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Nondefective Rotavirus Mutants with an NSP1 Gene Which Has a Deletion of 500 Nucleotides, Including a Cysteine-Rich Zinc Finger Motif-Encoding Region (Nucleotides 156 to 248), or Which Has a Nonsense Codon at Nucleotides 153 to 155

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We isolated two nondefective bovine rotavirus mutants (A5-10 and A5-16 clones) which have nonsense mutations in the early portion of the open reading frame of the NSP1 gene. In the NSP1 gene (1,587 bases long) of A5-10, a nonsense codon is present at nucleotides 153 to 155 just upstream of the coding region (nucleotides 156 to 230) of a cysteine-rich Zn finger motif. A5-16 gene 5 (1,087 bases long) was found to have a large deletion of 500 bases corresponding to nucleotides 142 to 641 of a parent A5-10 NSP1 gene and to have a nonsense codon at nucleotides 183 to 185, which resulted from the deletion. Expression of gene 5-specific NSP1 could not be detected in MA-104 cells infected with the A5-10 or A5-16 clone or in an in vitro translation system using the plasmids with gene 5 cDNA from A5-10 or A5-16. Nevertheless, both A5-10 and A5-16 replicated well in cultured cells, although the plaque size of A5-16 was extremely small.

The rotavirus genome is composed of 11 double-stranded RNA (dsRNA) segments. Since polyacrylamide gel electrophoresis (PAGE) readily enables identification of rotavirus strains, the rotavirus RNA profiles of a large number of strains collected worldwide have been well documented (5, 6, 14). Those studies point to an extensive genome diversity of the rotavirus. This diversity is thought to result from nucleotide substitutions in each RNA segment, genomic reassortment between different strains, and genome rearrangement (5, 14, 25). In particular, the phenomenon of rearrangement causes marked changes in the mobility of the RNA segment in PAGE. Genome rearrangement has been found in the rotaviruses isolated from both immunocompromised and immunocompetent children, from cultured cells infected at a high multiplicity of infection, and from animals such as calves, pigs, and rabbits (1, 8, 11–13, 15, 17, 24, 29). The rearrangement has been observed mostly in the RNA segments encoding nonstructural proteins NSP1 to NSP5. Since the function of nonstructural proteins has not been well elucidated, the analysis of their rearranged RNA segments would be useful for understanding their function and the mode of regulation in rotavirus RNA replication. In this study, we characterized mutants of bovine rotavirus with unusual structures of the NSP1 gene (RNA segment 5 or gene 5).

We have studied the antigenic and genomic properties of human and bovine rotaviruses in Thailand for several years (20–22, 26–28). During these studies, we isolated a bovine strain, A5, which has subgroup I, G serotype 8 (69M-like VP7), and P serotype 6 (NCDV-like VP4) specificities (26, 27). The RNA profile of the strain A5 showed a long migration pattern. However, it was of note that two RNA segments 5 having different mobilities were present in RNA preparations of unpurified strain A5 (Fig. 1). In order to separate A5 clones with distinct RNA segments 5, 48 plaques were picked in CV-1 cells and their RNA profiles were compared. From A5 virus stock, we obtained three clones (A5-10, A5-13, and A5-16) whose RNA segments 5 showed different migrations (Fig. 1). Each A5 clone was finally purified by at least six cycles of plaque-to-plaque purification. The versions of segment 5 in A5-10 and A5-13 match those of the fast- and slowly migrating ones in the original A5, respectively. Interestingly, clone A5-16 exhibited an unusual RNA profile; the segment corresponding to segment 5 was missing, and an RNA band migrating close to the RNA segment 7-9 complex appeared. Northern (RNA) blot hybridization analysis using full-length cDNA of A5-10 gene 5 showed that the very fast migrating RNA segment found in A5-16 was derived from RNA segment 5 of A5-10 (Fig. 2).

