Cloning and Characterization of Human Astrovirus Immunoreactive Epitopes

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We report the cloning of antigenic, protein-coding regions of human astrovirus serotype 1 that appear to be common to most, if not all, serotypes of human astrovirus. Screening of λ gt11 libraries identified three different but overlapping clones (A43, A35, and A1) and one independent clone (A14) that reacted with serum from a rabbit repeatedly immunized with purified astrovirus particles but not with its preimmunization serum. These clones were shown to be astrovirus specific. Of note, a radiolabeled probe representing the immunoreactive clones A43-A35-A1 hybridized exclusively to the 7.2-kb astrovirus genomic RNA, while a clone A14-specific probe hybridized with both the genomic and the 2.8-kb astrovirus subgenomic RNAs. This suggests that the immunoreactive epitopes, selected by antiserum to purified astrovirus particles, are encoded by the subgenomic RNA as well as other regions of the genomic RNA.

Gastroenteritis caused by astrovirus was first recognized as a clinical entity in infants in 1975 (13). Since then, five serotypes of human astrovirus and a variety of animal strains have been identified (10). Significant advances have been made in adapting the human astroviruses to growth in cell culture (12, 22) and in developing immunologic reagents to detect these viruses in clinical specimens (5). This, in addition to electron microscopy, has allowed the identification of astroviruses in outbreaks of gastroenteritis in adults and children worldwide. Recent surveys with an astrovirus enzyme immunoassay (EIA) indicate that astroviruses account for 7.3 to 8.6% of cases of diarrhea in young children in Guatemala (2) and Thailand (7).

We have been interested in the genomic organization of astroviruses and have approached this problem by cloning and characterizing immunoreactive regions encoded by human astrovirus serotype 1. We now present the sequences of two separate astrovirus-specific immunoreactive regions: one that appears to be encoded by genomic RNA and another that is common to both genomic and subgenomic RNAs. These clones are potentially useful for elucidating the remainder of the astrovirus genome sequence and developing diagnostic probes as well as renewable immunologic reagents derived from specific viral epitopes for use in the serological diagnosis of astrovirus infection.

Human astrovirus serotype 1 was propagated in LLCMK2 cells (ATCC CCL 7.1) as previously described (11, 12). For molecular cloning, astrovirus serotype 1-infected cell lysates were partially purified by fluorocarbon extraction and pel-

leting (3). Partially purified cell lysates were used because initial attempts to clone astrovirus from cesium chloride gradient-purified viral particles were unsuccessful. Viral antigen was monitored at each step by EIA and electron microscopy. Mock-infected cell lysates, prepared in parallel, served as the negative control.

The cloning and screening strategy used for human astrovirus serotype 1 closely paralleled that used for cloning and characterizing an immunoreactive portion of the Norwalk virus genome (14). Approximately 10 μ g of nucleic acid, extracted from infected cell lysates by a one-step guanidinium-phenol extraction protocol (1), was reverse transcribed into cDNA (4) with either oligo(dT) priming or random hexamers. The heterogeneous population of cDNA was amplified by sequence-independent, single-primer amplification (16) and inserted into bacteriophage λ gt11 vectors at the *Eco*RI site. In these experiments, as well as in those to obtain clones of the Norwalk (14) and hepatitis E viruses (20), the immunologically selected sequence-independent, single-primer amplification clones were relatively small (<500 bp). However, in the model used to develop and test the sequence-independent, single-primer amplification technique (16), clones that are >1,000 bp in length have been generated.

A typical recombinant λ gt11 library had a titer of 1×10^5 to 2.5×10^5 plaques per ml and a recombinant frequency in excess of 85%. Libraries were screened immunologically (24) with H-1 antiserum (1:400 dilution). H-1 antiserum had been prepared from a rabbit immunized with CsCl gradient-purified, cell culture-adapted astrovirus serotype 2. It is used as the detector antibody in the astrovirus EIA developed by Herrmann et al. (6) because of its ability to recognize all five serotypes of human astrovirus. In studies of viral neutralization, H-1 antiserum neutralizes only homologous, serotype 2

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A.

