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In Situ Detection of Hepatitis A Virus in Cell Cultures and Shellfish Tissues

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An in situ transcription method was developed to detect hepatitis A virus RNA in both cell cultures and shellfish tissues. Radiolabeled cDNA copies were synthesized in situ by reverse transcriptase-directed transcription after annealing with a specific primer to the viral RNA. Both tritium (^{3}H) and ^{35}S were useful in the in situ transcription reaction, but the use of ^{3}H resulted in a lower background and finer detail in the localization of viral particles. Application of the method to different organs of oysters which had bioaccumulated hepatitis A virus allowed the first in situ localization of the virus, specifically in stomach and hepatopancreatic tissues.

Bivalve shellfish have been implicated as vectors in the transmission of enteric diseases for many decades (13, 21). Periodic occurrences of shellfish-transmitted illness outbreaks have contributed to a public confidence problem over shellfish safety and resulted in important economic losses by the seafood industry (18). Currently, the sanitary quality of marketable shellfish is monitored by analyzing the levels of fecal coliforms in shellfish meat and growing waters (12). However, the accuracy of these bacteria as indicators of viral pollution has been widely questioned (2, 7, 9, 16).

Infectious hepatitis, caused by hepatitis A virus (HAV), is perhaps the most serious viral illness transmitted by the ingestion of raw or inappropriately cooked contaminated shellfish (20). HAV has a worldwide distribution and is endemic in the Mediterranean region and many developing countries (6). The lack of an efficient cell culture system for primary isolation of wild-type HAV has stimulated the development of a number of direct immunochemical methods for the detection of this noncultivable virus in feces and sera (5, 14, 15). However, the majority of these procedures have been unable to detect viral particles in shellfish and estuarine concentrates because of the low levels of virus in these environmental samples.

Recent biotechnology developments are making available rapid, sensitive, and specific tools for detecting HAV, and other food-borne viruses, in shellfish and shellfish growing waters. Gene probes and other nucleic acid techniques such as PCR have recently been used for the detection of enteric viruses in these types of samples (1–3, 11, 24, 25). Another new approach for the localization of different RNA types is in situ transcription (IST) (23). This technique uses the enzyme reverse transcriptase in tissue sections to construct radiolabeled cDNA. Although IST was initially developed to localize proopiomelanocortin mRNA in the rat pituitary, the method has been successfully used to detect coxsackievirus and echovirus RNA in cell cultures (4).

We report here the development of a modified IST procedure to detect HAV in both cell cultures and shellfish tissues. The anatomical localization of HAV RNA within different shellfish organs also was analyzed.

MATERIALS AND METHODS

Viruses and cells. The HM-175 strain of HAV used in this study had been adapted to rapid growth in the FRhK-4 (fetal rhesus monkey kidney) cell line (19). Monodisperse preparations were obtained as previously described (1) and stored at −70°C until used.

FRhK-4 cells were grown on sterile chambered slides (Lab-Tek, Naperville, Ill.) at 37°C in a 5% CO_2 incubator. When the cells reached confluence, they were washed once with phosphate-buffered-saline (PBS) and inoculated with HAV (final concentration, 10^3 PFU/ml). The virus inoculum was adsorbed to the cell monolayers for 1 h at 37°C in a 5% CO_2 atmosphere and then was removed and replaced with complete medium. Cultures were fixed after a 7- to 10-day incubation period by rinsing the slides in 100 mM NaCl and then immersing them in freshly prepared 4% paraformaldehyde in PBS for 1 h. Slides were subsequently dehydrated through a series of ethanol washes, dried, and stored desiccated at −20°C until use.