Sequencing of gene 5 of the three clones was carried out by dideoxynucleotide chain termination with a single-stranded RNA (ssRNA) transcript and cloned cDNA. Viral ssRNA was synthesized in vitro with endogenous RNA-dependent RNA polymerase present in single-shelled particles. A series of synthetic oligonucleotide primers (17- to 25-mers) was used to sequence ssRNA directly with reverse transcriptase from avian myeloblastosis virus (Seikagaku Kogyo) as described previously (7). Ten picomoles of synthetic oligonucleotide primer was mixed with 1 to 3 μl of ssRNA transcript, heated at 95°C for 1 min, and allowed to anneal at 42°C for 3 min. The mixture (7 μl) was added to 10 μl of reverse transcription buffer (125 mM Tris-HCl [pH 8.3], 125 mM KCl, 25 mM magnesium acetate, 25 mM dithiothreitol, 1 μCi [α-^{32}P]dATP [specific activity, 3,000 Ci/mmol; Amersham Corp.], 6U of reverse transcriptase), and then 4-μl aliquots were dispensed into four tubes containing 1 μl of termination mixture (40 μM dATP; 250 μM dCTP, dGTP, and dTTP; and 10 μM the appropriate dideoxynucleoside triphosphate). The reaction mixtures were incubated at 42°C for 30 min and then combined with 10 μl of sequencing gel sample buffer (95% formamide, 10 mM EDTA [pH 8.0], 2.5 mM Tris-HCl [pH 8.3], 0.1% xylene cyanol, 0.1% bromophenol blue), heated at 95°C for 3 min, and analyzed on 6% sequencing gels. The 3′-terminal 100 nucleotides were determined by using virion dsRNA in 50% dimethyl sulfoxide after being denatured by boiling for 2 min. For cloning gene 5

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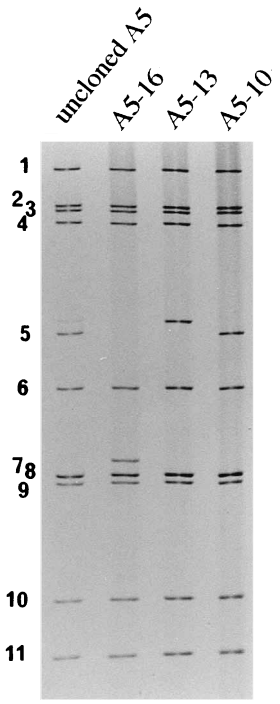

FIG. 1. RNA profile of three A5 clones with distinct RNA segments 5. Samples were subjected to electrophoresis in a 10% polyacrylamide gel; this was followed by silver staining. Numbers on the left represent the RNA segment order.

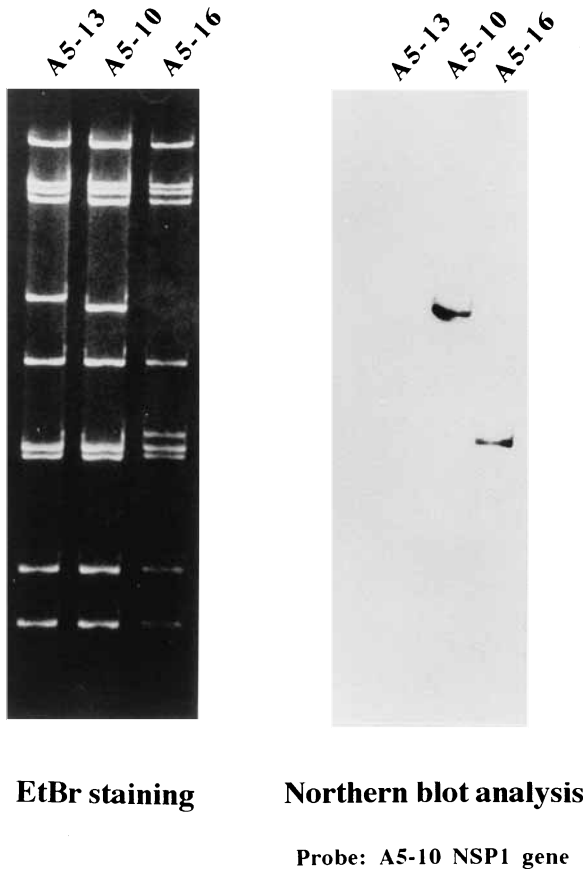

FIG. 2. Northern blot hybridization of peroxidase-labeled A5-10 full-length gene 5 cDNA to denatured genomic RNAs from three A5 clones. EtBr, ethidium bromide.

cDNA, rotaviral dsRNA was reverse transcribed and amplified by PCR as described previously (28, 30), using a pair of common oligonucleotides, 5′-GGCTTTTTTTATGAAAAGTC-3′ for the 5′ forward primer corresponding to the 5′ terminus sequence and 5′-GGTCACATTTTATGCTGCCTA-3′ for the 3′ reverse primer corresponding to the 3′ terminus sequence. PCR-amplified cDNA was ligated into the pCRII vector with a TA cloning kit (Invitrogen Corp). Cloned cDNA was sequenced by using a Sequenase version 2.0 kit (United States Biochemical Corp., Cleveland, Ohio) and appropriate oligonucleotide primers.

