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The 5′-Terminal Nucleotides of Hepatitis A Virus RNA, but Not Poliovirus RNA, Are Required for Infectivity

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A series of plasmids containing hepatitis A virus (HAV) cDNA was constructed such that positive-strand HAV RNA could be transcribed with T7 RNA polymerase. The plasmids differed in the number of 5′-terminal nucleotides representing the junctions between vectors and HAV sequences that were present in the transcripts. When these transcripts were used to transfect cultured BS-C-1 cells, it was found that only those transcripts that contained all of the 5′-terminal HAV nucleotides, in addition to one or more nucleotides from the vector, were capable of initiating an infectious cycle leading to production of progeny virus. Transcripts that contained one 5′-terminal nucleotide from the vector sequence but were missing two uridylate residues corresponding to the first two nucleotides of HAV sequences, or were missing U and C residues corresponding to nucleotides 2 and 3 of the HAV sequence, were not infectious. A similar plasmid containing poliovirus cDNA was engineered to produce transcripts similarly lacking the first two uridylate residues of the poliovirus RNA sequence. These transcripts were infectious.

Hepatitis A virus (HAV) is an atypical member of the picornavirus family. Although previously classified as an enterovirus (4, 13), it likely represents the only member of a new genus (24). All strains of HAV for which nucleotide sequence information is available show extensive similarity in both nucleotide and deduced amino acid sequence; they appear to be only distantly related, however, to all other picornaviruses (14). In addition, numerous aspects of HAV growth and replication in cultured cells differ markedly from those of the other, lytic members of the picornavirus family (for a review, see reference 23).

A common feature of HAV and all other picornaviruses is the presence of an unusually long 5′-untranslated region in the viral RNA, which is predicted to form extensive secondary structures (16, 17, 19, 20). This feature is thought to be important for (i) cap-independent, internal ribosome binding and translation, (ii) RNA polymerase binding and initiation of RNA synthesis, and (iii) perhaps assembly and packaging of viral RNA into virions. The specific sequences or portions of the 5′-untranslated region that are important for each of these different functions are beginning to be identified. With respect to ribosome binding, an internal ribosome entry site has been defined for poliovirus (15), rhinovirus (1), encephalomyocarditis virus (7, 8), and foot-and-mouth disease virus (12) RNAs and shown to be both necessary and sufficient for cap-independent translation in vitro and in vivo. The internal ribosome entry site consists of a segment of ~450 nucleotides located toward the 3′ end of the 5′-untranslated region and does not include the first several hundred nucleotides of the 5′ end of the RNA. With respect to initiation of RNA synthesis, specific sequences have not been identified, but it is likely that 5′-terminal sequences and/or structure is involved.

A combination of biochemical probing, computer-assisted folding predictions, and primary sequence comparisons has generated secondary structure maps for the various subgroups of picornaviruses (16, 17, 19, 20). All picornavirus RNAs appear to have stable duplex structures (hairpins) at or very near the 5′ termini. In most cases, the stem of the hairpin begins at around nucleotide 10; for HAV and the cardioviruses, the hairpin structure includes the very first 5′-terminal nucleotide. A deletion of one nucleotide comprising the terminal stem of poliovirus RNA reduced infectivity of virus carrying the mutation, and this phenotype was suppressed by a compensating mutation that restored base pairing (18). The data suggest that the secondary structure at the 5′ terminus of the viral RNA might be important for RNA replication.

RNA transcripts of cDNAs representing each group of picornavirus have been shown to be capable of initiating a complete virus replication cycle, leading to production of normal virions. In general, the construction of these plasmids mandated that the transcripts carry some extra nucleotides upstream of the viral RNA sequences. Although the number and perhaps the sequence of these extra nucleotides do affect the specific infectivities of the RNAs (25), the process of transcription and replication of these transfected RNAs results in regeneration of the authentic viral RNA sequences (25).

Recently, cDNA of coxsackievirus B3 (CVB3) as well as CVB3 transcripts of cDNA were shown to be infectious, even though they were lacking the two T residues corresponding to the first two U residues of CVB3 RNA (11). The virus resulting from transfection had restored the two U residues. This result has profound implications regarding the mechanism of viral RNA synthesis, since it might suggest that these two terminal uridylate residues are not templated. A possible mechanism for generating these two U residues on the 5′ end of the RNA by uridylylation of a VPg-containing protein, followed by template-dependent elongation of the nucleotidyl protein, has been proposed previously (21, 22).

