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Nucleotide Sequence Homology in Rotaviruses

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Rotaviruses의 염기배열 유사성 측정

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ABSTRACT: Nucleotide sequence homology between bovine, simian, and porcine rotavirus was determined by the RNA:RNA hybridization technique. Single stranded RNA, prepared *in vitro* with EDTA activated endogeneous viral RNA polymerase, was hybridized with tritium labeled bovine rotavirus genomic RNA. The heteroduplex RNA was treated with single stranded RNA specific ribonucleases and the RNase resistant hybrid RNA was precipitated, and collected by filtration on a filter paper. Seventy four percent RNA sequence homology between bovine and simian rotavirus and 8 percent RNA sequence homology between bovine and porcine rotavirus were observed. The low degree of sequence homology between bovine and porcine rotavirus was confirmed by hybridization between tritium labeled single stranded RNA and viral genomic RNA.
KEY WORDS□Rotavirus, Hybridization, RNA sequence homology.

Rotaviruses have been associated with diarrhea in a variety of animals (Flewett and Woode, 1978). Biochemical characterization has shown that the rotavirus genome consists of 11 double stranded RNA segment (Barnett *et al.*, 1978) which can be separated into 8 to 11 bands by polyacrylamide gel electrophoresis (Kalica *et al.*, 1976; Schnagl and Holmes, 1976; Verly and Cohen, 1977). Although rotaviruses from different species can be distinguished from each other by neutralization tests (Thouless *et al.*, 1977), human, calf, pig, and lamb rotaviruses share a group specific antigen which is demonstrable by complement fixation and immunofluorescence (Kapikian *et al.*, 1975; Woode *et al.*, 1976).

In spite of the close serological relationship between rotaviruses, differences in the electrophoretic mobility of the RNA genome segments have been demonstrated between isolates from different species (Kalica *et al.*, 1978; Rodger and Holmes, 1979) and the same species (Espejo *et al.*, 1977). These electropherotypes have been used to distinguish rotaviruses (Rodger and Holmes, 1979; Espejo *et al.*, 1977). However, the migration patterns depend on the size of the genome segment and not their base sequence. RNA sequence diversity among human rotavirus strains and between human and animal rotavirus strains has been studied by northern blot hybridization analysis (Street *et al.*, 1982; Schroeder *et al.*, 1982) and by using *in vitro* transcribed ss RNA (Matsuno and Nakajima, 1982).

In the present study, the nucleotide sequence homology between bovine, simian, and porcine

rotavirus RNA has been examined by the hybridization technique using single stranded RNA prepared from EDTA activated endogeneous viral RNA polymerase (Cohen *et al.*, 1979). The results provide quantitative information about genetic relationship between rotaviruses, and the degree of homology between each RNA segment may help to elucidate the epidemiology of rotavirus infection.

MATERIALS AND METHODS

Cells

Madin-darby bovine kidney (MDBK) cells (American Type Culture Collection, Rockville, MD) and Macacus rhesus monkey kidney (MA-104) cells (Dr. Mary Estes, Baylor College of Medicine, Houston, TX) were grown in Eagle's minimum essential medium (MEM) (Gibco) supplemented with 6% heat-inactivated fetal bovine serum (Sterile System, Inc.), 0.19% sodium bicarbonate, 50 units/m*l* penicillin-G (Sigma), and 100 ug/m*l* streptomycin sulfate (Sigma) at 37 °C with 5% CO_2.

Viruses

The vaccine strain of the Lincoln isolate of bovine rotavirus (BRV) obtained from Norden Laboratories (Lincoln, Nb) was propagated in MDBK cells. Porcine rotavirus (PRV), obtained from Dr. Edward Bohl, Ohio Agricultural Research and Development Center (Wooster, OH), and simian rotavirus (SRV), obtained from Dr. Mary Estes (address above), were grown in MA-104 cells.

