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Construction of a Genome-Length cDNA Clone for Human Astrovirus Serotype 1 and Synthesis of Infectious RNA Transcripts

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We have constructed a genome-length cDNA clone for human astrovirus serotype 1. When a human colon cancer-derived cell line, CaCo-2, is transfected with RNA transcribed in vitro from this cDNA clone, infectious virus is produced at titers close to those observed after infection with intact astrovirus. A rodent cell line, BHK, which is largely refractory to astrovirus infection, was found to support efficient growth of the virus if transfected with viral RNA. The high transfection efficiency seen in the BHK cells allows studies of the viral replication in the transfected cells and thus should prove useful for the characterization of noninfectious astroviral mutants.

Human astrovirus was first described in 1975 (15), and has since been recognized as one of the most common causes of viral gastroenteritis in young children (3, 8, 23). Recently, the nonenveloped, positive-strand RNA virus has been classified in its own family, the *Astroviridae* (19). The sequence of the polyadenylated 6.8-kb genomic RNA, which has been determined for serotypes 1 and 2 (9, 12, 29), comprises three open reading frames (ORF), ORF-1a, -1b, and -2. ORF-1a and -1b encode the viral nonstructural proteins and are directly translated from the 5′ two-thirds of the genomic RNA. ORF-1a contains a 3C-like serine protease motif (6), while ORF-1b displays sequence indicative of RNA-dependent RNA polymerase activity. ORF-1b is translated as part of a polyprotein with the ORF-1a gene product, dependent on the occurrence of a −1 ribosomal frameshift at the ORF-1a–ORF-1b junction (13, 16). How the ORF-1a and ORF-1a–ORF-1b gene products are proteolytically processed is still unclear.

ORF-2 is located on the 3′ terminal one-third of the viral genomic RNA and is likely expressed from a 2.5-kb subgenomic RNA which is coterminal with the genomic RNA at the 3′ end. Encoded in ORF-2 are the viral structural proteins, which are translated as an approximately 87-kDa protein precursor (12, 20). When virus is cultivated in the presence of trypsin, which is necessary for infectivity of viral particles (11), the structural protein precursor is processed into three cleavage products with sizes ranging from 24 to 33 kDa (25, 30). It is not known whether cleavage of the structural proteins occurs before, during, or after assembly of the viral capsid.

Since astrovirus contains an RNA genome, the availability of a genome-length cDNA clone that can be transcribed into infectious viral RNA would greatly facilitate and enhance studies on the replication strategy and pathogenesis of this virus. Here we report the construction of such a cDNA clone of human astrovirus serotype 1 and describe a cell system that should allow characterization of viral replication in first-passage cells transfected with RNA transcribed in vitro from the cDNA clone.

Sequential transfection-infection cell culture system for astrovirus. Using total cellular RNA from human astrovirus serotype 1-infected CaCo-2 cells (ATCC HTB-37) as a positive control test substrate, we set up a cell system that allowed us to study both cells transfected with astroviral RNA and cells infected with viral particles. Baby hamster kidney (BHK) cells (14) were grown in Dulbecco's minimal essential medium containing 10% fetal calf serum, and CaCo-2 cells were propagated in RPMI 1640 containing 10% fetal calf serum. Transfection of cells with viral RNA was performed using Lipofectin reagent (Bethesda Research Laboratories, Gaithersburg, Md.) according to the manufacturer's protocol, and transfection efficiencies were found to be reproducible for each cell type. After transfection, cells were incubated in medium supplemented with 2% (BHK) or 10% (CaCo-2) serum at 37°C overnight, and cells expressing astroviral structural protein(s) were detected by in situ immunostaining with monoclonal antibody 8E7 (5, 7). To recover infectious virus for passaging from the transfected cells, 1 ml of medium without serum was added to the cells after the lipofection step. After overnight incubation at 37°C, trypsin type IX (Sigma, St. Louis, Mo.) was added to a final concentration of 10 μg/ml, and cells and media were harvested after a 30-min (BHK) or overnight (CaCo-2) incubation at 37°C. The trypsin allowed easy harvesting of the cells, increased the release of the virus from the cells (21), and was necessary to activate the virus so as to render it infectious (11, 30). In the case of the highly infectable CaCo-2 cells, reinfection of the cells with virus released from transfected cells is assumed to occur. Since BHK cells are much less susceptible to infection by astrovirus (see below), overnight incubation in the presence of trypsin did not affect the titer of astrovirus released. Cells were lysed by three cycles of freeze/thawing, and the medium-cell lysate was used for passaging on CaCo-2 cells. CaCo-2 cells were infected in the presence of 5 or 10 μg of trypsin type IX/ml for in situ immunostaining of infected cells or recovery of infectious virus, respectively. After incubation at 37°C for 1 h, the trypsin was either inactivated by addition of 10% fetal calf serum for in situ detection of infected cells after overnight incubation at 37°C or maintained at