In this report, we describe the construction and analysis of several HAV RNAs, as well as one poliovirus RNA, altered at their 5′ termini. These RNAs were directly introduced into cultured BS-C-1 cells, and infectivity was determined by measuring the production of viral antigens after subsequent

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TABLE 1. Sequences at the junction of plasmid and viral cDNA

Junction	Plasmid	Plasmid-linker sequence	Virus sequence
T7 promoter and HAV sequences	pT7-HAV1	...cactatagtagc	TTCAAGAGGGG....
	pT7-HAV2	...cactatagt	TTCAAGAGGGG....
	pT7-HAV3	...cactatag	CAAGAGGGG....
	pT7-HAV4	...cactatagt	AAGAGGGG....
T7 promoter and poliovirus sequences	pT7(T)-PV	...cactatag	TTAAAACAGCT....
	pT7(T)-Δ5'PV	...cactatag	AAAACAGCT....

passage of virions isolated from the transfected cells. The results show that deletion of HAV nucleotides 1 and 2 or 2 and 3 abolishes the infectivity of the RNA. As previously shown, the presence of additional nucleotides on the 5' end of the HAV sequence is tolerated. In contrast to these data for HAV RNA, but consistent with the previous data reported for CVB3 RNA, poliovirus RNA lacking the first two terminal uridylate residues is infectious.

HAV mutant construction. A plasmid, pHAV/7 (2), containing the entire HAV cDNA, was used as the source of HAV sequences. The plasmid was cut with *Hin*dIII and *Bam*HI to excise a 632-bp fragment comprising the sequence for the 5' end of HAV. The fragment was inserted into pGEM-2 (Promega) so that it was under T7 promoter control. A synthetic nucleotide (5'-CTGGCTTATCGAAATTA ATACGACTCACTATAGT-3') and its complementary oligonucleotide were annealed; this double-stranded oligonucleotide contained the T7 promoter and transcription start site for juxtaposition to the HAV cDNA. The chimeric plasmid was cut in the *Pvu*II site of the vector, and the aforementioned oligonucleotide was ligated at this site; the vector segment between the *Pvu*II and *Hin*dIII sites was excised. Mung bean nuclease was used to eliminate nucleotides at the 5' end of the overhanging strand at the *Hin*dIII site, and the plasmid was blunted and closed for transformation.

Variability in the mung bean nuclease activity on different molecules generated a series of plasmids which differed in their precise sequences at the linker-HAV junction (Table 1). Sequences at these junctions were determined by DNA sequencing of plasmid preparations from a number of transformant colonies. Seven different mutations were found, and four were chosen as templates for making transcripts containing HAV sequences with five or two extra nucleotides at the 5' terminus or containing HAV sequences lacking the 5'-terminal two nucleotides (or nucleotides 2 and 3).

Subsequently, the remaining downstream sequences of HAV cDNA were introduced into these plasmids at the *Bam*HI site to produce plasmids of about 10,330 bp. The presence of full-length HAV sequences was confirmed by restriction mapping.

Infectivity of transcripts in BS-C-1 cells. Plasmid DNA from each of these clones was linearized with the appropriate restriction enzyme, extracted, and ethanol precipitated. Control plasmids pHAV/7 (wild type) and pHAV/7-μ3C, which contains three nucleotide substitutions in the 3C-coding region of pHAV/7 (9) which lead to two amino acid changes and inactivation of the 3C protease function, were treated similarly. Transcripts generated by T7 RNA polymerase or SP6 RNA polymerase were prepared from 5 μg of DNA, producing 10 to 25 μg of RNA. In general, the yield of RNA from pT7-HAV2 was significantly lower than from all other plasmids. These transcripts were used for transfection of BS-C-1 cells (prewashed with medium lacking serum) by the DEAE-dextran method of Cohen et al. (2) in a total volume of 0.5 ml. After 30 min at room temperature, 2 ml of minimal essential medium with 10% fetal calf serum was added, cells were incubated at 35°C for 3 h, and the medium was replaced. After 1 week, cells were split 1:2, and at 2 weeks, medium was replaced with minimal essential medium containing 2% serum.

At 3 weeks posttransfection, cells were collected, frozen and thawed three times, and sonicated, and the cell lysate was clarified by centrifugation (10,000 × *g*). The supernatant was centrifuged at 180,000 × *g* for 90 min, and the pellet was suspended in minimal essential medium, filtered, and used as a source of putative virus. BS-C-1 cells were infected with this virus, and cells were collected after 2 weeks for isolation of virus progeny, as above (first amplification). The virus progeny was again used to infect BS-C-1 cells (second and third amplifications) for isolation of HAV after 2 weeks for each amplification. Virus after the second and third amplifications was used for analyses.

