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Detection of Hepatitis A Virus by Hybridization with Single-Stranded RNA Probes

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An improved method of dot-blot hybridization to detect hepatitis A virus (HAV) was developed with single-stranded RNA (ssRNA) probes. Radioactive and nonradioactive ssRNA probes were generated by in vitro transcription of HAV templates inserted into the plasmid pGEM-1. ^{32}P-labeled ssRNA probes were at least eightfold more sensitive than the ^{32}P-labeled double-stranded cDNA counterparts, whereas biotin-labeled ssRNA probes showed a sensitivity comparable with that of the ^{32}P-labeled double-stranded cDNA counterparts. Hybridization of HAV with the ssRNA probes at high stringency revealed specific reactions with a high signal-to-noise ratio. The differential hybridization reactions seen with probes of positive and negative sense (compared with HAV genomic RNA) were used to detect HAV in clinical and field samples. A positive/negative ratio was introduced as an indicator that permitted a semiquantitative expression of a positive HAV reaction. Good agreement of this indicator was observed with normal stool samples and with HAV-seeded samples. By using this system, HAV was detected in estuarine and freshwater samples collected from a sewage-polluted bayou in Houston and a saltwater tributary of Galveston Bay.

Hepatitis A virus (HAV) has long been recognized as a water- and shellfish-transmitted pathogen responsible for outbreaks of illness with associated potentially serious public health and economic impacts (12, 27). Infected individuals may be unable to work productively for weeks to months, and the shellfish industry along with the seafood restaurant trade can suffer substantial financial losses.

Interdiction of virus transmission routes has had to await the onset of illness and the clinical recognition of hepatitis infection before attempts to locate the source of virus could begin. Although epidemiologic studies have contributed greatly to our knowledge of virus source and transmission routes, they have had to rely upon a time-consuming information-gathering process independent of a rapid, direct virus detection capability.

A number of difficulties are encountered in attempts to detect wild-type strains of HAV in samples from environmental sources. First, virus quantities are low. Second, wild-type strains of HAV grow slowly, if at all, in cell culture, and no visible cytopathic effects are found. Radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), or immunoelectron microscopy methods used to detect the usually abundant quantities of HAV in clinical samples lack the degree of sensitivity required to detect the low quantities of virus expected in samples from environmental sources. An exception to this general rule occurred in an outbreak of type A hepatitis in Georgetown, Tex., where radioimmunoassays revealed the presence of HAV antigen in heavily sewage-contaminated drinking water (9). Finally, the presence of humic or fulvic acids, which are often present in environmental samples, is known to reduce the effectiveness of virus collection and concentration processes and consequently may adversely influence detection test efficiency (22).

Immunofluorescence or radioimmunofocus assays, which depend upon virus replication in cell culture that is adequate for production of detectable levels of progeny virus antigen, are of no value for rapid detection of wild-type strains of HAV that grow slowly, if at all, in cell culture. These tests have been used to detect HAV in sewage-polluted drinking water but only after 3 weeks of culturing samples in African green monkey kidney cultures (23). We have turned to molecular hybridization as an alternative rapid and sensitive HAV detection method in an effort to overcome the limitations of the above methods.

Nucleic acid hybridization based on genetic engineering techniques is of increasing value for the clinical diagnosis of viral diseases (11, 18, 20, 28). We have developed a method that uses ^{32}P-labeled cDNA probes to detect HAV in seeded estuarine samples (10). Although the method is of promising sensitivity, a specter of false-positive results is raised by the possible presence of plasmid DNA or homologous sequences of plasmid DNA in sewage-polluted waters in a manner similar to their occurrence in clinical specimens (1, 5, 16, 20). These sequences can lead to false-positive results when cDNA probes are contaminated with vector DNAs.

We attempted to avoid the potential problems associated with the use of cDNA probes by turning to a probe devoid of vector sequences. Development of single-stranded RNA (ssRNA) probes that lack vector sequences has become possible with the introduction of a new in vitro transcription system. ssRNA probes have several advantages over cDNA probes (25; M. K. Estes and T. Tanaka, *in* F. Tenover, ed., *DNA Probes for Infectious Diseases*, in press). (i) RNA-RNA hybrids are more stable than DNA-DNA or DNA-RNA hybrids, allowing use of a higher stringency of hybridization with a concomitant reduction of background reactivity. (ii) ssRNA probes avoid complementary probe sequence competition (a problem in all hybridizations with double-stranded cDNA [dscDNA] probes) and allow greater test sensitivity and speed. (iii) De novo probe synthesis results in a higher specific activity of ssRNA probes. (iv) Last and most important, the existence of two independent promoter sequences (SP6 and T7) that flank each side of the inserted HAV DNA in some transcription vectors makes it possible to generate two separate ssRNAs with opposite but complementary sequences. The ssRNA strand complementary to the HAV genome (cRNA) hybrid-

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izes with HAV RNA. vRNA (the ssRNA strand with sequences identical to the HAV genomic RNA) does not react with HAV RNA. Thus, the vRNA probe serves as an internal control, making it possible to distinguish positive reactions (cRNA probe) from nonspecific hybridization reactions (vRNA).

In this paper, we describe the development of a dot-blot hybridization test that uses cRNA and vRNA probes to detect HAV and to distinguish between positive and false-positive reactions. The sensitivity and specificity of ssRNA probes were determined and compared with the corresponding reactivities of cDNA probes. The potential use of ssRNA probes to detect wild-type strains of HAV in clinical and environmental samples was evaluated with normal stool samples and water samples collected from a bayou in Houston and a tributary of Galveston Bay.

MATERIALS AND METHODS

HAV. Virus obtained by cultivation of strain HM175 in persistently infected African green monkey kidney cultures was concentrated and purified, and physical particle counts were made in an electron microscope as previously described (6, 10). Briefly, physical particle counts were made in a calibrated RCA model EMU 3F electron microscope. Virus on Parlodion (Mallinckrodt, Inc., St. Louis, Mo.) (0.75% in amyl acetate) films formed on agar disks (2% special Noble agar [Difco Laboratories, Detroit, Mich.] in 0.85% saline) containing an original added virus sample was stained with 1% ammonium molybdate and transferred to a grid, and particle counts were made. These particle counts were used to determine the numbers of physical particles immobilized onto membranes for hybridization tests.

