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Combined Immunoaffinity cDNA-RNA Hybridization Assay for Detection of Hepatitis A Virus in Clinical Specimens

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To apply cDNA-RNA hybridization methods to the detection of hepatitis A virus (HAV) in clinical materials, we developed a two-step method in which a microtiter-based, solid-phase immunoadsorption procedure incorporating a monoclonal anti-HAV capture antibody was followed by direct blotting of virus eluates to nitrocellulose and hybridization with ^{32}P-labeled recombinant HAV cDNA. This immunoaffinity hybridization method is simple and involves few sample manipulations, yet it retains high sensitivity (10- to 30-fold more than radioimmunoassay) and is capable of detecting approximately 1×10^5 to 2×10^5 genome copies of virus. The inclusion of the immunoaffinity step removes most contaminating proteins and thus facilitates subsequent immobilization of the virus for hybridization. It also permits positive hybridization signals to be related to specific antigens and adds a level of specificity to the hybridization procedure. When the method was applied to 23 fecal specimens collected from individuals during week 1 of symptoms due to hepatitis A, 13 specimens were found to be reproducibly positive for HAV RNA by immunoaffinity hybridization, whereas only 11 contained viral antigen detectable by radioimmunoassay.

Wild-type hepatitis A virus (HAV) grows only poorly in cell culture, and primary isolation of the virus is slow and unpredictable (3, 20). Other methods, such as immune electron microscopy (8) or solid-phase immunoassays (22), have therefore been used for the detection of HAV in clinical specimens or in specimens collected from experimentally infected nonhuman primates. Compared with these older immunologically based methods for detection of the virus, the recent application of cDNA-RNA hybridization by using ^{32}P-labeled recombinant cDNA probes appears to offer enhanced sensitivity (21). However, this potential advantage may be offset by the presence in crude fecal suspensions of contaminating nucleic acids or proteins that may result in false-positive hybridization signals (1, 5). Even the use of highly purified cDNA insert probes may not totally overcome this limitation, which is not restricted to the HAV system (1). In addition, the preparation of RNA contained in clinical specimens for hybridization may involve several steps, including denaturation and protease digestion of proteins followed by chemical extraction of viral RNA (21), and thus often entails multiple manipulations of each specimen. This makes the procedure difficult to apply to many samples and is an additional factor hindering the application of hybridization techniques to clinical materials.

We avoided the problems inherent in cDNA-RNA hybridization of grossly contaminated clinical specimens, such as fecal suspensions, by the incorporation of a simple solid-phase immunoadsorption procedure before the immobilization of viral RNA on nitrocellulose paper. The technical simplicity of this procedure is further enhanced by the fact that HAV, when suspended in a medium with low protein content, may be blotted directly onto nitrocellulose paper, without prior protease digestion or extraction with organic solvents, and subsequently is detected by cDNA-RNA hybridization without loss of sensitivity. In this paper, we describe this combined immunoaffinity hybridization approach to the detection of HAV and its application to human fecal specimens.

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MATERIALS AND METHODS

HAV. Cell culture-adapted HAV was purified from infected BS-C-1 cell culture supernatant fluids by polyethylene glycol precipitation and repetitive isopycnic and rate-zonal ultracentrifugation, as described previously (14).

Antibody. Monoclonal antibody to HAV was the generous gift of A. G. Coulepis and I. D. Gust, Fairfield Hospital, Melbourne, Australia (17). Monoclonal antibody to mycoplasma attachment protein was obtained from P.-C. Hu, University of North Carolina School of Medicine, Chapel Hill, and was used as a specificity control.

Clinical material. Fecal samples were obtained from New World owl monkeys (11) and human volunteers (4) undergoing experimental infection with HAV, as well as from individuals involved in natural outbreaks of hepatitis A occurring in Kansas and the Federal Republic of Germany (10). Fecal specimens were tested as 10 to 20% suspensions made in normal saline or Hanks balanced salt solution and clarified at 7,000 × *g* for 30 min.