The nucleotide sequences determined with the different templates, mRNA or dsRNA and cDNA, were completely the same, except that the sequences which corresponded to the primers used in PCR could not be determined for cDNA. Figure 3 shows the complete nucleotide sequences of the genes 5 of three A5 clones. The A5-10 and A5-13 genes 5 are 1,587 and 1,579 bases in length, respectively. The nucleotide identity between A5-13 and A5-10 genes 5 was 73.2%. In contrast, the gene 5 of A5-16, which migrated very fast in PAGE, is only 1,087 bases long. The A5-16 gene 5 was found to be derived from the A5-10 gene 5, and 500 bases corresponding to nucleotides 142 to 641 of the A5-10 gene 5 were deleted in the A5-16 gene 5. Except for this large deletion, the nucleotide sequences of the parent A5-10 gene 5 and the A5-16 gene 5 were 100% homologous. Since the nucleotide sequence encoding a cysteine-rich region is located at nucleotides 156 to 248 of the A5-10 gene 5, the A5-16 gene 5 loses the entire sequence encoding the cysteine-rich region.

The mechanism for the occurrence of such a large deletion is not known. However, it is of note that sequence AACAA just before the deletion site is found at the end of the deletion sequence, and sequences GAAUGG and CACCAAU are also observed upstream of the deletion site and at the end of the deletion sequence. Similar direct sequence repeats in the rearranged genomes have been indicated in previous reports (1, 11). In the rearranged genes of bovine rotavirus strain brvA and human rotavirus strain A28, both of which have a partial head-to-tail duplication, sequences UGAA and AAUGUGA were found to occur at the junction site and reinitiation site, respectively. A similar mechanism might be at work in the present example.

Since gene 5 of A5-13 has an initiation codon AUG at nucleotides 33 to 35 which conforms to the consensus sequence for strong initiation (ANNAUGG) and a termination codon at 1,506 to 1,508 as found in other reported sequences of genes 5 of various rotavirus strains, the NSP1 protein of A5-13 is deduced to consist of 491 amino acid residues, with an estimated molecular mass of 59 kDa. Three basic regions at amino acids 10 to 39, 79 to 91, and 111 to 126 are conserved in A5-13 NSP1 (data not shown). NSP1 of A5-13 has a cysteine-rich region (amino acids 42 to 75) which is suggested to represent a Zn finger motif. The second cysteine-rich region (amino acids 314 to 327) found in bovine strains and a feline strain (Cat2) (14a) is not conserved in A5-13 NSP1. A5-13 NSP1 is more related to those of bovine strains (UK, RF, and A44; homology values of 69.3 to 71.3%) than to those of rotaviruses from humans and other animal species (a homology of less than 60.1%).

However, when the amino acid sequences of NSP1 of both A5-10 and A5-16 are deduced, nonsense codons at the early portions of their genes 5 are found. Figure 4A shows an open

