Nucleic Acids Research, 1993, Vol. 21, No. 8 ***1949–1957***

5′ and 3′ untranslated regions of pestivirus genome: primary and secondary structure analyses

Ruitang Deng[+] and Kenny V.Brock*
Ohio Agricultural Research and Development Center, Food Animal Health Research Program, 1680 Madison Avenue, Wooster, OH 44691, USA

Received November 6, 1992; Revised and Accepted March 10, 1993 GenBank accession no. M96751

ABSTRACT

Within the conserved 5′ untranslated region (UTR) of the pestivirus genome three highly variable regions were identified. Preceding the polyprotein start codon, multiple cryptic AUG codons and several small open reading frames are characteristic for all the five pestiviruses. Inspection of the context of AUGs revealed that the polyprotein initiation AUG of pestivirus has a weak context for efficient translation initiation. The most favorable context was found in two of the cryptic AUGs. Two oligopyrimidine-rich tracts upstream to the conserved either cryptic or authentic AUG in the 5′-UTR of pestivirus were identified and 83.3% of their nucleotide sequences are complementary to the consensus sequence at the 3′ terminus of eucaryotic 18S rRNA. A secondary structure model for the 5′-UTR of pestivirus was predicted. Nucleotide sequence comparison among five pestiviruses led to the identification of a variable region and a conserved region in the 3′-UTR. A deletion of 41 nucleotides was found within the variable region in Osloss. A secondary structure model for the 3′-UTR was also predicted. The structural similarity of the 5′-UTR between pestiviruses and picornaviruses and hepatitis C viruses was demonstrated and the possible implications of features of the 5′ and 3′-UTR of pestiviruses are discussed.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) of cattle, hog cholera virus (HoCV) of swine and border disease virus (BDV) of sheep make up the pestivirus group. The genome of pesitivirus is a single stranded, positive sense and nonpolyadenylylated RNA molecule with a size of about 12.5 kb (1, 2). It encodes a larger polyprotein which is proteolytically processed during virus replication to produce a series of structural and nonstructural proteins (3). The complete genomic RNA sequence of cytopathic (CP) BVDV strain NADL (2, 4) and noncytopathic (NCP) BVDV strain SD-1 (5) have been reported. In addition, almost complete genomic RNA sequences of other 3 strains of pestivirus: CP BVDV strain Osloss (6), HoCV strains Alfort (7) and Brescia (8), have been determined. It is known that the nucleotide sequence at the termini of the viral RNA molecules harbors specific signals for viral RNA replication, transcription and translation (9). The signals origniate from both primary and secondary structure and are specifically recognized by a virus-specific replicase (RNA dependent RNA polymerase) and cellular translational complexes. Recently, a specific internal sequence within the 5′ untranslated region (UTR) of picornavirus, called internal ribosome entry site (IRES), was reported to mediate the direct binding of ribosome to viral RNA template and initiate viral RNA translation (10–15). A similar IRES sequence within the 5′-UTR of hepatitis C virus (HCV) (16) and some cellular mRNAs have also been identified (17). In addition, it has been reported that the consensus primary and secondary structures in the 5′- and 3′-UTR are functionally important for replication, phenotype and virulence of the viruses (9, 18–21).

Although some of the primary structural features of the 5′ and 3′-UTR of pestiviruses have been described previously (2, 8, 22), no comprehensive and comparative analyses were made. With the determination of the complete 5′ and 3′ terminal nucleotide sequence of BVDV strain NADL (4) and SD-1 (5), structural analysis for the entire 5′ and 3′-UTR is possible. In this report, the primary sequence and secondary structure of the 5′ and 3′-UTR of five pestiviruses were compared and analyzed. A secondary structure model for the 5′ and 3′-UTR of pestivirus RNA is proposed. In addition, the structural similarity of the 5′-UTR of pestivirus RNA to picornavirus and hepatitis C virus RNA is demonstrated.

