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Astrovirus Ribosomal Frameshifting in an Infection-Transfection Transient Expression System

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Different regions of the human astrovirus frameshift signal were cloned into the rhesus rotavirus VP4 gene and evaluated in an infection-transfection transient expression cell culture system. BHK-21 cells, infected with a vaccinia virus that expresses T7 RNA polymerase (vTF7-3), were transfected with the various astrovirus-VP4 constructs. All constructs were driven by a T7 promoter and contained an internal ribosome entry site. Frameshifted and nonframeshifted protein products were immunoprecipitated with VP4 amino- and carboxy-terminal-specific monoclonal antibodies, and their ratios were determined by PhosphorImager analysis. The efficiency of frameshifting was 25 to 28%, significantly greater than the 5 to 7% efficiency reported previously in a cell-free translation system. Coupling of transcription and translation in a cell-free system yielded frameshifting efficiencies threefold greater than that of the uncoupled in vitro system. The presence of the shifty heptamer was an absolute requirement for frameshifting in both cell-free and intact-cell systems, while deletion of the potential downstream pseudoknot region did not affect the efficiency of frameshifting.

Astroviruses are small (28 to 32 nm in diameter) nonenveloped particles with a single-stranded plus-sense RNA genome, approximately 6,800 nucleotides (nt) in length (19, 22, 39). Human astroviruses are recognized as a cause of acute gastroenteritis in young children (8, 14, 25), elderly patients (21, 33), and immunocompromised hosts (7, 13). On the basis of analysis of the complete sequences from two strains of human astrovirus serotype 1 and one strain of serotype 2 as well as expression studies, human and morphologically similar animal astroviruses have recently been classified as members of a new viral family, the *Astroviridae* (19, 22, 27–30, 39, 40). The viral genome consists of three long open reading frames (ORFs): 5′-ORF1a-ORF1b-ORF2-3′. ORF2 spans the 3′ third of the genome and encodes a viral structural protein(s) that is likely expressed from a subgenomic RNA (22, 29, 40). ORF1a and -1b contain nonstructural protein coding motifs, including a 3C-like serine protease motif and a viral RNA-dependent RNA polymerase motif, respectively.

The astrovirus viral RNA-dependent RNA polymerase is likely expressed by a −1 ribosomal frameshift (19, 22, 39); −1 ribosomal frameshifting is a mechanism of regulation at the level of translation that was first described for higher eukaryotes in 1985 as the means by which the Rous sarcoma virus *pol* gene is expressed (18). Translational regulation by −1 ribosomal frameshifting has been demonstrated subsequently in other retroviruses as well as in coronaviruses (infectious bronchitis virus [IBV] and mouse hepatitis virus) and a torovirus (Berne virus) (1–3, 15, 16, 38). Two fundamental *cis*-acting sequence elements are required in a mammalian ribosomal frameshift signal: a shifty heptamer with the consensus sequence X XXY YYZ followed by a downstream RNA secondary structure in the form of a stem-loop, or a more complex configuration called a pseudoknot (reviewed in references 2 and 15).

On the basis of the following observations, −1 ribosomal frameshifting was proposed as the mechanism for translation of the astrovirus viral RNA-dependent RNA polymerase. The first potential start codon in ORF1b is located at nt 3,229 to 3,231 (455 nt into ORF1b) in a suboptimal context for initiation according to Kozak's rules (20). The astrovirus ORF1a/1b overlap region, comprising 70 nt, contains both *cis*-acting signals required for −1 ribosomal frameshifting: a shifty heptamer with the sequence A AAA AAC and, 6 nt downstream, a potential stem-loop structure composed of a 14-nt GC-rich stem and 10-nt loop (19, 22, 39). Both the shifty heptamer and the stem-loop are completely conserved among astrovirus serotypes 1, 2, and 5, the only serotypes completely sequenced in this region (22).

Recently, two groups demonstrated that the astrovirus frameshift signal presented in an heterologous context can induce frameshifting in a cell-free transcription-translation system (23, 26). The efficiency of frameshifting was determined to be 5 to 7% in these uncoupled cell-free systems. These studies demonstrated the absolute requirement for both the shifty heptamer (23) and the stem-loop (26). However, pseudoknot formation was not found to be essential, since a 37-nt minimal sequence that spans astrovirus sequence from the shifty heptamer through the stem-loop induces frameshifting with the same efficiency as much larger constructs containing long flanking astrovirus sequences proximal to the shifty heptamer and distal to the stem-loop (26). A pseudoknot structure is also not required in at least two other viral systems, human immunodeficiency virus type 1 (HIV-1) and human T-cell leukemia virus type 2 (10, 34, 36, 41). In contrast, the avian coronavirus IBV depends on the formation of a pseudoknot structure to achieve optimal frameshifting efficiencies (4, 5).