RNA extractions. Total cellular RNA was extracted from normal and HAV-infected BS-C-1 cells by the guanidinium thiocyanate-cesium chloride method (18). Virion RNA was obtained by treatment of gradient-purified HAV with proteinase K and sodium dodecyl sulfate (SDS), followed by phenol-chloroform extraction and ethanol precipitation (25). For the extraction of RNA from fecal suspensions, samples were subjected to 1% SDS and proteinase K digestion for 30 min at 37°C, followed by extraction with phenol-chloroform and subsequent blotting to nitrocellulose.

Solid-phase immunoaffinity purification of HAV for hybridization. Individual wells of polyvinyl chloride "U" bottom microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 100 μl of murine ascitic fluid (K3-2F2) containing monoclonal antibody to HAV (17) diluted 1:1,000 in 50 mM sodium carbonate buffer (pH 9.6). After 4 h of incubation at 37°C, 100 μl of 1% bovine serum albumin, also

diluted in carbonate buffer, was added to each well, and the microtiter plates were incubated for an additional hour at 37°C. The microtiter wells were then washed twice with phosphate-buffered saline containing 0.05% Tween 80 and loaded with samples (50 to 100 μl) of tissue culture fluids, gradient fractions, or fecal suspensions containing HAV. After an overnight incubation at 4°C, the wells were washed three times with phosphate-buffered saline, and the virus bound to the solid-phase support was eluted by the addition of 100 μl of 0.1 N HCl. After 30 min at room temperature, the eluate was aspirated from the wells and applied directly to nitrocellulose for hybridization as described below.

Blotting of HAV to nitrocellulose paper. RNA extracts were diluted with 3 volumes of 10 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) in 6.15 M formaldehyde (G. Wahl, "Sequences" application update 371, Schleicher & Schuell, Inc., Keene, N.H.) and were heated before being blotted for 15 min at 65°C. For blotting of whole-virus samples, specimens were diluted into either 500 μl of 7.5 × SSC in 4.6 M formaldehyde (blotting buffer, for samples of 50 μl or less) or 3 volumes of 10 × SSC in 6.15 M formaldehyde (for all other whole-virus samples, including HCl-released immunoaffinity eluates). Blotting consisted of direct application to BA85 nitrocellulose paper (Schleicher & Schuell) in the Minifold II slot blotter (Schleicher & Schuell), followed by one wash with 10 × SSC. After air drying for at least 30 min, the nitrocellulose papers were baked at 80°C in a vacuum oven for at least 2 h.

HAV cDNA probes. *Escherichia coli* cultures containing recombinant pBR322 plasmids $pHAV_{LB}1307$, $pHAV_{LB}148$, and $pHAV_{LB}228$ which contain inserts of cDNA complementary to portions of wild-type (6th marmoset passage) HM-175 strain virion RNA were obtained from J. R. Ticehurst of the National Institute of Allergy and Infectious Diseases, Bethesda, Md. (25). After large-scale culture growth and plasmid amplification by standard procedures (18), plasmid DNA was isolated from lysed *E. coli* cultures by repetitive banding in CsCl-ethidium bromide gradients. HAV cDNA inserts were excised from plasmid DNA by digestion with *Pst*I, electrophoretically separated from pBR322 DNA in a low-melting-point agarose gel (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), and recovered as previously described (7). Three different probes were used for most of the described work. These included probe 1307-2 (approximately 700 bases long, derived from the 5′ end of the HAV genome, and extending approximately 44 bases into the putative VP2 coding sequence), probe 1307-1 (approximately 1,900 bases long, starting 152 bases 3′ of probe 1307-2 and spanning most of the putative capsid sequence region), and probe 228 (approximately 2,200 bases long and spanning the central portion of the genome) (2, 25). ^{32}P-labeled probes were prepared from purified insert DNA by partial denaturation followed by random priming with calf thymus oligodeoxynucleotide fragments and *E. coli* DNA polymerase I (24). Labeled DNA was precipitated with ethanol, dissolved in water, and used directly in the hybridization assay described below.