MATERIALS AND METHODS

Computer analysis

Primary structural analysis was made with HIBIO DNASIS (Hitachi Software Engineering Co., Ltd, Brisbane, CA) (23) to determine nucleotide sequence homology, nucleotide sequence repetition and open reading frame prediction. One limitation of this program imposed when performing nucleotide sequence

* To whom correspondence should be addressed

[+] Present address: Molecular Genetics and Microbiology Department, University of Massachusetts Medical School, 55 Lake Avenue, North Worcester, MA 01655, USA

homology searches is that a deletion or insertion of more than 10 nucleotides can not be aligned by the program. In this case, the sequence upstream and downstream the deletion or insertion must be aligned separately.

Secondary structure prediction was done using the FOLD program (24) and HIBIO DNASIS program (24). The first 500 nucleotides of the genome, which included the entire 5′-UTR and part of the coding sequence, was folded to predict the secondary structure of the 5′-UTR. The nucleotide sequence downstream of the stop codon of the viral polyprotein was folded to predict the secondary structure of the 3′-UTR. To derive a consensus model of the secondary structure, the nucleotide sequences of the 5′-UTR and 3′-UTR from five pestiviruses were folded separately and elements common to or dominant in these five pestiviruses were retained. Next, the intervening segments were refolded until a consensus structure maximizing total base pairing was achieved. Taking into account the conservation of primary structure, the substitution of one base pair for another and the thermodynamic stability of the structure; some adjustments were made to maintain the structural details among all the five pestiviruses.

Source of sequence information

Primary structures of the pestivirus genome were taken from the references indicated in the parentheses: CP BVDV strain Osloss (6), CP BVDV strain NADL (2, 4), NCP BVDV strain SD-1 (5), HoCV strain Alfort (7) and HoCV strain Brescia (8).

RESULTS AND DISCUSSION

Primary structural feature of 5′-UTR of pestivirus genome

The genome of the pestiviruses has a relatively long 5′-UTR, ranging from 360 to 363 nucleotides for HoCV (7, 8) and 383 to 385 nucleotides for BVDV (2, 5, 22). The 5′-UTR is the most conserved nucleotide sequence among five pestiviruses (5). Within the 5′-UTR three highly variable regions (designated as region I, II and III) were identified when the nucleotide sequence of the 5′-UTR from five pestiviruses was compared (Fig. 1). Region I extended from nucleotides 1 to 72, Region II from nucleotides 206 to 221 and Region III from nucleotides 280 to 320. One of the characteristic features of the pestiviral 5′-UTR is the presence of multiple cryptic AUG start codons ranging from 5 to 8. Two of them, one located at nucleotides 108 to 110 and the other 7 bases upstream of the polyprotein initiation AUG at nucleotides 379 to 381, are conserved among all the five pestiviruses. Most of these cryptic AUGs can potentially open a reading frame containing 7 to 57 amino acid residues (Fig. 1). Studies on the small ORFs in the 5′-UTR of poliovirus RNA indicate that the upstream ORFs are not essential for virus replication (25). However, the utilization of upstream ORFs in the 5′-UTR of human rhinovirus has been demonstrated by the fact that a number of unexpected polypeptides appeared early in time-course experiment in addition to the expected products (15). It has been also reported that two AUG start codons, separated by 84 bases, were used to initiate viral protein synthesis of foot-and-mouth disease virus (FMDV) both *in vivo* and *in vitro* (26). Limited data of *in vitro* translation of BVDV RNA revealed that in addition to viral specific proteins, a large number of other proteins evidenced by high levels of background were synthesized in a cell free translation system (27), possibly suggesting initiations of viral RNA translation at upstream cryptic AUGs. Although it was believed that these small ORFs had no coding function because most of these small ORFs are poorly conserved among pestiviruses and no virus specific polypeptides have been detected *in vivo* or *in vitro* from this region (3, 7), further experiments are required to determine the function of these small ORFs.

