In Situ Hybridization for Quantitative Assay of Infectious Hepatitis A Virus

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A method of in situ hybridization using single-stranded RNA probes of opposite polarity for quantitative enumeration of hepatitis A virus (HAV) in infected cells has been developed. Kinetic experiments showed that foci of infected cells appeared as early as day 2 postinfection. The absence of foci in cells examined immediately after virus adsorption indicated that foci detected subsequently were related to viral replication. Foci were detected by hybridization with RNA probes complementary to HAV genomic RNA but not with RNA probes identical to HAV genomic RNA. The number of foci observed was linearly related to the HAV dose inoculated. Focus formation was reduced when a virus inoculum was pretreated with guinea pig anti-HAV hyperimmune serum but not when it was pretreated with preimmune serum. The high resolution of hybridization signals and relative rapidity of the test indicated that this technique will be useful for measuring serum neutralizing antibodies and for quantitative assay of infectious HAV.

Assay of infectious hepatitis A virus (HAV) for cell culture-adapted strains has depended on immunologically based methods such as radioimmunofocus assay or immunofluorescence tests (2, 4, 8). Fast-growing HAV variants that produce plaques under agar overlay (1, 3) or cytopathic effects under liquid overlay (11) have been reported.

Nucleic-acid-based dot blot hybridization tests have been used to detect cell culture-adapted HAV (6) and naturally occurring HAV (7). However, because dot blot tests do not distinguish between infectious and noninfectious virus, we turned to in situ hybridization for quantification of infectious cell culture-adapted HAV. Results of hybridization-based assays are obtained within 3 to 4 days, which contrasts with reported longer times for radioimmunofocus (6 to 21 days) and for immunofluorescence (7 days) assays. Rapid in situ hybridization assays should facilitate basic biologic studies of what influences interaction(s) between HAV and host cells, survival of virus in the environment, and susceptibility of virus to disinfectants. These assays may also be useful for clinical diagnostic purposes. For example, tests for serum neutralizing antibody from HAV patients or for monitoring development of neutralizing antibodies following vaccine trials can be carried out quickly.

This study describes the development of an in situ hybridization test using HAV-specific single-stranded RNA (ssRNA) probes and the effectiveness of this method for the rapid detection of infectious HAV.

MATERIALS AND METHODS

Cells. Primary African green monkey kidney (AGMK) cells were purchased from Earl-Clay (Novato, Calif.) or from Whittaker M.A. (Walkersville, Md.). Cells were grown in 850-cm² roller bottles in Earle minimal essential medium-L15 medium (50:50, vol/vol) supplemented with 0.075% glutamine and 10% fetal calf serum and containing 100 µg of penicillin and 100 µg of streptomycin per ml. Cells harvested at passage 1 or 2 ($\approx 10^6$ /ml) were stored in liquid nitrogen for later use.

Virus. AGMK cells persistently infected with HAV (strain HM-175) were maintained in minimal essential medium-L15

medium with 2% fetal calf serum at 35°C for 22 days before harvesting. Virus in cell lysates was collected after three cycles of freezing and thawing and was partially purified by chloroform extraction (7). Aliquots of virus were kept at -70°C.

HAV RNA probes. HAV-specific RNA probes were generated by in vitro transcription by using linearized pGHAV 1307B as template as described previously (6). These probes represent 600 bases at the 5' end of the HAV genome (6, 13). Radiolabeled ssRNA probes were produced by incorporation of [³²P]GTP during the transcription reaction, and nonradiolabeled RNA probes were generated by incorporation of biotin-11-UTP (6). Probes with opposite polarities (identical [vRNA] and complementary [cRNA] to HAV genomic RNA) were synthesized in separate reactions by using T7 or SP6 RNA polymerase, respectively.

Infection of cells grown on cover slips. Cover slips were boiled in 0.1 N HCl for 10 min, rinsed with distilled water, and then autoclaved in 0.5% gelatin solution (12). Two types of cover slips were used in this study. Cover slips of 2.5-cm diameter were used for hybridization focus assays with ³²P-labeled probes; cover slips of 1.2-cm diameter were used with biotinylated probes when hybridization was detected by fluorescence staining. In some experiments, hybridization also was performed with cells grown in monolayers on petri dishes; results were similar to those obtained with cover slips, although the cover slips were handled easily compared with the petri dishes.

