Bombyx larva. The PTTH gene transcript was localized to two pairs of dorsolateral neurosecretory cells of *Bombyx* brain (Fig. 2), indicating that PTTH is synthesized by these cells. The same cells react immunohistochemically with a monoclonal antibody that recognizes PTTH (*13*). In *Manduca sexta*, two pairs of brain neurosecretory cells at a similar position have been immunohistochemically identified as the PTTH-producing cells (*14*).

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5. The library was further screened with a ^{32}P-labeled DNA fragment that was derived from the P1 clone and contained nucleotides 34 to 776 of Fig. 1b and an extra 34-bp sequence at the 5′ end, an inverted duplicate of the nucleotides 34 to 67 generated as a cloning artifact. Hybridization and washing were performed at 60°C in the presence of 6× standard saline citrate (SSC) (0.9*M* NaCl, 0.09*M* sodium citrate) and 2× SSC, respectively. Four positive clones (C2, C9, C19, and C21) were obtained. Nucleotide sequencing of these clones disclosed two types of cDNA, P1-type (P1, P2, C2, C9, and C19) and P4-type (P4 and C21). Sequencing was done by the dideoxynucleotide method.
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12. *Bombyx* brains were fixed for 0.5 to 1 hour in a fixative containing 85% ethanol, 4% formaldehyde, and 5% acetic acid at room temperature, and washed in PBS containing 15% sucrose at 4°C overnight [C. E. Bandtlow, R. Heumann, M. E. Schwab, *EMBO J.* 6, 891 (1987)]. After dehydration, the brains were embedded in paraffin wax, sectioned at 7 μm, and pretreated for hybridization as described [E. Hafen, M. Levine, R. L. Garber, W. J. Gehring, *ibid.* **2**, 617 (1983); P. W. Ingham, K. R. Howard, D. Ish-Horowicz, *Nature* **318**, 439 (1985)]. The DNA fragment of clone P1 that was used for screening the library (see legend to Fig. 1) was subcloned into Eco RI–Hind III sites of Bluescript-SK vector (Stratagene), and ^{35}S-labeled RNA was synthesized with T7 RNA polymerase. Final specific activity was 1.0×10^9 dpm/μg. The probe was used at a final concentration of 200 ng per milliliter in 50% formamide, 5% dextran sulfate, 2× SSC, 1× Denhardt's solution, 10 m*M* dithiothreitol (DTT), and yeast tRNA at 0.1 mg/ml. After hybridization to probe, the slides were washed in 0.1× SSC for 1 hour at 50°C and treated with ribonuclease (RNase) A at 20 μg/ml in 0.5*M* NaCl, 10 m*M* tris-HCl, *p*H 8.0, and 1 m*M* EDTA for 30 min at 37°C. Subsequently, the slides were washed in 0.1× SSC for 1 hour at 65°C. After dehydration, slides were coated with Sakura NR-M2 emulsion, exposed at 4°C for 5 days, and developed. The tissue was counterstained with hematoxylin.
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Isolation of a cDNA from the Virus Responsible for Enterically Transmitted Non–A, Non–B Hepatitis

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Major epidemic outbreaks of viral hepatitis in underdeveloped countries result from a type of non–A, non–B hepatitis distinct from the parenterally transmitted form. The viral agent responsible for this form of epidemic, or enterically transmitted non–A, non–B hepatitis (ET-NANBH), has been serially transmitted in cynomolgus macaques (cynos) and has resulted in typical elevation in liver enzymes and the detection of characteristic virus-like particles (VLPs) in both feces and bile. Infectious bile was used for the construction of recombinant complementary DNA libraries. One clone, ET1.1, was exogenous to uninfected human and cyno genomic liver DNA, as well as to genomic DNA from infected cyno liver. ET1.1 did however, hybridize to an approximately 7.6-kilobase RNA species present only in infected cyno liver. The translated nucleic acid sequence of a portion of ET1.1 had a consensus amino acid motif consistent with an RNA-directed RNA polymerase; this enzyme is present in all positive strand RNA viruses. Furthermore, ET1.1 specifically identified similar sequences in complementary DNA prepared from infected human fecal samples collected from five geographically distinct ET-NANBH outbreaks. Therefore, ET1.1 represents a portion of the genome of the principal viral agent, to be named hepatitis E virus, which is responsible for epidemic outbreaks of ET-NANBH.