It has been reported that insertion of a tract with an AUG codon upstream to the authentic AUG reduced virus growth, conferring a small plaque phenotype and all the large plaque revertants had lost the inserted AUG codon as a result of point mutations (28). It has also been reported that an AUG, created by point mutation 8 bases upstream the authentic AUG, acted as a barrier to initiating at the downstream authentic AUG (29). In the 5′-UTR of pestivirus, just 7 bases upstream of the polyprotein initiation AUG is a conserved cryptic AUG in a different reading phase. It remains to be determined whether this closely located cryptic AUG could function as a negative regulator to the downstream

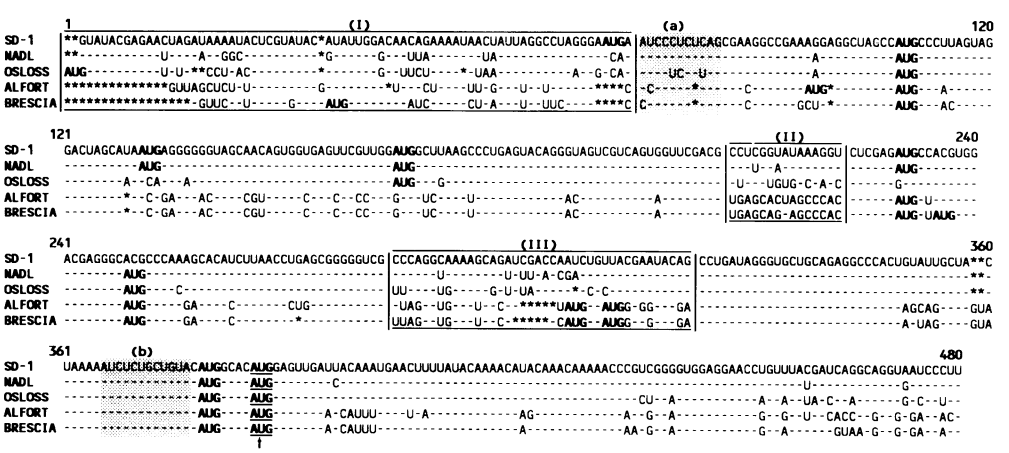


Figure 1. Primary structure for 5′-UTRs of five pestiviruses. The sequence shown in the top line is for SD-1 and the coordinates of the nucleotides for SD-1 are given above the sequence. Hyphens indicate the identity with SD-1 sequence. Asterisks denote the deletions compared with one another. AUG codons within the 5′-UTR are bolded. The polyprotein initiation AUGs are underlined. Three variable regions (I, II and III) are boxed. Two oligopyrimidine-rich tracts (a and b) are shadowed.

authentic AUG and decrease the initiation efficiency of viral RNA translation, resulting in relatively poor growth of pestivirus *in vitro* (30) and possibly *in vivo*.

Compared with the context of AUG start codon for efficient initiation (29), the authentic AUG of pestivirus has a weak context for initiation, where the nucleotide at position −4 is C rather than A or G. The weak context of the authentic AUG may be one of the reasons that pestivirus can not efficiently replicate *in vitro* (30) and possibly *in vivo*. The most favorable context was found in two of the cryptic AUGs. The one at nucleotides 131 to 133 can potentially open a reading frame with 45 amino acid residues in both BVDV NADL (2) and SD-1. The other at nucleotides 312 to 314 can potentially open a reading frame with 17 amino acid residues in both HoCV Alfort and Brescia. The context similar to that of the polyprotein initiation AUG was also found in another two cryptic AUGs which are located at nucleotides 165 to 168 and 369 to 371, respectively. It should be noted that the nucleotide sequence around the authentic AUG from −25 to +10 is identical among all the five pestiviruses (Fig.1), indicating the importance of that sequence for viral RNA translation.

```
Pestivirus (tract a)
      18S rRNA  3'  AUUACUAGGAAGGCGUCCAACUGGAUGCCUUU   5'
                      **** *** ***    *** ***
      SD-1      5' (74) AUCCCUCU CAG 20 GCC AUGC (111) 3'
      NADL      5' (74) AUCCCUCU CAG 20 GCC AUGC (111) 3'
      OSLOSS    5' (73) AUCCUCCU UAG 20 GCC AUGC (110) 3'
      ALFORT    5' (58) ACCCCUC- CAG 19 GCC AUGC (94)  3'
      BRESCIA   5' (56) CUCCCUC- CAG 19 GCC AUGC (91)  3'

Pestivirus (tract b)
     18S rRNA   3'  AUUACUAGGAA GGCGUCCAACUGGAUGCCUUU   5'
                      **** * *****   ****  *****
      SD-1      5' (366) AUCUCUGCUGUAC AUGGCACAUGGA (390) 3'
      NADL      5' (366) AUCUCUGCUGUAC AUGGCACAUGGA (390) 3'
      OSLOSS    5' (364) AUCUCUGCUGUAC AUGGCACAUGGA (388) 3'
      ALFORT    5' (344) AUCUCUGCUGUAC AUGGCACAUGGA (368) 3'
      BRESCIA   5' (341) AUCUCUGCUGUAC AUGGCACAUGGA (365) 3'

Picornavirus
      18S rRNA  3'   AUUACUAGGAAGGCGUCCAACUGGAUGCCUUU   5'
                     ** *****         ********
      PV1M      5' (556) GUGUUUCC   18   GCUUAUGG (589) 3'
      COXB1     5' (559) GUGUUUCA   19   GCUUAUGG (593) 3'
      RHINO1B   5' (557) GUGUUUCA   18   GCUUAUGG (590) 3'
      EMCV-R    5' (809) UUUCCUUU   14   UAAUAUGG (837) 3'

Hepatitis C virus
      18S rRNA      3'   AUUACUAGGAAGGCGUCCAACUGGAUGCCUUU   5'
                         ***  *** **        * ** ***
      HCV-1     5' (190) GGUCCUUUCUU   10   GCUCAAUGC (219) 3'
      HCV-J     5' (177) GGUCCUUUCUU   10   GCUCAAUGC (206) 3'
      HCV-H     5' (189) GGUCCUUUCUU   10   GCUCAAUGC (218) 3'
```