To determine whether additional controls operate in intact cells, we examined the efficiency of astrovirus frameshifting and the requirement for a pseudoknot in a vaccinia virus infection-transfection system. The heterologous constructs described previously were among those used in the current stud-

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ies (23). Briefly, astrovirus frameshift inserts of various lengths were cloned into the rhesus rotavirus (RRV) outer capsid gene VP4, and frameshifted and nonframeshifted products were distinguished by VP4 monoclonal antibodies (MAbs) specific for either the amino or carboxy terminus. This strategy was necessary, since no such serological reagents currently exist for astrovirus. The heterologous constructs were introduced into an infection-transfection transient expression assay, in which plasmids containing an internal ribosome entry site (IRES) and a T7 promoter were transfected into cells infected with a recombinant vaccinia virus expressing T7 RNA polymerase (9, 11, 32). The efficiency of frameshifting induced by the astrovirus frameshift signal was examined and compared with that found in an uncoupled cell-free system, a coupled cell-free system, and infection-transfection assays for HIV-1 and IBV.

MATERIALS AND METHODS

Expression constructs. Heterologous astrovirus serotype 1 frameshift plasmids (constructs A, B, C, and D) previously used in an in vitro expression system (23) were adapted for use in the infection-transfection system, as depicted in Fig. 1a. Astrovirus inserts in constructs A (367 nt) and B (191 nt) contain the shifty heptamer, stem-loop, and downstream sequences potentially involved in pseudoknot formation. In construct C (179 nt), the downstream potential pseudoknot region is deleted, but the shifty heptamer and stem-loop are intact. Construct D (313 nt) contains the stem-loop and downstream potential pseudoknot-forming region, but the shifty heptamer is deleted. Constructs A, B, C, and D as well as full-length VP4 cDNA were subcloned into DNA plasmid pTM1 (kindly provided by B. Moss), which contains a multiple cloning region immediately 3′ to a T7 promoter and an encephalomyocarditis virus internal ribosome entry site (IRES) (32) (Fig. 1a). The infection-transfection system supports high levels of transient protein expression through the use of recombinant vaccinia virus vTF7-3, which expresses T7 DNA-dependent RNA polymerase throughout its replication cycle (9, 11). Since optimal utilization of the IRES requires the VP4 start codon (ATG) to be in the unique *Nco*I site, we devised a partial PCR strategy for subcloning into the pTM1 vector. A 5′ oligomer containing an *Nco*I site and 23 nt of the 5′ end of the VP4 coding region (5′-CCCC**ATG**GCTTCG CTCATTTATAGACA-3′) and an oligomer 3′ of a unique VP4 *Hpa*I site (Fig. 1a) were used for PCR with cloned VP4 as the template. The resulting 1.0-kb PCR product was made blunt ended with T4 DNA polymerase, digested with *Nco*I, and ligated into pTM1 that was digested with *Nco*I and *Sma*I. This construct was subsequently digested with *Hpa*I-*Sal*I, and the VP4 PCR fragment 3′ of the *Hpa*I site was discarded. This resulted in an *Hpa*I-*Sal*I plasmid, pTM1, that contains the 5′ 144 nt of VP4. The *Hpa*I-*Sal*I fragment from VP4 and from constructs A, B, C, and D was then ligated into *Hpa*I-*Sal*I pTM1 (Fig. 1a). The final constructs contained 144 nt of VP4 PCR product (*Nco*I to *Hpa*I), with the remainder (*Hpa*I to *Sal*I) derived from previously well-characterized clones. The entire region derived by PCR, including junctions, was completely sequenced to ensure that no PCR artifacts or errors were introduced during cloning steps.

Minimal heterologous frameshift constructs that contained the shifty heptamer and stem-loop sequences of astrovirus (26), HIV-1 (17), and the avian coronavirus IBV (4) (Fig. 1b) were synthesized as complementary oligomers, annealed, and inserted into the *Sna*BI-digested VP4 cloned into pTM1 (23). The astrovirus sense oligomer for construct C_m consisted of 37 nt of astrovirus-specific sequence plus an introduced 0-frame stop codon (indicated in boldface): 5′-AAAAAACTACAAAGGGCCCCAGAAGACCAAGGGGCCC**TAG**T-3′. Similarly, the sense oligomer for HIV-1 consisted of 43 nt of HIV-1 sequence plus an introduced 0-frame stop codon (in boldface): 5′-TTTTTTAGGGAA GATCTGGCCTTCCTACAAGGGAAGGCCAGGG**TAG**T-3′. The minimal sequence selected for IBV also includes the shifty heptamer and stem-loop, but not the region downstream that is involved in pseudoknot formation (4, 5). The IBV sense oligomer 5′-TTTAAACGGGTACGGGGTAGCAGTGA GGCCTCGGC**TGA**TACCCCT-3′ contains the authentic stop codon (in boldface). The sequence and orientation of the inserts and all flanking regions were confirmed by sequencing.

