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In Vitro Synthesis of an Infectious RNA from cDNA Clones of Human Rhinovirus Type 14

SATOSHI MIZUTANI AND RICHARD J. COLONNO*

Virus and Cell Biology Research, Merck Sharp & Dohme Research Laboratories, West Point, Pennsylvania 19486

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Development of a novel infectious cDNA assay is described for human rhinovirus type 14. A full-length cDNA clone of the human rhinovirus type 14 genome RNA was assembled and transcribed in vitro by using the SP6 transcription system. Transfection of HeLa cells with the nascent RNA resulted in the production of rhinovirus indistinguishable from the parental virus by both immunological and polyacrylamide gel analysis.

Human rhinoviruses (HRVs) consist of a group of at least 115 antigenically distinct serotypes that belong to the family *Picornaviridae* and are the major causative agent of the common cold in humans (7). Because of the vast number of serotypes, classic approaches to prevent HRV infections do not appear to be feasible. A better understanding of the biochemical mechanisms used during viral infection will be needed to develop a novel approach to control HRV infections. Recently, the complete nucleotide sequence of cDNA representing the HRV-14 genome RNA was determined (4, 15). This information has allowed us to determine the primary sequence of all the HRV-14 proteins and has demonstrated that a strong homology exists between HRV-14 and poliovirus at the amino acid level. Of even greater value is the ability to use these cDNA clones in the construction of an infectious cDNA clone. Previous studies with poliovirus (11, 13, 14) have demonstrated that a full-length cDNA clone, representing the entire genome RNA, could be used to generate progeny virus upon transfection of susceptible cells. The construction of a biologically functional cDNA of HRV-14 would enable manipulation of specific viral genes to better understand gene function, serotype diversity, and virulence. We now report the successful construction and functional expression of a biologically active cDNA of HRV-14.

Four cDNA clones, designated 198 (nucleotides 13 to 880), 7 (nucleotides 443 to 3672), 57 (nucleotides 2600 to 4600), and 186 [nucleotides 4100 to 7212-(deoxyribosyladenine [dA])$_{15}$-(deoxyribosylcytidine [dC])$_{25}$], were sequenced previously and were shown to represent virtually the entire genome RNA (4). These cDNA clones were originally inserted into pBR322 at the *Pst*I site by deoxyribosylguanine (dG) and dC tailing. Since there are no *Pst*I sites in the HRV-14 sequence, the four inserts were recloned into the *Pst*I site of the high-copy-number plasmid pUC9 (17). The orientation of the cDNA inserts was opposite to that of the *lacZ* gene in pUC9. A double-stranded deoxyoligonucleotide representing genome nucleotides 1 through 30 was synthesized with overhanging ends to accomodate ligation into a *Pst*I site at the 5′ end and a *Taq*I site at the 3′ end. The oligonucleotide was ligated to the cDNA sequence of clone 198 through the *Taq*I site to generate clone 36 (nucleotides 1 to 880) (Fig. 1A). By this manipulation the oligo(dG) sequence between the *Pst*I site and genome nucleotide 1 was removed. The predicted sequence at the 5′ end of the cDNA junction of clone 36 was confirmed by DNA sequencing (data not shown). Clones 57 and 186 were then joined through the common *Nco*I site at position 4310 to generate clone 371 [nucleotides 2600 to 7212-(dA)$_{15}$-(dC)$_{25}$]. Clones 7 and 371 were then connected through another common *Nde*I site at position 2989 to generate clone 30 [nucleotides 443 to 7212-(dA)$_{15}$-(dC)$_{25}$]. The 3′ end of clone 30 was modified by removing the (dC)$_{25}$ tail with Bal31 nuclease and extending the dA tail to a length of more than 100 bases with terminal deoxynucleotidyl transferase. The resulting 3′ end sequence was also confirmed by DNA sequencing to ensure that no 3′-encoded nucleotides had been removed. As a final step, clones 36 and 30 were connected through a *Bgl*II site at position 803 to generate clone pRV14 [nucleotides 1 to 7212-(dA)$_{100}$] (Fig. 1A).