The minimum numbers of physical particles required for positive antigen- and infectious-virus-based test results were determined by the correlation of electron microscope counts and assay endpoints (unpublished data). Radioimmunoassay sensitivity was 5×10^5 physical particles per 75-μl test sample, amplified ELISA (A-ELISA) sensitivity was 2×10^5 particles per 75-μl test sample, and radioimmunofocus assay sensitivity was 1×10^3 particles per 200-μl test sample.

Subcloning HAV cDNA into pGEM. Four HAV cDNAs (pHAV1307, pHAV228, pHAV148, and pHAV207) received from John Ticehurst, National Institutes of Health (25), were purified from the bacterial hosts and digested with *Pst*I. The HAV inserts were separated from the vector DNA by agarose gel electrophoresis and were recovered by electroelution as previously described (11). The purified HAV cDNA fragments were ligated with the in vitro transcription vector pGEM-1 (Promega Biotec, Madison, Wis.), which had been previously linearized by *Pst*I digestion. The ligation reaction was carried out by using different ratios of concentrations of the HAV insert and vector DNA (from 1:1 to 50:1) in the presence of T4 ligase. *Escherichia coli* DH-1 was used as the host bacterium for the propagation of plasmid DNA. The colonies of transformed bacteria on L broth agar plates containing ampicillin (50 μg/ml) were screened for the presence of HAV inserts by electrophoretic analysis of DNA samples extracted from small volumes of culture. The orientations of the HAV inserts in the plasmids were determined by restriction enzyme analysis based on the restriction map of the HAV genome (2, 26). These HAV subclones were designated pGHAV.

Preparation of ssRNA probes. The in vitro transcription system of pGHAV that was used contains promoter sequences for SP6 or T7 RNA polymerase in the plasmid. Linearized plasmid DNAs made by cutting at the end of the inserted HAV DNA were used as templates in the transcription reactions. The reactions were performed by using the protocol of Promega Biotec with minor modifications. Briefly, a 50-μl reaction mixture that contained 1 to 4 μg of linearized DNA template, 4 mM NaCl, 40 mM Tris hydrochloride (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiotreitol, 1 mM each ATP, CTP, and UTP, 100 U of RNasin, SP6, or T7 RNA polymerase (5 to 10 U/μg of DNA), and 12 μM [^{32}P]GTP (10 mCi/ml and 410 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) was incubated at 37°C for 1 to 2 h. After incubation, the template DNA was removed by DNase digestion (1 to 2 U/μg of DNA) at 37°C for 15 min in the presence of RNasin (1 U/μl). Unincorporated ribonucleoside triphosphates were removed by running the reaction products over Sepharose G-50 columns in 10 mM Tris (pH 7)–1 mM EDTA–0.1% sodium dodecyl sulfate (SDS). The RNAs were recovered by sodium acetate-ethanol precipitation, suspended in sterile water, and kept at −20°C for use as probes in the hybridization experiments.

Biotin-labeled ssRNAs were generated by using the same technique as above except that biotin-11-UTP (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) replaced the UTP.

Stool samples. Stool samples were collected from children hospitalized at Texas Children's Hospital in Houston. These samples, which had been submitted for testing for viruses by electron microscopy, were prepared as 10% extracts and were extracted with trichlorotrifluoroethane as previously described (6). For analysis in the HAV hybridization assays, RNA from stool samples that lacked viruses by electron microscopy (7) was tested. For RNA purification, 0.5 ml of the Genetron (Fisher Scientific Co., Pittsburgh, Pa.) extracts were extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol]), precipitated with ethanol, and then suspended in 250 μl of 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0). A 50-μl amount was applied to each well for hybridization.

Field sample collection and processing. Estuarine samples to be analyzed for HAV included water, suspended solids, and fluffy sediments from a polluted tributary of Galveston Bay. Freshwater samples were collected from Buffalo Bayou in Houston. Water with suspended solids was treated as follows. Water (≥100 gallons) and solids were pumped into a large tank, and the pH was adjusted to 3.5. $AlCl_3$ was added to a final concentration of 0.0015 M, and the water and solids were pumped out through a 3.0- and 0.45-μm-pore-size filter assembly (Filterite Corp., Timonium, Md.). Viruses and solids that were adsorbed by these filters were recovered by elution with 3% beef extract–10% tryptose phosphate broth–0.05 M glycine, pH 9.5. Filters were held in the eluent for 30 min before backflushing and collection of the eluate for further concentration. Samples of ≥100 gallons of water and suspended solids were also passed through Mikroklean filters (AMF/CUNO, Meriden, Conn.). In these instances, the water pH was adjusted to 6.0 prior to passage through the filters and $AlCl_3$ was not added. The filters were eluted exactly as described for the Filterite filters.

The eluates were reconcentrated by organic flocculation at pH 3.5 (3), precipitation with 8% polyethylene glycol 6000 at pH 7.5 (13), or adsorption with magnetic iron oxide at pH 5.5 (19). The precipitates were suspended in 0.15 M Na_2HPO_4, and 5- to 15-ml final test samples were treated with antibiotics or adjusted to pH 9.0 and immediately filtered through serum-treated, 0.45-μm filters to eliminate microorganisms. The final samples were stored at −70°C until tested.

