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An astrovirus frameshift signal induces ribosomal frameshifting in vitro*

Brief Report

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Summary. Expression of the astrovirus RNA-dependent RNA polymerase has been hypothesized to be regulated by (−1) ribosomal frameshifting. Sequence analysis of the 70 nucleotide region between open reading frames 1a and 1b indicates the presence of a shifty heptamer consensus sequence and downstream sequences that may be needed for ribosomal frameshifting. We constructed four astrovirus cassettes that spanned this region and inserted each into the rhesus rotavirus VP4 gene. The constructs were expressed in an in vitro system, and products were immunoprecipitated by rotavirus amino and carboxy terminal-specific monoclonal antibodies. Ribosomal frameshifting, at an efficiency of 6–7%, was demonstrated in all constructs containing the shifty heptamer and stem-loop. Deletion of the downstream sequence potentially involved in pseudoknot formation did not affect frameshifting efficiency. However, deletion of the shifty heptamer resulted in no detectable frameshift activity.

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Astrovirus was first described in 1975 by Madeley and Cosgrove as a human pathogen in a diarrhoeal outbreak among newborns [24]. Seven tissue culture-adapted human serotypes have been identified to date [18–20]. As epidemiologic information accumulates, astrovirus is emerging as a medically important pathogen in infants and children in developing countries [6, 12]. Furthermore, astrovirus appears to be a significant pathogen in immunocompromised

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individuals, including bone marrow transplant recipients [5] and HIV-infected patients [9].

Astrovirus particles are nonenveloped and 28–32 nm in diameter, with a characteristic 5–6 point star-like surface morphology by electron microscopy. Recent molecular information obtained from the complete sequence of two strains of astrovirus serotype 1 [21, 31] and one strain of serotype 2 [13], as well as expression studies [21, 32], supports their classification into a new viral family, the *Astroviridae* [27]. The RNA genome for astrovirus is 6.8 kb in length and comprises three open reading frames (ORF) [13, 21, 31]. ORF1a, encodes viral nonstructural proteins including a 3C-like serine protease motif and ORF1b, which is in the (−1) frame with respect to ORF1a, encodes a viral RNA–dependent RNA polymerase (RDRP) motif. ORF2 encodes a viral structural protein and is probably expressed from a 2.4 kb subgenomic RNA detected in astrovirus-infected cells [21, 26–28, 32].

The mechanism by which the viral RDRP is expressed is not immediately apparent since the codon for the first methionine encoded by ORF1b is at nucleotide (nt) 453–455. This places the first methionine well into the RDRP motif and in a suboptimal context for initiation according to Kozak's rules [17]. Thus, the possibility of an internal ribosome entry site as well as a yet unidentified subgenomic message for polymerase expression are unlikely. Detailed sequence analysis of the overlap region between ORFs 1a and 1b led several investigators to propose ribosomal frameshifting as a mechanism for expression of the astrovirus RDRP gene [13, 21, 31]. Regulation at the level of translation by ribosomal frameshifting was first described for higher eukaryotes by Jacks and Varmus in 1985 for the expression of the Rous sarcoma virus (RSV) Pol gene [16]. Since then, ribosomal frameshifting has been described for many systems including several retroviruses [14], coronaviruses [2, 4], yeast double stranded RNA viruses [7], retrotransposons [1] and most recently astroviruses [25]. A (−1) frameshift at the astrovirus ORF1a/1b overlap would allow the expression of an ORF1a/1b fusion protein containing the viral polymerase. This viral fusion protein is probably cleaved by viral and/or cellular proteases into active peptides.