Confluent monolayers of either MA-104 or MDBK cells were washed twice with MEM without serum and inoculated with virus previously treated with 10 ug/m*l* of trypsin for 30 min at 37 °C. After adsorption for 1 h, MEM without serum was added. Virus was extracted from infected cells and fluids by homogenizing in a Virtis 60 K homogenizer for 3 min at 10,000 rpm with Freon 113 (25%, v/v) and purified by two cycles of isopycnic banding in CsCl density gradients. Purified virus was dialyzed extensively against 0.01 M Tris-HCl, pH 8.0 and stored at -20 °C until used.

Preparation of single stranded (ss) RNA

Single stranded RNA was prepared as described by Cohen and Dobos (1979). Purified virus was treated with 10 mM EDTA for 30 min at 37 °C. EDTA treated virus was pelleted in a fixed angle IEC rotor for 2 h at 35,000 rpm and added into the reaction mixture which had the following composition; 0.1 mM Tris-HCl buffer, pH 8.0; 2 mM ATP, 2 mM UTP, 2 mM CTP, and 2 mM GTP (Sigma Chemical); 10 mM $MgCl_2$; 5 mg/m*l* of phosphoenol pyruvate (Sigma); and 0.5 mg/m*l* of pyruvate kinase(Calbiochem-Behring Corp.). After 6 h of incubation at 37 °C, the virus particles were pelleted in a fixed angle IEC rotor for 2 h at 35,000 rpm.

The single stranded (ss) RNA was extracted twice with an equal volume of water saturated phenol and precipitated with two volumes of 95% ethanol at -20 °C. The ss RNA precipitate was redissolved in sterile distilled water, and precipitated with 3 M sodium acetate at 4 °C. The purified ss RNA was dissolved in sterile distilled water and stored at -20 °C. For preparation of labeled ss RNA, 25 uci/m*l* of tritiated UTP (ICN) instead of UTP was added. The amount of RNA was estimated by using the following relation; one unit of optical density at 260 nm = 44 ug of ss RNA per m*l* (J. Cohen, Personal communication).

Preparation of genomic double stranded (ds) RNA

Purified virus was disrupted with 1% sodium dodecyl sulfate (SDS) for 30 min at 37 °C. RNA was extracted twice with phenol and residual phenol was removed by ether. The RNA was precipitated with two volumes of 95% ethanol at -20 °C, pelleted by centrifugation, washed two times with 75% ethanol, vacuum dried, and dissolved in 2.25 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2).

Preparation of ^{3}H-labeled genomic RNA

Confluent monolayers of cells were infected with rotavirus. After 1 h of adsorption, the cells were washed three times and maintained in MEM without serum. After 4 h of infection, the medium was changed to MEM that contained 120 uci/m*l* of tritiated uridine (ICN), and 0.5 ug/m*l* of Actinomycin D (Sigma). Virus purification and RNA extraction were done as described above.

Hybridization

The RNA:RNA hybridization was performed according to the method of Yin *et al.* (1978) with a minor modification. Tritium labeled genomic RNA was incubated at 98 °C for 5 min and quickly quenched in an ice water bath. Approximately 1000 counts per minute (cpm) of the denatured RNA was mixed with various amounts of ss RNA in 1 m*l* of 2.25 × SSC. After 2 h of incubation at 67 °C, RNase A and T1 (Worthington Biochemical Corp.) were added to final concentrations of 20 ug/m*l* and 50 units/m*l* respectively and the mixture was incubated at 37 °C for 30 min. Hybrid RNA was precipitated with an equal volume of cold 10% trichloroacetic acid (TCA), collected by filtration on a filter paper (GF/C, Whatman Grade), and washed two times with 5 m*l* of cold 5% TCA. Filters were dried and counted in a liquid scintillation spectrometer. It was assumed that trichloroacetic acid insoluble counts represented ribonuclease resistant hybrid RNA.

RESULTS

Characterization of ^{3}H-labeled BRV ds RNA

The native and heat-denatured ^{3}H-labeled BRV ds RNAs were digested with single stranded RNA specific RNase A and T1. The uncleaved ds RNAs were precipitated by adding cold 10% TCA, collected on a filter paper, and counted on the liquid scintillation spectrometer. The native BRV ds RNA was resistant to the digestion by RNase A and T1 while 96-90% of the heat-denatured BRV ds RNA was susceptible to the digestion by RNase A and T1.