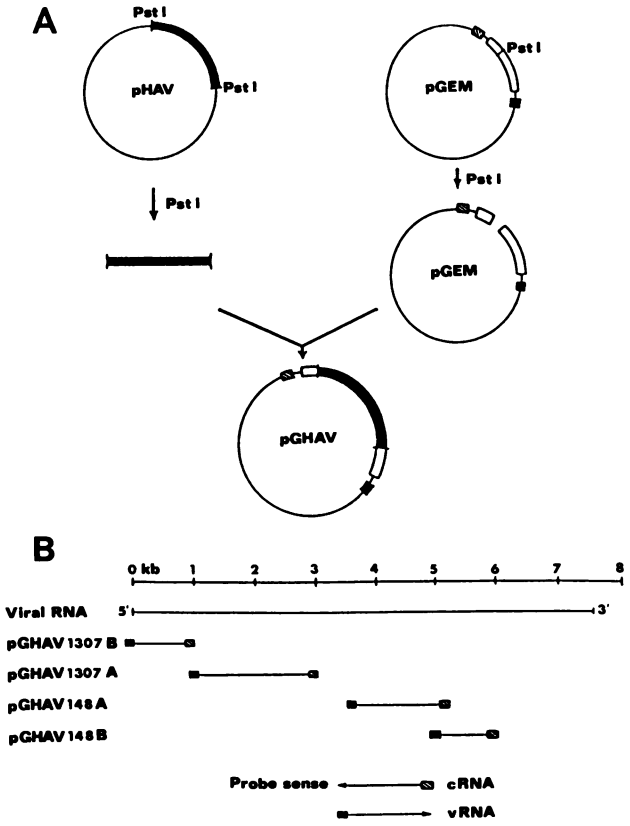

FIG. 1. (A) Construction of pGHAV subclones. (B) Genomic map of four pGHAV subclones. ■, T7 promoter sequences; ▨, SP6 promoter sequences. Negative-sense ssRNA (cRNA) probes were made by using the SP6 polymerase that contained sequences that were complementary to HAV genomic RNA; positive-sense ssRNA (vRNA) probes were made by using the T7 polymerase that contained sequences that were identical to those in HAV genomic RNA. kb, Kilobase.

Dot hybridization with ssRNA probes. Field samples and stool samples were treated with proteinase K (400 μg/ml) for 30 min at 37°C, followed by extraction with phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol]) and precipitation with ethanol as previously described (10). The RNA pellets were suspended in 5× SSC and dotted onto nylon membranes (Zetabind; AMF/CUNO) by using a manifold apparatus with vacuum suction (Minifold; Schleicher & Schuell, Inc., Keene, N.H.). The filters were baked in a vacuum oven for 2 h at 80°C before hybridization.

Hybridization with ssRNA probes was performed at higher stringency than the hybridization with cDNA probes that was described previously (10). Filters with immobilized nucleic acids were prehybridized for 2 h at 50°C in a solution containing 4× SSC, 2.67× Denhardt solution, 0.1% SDS, 1.83 mM EDTA, and 83 μg of tRNA per ml. Hybridizations were performed at 50°C in the same solution but with the addition of ^{32}P-labeled ssRNA (4×10^6 to 8×10^6 cpm/ml). After hybridization, the filters were washed three times in 2× SSC–0.2% SDS and three times in 2× SSC without SDS at 50°C. Air-dried filters were exposed to Kodak XAR film with intensifying screens at −70°C. In some experiments, the radioactivity of each dot was quantitated by counting in a liquid scintillation spectrometer.

Hybridization with a biotin-labeled ssRNA probe was carried out by using a protocol developed in our laboratory. Briefly, viral RNAs were immobilized on GeneScreen Plus filters (New England Nuclear Corp., Boston, Mass.) in the presence of 5× SSC. The filters were air dried and baked in a vacuum oven for 30 min at 80°C. Prehybridization was carried out in a solution containing 5× SSC, 0.5% bovine serum albumin, 0.5% polyvinylpyrrolidone, and 1% SDS at 55°C for 30 min. Hybridization was performed at 55°C for 1 to 2 h in the same solution with 700 ng of biotin-labeled ssRNA per ml. After hybridization, the filters were rinsed three times with phosphate-buffered saline and incubated with Detek I-acp complex (Enzo Biochem Inc., New York, N.Y.) for 30 min at room temperature. The filters then were washed twice with 1× SSC–1% SDS at room temperature, twice with 1× SSC–1% Triton X-100 at 55°C, twice with 1× SSC–1% Triton X-100 at room temperature, and twice with 1× SSC at room temperature (with 5 min for each change). Positive signals were detected according to instructions provided with the Detek I-acp kit.

RESULTS

Production and characterization of ssRNA probes. Seven HAV *Pst*I fragments (1307A, 1307B, 228, 148A, 148B, 207A, and 207B) were subcloned into the in vitro transcription vector pGEM-1 (Fig. 1A). The orientation of the HAV cDNA in the plasmid was determined by restriction enzyme analysis. Four of the seven clones had the same orientation, in which transcription from the SP6 promoter sequences in pGEM-1 resulted in the synthesis of transcripts complementary (cRNA) to the HAV genomic RNA. The other three clones had the opposite orientation. The former four clones (covering 60% of the HAV genome) were used in this study as templates for in vitro transcription to generate ssRNA probes (Fig. 1B).

After in vitro transcription with linearized DNA templates of the four clones and SP6 polymerase, the length of each [^{32}P]GTP-labeled transcript was determined by polyacrylamide gel electrophoresis (8) followed by autoradiography of the wet gel (Fig. 2). The migration patterns of the RNA transcripts showed that each of the RNA products had a molecular weight very close to the predicted molecular weight. An extra, small-molecular-weight band was seen in the RNA transcripts of pGHAV148A. This band was reproducible, although the mechanism of generating it is unknown.

A specific activity of 6.6×10^8 cpm/μg of RNA was expected by in vitro transcription with [^{32}P]GTP (410 Ci/mmol) (protocol no. 1, Riboprobe system transcription of cloned DNA; Promega Biotec). In our experiments, we usually generated 1 μg of ssRNA with a specific activity of 3×10^8 to 4×10^8 cpm/μg from a 50-μl reaction mixture containing 2 to 4 μg of template DNA and incubated at 37°C for 1 h. Based on our optimal conditions of hybridization, this amount of RNA is suitable for testing at least 500 dots. If maintained at 4°C, probes in the hybridization solution could be reused at least five times without loss of hybridization effectiveness.

