the synthesis of the major product, which corresponded to cRNA of the template RNA (Fig. 5C, lanes 2 to 4). Interestingly, there was no effect on RNA production with the reaction containing the dinucleotide primer UpU (Fig. 5C, lanes 5 to 7). From these results, we concluded that NV RdRp could synthesize RNA comple-

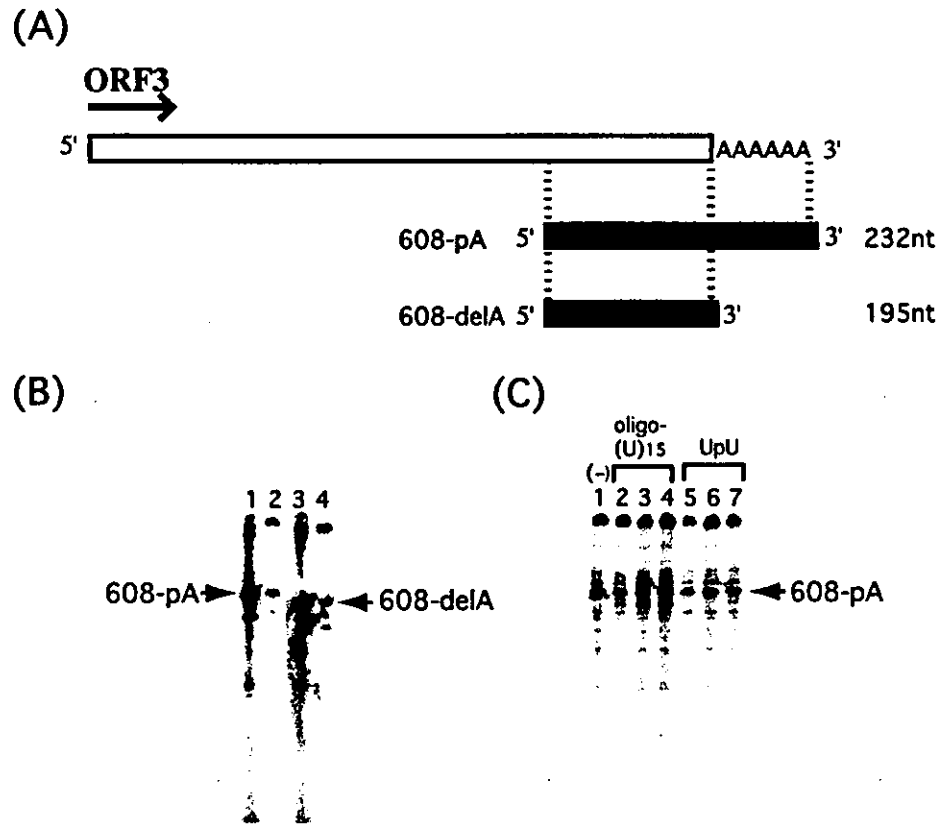

FIG. 5. RdRp activity was not dependent on RNA primer and poly(A) sequence. (A) Genome structure downstream of NV ORF3 with poly(A). The RNA templates used for RdRp assays are represented by black bars. (B) The RdRp products from reactions with template RNA 608-pA (lane 2) and 608-delA (lane 4), together with labeled RNA from a T7 polymerase reaction of 608-pA (lane 1) and 608-delA (lane 3), are represented. (C) Effect of RNA primer on RdRp activity. RdRp reactions were performed with 608-pA RNA as a template, with 1.0 pmol (lane 2), 5.0 pmol (lane 3), or 20.0 pmol (lane 4) of oligo$(U)_{15}$; 10.0 pmol (lane 5), 1.0 nmol (lane 6), or 10.0 nmol (lane 7) of UpU dinucleotide primer; or no primer (lane 1). RNA products were analyzed by methods similar to those described for Fig. 2.

mentary to the NV genomic RNA in a primer- and poly(A)-independent manner.

Effect of RdRp inhibitors. The assay system described above allowed us to screen for potential NV RdRp inhibitors. Various concentrations of gliotoxin or PAA, which inhibits the activities of other viral RdRp proteins, were added to the NV RdRp reaction. PAA inhibited the RdRp activity (Fig. 6). The estimated 50% inhibitory concentration of PAA for NV RdRp activity was <20 μM. No inhibitory effect was observed with gliotoxin, even at concentrations as high as 250 μM.

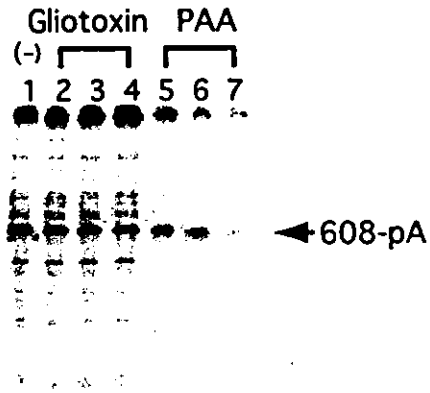

FIG. 6. Effects of RdRp inhibitors. RdRp reactions were performed with 608-pA RNA as a template, with 25 μM (lane 2), 100 μM (lane 3), or 250 μM (lane 4) gliotoxin; 25 μM (lane 5), 100 μM (lane 6), or 250 μM (lane 7) PAA; or no inhibitors. The 232-nucleotide band, identified by an arrow, disappeared gradually upon PAA treatment. RNA products were analyzed by methods similar to those described for Fig. 2.