Figure 1 also showes that the susceptibility of heat-denatured BRV ds RNA to RNase A. As the amount of RNase A was increased, the sensitivity of the RNA to the RNase A was also increased. About 20 ug/m*l* was more than enough to digest heat-denatured BRV ds RNA

Preparation and characterization of BRV ss RNA

The endogeneous rotavirus RNA polymerase could be activated by EDTA treatment (15). Single stranded RNAs synthesized *in vitro* by endogeneous RNA polymerase consist of 11 segments

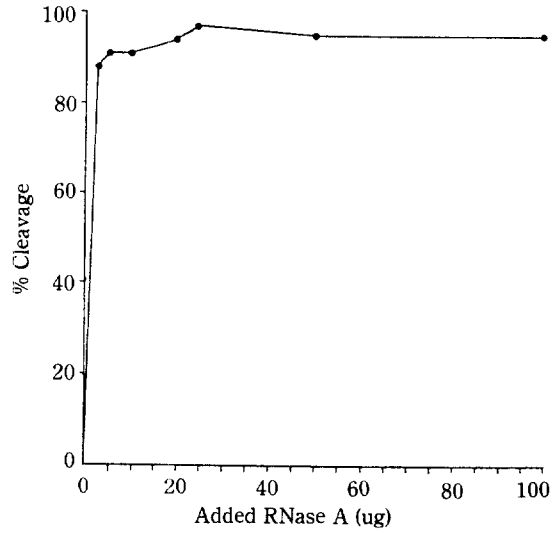

Fig. 1. *Susceptibility of denatured BRV genomic ds RNA to RNase A.*
^{3}H-labeled BRV ds RNA (about 1,000 cpm) was denatured at 98 °C for 5 min, quickly quenched in ice water bath, and treated with RNase A for 30 min at 37 °C. The RNase A resistant hybrid RNA was collected and counted as described in Materials and Methods.

that hybridize with denatured genomic ds RNA (20). Over 1 mg of ss RNA was routinely obtained from the EDTA treated BRV. ^{3}H-labeled heat-denatured BRV ds RNA was hybridized with BRV ss RNA in 2.25 × SSC. After 2h of incubation at 67 °C, the reaction mixture was treated with RNase A and T1 for 30 min at 37 °C and RNase resistant hybrid RNA was precipitated and collected on a filter paper.

Figure 2 showes that the degree of hybrid RNA formation was increased as the quantities of the added BRV ss RNA was increased. When more than 4 u*l* of ss RNA (equivalent to 5 ug of ss RNA) was added, the degree of hybrid RNA formation reached to a maximum level. The maximum percentage of the RNase resistant hybrid RNA formation was about 44%.

Determination of RNA sequence homology between rotaviruses

For determining RNA sequence homology between BRV and SRV, BRV and PRV, 11 ug each of BRV ss RNA, SRV ss RNA, and PRV ss RNA, which is twice the amount needed for saturation, was hybridized with ^{3}H-labeled heat-denatured