THE ABILITY TO SEROLOGICALLY DIagnose viral hepatitis caused by infection with hepatitis A virus (HAV) or hepatitis B virus (HBV) led to the recognition of other viral hepatitis agents transmitted either by the percutaneous (blood) or the fecal-oral routes (*1*). Viral hepatitis resulting from viruses other than HAV or HBV, other well-characterized viruses, or predisposing conditions has been referred to collectively as non–A, non–B hepatitis (NANBH) and until recently has been a clinical diagnosis of exclusion. The molecular cloning of a parenterally transmitted form of viral NANBH, referred to as hepatitis C virus (HCV), has recently been de-

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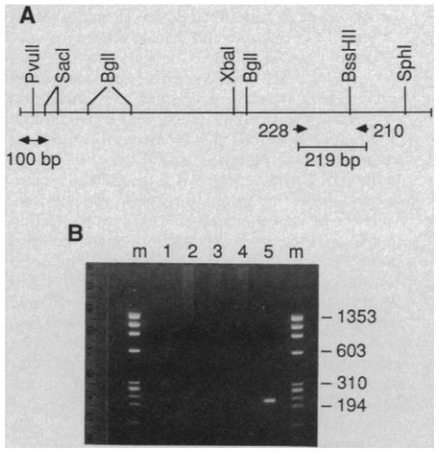

Fig. 1. ET1.1 is an exogenous sequence. (**A**) A restriction endonuclease map for the ET1.1 clone and the position of primers (228 and 210). (**B**) Cellular DNAs prepared from the livers of infected (lane 1) and uninfected (lane 2) cynos, normal human fetal liver (lane 3), a common laboratory strain (1088) of *E. coli* (lane 4), and clone ET1.1 as a positive control (lane 5) were tested by direct PCR with the indicated primer set. Oligonucleotides were synthesized by an Applied Biosystems 380 B DNA Synthesizer and purified for use in PCR with disposable Applied Biosystem purification cartridges (Applied Biosystems, Foster City, California). The sequence of the specific primers was as follows: primer 210: 5′-GGGCCCCAATTCTTCT-3′; primer 228: 5′-TTTTCAGGTGGCTGCC-3′. Standard PCR with ET1.1 primers was performed with 1 μ*M* of the specific primer and 1 μg of the indicated test DNA or 1 ng of the positive control template (clone ET1.1). The reaction conditions used for melting (94°C, 1 min), annealing (37°C, 2 min), and extension (72°C, 4 min) were repeated for 30 cycles. The PCR products were analyzed on 1.5% agarose gels.

described (*2*) and, by virtue of its bloodborne and other routes of transmission, is the recognized cause of the majority of NANBH in the developed world. A second epidemiologically distinct form of NANBH, transmitted by the fecal-oral route, has been documented as causing extremely large epidemic outbreaks of viral hepatitis, particularly in areas with inadequate public sanitation or malnourished populations (*3*). This form of hepatitis has been referred to as water-borne hepatitis, epidemic hepatitis, or enterically transmitted non–A, non–B hepatitis (ET-NANBH) (*3*). ET-NANBH is a major public health problem in those areas of the world where it is endemic. Outbreaks of ET-NANBH can generally be traced to contaminated water supplies. The unique properties of this NANBH pathogen, aside from its ability to cause major epidemic outbreaks, include its associated mortality in pregnant women, ranging in some studies to as high as 20% (*3*). Our strategy for isolation of a molecular clone of ET-NANBH involved using the cynomolgus macaque. The experimental transmission of ET-NANBH has been described in this macaque with detection of characteristic 27- to 34-nm virus-like particles (VLPs) and the induction of serologically confirmed ET-NANBH (*4, 5*). In the absence of any concomitant infectious process in the liver and biliary system, bile collected directly from the gall bladder of infected animals should have a lower nucleic acid sequence complexity than samples collected after passage into the intestinal tract. Human fecal material from a Burmese patient had been used to inoculate cynos (first passage) (*5*). A 10% w/v stool suspension prepared from pooled second-passage cyno #37 feces was used as an intravenous inoculum for third-passage infection of cynos as described (*5*). Gall bladder bile collected at necropsy from one third-passage animal (cyno #121) was found by immunoelectron microscopy (IEM) to contain large numbers of morphologically characteristic 32- to 34-nm VLPs that were serologically specific for ET-NANBH (*6*). This virus-enriched gall bladder bile induced serologically confirmed ET-NANBH in two cynos after intravenous inoculation of 1:10 diluted bile preparation (*6*). Both cyno recipients developed antibody to the VLPs found in bile and expressed virus-specific antigen in their livers (*7*). No other morphologically distinct VLPs were detected by IEM in gall bladder bile obtained from cyno #121; this bile was used for cDNA cloning.