Comparison of hybridization parameters with ssRNA and dscDNA probes. Hybridization tests with both ssRNA and cDNA probes were compared to determine the optimal conditions of hybridization with the ssRNA probes. We first determined profiles of the thermal kinetics of association after hybridization with ssRNA and dscDNA. Zetabind strips with immobilized HAV particles (6.7×10^7 physical particles per dot) were hybridized at different temperatures

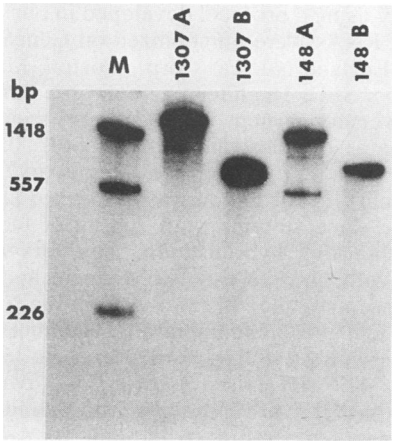

FIG. 2. Characterization of transcription products of pGHAV. In vitro transcription of pGHAV was carried out with linearized DNA templates by cutting at the 3′ end of the HAV inserts in the presence of SP6 RNA polymerase and [^{32}P]GTP. After incubation at 37°C for 30 min, the reaction mixtures were heated at 65°C for 5 min. The transcription products were immediately loaded onto a 5% polyacrylamide gel and were electrophoresed for 16 h. The resulting wet gel was exposed directly to X-ray film for 45 min. The marker (M) bands were RNA transcripts of DNA templates that were purchased from Promega Biotec, and the in vitro transcription was performed by using the same conditions as for pGHAV. Sizes are given in base pairs (bp).

for 12 h in a solution containing the same amount (4×10^6 cpm/ml) of either ssRNA or cDNA probes (Fig. 3). After the filters were washed, the radioactivity bound on the filters (examined by autoradiography) was quantitated by counting in a scintillation counter.

Higher signals (a peak of 1.1×10^3 cpm detected by the ssRNA probes as compared with a peak of 2.9×10^2 cpm detected by the cDNA probes) were observed at each temperature. Two curves were obtained when the percentages of counts per minute detected by the two probes were plotted. The apparent differences at the peak reaction as well as at the 50% reaction between the two probes suggested that a higher stringency of hybridization with ssRNA probes could be used to increase the test specificity without loss of sensitivity. A hybridization test in 50% formamide at 42°C has been proved to be optimal for dscDNA probes. We used the same concentration of formamide but increased the temperature to 50°C for hybridization with the ssRNA probe, and our results showed that this condition was optimal.

We performed a kinetic experiment to determine the optimal input of ssRNA for hybridization. Duplicates of purified HAV RNA were dotted onto Zetabind filters and were hybridized with the ssRNA probes at different concentrations for different periods of time (Fig. 4). At 12 h posthybridization, the detected signals reached a maximum for all four conditions of probes used. Significant differences of signal detected by the various probe concentrations were observed.

Acceptable background on all the filters that were tested with ssRNA probes was seen, even at an input concentration of 1.6×10^7 cpm/ml, although this was not the case when high concentrations of dscDNA probes were used (6; data not shown). As the kinetics of hybridization with ssRNA probes follows a first-order reaction, a high probe concentration (8×10^6 cpm/ml or higher) is recommended to facilitate detection sensitivity and speed.

We tested the sensitivities of the two types of probes to detect HAV by using the optimal condition for ssRNA probes determined in these studies and that for dscDNA probes described previously (10). The specific activities of the ssRNA and the dscDNA probes used were 3.1×10^8 and 1×10^8 cpm/μg, respectively. The level of detection of HAV by the dscDNA probes (Fig. 5) confirmed our previous results. The sensitivity of ssRNA probes was much higher (approximately 3.9×10^4 physical particles for a single probe and 1.8×10^4 physical particles for pooled probes) and the background on the filters was lower compared with those of the dscDNA probes. We also developed a biotin-labeled ssRNA probe that had a sensitivity comparable with that of

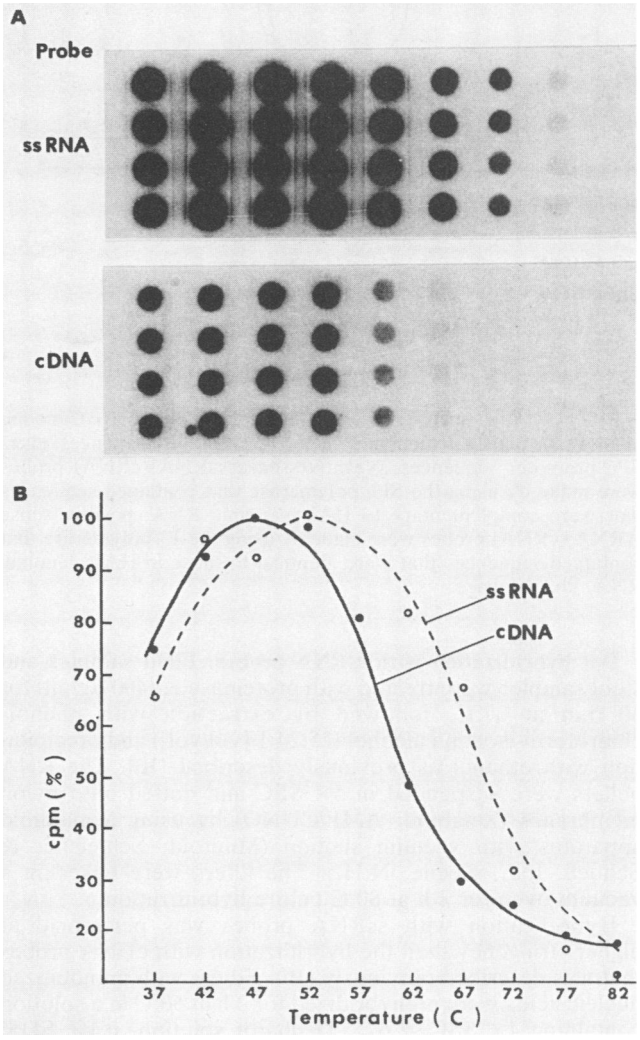

FIG. 3. Thermal association profiles of ssRNA and dscDNA probes. Duplicate strips containing immobilized HAV RNA (6.7×10^7 physical particles per dot) were hybridized at various temperatures with ^{32}P-labeled ssRNA and dscDNA probes in the solution described in Materials and Methods. The specific activities of the ssRNA and dscDNA probes were 3.1×10^8 and 1×10^8 cpm/μg, respectively. After hybridization, each strip was examined by autoradiography (A) or the percentages of counts per minute detected at each temperature were determined and plotted (B). The counts that were 100% for the ssRNA probe were 1.1×10^3 cpm, and those for the cDNA probe were 2.9×10^2 cpm.