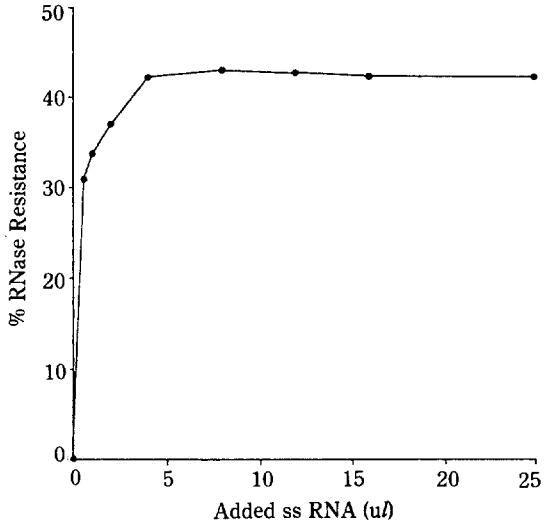

Fig. 2. *Dependence of the extent of hybridization upon the concentration of ss RNA.*
^{3}H-labeled BRV ds RNA (about 1,000 cpm) was denatured, hybridized with BRV ss RNA in 2.25 × SSC for 2 h at 67 °C, and treated with 20 ug/ml of RNase A and 50 units/ml of RNase T1 for 30 min at 37 °C. The RNase resistant hybrid RNA was precipitated by adding equal volume of cold 10% TCA, and collected on glass sintered filter paper. The radioactivity of the air-dried filter paper was counted in a liquid scintillation spectrometer.

BRV ds RNA. The RNA sequence homology was determined as following rules (1) substract the counts of RNase-treated BRV ds RNA, that is first denatured and then self-annealed under the hybridization conditions employed, from all the samples (2) assume that RNase resistant counts of homologous RNA:RNA hybrid is 100% sequence homology (3) RNase resistant counts of heterologous RNA:RNA hybrid divide by RNase resistant counts of homologous RNA:RNA hybrid is the percent sequence homology for heterologous RNA.

A 74% homology between BRV and SRV, and an 8% homology between BRV and PRV were observed (Table 1). It was unexpected that SRV was more closely related to BRV than PRV was to BRV. To confirm the above results, ^{3}H-labeled PRV genomic ds RNA was heat-denatured and hybridized with increasing amounts of BRV ss RNA, SRV ss RNA, and PRV ss RNA respectively (Fig. 3). No more than 5 percent homology between PRV and SRV were observed.

Table 1. *Percent RNA sequence homologies between BRV, SRV, and PRV.*

	Radioactivity of the RNase resistant hybrid RNA (cpm)[1]	RNA sequence homology to BRV ds RNA (%)[2]
BRV	560	100
SRV	410	74
PRV	45	8

1. ^{3}H-labeled BRV ds RNA (about 1,800 cpm) was denatured, and hybridized with 11 ug each of BRV ss RNA, SRV ss RNA, and PRV ss RNA. The counts of RNase resistant hybrid RNA was determined as described in Fig. 2 legend.
2. % sequence homology was determined as described in the text.

Hybridization of in vitro prepared ^{3}H-labeled ss RNA with genomic ds RNA

^{3}H-labeled BRV ss RNA, SRV ss RNA, and PRV ss RNA, prepared from EDTA treated BRV, SRV, and PRV, were hybridized with denatured genomic RNA and the % RNase resistances were

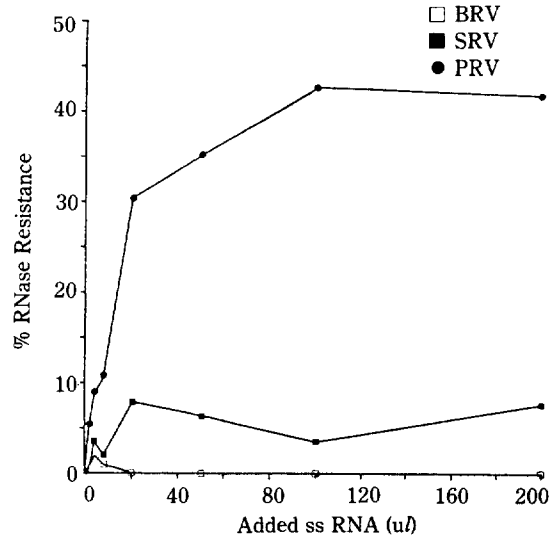

Fig. 3. *Hybridization between PRV ds RNA and PRV ss RNA, PRV ds RNA and BRV ss RNA, and PRV ds RNA and SRV ss RNA.*
^{3}H-labeled PRV ds RNA (about 700 cpm) was denatured and hybridized with BRV ss RNA, SRV ss RNA, and PRV ss RNA in 2.25 × SSC. The % RNase resistance was determined as described in Fig. 2 legend.