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TABLE 1. Transfection of BHK and CaCo-2 cells with astroviral RNA isolated from astrovirus serotype 1-infected CaCo-2 cells and recovery of infectious virus[a]

Cell type transfected	% Transfected cells	Recovery of infectious virus from:	
		Transfected cells (1st passage) (IU/ml)	Infected CaCo-2 cells (2nd passage) (IU/ml)
CaCo-2	0.7	6.7×10^8	7.5×10^8
BHK	17	3.7×10^8	7.6×10^8

[a] Total cellular RNA from virus-infected cells was isolated with TriReagent (Molecular Research Inc., Cincinnati, Ohio).

TABLE 2. Transfection of BHK cells with RNA isolated from partially purified astroviral particles[a]

Presence of RNase	% BHK cells transfected	Infectious virus recovered from transfected BHK cells (IU/ml)
No	10	1.5×10^7
Yes	0.00003[b]	0

[a] RNA was isolated from partially purified viral particles (25) by repeated extraction with phenol-chloroform (1:1) and chloroform. Before transfection, the RNA was incubated for 30 min at 37°C in the absence or presence of 40 μg of RNase A per ml in 100 mM Tris-HCl (pH 7.5)–150 mM NaCl.

[b] Represents one positive cell detected.

a level of 10 μg/ml for an additional 2 days at 37°C to allow repeated reinfection of the cells and therefore maximal recovery of virus. Every transfection experiment included a negative control where cells were mock transfected in the absence of RNA. Lysates from mock-transfected cells were used as a negative control for subsequent infection of cells. In no case were mock-transfected or mock-infected cells found to express astroviral structural proteins.

CaCo-2 cells are known to be readily infectable with astrovirus (30) and allow growth of the virus to a titer of 10^8 infectious units per ml (IU/ml). However, transfection of CaCo-2 cells by both lipofection (0.7% [Table 1]) and electroporation (data not shown) proved to be very inefficient. BHK cells are easier to transfect but our studies have shown that BHK cells are far less susceptible to infection with astrovirus particles than CaCo-2 cells, with the apparent titer on BHK cells 10^4-fold less than on CaCo-2 cells (5×10^3 and 4×10^7 IU/ml found with the same virus preparation on BHK and CaCo-2 cells, respectively). When transfected with RNA extracted from infected cells, however, 24 times more of the BHK cells (17%) expressed astroviral structural protein(s) compared to CaCo-2 cells (0.7% [Table 1]). These studies also demonstrated that transfected BHK cells give rise to infectious astrovirus at titers close to those seen for infected CaCo-2 cells (3.7×10^8 versus 7.5×10^8 IU/ml, respectively [Table 1]).