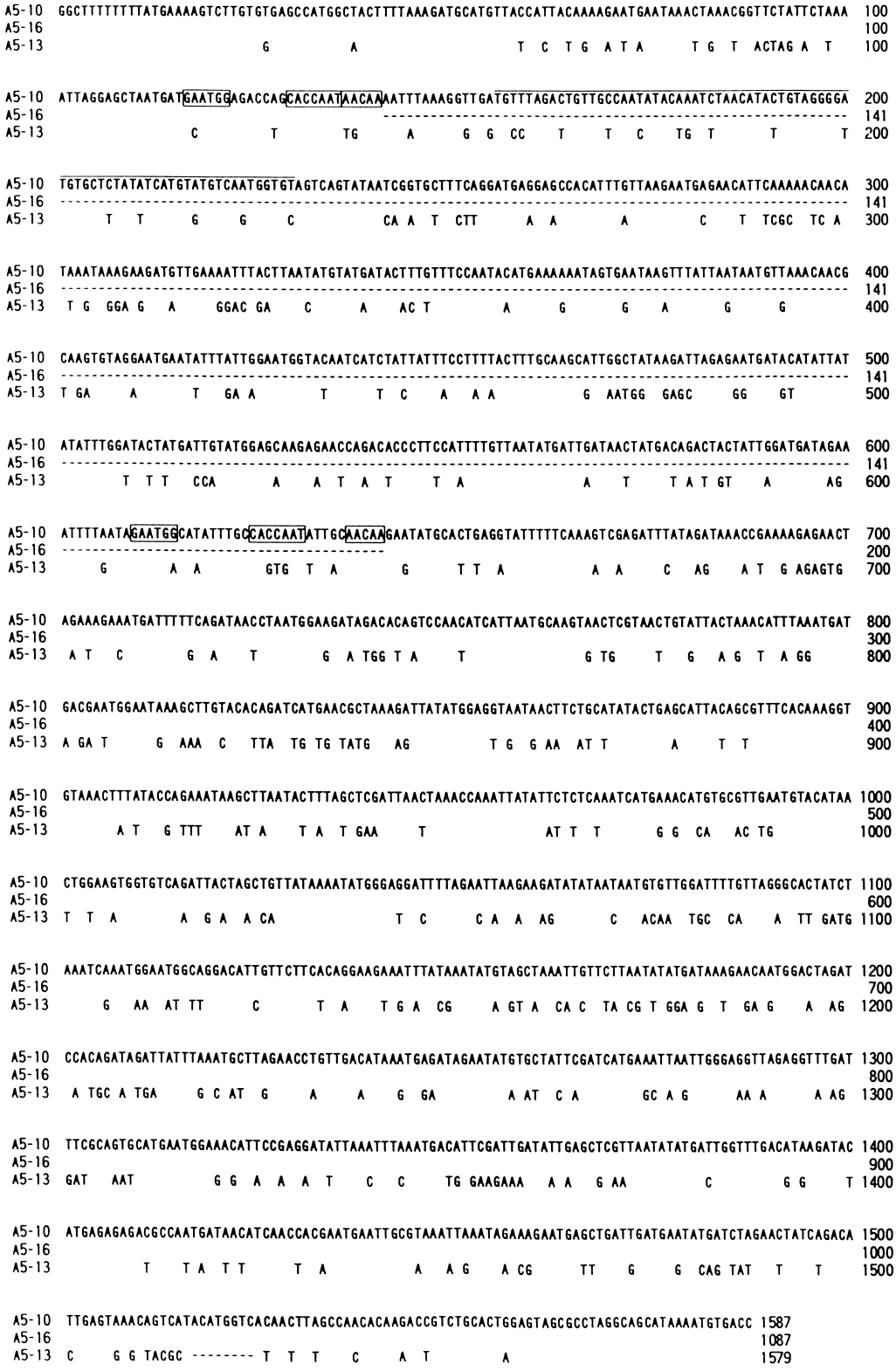

FIG. 3. Complete nucleotide sequences of the NSP1 genes of three A5 clones (A5-10, A5-13, and A5-16). The sequence of the A5-10 NSP1 gene is shown in its entirety; for the other clones, only the differences from A5-10 are indicated. Dashes show the deletion sequence. Three repeated sequences, GAATGG, CACCAAT, and AACAA, found upstream of the deletion site and at the end of the deletion sequence are boxed. Sequences encoding the cysteine-rich region are overlined.