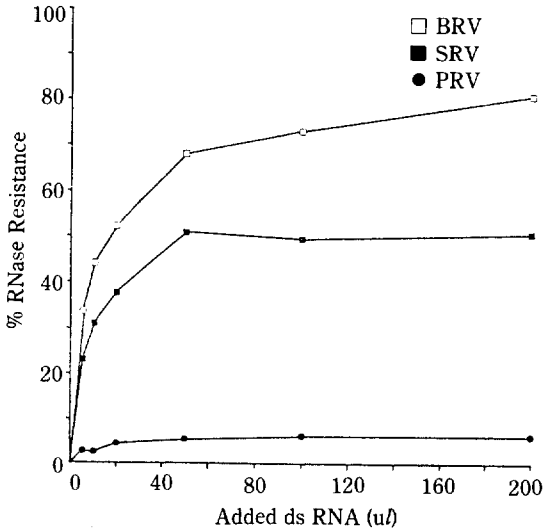

Fig. 4. *Dependence of the extent of hybridization upon the concentration of ds RNA.*
^{3}H-labeled BRV ss RNA (about 6,000 cpm) was hybridized with denatured BRV ds RNA, SRV ds RNA, and PRV ds RNA in 2.25 × SSC. The % RNase resistance was determined as described in Fig. 2 legend.

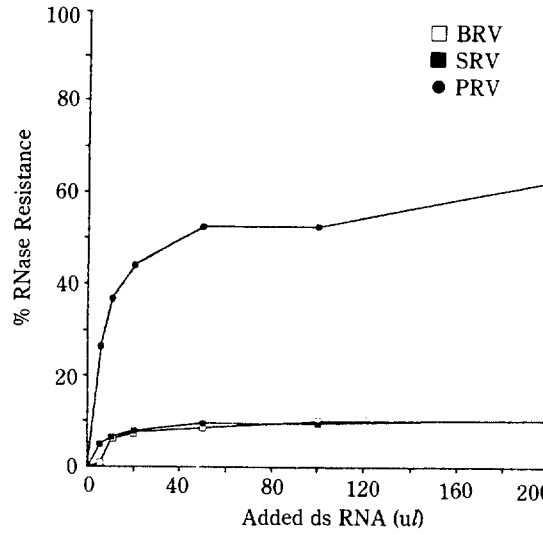

Fig. 5. *Dependence of the extent of hybridization upon the concentration of ds RNA.*
^{3}H-labeled PRV ss RNA (about 4,000 cpm) was hybridized with denatured BRV ds RNA, SRV ds RNA, and PRV ds RNA in 2.25 × SSC. The % RNase resistance was determined as described in Fig. 2 legend.

determined. Although the maximum hybrid RNA formation was less than expected, plateaus were formed which suggests that excess amount of ds RNA was provided to the saturation level. The results agree with the previous observations confirming that SRV is closely related to BRV compared to RNA and BRV (Figures 4,5, and 6)

DISCUSSION

Electrophoretic migration pattern of viral nucleic acid has been considered to be a useful tool for studying the epidemiology and distribution of rotaviruses (Espejo *et al.*, 1977; Roger *et al.*, 1978). There is, however, little information upon which to build any conclusion about the degree of genetic relatedness among the rotaviruses. The hybridization procedure which makes use of essentially all sequences present in RNA allows a comparison of the total genomic RNA.

Strains of bovine, simian, and porcine rotaviruses have been cell culture adapted and grow to high titer (Clark *et al.*, 1979). EDTA treatment of these viruses activates the endogeneous RNA dependent RNA polymerase from which ss RNA can be prepared (Cohen *et al.*, 1979; Mason *et al.*, 1980). The ss RNA, highly susceptible to RNase