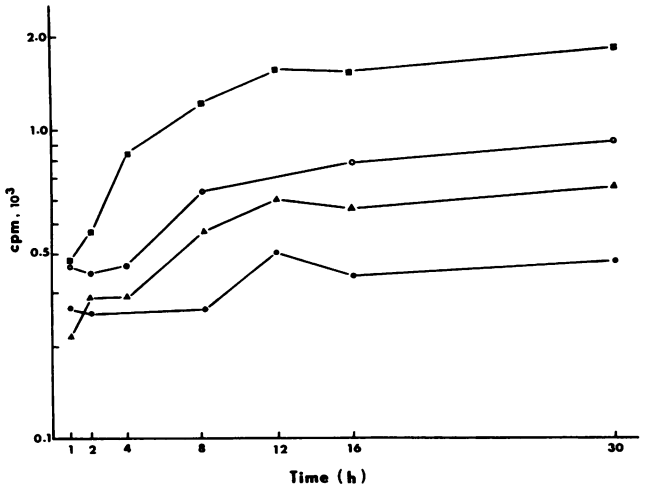

FIG. 4. Kinetics of hybridization with ssRNA HAV probes. Purified HAV RNA was immobilized on Zetabind filters and hybridized with a ^{32}P-labeled HAV ssRNA probe at different concentrations (●, 2 × 10^6 cpm/ml; ▲, 4 × 10^6 cpm/ml; ○, 8 × 10^6 cpm/ml; ■, 1.6 × 10^7 cpm/ml) with the stringency described in Materials and Methods. Duplicate strips were removed at each time point posthybridization, washed, and examined by autoradiography and scintillation counting.

a ^{32}P-labeled dscDNA probe but not with that of a ^{32}P-labeled ssRNA probe (Fig. 5).

Test specificity with the ssRNA probes. The specificity of the HAV ssRNA probes was determined by hybridization with homologous and heterologous nucleic acids as well as

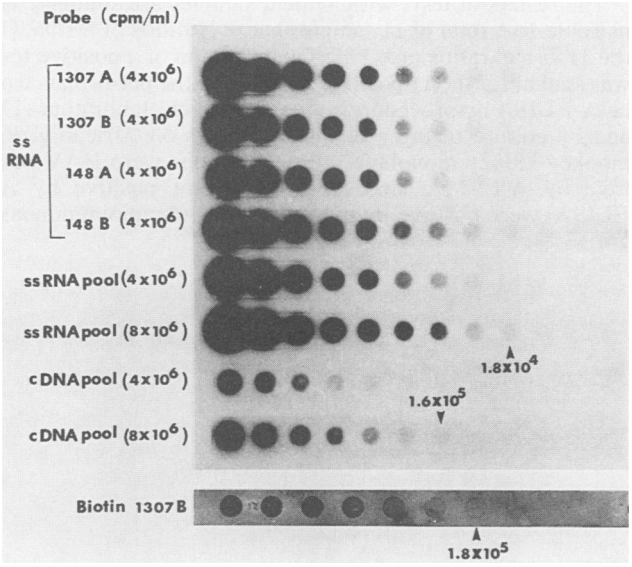

FIG. 5. Test sensitivities of HAV ssRNA and dscDNA probes. Twofold dilutions of purified HAV (10^7 physical particles per 100 μl) were loaded onto a Zetabind filter. Duplicate strips were hybridized with the four ssRNA probes (Fig. 1), separately or pooled, or with four pooled dscDNA probes at the indicated concentration (counts per minute per milliliter) of probe input. The hybridization was carried out as described in Materials and Methods, with hybridization temperatures at 50°C for the RNA probes and 42°C for the cDNA probes. The specific activities of the RNA and cDNA probes were 3.1 × 10^8 and 1 × 10^8 cpm/μg, respectively. One strip was hybridized with a biotin-labeled ssRNA probe.

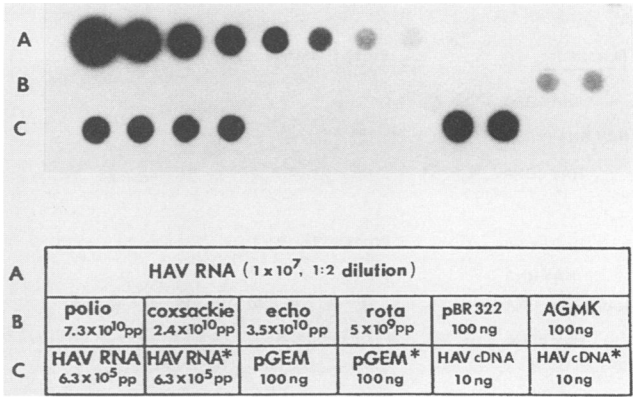

FIG. 6. Specificity of HAV ssRNA probes with homologous, heterologous, and vector nucleic acids. The origin and amount of nucleic acids or physical particles of virus immobilized in each dot are shown in the bottom panel. Asterisks indicate the samples that were treated with DNase (2.1 U per 100 μl of sample) by digestion at 37°C for 30 min prior to loading onto the filters. AGMK, African green monkey kidney cells; pGEM, pGEM-1.

with vector DNAs. The ssRNA probes showed specific hybridization with purified HAV RNA and with HAV cDNA but did not react with either heterologous viral RNAs (poliovirus, coxsackievirus, echovirus, and rotavirus) or vector DNAs (pGEM-1 and pBR322) (Fig. 6). A slight reaction was observed between the ssRNA probe and cellular RNA. This nonspecific reaction in HAV detection was not seen by differential hybridization with both positive and negative probes (data not shown; discussed below).