In general, two cis-acting elements are required for (−1) ribosomal frameshifting: a shifty heptamer with the consensus sequence, X XXY YYZ, followed by potential downstream RNA secondary structures in the form of a stem-loop or more complex pseudoknots [3, 14]. For the astrovirus serotype 1 (Oxford strain) we study, a shifty heptamer, A AAA AAC, at nt 2794–2800 (GenBank Accession Number L23513) of the viral genome was identified. A second potential shifty heptamer (also A AAA AAC at nt 2779–2785) was considered but deemed unlikely since it was not fully conserved among all serotypes reported previously (Newcastle serotype 1 and Oxford serotypes 2 and 5) [21, 31]. The latter serotypes contain the sequence (G AAA AAC) which does not conform to the shifty heptamer consensus sequence described above.

A potential stem loop, located 6 nt downstream from the predicted shifty heptamer (nt 2794–2800), contains a 14 nt GC- rich stem and 10 nt loop (Fig. 1).

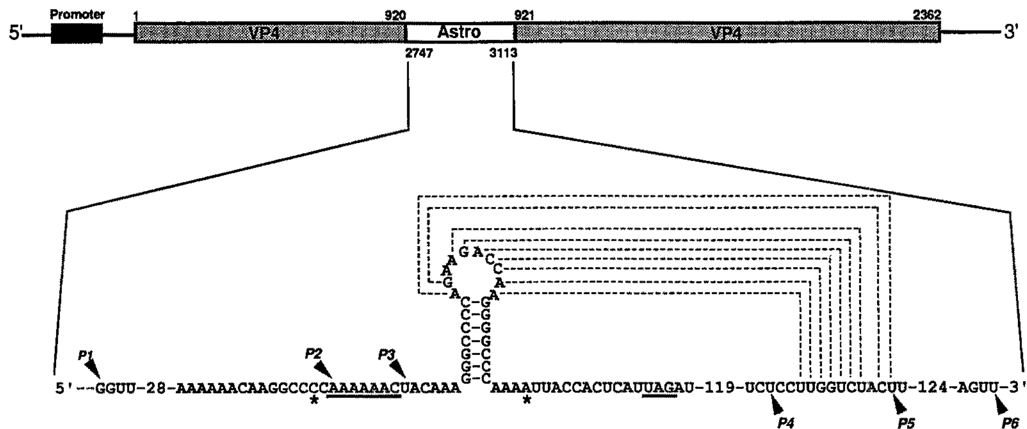

Fig 1. The astrovirus-rotavirus VP4 gene construct. The upper panel depicts the 367 nt astrovirus frameshift cassette (nt 2747–3113), inserted into the SnaB I site of RRV VP4 cDNA. The lower panel shows the RNA sequence of the astrovirus insert, with emphasis on representing the potentially important cis-acting elements for ribosomal frameshifting that include the shifty heptamer (*A AAA AAC*), the downstream stem-loop, and the possible pseudoknot forming region (dashed lines between nt in the loop of the stem-loop and downstream complementary sequence). The (0) frame stop codon (*UAG*), and the 5′ ends of the forward (P1, P2, P3 arrowheads) and reverse (P4, P5, P6 arrowheads) primers used to generate constructs A–D are also shown. The sequence between the asterisks is completely conserved among the three astrovirus serotypes tested to date [21]

The sequence between nt 2792–2834 includes both the shifty heptamer and the potential stem-loop and is completely conserved among the human astrovirus serotypes 1, 2, and 5 that have been evaluated to date [21]. This suggests that this 43 nt region, defined by nt 2792–2834, may encode an important function for human astroviruses. The highest region of complementarity for pseudoknot formation is found between 9 of 10 nt of the loop (nt 2814–2823) of the stem-loop and nt 2975–2984, located 151 nt downstream. Sequences between the stem-loop and proposed pseudoknot forming regions appear to have lower degrees of complementarity with the 10 base loop (see below).