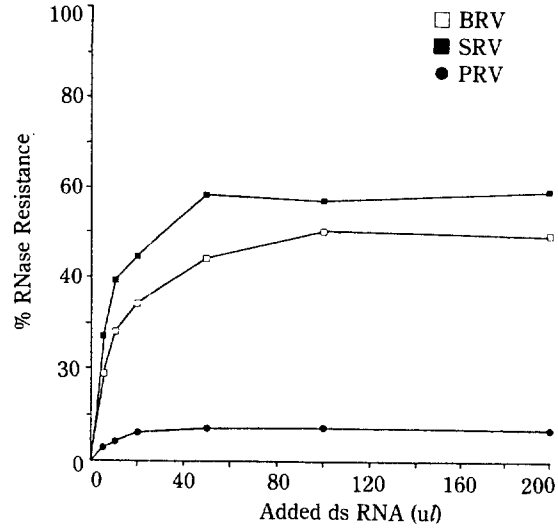

Fig. 6. *Dependence of the extent of hybridization upon the concentration of ds RNA.*
^{3}H-labeled SRV ss RNA (about 5,500 cpm) was hybridized with denatured BRV ds RNA, SRV ds RNA, and PRV ds RNA in 2.25 × SSC. The % RNase resistance was determined as described in Fig. 2 legend.

A and T1, is complementary to only one strand of genomic ds RNA and displays messenger activity in the rabbit reticulocyte lysate translation system (Cohen and Dobos, 1979).

We have examined rotavirus RNA sequence homology by RNA-RNA hybridization with ^{3}H-labeled genomic ds RNA and *in vitro* prepared ss RNA. Seventy four percent homology between BRV and SRV RNA, and 8% homology between BRV and PRV RNA were observed. The similar results were also observed in the hybridization between ^{3}H-labeled ss RNA and genomic ds RNA. Young *et al.* found 74% RNA sequence homology between polioviruses type 1 Brunhilde and type 1 Mahoney, while 22% RNA sequence homology was found between type 1, type 2, and type 3 polioviruses, which does not share common neutralization determinants (Young *et al.*, 1968). Seventy percent homology was observed between human rhinovirus type 1A and 1B (Yin *et al.*, 1973). They also reported that no more than 4% homology was observed between heterologous pairs, such as type 2 and 1A. The genomes from reovirus type 1 and 3 have been shown to share 90% sequence homology, whereas type 2 is related to the other two types to the extent of about 10% sequence homology (Joklik, 1980).

On the basis of RNA sequence homology SRV is more closely related to BRV than PRV is to BRV. These results well agree with the RNA migration patterns on electrophoretic gel analysis. Coelectrophoresis of PRV and BRV RNAs revealed differences in the mobilities of 5 segments, (1,4,5,7, and 10) whereas comparison of SRV and BRV RNAs revealed the differences in the mobility of 2 segments (3 and 10) (data not shown).

Matsuno and Nakajima (1982) reported that BRV RNA did not hybridize with HRV RNA, but hybridized with SRV RNA at the level of 30%. Their results confirm the close relationship of BRV to SRV rather than to HRV although 62% sequence homology between BRV ss RNA and SRV ds RNA was observed in this study. The disparity between these two studies may be as a result of the difference in the way that the labeled total ss RNA was produced. That is, we used the total product of the endogeneous viral RNA polymerase reaction whereas Matsuno and Nakajima prepared their total as RNA by mixing equal counts per minute of each of the 11 segments. It is also possible that the strains of simian rotavirus may be slightly different. Hybridization studies using the individual segment of RNA will help in studying the RNA segments responsible for group and serotype specificity. Therefore, nucleotide sequence homology may provide useful information for the elucidation of the epidemiology and the relationships among the rotaviruses.

적 요

소, 원숭이, 돼지 rotavirus의 염기배열 유사성을 리보핵산간의 hybridization 방법으로 측정하였다. EDTA로 활성화시킨 바이러스의 리보핵산 중합효소를 사용하여 외가닥 리보핵산을 시험관에서 합성한 후 방사능 동위원소로 표지된 소 rotavirus의 유전자 리보핵산(쌍가닥)과 hybridization시켰다. 소와 원숭이 rotavirus간의 염기배열 유사성은 74%인 반면 소와 돼지 rotavirus간은 8%였다. 이 실험결과는 방사능 동위원소로 표지된 외가닥 리보핵산과 각 rotavirus의 유전자 리보핵산을 hybridization시킨 결과와 일치하였다.

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