As it had been previously postulated that the virus of ET-NANBH was an RNA virus (*8*), the identification of a molecular clone from bile would involve the construction of a cDNA library (*9*). The ET-NANBH cDNA library was constructed in λgt10 and screened at a low density (10^3 plaques per 150-mm plate) by hybridization to random-primed ^{32}P-labeled cDNA probes derived from either infected (cyno #121) or uninfected (cyno #126) bile. A probe complexity of one part per 10^3 would detect the presence of a specific marker sequence (*10*). As the complexity approached 1:10^4, differential hybridization was incapable of detecting target sequences. We postulated that differential hybridization might, therefore, have the required sensitivity to detect a unique cDNA species when cloning from bile. Approximately 1×10^6 to 2.5×10^6 cpm (5×10^7 to 10×10^7 cpm/μg) of probe was added per filter for hybridization at 37°C, 36 hours in 10% dextran sulfate and 50% formamide (*11*). The application of differential hybridization yielded 16 putative ET-NANBH molecular clones after two cycles of screening. Analysis of 12 of these clones indicated sizes ranging from approximately 75 bp to 2.5 kb (*12*). Of six cloned inserts tested, only the 1.3-kb cDNA derived from clone ET1.1, detected a uniquely hybridizing band in DNA prepared from the ET-NANBH library when compared with normal cyno bile and control cDNA libraries (*12*). It was later determined that ET1.1 cross-hybridized with three other

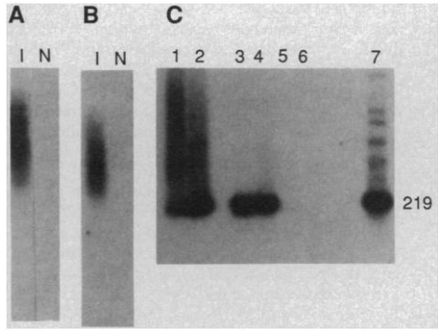

Fig. 2. Hybridization analysis of ET-NANBH cDNA. The cDNA prepared from infected (I) and uninfected (N) cyno bile, was amplified by SISPA (*16*), Southern-blotted (*17*), and hybridized with clone ET1.1 as probe (**A**). A similar experiment was performed with cDNA prepared from infected Mexican (Mex #14) and uninfected human feces (**B**). SISPA-amplified cDNAs were prepared from ET-NANBH fecal material that had been collected from outbreaks in Pakistan (Pak #1; lane 1), Borneo (IM35A; lane 2), Somalia (#020; lane 3), and Tashkent (#1435; lane 4), as well as from a pool of three normal individuals (lane 5). These samples, along with a negative control (primer alone, lane 6), and a positive control with clone ET1.1 (lane 7), were tested after PCR with primers 228 and 210 by hybridization with the expected 219-bp band isolated from the ET1.1 clone (**C**). Feces were prepared as 10% suspensions with phosphate-buffered saline (*p*H 6.6) and then vortexed for 2 min after addition of five 5-mm glass beads (American Scientific Products). The suspensions were clarified by centrifugation in an HS4 rotor (5000 rev/min, 45 min, 5°C). The resulting supernatant was either stored at −20°C or extracted immediately for RNA. Briefly, 0.5 ml of suspension was mixed with 0.5 ml of 2× solution D (*35*), 100 μl of 2*M* sodium acetate, 1 ml of water-saturated phenol, and 200 μl of chloroform:isoamyl alcohol (49:1). After 15 min on ice, the phases were separated by centrifugation (10,000 rev/min, 20 min, 4°C) and the RNA precipitated with two volumes of ethanol. The pelleted RNA was converted to cDNA by a commercially available kit (Boehringer Mannheim Biochemicals) and random priming of the first strand. Portions of cDNA were modified for SISPA by the ligation of AB/linker/primers (*16*). After Nru I cleavage (to remove any linker/primer dimers), an aliquot of the reaction was subjected to 30 cycles of Taq polymerase–mediated amplification. The reaction conditions used for melting (94°C, 1 min), annealing (50°C, 2 min), and extension (72°C, 6 min) included 1 μ*M* of "A" primer (5′-GGAATTCGCGGCCGCTCG-3′) for 1 pg of cDNA. After amplification, 10% of the SISPA cDNA was analyzed by agarose gel electrophoresis and Southern blotting (*17*).