The strategy we adopted to prove that these sequences are involved in frameshifting in astroviruses was to clone the suspected frameshift region into the rhesus rotavirus (RRV) VP4 gene. RRV VP4 encodes an 86.5 kDa protein that serves as the outer capsid spike protein for rotavirus [22]. The VP4 gene was selected since well-characterized monoclonal antibodies (MAbs) specific for both the amino- or carboxy terminal-specific portions of the VP4 protein are available [23]. For the astrovirus-VP4 constructs used in the current studies, these MAbs allowed the differentiation of full-length protein translated by (−1) ribosomal frameshifting from a truncated, nonframeshifted protein when translation stops in the (0) frame, as described below. Direct analysis of frameshifting in astrovirus-infected cells was not possible since immunological reagents have not yet been developed for specific astrovirus proteins. The strategy of cloning a suspected ribosomal frameshift region into a foreign gene has also been used to demon-

strate frameshifting in the coronavirus, infectious bronchitis virus (IBV) [4], and some of the retroviruses such as human immunodeficiency virus [29]. Marczinke et al also adopted a similar scheme using the influenza genes PB1 and PB2 and amino terminal-specific polyclonal antibodies to evaluate the ORF1a/1b overlap region of the Newcastle strain of astrovirus serotype 1 [25].

The astrovirus frameshift cassettes were generated by the polymerase chain reaction (PCR) using a cloned astrovirus serotype 1 fragment as the template. The PCR and blunt-end ligations were done as previously described [21]. The astrovirus cassettes were cloned into a unique *Sna*B I site (nt 918–923) of RRV VP4 such that the amino terminus of VP4 is translated from the (0) frame and the carboxy terminus from the (−1) frame. All frameshift constructs were verified by sequencing the entire astrovirus insert as well as flanking VP4 sequences. The chimeric constructs as well as the original VP4 construct were linearized with *Sal*I followed by run-off transcription with T7 RNA polymerase. These transcripts were translated in vitro in a rabbit reticulocyte lysate system. The proteins synthesized in vitro were immunoprecipitated as described previously [21], using the VP4 amino terminal-specific MAb 7A12 [30], and the carboxy terminal-specific MAb HS2 (kindly provided by Dr. H. B. Greenberg).

The first VP4-astrovirus chimeric clone studied was designated construct A. Construct A contained 367 nt of astrovirus sequence (nt 2747–3113) and spanned the region from 47 nt upstream from the shifty heptamer to 129 nt downstream of the sequence suspected of forming a pseudoknot (Fig. 1). The PCR primers used to generate the construct A cassette were forward primer, P1 (5′-GGTTTGG-AAGGTTTCCTCCAA-3′), which contained one VP4 nt (G) at the 5′-end to maintain the frame, and reverse primer, P6 (5′- AACTGTGGATATTCTGA-GAAAAAGT-3′). We hypothesized that if this sequence induces frameshifting, amino terminal-specific MAb 7A12 would immunoprecipitate two products from the in vitro translation mixture: a 40 kDa truncated protein where translation stops in the (0) frame and a 100 kDa chimeric protein in which translation continues in the (−1) frame avoiding the (0) frame stop codon, 45 nt from the shifty heptamer (Fig. 1). These two products were detected by MAb 7A12 indicating a (−1) ribosomal frameshift has occurred (Fig. 2A, lane 3). An 86.5 kDa protein translated in vitro from the VP4 construct with no astrovirus insertion was immunoprecipitated by MAb 7A12 (Fig. 2A, lane 4). The band below the 100 kDa chimeric protein and 86.5 kDa VP4 is likely an internal initiation product or a partial degradation product and was also detected from RRV-infected cell lysates (data not shown). The carboxy terminal-specific MAb HS2 immunoprecipitated the 100 kDa chimeric protein, but the 40 kDa protein was not detected, as predicted (Fig. 2A, lane 1). This verifies the carboxy terminal-specific portion of the translated chimeric protein is VP4. VP4, translated from the VP4 construct, was also immunoprecipitated by MAb HS2 (Fig. 2A, lane 2). The two bands directly below the uppermost bands in lanes 1 and 2 likely represent internal initiation products in this in vitro system. The identity of the other bands (25–50 kDa) detected by MAb HS2 (Fig. 2A, lanes 1 and 2) were not characterized further but are found in both the VP4 and