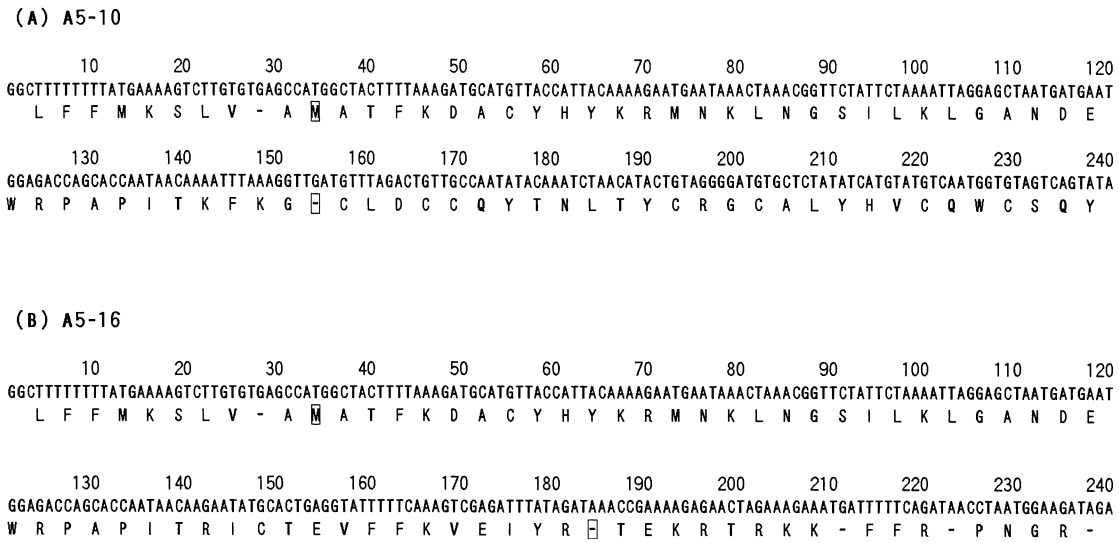

FIG. 4. An open reading frame for translation of a part (nucleotides 1 to 240) of the NSP1 genes from A5-10 (A) and A5-16 (B). Dashes indicate the position corresponding to the termination codon. Methionine residues corresponding to the AUG which conforms to Kozak's rule and the predicted end of the reading frame are boxed.

reading frame of a part (nucleotides 1 to 240) of the A5-10 gene 5. At nucleotides 33 to 35, a strong initiation codon is present, as found in the cognate gene of other rotaviruses. In this frame, a termination codon UGA appears at nucleotides 153 to 155 just upstream of the coding region (nucleotides 156 to 230) of a cysteine-rich Zn finger motif. If this termination codon works, the A5-10 gene 5 product is only 40 amino acids and the molecular weight of the product is estimated to be 4,615. Otherwise, if a readthrough occurs at this termination codon, the A5-10 gene 5 goes on to produce the normal-size NSP1. In the nucleotide sequence of the A5-16 gene 5 with a 500-base deletion, a deduced coding region also starts from the initiation codon at nucleotides 33 to 35 and ends at nucleotides 183 to 185 (Fig. 4B), since the junction causes a frameshift. If this termination codon works, the A5-16 gene 5 forms only 50-amino-acid peptides (estimated molecular weight, 5,940). Alternatively, there might be a frameshift around the junction site to produce a longer NSP1. The A5-10 NSP1 amino acid sequence (if missing the nonsense codon) has a relatively low homology (66.3%) with that of A5-13.

Protein expression of genes 5 from mutants A5-10 and A5-16 was investigated. A5-10, A5-13, and A5-16 clones were inoculated onto MA-104 cells, and after 5 or 7 h of incubation, viral proteins were labeled with [^{35}S]methionine or ^{14}C-amino acid mixtures (Amersham) for 2 h. Whole cells, the cytosol fraction, and the cytoskeleton fraction were prepared as described by Hua and Patton (11). The cells were washed with isotonic buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl), harvested with a plastic scraper, and pelleted by low-speed centrifugation. The cells were then suspended in lysis buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.6], 150 mM NaCl, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100). One-third of the lysed cells were analyzed by sodium dodecyl sulfate (SDS)-PAGE as is. The remainder were centrifuged; the supernatant (cytosol fraction) and the pellet (cytoskeleton fraction), suspended in the isotonic buffer and dispersed by ultrasonication, were analyzed by SDS-PAGE. Figure 5 shows the patterns of protein synthesis in the cytoskeleton fraction of the MA-104 cells infected with each rotavirus. The whole-cell fraction and cytosol fraction also showed similar results, although the relative amount of NSP1 in these fractions was smaller for the cells infected with A5-13 and reference strains than that in the cytoskeleton fraction. Although NSP1 is reported to be produced at a low level in the cells, a strong protein band of 59 kDa corresponding to NSP1 was clearly observed in the cells infected with A5-13. Also in the cells infected with reference