1	K P I P A L R T T K P K T C P E P E	18
1	AAAACCAATTCCTGCGCTGAGAAACCAACCAAGCCAAACTTGCCCGAACCAGAA	55
19	V E S Q P L D L S Q K K E K Q S E Y	36
56	GTCGAATCACAACCACTTGATTGTCCCAAGAGAGAGAAACATCAGAAATAT	109
37	E Q Q V V K S I K P Q K S E P Q P Y	54
110	GAACAACAAGTGGTGAAGTCTATCAAGCTCAGAGAGTGAGCCCAACCATAC	163
55	S Q T Y G K A P I W E S Y D F D W N	72
164	<u>TACAAACTTACGGCAAGGCACCAATCTGGGAATCTTACGATTTTACTGGAAT</u>	217
73	E D D A K F I L P A P Y R L T K A D	90
218	GAGGATGATGCCAAGTTTATTCGCGAGCCATACCGGTTGACTAAGGCAGAT	271
91	E I V L G S K I V K L R T I I E T A	108
272	GAATAGTCCTTGGATCTAAATCGTCAAGCTTAGAACGATTATTGAAACAGCC	325
109	I K T Q N Y S A L P E A V F E L D K	126
326	ATAAAGACTCAGAATTATAGTGCATTACCTGAAGCAGTATTGAGCTCGACAAA	379
127	A A Y E A G L E G F L Q R V K S K K	144
380	GCAGCTTATGAAGCAGGTTTGAAGGTTTCTCCAAAGGTTAAATCAAAAAA	434

B.

1	R N P V T T T L Q F T Q M N Q P S L	18
1	GCGGAATCCTGTGACACCAACCTTACAGTTCACACAAATGAACCACTAGTCTA	55
19	G H G E A P A A I G R S I P A P G E	36
56	GGCAGCGTGAAGCACCAGCTGCGATTGGTAGATCCATTCCAGCACCTGGTGAG	109
37	E Y K V V L T F G S P M S P N A N N	54
110	GAGTATAAAGTTGCTCTACATTTGGATCCCAATGAGCCCTAATGCAAAATAC	163
55	K Q T W V N K P L D A P S G H Y N V	72
164	AAACAGACTTTGGGTTAATAAACCTCTTGATGCGCTTCGGGCCATTACGATGTG	217
73	K I A K D V D H Y L T M Q G F T S I	90
218	AAATTCGAAGGATGTGACCACTATCTAACCATGCAAGGTTTCACTTCTATA	271
91	A S V D S R	96
272	<u>GCACTCTGTGACTCGAGG</u>	289

C.

A14	227	AAGGATGTTGACCACTATCTAACCATGCAAGGTTTCACTTCTATAGCA	274
(21)	1	-----T	48
A14	275	TCTGTTGACTCGAGG	289
(21)	49	-----G-TAC	63

FIG. 1. Nucleic and amino acid sequences of overlapping clones A43-A35-A1 and independent immunoreactive clone A14. (A) Clone A43 (nt 1 to 184; amino acids 1 to 61), clone A35 (nt 95 to 325; amino acids 32 to 108), and clone A1 (nt 299 to 434; amino acids 100 to 144). Clone A43-specific probes were produced in polymerase chain reactions (17) with the following primers that span nt 5 to 24 and nt 163 to 183 (underlined), encompassing nearly all of the A43 clone and the 5' half of the A35 clone. Overlapping regions between clones are depicted in italics (nt 100 to 185 and nt 299 to 326). (B) Clone A14-specific primers used to generate probes are underlined (nt 10 to 29 and nt 255 to 274). These probes do not hybridize with clone A43, A35, or A1. (C) The 63-bp region of overlap between the 3' end of clone A14 and the 5' end of the 3' terminal sequence (21). There is 92% nucleotide identity, as shown, and 90.5% (19 of 21 residues) amino acid identity (data not shown).

astrovirus (6, 8). A total of 1.2×10^5 recombinant phage were screened from the oligo(dT)-primed cDNA library, and an additional 2.3×10^5 recombinant phage were screened from the random-primed cDNA library. After two subsequent rounds of immunoscreening, followed by plaque purification and a final screening of H-1-reactive clones with preimmunization rabbit serum, three immunoreactive, oligo(dT)-primed (A35, A39, and A43) and five random-primed (A1, A11, A14, A21, and A33) cDNA clones were identified. cDNAs were subcloned into Bluescript SK+ (Stratagene Inc., La Jolla, Calif.) for sequencing (18).

Cross-hybridization between clones A43 and A35 and between clones A35 and A1 was found. The sequence of the overlapping clones A43-A35-A1 is shown in Fig. 1A. These clones and the nonoverlapping clone A14 were characterized further. Clones A11, A21, A33, and A39 have yet to be characterized fully.