clones initially identified by the differential hybridization procedure. The presence of ET1.1 in the original library indicates that the clone was not an artifact associated with recloning.

These experiments did not, however, directly address the possibility that an induced, endogenous agent might be the source for the cloned ET1.1 sequence. The library hybridization results might also be attributed to minute common contamination of the human feces or cyno bile with bacterial or phage DNA introduced during the numerous manipulations. To test these possibilities, oligonucleotide primers derived from the ET1.1 sequence (*13*) were used in the polymerase chain reaction (PCR) (*14*) to assay various sources for the presence of similar sequences to ET1.1. A restriction endonuclease map of the ET1.1 molecular clone and the localization of these primers is shown in Fig. 1A. Genomic DNAs obtained from infected and uninfected cyno liver, normal human liver, and other bacterial and bacteriophage sources were tested and found negative by this PCR analysis (Fig. 1B). As this procedure would detect less than one copy per cell (*15*), we conclude that ET1.1 is exogenous to the human and cyno genomes and is unrelated to several potential sources of exogenous sequence contamination.

A procedure for the amplification of DNA molecules, independent of their sequence, has recently been developed and was utilized in the analysis of genomic DNA for the presence of ET1.1 sequences. Sequence-independent single primer amplification (SISPA) (*16*), is a primer-initiated, nonselective technique that requires modification of target sequences to achieve amplification of heterogeneous cDNA populations. This approach is designed to amplify messages (as cDNA) derived from minute amounts of material for which nucleotide sequence information is not currently available. In brief, SISPA relies on the directional ligation onto cDNA of an asymmetric (one end blunt and the other staggered), double-stranded linker/primer oligonucleotide (AB), which provides a common end sequence to all cDNA molecules. We used this procedure to evaluate the specificity of clone ET1.1 for ET-NANBH.

As a first experiment we tested cDNA from infected and uninfected cyno bile sources. After SISPA, the amplified cDNA was separated by electrophoresis, and then probed with ET1.1 on Southern blots (Fig. 2A) (*17*). A smear of hybridization was evident only in the SISPA cDNA derived from the infected cyno bile. This result indicates that clone ET1.1 was derived from the infected bile source and again excludes the possibility of a cloning artifact (*9*). The hybridization smear indicates ET1.1 similarity to a heterogeneously sized population of amplified cDNA molecules. The stringent conditions of both the hybridization and wash indicate that ET1.1 hybridized specifically to SISPA cDNA from the infected cyno and exclude the possibility of nonspecific annealing of probe to target.

The testing of human material would eliminate the possibility that the third passage Burma isolate in cyno #121 had induced the expression of a preexisting nonhuman pathogen or that some cyno pathogen other than ET-NANBH had been introduced and serially passaged. Normal (pool of three individuals) and ET-NANBH–infected (Mex #14) human feces were prepared for cDNA synthesis and subsequent SISPA (Fig. 2B). Hybridization of the ET1.1 clone to the ET-NANBH–infected feces again confirmed that the presumptive ET-NANBH clone was specific to the infected source and excluded the possibility of an experimental artifact associated with the sequential passage of VLPs in the cyno.