Direct transfection (12) of HeLa R-19 cells (1) with either pHRV14 DNA or pHRV14 DNA in which the simian virus 40 origin of replication and enhancer sequences had been inserted upstream repeatedly failed to yield progeny virus. However, control transfections with a full-length cDNA clone of poliovirus (14), supplied by E. Wimmer, State University of New York, did yield infectious progeny virus. The use of the more efficient COS cells as reported by Semler et al. (14) was not applicable to HRV-14 because these cells do not possess cellular receptors for attachment of HRVs (unpublished data). Since unknown inhibitory factors appeared to be involved in direct transfection of HRV-14 cDNA, we decided to try to bypass the cell nucleus and synthesize full-length genome RNAs in vitro. Previous studies have demonstrated that HRV genome RNA is infectious (6). In addition, recent studies (2) have shown infectivity of brome mosaic virus RNA synthesized in vitro from cDNA clones.

HRV-14 cDNA isolated from pHRV14 by digestion with *Pst*I was inserted into plasmid pSP64 (10) at the *Pst*I site (Fig. 1B) for in vitro transcription, using the *Salmonella typhimurium* phage SP6 promoter and the promoter-specific RNA polymerase (Riboprobe; Promega Biotec, Madison, Wis.). The plasmid was linearized by digestion with *Sal*I and was used as a template for in vitro RNA synthesis with the SP6 RNA polymerase. No *Sal*I sites exist in the HRV-14 sequence. The resulting RNA transcripts were analyzed by 6 M urea–2% agarose gel electrophoresis (3). After autoradi-

* Corresponding author.

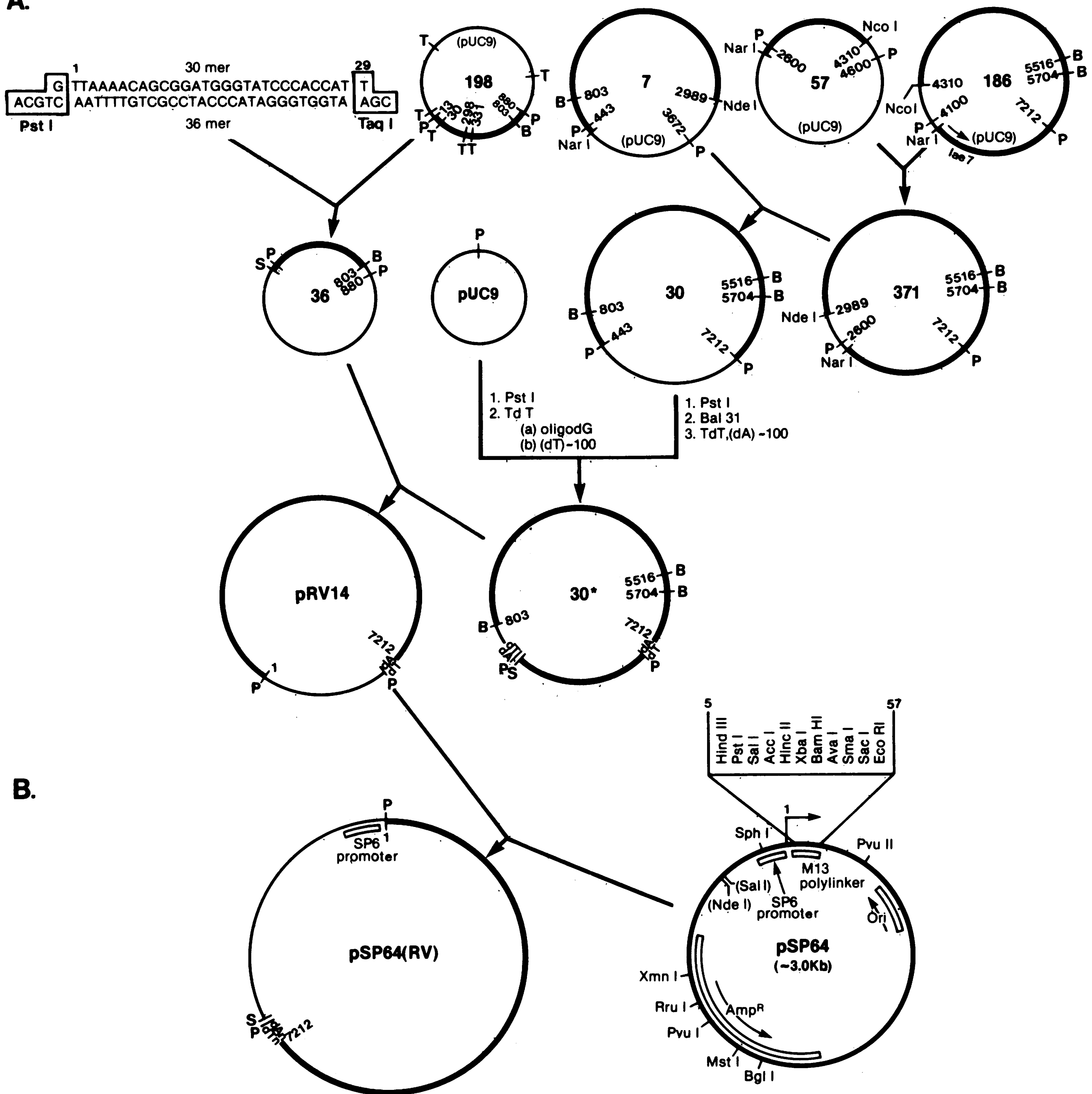
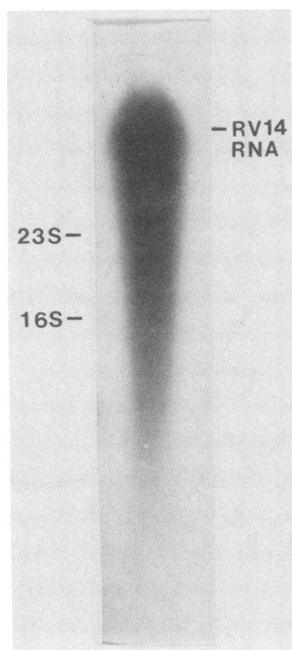