DISCUSSION

Previous studies of viral homology have indicated that the C terminus of NV ORF1 contains a motif in the RdRp that is conserved among various plus-strand RNA viruses. In this study, we report that the 3D region of the NV genome contains an active RdRp. We expressed a soluble recombinant RdRp encoded by the NV 3D genomic region in insect cells without a tagged sequence but with a single additional methionine residue at the N terminus. We purified the protein and established a system for measuring its RNA polymerase activity. That activity was not due to contamination by an endogenous

insect polymerase, and actinomycin D did not inhibit the RdRp reaction, also indicating a lack of endogenous RdRp.

We focused on the conserved GDD motif in the 3D region. Found in most RdRps of positive-strand RNA viruses, GDD is important for metal binding and is considered to be the catalytic site of the enzyme (14). When we replaced the GDD motif in RdRp, the RNA polymerase activity was lost, and the expressed 3B protein of NV also lacked activity. The loss of RdRp activity in these mutant RdRp proteins indicated that labeled RNA products obtained by a reaction with the wild-type RdRp protein were driven by an RNA polymerase-related activity and maybe by an intrinsic activity of the expressed NV RdRp protein. The enzymatic activity of NV RdRp depended on Mn^{2+}: the optimal Mn^{2+} concentration was 2.0 mM. This requirement for divalent cations is similar to that of the primer-independent de novo RNA polymerase activity of poliovirus (1), HCV (18, 21), and human rhinovirus 16 (9). In the presence of Mn^{2+} ions, RdRp from RHDV is thought to form an active structure (19). Our observations suggest that Mn^{2+} ions promote conformational changes in NV RdRp that are similar to those of other positive-strand RNA viruses, such as picornaviruses, HCV, and RHDV. We believe that the enzymatic activity in the presence of Mn^{2+} may reflect a primary biological function of RdRp, and we used Mn^{2+} for all of our RdRp characterizations.

Several investigators have shown that in vitro viral RdRp reactions generate cRNA by a "copy-back" mechanism (3, 26). These reactions produce dimer-sized RNAs, presumably as a result of a self-priming event at the extreme 3′ terminus of the template. In our study, dimer-sized RNAs were not seen when the RdRp products were separated in denatured gels. We speculate that NV RdRp does not have a copy-back mechanism for cRNA synthesis. This notion is supported by the absence of self-priming by an additional oligo(U) at the 3′ end of the template RNA mediated by TNTase activity.

Interestingly, our results indicate that NV RdRp may not recognize poly(A). RNA production was as efficient without a poly(A) tail at the 3′-terminal region of the template RNA as it was with the tail. Furthermore, priming by synthetic oligo(U) or UpU had little effect on the RdRp activity. RdRp was able to synthesize cRNA without a poly(A) tail. Therefore, as reported for HCV RdRp (12), there must be a specific sequence requirement for *cis*-acting signals for RNA synthesis when NV genomic RNA is used. Additional studies are needed to determine the specific sequence.

Our results revealed that NV RdRp has the activity of de novo RNA synthesis with the NV genomic RNA as a template. Like those of other RNA viruses (1, 12, 18), NV RdRp directs minus-strand RNA synthesis in a primer-independent manner. We also showed an interesting feature of NV, namely that the activity of minus-strand synthesis is independent of a poly(A) tail. The NV genome encodes a VPg-like protein on the genome (11, 15). In picornaviruses, the VPg protein is thought to link to the 5′ end of the genome and serve as a primer for RdRp (23). In the *Caliciviridae*, a genome-linked VPg protein has also been reported for FCV (8). Therefore, it is important to determine whether the VPg protein is linked to the NV genome and has a role in cRNA synthesis.

Finally, our in vitro RdRp assay may be useful in further studies to develop drugs to treat NV, and the NV RdRp may itself be a useful target for antiviral drugs. It was strongly inhibited by PAA, which interferes with the replication of DNA viruses (4). The 50% inhibitory concentration (<20 μM) for NV RdRp was significantly lower than that reported for HCV RdRp (10), indicating a specific inhibition of NV RdRp activity by PAA. In contrast, gliotoxin, a known inhibitor of poliovirus and HCV RdRps (5, 22), had little effect on NV RdRp activity, despite the high concentrations tested. The differences in sensitivities may be attributed to the specific nature of NV RdRp, which synthesizes minus-strand RNA in a primer- and poly(A)-independent manner.

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