To determine if a common pathogen was associated with ET-NANBH, additional fecal specimens from five, geographically isolated epidemics were tested by hybridization with ET1.1 after application of the SISPA technique. The presence of the characteristic VLP had been previously confirmed in the primary human fecal specimens obtained from Mexico (Mex #14), Tashkent (#1435), Somalia (#020), and Pakistan (Pak #1) (*18*). In an initial hybridization analysis similar to that shown in Fig. 2B, two ET-NANBH specimens (Tashkent and Borneo) tested positive and uninfected controls negative. All SISPA-cDNAs were subsequently tested by direct PCR with primers

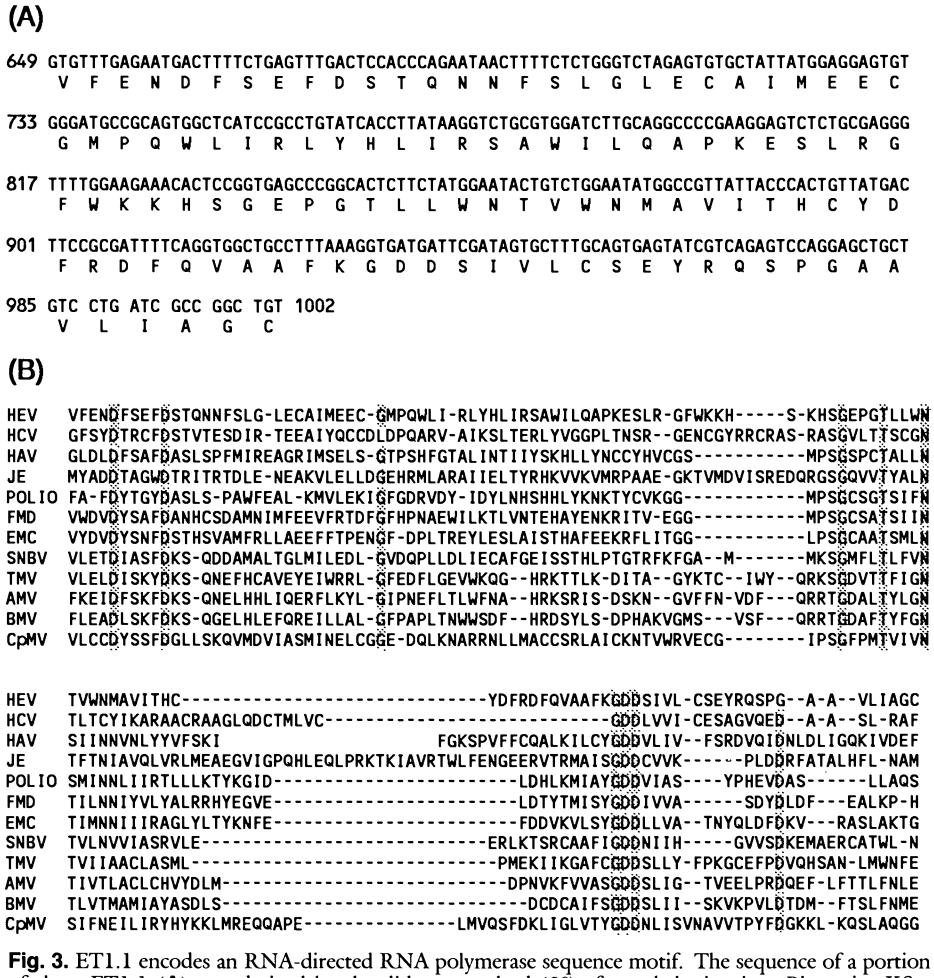

Fig. 3. ET1.1 encodes an RNA-directed RNA polymerase sequence motif. The sequence of a portion of clone ET1.1 (**A**) was derived by the dideoxy method (*29*) after subcloning into Bluescript KS+ (Stratagene, La Jolla, CA). The localization of this sequence within the ~1300-bp ET1.1 clone is indicated by the nucleotide numbering. The translated amino acid sequence was derived by means of the DM program (*30*). Consensus amino acid residues thought to encode the putative RNA-directed RNA polymerase (*20*) are identified by stippling in (**B**) for the hepatitis E virus (HEV), hepatitis C virus (HCV) (*31*), hepatitis A virus (HAV) (*32*), Japanese encephalitis virus (JE) (*33*), polio virus (polio) (*34*), foot-and-mouth disease virus (FMD) (*35*), encephalomyocarditis virus (EMC) (*36*), Sindbis virus (SNBV) (*37*), tobacco mosaic virus (TMV) (*38*), alfalfa mosaic virus (AMV) (*39*), brome mosaic virus (BMV) (*40*), and cowpea mosaic virus (CpMV) (*41*). The Genbank accession number for the sequence of clone ET1.1 is M32400.