Figure 2. The complementarity of oligopyrimidine-rich tracts in pestivirus, picornavirus and hepatitis C virus to the eucaryotic 18S rRNA. The 18S rRNA sequence was taken from Hagenbüchle et al. (1978). The viral names are abbreviated as follows: PV1M, poliovirus type 1 strain Mahoney; COXB1, coxsackievirus strain B1; RHINO1B, rhinovirus type 1 strain B; EMCV-R, encephalomyocarditis virus strain Rueckert; HCV-1, HCV-J and HCV-H, hepatitis C virus strain 1, J and H, respectively. The viral nucleotide sequences are taken from the following sources: PV1M, COXB1 and RHINO1B, Pilipenko et al. (1992); EMCV-R, Duke et al. (1992); HCV-1, Choo et al. (1991); HCV-J, Kato et al. (1990); HCV-H, Inchauspe et al. (1991). The coordinates of the first and last nucleotides of the oligopyrimidine-rich tracts are given parenthetically. The conserved AUG codons are shadowed. The numbers of omitted nucleotides in the middle of the tracts are given. Asterisks denote the possibility of base pairing between 18S rRNA and the viral RNA sequences.

Comparison of the consensus sequence at the 3′ terminus of eucaryotic 18S rRNA (31) with the 5′-UTR of pestiviruses revealed that two oligopyrimidine-rich tracts (designated as track a and b) in the 5′-UTR of pestiviruses were complementary to that consensus sequence by 83.3% when G:U base pairing was speculated (Fig. 2). Tract a is located 34 bases upstream of the cryptic AUG at position 108 to 110 and track b 13 bases upstream of the cryptic AUG at position 379−381 and 20 bases upstream of the authentic AUG. It has been proposed that, similar to the Shine−Dalgarno sequence in procaryotic mRNA, an oligopyrimidine-rich tract upstream of the authentic AUG in a number of eucaryotic mRNAs mediates base-pairing with the consensus sequence at the 3′ terminus of eucaryotic 18S rRNA and directs the translation initiation (31−32). Recently, an essential *cis*-acting oligopyrimidine element in the 5′-UTR of picornaviruses has been described to be important for directing initiation of viral RNA translation (33−35). Whether either of the oligopyrimidine-rich tracts identified in the 5′-UTR of pestiviruses mediate ribosome binding to and direct the translation initiation at the downstream AUG sites remains to be determined.

Comparison between pestiviruses and other related viruses revealed that the primary structural features of the 5′-UTR of pestivirus resemble that of picornavirus (25, 33−36) and hepatitis C virus (16, 37−40) rather than a number of Flaviviridae (41) (Table 1 and Fig. 2).