Shellfish preparations. Oysters (*Crassostrea virginica*), obtained as shell stock from approved commercial sources, were used in viral bioaccumulation experiments as described elsewhere (1, 17). Briefly, virus adsorbed to human feces (approximately 10^6 PFU) was added to 4 to 6 liters of seawater (18 to 25‰ salinity) in an aquarium holding from 6 to 10 shellfish. The feces-associated virus was continuously mixed in seawater by a magnetic stir bar, and the water was aerated by aquarium air pumps. Pulverized algae wafers (Hikari Tropical; Kyorin Co. Ltd.) were added along with feces-associated virus. Bioaccumulation times varied from 4 to 48 h from the time the shellfish were observed to be pumping. After bioaccumulation assays, shellfish were shucked, drained of excess fluid, and

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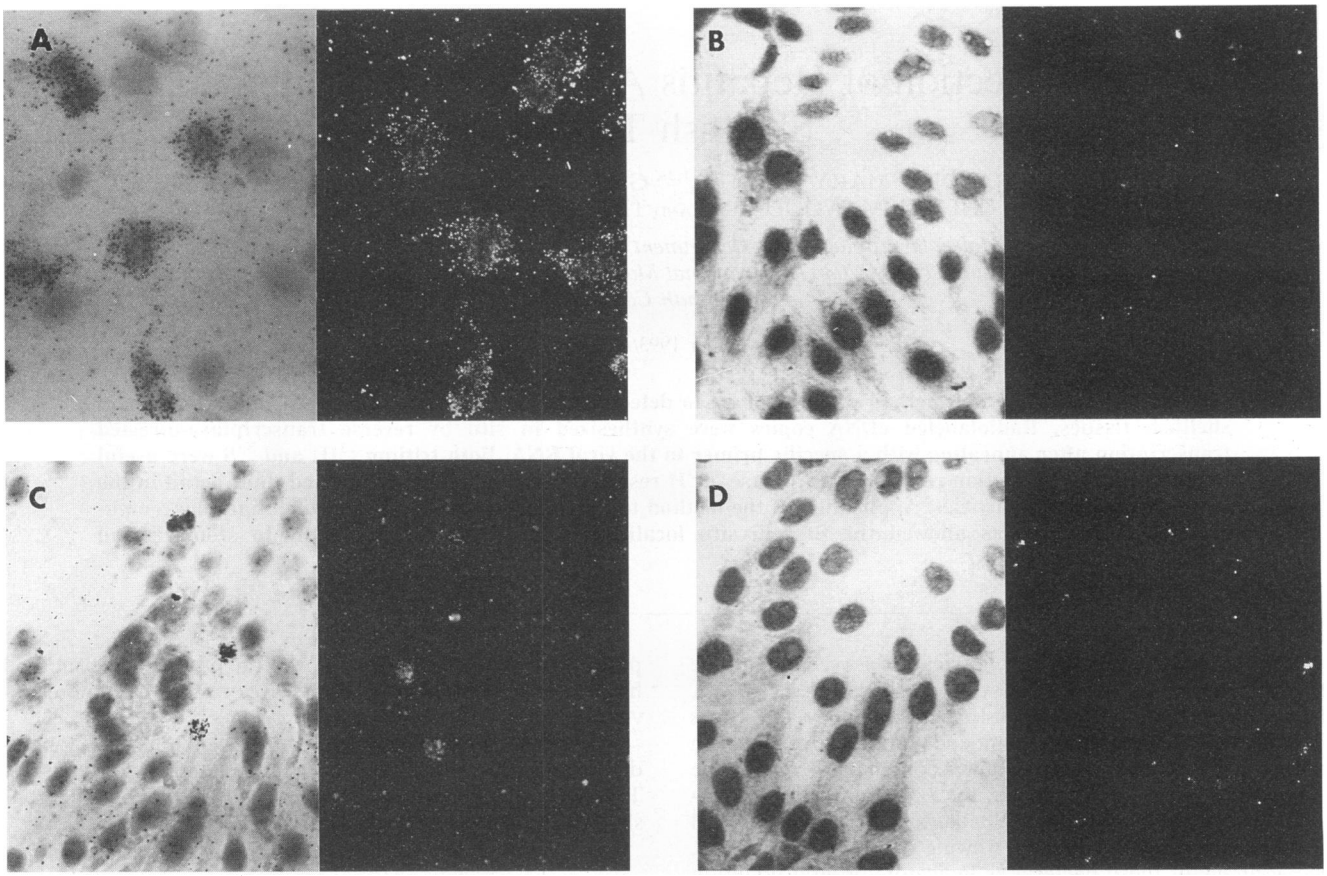

FIG. 1. Detection of HAV RNA in FRhK-4 cells by IST. HAV-specific primer with infected (A) and uninfected (B) cells; no exogenous primer with infected (C) and uninfected (D) cells. Left panels are bright-light photographs, and right panels are mirror images of the corresponding fields with dark-field photography. Magnification, ×292.

dissected to obtain individual tissues (stomach, digestive diverticula, and adductor muscle). Tissues were fixed in 4% paraformaldehyde in PBS for 8 h and embedded in paraffin. Sections were cut from the embedded tissues and affixed to poly-L-lysine-treated glass slides with 1% Elmer's glue (10).