(Fig. 1) based on the ET1.1 sequence (*13*). In this analysis, SISPA cDNA of specimens isolated from epidemics in Somalia and Pakistan tested positive by the application of sequence-specific PCR on SISPA cDNAs. The specificity of the appropriately sized fragment was demonstrated by hybridization with the isolated 219-bp fragment primed from the ET1.1 clone (Fig. 2C). These results not only indicate a molecular epidemiologic association between ET1.1 and outbreaks of ET-NANBH, but also strongly suggest that a single conserved agent is responsible for the majority of the ET-NANBH observed worldwide.

A partial nucleotide sequence of the ET1.1 clone is presented in Fig. 3A. A survey of the GenBank version 61-nucleotide sequence library did not show similarity to any entries when searched by the FASTA program (*19*). However, the translated nucleotide sequence exhibited a single open reading frame that is similar to an amino acid sequence motif that is present in all positive strand RNA viruses (Fig. 3B) and is believed to encode the RNA-dependent RNA polymerase (*20*). These initial sequence alignments were verified by the FASTDB algorithm (*21*).

Table 1. Summary of samples with sequence content similar to ET1.1

Source*	Material†	Target‡	ET1.1§
		Cyno	
#37 Pre	Feces	cDNA/SISPA	−P, Hf
#37 Post	Feces	cDNA/SISPA	+P, Hf
#121 Pre	Feces	cDNA/SISPA	−P, Hf
#121 Post	Bile	cDNA/SISPA	+Hi
#126	Bile	cDNA/SISPA	−Hi
#121 Post	Liver	RNA	+Hi
#126	Liver	RNA	−Hi
#121 Post	Liver	DNA	−P, Hf
#126	Liver	DNA	−P, Hf
		Human	
Borneo	Feces	cDNA/SISPA	+P, Hf
Mexico	Feces	cDNA/SISPA	+Hi
Pakistan	Feces	cDNA/SISPA	+P, Hf
Somalia	Feces	cDNA/SISPA	+P, Hf
Tashkent	Feces	cDNA/SISPA	+P, Hf
Normal	Feces	cDNA/SISPA	−P, Hf

*Source: cyno #37 was the recipient of a second-passage fecal inoculum (10% w/v) from an animal that had received the original inoculum from a Burmese patient (*5*). Cyno #37 was the source for the third-passage inoculum used in cyno #121. Cyno #126 was an uninfected negative control. The cDNA preparation was as described (*9*) (see legend to Fig. 2) except for cyno #37 where cDNA was prepared by specific priming with the 233 oligonucleotide (sequence, 5′-AGGTTATGAA-CGAGTCC-3′) (*13*), followed by amplification by SISPA (*16*) and then specific priming with the 210 and 228 primer pairs (see legend to Fig. 1). The origins and infectivity of the original human inocula used in these studies have been previously described (*18*). Pre, preinoculation material; post, material taken after onset of ET-NANBH. †Feces were processed as described in Fig. 2. The extraction of the RNA from liver is described in Fig. 4. ‡The amplification of cDNA by the SISPA protocol (cDNA/SISPA) is described in the legend to Fig. 2. §The presence (+) or absence (−) of ET1.1 sequences was determined by a confirmatory direct hybridization with either the isolated 219-bp PCR fragment (Hf), or the full-length (1.3 kb) ET1.1 insert (Hi) on the indicated target either directly or after specific priming (P) with primers 228 and 210 (Fig. 1).

A transcript of approximately 7.6 kb was detected only in the total RNA from infected cyno liver when hybridized with ET1.1 (Fig. 4). A similarly sized transcript was also detected in polyadenylate-selected RNA (*22*). No other distinctly hybridizing RNA species were identified by ET1.1. Specific hybridization has been detected against two other infected livers as well as to RNA extracted from feces (*13*).