Because the number of transfected cells is higher for the BHK than for the CaCo-2 cells, the apparent yield of infectious virus per transfected cell is lower for BHK cells. However, CaCo-2 cells are much more susceptible to infection by astrovirus than BHK cells, and can therefore be reinfected with astrovirus released from the transfected cells. Therefore, the astroviral titer determined for the transfected CaCo-2 cells does not represent a true measure of the yield of infectious virus per transfected cell and thus does not reflect the efficiency of virus production in the transfected CaCo-2 cell. In a control experiment, virus was harvested from transfected CaCo-2 cells before reinfection with the released virus could occur (30 min after addition of trypsin). In this case, the estimated yield of virus per transfected CaCo-2 cells mimics more closely that seen for the transfected BHK cells in Table 1 (data not shown). In summary, these findings indicate that BHK cells transfected with astroviral RNA support efficient replication of the virus.

Due to their high transfection efficiency, BHK cells, in contrast to CaCo-2 cells, allow the study of first-passage viral replication in transfected cells. Such a system should prove useful for characterizing viral mutants that have lost their infectivity and which therefore can only be studied in the initially transfected cells.

Since astrovirus can replicate efficiently in BHK cells, the reduced infectability of BHK compared to CaCo-2 cells is probably due to a block at the level of binding, entry, or uncoating rather than at a later step in the viral life cycle.

Demonstrating infectivity of the astroviral genomic RNA. It has been assumed, but not yet been proven, that the genomic RNA of astrovirus by itself is infectious. To address this question, we transfected BHK cells with RNA that was extracted from partially purified astrovirus (25). The transfected cells expressed viral structural protein(s), as detected by in situ immunostaining, and gave rise to virus that was able to infect CaCo-2 cells (Table 2). After pretreatment of the viral RNA with RNase, the number of transfected cells expressing viral structural protein(s) was reduced by 10^5-fold, and no infectious virus could be recovered (Table 2). Thus the genomic RNA of astrovirus by itself appears to be infectious.

Construction of a full-length cDNA clone for human astrovirus serotype 1. It has been previously reported that genomic cDNA clones of other RNA viruses rapidly accumulate deleterious mutations in high-copy-number vectors (4, 24, 28). As a precaution, the astroviral cDNA was assembled in a pBS-KS vector (Stratagene, La Jolla, Calif.) that had been modified to substantially reduce its copy number. A fragment of the pUC-derived pBS-KS vector extending from the *Sca*I site in the ampicillin resistance gene to the *Kpn*I site in the polylinker was replaced by a corresponding fragment from pBR322, containing the origin of replication as well as the *rop* gene, which regulates plasmid copy number. In the modified vector, pBS^{lr} KS , the viral T3 DNA-dependent RNA polymerase promoter is deleted, and the copy number exhibited is close to that of pBR322 (at least 10-fold lower than unmodified pBS-KS).

A full-length cDNA copy of the genomic RNA of human astrovirus serotype 1 was assembled in pBS^{lr}KS by sequentially linking overlapping fragments of astroviral cDNA at unique restriction sites (Fig. 1). These overlapping cDNA fragments were generated by reverse transcription (RT)-PCR by using the total cellular RNA from astrovirus serotype 1-infected LLC-MK$_2$ cells (ATCC CCL-7.1) or CaCo-2 cells (Fig. 1) as template. First-strand cDNA synthesis was performed with Superscript II reverse transcriptase (Bethesda Research Laboratories), and PCRs were conducted with Vent polymerase (New England Biolabs, Beverly, Mass.) under the conditions recommended by the manufacturers. Table 3 lists the primers used for generation of the RT-PCR products. Two of the cDNA fragments, nucleotides 2720 to 3333 and nucleotides 3290 to 6679, respectively, were generously provided as M13 subclones by T. Lewis (USDA/ARS, Plum Island, N.Y.). The 5′-most primer on the viral genome comprised a T7 promoter as well as a *Xho*I site. The viral 3′-terminal sequence including a poly(A)$_{39}$ stretch was obtained by using in vitro-ligated genomic RNA as a template for first-strand cDNA synthesis. The 3′ primer used for the subsequent PCR was complementary to the viral 5′ sequence and contained a *Not*I site. All