cDNA-RNA hybridization. Nitrocellulose papers were prehybridized for at least 3 h at 42°C in a solution containing 50% formamide (MCB Manufacturing Chemists Inc., Darmstadt, Federal Republic of Germany), 2.5 × Denhardt solution, 5 × SSC, 0.1% SDS, and denatured calf thymus DNA at 100 μg/ml. For hybridization, the prehybridization solution was replaced with a similar solution containing only 1 × Denhardt solution and ^{32}P-labeled probe at a concentration of 0.5×10^6 to 2.5×10^6 cpm/ml. The probe was boiled for 3 min immediately before dilution into hybridization solutions. Hybridization was performed at 42°C in the presence of 50% formamide with gentle shaking for at least 22 h. The nitrocellulose was washed twice with 2 × SSC–0.1% SDS at room temperature for 15 to 30 min per wash and twice with 0.1 × SSC–0.1% SDS at 52°C for 20 to 30 min per wash. After brief blotting on Whatman 3MM filter paper, the nitrocellulose papers were sealed in plastic wrap and placed in film cassettes containing Kodak XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) and Kodak X-Omatic intensifying screens. The films were developed after exposure for 16 h to 8 days at −70°C. The intensity of hybridization signals was assessed by laser densitometry with an LKB Ultroscan (LKB Instruments, Turku, Finland).

Solid-phase radioimmunoassay for HAV. HAV antigen was detected in fecal suspensions and gradient fractions by a solid-phase radioimmunoassay described previously (15). A positive result was defined in this assay by a specimen/control cpm ratio of 2.1 or greater and was considered specific for HAV in fecal specimens only if positive results were competitively blocked 50% or more after the addition of convalescent chimpanzee serum to the radiolabeled detector antibody. When noted, monoclonal anti-HAV murine ascitic fluid (17), diluted 1:1,000 in carbonate buffer, replaced the polyclonal capture antibody normally used in this assay.

RESULTS

cDNA-RNA hybridization after direct blotting of intact HAV. In an attempt to bypass protease and detergent treatments and phenol-chloroform extractions for the preparation of HAV RNA, we blotted suspensions of purified virions directly onto nitrocellulose. Purified virion suspensions (14) (either untreated or treated with various combinations of proteinase K digestion and SDS denaturation), diluted into 7.5 × SSC–4.6 M formaldehyde blotting buffer and applied directly to nitrocellulose, gave a positive hybridization signal. The intensity of this signal was not enhanced by prior treatment of the virus with either proteinase K or SDS (data not shown). To compare the sensitivity of direct blotting of virus with blotting of RNA prepared by a proteinase K-SDS-phenol-chloroform extraction procedure (25), both methods were applied to a series of dilutions made from sucrose gradient-purified virus (Fig. 1, lanes A and C) and a positive human fecal specimen (Fig. 1, lanes D and F). In several different experiments, direct blotting of virus proved 3 to 30 times more sensitive than blotting of extracted RNA in terms of the highest sample dilution yielding a positive hybridization signal. The lower ratio, 3, was obtained when 1 mM aurintricarboxylic acid, a potent RNase inhibitor, was included in the RNA extraction mixture. Incubation of whole-virus blots with RNase after the baking of the nitrocellulose paper but before the prehybridization step eliminated the positive hybridization signal, indicating that immobilized ^{32}P was due to cDNA-RNA hybridization and not to nonspecific binding or trapping of probe by viral or other proteins (data not shown). By quantitatively comparing blot intensities obtained when probe 1307-2 was hybridized in the same experiment against a known quantity of purified plasmid $pHAV_{LB}113$ DNA (containing sequences identical to those in the probe) (25), the minimal quantity of virus detectable with the direct blot method was estimated to be 1×10^5 to 2×10^5 HAV genome copies, or less than 5×10^3 radioimmunofocus-forming units of virus (12).