IST. Virus-infected and uninfected cell cultures and oyster tissue sections were subjected to IST according to the procedures of Tecott et al. (23) with some modifications. Basically, cell cultures and deparaffinized tissues were rehydrated in 0.9% NaCl and subjected to sequential treatments with 0.2 N HCl (15 min at room temperature) and proteinase K (20 μg/ml in 50 mM Tris-HCl [pH 7.6]–5 mM EDTA) (7 min at room temperature). After a postfixation step in 4% paraformaldehyde-PBS for 10 min, a specific negative-sense primer (5′-GGAAATGTCTCAGGTACTTTCTTTG-3′), designed to bind VP1 nucleotides (22), was added at a final concentration of 1 ng/25 μl in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)—50% formamide, and annealing was allowed to proceed during a 20-h incubation at room temperature. The slides were washed in 2× SSC for 30 min and 0.5× SSC for 2 h, and then the reverse transcription mix containing RT buffer (50 mM Tris-HCl [pH 8.3], 6 mM $MgCl_2$, 120 mM KCl); dATP, dGTP, and dTTP (each 250 μM); dithiothreitol (7.5 mM); recombinant RNasin (0.12 U/μl); ^{3}H-dCTP (60 μCi/ml; 25 Ci/mmol) (Dupont NEN, Wilmington, Del.); and avian myeloblastosis virus reverse transcriptase (600 U/ml) (Life Sciences, Inc., St. Petersburg, Fla.) was added. Reverse transcription was performed for 1 h at 43°C. The slides then were washed in 2× SSC and 0.5× SSC as before, dehydrated through ethanol washes, and dipped in autoradiography emulsion NTB-2 (Eastman Kodak, Rochester, N.Y.). After a 72-h exposure, the autoradiogram was developed and fixed following the manufacturer's instructions. After counterstaining with 0.6% hematoxylin for 2 to 3 min, the slides were examined under transmitted-light and dark-field conditions with a Zeiss Axiophot microscope.

In some experiments, the hybridization step was omitted and the primer was added simultaneously within the reverse transcription mixture. In some assays, 250 μM of each nucleotide (dCTP, dTTP, and dGTP) and ^{35}S-dATP (300 μCi/ml; 1,000 Ci/mmol) (Amersham, Arlington Heights, Ill.) were employed. Endogenous priming activity was monitored by performing control experiments without adding exogenous primers. All experiments were done at least in triplicate, with serial tissue sections obtained from 12 (4 negative controls and 8 infected) oysters.

RESULTS

Optimization of IST to detect HAV in cell cultures. The IST procedure was developed in FRhK-4 monolayers by examining the presence of HAV RNA. A strong signal was detected in the HAV-infected cells (Fig. 1A) after annealing with the specific