FIG. 2. Clone A43-specific probes hybridize to nucleic acid extracted from cells infected with astrovirus of four different serotypes (spots I-1, I-2, I-4, and I-5). Each spot contains ~20 µg of total RNA. These probes hybridize most prominently with nucleic acid from cells infected with homologous serotype 1 astrovirus (spot I-1). Serotype 3 astrovirus was not available for testing. No hybridization was seen with nucleic acid extracted from uninfected cells (spot U). A similar pattern of hybridization was seen with clone A14-specific probes.

In order to assess the specificities of the selected clones for astrovirus, several approaches were undertaken. The clones were first tested for any sequence similarities with potential contaminants from the laboratory. Clone-specific probes did not hybridize to Southern blots of human or *Escherichia coli* DNA. Furthermore, a radiolabeled A43 clone-specific probe did not hybridize to NCDV rotavirus RNA or Norwalk virus cDNA (data not shown). Direct comparison of the sequences of clones A43-A35-A1 and A14, shown in Fig. 1A and B, with the hepatitis E virus sequence (20) and GenBank data base searches failed to reveal any similarities, with one exception (see below).

The clones were established to be astrovirus specific in four ways. First, radiolabeled clone-specific probes hybridized to RNAs extracted from serotype 1 human astrovirus-infected cells, as well as RNAs from cells infected with human astrovirus serotypes 2, 4, and 5 (Fig. 2). Human astrovirus serotype 3 was not available for testing. Equal quantities of total RNA from lysate were spotted in each of the wells. No hybridization was seen with RNAs from mock-infected cells. Of note was the greater intensity of the hybridization signal to RNAs extracted from the astrovirus serotype 1 from which the molecular clones were isolated.

Next, clone-specific probes were found to hybridize specifically to RNA extracted from purified astrovirus particles isolated on a cesium chloride density gradient (Fig. 3). An intense hybridization signal, seen in Fig. 3, fraction 5, corresponded to a buoyant density of 1.37 g/ml and correlated with peak astrovirus antigenic activity as detected by EIA. A much less intense signal was observed in fraction 6 (1.35 g/ml) after longer exposure. In a parallel CsCl gradient, intact virions were localized to a fraction of the gradient at a density of 1.37 g/ml by electron microscopy. A second peak of antigenic reactivity was found in fraction 7 (buoyant density, ~1.33 g/ml), without a detectable, corresponding RNA-probe hybridization signal, and this peak probably represents empty viral capsids that are devoid of genomic RNA.

Third, Northern (RNA) blot analysis (9) revealed hybridization of clone-specific probes to astrovirus-specific RNAs generated during infection of LLCMK2 cells. An unexpected outcome of these experiments, however, was that clone-specific probes showed different patterns of hybridization. Probes specific for both A43-A35-A1 and A14 hybridized to a 7.2-kb, poly(A)⁺ RNA from astrovirus-infected cells (Fig. 4, lanes 1 and 3) that is thought to be astrovirus full-length genomic RNA (15). In addition, only the A14-specific probe (Fig. 4, lane 3) hybridized to a 2.8-kb RNA that had been described previously as an astrovirus subgenomic RNA coterminal with the 3' end of the genomic RNA (15). These patterns of hybridization allowed the tentative

FIG. 3. Clone A43-specific probes hybridize with nucleic acid extracted from CsCl gradient-purified astrovirus particles. Fractions (250 μ l each) were collected, and RNA was extracted and spotted on nitrocellulose. Specific hybridization is seen with fraction 5, the fraction with the greatest level of astrovirus antigenic activity by EIA. The buoyant density of this fraction was 1.37 g/ml. After longer exposure, faint hybridization in fraction 6 is also seen (1.35 g/ml). A similar pattern of hybridization was seen with clone A14-specific probes.

localization of the sequences encoding the immunoreactive epitopes to two distinct regions of the viral genome: clone A14 to the 3' end of the genome, within the subgenomic RNA, and A43-A35-A1 to the genomic RNA (5' to the subgenomic RNA).