By taking advantage of the two different promoter sequences (SP6 and T7) flanking each side of the HAV inserts in pGHAV, we were able to generate separately two strands of HAV ssRNA with opposite polarities. One strand of the RNA transcripts was complementary to the HAV genome ([negative-sense] cRNA probe), and the other strand was identical to the HAV genome ([positive-sense] vRNA probe). As expected, the cRNA probe showed a highly specific reaction with HAV RNA, whereas the vRNA probe did not hybridize with the viral RNA at all. Both probes reacted equally with the dscDNA (Fig. 7A).

The differential reactivities of the two probes with HAV RNA suggested that this difference would be useful for evaluating the specific detection of HAV. Graphical plotting of the counts hybridized with the cRNA and vRNA probes (Fig. 7B) showed the consistent low backgrounds observed with the vRNA probes. These plots suggested the use of a positive/negative (P/N) ratio for data analysis that is commonly used for serologic tests. We used a P/N ratio in the dot-blot hybridization as an endpoint titration of positive results. P and N represent the counts per minute of a sample detected by a positive probe and a negative probe, respectively. A good correlation was seen between the concentration of HAV and the P/N ratio after hybridization of purified HAV duplicates with both cRNA and vRNA probes (Fig. 7B).

To determine the negative (background) and the cutoff values of the test, 130 stool extracts were tested for HAV. These samples had been taken from hospitalized children and were submitted for electron microscopic analysis. All of the stool samples were negative for virus detection by electron microscopy. The P/N values from testing these samples are shown in Fig. 8. The average P/N ratio was 0.96,

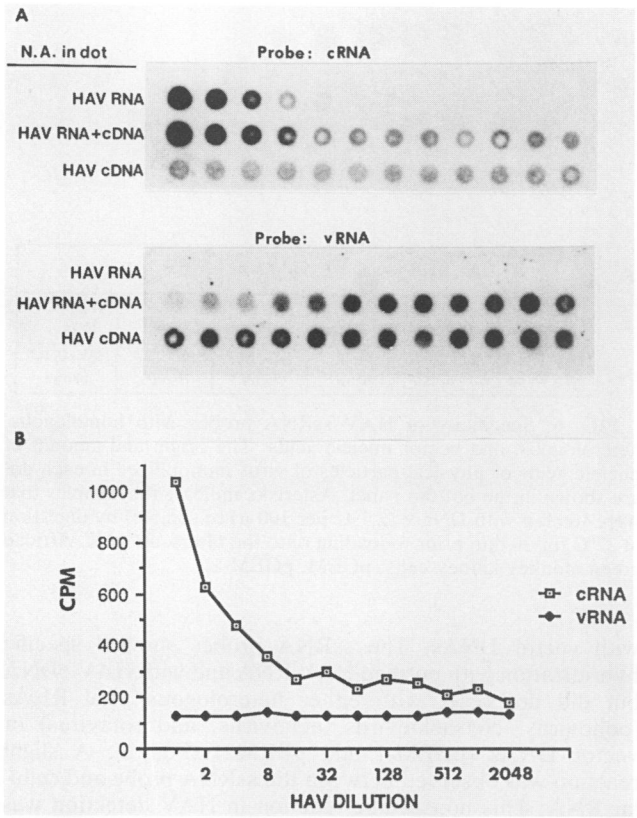

FIG. 7. Use of positive- and negative-sense probes to evaluate test specificity. Twofold dilutions of HAV RNA alone (top row), a constant amount of HAV plasmid dscDNA (bottom row), or mixtures of these (middle row) were dotted onto Zetabind filters and hybridized with a positive (complementary strand of HAV RNA) probe and a negative (genomic strand of HAV RNA) probe. After hybridization, autoradiographs of the filters were obtained (A), and the radioactivity determined in each dot was plotted for reactivity with the HAV RNA (B). N.A., Nucleic acid.

and the standard deviation (SD) was 0.30. The range of the individual ratios under 3 SD (99.73% of the population) was $0.06 < \mu < 1.86$. Of the 130 stool samples tested, only one sample had a P/N ratio of >1.86 (3.49). When the hospital record of this child was analyzed retrospectively, it was found that this child had hepatitis. Analysis of this sample by immune electron microscopy with specific anti-HAV serum also demonstrated virus particles (data not shown). The P/N ratios of all other samples were <1.86. In the hybridization of the purified HAV samples, amounts of 2×10^4 physical particles or greater had P/N ratios of >1.86 (with the exception of one point for the 1:64 dilution). This P/N value was used as a threshold for positive results in the field study, and our results suggested that it is a good criterion for specific HAV detection.

Detection of HAV in field samples. Field samples collected from April to October 1986 were prepared and tested in duplicate with both cRNA and vRNA probes. Representative dot-blot results for 18 field samples on filter paper are shown in Fig. 9. Visual examination shows 8 strongly reactive and 2 weakly reactive results with the cRNA probe and 15 weakly reactive results with the vRNA probe. Three samples showed no reactivity with either probe, and five samples that were weakly reactive with the vRNA probe