FIG. 1. Schematic diagram of steps used in the assembly of a full-length HRV-14 cDNA clone. Plasmid designations or identities are shown in the center of each plasmid. Numbered positions within each plasmid refer to nucleotide positions within the HRV-14 sequence (4). Letters indicate the restriction sites used during the construction: B, *Bgl*II; P, *Pst*I; S, *Sal*I; T, *Taq*I. DNA fragments indicated by thick lines in each plasmid were ligated through the common restriction sites as indicated. (A) Assembly of a full-length cDNA sequence. (B) Insertion of a full-length cDNA copy into an SP6 in vitro transcription plasmid, pSP64.

ography (Fig. 2), the area in the gel that corresponded to full-length HRV-14 RNA was recovered from the agarose by heating. Of the total trichloroacetic acid-precipitable counts per minute incorporated into the in vitro transcripts, 39% were found in the area of full-length HRV-14 RNA. About 20 pmol of full-length HRV-14 transcripts was synthesized from 1.5 pmol of template DNA in these particular experiments.

HRV-14 in vitro RNA transcripts were treated with pancreatic DNase I, extracted with phenol-chloroform (1:1), and then ethanol precipitated. Authentic HRV-14 genome RNA was purified from infectious virus by phenol-chloroform extraction and served as a positive control (6). Equivalent amounts of HRV-14 RNA and in vitro RNA transcript (1 μg by calculation) were dissolved in 15 μl of diethylpyrocarbonate-treated water. The solutions were diluted by fivefold serial dilution in water, heated at 100°C for 2 min, and



quickly chilled. Then 10 μl of each dilution was mixed with 0.4 ml of IRA buffer (0.14 M LiCl, 1 mM $MgCl_2$, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.5], 1.2 mg of DEAE-dextran per ml). This mixture (200 μl) was plated in duplicate onto 60-mm plastic plates containing confluent HeLa R-19 cells and was rocked for 20 min at 34°C. The plates were overlaid with 5 ml of 0.4% agar in McCoy medium containing 5% fetal calf serum and were incubated at 34°C. By day 3 after transfection, plaques of sufficient size were observed by visual examination to allow virus to be recovered from isolated plaques before staining the plates with 1% crystal violet (Fig. 3).