Immunoaffinity hybridization. Whereas direct blotting provides a simple and efficient means of detecting HAV in cell

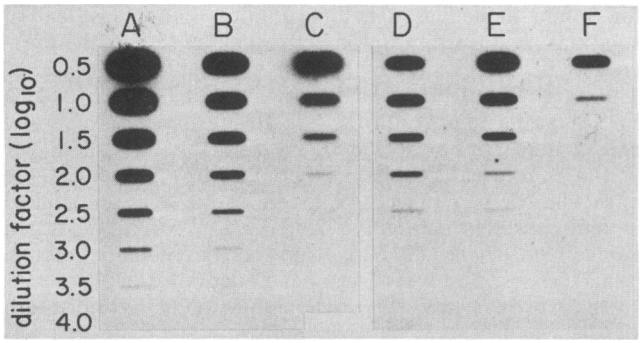

FIG. 1. Comparison of different methods for the detection of HAV RNA in a gradient-purified, cell culture-adapted virus preparation (lanes A to C) and a representative virus-positive fecal specimen (GR8 [Tables 2 and 3]; lanes D to F). Indicated dilutions were prepared for both samples and then subjected to direct blotting (lanes A and D), immunoaffinity hybridization (lanes B and E), or RNA extraction followed by RNA blotting (lanes C and F). The probe was 1307-1; autoradiographs were exposed for 72 h. Undiluted gradient-purified virus was estimated to contain 4×10^9 genome copies per ml, and the 20% GR8 fecal suspension contained about 4×10^8 genome copies per ml (based on a comparison with purified plasmid DNA).

culture supernatant fluids and gradient fractions, its application to the detection of viral RNA in fecal suspensions is not practical. In concentrated fecal suspensions (20%), contaminating proteins and other debris frequently clog the pores of the nitrocellulose paper to such a degree that sample flow is substantially impeded (see below). Moreover, the binding of probe to extraneous proteins and nucleic acids (possibly including common DNA vector sequences) may lead to false-positive hybridization signals (1, 5). These problems were eliminated by the introduction of a simple solid-phase immunoaffinity purification step which removed contaminating materials before blotting. The wells of plastic microtiter plates were coated with monoclonal antibody to HAV (17) at a dilution that was determined to be optimal, and the resulting solid-phase immunoadsorbent was used to bind the virus present in clinical specimens. (In preliminary experiments with a solid-phase radioimmunoassay, we had confirmed that this monoclonal antibody [K3-2F2] was broadly reactive against several HAV strains and capable of efficiently binding the HM-175, PA-21, GR-8, LV-374, and MS-1 strains of the virus.)

Several different elution buffers or denaturing solutions were assessed for their ability to remove virus bound to the antibody-coated solid phase (Table 1). The efficiency of each elution buffer was assessed by direct blotting of the eluate (diluted 10-fold in blotting buffer) to nitrocellulose, followed by hybridization with the HAV cDNA probe, and by reacting the residual virus remaining bound to the solid-phase with ^{125}I-anti-HAV (13). The strongest hybridization signals were achieved when virus was eluted with 1% SDS or 0.1 N HCl, although 0.1 N HCl resulted in more complete removal of virus from the solid phase. Blot intensities achieved after immunoaffinity purification of the virus and elution with 1% SDS or 0.1 N HCl were somewhat greater than those achieved with direct blotting of the virus suspension, and this may reflect the removal of 0.1% bovine serum albumin present in the original virus diluent. Because 0.1 N HCl resulted in more consistent hybridization signals than the use of 1% SDS, 0.1 N HCl was used in subsequent experiments. However, preliminary results suggested that 1% SDS might be a reasonable alternative for use with other, acid-sensitive picornaviruses.