In situ hybridization. The protocol of in situ hybridization developed by Singer et al. (12) was followed, but with modifications. AGMK cells from liquid nitrogen storage were thawed and seeded on cover slips in 6-well plastic plates. The plates were incubated at 37°C in a 5% CO₂ incubator. After 3 to 4 days of incubation, the cells reached confluence and were inoculated with HAV. Before inoculation with virus, cell monolayers were washed once with phosphate-buffered saline (PBS). The virus inoculum (0.2 ml per 2.5-cm-diameter cover slip and 0.1 ml per 1.2-cm-diameter cover slip) was diluted in 0.01 M PBS, pH 7.2, and was allowed to adsorb to the cell monolayers for 1 h at 37°C in a 5% CO₂ incubator. The inoculum was removed, and the cultures were overlaid with maintenance medium containing

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0.75% agarose (SeaPlaque; FMC BioProducts, Rockland, Maine) at 37°C. After the agarose solidified, the cultures were incubated at 37°C in a 5% CO₂ incubator. The cultures were usually held for 3 to 5 days before processing for in situ hybridization.

The cover slips were harvested by removing the agarose gel from the plates, washing the monolayer once with 0.01 M PBS containing 5 mM MgCl₂, and fixing the cells with 4% paraformaldehyde in PBS-MgCl₂ for 15 min at room temperature. After fixation, the cells were used immediately for hybridization or they were stored in PBS-MgCl₂ solution at 4°C for 1 to 2 weeks. For longer storage, the cells were kept in 70% alcohol at 4°C.

Before hybridization, cover slips were soaked in a solution containing 50% formamide and 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) and then heated at 65°C for 10 min. The hybridization solution contained 2× SSC, 0.2% bovine serum albumin, 1 mg of tRNA per ml, 50% formamide, and 4 × 10⁶ cpm of ³²P-labeled ssRNA per ml. A total of 100 µl of the hybridization solution was put on Parafilm, and a cover slip was carefully laid on top of this solution. Hybridization was carried out at 50°C for 4 h in a humidified chamber. After hybridization, the cover slips were transferred to plates and washed in 2× SSC for 10 min with six changes. The washed cover slips were air dried and exposed to Kodak XAR-5 film (without intensifying screens) at -70°C.

Hybridization with biotinylated ssRNA probes was carried out under the same conditions as described above for the ³²P-labeled probes, except that an avidin-fluorochrome reporter was used to detect a positive reaction. After six posthybridization washes with 2× SSC, the cover slips were incubated for 30 min with 4× SSC, 1% bovine serum albumin, and avidin (2 mg/ml) conjugated to fluorescein isothiocyanate (FITC) (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The cells were then washed three times in 2× SSC at room temperature and air dried. After being mounted in Tris-glycerol (9:1, vol/vol) on microscope slides, the cover slips were viewed with a UV microscope.

Hybridization of HAV transcripts in agarose gels. Confluent AGMK cells in 25-cm² flasks were infected with HAV and harvested at different times postinfection. Monolayers were freeze-thawed three times, and the total nucleic acids were extracted with phenol-chloroform and then ethanol precipitation. These partially purified viral RNAs were electrophoresed on a 1% agarose gel (SeaKem; FMC BioProducts). After ethidium bromide staining, the gel was dried at 60°C for 2 h and then hybridized directly with HAV ssRNA probes. The conditions of hybridization with the dried gel were the same as those for hybridization with nylon membranes described previously (6, 7).

Antibodies. HAV hyperimmune antiserum was prepared in guinea pigs. Virus was grown in AGMK cells, partially purified by chloroform extraction, and then subjected to centrifugation in CsCl density gradients (7). One dose of virus (0.5 ml containing 5 × 10⁹ physical particles per ml) in complete Freund adjuvant was administered intramuscularly, and this dose was followed by three intramuscular boosters of the same virus preparation in incomplete Freund adjuvant at 2-week intervals. Anti-HAV monoclonal antibody (K3-2F2) was purchased from Fairfield Hospital, Melbourne, Australia.

RESULTS

Detection of HAV-infected cells by hybridization with ssRNA probes. In situ hybridization was examined as a way

of detecting HAV-infected cells in the absence of cytopathic effects. Initially, AGMK cover slip cultures infected with HAV were hybridized with biotinylated HAV ssRNA probes. Hybridized probes were detected with an avidin-FITC conjugate. Bright granular-like cytoplasmic staining was seen 2 days postinfection (Fig. 1A). Single-stained cells and foci of stained cells were evenly distributed on the cover slips. No staining was observed in uninfected cells (Fig. 1B) or on cover slips hybridized with vRNA probes (data not shown). These results indicated that in situ hybridization was a rapid and specific way of detecting HAV-infected cells.