FIG 2. Characterization of the in vitro RNA transcript by agarose-urea gel electrophoresis. An RNA transcript was synthesized according to the method described by Melton et al. (10) with 1.5 pmol of a *Sal*I digest of pSP64(RV). The transcript was analyzed (5×10^5 cpm) by 2% agarose gel in 6 M urea (3). *Escherichia coli* rRNAs and HRV-14 RNA were electrophoresed in adjacent wells and were visualized by staining with ethidium bromide and UV shadowing. The positions of RNA bands are shown in the autoradiogram.

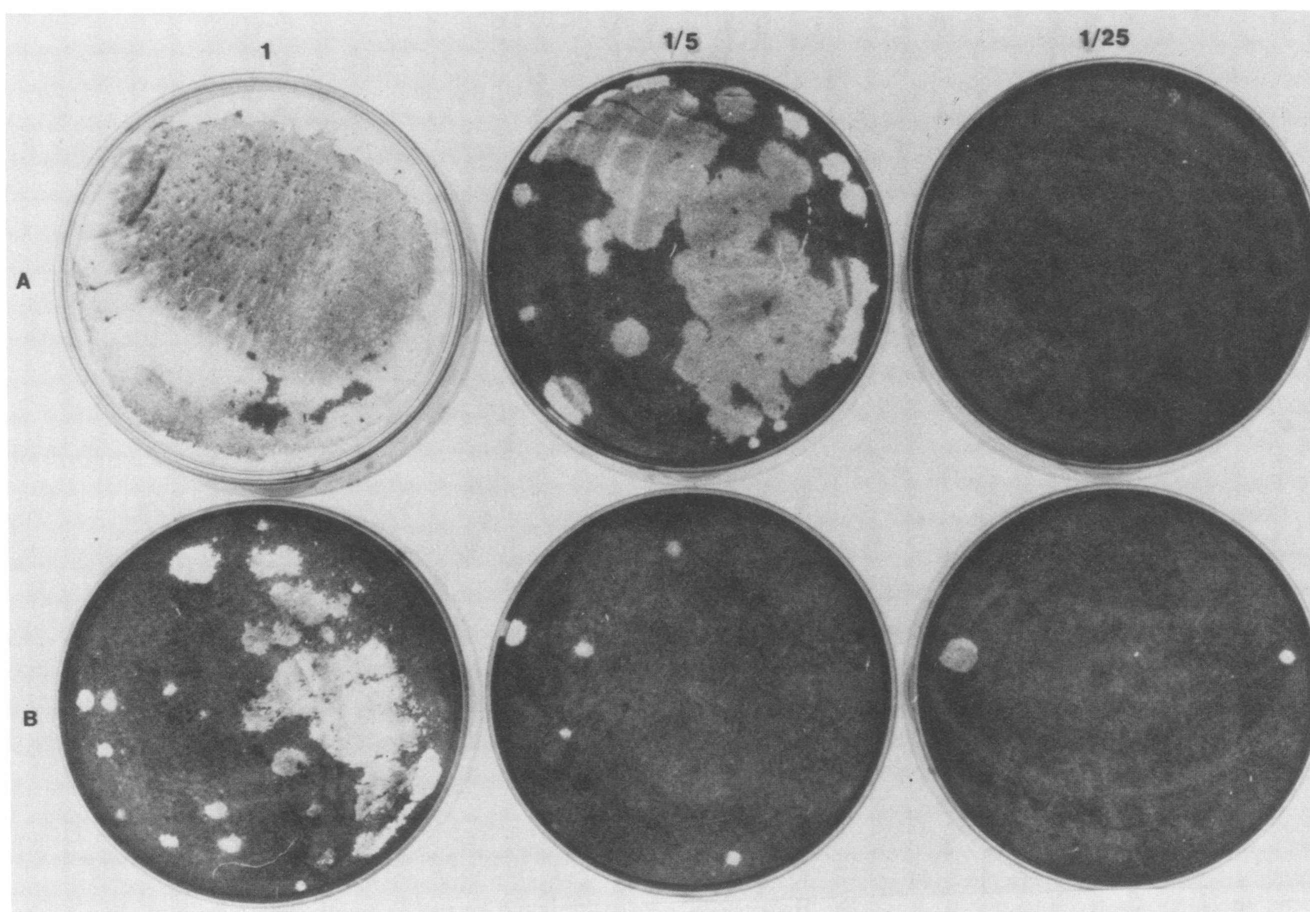


FIG. 3. Infectious RNA assay of in vitro transcript RNA. HeLa R-19 cells and procedures used for the growth, purification, and assay of HRVs have been described previously (1). Confluent HeLa R-19 monolayers in 60-mm plates were transfected with HRV-14 RNA (row A) or in vitro transcript RNA (row B) in 0.2 ml of IRA buffer. After 3 days of incubation at 34°C, the plates were stained with crystal violet to visualize plaque formation.