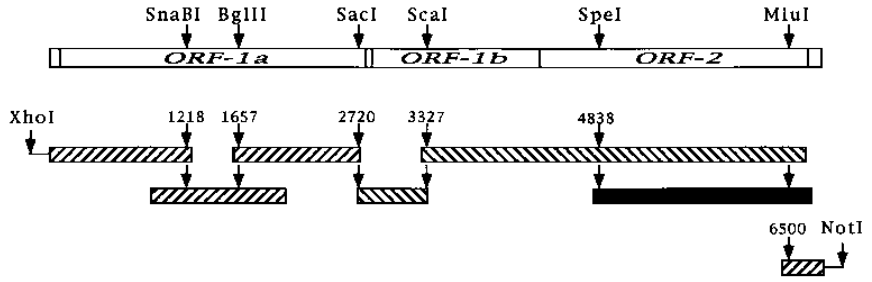

FIG. 1. Assembly of the full-length astroviral cDNA clone. All boxes represent cDNA fragments. Open box, full-length viral cDNA; hatched boxes, fragments derived by RT-PCR (see Table 3 and text) with RNA from astrovirus serotype 1-infected LLC-MK_2 cells as template; solid box, fragment derived by RT-PCR (see Table 3) with RNA from astrovirus serotype 1-infected CaCo-2 cells as template. CaCo-2 cells were infected with astrovirus serotype 1 at a 0.1 multiplicity of infection as a precaution against the potential accumulation of defective interfering RNAs. Numbering is according to the astroviral genomic sequence (GenBank accession number L23513 [12]).

PCR-derived fragments used for the assembly of the full-length cDNA clone were sequenced as cloned cDNA by using the Sequenase version II kit (U.S. Biochemicals, Cleveland, Ohio) according to the manufacturer's protocol. Vector-insert junctions created by blunt-end ligations were also verified by sequencing.

The $pBS^{lr}KS$ clone containing the full-length astroviral cDNA between the *Xho*I and the *Not*I sites was called pAVIC. This plasmid harbors two T7 promoters organized in opposite directions, one derived from the pBS vector and the other from the 5′-most PCR primer. The existing *Xho*I site in pAVIC was then destroyed, and a new *Xho*I site was inserted immediately downstream of the viral poly$(A)_{39}$ stretch by using a PCR-based strategy (for the primer sequence, see Table 3).

Table 4 lists the nucleotide changes in pAVIC compared to the published astrovirus sequence (12; GenBank accession number L23513). To allow positive identification of astrovirus derived from our cDNA clone, two silent point mutations (A to T at nucleotide 2716 and T to C at nucleotide 2719) were introduced by PCR. Half of the unintended sequence differences identified probably result from either errors introduced by PCR or the sequence heterogeneity inherent to an RNA virus. However, the other seven of the nucleotide changes seen in the full-length cDNA clone are reported as the genomic sequence for astrovirus serotype 1 by Willcocks et al. (29; GenBank accession number Z25771). It is noteworthy that six of these seven changes are located in ORF-2. While Willcocks et al. (29) determined the astroviral sequence from virus isolated directly in CaCo-2 cells, Lewis et al. (12) used RNA from LLC-MK_2 cell-adapted virus as a template. Our cDNA clone was constructed mainly from RNA from astrovirus serotype 1-infected LLC-MK_2 cells, and only parts of ORF-2 have been derived from virus grown in CaCo-2 cells (Fig. 1). The differences in the ORF-2 region between the two published genomic astroviral sequences may therefore reflect adaptation of the virus to LLC-MK_2 cells.