Because previous data for dot blot hybridization showed radiolabels to be more sensitive than biotin labels, the effectiveness of ³²P-labeled probes for quantitative enumeration of infectious virus by in situ hybridization was determined. Cover slip cultures of HAV-infected AGMK cells were harvested 4 days postinoculation and hybridized with ³²P-labeled HAV cRNA probes. Autoradiograms of these cover slips showed clear hybridization foci (Fig. 2). At the highest concentration of virus used (dilution of 10^{-1.5}), the entire cover slip monolayer reacted strongly with the probes and discernible foci were not seen. Individual foci became visible at lower virus concentrations. The sizes of foci on cover slips varied from pinpoint to 1 mm. No foci were seen in uninfected cells. Because the background reactivity was very low, more than 500 foci could be counted on a 2.5-cm-diameter cover slip without difficulty. A linear relationship between the dose of virus inoculated and the number of foci seen on each cover slip was observed (Fig. 3). The number of foci observed was highly reproducible.

Specificity of hybridization foci. In all of the experiments described above, foci appeared on cover slips hybridized with cRNA probes but not on those hybridized with vRNA probes, showing in situ hybridization to be highly specific. Test specificity was also evaluated by determining the reduction of hybridization-indicated foci obtained from incubation of HAV with anti-HAV-specific antibody prior to inoculation of monolayers (Fig. 4). Two anti-HAV serum specimens were tested, including a guinea pig hyperimmune serum and a monoclonal antibody, K3-2F2. A preimmune guinea pig serum was used as a control. Complete reduction of foci, which was observed at high concentration of the anti-HAV sera but not with the preimmune serum, indicated that the foci were HAV specific. The neutralizing titers of the antibodies were determined by foci reduction and were 1:10,000 for the guinea pig hyperimmune serum and 1:100,000 for the monoclonal antibody K3-2F2.

Kinetics of hybridization focus development. The kinetics of foci development was determined in HAV-infected AGMK cells harvested at intervals from 0 h to 9 days after infection. No foci were visible at 0 h and day 1, indicating that foci appearing at later incubation times resulted from viral RNA replication and not from direct detection of virus inocula. Foci could be detected on day 2 but were barely visible at that time. Clear foci were observed on day 3 and subsequently to day 9. The number of foci on each cover slip did not increase after 4 to 5 days of incubation, although focus signals were intensified slightly.

The kinetics of HAV RNA replication in infected cells was examined by using a gel hybridization procedure; these results were consistent with those of the in situ hybridization assays. Nucleic acids extracted from HAV-infected AGMK cells from 1 to 15 days postinfection were electrophoresed and hybridized with ³²P-labeled HAV ssRNA. Figure 5 shows ethidium bromide-stained gels and autoradiograms of

FIG. 1. In situ hybridization with biotinylated ssRNA probes. AGMK cells grown on cover slips were mock infected (B) or infected with HAV (HM-175) (A), hybridized with HAV-specific biotinylated ssRNA probes on day 2 postinfection, and then stained with an avidin-FITC conjugate. The hybridized signals were visualized with a UV microscope.

the gels after hybridization with both HAV cRNA and vRNA probes. A band of HAV-specific RNA transcripts was detected with the cRNA probes on day 2 postinfection, and the amount of RNA in the band increased until day 15 postinfection (Fig. 5B, large arrowhead). This band was totally absent from uninfected cells, including those held at 37°C for 15 days. A second band, seen in both infected and uninfected cells which comigrated with cellular 28S rRNA molecular weight markers, also reacted with this probe. This reactivity may have resulted from trapping of the probe by the large amounts of rRNA in the gels. Alternatively, this reactivity may indicate that some homologous sequences exist between the viral genome and cellular nucleic acids.

No virus-specific bands were detected with the vRNA probe, but two bands, representing the 18S and 28S cellular rRNA, were seen with this probe. These bands reacted equally well with the vRNA probe in both infected and uninfected cells.