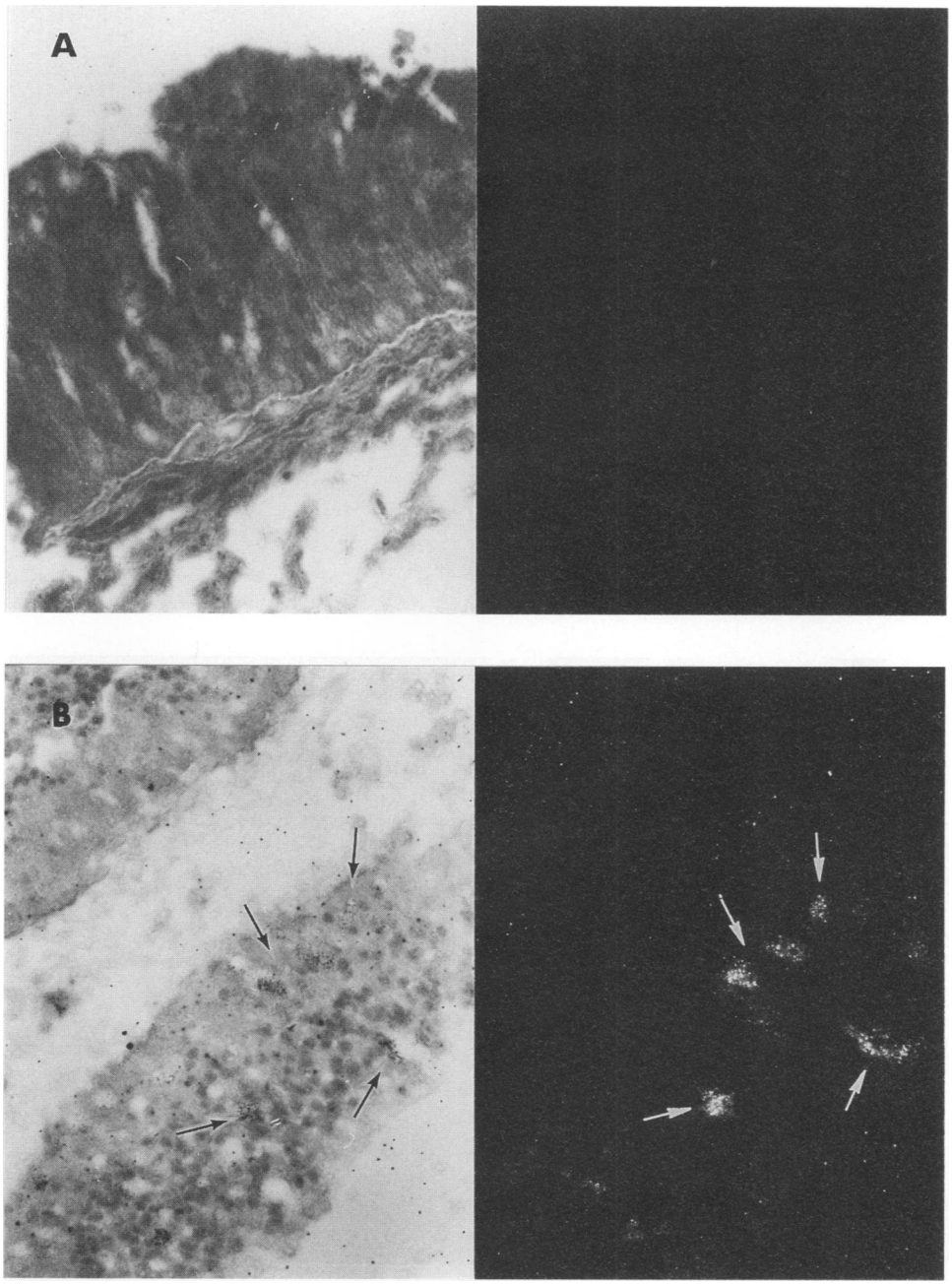

FIG. 2. IST in shellfish tissues. Stomach from a negative control oyster (A) and an experimentally HAV-infected oyster after 48-h bioaccumulation (B). Left panels are transmitted-light pictures, and right panels are the corresponding dark-field photographs. Arrows indicate positive signals. Magnification, ×328.

primer and reverse transcription. No signal above a very weak background was observed in uninfected cells analyzed in parallel (Fig. 1B). Moreover, this signal was not detected when the enzyme, reverse transcriptase, was omitted from the reaction mixture or was temperature inactivated (data not shown). An input of as few as 0.5 to 5 PFU of HAV could be detected in tissue culture monolayers.

The specificity of the IST results was examined in several ways. First, negative control reactions which omitted the primer in the hybridization step in HAV-infected and uninfected cells were performed (Fig. 1C and D). In the infected FRhK-4 cells, a slightly increased background was seen (Fig. 1C), suggesting some endogenous priming could occur. However, this background could be easily avoided by performing a reverse transcription reaction with unlabeled nucleoside triphosphates prior to the specific primer annealing and transcription (data not shown). No endogenous priming activity was seen in the uninfected cells, indicating that it was probably due to duplexes formed during virus replication.