Infection-transfection transient expression assay. All eight plasmid constructs described above were used in the infection-transfection transient expression assay. Baby hamster kidney cells (BHK-21) were infected with vTF7-3 at a multiplicity of infection of 10 in minimal essential medium (MEM) containing 5% fetal bovine serum at 37°C. After a 30-min adsorption, the inoculum was removed, and the cells were washed with MEM lacking serum. The cells were then transfected with 2 μg of plasmid DNA in 10 μg of Lipofectamine (Gibco-BRL) and 0.5 ml of MEM per well (9.6 cm^2). After 2 h, the transfection mixture was removed and the cells were washed with methionine-free MEM. The cells were then incubated for 4 h at 37°C in 0.5 ml of methionine-free MEM containing 2% fetal bovine serum and 40 μCi of [^{35}S]methionine (Amersham) per well. As a negative control, cells were treated as described above, but no plasmid DNA was transfected (mock transfection).

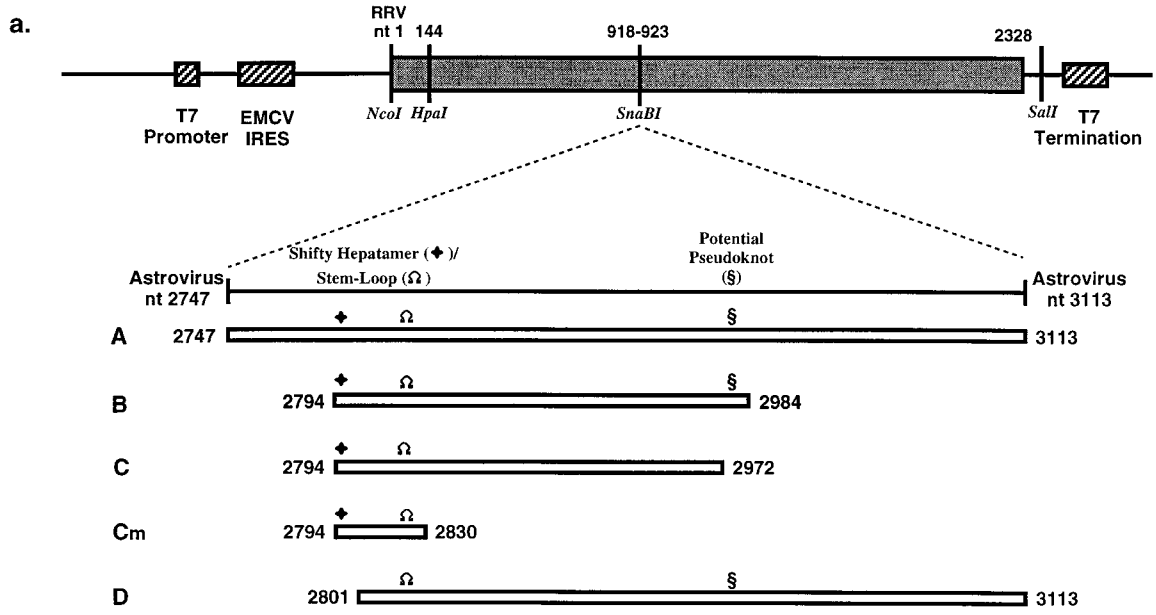
In vitro transcription-translation. The astrovirus, IBV, and HIV-1 minimal frameshift constructs as well as astrovirus construct A and VP4 were transcribed and translated in vitro in both an uncoupled cell-free system, as previously described (22, 23), and a coupled system. In the uncoupled system, the constructs were linearized with *Sal*I and underwent runoff transcription with T7 RNA polymerase at 37°C. The transcripts were translated in vitro in rabbit reticulocyte lysates at 30°C. The in vitro coupled transcription-translation reactions were performed with the TnT kit (Promega) with T7 RNA polymerase under conditions recommended by the manufacturer. All proteins produced in vitro were labeled with [^{35}S]methionine.

Cell lysis and radioimmunoprecipitation. Cell lysates were prepared for radioimmunoprecipitation as previously described (22). Untreated lysates were examined to ensure that relatively equal quantities were used for immunoprecipitation (Fig. 2a). To immunoprecipitate VP4-specific proteins from the total protein produced in the infection-transfection system, 1 ml of radioimmunoprecipitation assay buffer, followed by 1 μl of terminal-specific MAb 7A12 (amino) or HS2 (carboxy), was added to 25 to 50 μl of BHK-21 cell lysate (22, 24, 37). For immunoprecipitation of the protein(s) produced in vitro, 1 ml of radioimmunoprecipitation assay buffer and 1 μl of MAb 7A12 were added to 30 μl of total lysate for the uncoupled system and 50 μl of total lysate for the coupled system. After incubation at room temperature for 1 h, immune complexes were precipitated with protein A conjugated to Sepharose beads. Immunoprecipitates were washed six times with 1.0 ml of radioimmunoprecipitation assay buffer, followed by two washes with 1.0 ml of buffer containing 150 mM NaCl and 50 mM Tris-HCl (pH 7.5). Immunoprecipitates were solubilized in gel loading buffer with β-mercaptoethanol and sodium dodecyl sulfate (SDS) and separated on an SDS–12% polyacrylamide minigel. The gel was fixed, enhanced with Entensify (DuPont), dried, and autoradiographed. To quantify radioactivity in individual bands, gels were analyzed by PhosphorImager (Molecular Dynamics).