DISCUSSION

An in situ hybridization technique which uses ssRNA probes to detect and quantify HAV-infected cells in monolayer culture is described. This technique fulfills most of the criteria needed for a reproducible viral plaque assay, including (i) formation of hybridization foci that is viral RNA

FIG. 2. In situ hybridization with ^{32}P -labeled ssRNA probes. AGMK cells grown on 2.5-cm-diameter cover slips were inoculated with PBS (-) or with 0.2 ml of serial dilutions of HAV, as indicated. After virus adsorption, the monolayers were overlaid with agarose-containing maintenance medium. After a 4-day incubation, the cover slips were hybridized with HAV cRNA probes as described in Materials and Methods. After being washed, the cover slips were air dried and exposed to X-ray film overnight at -70°C without intensifying screens.

replication dependent, (ii) absence of focus development in the absence of HAV, (iii) linear relationship between foci observed and quantity of virus inoculated, (iv) specific inhibition of focus formation by anti-HAV antibodies, and (v) assay reproducibility.

The in situ hybridization technique described here has several advantages over immunological assay methods for detection of HAV. First, the technique is rapid, since foci of infected cells can be detected at early stages of virus replication. A minimum of 3 to 4 days is required to complete an in situ hybridization test, whereas the radioimmunoassay (with the same virus strain) requires a longer time (1 to 2 weeks) to obtain satisfactory results. This may be due to the fact that a high copy number of progeny viral genomes is present relatively early in cells. Second, the use of ssRNA probes and high-stringency conditions for hybridization results in a high signal-to-noise ratio in the test. The use of ^{32}P -labeled probes increases the intensity of hybridization signals and makes early detection possible. In addition, the use of vRNA probes as an internal negative control allows direct evaluation of test specificity. Third, the clear resolu-

tion of individual foci on the cover slips contributes to high test sensitivity and accuracy of results. Hybridization of the same cover slips with radiolabeled and biotin-FITC-labeled probes indicated that ^{32}P -associated foci and fluorescing foci were virtually interchangeable and led us to believe that a single infected cell can be detected. Omission of intensifying screens during autoradiography enhances resolution of a test.

The patterns of HAV RNA transcripts detected in infected cells in this study are different from those of a previous report (5). We detected two types of nucleic acid bands in cells. One of these was detected only in infected cells, while the other was observed in both infected and uninfected cells. We believe that the unique band seen only in infected cells represents viral RNA replication, while the bands detected uniformly in both infected and uninfected cells may be due to the existence of homologous sequences between the virus and cellular rRNA, as has been reported for other picornaviruses (9, 10).

FIG. 3. Relationship between hybridization foci and virus inoculum dose. Each point represents the mean of three duplicates, and the bars show the range of values for replicate cultures.

FIG. 4. Hybridization focus reduction by HAV-specific antibodies. Serial dilutions of anti-HAV antibodies were mixed with each HAV inoculum (approximately 100 foci). The mixtures were incubated at 4°C overnight and then at 37°C for 1 h before inoculation onto cells on cover slips. The in situ hybridization test was performed as described in Materials and Methods. P-, Preimmune guinea pig serum; H-, hyperimmune guinea pig serum; MAb, monoclonal anti-HAV antibody K3-2F2.

FIG. 5. Hybridization with nucleic acids in infected cells. Total nucleic acids extracted from HAV-infected AGMK cells were purified at the indicated day postinfection and were electrophoresed on 1% agarose gels. The gels were then dried and hybridized with ^{32}P -labeled ssRNA probes as described in Materials and Methods. Ethidium bromide-stained gels (A) and autoradiograms of the same gels (B) hybridized with cRNA (left) and vRNA (right) probes are shown. The large arrowhead indicates HAV-specific RNA transcripts, and small arrowheads highlight 28S and 18S cellular RNA.

One disadvantage of using radiolabels is the short shelf life of the radiolabeled probes. The effectiveness of biotinylated probes containing FITC reporters suggests that replacement of radioactive probes by nonradioactive probes will be made in the future. Further study of this potential test improvement is being carried out.

Adaptation of in situ hybridization to the detection of natural HAV in clinical or environmental samples depends on the status of infectious virus replicative events. If the limited growth of natural HAV in cell cultures is the result of some biosynthetic block that occurs after RNA replication, then RNA adequate for detection may be present and a positive test may be obtained.

In situ hybridization methods available for use in molecular biology and virology have found only limited application when tritium-labeled probes have been used. These probes are relatively insensitive. The tests are labor intensive, involve multiple steps, require weeks to months to obtain results, and depend on microscopy for visualization of results. The technique described here differs because it is simple, yet sensitive, specific, and accurate. It should be suitable for wide use in molecular biology studies to monitor gene expression in cells when combined with transfection techniques. In addition, the method may be applicable to the study of pathogenesis by allowing direct examination of tissue sections. It promises to be useful for detection of neutralizing antibody in clinical studies as well as for monitoring the development of antibody during vaccine trials.

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