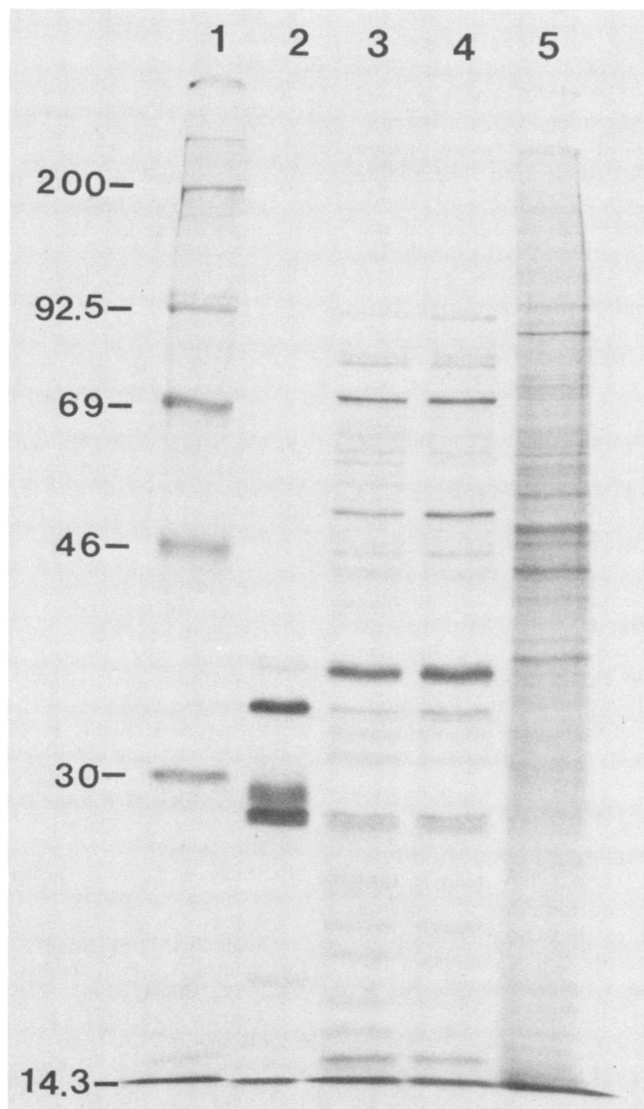


FIG. 4. Comparison of infected cell extracts by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. HeLa cells were infected with wild-type HRV-14 or the recovered virus as described in the text. At 6 h postinfection, cells were pulsed for 1 h with L-[^{35}S]methionine, washed twice with phosphate-buffered saline, and lysed in Laemmli sample buffer before analysis on a 10% acrylamide gel (8). Lanes: 1, ^{14}C-protein molecular weight standards (Amersham) (myosin, 200,000; phosphorylase-b, 92,500; bovine serum albumin, 69,000; ovalbumin, 46,000; carbonic anhydrase, 30,000; lysozyme, 14,300); 2, purified ^{35}S-labeled HRV-14 virions; 3, cytoplasmic extract from HRV-14 infected cells; 4, cytoplasmic extract from cells infected with virus recovered from in vitro-synthesized RNA transfections; 5, cytoplasmic extract from uninfected HeLa R-19 cells.

Pretreatment of authentic HRV-14 RNA and in vitro-synthesized RNA with RNase completely destroyed their infectivity (data not shown). Rescued viruses were propagated by several passages in HeLa R-19 cells and were tested for neutralization with specific neutralizing antiserum (American Type Culture Collection, Rockville, Md.) to HRV-14 by plaque assay. Viruses recovered from HeLa R-19 cells, transfected with authentic genome RNA or in vitro-transcribed RNA, were equally neutralized with anti-HRV-14 antiserum (data not shown).

These experiments clearly established that the assembled cDNA sequence of HRV-14 was biologically active and that the anti-HRV-14 antiserum could not distinguish the rescued viruses from the parental HRV-14. Quantitative dilution of the infectious RNA could not be achieved because of aggregation of the RNA molecules. To further illustrate the integrity of the in vitro transcription method, cells were infected at a multiplicity of infection of 100 in Hanks balanced salt solution supplemented with essential amino acids minus methionine with both parental virus and virus recovered from in vitro-synthesized transcripts. At 6 h postinfection, cells were pulsed with [^{35}S]methionine (1,200 Ci/mmol, 100 μCi/ml; Amersham Corp., Arlington Heights, Ill.) for 1 h, and cytoplasmic extracts were compared by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8). No detectable changes in any of the HRV-14 structural or nonstructural proteins were observed (Fig. 4). These experiments clearly showed no physical or structural differences between the parental HRV-14 and the recovered virus as a result of nucleic acid manipulations.