Analyses of progeny virus. Pellets containing amplified virus were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with anti-HAV VP1 serum (raised against a recombinant baculovirus protein [6]) to detect production of HAV. The results are shown in Fig. 1. Cells transfected with HAV transcripts containing the total HAV sequence plus five or two extra nucleotides at the 5' end reproducibly showed production of viral antigen (Fig. 1, lanes 4 and 5). Virus was also derived from transcripts of the parental plasmid, pHAV/7 (Fig. 1, lane 6), as was previously reported (2). On the other hand, cells transfected with HAV transcripts lacking the two uridylate residues corresponding to HAV nucleotides 1 and 2 or uridylate and cytidylate residues corresponding to HAV nucleotides 2 and 3 from the 5' terminus did not give evidence for production of viral antigen (Fig. 1, lanes 1 and 2). Similarly, transcripts of pHAV/7-μ3C, containing a mutated HAV sequence, were negative (Fig. 1, lane 3). Results were the same after two or three amplifications, suggesting that the initial failure to detect virus was not simply due to low virus titer.

The conclusions drawn from the immunoblot analysis were confirmed by immunofluorescence microscopy. BS-C-1 cells were infected with the putative virus pellets for 7 days, and the cells were washed and fixed with methanol-acetone (5) at 4°C. Convalescent human antiserum (obtained from the Centers for Disease Control, Atlanta, Ga.) was used to detect HAV proteins, and this immune complex was visualized by goat anti-human fluorescein isothiocyanate. Cells infected with virus preparations derived from transcripts containing the entire HAV sequence, including all 5'-terminal nucleotides, showed positive immunofluorescence, whereas those infected with preparations derived

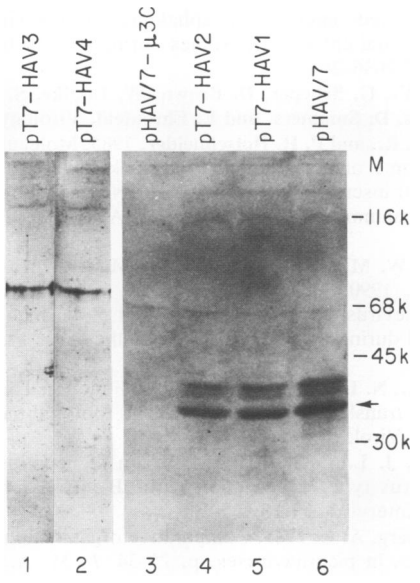

FIG. 1. Immunoblot analysis of HAV originating from RNA transfection of BS-C-1 cells. Virus from a second amplification (see text) was boiled in sample buffer, and viral proteins were fractionated on an SDS–10% polyacrylamide gel. Proteins were blotted onto nitrocellulose, and HAV VP1 was detected (arrow) by anti-HAV VP1 serum. The plasmids used to generate transcripts for the original transfections are indicated at the top of each lane and are discussed in the text. The mobilities (M) of proteins used as molecular-mass markers are indicated on the right.

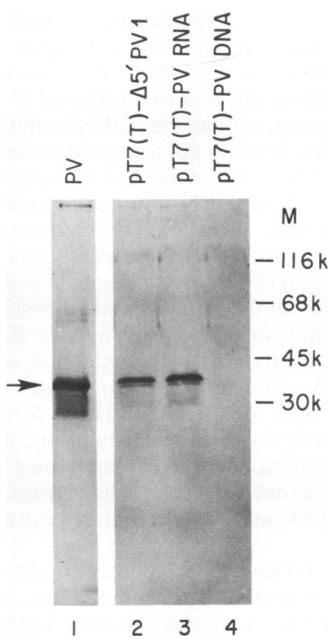

FIG. 2. Immunoblot analysis of poliovirus originating from transfection of BS-C-1 cells. Virus obtained at 48 h after transfection was boiled in sample buffer, fractionated in SDS–10% polyacrylamide gels, and immunoblotted with human anti-poliovirus serum. Abbreviations: PV (lane 1), poliovirus from a normal infection; pT7(T)-Δ5′PV1 (lane 2), transcripts which lacked the 5′-terminal two uridylate residues of poliovirus RNA; pT7(T)-PV RNA (lane 3), transcripts having the total complement of poliovirus RNA; and pT7(T)-PV DNA (lane 4), linearized plasmid DNA from which the wild-type transcripts were obtained. The mobilities (M) of marker proteins are indicated on the right.

from transcripts deleted for any HAV nucleotides or transcripts coding only for the P1 region were negative (data not shown).

The results remained negative for pT7-HAV3 and pT7-HAV4 (Table 1) when transfections and putative viral infections were done at 32°C, as examined by Western immunoblots or by immunofluorescence. Thus, pT7-HAV3 and pT7-HAV4 transcripts consistently failed to induce production of viral antigen by all criteria measured.