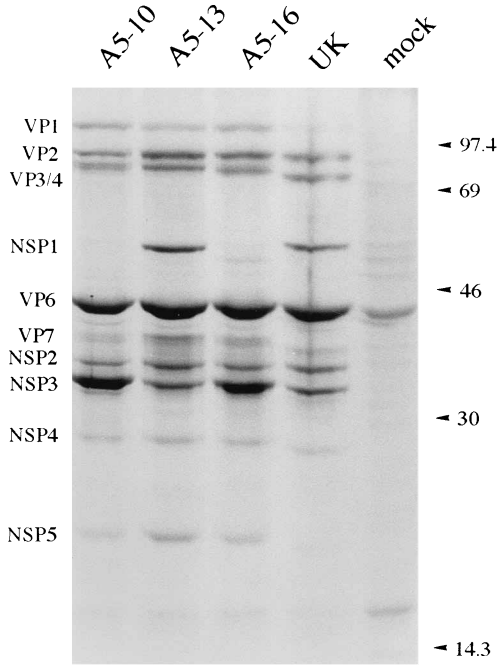

FIG. 5. Protein synthesis in MA-104 cells infected with each of three A5 clones and the bovine UK rotavirus strain. The numbers on the right are molecular sizes in kilodaltons: phosphorylase *b* (97.4 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and lysozyme (14.3 kDa).

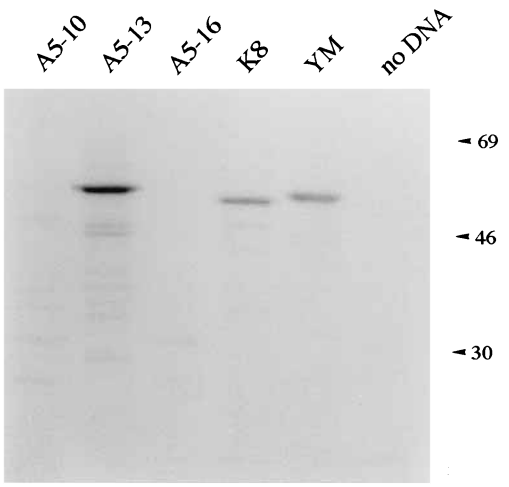

FIG. 6. In vitro translation of NSP1-specific transcript from each of three A5 clones, human strain K8 (G serotype 1, P serotype 3) and porcine strain YM (G serotype 11, P serotype 7). The numbers on the right are molecular sizes in kilodaltons: bovine serum albumin (69 kDa), ovalbumin (46 kDa), and carbonic anhydrase (30 kDa).

bovine strain UK, a similar band corresponding to NSP1 was found. In contrast, no corresponding proteins were observed in the cells infected with the A5-10 or A5-16 clone. However, the protein band corresponding to NSP3 was much stronger in the cells infected with the A5-10 or A5-16 clone than in those infected with the A5-13 clone (Fig. 5).

Even when the dried gels were exposed to X-ray films for a long time (2 weeks or 1 month instead of the usual 1 day) and even in the case of a very short run (30 min instead of the usual 2.5 h) of PAGE to see the predicted low-molecular-weight products, no protein band was observed (data not shown). The NSP1 genes of A5-10 and A5-16 might express no proteins, or the truncated NSP1 they express might be unstable. There is also a possibility that the A5-10 and A5-16 genes 5 produce proteins with molecular weights similar to that of NSP3, though this seems quite unlikely since a truncated protein with a molecular weight equivalent to that of NSP3 cannot be deduced from the sequence of A5-10 or A5-16. Immunoprecipitation analysis using a rabbit antiserum to the peptide NH_2-DKPKRELERNDFSDNLME-COOH corresponding to amino acids 217 to 234 of the assumed full-length gene 5 product of A5-10 failed to identify any proteins as the gene 5 product of A5-10 or A5-16 (data not shown). The peptide NH_2-NKLNG-SILKLGAND-COOH (amino acids 15 to 28 of A5-10 and A5-16) synthesized by a multiple antigen peptide method (23) was nonimmunogenic in guinea pigs. It will be interesting to explore the possibility that the gene 5 or the unidentified products of the A5-10 and A5-16 mutants enhance the translation of NSP3, since NSP1 and NSP3 have several features in common: RNA binding activity, association with replication intermediates, and cytoskeleton localization (16, 19).

Using a coupled transcription-translation system, the TNT reticulocyte lysate system (Promega, Madison, Wis.), we examined the in vitro translation of gene 5-specific transcripts. In this system, proteins can be produced from protein-encoding DNA sequences cloned downstream of an SP6 RNA polymerase promoter. When we used the plasmid containing A5-13 gene 5 cDNA or reference K8 or YM gene 5 cDNA, a 55- to 58-kDa protein corresponding to NSP1 was detected. However, in the reaction using the plasmid containing the A5-10 gene 5 or the A5-16 gene 5, no significant strong protein bands were detected (Fig. 6), even in a long exposure of the gels or in a short run of PAGE, as found in the cells infected with A5-10 or A5-16. Again, no proteins were detected by immunoprecipitation analysis with the antipeptide rabbit serum (data not shown).