RESULTS

Infection-transfection transient expression assay. To study astrovirus frameshifting in cell culture, we used heterologous astrovirus-rotavirus constructs in an infection-transfection transient expression assay. In this assay, the heterologous frameshift constructs are introduced into cells as DNA. RNA is transcribed in the cytoplasm by the T7 DNA-dependent RNA polymerase that is provided by recombinant vaccinia virus vTF7-3 infection in BHK-21 cells (11). The majority of transcripts are uncapped but contain the encephalomyocarditis virus 5′ IRES to achieve efficient cap-independent translation (9). MAbs that are specific for either the amino or carboxy terminus of RRV VP4 were used to differentiate frameshifted from nonframeshifted radiolabeled proteins by immunoprecipitation as described previously (22, 23). Briefly, if translation of the heterologous astrovirus-rotavirus constructs terminates at the 0-frame stop codon, a ca. 40-kDa truncated protein whose amino terminus is detectable by MAb 7A12, but not by MAb HS2, is produced. If a −1 ribosomal frameshift occurs, a chimeric protein with a molecular mass of ca. 100 kDa is produced and its amino and carboxy termini are detectable by MAbs 7A12 and HS2, respectively.

(i) Astrovirus frameshift constructs. vTF7-3-infected cells were mock transfected or transfected with the VP4 or astrovirus frameshift constructs (A, B, C, C_m [astrovirus minimal frameshift sequence from shifty heptamer through stem-loop], and D). Lysates were immunoprecipitated with the amino-terminal-specific MAb 7A12 (Fig. 2b). For all constructs containing the astrovirus frameshift sequence, a ca. 40-kDa truncated nonframeshifted protein was detectable, indicating that translation had stopped at the termination codon in the 0-frame. In addition, a ca. 100-kDa chimeric protein was immunoprecipitated by MAb 7A12 from lysates (of cells transfected with constructs) A, B, C, and C_m, demonstrating that −1 ribosomal frameshifting had occurred. No protein was detected in mock-transfected cells, while only the truncated protein was detected in lysates from construct D. When the carboxy-terminal-specific MAb HS2 was used for immunoprecipitation, a ca. 100-kDa frameshifted protein was detected in lysates A, B, C, and C_m, thus verifying the carboxy terminus as VP4 (Fig. 2c). No protein was immunoprecipitated by MAb



