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EXPRESSION AND PROCESSING OF NONSTRUCTURAL PROTEINS OF THE HUMAN ASTROVIRUSES

C. A. Gibson,[1,3] J. Chen,[4] S. A. Monroe,[4] and M. R. Denison[1,2,3]

[1]Department of Microbiology and Immunology
[2]Department of Pediatrics
Vanderbilt University
[3]Elizabeth B. Lamb Center for Pediatric Research
Vanderbilt University Medical Center
Nashville, Tennessee
[4]Centers for Disease Control
Atlanta, Georgia

1. ABSTRACT

The human astroviruses (HAst) are increasingly recognized as an important cause of gastroenteritis. These viruses contain a 6.8-kb positive-sense, single-stranded RNA molecule that is infectious when transfected into permissive cells. The HAst gene 1 is composed of two open reading frames (ORFs 1a and 1b) connected by a ribosomal frameshift. Gene 1 is predicted to encode two nonstructural polyproteins (pp1a and pp1ab), and analysis of the HAst gene 1 sequence has resulted in predictions of a serine proteinase within the ORF1a polyprotein. However, none of the gene 1 proteins have been identified. To examine the expression and processing of the HAst2 gene 1 polyprotein, we have translated pp1a and pp1ab *in vitro*. These ongoing studies will provide the foundation for correlating gene 1 expression *in vitro* with proteins expressed in virus-infected cells.

2. INTRODUCTION

Astroviruses were first described in 1975 as star-shaped particles in electron micrographs from diarrheal stools of infants (Madeley *et al*). Members of the Astroviridae are now known to be involved in endemic, epidemic and common-source outbreaks of diarrhea, and may especially impact immunocompromised individuals. Of the eight as-

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trovirus serotypes that cause human disease, types 1 and 2 (HAst1 and HAst2) are associated with the majority of infections and clinical gastroenteritis. Complete sequences for both of these serotypes are known.

The astrovirus virion particle contains a 6.8kb single-stranded, positive-sense RNA molecule (RNA 1) that is infectious when directly transfected into permissive cells (Geigenmuller *et al*, 1997). During infection a single subgenomic RNA (RNA 2) is generated; this RNA is 3' coterminal with RNA1 (Monroe *et al*, 1991; Monroe *et al*, 1993). The HAst2 gene 1 consists of two out of frame open reading frames (ORFs 1a and 1b) that are connected by a ribosomal frameshift (Figure 1) (Jiang *et al*, 1993; Marczinke *et al*, 1995; Lewis *et al*, 1996). The gene 1 polyprotein is predicted to be cleaved by the putative serine proteinase (Spro). Sequence analyses have also predicted a nuclear localization signal (NLS) and an immune response element (IRE) as well as membrane spanning domains (MB) and an RNA-dependent-RNA polymerase (Pol) (Matsui *et al*, 1993; Carter, 1994; Lewis *et al*, 1994). RNA 2 is translated as a 90 kDa capsid precursor that is processed to mature capsid proteins, but the mechanism of subgenomic RNA 2 transcription is unknown.

Nothing is known about the pattern of gene 1 polyprotein expression and processing, nor of the role of viral nonstructural proteins in initiating virus replication. We have combined *in vitro* and *in cyto* approaches to begin to define the steps in replication occurring immediately after virus entry. These studies will allow us to compare astrovirus strategies of replication with those of other positive–sense RNA viruses sharing similar genome organization.

3. MATERIALS AND METHODS

3.1. Construction and Expression of the HAst2 Gene 1 Clones

Fragments incorporating ORFs 1a and 1ab were obtained by single step RT-PCR of HAst2-infected cell lysates and were cloned into pBluescript II SK- to generate the Mono

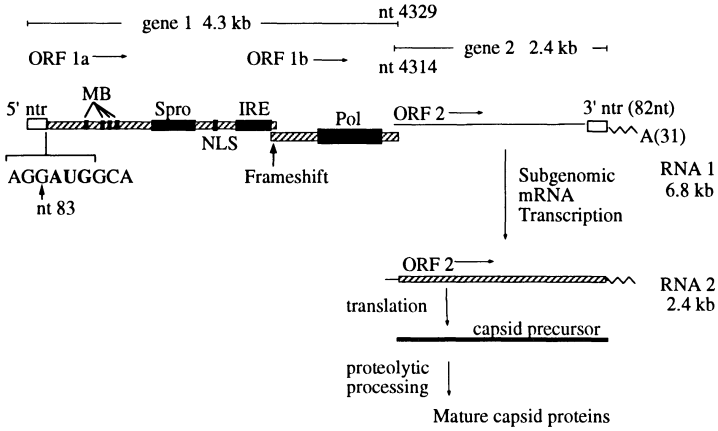

Figure 1. HAst2 genome organization and predicted replication strategy.