TABLE 1. Efficiency of various buffers in eluting HAV in the solid-phase immunoadsorption procedure

Elution buffer	Blot intensity (%)[a]	Residual bound antigen (cpm) (%)[b]
5% TCA[c]	0 (0)	112 (3)
1 N HCl	0 (0)	72 (2)
0.1 N HCl	1,147 (145)	202 (5)
4 M Urea	410 (52)	2,201 (54)
0.5 M Urea	27 (3)	3,196 (79)
4 M GTC[d]	228 (29)	122 (3)
1% SDS	1,439 (182)	2,091 (51)

[a] cDNA-RNA hybridization signal intensity measured by laser densitometry (percent of blot intensity obtained with direct blotting of the same virus sample). In each case, virus was 50 μl of gradient-purified HAV suspended in 0.1% bovine serum albumin; direct blotting of this material gave a blot intensity of 787. The probe was whole plasmid $pHAV_{LB}148$ (25).

[b] Residual HAV antigen bound to the solid-phase support after the elution of virus detected with ^{125}I-anti-HAV (percent of total virus sample counts per minute [4,066 cpm]).

[c] TCA, Trichloroacetic acid.

[d] GTC, Guanidinium thiocyanate.

The sensitivity of the combined immunoaffinity hybridization procedure was compared with that of direct blotting of virus present in dilutions made from both gradient-purified virus (Fig. 1, lanes A and B) and a positive human fecal suspension (Fig. 1, lanes D and E). These two procedures gave comparable results in terms of the highest dilutions at which viral RNA could be detected, although hybridization signal intensities were somewhat greater with the direct blotting method at the highest dilutions. In terms of virus titer determined by endpoint dilution, both hybridization procedures were 10- to 30-fold more sensitive than a conventional solid-phase radioimmunoassay (15) or a similar assay incorporating a monoclonal antibody (K3-2F2) for antigen capture (Table 2). The difference between the immunoassay and hybridization methods was less impressive with the crude fecal suspension than with gradient purified virus, possibly owing to the presence of viral antigen that was not associated with RNA in the fecal specimen.

Despite the fact that comparable endpoint titers were obtained when the direct blotting and immunoaffinity hybridization methods were applied to the specimens shown in Fig. 1, there was a substantial difference in the blot intensities at

TABLE 2. Sensitivity of direct blot, immunoaffinity hybridization, and solid-phase immunoassays for detection of HAV

Method	Log_{10} endpoint titer with:	
	Purified HAV[a]	Fecal specimen GR8[a]
Immunoaffinity hybridization[b]	>4.0	3.5
Direct blot, hybridization	>4.0	3.5
RNA blot, hybridization	3.0	2.0
Solid-phase radioimmunoassay		
Monoclonal capture antibody	3.0	3.0
Polyclonal capture antibody	2.5	3.0

[a] Purified HAV was estimated to contain 4×10^9 genome copies per ml, whereas fecal specimen GR8 contained about 4×10^8 genome copies per ml.

[b] The probe was 1307-1; autoradiograph exposure time was 72 h.

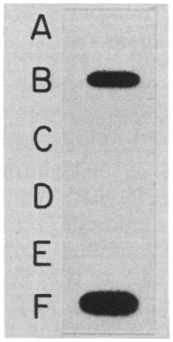

FIG. 2. Specificity of the cDNA probe and the immunoaffinity hybridization procedure for HAV. Slots A and B represent DNA-DNA hybridization in which DNA from 2×10^7 *E. coli* cells was immobilized on nitrocellulose and hybridized with the HAV insert probe. (A) *E. coli* containing plasmid pBR322. (B) *E. coli* containing the related plasmid $pHAV_{LB}1307$ (25). Slots C to E represent immunoaffinity hybridization blots of a normal, virus-negative fecal specimen spiked with 8×10^7 (C), 4×10^8 (D), or 2×10^9 (E) CFU of *E. coli* containing $pHAV_{LB}1307$ per ml. Slot F is a direct blot of cell culture supernatant containing approximately 10^4 radioimmunofocus-forming units of cell culture-adapted HAV (12). The probe was 1307-1; autoradiographs were exposed for 72 h.