Secondary structure of 5′-UTR of pestivirus genome

With the completion of the genomic sequence of BVDV NADL and SD-1, we have now been able to perform secondary structural analysis for the entire 5′-UTR of the pestivirus genome. The RNA secondary structure model for the 5′-UTR of pestivirus was created through the FOLD program of Zuker and Stiegler (24).

Table 1. Primary structure features of 5′-UTR of pestiviruses and other related viruses*

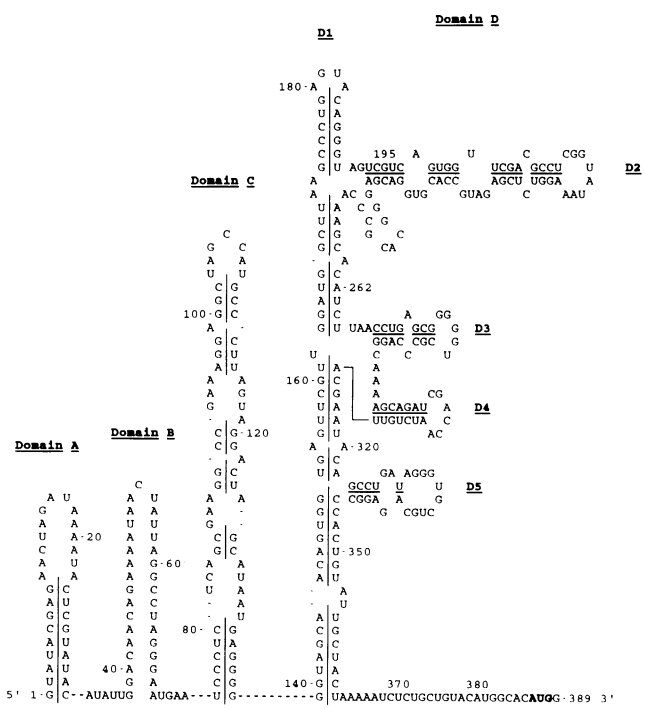
Virus	5′Cap	Size	Number of AUGs	Number of ORFs	Initiator AUG context	References
Pestiviruses						
BVDV SD-1	No	385nt	6	4	CxxAUGG	Deng and Brock, 1992
BVDV NADL	No	385nt	6	4	CxxAUGG	Collett et al., 1988a
BVDV Osloss	No	383nt	5	5	CxxAUGG	Renard et al., 1987b
HoCV Alfort	No	363nt	7	5	CxxAUGG	Meyers et al., 1989
HoCV Brescia	No	360nt	8	6	CxxAUGG	Moormznn et al., 1990
Hepatitis C Virus						
HCV1	?	341nt	5	3	AxxAUGA	Han et al., 1991
Piconavirus						
EMCV-R	No	834nt	10	6	AxxAUGG	Duke et al., 1992
Flavivirus						
YFV	Yes	119nt	0	0	AxxAUGU	Brinton et al., 1988

*The viral names are abbreviated as follows: HCV1, hepatitis C virus strain 1; EMCV-R, encephalomyocarditis virus strain Rueckert; YFV, yellow fever virus.