Since these clones had been selected by antiserum (H-1) prepared to purified astrovirus particles, we initially postulated that they represented immunoreactive epitopes of astrovirus structural proteins. In the alphavirus family,

excess production of subgenomic RNAs appears to be necessary to produce the large numbers of capsid units required to package viral genomic RNA molecules into virions (19). Modulated expression of subgenomic RNAs in hepatitis E virus has been observed as well (20). By analogy to the alphaviruses and caliciviruses, it is likely that the astrovirus subgenomic RNA also encodes the virion structural proteins. However, because all of the astrovirus clones that were immunoselected with hyperimmune serum H-1 could not be localized exclusively to the subgenomic RNA, it appears that the astrovirus particle may be composed of proteins encoded by both the genomic and the subgenomic RNAs. Of note, a highly immunogenic epitope in hepatitis E virus (open reading frame 3) appears to be encoded in a small open reading frame that is 5' to (and overlaps with) the subgenomic RNA that encodes the major capsid protein (open reading frame 2) (20, 23). An alternative explanation for our findings may be that the H-1 hyperimmune serum contains antibody to nonstructural proteins that copurified with virion and that clone A43-A35-A1 encodes such an epitope. The precise localization of the clone A43-A35-A1 sequence on the astrovirus genome and determination of the type of protein that it encodes require further study. Issues concerning the organization of the astrovirus genome and whether its replication strategies are similar to those of caliciviruses, alphaviruses, or other RNA viruses remain uninvestigated. At present, it appears that at least two serotypes of human astrovirus, type 1 (characterized here) and type 2 (studied by Monroe et al. [15]), synthesize at least one subgenomic RNA during infection *in vitro*.

A fourth proof of clone specificity was provided by comparing the sequences of clones A14 and A43-A35-A1 with the astrovirus 3' end sequence recently published by Willcocks and Carter (21). The 3' end of clone A14 (nucleotides [nt] 227 to 289) and the 5' end of the Willcocks sequence (nt 1 to 63) were 92% identical over 63 bases, as shown in Fig. 1C. The Northern blot hybridization data were additionally supported by the observation that a probe representing the Willcocks sequence hybridized in a pattern (data not shown) identical to that of the A14 clone-specific probe (Fig. 4, lane 3). There was no homology between the A43-A35-A1 sequence and the Willcocks sequence.

The clone-specific probes have also been useful in confirming previous assertions that the astrovirus genome is composed of plus-sense, single-stranded RNA. As shown in Fig. 5A, astrovirus clone-specific probes do not hybridize with RNase-treated nucleic acid from infected cells, while hybridization signals between DNase-treated and untreated control nucleic acid from infected cells are present and indistinguishable. Hybridization studies with single-stranded, clone-specific probes of opposite polarities demonstrated that only the minus-sense probe hybridized to astrovirus RNAs extracted from infected cell lysates. This indicates that the predominant RNA species produced is plus sense and suggests that genomic RNA is messenger sense or plus sense (Fig. 5B).

The identification of four independently selected immunoreactive clones representing two distinct regions of the astrovirus genome provides some basic information needed to clone and sequence the remainder of the genome. These clones may also be useful in producing antibodies to recombinant antigens that recognize specific structural and non-structural epitopes and may facilitate identification of astrovirus in field specimens.

FIG. 4. Northern blot of poly(A)⁺ RNAs from astrovirus-infected (lanes 1 and 3) and uninfected (lanes 2 and 4) cells. Clone A43-specific probes hybridized to the 7.2-kb genomic RNA alone (lane 1), while clone A14-specific probes hybridized to both the genomic and the 2.8-kb subgenomic RNAs (lane 3). A random-primed probe representing the 3' terminal sequence (21) also hybridized to this blot (data not shown) in a pattern identical to that of clone A14-specific probes (lane 3). The faint band at ~4 kb in lane 3 is not constantly observed, and its significance is unclear. Neither of the probes hybridized to RNAs from uninfected cells (lanes 2 and 4). RNA size markers (in kilobases) are indicated on the left.

FIG. 5. Clone A43-specific probes confirm that the astrovirus genome is composed of plus-sense, single-stranded RNA. (A) Clone A43-specific probes hybridized to untreated (spot U) and DNase I-treated (spot D) nucleic acids from infected cells. RNase A treatment (spot R) obliterated the hybridization signal. (B) Only minus-sense probes, as determined by the available sequence information that defined the coding strand of the cDNA insert, hybridized with RNAs extracted from infected cells.

Nucleotide sequence accession numbers. The GenBank accession numbers of the sequences in Fig. 1A and B are L08014 and L08015, respectively.

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