Generation of poliovirus RNA lacking the 5′-terminal two UMP residues. The findings described above for HAV are in marked contrast to the results of similar experiments performed with CVB3 sequences, which showed no infectivity requirement for the two 5′-terminal uridylate residues (10, 11). We therefore constructed a similar mutation for a third picornavirus, poliovirus, whose 5′-terminal nucleotide sequence is quite similar to that of CVB3 (11). The plasmid pT7(T)-PV (provided by Bert Semler, University of California, Irvine) contains the complete poliovirus cDNA regulated by a T7 promoter to produce a transcript with a single nonviral 5′-terminal GMP residue. This plasmid was linearized with *Bgl*II and hybridized with two synthetic primers: 5′-CCTTAATACGACTCACTATAGAAAA CAGCT-3′, which places the T7 promoter adjacent to a poliovirus 5′ terminus lacking two T residues, and 5′-GTTGAGTGCTGAGCGCAACGA-3′, which spans an *Esp*I site at position 287 in the poliovirus sequence. A 320-bp fragment of sequences between the primers was generated by the *Taq* polymerase chain reaction, and this fragment was cut with *Esp*I to release a 305-bp fragment which was gel purified. The 305-bp fragment was inserted into pT7(T)-PV at the *Stu*I and *Esp*I sites to generate a 10.4-kb plasmid containing the entire poliovirus genome code but lacking the two 5′-terminal base pairs. *Escherichia coli* DH5α was transformed, plasmid DNA was isolated from selected colonies, and mutations were confirmed by restriction mapping and DNA sequence analysis through the T7 promoter-poliovirus cDNA junction. The resulting plasmid, pT7(T)-PV-Δ5′, had one 5′-terminal G at the transcription start site but lacked the two succeeding T residues corresponding to the first two virus-specific nucleotides, whereas the parental pT7(T)-PV contained the initiating G residue plus the two succeeding T residues.

Infectivity of poliovirus RNA lacking 5′-terminal two U residues. Plasmid DNAs were linearized and transcripts were synthesized with T7 RNA polymerase (Table 1). Transfection was performed by the DEAE-dextran method, as for HAV transcripts. As additional controls, BS-C-1 cells were mock transfected or transfected with nontranscribed linearized plasmid DNA. Within 48 h, the majority of cells receiving either mutant or wild-type RNA were lysed, whereas cells receiving no nucleic acid or untranscribed DNA appeared unaffected.

Cells plus medium from each transfected sample were collected, frozen and thawed three times, and adjusted to 1% Nonidet P-40 before clarification and then centrifugation at 180,000 × *g* for 90 min to pellet virus. This putative virus preparation was dissociated by boiling in sample buffer, analyzed by SDS-PAGE, and then immunoblotted with human anti-poliovirus serum (Fig. 2). Virus preparations derived from mutant and wild-type transcripts all showed positive reactivity of capsid protein VP1 by immunoblotting

(Fig. 2, lanes 2 and 3). Cells transfected with DNA alone or no nucleic acid were negative for poliovirus antigen.

The results presented here show that poliovirus RNA, like CVB3 RNA, lacking the first two terminal U residues of the viral RNA sequence, retains the ability to initiate a complete replication cycle, leading to the production of infectious progeny virus. This virus was used to infect BS-C-1 cells, and the 5′ ends of viral RNA in virions pelleted from the infected cell lysates were directly sequenced by primer extension with avian myeloblastosis virus reverse transcriptase (Promega) (3). As was demonstrated for CVB3, the virus generated from the initial transfection had restored the two U residues and generated progeny viral RNA identical in sequence to that derived from transfection with the control pT7(T)-PV RNA (data not shown). It was therefore quite surprising that both deletions in HAV RNA constructed here, lacking the first two U residues or lacking the U and C residues representing the second and third nucleotides of HAV RNA, were not infective. The reason for this difference between HAV and the two enteroviruses is not understood.

The data for poliovirus from this report and those for coxsackievirus reported by Klump et al. (11) suggest a nontemplated addition of the terminal uridylate residues, perhaps to VPg or VPg precursor, to initiate RNA synthesis. If HAV is unable to support this reaction, it might suggest that the mechanism of initiation of RNA synthesis is different from that used by the enteroviruses, although we consider this unlikely. Alternatively, the different structures of the 5′-terminal hairpin, starting at nucleotide 1 for HAV and nucleotide 10 for poliovirus and CVB3, might lead to different forms of protein binding activities. It would be of interest to know whether cardioviral RNAs lacking the terminal U residues can participate in RNA replication, since these RNAs also initiate the 5′-terminal hairpin structure with the first nucleotide.

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