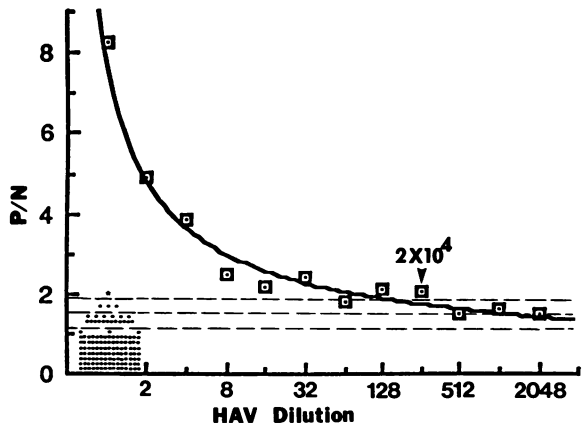

FIG. 8. Hybridization of stool samples with positive and negative probes. Twofold dilutions of HAV RNA and the nucleic acid in 130 stool samples were dotted onto Zetabind filters and hybridized with the positive (complementary strand of HAV RNA) and the negative (genomic strand of HAV RNA) RNA probes. After hybridization and autoradiography, the radioactivity in each dot was determined and a P/N ratio of each duplicate dot was calculated and plotted. P, Counts per minute detected by the positive probe; N, counts per minute detected by the negative probe. ··, Distribution of P/N ratios of the 130 stool samples tested by both positive and negative HAV ssRNA probes; – –, 1, 2, and 3 SD (from the bottom to the top) determined from the normal stool sample population; *, sample from the child subsequently found to have hepatitis.

showed no reactivity with the cRNA probe. Although the data are not shown, P/N ratios of >1.86 were obtained for each of the 8 strongly reactive samples, whereas P/N ratios of <1.2 were obtained for the remaining 10 samples.

The results of tests with 43 field samples are summarized in Table 1. A total of 11 samples had P/N ratios of ≥1.96. Of the 11, 7 had ratios of ≥3.17. Confirmation of a positive test was sought for the 11 positive samples by the performance of an A-ELISA by procedures developed in our laboratory (17) and by culture of three of these samples on African green monkey kidney monolayers. Four samples were HAV positive by A-ELISA, and two others (not positive by A-ELISA) were positive by hybridization and radioimmunoas-

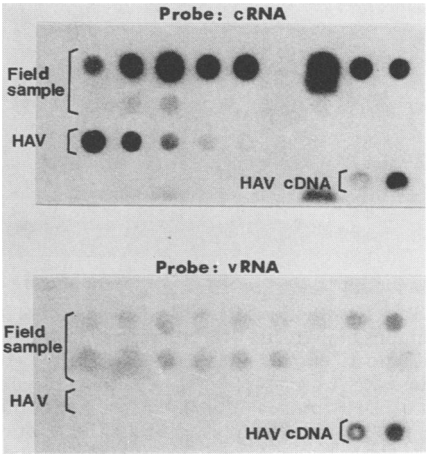

FIG. 9. Hybridization of field samples with cRNA and vRNA probes. The nucleic acid from 18 field samples was dotted on the Zetabind filters and hybridized with cRNA or vRNA probes. HAV RNA and HAV cDNA were included as positive controls.

TABLE 1. Detection of HAV in water samples by hybridization with ssRNA probes

Location[a]	Date sample collected[b]	P/N[c]	Detection of HAV
Cypress Creek	4/24	1.19	−
	5/20	1.30	−
	6/4	0.93	−
	7/2	0.71	−
Clear Lake	4/9	2.29	+[d]
	4/9	2.31	+
	4/9	3.44	+
	4/17	8.45	+[e]
	4/17	5.65	+[e]
	4/29	1.22	−
	4/29	3.18	+
	7/24	0.97	−
	8/1	0.91	−
	8/1	0.74	−
	8/1	0.51	−
	8/5	0.76	−
	8/14	1.19	−
	8/14	1.32	−
	8/14	0.57	−
	9/29	0.83	−
	9/29	0.96	−
	9/29	1.03	−
	9/29	1.02	−
Seabrook	6/11	3.70	+
	6/11	1.64	−
	7/10	1.30	−
	7/10	1.06	−
	9/11	0.96	−
	9/11	1.19	−
Buffalo Bayou	9/15	0.89	−
	9/15	0.84	−
	9/15	1.20	−
	9/15	1.64	−
	9/22	3.17	+[d]
	9/22	3.23	+
	9/22	0.49	−
	9/22	1.52	−
	10/6	1.96	+[d]
	10/6	2.14	+[d]
	10/20	1.36	−
	10/20	1.80	−
	10/20	0.92	−
	10/20	0.77	−

[a] Cypress Creek drains the northwest suburbs of Houston and empties into the San Jacinto River. Clear Lake drains into Galveston Bay at the communities of Seabrook-Kemah, which are located on the western shore of the bay. Buffalo Bayou runs through central Houston and suburbs from west to east and empties into the Houston ship channel. All Cypress Creek samples were water with suspended solids. All Clear Lake samples were water with suspended solids, except for one suspended solids sample per se. All Seabrook samples were water with suspended solids, except for one fluffy sediment sample. All Buffalo Bayou samples were water with suspended solids.

[b] The samples were collected from April through October 1986.

[c] P, Counts per minute detected by cRNA probe; N, counts per minute detected by vRNA probe.

[d] Positive by A-ELISA.

[e] Positive by cell culture.

say tests after two 14-day passages in African green monkey kidney monolayer cultures. Thus, 6 of the 11 hybridization-positive samples were confirmed by A-ELISA, cell-culture-magnified radioimmunoassay, or hybridization tests.

DISCUSSION

In this study, HAV-specific ssRNA probes were used in dot-blot hybridization tests to detect HAV in environmental samples. These probes have been shown to be the most sensitive as compared with radiolabeled dscDNA probes. By using this technique we were able to detect naturally occurring HAV in polluted estuarine and freshwater samples as well as in clinical stool samples.

The ssRNA probe showed a high specificity when it was tested with homologous and heterologous nucleic acids. We did not see a high background when field samples were properly treated with proteinase K followed by extraction with phenol and chloroform. A mild cross-reaction between the RNA probes and cellular rRNA was observed when nucleic acid from uninfected monkey kidney cells was tested by using the cRNA probes. Similar observations have been reported in other viral systems (14, 15), and this was believed to be caused by small homologous regions between viral and cellular RNAs. Our results suggest that this potential problem can be solved by performing simultaneous hybridizations by using cRNA and vRNA HAV ssRNA probes separately with duplicates of dotted samples.