the lowest dilution of the fecal specimen. At this dilution, at which the concentration of fecal contaminants is maximal, significantly greater blot intensity was achieved with the immunoaffinity procedure than with direct blotting (Fig. 1, lanes D and E; 0.5 log dilution). This difference was not evident when the applied virus was first gradient purified (Fig. 1, lanes A and B). The reduced signal achieved with direct blotting of the concentrated fecal specimen was in part due to difficulties encountered in filtering it through the nitrocellulose but possibly was also related to contaminating proteins or nucleic acids or both interfering with the binding of filtered virus to the nitrocellulose. Together, these results suggest that the immunoaffinity hybridization procedure may be especially useful for fecal specimens containing amounts of virus that are too low to be detectable by direct blotting after the sample has been diluted to a point at which filtration may be efficiently accomplished.

Specificity of immunoaffinity hybridization. An important advantage of the immunoaffinity hybridization procedure over direct blotting or more conventional approaches to the detection of virus by nucleic acid hybridization is the potential for enhanced specificity afforded by the monoclonal capture antibody. To assess the role played by the capture antibody in determining the specificity of the assay method, the monoclonal antibody coating the solid phase was replaced with an equal amount of murine monoclonal antibody directed against an unrelated antigen (mycoplasma) or carbonate buffer alone. Both modifications resulted in the complete elimination of the hybridization signal achieved with samples containing HAV (data not shown). To further assess specificity, a human fecal specimen that did not contain detectable HAV nucleic acid or antigen was spiked with a suspension of *E. coli* cells containing the plasmid $pHAV_{LB}1307$ (pBR322 with a 2.8-kilobase HAV cDNA insert at the *PstI* site) (25). This mixture was subjected to immunoaffinity hybridization, using probe 1307-1 (Fig. 2, slots C to E). As an additional control, a second sample of the fecal suspension was spiked with an equal amount of *E. coli* containing pBR322 (with no HAV insert) and tested in a similar fashion. In these experiments, the addition of 2×10^9 CFU of either bacterial strain per ml to the normal fecal suspension did not result in a positive hybridization signal, confirming a high degree of specificity conferred by the immunoaffinity step. When DNA was extracted from both strains of *E. coli* (2×10^7 CFU) and subjected to slot blotting and hybridization with the HAV probe, only DNA from the bacterial strain containing plasmid $pHAV_{LB}1307$ evoked a positive hybridization response (Fig. 2, slots A and B). This result indicated that the HAV cDNA probe had a high degree of specificity for HAV relative to pBR322 sequences. Thus, the overall immunoaffinity hybridization procedure had specificity conferred at two levels, the first being the monoclonal capture antibody and the second being the probe itself.

Detection of HAV RNA in human fecal specimens. Positive results were obtained with the immunoaffinity hybridization procedure when it was applied to known positive fecal specimens containing either strain MS-1 (4) or strain HM-175 (11) of the virus. In addition, virus was detected by this method in fecal specimens obtained from two recent outbreaks of hepatitis A, one of which occurred in North America and the other of which occurred in Europe (10). Thus, the combination of the monoclonal antibody and probe used in the procedure appears broadly reactive with a number of HAV strains. This is not surprising given the conditions of selection of the monoclonal antibody (17) and the degree of conservation apparent in the nucleotide sequence of several different HAV strains (2, 16, 19).

Immunoaffinity hybridization was compared with the solid-phase radioimmunoassay for detection of HAV in fecal specimens collected during week 1 of illness from 23 men experiencing acute hepatitis A (10) (Table 3). Of these 23

TABLE 3. Comparison of immunoaffinity hybridization and solid-phase radioimmunoassay for detection of HAV in human fecal samples