Based on the construction strategy described above, the resulting in vitro RNA should have 21 additional nucleotides at its 5′ end and an extended poly(A) tail at its 3′ end. The predicted 5′ end would be 5′(GAAUACAAGCUUGG GCUGCAG)UUAA . . . 3′. Recent evidence (5, 16) strongly suggests that plus- and minus-strand syntheses are primed by VPg-U-U during poliovirus RNA replication. The finding (4, 15) of identical nucleotide sequences at the 5′ and 3′ ends of HRV-14 and poliovirus suggests that a similar, if not identical, mechanism of RNA replication also occurs during HRV infection. It is unknown at this time which of the UU sequences at the predicted 5′ end is utilized during viral replication. The predicted 3′ end of the in vitro transcripts contains a poly(A) tail of about 100 bases instead of the approximately 90 bases found in HRV (9). In addition, two guanosine residues would follow the poly(A) tract at the 3′ end. It is doubtful that the guanosine residues would be maintained during viral replication.

Development of an infectious cDNA system for HRV-14 used a novel approach that is applicable to other picornaviruses, such as poliovirus. The use of in vitro RNA transcripts has a major advantage over direct cDNA transfection in that it completely avoids involvement of the cell nucleus and its potential problems regarding in vivo RNA transcription, potential RNA splicing, and transport to the cytoplasm while still allowing easy alteration at the DNA level by in vitro site-directed mutagenesis. In addition, the infectious RNA system allows transfection of cells that are normally used in the propagation and study of these viruses.

ADDENDUM IN PROOF

Sequence analysis of HRV-14 genome RNAs of wild-type and recovered viruses from in vitro RNA transfection indicated that both viruses contained identical 5′ terminal sequences. This result demonstrates that there is selective pressure to eliminate extra nucleotides upstream from the normal 5′ sequence and suggests a functional role for 5′ terminal sequence beyond the 5′ UU dinucleotide.

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