We also compared the use of ^{3}H and ^{35}S as the radiolabeled tracer. Although both isotopes were useful in the detection of HAV by IST, the background level was notably higher with ^{35}S

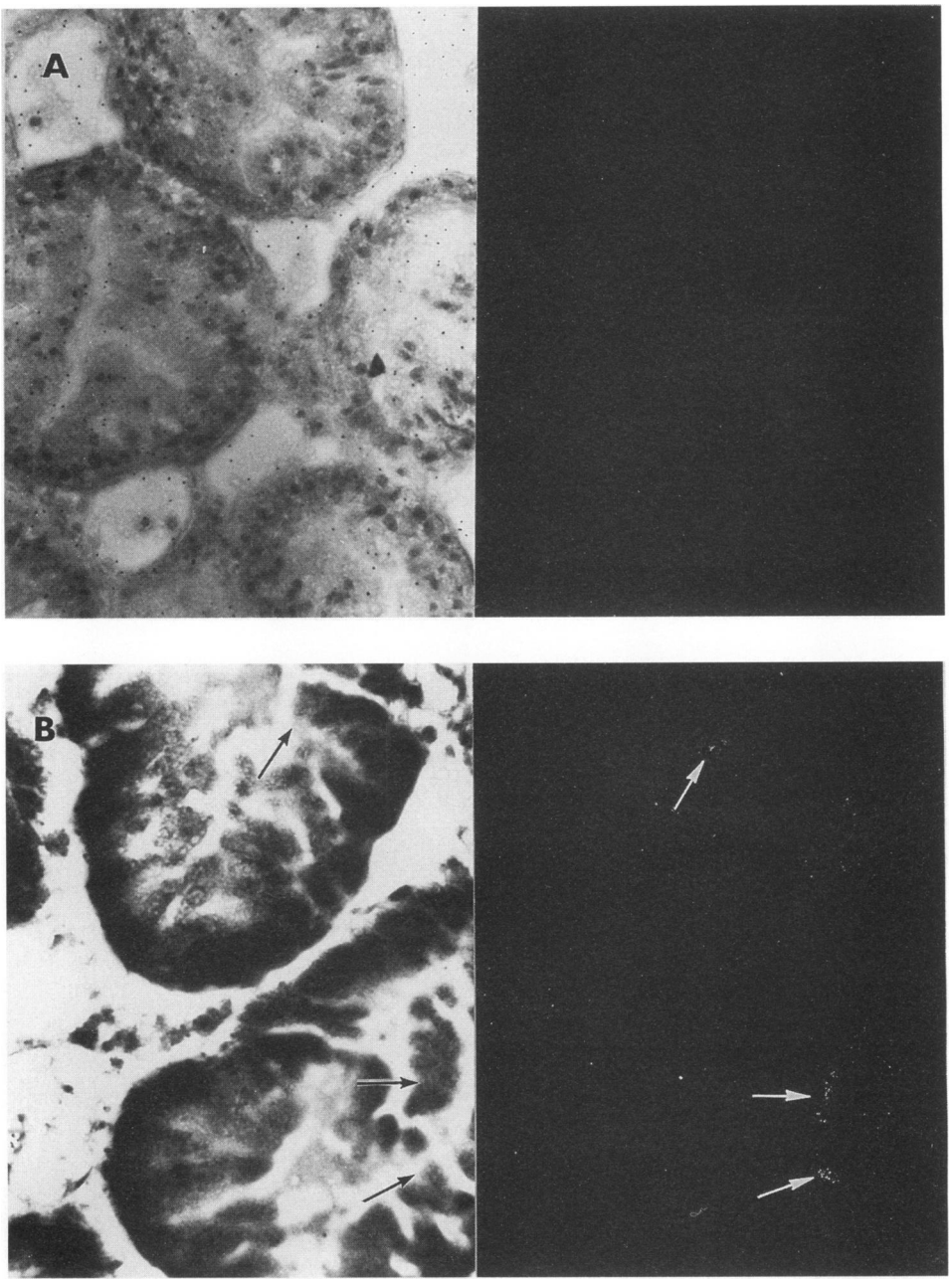

FIG. 3. Localization of HAV RNA sequences in oyster hepatopancreas (digestive diverticula) from a negative control oyster (A) and an experimentally HAV-infected oyster after 24-h bioaccumulation (B). Left panels are bright-light pictures, and right panels are the corresponding dark-field photographs. Arrows indicate positive signals. Magnification, ×328.

than with ^{3}H. In addition, tritium gave a finer grain deposition that facilitated the localization of viral RNA within the cells.