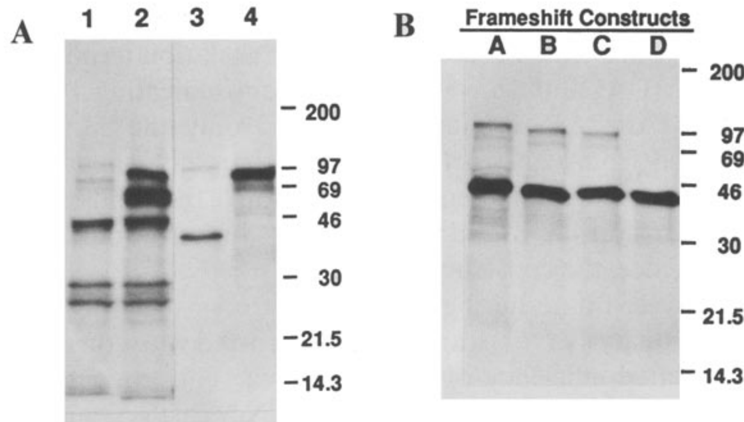

Fig 2. **A** Demonstration of ribosomal frameshifting by immunoprecipitation analysis of in vitro translation products: Construct A lanes 1 and 3, and a VP4 construct that does not contain an astrovirus insert, lanes 2 and 4. The VP4 carboxy terminal-specific MAb HS2 was used for immunoprecipitation in *1* and *2*, and the VP4 amino terminal-specific MAb 7A12 in *3* and *4*. The polypeptides were labeled with ^{35}S-methionine and separated on an 12% SDS-PAGE gel followed by autoradiography. **B** Identification of important astrovirus sequences in ribosomal frameshifting. The in vitro translation products of constructs *A*, *B*, *C*, and *D* were immunoprecipitated with the VP4 amino terminal-specific MAb 7A12. The polypeptides were labeled with ^{35}S-methionine and separated on a 12% SDS-PAGE gel followed by autoradiography. This gel was analyzed quantitatively using a phosphorimager to determine the efficiency of frameshifting

frameshift constructs. We speculate that these bands represent degradation products not detectable by MAb 7A12.

In order to determine what the shifty heptamer and downstream sequence complementary to the loop contributed to frameshifing, three additional constructs (B, C, and D) were designed and generated byPCR. Construct B (191 nt astrovirus cassette) is an abbreviated version of the original construct A, with the shifty heptamer sequence (nt 2794–2800) at its upstream border and the hypothetical pseudoknot sequence at its downstream border. The upstream potential, but less likely, shifty heptamer (nt 2779– 2785) was not included in this construct. Construct C (179 nt) was further shortened by deleting the downstream sequence potentially involved in pseudoknot formation. In construct D (313 nt), the shifty heptamer was eliminated but all downstream sequences (including the potential pseudoknot sequence) found in construct A were maintained. The forward and reverse PCR primers, respectively, used to generate the astrovirus cassettes in constructs B–D were: P2 (5′-AAAAAACTACAAAGGGCCCCAGA-3′) and P5 (5′-AGTAGACCAAGGAGATCGT-3′) for construct B; P2 and P4 (5′-AGATCGTCCCTGGGTTTCTT-3′) for construct C; and P3 (5′- GTACAAAGGGCCCCAGAAGA-3′) and P6 for construct D. The location of the 5′-end of each primer is indicated by arrowheads in Fig. 1.

Constructs A–D were transcribed and translated in vitro, then immunoprecipitated with the VP4 amino terminal-specific MAb 7A12. The pattern of immunoprecipitation of the protein products from constructs B and C were

similar to that of construct A (Fig. 2B). MAb 7A12 immunoprecipitated two proteins, a 38 kDa truncated protein in which translation terminated at the (0) frame stop codon (UAG), and a ~93.5 kDa protein, indicating a (−1) frameshift had occurred. MAb 7A12 immunoprecipitated only the 38 kDa truncated protein translated from construct D in which the shifty heptamer has been deleted (Fig. 2B). The slight differences in migration of the bands representing the chimeric protein in lanes A, B, and C of Fig. 2B is due to the difference in sizes of the inserts and hence, the construct products.