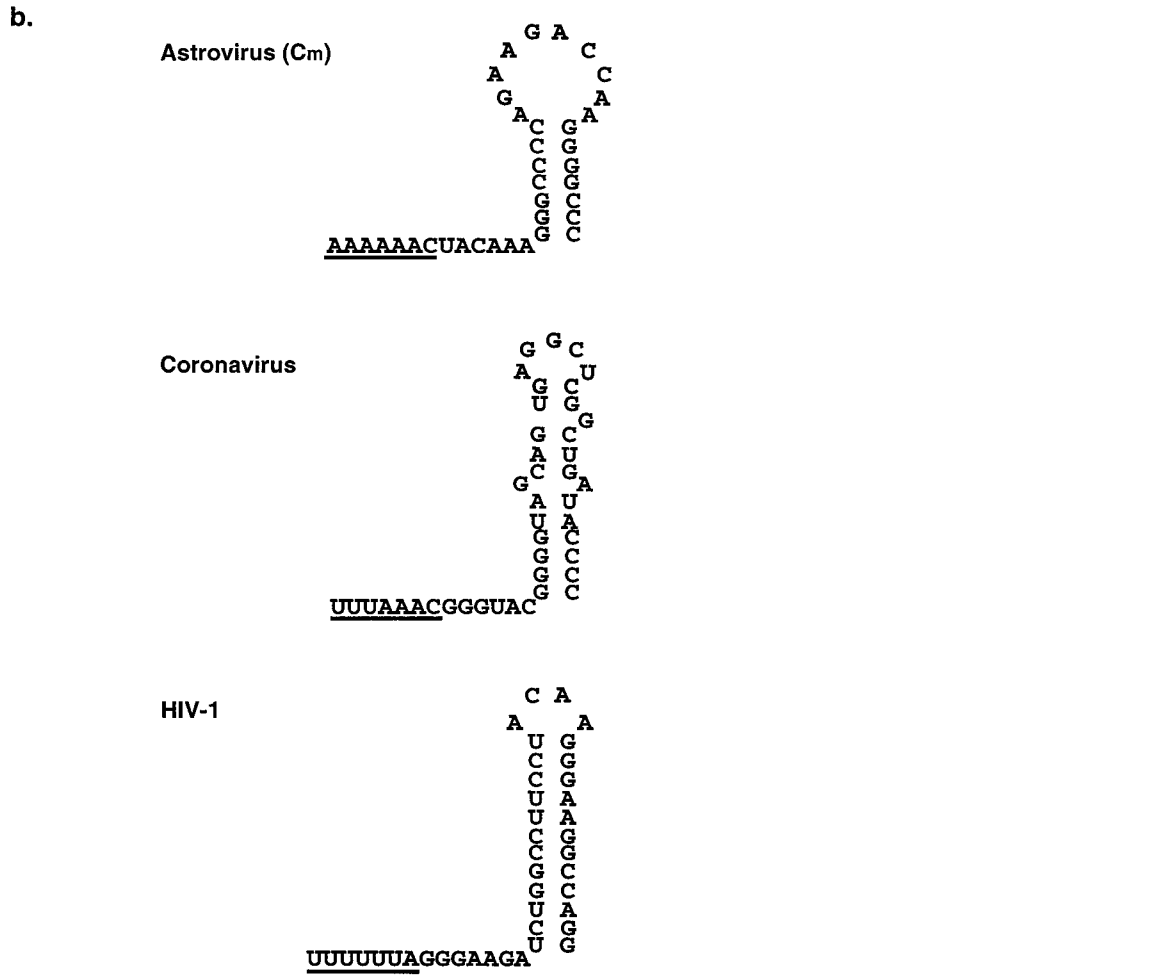


FIG. 1. Schematic diagram of the frameshift expression constructs. (a) RRV VP4 gene (shaded region) cloned into the *Nco*I-*Sal*I restriction sites of the pTM1 vector. The pTM1 vector contains a T7 promoter, an IRES, and a T7 terminator (hatched boxes). A, B, C, C_m (Cm), and D represent the astrovirus fragments (astrovirus genome locations are noted by nucleotide number) cloned as cDNA into the *Sna*BI site of VP4 and expressed both in vitro (uncoupled and coupled) and in the infection-transfection transient expression assay. The elements of the astrovirus frameshift signal that were tested by these constructs are the shifty heptamer (✦), stem-loop (Ω), and potential pseudoknot-forming region (§) complementary to the loop of the stem-loop. EMCV, encephalomyocarditis virus. (b) Minimal frameshift signals, including the shifty heptamer and the stem-loop, from astrovirus (C_m), coronavirus (IBV), and HIV-1. These frameshift regions were ligated into the *Sna*BI site of VP4.