The specificity of the clone for RNA or cDNA from infected humans or cynos is summarized in Table 1. Specifically, clone ET1.1 only identified similar sequences in the feces, bile, and liver taken from infected cyno sources. Additionally, the serial passage of ET-NANBH in the cyno was accompanied by the serial passage of the ET1.1 sequence as demonstrated by a retrospective analysis of ET-NANBH feces prepared from the second passage animal (cyno #37). Cyno #37 was the source for the inoculum used in the third passage studies in cyno #121. The ET1.1 clone, derived from the bile of cyno #121, was also absent from a pre-inoculation fecal specimen collected from cyno #121 (Table 1). Only RNA or cDNA derived from post-inoculation samples (cynos #37 and #121) contained the ET1.1 sequence. Although the RNA from post-inoculation cyno #121 was positive for the ET1.1 sequence (Fig. 4), DNA from the same liver was negative by PCR analysis (Fig. 1B). These findings as well as the absence of specific priming from genomic DNA from infected liver (Fig. 1B) suggests that the infectious agent of ET-NANBH carries an RNA genome, that is not being integrated into host DNA as a provirus. We have subsequently determined by experimentation with strand-specific oligonucleotide probes that the viral genome is a positive sense, single-stranded RNA genome (*13*).

Our results indicate that the virus of ET-NANBH has a polyadenylated, plus-stranded RNA genome of approximately 7.6 kb. No similarity to any known viral or nonviral sequence was detected (*13*) aside from the consensus RNA-directed RNA polymerase motif. As the data indicate that this is a unique viral entity, we propose the name hepatitis E virus or HEV, as previously suggested (*23*). It has been hypothesized that HEV is a member of the calicivirus family (*8*). The virus particle size of 32 to 34 nm (*24*) compares quite closely to that of feline calicivirus particles (*25*). In addition, the sedimentation coefficient of 183*S* for HEV (*24*) is also similar to that determined for the nonenveloped caliciviruses (*26*). The viral particles of HEV, like those of other characterized caliciviruses, have also been found to be sensitive to CsCl (*27*). Other studies have confirmed that HEV is unrelated both antigenically and biophysically to HAV and other picornaviruses (*28*).

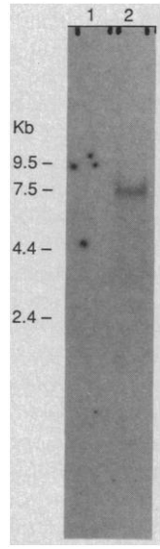

Fig. 4. Northern blot hybridization. RNAs were extracted from the liver of uninfected cyno #126 (lane 1) and infected cyno #121 (lane 2) for hybridization analysis utilizing clone ET1.1 as probe. Total RNA (20 μg per lane), was prepared from cyno liver tissue by solubilizing in 2.5*M* guanidinium isothiocyanate followed by extraction with water-saturated phenol (*42*). The RNA was precipitated with two volumes of ethanol and the resulting pellet was washed in 70% ethanol, dried briefly, dissolved in water, and stored at −70°C prior to electrophoresis on formaldehyde agarose gels (*43*), transfer to nitrocellulose, and hybridization with the ET1.1 clone (10^7 cpm) as the probe.

The detection of ET1.1 sequences in human fecal material collected from diverse geographic and temporally distinct outbreaks of epidemic ET-NANBH suggests that a single virus is responsible for the majority of ET-NANBH seen worldwide. Serological data also support this conclusion, as it has been determined that convalescent serum from one outbreak will aggregate virus from case stool specimens derived from altogether different outbreaks (*5*). This serologic association may be confirmed by the expression of recombinant proteins from the cloned sequence for diagnostic assay development.

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acetate, pH 4.8. After addition of 1/10 volume 5*M* sodium acetate, pH 5.8, the resulting RNA was precipitated from the aqueous phase with two volumes of 100% ethanol. The RNA precipitate was collected by centrifugation, washed in 70% ethanol, and dried briefly prior to first strand cDNA synthesis by random priming and a commercially available kit (Boehringer Mannheim, Indianapolis, IN). After the completion of second strand synthesis, aliquots of the cDNA were modified by addition of Eco RI linkers (New England Biolabs, Beverly, MA) for the construction of cDNA libraries in the phage vector λgt10 (Promega Biotec, Madison, WI). The initial λgt10 library contained 9.1×10^6 apparent recombinant plaques when titered on the *Escherichia coli* hfl strain HG415. Analysis of random plaque pure clones indicated, however, that the actual recombinant frequency was less than 5%. This library was, therefore, plated and total phage DNA was prepared. The phage DNA was cleaved with Eco RI, fractionated on agarose gels, and inserts in the 500- to 4000-bp size range were recovered on NA45 paper for recloning into λgt10. This resulted in a library with a recombinant frequency of greater than 90%.