Fecal specimen	cDNA-RNA hybridization blot intensity[a]	Radioimmunoassay P/N ratio[b]
Hybridization position		
GR15	6,419	59.0
GR8	4,582	13.8
GR7	2,694	42.4
GR19	2,470	30.0
GR5	2,428	33.8
GR6	131	2.7
GR1	123	21.2
GR4	78	3.1
GR14	64	3.5
GR2	45	1.9 (−)
GR17	17	5.9
GR9	1	2.2
GR18	1	1.8 (−)
GR17	+/−[c]	1.0 (−)
GR21	+/−	2.0 (−)
Hybridization negative		
GR3	−	1.4 (−)
GR10	−	1.1 (−)
GR11	−	1.1 (−)
GR12	−	1.1 (−)
GR13	−	0.8 (−)
GR16	−	1.7 (−)
GR23	−	0.8 (−)
GR24	−	1.0 (−)

[a] The probe was 228; autoradiograph exposure time was 8 days.
[b] P/N ratio, Positive/negative ratio.
[c] +/−, Not reproducibly positive on repeat testing.

fecal specimens, 13 were reproducibly positive for HAV RNA by the hybridization method, whereas 2 others gave weakly positive results that were not confirmed on repeat testing. Two of the 13 reproducibly positive specimens and both of the questionably positive specimens were negative for HAV antigen by radioimmunoassay. Whereas the most intense hybridization signal was generally found with those specimens having the highest radioimmunoassay positive/negative values (highest antigen content), exceptions were noted in both directions (Table 3).

To confirm the specificity of the hybridization results, fecal specimens were tested by immunoaffinity hybridization by using both anti-HAV and an unrelated murine monoclonal antibody immobilized on the solid phase as capture antibodies; positive hybridization signals occurred only with the anti-HAV monoclonal antibody (Fig. 3).

For the sake of comparison, we attempted to directly blot the fecal suspensions. Substantial problems were encountered in accomplishing the filtration of sufficient quantities of these suspensions, owing to their high protein content. Although it was possible to subject 100 μl of each fecal suspension to testing by the immunoaffinity hybridization method, with 5 of the 23 specimens we were unable to completely filter 10 μl of a 20% suspension (even after dilution in 200 μl of blotting buffer). Five other specimens were successfully filtered only after an extended period, and only 6 of the 23 original specimens gave positive hybridization results with this approach (compared with 13 that were positive by immunoaffinity hybridization). Not surprisingly, these six positive specimens were those that were most strongly positive in the immunoaffinity hybridization assay (GR15, GR8, GR7, GR19, GR5, and GR6; Table 3). It is important to note that the greater sensitivity of the immunoaffinity hybridization method, when compared with the direct blotting of fecal specimens, may be largely related to the fact that the method makes possible the testing of a larger volume of the stool suspension.

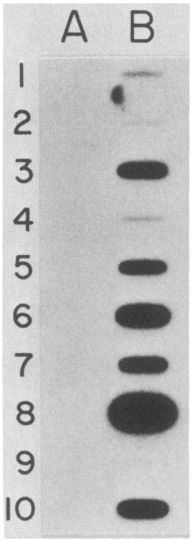

FIG. 3. Immunoaffinity hybridization of HAV present in suspensions of 10 human fecal specimens collected during week 1 of symptoms due to hepatitis A (10). Lane A, Blots of eluates from control microtiter plate wells coated with a murine monoclonal antibody to an unrelated, mycoplasmal antigen; lane B, blots of eluates from microtiter plate wells coated with the anti-HAV monoclone K3-2F2 (17). The probe was 1307-2; autoradiographs were exposed for 8 days.

DISCUSSION

The recent molecular cloning of the HAV genome (16, 19, 25) provided important new reagents that now permit the detection of this virus in cell culture samples as well as clinical specimens by cDNA-RNA hybridization (21). However, the successful detection of viral nucleic acid by hybridization has previously entailed multiple manipulations of individual specimens, and we therefore sought to develop a simplified approach that would facilitate the application of cDNA-RNA hybridization to many specimens.