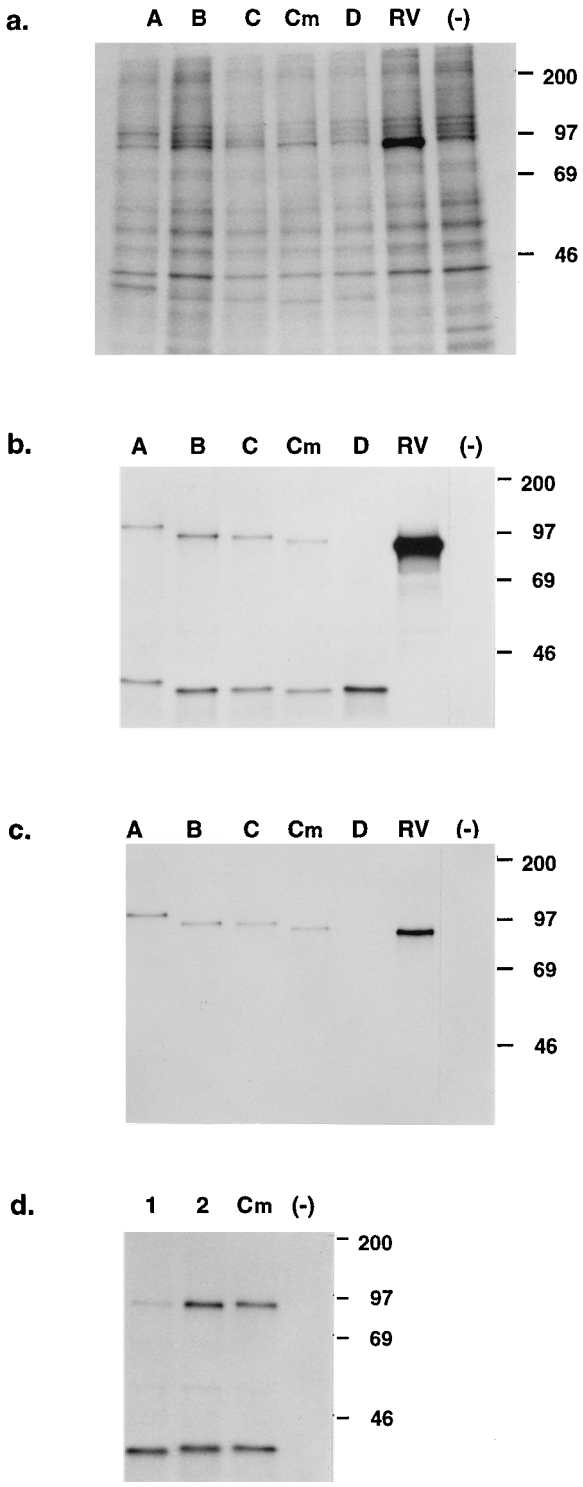


FIG. 2. Ribosomal frameshifting in intact cells. The various frameshift constructs described in Fig. 1 were expressed in cells by the infection-transfection transient expression system. Proteins were labeled by incorporation of [^{35}S]methionine and, after cell lysis, analyzed on an SDS–12% polyacrylamide gel directly (a) or after immunoprecipitation with the amino-terminal-specific MAb 7A12 (b and d) or the carboxy-terminal-specific MAb HS2 (c). Lysates were derived from mock-transfected cells [lane (−)]; cells transfected with VP4 containing no insert (lane RV); the astrovirus frameshift constructs A, B, C_m (Cm), and D; or the minimal frameshift sequences from IBV (lane 1) and HIV-1 (lane 2).

HS2 from lysates of mock-transfected cells or cells transfected with construct D. This confirms that the shifty heptamer is essential for ribosomal frameshifting. The 87-kDa RRV VP4 was specifically immunoprecipitated by both terminal-specific MAbs 7A12 and HS2 (Fig. 2b and c).

The efficiency of −1 ribosomal frameshifting was calculated as the amount of radioactivity detectable in the ca. 100-kDa frameshifted protein band compared with the total amount of radioactivity in the frameshifted protein and nonframeshifted protein bands. All values were adjusted for the number of methionine residues in the frameshifted and truncated proteins. Constructs A, B, C, and C_m all induced frameshifting with similar efficiencies of 25 to 28%. Since C_m only contains the shifty heptamer and the stem-loop (37 nt of astrovirus sequence), the potential pseudoknot-forming sequences as well as other regions distal to the stem-loop are not required for frameshifting in intact cells. Unexpectedly, however, the frameshifting efficiency we observed in intact cells is four to five times higher than that reported for comparable constructs in cell-free studies (23, 26).

(ii) Infection-transfection assay of IBV and HIV-1 minimal frameshift constructs. The minimal frameshift constructs from astrovirus (C_m), IBV, and HIV-1 (Fig. 1b) were tested in the infection-transfection system. Lysates were immunoprecipitated with amino-terminal-specific MAb 7A12 (Fig. 2d). All of the plasmids transfected above induced ribosomal frameshifting, as indicated by detection of the ca. 100-kDa frameshifted protein. As expected, the nonframeshifted protein (ca. 40 kDa) produced when translation stops in the 0-frame was also detected. No proteins were detected in the lysates from mock-transfected cells. The efficiencies of frameshifting were determined to be 25 to 28% for astrovirus (C_m), 8 to 9% for IBV, and 28 to 30% for HIV-1. The calculated efficiency we observed for the HIV-1 construct is approximately 10 times higher than those previously reported for other expression systems in which transcription occurred in the nucleus rather than in the cytoplasm as in our studies (6, 31, 35, 36). The 8 to 9% frameshifting efficiency determined in our experiment for the IBV construct is three times lower than that previously reported (3). These prior studies examined the impact of larger IBV constructs that contain the pseudoknot region known to contribute to efficient frameshifting of IBV (4, 5). In contrast, we examined only the effect of the IBV shifty heptamer and stem-loop region, independent of the downstream pseudoknot-forming region.

In vitro transcription and translation. Because of the marked increase in frameshifting efficiency for the astrovirus minimal frameshift construct in the infection-transfection system, the constructs were tested in an uncoupled cell-free system to determine whether any features of the pTM1 vector, such as the IRES, have any effect on the level of frameshifting. A cell-free system in which transcription and translation are coupled was also tested to investigate whether spatial coupling and temporal coupling influence the efficiency of frameshifting.