The efficiency of frameshifting in this in vitro system was determined by phosphorimager analysis. (−1) frameshifting occurred with constructs A, B, and C, with a calculated efficiency of 6–7% in each. This concurs with the 5% efficiency determined by densitometry for the Newcastle strain of astrovirus serotype 1 [25] and is similar to that reported for RSV [16], but is significantly lower than the 20–30% efficiency reported for IBV [4]. It is notable that, construct B, which included the most likely shifty heptamer (nt 2794–2800) but not the upstream potential shifty heptamer (nt 2779–2785), shifted with an efficiency indistinguishable from construct A in which both potential shifty heptamers were included.

Several factors may explain why construct C, which lacked the downstream sequence potentially involved in pseudoknot formation, shifted with an efficiency equal to A and B. First, our studies were conducted in an in vitro system which may not mimic in vivo conditions, where regulation of frameshifting may be more subtle. Second, the downstream sequence that may be involved in pseudoknot formation is 151 nt from the loop of the upstream stem loop. The distance between the loop and its complementary downstream sequence is greater than that described for pseudoknot structures in other viruses [3, 10]. Jiang et al. suggest a sequence 29 nt from the loop as potentially being involved in pseudoknot formation for astrovirus serotype 2 [13]. This sequence complements the loop of the upstream stem-loop in 5 of 10 nt for serotype 2 and only 4 of 10 nt for both strains of serotype 1. The sequence 151 nt downstream has a homology of 7 of 10 nt for serotype 2, 8 of 10 nt for the Newcastle strain of serotype 1 [31], and 9 of 10 nt for the Oxford strain of serotype 1 used in this study. A third possibility is that the system we studied does not require pseudoknot formation. This is most likely the case since Marczinke et al. demonstrated that a 37 nt oligonucleotide from the Newcastle strain of astrovirus serotype 1 which contains only the shifty heptamer and stem-loop regions induces frameshifting at an efficiency equal to their largest construct of 2000 nt. Other viral frameshift systems, such as HIV-1 [29] and HTLV-II [8], also require only a shifty heptamer and stem loop for frameshifting.

Construct D demonstrates that the shifty heptamer at nt 2794–2800 is required for (−1) ribosomal frameshifting and the shifty heptamer upstream at nt 2779–2785 is not utilized since its deletion (construct B) does not affect the efficiency of frameshifting. The phosphorimager analysis detected little or no chimeric protein from construct D, and no band was detected by autoradiography as shown in Fig. 2B or with longer exposure (not shown).

The astrovirus shifty heptamer sequence (A AAA AAC) is identical to the slippery sequence utilized by mouse mammary tumor virus, human T-cell leukemia virus 1 and 2, equine infectious anemia virus, and simian T-cell leukemia virus [10, 11, 15]. In the astrovirus system, a (−1) frameshift would result in no mismatches in the ribosome P-site codon with its cognate tRNA-Lys. In the ribosome A-site, a (−1) shift would result in a one base mismatch between the tRNA-Asn and the AAA (−1) frame codon, which is AAC in the (0) frame.

Development of immunologic reagents that are specific for protein products of several regions of the astrovirus genome should provide important tools for studying astrovirus genomic regulation. This includes the determination of frameshifting efficiency in susceptible cells and the effect on the astrovirus replication cycle. In conclusion, we have identified a sequence in astrovirus that causes ribosomal frameshifting in the foreign gene, rhesus rotavirus VP4. With the addition of the nonenveloped astroviruses, there are now three distinct animal virus families that utilize (−1) ribosomal frameshifting as means of regulation at the level of translation: the *Retroviridae*, the *Coronaviridae*, and the *Astroviridae*.

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