In an attempt to shorten the method, we tried to omit the hybridization step by adding the primer in the reverse transcription mixture. However, in these experiments, the background increased to a level that in some cases was indistinguishable from the positive signal (data not shown). A possible explanation for this fact could be the increase of nonspecific binding of the primer since, in this way, the primer is not washed out from the sections prior to the transcription reaction.

Application of the IST method to detect HAV in shellfish. The optimized method, including the separate hybridization step and the use of tritium, next was carried out in oyster tissues dissected from shellfish subjected to bioaccumulation experiments. No signal was detected in the tissue sections from negative control shellfish (Fig. 2A, 3A, and 4A). In oysters which had bioaccumulated HAV, grain deposition was observed in the stomach and digestive diverticula (Fig. 2B and 3B) but not in the adductor muscle (Fig. 4B). HAV RNA was primarily localized in the internal area of the digestive diverticula, near the lumen, and in the basal cells of the ciliated epithelium of the stomach (Fig. 2B and 3B).

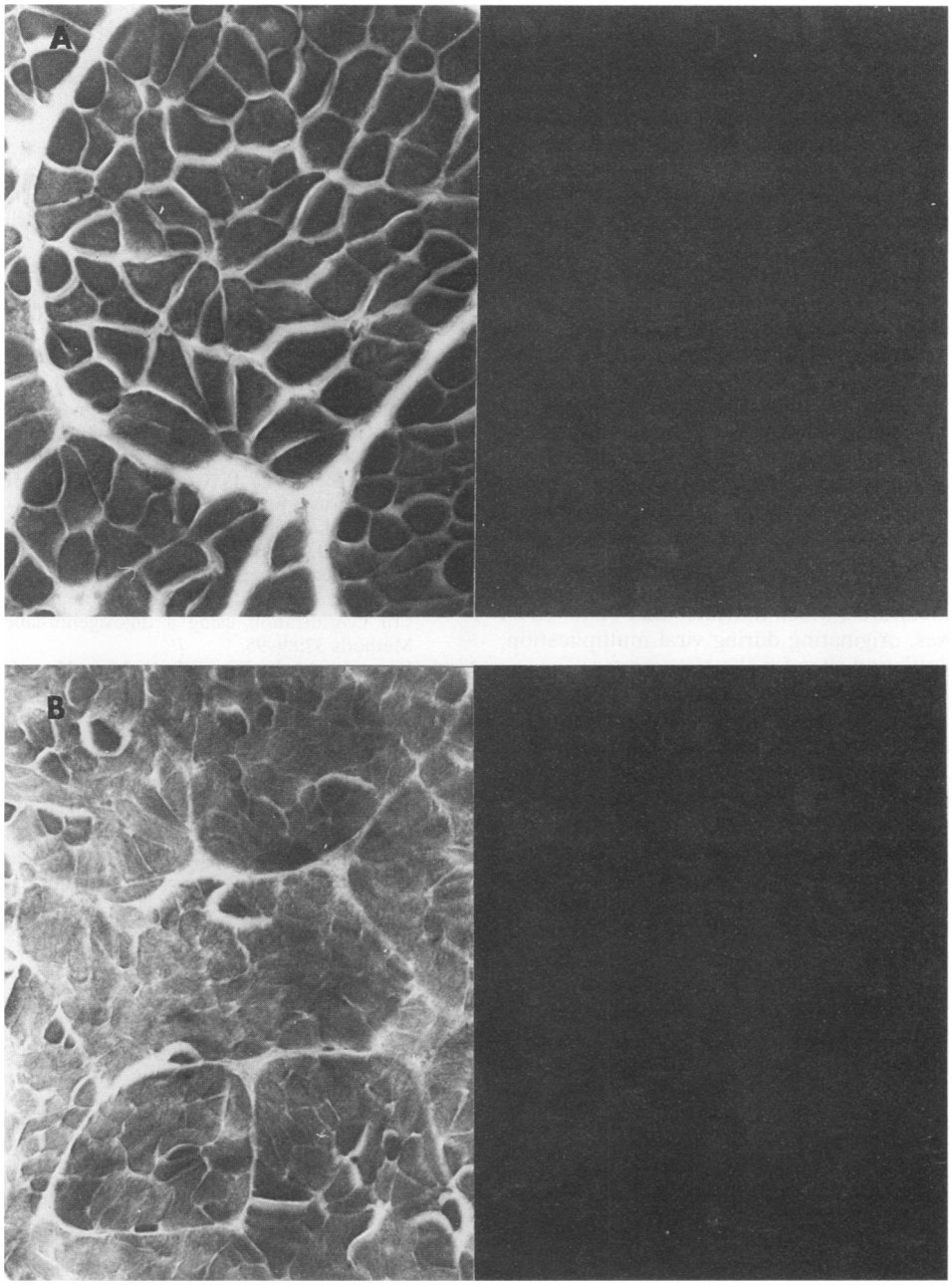

FIG. 4. Localization of HAV RNA sequences in oyster adductor muscle from a negative control oyster (A) and an experimentally HAV-infected oyster after 24-h bioaccumulation (B). Left panels are transmitted-light pictures, and right panels are the corresponding dark-field photographs. Magnification, ×328.

No endogenous priming activity was detected in any of the shellfish tissues analyzed when IST without a specific primer was performed (data not shown). This presumably was due to the fact that HAV does not replicate within the shellfish and, consequently, no RNA duplexes were present in the shellfish tissues. Moreover, the signal achieved in the same sections when the specific primer was present support the specificity of the procedure. The identical localization of the accumulation sites in serial sections of each positive sample (data not shown) also supports the specificity of the method.

DISCUSSION

The importance of developing sensitive and specific methods for the detection of nonculturable human viral pathogens in shellfish is clear if one considers the large number of illness outbreaks caused by shellfish consumption and the serious financial losses by the seafood business due to these outbreaks (18, 20, 21).

This paper reports the establishment of a rapid and reliable procedure for the in situ localization of HAV within cell cultures and shellfish tissues. This method (based on the