(i) Uncoupled in vitro transcription and translation. The minimal constructs for IBV and HIV-1, as well as the astrovirus constructs C_m and A, were transcribed and translated in vitro in an uncoupled manner. The lysates were immunoprecipitated with MAb 7A12 (Fig. 3a). The efficiency of frameshifting of the astrovirus constructs A and C_m was 4 to 5%, that of the IBV construct was 2.6%, and that of the HIV-1 construct was 5%. These values are within the range of efficiencies reported previously for these viruses when examined in vitro and indicate that the pTM1 vector does not have an effect on frameshifting efficiency (4, 23, 26, 34, 41).

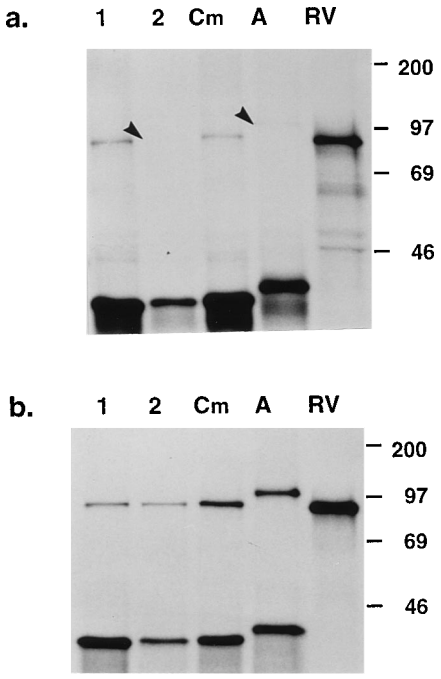


FIG. 3. Frameshifting in cell-free systems. The minimal frameshift constructs from IBV (lane 1), HIV-1 (lane 2), and astrovirus (C_m [Cm]), as well as the astrovirus construct A and the VP4 construct with no insert (lane RV), were expressed in both uncoupled and coupled in vitro systems. The amino terminal-specific MAb 7A12 was used to immunoprecipitate lysates from the uncoupled (a) and coupled (b) cell-free systems.

(ii) Coupled in vitro transcription and translation. The constructs described above (IBV, HIV-1, A, and C_m) were transcribed and translated in a coupled in vitro transcription and translation system. The lysates from the coupled transcription-translation reactions were also immunoprecipitated with MAb 7A12 (Fig. 3b). The efficiency of frameshifting was significantly increased over that observed in the uncoupled cell-free assay. Astrovirus constructs A and C_m induced frameshifting at an efficiency of 15 to 16%, the IBV coronavirus construct did so at 5%, and the HIV-1 construct did so at 11% (Table 1). The astrovirus constructs induced frameshifting with an efficiency nearly three times higher in the coupled cell-free system than in the previously reported uncoupled system (23, 26). The frameshifting efficiency induced by the IBV coronavirus construct in the coupled system was nearly two times higher than that reported previously (4), while the frameshifting efficiency observed for the HIV-1 construct in the coupled system was nearly two times higher than that determined above in the uncoupled in vitro system but is within the range previously reported (17, 34, 36, 41). These results indicate that coupling of transcription and translation in a cell-free system induces a higher level of ribosomal frameshifting but does not achieve the level of frameshifting efficiency observed in the infection-transfection system.

DISCUSSION

Astrovirus −1 ribosomal frameshifting has been demonstrated previously in a cell-free uncoupled transcription-translation system in which the astrovirus frameshift signal was presented in a heterologous context (23, 26). We now report the use of an infection-transfection cell culture system to examine the efficiency of frameshifting induced by the astrovirus frameshift signal in the same heterologous context. Given that the reagents required to study frameshifting directly in astrovirus-infected cells are not available at this time, we hypothesized that the infection-transfection cell culture system, in contrast to cell-free systems, would provide a more accurate reflection of frameshifting in a natural astrovirus infection. In addition, studying the astrovirus frameshift signal in a heterologous context allows direct investigation of the process of ribosomal frameshifting while avoiding problems that may be encountered in astrovirus-infected cells, such as detection of proteins that may be short-lived in natural infections and accurate quantitation of products of proteolytic cleavage. The high level of transient gene expression achieved in BHK-21 cells has made the vaccinia virus infection-transfection system adaptable for studying many diverse viruses (12, 32). An attractive feature of this system relevant to studying astrovirus replication is that gene expression is cytoplasmic and does not require the transfected plasmid to enter the nucleus. Since astrovirus is an RNA virus that likely replicates in the cytoplasm, the infection-transfection system may provide an environment more suitable than cell-free systems for examining the efficiency of astrovirus ribosomal frameshifting.