Although we could not identify the gene 5 authentic product of A5-10 or A5-16, clones A5-10 and A5-16 are both nondefective and infectious. The virus titers of these clones were comparable to those of other animal rotaviruses: virus yields were 1.8×10^7 PFU/ml for A5-10 and 4.7×10^6 PFU/ml for A5-16. The plaque size of A5-13 was extremely large (4.48 ± 1.27 mm, in counting 20 plaques). A5-10 produced medium-size plaques (2.35 ± 0.75 mm). In contrast, the plaque size of A5-16, whose gene 5 has a 500-base deletion from the A5-10 gene 5, was very small (0.29 ± 0.14 mm). These results suggest that gene 5 affects the efficiency of virus growth. However, the difference in plaque size between A5-10 and A5-16 might indicate the possible presence of a minor variation in a gene or genes other than gene 5. Preparation of monoreassortants with gene 5 from A5-10 or A5-16 and their characterization are required to more precisely assess the association of this gene with growth characteristics. Concerning the possibility of the presence of a small amount of the A5-13 clone in the A5-10 or A5-16 preparation as a helper, we performed PCR with a pair of primers specific to the A5-13 NSP1 gene (corresponding to nucleotides 76 to 93 and 470 to 487), and we could exclude this possibility since no amplified DNA was detected in the RNA preparation of A5-10 or A5-16 (data not shown).

The gene 5 product NSP1 is poorly conserved among mammalian group A rotaviruses (2, 4, 10, 14a, 18, 31). Despite the lack of overall identity, a cysteine-rich region near the amino terminus of NSP1, which is proposed to fold in such a way as to form one or two zinc fingers, is highly conserved among various strains. The second cysteine-rich region (amino acids 314 to 327) detected in bovine strains and a feline strain, Cat2, was not conserved in the NSP1 of A5-13 or A5-10 (if missing the nonsense codon). NSP1 has been suggested to be responsible for RNA binding, assortment of RNA segments in morphogenesis, and RNA replication (2, 3, 9, 11). Such functions are assumed to be located in the N-terminal half including the cysteine-rich region of NSP1. Hua et al. (9) employed deletion mutagenesis to map the location of the RNA binding domain associated with viral RNA packaging and genome replication and the intracellular (cytoskeleton) localization domain at the first 81 amino acid positions and at amino acid positions 84 to 176, respectively; their findings contradict our present data. Comparable growth in cell culture and plaque formation, though with a low level of efficiency, were found with A5-16, whose gene 5 lacks the region encoding the cysteine-rich amino acid sequence and which has a nonsense codon at nucleotides 183 to 185. Further, the same was the case with A5-10, whose gene 5 has a nonsense codon at nucleotides 153 to 155. These results imply that the cysteine-rich region is not necessarily required for virus replication, at least in vitro. In a preliminary experiment, we observed that the A5-16 clone could induce diarrhea in each of the 10 5-day-old BALB/c suckling mice studied after inoculation with 5×10^4 PFU per mouse (28a). Further studies will be needed to answer the following questions. Do gene 5 in A5-10 and gene 5 in A5-16 produce any truncated proteins? Are such products too labile to be detected under common experimental conditions? Is the gene 5 product itself actually necessary for virus replication?

The nucleotide sequence of the A5-13 NSP1 gene is bovine-like, although it has a relatively low homology (73.2% at the nucleotide level) with that of A5-10. The A5-13 NSP1 gene might be derived from an unidentified bovine strain X. The

cow from which A5 feces were collected might have been coinfected with A5-10 and the strain X having the A5-13 NSP1 gene. Reassortment might have occurred in the cow between A5-10 and strain X, and A5-10 and a single-gene reassortant with the A5-13 NSP1 gene and the other 10 genes from A5-10 might have been excreted in the feces. However, it is not known whether the coexistence of the multiple NSP1 genes in the cells influenced the occurrence of the mutations of the NSP1 gene of A5-10.

Nucleotide sequence accession numbers. Sequence data from this study have been deposited with the DDBJ and EMBL-GenBank data libraries under accession no. D38147 (A5-10), D38148 (A5-13), and D38149 (A5-16).

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