The use of cRNA and vRNA probes showed the ability to distinguish false-positive results in the detection of ssRNA as well as ssDNA viruses. The reaction with the cRNA probes shows specific virus detection, whereas the reaction with the vRNA probes serves as an internal control for background and specificity. The P/N ratio introduced here was found to be a sensitive and specific indicator of virus detection. We have used an endpoint of 3 SD of the P/N ratio based on a study of 130 normal stool samples to determine positive and negative HAV detection in field samples. Although these data are preliminary and this value may not be optimal for environmental samples, good agreement was obtained when HAV samples that were reconstituted in the laboratory were tested and the clinical histories of these children were reviewed.

To our knowledge this is the first study that demonstrates the use of hybridization with positive- and negative-sense probes to detect an ssRNA virus. However, recently, De Chastonay and Siegl (4) reported a study of HAV replication in cells by using the same type of probes. Comparison of results with both probes offers an excellent internal control for test specificity that will continue to be critical in helping develop these methods further and in helping gain confidence in their use for rapid virus detection in environmental, clinical, and food samples. Although this report describes the use of ssRNA probes for HAV, modification and application of this system to detect other RNA virus pathogens (e.g., astroviruses, Norwalk-like viruses, caliciviruses, coronaviruses, etc.) are obvious and will be straightforward as the appropriate probes become available.

Our finding of HAV in the stool sample from a child with clinical symptoms of hepatitis provided further confidence in our assay. In retrospect, this result is not unexpected, as HAV can be excreted from 2 weeks before and up to 16 days after the onset of dark urine (24). Our detection of HAV with these ssRNA probes in this stool sample, in which virus initially was not detected by direct electron microscopy (but was subsequently detected by immune electron microscopy), also confirms the higher sensitivity of hybridization for virus detection. This has been shown recently by using cDNA probes (21, 24). It would be of interest to determine if the ssRNA probes would show different patterns of HAV shedding as compared with results obtained with the cDNA probes (21, 24). Importantly, the demonstration by Tassopoulos et al. (24) and Ticehurst et al. (25) that these HAV probes detect several, if not all, strains of HAV should encourage their use to detect and possibly help prevent HAV

transmission on a worldwide basis. This could be particularly important in developing countries where HAV infection is endemic or in planned vaccine trials to monitor excretion of virus from vaccinees by the most sensitive techniques.

Our long-term goal is to develop a rapid and simple method to detect human enteroviruses in environmental samples for public health safety. For this purpose, it would be optimal to replace radioactively labeled probes with nonradioactive probes. Our current results indicate that radiolabeled or nonradioactive double-stranded probes are not as good as single-stranded probes. For the biotin-avidin detection system, the requirement of denaturation of the dscDNA probe before use for hybridization causes low test efficiency because of the instability of the biotinylated DNA probe under the conditions of denaturation. The use of biotin-labeled ssRNA probes not only avoids the need for denaturation but also allows efficient generation of the probe. However, the sensitivity of this detection system is not as high as that achieved by using radiolabeled ssRNA probes, and further work with nonradioactive probes and environmental samples is needed to eliminate false-positive reactions.

One potential disadvantage of ssRNA probes could be the susceptibility of such probes to digestion with contaminating RNase in the samples. RNase-free equipment and reagents are required for all procedures during the hybridization test. However, in practice, we have not found this to be a problem when an RNase inhibitor is present during the in vitro transcription reactions as well as for storage of the probe. The use of dextran sulfate in the hybridization solution also resulted in reduced degradation of the probe during the hybridization reactions. The use of ssRNA probes is also economical because the probe can be reused several times (if it is carefully preserved) without loss of test effectiveness.

The preliminary data on the use of these probes to detect HAV in environmental samples yielded a somewhat unexpectedly high number of positive results (11 or 43 tested). Some of these positive samples were duplicate water and suspended solid samples taken on the same day at the same location, and some were taken at different times at the same location. We have confidence in these results because (i) not all samples tested at all locations were positive and (ii) the cRNA and vRNA probes gave very clear positive and negative reactions. These samples were also tested for the presence of rotavirus and other enteroviruses, and only some samples contained rotavirus.

The absolute level of HAV pollution in positive field samples was not determined in this study, but it was undoubtedly less than the 20,000 physical particles represented by the 3 SD threshold value. If our unpublished estimate that 1,000 particles of purified HAV are needed for a positive radioimmunofocus assay is correct and applicable to field study conditions, one can speculate that there are 20 infectious particles in these hybridization assays by using 3 SD as the cutoff value. Accurate knowledge of the level of HAV pollution and its significance will require future determinations of (i) the total number of particles, (ii) the number of particles that lack nucleic acid, and (iii) the number of infectious particles present in such samples.

Further use of this technique in the field is clearly required to monitor the prevalence of HAV in such samples and to evaluate the public health significance of a hybridization-positive test. It is interesting that the presence of HAV in the stools of hospitalized patients does not necessarily imply risk of communicability (21, 24). It will be important to determine whether the HAV detected in these environmental samples is infectious or present at levels sufficient to transmit infection and whether its appearance is seasonal. Until this information is known, we believe that initially a positive test should carry the connotation of an existing public health hazard, even though it cannot be determined whether the reacting nucleic acid was associated with infectious virus. We favor an a priori interpretation of the presence of a possible public health hazard in the interest of offering protection against a virus causing a potentially serious illness that cannot otherwise be detected. No method capable of the rapid detection of infectious wild-type strains of HAV is currently available, and no antigen or other direct test with the detection sensitivity of the hybridization procedure exists. In situ hybridization methods may eventually be helpful, but issues of test sensitivity and ability of wild-type strains to replicate adequately in cell culture for the detection of virus must first be resolved. In the meantime, failure to take advantage of a sensitive detection method to warn against a possible hepatitis A illness would be irresponsible, given the well-recognized survival capability of HAV in the environment, the strong likelihood of infectious virus present in samples testing positive, and the low number of virions believed able to cause illness.

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