Using the infection-transfection system, we determined that the efficiency of frameshifting induced by the astrovirus frameshift signal is at least fourfold greater than the level achieved in uncoupled cell-free studies confirmed here and reported previously (23, 26). The higher level of ribosomal frameshifting in cells compared with in vitro cell-free systems has not been demonstrated in other viruses that use ribosomal frameshifting to regulate synthesis of the viral polymerase gene. Studies of other viruses such as IBV and HIV-1, however, were conducted with different cell culture systems. IBV RNA transcripts were transfected into *Xenopus* oocytes, in which the efficiency of frameshifting was found to be identical to that determined in an uncoupled cell-free system (4). For HIV-1, several nuclear expression strategies were used previously, and the level of efficiency of frameshifting in these systems was slightly lower than that reported in an uncoupled cell-free

TABLE 1. Ribosomal frameshifting efficiencies from three different systems

Virus	% (range [reference]) frameshifting efficiency with system[a]		
	In vitro		Intact cells (infection and transfection)
	Uncoupled	Coupled (TnT)	
Astrovirus	4–5 (5–7 [23, 26])	15–16 (NR)[b]	25–28 (NR)
Coronavirus (IBV)	2.6 (2–3 [4])	5 (NR)	8–9 (NR)
HIV-1	5 (2–11 [17, 34, 36, 41])	11 (NR)	28–30 (0.65–4 [6, 31, 34, 35, 36])

[a] Numbers in parentheses reflect ribosomal frameshifting efficiencies reported in the literature, as referenced.
[b] NR, not previously reported in the literature.

system (6, 31, 34–36). It should be noted that in previous studies of both IBV and HIV-1; RNA transcription and translation were uncoupled. A similar vaccinia virus infection-transfection transient expression system was used to examine the ribosomal frameshift signal in Berne virus, the prototypical member of the proposed family *Toroviridae* (38). In these studies, HeLa cells, instead of BHK-21 cells, were used and the transfected constructs did not contain an IRES sequence. The efficiency of frameshifting for Berne virus in cell culture ranged from 20 to 30% and was found to be no different from that determined in an uncoupled cell-free system.

In our infection-transfection studies, the heterologous astrovirus constructs were expressed from the pTM1 vector that contains an IRES. To ensure that neither this nor other features of the vector had any effect on the level of ribosomal frameshifting, uncoupled cell-free transcription and translation assays were performed with the infection-transfection astrovirus constructs A and C_m as well as the minimal frameshift constructs from IBV (pseudoknot excluded) and HIV-1 as positive controls. The astrovirus, IBV, and HIV-1 constructs induced frameshifting within the range previously reported for each of these viruses in cell-free systems (Table 1). This indicates that the increased frameshifting efficiency determined in the infection-transfection assay is not merely an artifact of the pTM1 vector or its IRES sequence and that other factors may be playing a role.

One such factor may be the coupling of transcription and translation in the infection-transfection assay. To examine the effect of this factor, frameshifting efficiency was studied in a coupled cell-free system. Compared with the uncoupled cell-free system that we also examined, the levels of frameshifting were approximately threefold higher with astrovirus constructs A and C_m and approximately twofold higher with the IBV and HIV-1 constructs in the coupled cell-free system. Thus, frameshifting efficiency was increased in a cell-free system in which transcription and translation are coupled. While it is not clear how coupling of transcription and translation influences ribosomal frameshifting, the importance of coupled transcription and translation may be that this system more accurately reflects what occurs in the cytoplasm during replication of an RNA virus. It is interesting that the level of HIV-1 frameshifting was also substantially increased in the infection-transfection assay compared with results previously reported in other cell culture systems. However, it is difficult to interpret the relevance of this finding, since HIV-1 undergoes transcription in the nucleus.

The coupling of transcription and translation can account for some, but not all, of the increased level of ribosomal frameshifting observed in the infection-transfection system. The effect of the recombinant vaccinia virus or the high levels of RNA production found in this system cannot be excluded. However, identical frameshifting efficiencies were reported for Berne virus in both an uncoupled cell-free system and a vaccinia virus-based infection-transfection assay similar to the system we employed. These observations suggest that neither the recombinant vaccinia virus nor the high levels of RNA production inhibit or enhance the level of ribosomal frameshifting (38). Another variable between the cell-free and cell culture studies was the temperature at which the assays were performed: 30°C in the cell-free system and 37°C in the infection-transfection assay. The lower temperature of the cell-free assay may not provide a favorable environment for subtle RNA interactions that are involved in ribosomal frameshifting in cells. Finally, it is possible that certain *trans*-acting factor(s) provided by the host BHK-21 cells are involved in ribosomal frameshifting, but no such factors have been reported for any system to date.

In these studies, we used the rotavirus VP4 gene containing the astrovirus frameshifting signal insertion and demonstrated a four- to fivefold increase in ribosomal frameshifting efficiency in cell culture compared with those in previously reported uncoupled cell-free systems (23, 26). The increased level of frameshifting in cell culture may have important implications in the astrovirus replication cycle, including viral protein synthesis and processing. Detailed study of ribosomal frameshifting in astrovirus-infected cells awaits the development of astrovirus-specific reagents.

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