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Calcium-Induced Movement of Troponin-I Relative to Actin in Skeletal Muscle Thin Filaments

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The role of troponin-I (the inhibitory subunit of troponin) in the regulation by Ca^{2+} of skeletal muscle contraction was investigated with resonance energy transfer and photo cross-linking techniques. The effect of Ca^{2+} on the proximity of troponin-I to actin in reconstituted rabbit skeletal thin filaments was determined. The distance between the cysteine residue at position 133 (Cys^{133}) of troponin-I and Cys^{374} of actin increases by approximately 15 angstroms on binding of Ca^{2+} to troponin-C. Also, troponin-I labeled at Cys^{133} with benzophenone-4-maleimide could be photo cross-linked to actin in the absence of Ca^{2+}, but not in its presence. These results suggest that troponin-I is attached to actin in the Ca^{2+}-free or relaxed state of muscle, and that it detaches from actin on Ca^{2+} activation of contraction. Thus, troponin-I may function as a Ca^{2+}-dependent molecular switch in regulation of skeletal muscle contraction.

CONTRACTION OF VERTEBRATE STRIated muscle is regulated by Ca^{2+} and requires the regulatory proteins troponin (Tn) and tropomyosin (Tm) (*1*). Troponin is composed of three subunits, the Ca^{2+}-binding (TnC), inhibitory (TnI), and Tm-binding (TnT) subunits. TnI inhibits actomyosin Mg^{2+}-dependent adenosine triphosphatase (Mg-ATPase) activity by itself (*2, 3*). It binds weakly to filamentous actin (F-actin) (*4, 5*) near the NH_2-terminus of actin (*6*) and more strongly to a complex of Tm and F-actin (Tm–F-actin); TnC reverses the binding of TnI to Tm–F-actin in the presence of Ca^{2+}, but not in its absence (*4, 5*). On the basis of these and other findings, a model for regulation by Ca^{2+} of skeletal muscle contraction has been proposed (*4, 7, 8*). This model postulates that in the relaxed state (absence of Ca^{2+}), TnI is attached to actin and anchors Tm in a position or state that inhibits one of the steps in the actin-myosin interaction cycle. In the activated state (presence of Ca^{2+}), the binding of Ca^{2+} to TnC causes TnI to detach from actin, with the result that the inhibitory effect of Tm is reversed. With the use of reconstituted rabbit skeletal muscle thin filaments composed of Tn, Tm, and F-actin, we have directly examined how TnI functions in the proposed mechanism of Ca^{2+} regulation.

TnI labeled at Cys^{133} with the fluorescent donor 1,5-IAEDANS [*N*-iodoacetyl-*N*′-(5-sulfo-1-naphthyl)ethylenediamine] and F-actin labeled at Cys^{374} with the nonfluorescent acceptor DAB-Mal (4-dimethylaminophenylazophenyl-4′-maleimide) were reconstituted with the other thin filament proteins to form the (TnC-TnI^{DAN}-TnT)–Tm–F-actin and (TnC-TnI^{DAN}-TnT)–Tm–F-$actin^{DAB}$ complexes (where TnI^{DAN} is donor-labeled TnI and F-$actin^{DAB}$ is acceptor-labeled F-actin). Resonance energy transfer distances were measured in these complexes in the following metal-bound states: (i) the Ca^{2+}-state, in which both the high- and low-affinity metal-binding sites of TnC are saturated with Ca^{2+}, simulating the in vivo activated state; (ii) the Mg^{2+}-state, in which only the high-affinity sites are occupied by Mg^{2+}, simulating the in vivo relaxed state; and (iii) the apo-state, in which none of the sites are occupied.

Our results show that the extent of resonance energy transfer is markedly Ca^{2+}-dependent (Fig. 1 and Tables 1 and 2), the transfer yield being significantly lower in the Ca^{2+}-saturated state than in either of the two Ca^{2+}-free states. Measurements carried out in the presence of both Ca^{2+} (0.2 m*M*) and Mg^{2+} (4 m*M*) yielded the same results as those carried out in the Ca^{2+}-state (*9*). If we assume that the orientation factor, κ^2, is the isotropically averaged value of 2/3, dis-

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