synthesis of cDNA copies of the target RNA within fixed tissue sections) achieved excellent sensitivity and specificity. Since no extraction of RNA is needed, it avoids the potential decrease in sensitivity that any extraction procedure must overcome. In fact, it has been reported to be at least as sensitive as in situ hybridization techniques (4), while being more rapid and simple. The method also possesses other advantages, such as versatility, since it can be used for the detection of different shellfish-transmitted nonculturable human viral pathogens by changing only the specific primer in the hybridization step. In addition, IST can be performed on paraffin-embedded stored material, allowing retrospective analyses.

We optimized this IST method by using a growth-adapted HAV strain and fetal monkey kidney cells (FRhK-4). In this system, we observed that the use of tritium improved the results by lowering the background levels and also permitting better localization of the transcripts. These results are in agreement with those obtained by other authors with both cell cultures and tissue sections (4, 23). Endogenous priming activity was detected in some experiments. Others have suggested that any kind of nucleic acid hybrids (DNA-DNA, DNA-RNA, or RNA-RNA) could serve as initiation sites for transcription (23). However, in our case, this activity was seen only in infected cell cultures, which indicates that HAV RNA replication complexes, originating during viral multiplication, were probably the initiation sites for the transcriptase.

When the method was applied to oyster tissue sections, we were able to localize HAV-specific genomic RNA in the stomach and hepatopancreas but not in the adductor muscle. Similar findings were seen in a preliminary study with clams (data not shown). These results are in agreement with those obtained by Enriquez et al. (8) in experimentally contaminated mussels with a cell culture system for HAV detection. However, in their study, these authors divided the shellfish in only two parts, filtration apparatus and digestive system, without analyzing individual tissues. Moreover, these findings support our previous hypothesis, based on observations with other enteroviruses, that the accumulation and sequestering of virus in tissues other than the alimentary tract are possible (16). Studies to evaluate the influence of the bioaccumulation sites on the effectiveness of the depuration processes are in progress.

In summary, the IST procedure reported here provides a powerful tool to study and better understand the viral bioaccumulation dynamics in filter-feeding bivalve mollusks. It also will be useful for direct detection of HAV and other viral pathogens in shellfish to assess the sanitary quality of this type of seafood.

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