Demonstrating infectivity of RNA transcribed from the cDNA clone of human astrovirus serotype 1. The astroviral cDNA was positioned downstream of a viral T7 DNA-dependent RNA polymerase promoter, and a unique *Xho*I restriction site allowed linearization of the DNA immediately downstream of the viral poly(A) sequence. In vitro transcription with T7 polymerase (Promega, Madison, Wis.) was performed according to the manufacturer's recommendations in the presence of an $m^7G(5')ppp(5')G$ RNA cap structure analog and was followed by treatment with an RNase-free DNase. This resulted in a genomic-length viral RNA that contained six extra nucleotides beyond the 3′ terminal poly(A) sequence and one additional G residue linked to an m^7GTP cap structure at its 5′ end. The cap structure was added to increase the stability of

TABLE 3. PCR primers used to generate astroviral cDNA fragments for the assembly of a full-length cDNA clone[a]

Primer and position on astroviral genome (nt)[b]	Sequence (5′→3′)
5′	
1–21	ccgcgctcgagtaatacgactcactatagCCAAGAGGGGGGTGGTGATTG
887–906	GGTACAGACTTGATTACACT
1625–1645	AGTTGTGTCACAGTTATGGCT
4805–4824	GTCTCACTTAACCCCACATC
6454–6474	CCGTGTAACCCTCCTCTCAAC
3′	
1246–1225	GGTGCCAATGAAAACGGTTGCC
2070–2049	AATGGTGCAAGTATTCCATTGA
2732–2699	CTTTGTCGAGCTCgAAaACTGCTTCAGGTAATGC
6723–6695	AGACAGAAAAGAAGGAAGCTGTACCCTCG
17–1	cgcgctcgagcggccgCACCACCCCCCTCgcGGTTTT
17–1	gcgcgcgcgcggccgCACCACCCCCCTCgaGGTTTTT

[a] Uppercase letters denote primer sequence corresponding to (5′ primer) or complementary to (3′ primer) the astroviral genomic sequence. Lowercase letters denote positions of noncomplementarity in the primer sequence used to introduce restriction sites, T7 promoter sequence, or targeted mutations into the sequence of the PCR product. Primer pairs for PCR amplification were the first 5′ and 3′ primers, the second 5′ and 3′ primers, etc. The fifth 5′ primer was used in combination with both the fifth and the sixth 3′ primers.

[b] nt, nucleotide.

TABLE 4. Changes in the astroviral cDNA sequence relative to the published wild-type sequence[a]

Nt position in astroviral genome[b]	ORF affected	Nt change	Amino acid change
913	ORF-1a	G to A[c]	None
2191	ORF-1a	T to C	None
2335	ORF-1a	A to G	None
2716[d]	ORF-1a	A to T[d]	None
2719[d]	ORF-1a	T to C[d]	None
2936	ORF-1b	G to A	Arg to Lys
3213	ORF-1b	A to G	None
3465	ORF-1b	G to C	Leu to Phe
3986	ORF-1b	G to T	Trp to Leu
5006	ORF-2	T to A[c]	Ser to Thr
5102	ORF-2	T to G[c]	Ser to Ala
5586	ORF-2	T to C[c]	Val to Ala
5745	ORF-2	G to A[c]	Gly to Asp
6010	ORF-2	C to T	None
6548	ORF-2	G to A[c]	Gly to Arg
6562	ORF-2	T to A[c]	None

[a] See reference 12 for wild-type sequence.
[b] Nt, nucleotide.
[c] Change present in pAVIC also reported for astrovirus serotype 1 genomic sequence by Willcocks et al. (29) (GenBank accession number Z25771).
[d] Mutations deliberately introduced by PCR primer.

the RNA. However, activity of this capped in vitro-transcribed RNA does not allow any conclusions regarding the status of the 5′ end of the genomic RNA found within viral particles. A capped RNA transcribed from a feline calicivirus cDNA clone has been shown to be infectious (27), although feline calicivirus RNA is known to be linked to a VpG protein (2).