In comparing different methods for blotting HAV to nitrocellulose for subsequent detection by cDNA-RNA hybridization, we found that the direct application of virus suspended in a 7.5 × SSC–4.2 M formaldehyde buffer resulted in a higher level of sensitivity than the blotting of RNA extracted from samples by a procedure involving SDS denaturation, proteinase K digestion, and extraction with organic solvents (Fig. 1). We suspect that under the conditions used for direct blotting, virions attached to the nitrocellulose are disrupted with the release of RNA onto the nitrocellulose during the baking of the paper. The greater sensitivity of the direct blotting method, compared with the blotting of chemically extracted RNA (Fig. 1), may be related either to the more efficient binding of virus to the nitrocellulose or to the physical loss or degradation of RNA during the extraction procedure. Regarding this latter possibility, preliminary experiments involving the extraction of RNA from virions that were intrinsically labeled with [^{3}H]uridine have suggested that at least 50% of the virion RNA is recovered as acid-precipitable material. Previous investigators have noted the feasibility of directly blotting picornaviruses present in cell culture lysates to nylon membrane filters (23), and this may be a general attribute of this group of viruses. We used nitrocellulose exclusively for the experiments described in this paper. More recent experiments suggest that, whereas nylon filters such as Nytran (Schleicher & Schuell) and Biodyne (ICN Biomedicals, Inc., Irvine, Calif.) may be substituted for nitrocellulose paper in the immunoaffinity hybridization procedure, they may be less efficient in binding intact virions.

Although we have found the direct blotting of virus to be extremely useful in monitoring the release of HAV into cell culture supernatant fluids or in tracking virus during purification procedures (14), it is not generally applicable to clinical specimens, owing to the frequent presence of large amounts of contaminating materials that interfere with the blotting process. We therefore developed a combined immunoaffinity hybridization method in an effort to arrive at a simple and practical yet sensitive method of applying cDNA hybridization to the detection of HAV in clinical materials. The new method offers significantly greater sensitivity than the conventional solid-phase radioimmunoassay for the detection of HAV in fecal specimens collected from acutely infected individuals (Tables 2 and 3). This added sensitivity was achieved with a minimum increase in the number of manipulations for each specimen. In addition, specificity problems that may be encountered in detecting viral nucleic acids in grossly contaminated specimens (1, 5) have been largely overcome by the combined use of highly purified insert probes and the incorporation of a simple solid-phase immunoaffinity purification step before the immobilization of the viral RNA. This additional step significantly enhances the specificity of the hybridization assay (without substan-

tially reducing sensitivity) and permits positive hybridization signals to be related to specific antigens (Fig. 3). At the same time, the immunoaffinity step efficiently removes most contaminating proteins and results in the suspension of HAV particles in a medium that permits their direct blotting to nitrocellulose.

The specific immunoaffinity hybridization procedure we developed for the detection of HAV was intended for use in the research laboratory and is likely to prove useful in future studies of the pathogenesis of hepatitis A in naturally infected humans or experimentally infected nonhuman primates. It is unlikely that any application in the clinical diagnostic laboratory will be found, as the diagnosis of acute hepatitis A is made more rapidly and with greater ease and sensitivity by the detection of virus-specific immunoglobulin M antibodies (6, 13). Nonetheless, with appropriate modifications, the method described in this paper should prove generally applicable to other picornaviruses, including other enteroviruses. Antigenically distinct enteroviruses may share extensive nucleic acid homology, both at the 5′ end of the genome in regions outside the open reading frame and within the 3′ region of the genome which encodes the RNA polymerase (2, 9, 23). This shared homology has been exploited by the successful use of poliovirus and coxsackievirus B cDNA probes for the detection of antigenically unrelated enteroviruses in cell culture (9, 23). The inclusion of a simple immunoaffinity step, such as that described in this paper, before the immobilization of the virus for hybridization would provide a rapid means of establishing the immunologic identity of viruses detected with any of several different probes and could result in substantially improved methods for the detection of picornaviruses in clinical materials. Such an approach would permit the serotyping of enteroviruses and, potentially, the distinction of vaccine strain and wild-type polioviruses at a level of sensitivity offered only by cDNA hybridization.

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