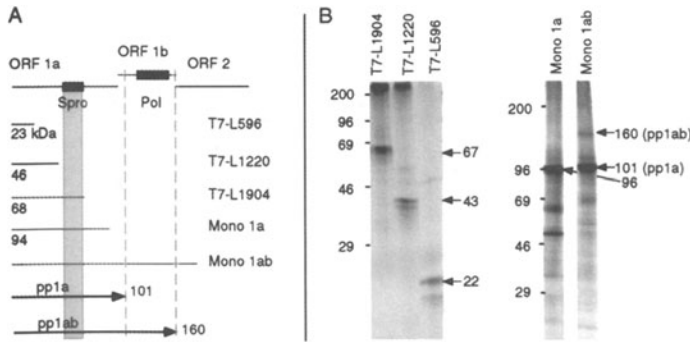

Figure 2. *In vitro* expression of HAst2 gene 1 clones.

1a and Mono 1ab constructs (Figure 2A). The "T7" clones were subcloned from Mono 1ab cDNA using a common 5' primer immediately upstream of the 5' nontranslated region. The masses of the expected proteins (kDa) are shown beneath each clone. The vertical gray box demarcates the putative Spro region. The predicted polyproteins (pp1a and pp1ab) resulting from translation of ORF 1a or ORFs1a/b are shown below the Mono 1ab clone.

The partial and full-length gene 1 cDNA constructs were used to express proteins, using a combined transcription/translation rabbit reticulocyte lysate system (TnT, Promega) with [^{35}S]-methionine (Figure 2B). Numbers to the left of the gels indicate the molecular mass of the marker proteins. Apparent mass (kDa) of individual proteins is shown to the right of the gels.

3.2. Induction of Antibodies against Gene 1 Proteins

Antigenic domains in the gene 1 polyprotein sequence were identified, and synthetic peptides were synthesized (Figure 3). Peptides were coupled to keyhole limpet hemocyanin

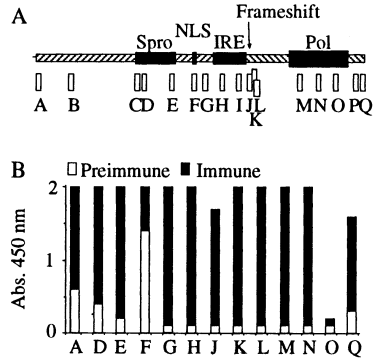

Figure 3. Antigenic domains in gene 1, synthesis of peptides, and immune response in rabbits.

and were used to induce antibodies in rabbits. The HAst2 peptides were used as capture antigens ELISAs with the corresponding preimmune and immune sera (Figure 3B). Sera were diluted 10^2 to 10^7, and the 450nm absorbance reading at the 10^2 dilution was plotted.

3.3. Construction of a Full Length HAst2 Clone

A full length 7kb HAst2 cDNA was generated by single step RT-PCR of HAst2-infected cell lysates. The insert was cloned into pBluescript II SK- by restriction digest at the 5' Cla 1 and 3' BamH1 sites to generate the pAst2 construct.

4. RESULTS AND DISCUSSION

4.1. Expression of the HAst2 Gene 1 Clones

The clone extending from the 5' end of the genome to 214 nucleotides upstream from the ribosomal frameshift (Mono 1a) directed expression of a 96 kDa protein, the expected size for full-length translation of this clone (Fig 2). The Mono 1ab clone expressed a 101 kDa protein consistent with translation termination at the frameshift and also expressed a less prominent protein of 160 kDa, identical to the predicted size of the frameshifted gene 1 fusion polyprotein. These data indicate that ORF 1b is expressed only as a 1ab polyprotein (pp1ab) and that frameshifting occurs in the context of all of gene 1 with an efficiency similar to that observed in subclones containing the frameshift region (Lewis and Matsui, 1996).

Since several of the clones expressed the putative Spro domain, it was surprising that full-length translation products were detected. The reason for the lack of processing is not known but may be due to a need for higher concentrations of viral proteinase than can be synthesized *in vitro*. Alternatively, this result could indicate that *in vitro* processing of the polyprotein is delayed, or that cellular factors not present in the reticulocyte lysate, such as membranes or cellular proteinases, may be needed to initiate or facilitate polyprotein processing.

4.2. Specificity of the Polyclonal Antisera Directed against HAst2 Gene 1 Peptides

Antisera raised to peptides A, D, G, and H when diluted up to 10^5 retained optical density readings at least 3-fold higher than readings for the corresponding preimmune sera. Antisera raised to peptides K, L, M, and N were detectable over background when diluted up to 10^3. Antisera raised against the D peptide are able to detect E. coli-expressed recombinant Spro (rSpro) by Western blot analysis (data not shown).

4.3. Generation of a Full Length HAst2 Clone

In parallel with the *in vitro* expression studies, we are developing an infectious clone for the human serotype 2 astroviruses in order to further characterize the role of the nonstructural polyproteins in HAst2 replication. A full-length HAst2 cDNA clone has been obtained. Preliminary nucleotide sequencing data have revealed several independent point mutations among the clones, which may yield new insights into regions of the genome that are essential for infectivity. The panel of polyclonal antisera will be a tremendous asset to these studies.

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