BHK or CaCo-2 cells were transfected with RNA transcribed in vitro from pAVIC (AVIC RNA). Both cell types were found to express the astroviral structural protein(s), as detected by immunostaining (Table 5), and gave rise to a high titer of infectious virus (5.8×10^7 and 6.1×10^7 IU/ml, respectively). The titers observed were close to those seen in control experiments where RNA extracted from wild-type virus-infected cells was used for transfection (see Table 1). Virus derived from the infectious in vitro-transcribed RNA could be repeatedly passaged on CaCo-2 cells (Table 5). When RNA transcribed in the absence of the m^7GTP cap structure analog was used for transfection of BHK cells, no expression of astroviral structural proteins was detected in transfected cells and no infectious virus could be recovered. This loss of activity is probably due to the decreased stability of the uncapped RNA transcript in transfected cells and clearly argues that the RNA rather than any residual DNA template is the infectious agent.

TABLE 5. Transfection of BHK and CaCo-2 cells with RNA transcribed in vitro from pAVIC and recovery of infectious virus[a]

Cell type transfected	% Transfected cells	Recovery of infectious virus from:	
		Transfected cells (1st passage) (IU/ml)	Infected CaCo-2 cells (2nd passage) (IU/ml)
CaCo-2	0.9	6.1×10^7	1.1×10^8
BHK	15	5.8×10^7	1.8×10^8

[a] RNA was transcribed in the presence of m^7G(5′)ppp(5′)G RNA cap-structure analog. For transfection, the in vitro transcription reaction containing the newly transcribed RNA was generally used without further purification. The transfection efficiency achieved using 5 μl of unpurified in vitro transcription reaction mixture corresponded to that seen with 2 μg of in vitro-transcribed RNA which had been purified after transcription by phenol-chloroform extraction, ethanol precipitation, and passage over a G-50 Sephadex Quick Spin column (Boehringer Mannheim, Indianapolis, Ind.).

To prove definitively that infectious virus was derived from RNA transcribed from the cDNA clone and not due to contaminating wild-type virus, we infected CaCo-2 cells with lysates from BHK cells transfected with AVIC RNA or wild-type astroviral RNA and isolated the total cellular RNA. We then amplified the region covering positions 2716 and 2719 in the genomic RNA by RT-PCR and directly sequenced both strands of the resulting uncloned cDNA fragments (fmol DNA sequencing system, Promega). The sequence obtained unambiguously showed that the two point mutations introduced at positions 2716 and 2719 of the cDNA clone were present in the virus derived from AVIC RNA but not in that derived from wild-type astroviral RNA. Thus, the infectious virus observed does indeed originate from the astroviral cDNA clone.

Genomic cDNA clones from which infectious RNA can be transcribed have already been described for numerous other RNA viruses (1, 17, 18, 22, 27). Here we describe for the first time the construction of such an "infectious cDNA" clone for human astrovirus serotype 1, a member of the new viral family *Astroviridae*. When RNA transcribed in vitro from this cDNA is transfected into BHK or CaCo-2 cells, infectious virus is produced that is indistinguishable in its growth characteristics from wild-type astrovirus. The cDNA clone will allow the analysis of the viral life cycle by targeted introduction of mutations into the viral genome. Viral mutants that have lost their infectivity, however, can only be characterized in the initially transfected cells. This is made possible by the BHK cell system described here because of its high transfection efficiency. Availability of the infectious cDNA clone should greatly facilitate research on the molecular biology of astrovirus.

Members of the Togavirus family, which share genomic organization and important elements of their replication strategy with the *Astroviridae*, have been successfully exploited as eukaryotic expression vectors (26). The similarities between these two viral families suggest that astrovirus can also be modified to serve as a vector. Astrovirus has several unique features that would be attractive in a eukaryotic expression vector: it is a human virus which shows high specificity for intestinal cells and causes a self-limiting infection. One could therefore envision the use of astrovirus as a vehicle for presenting immunogenic epitopes of other, more severe gastrointestinal pathogens at their shared site of action, the gastrointestinal tract. This immunization strategy should serve to elicit mucosal immunity, which has been shown to be most effective against gastrointestinal pathogens (10). The astroviral cDNA clone described here should prove useful for defining the minimal and optimal requirements for the use of astrovirus as an expression vector, and for developing a packaging system for vector RNA.

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