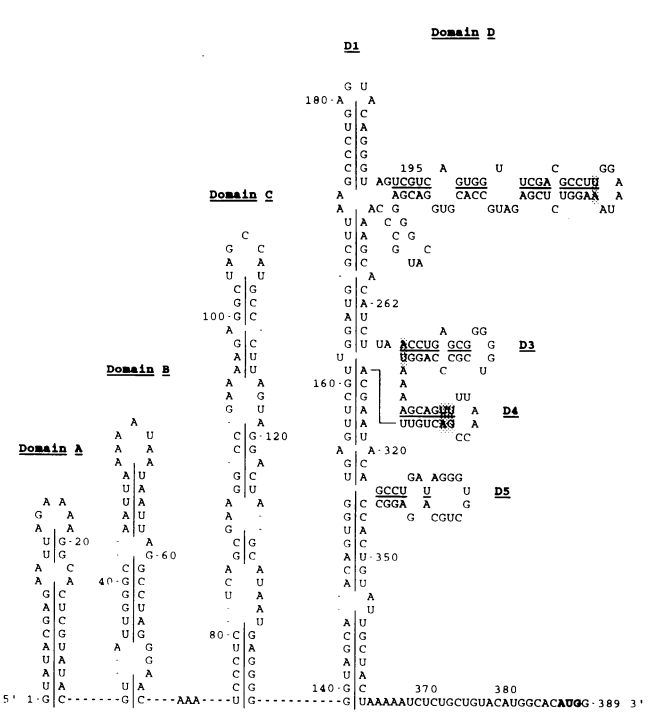
The model shows a series of stem-loop structures which were divided into 4 domains: domain A, B, C and D (Fig. 3). About 70% of the nucleotides in the 5′-UTR participate in the base pairing to conform to the secondary structure. The optimal ΔG values of the structures calculated by the FOLD program are −103.8 Kcal/mol, −109.6 Kcal/mol, −117.3 Kcal/mol, −110.8 Kcal/mol and −102 Kcal/mol for BVDV SD-1, BVDV NADL, BVDV Osloss, HoCV Alfort and HoCV Brescia, respectively. Domain A is a stable and conserved stem-loop structure in the 3 BVDV strains. The variable nucleotide sequences including base substitutions and deletions are exclusively located in the loop of this domain. The complete nucleotide sequence at the extreme 5′ termini of both HoCV Alfort (7) and HoCV Brescia (8) has not been established and the published sequence was used to predict the secondary structure, it was, therefore, not surprising that no similar domain A structure was found in HoCV. Domain B is located in the variable region I of the primary sequence and shows little conservation among the pestiviruses. Domain C is a conserved, relatively large and imperfect stem-loop structure. The most significant is Domain D which covered two thirds of the 5′-UTR sequence from nucleotides 139 to 361 and is conserved among all the five pestiviruses. It is a stable, multiple stem-loop structure with ΔG values of −74.2 Kcal/mol, −76.6 Kcal/mol, −82.8 Kcal/mol, −78.1 Kcal/mol and −72.7 Kcal/mol for SD-1, NADL, Osloss, Alfort and Brescia respectively. Although two of the variable regions (II and III) of primary sequence are located in this domain, the secondary structure is almost identical among the pestiviruses. The conserved part of primary nucleotide sequence in this region is a factor contributing to the maintenance of the common secondary structure. The other, perhaps more important, contributing factor is a large number of compensatory mutations, that is the simultaneous alterations of two noncontiguous nucleotides that result in the substitution of one base pair for another. When comparing the structure between BVDV and HoCV, about 36% of the base pairs maintaining the common secondary structure are substituted in this domain. In addition to the compensatory substitutions, the other heterogenous nucleotide sequence containing a number of base substitutions and small stretches of nucleotide deletions is exclusively located in the loop structure of this domain, mainly in the loops of D2 and D4 domain. It should be noted that the oligopyrimidine-rich tract b described before does not participate in the base pairing to conform to the secondary structure of 5′-UTR and, as linear sequence this tract is located 4 bases downstream to domain D, indicating the ease accessibility of this stretch to the eucaryotic 18S rRNA to mediate base pairing.

Although the secondary structure model presented has not been demonstrated by experiments, the high conservation of the structure through a number of compensatory mutations among all the pestiviruses indicates the possibility of the existence of such a structure *in vivo*, particularly, domain D. However, it is clear that predictions for the folding of RNA molecules are still in the early stages because different computer programs and length of input sequence influenced the predicted structure and secondary structure predictions are only a first approximation, giving no consideration to tertiary interactions.

Recent evidence suggests that picornavirus behaves unlike most other eucaryotic mRNA in its translational strategy in that it appears to initiate translation internally through the internal ribosome entry site (IRES) within 5′-UTR (10−15). It also has been demonstrated that the translation initiation of HCV occurs by direct entry of ribosomes to the internal sequence (IRES) within 5′-UTR (16). Analysis of the primary and secondary structure of 5′-UTR of pestivirus revealed that the features of 5′ -UTR of pestiviruses resemble that of picornaviruses and HCV.



BVDV SD-1



BVDV NADL

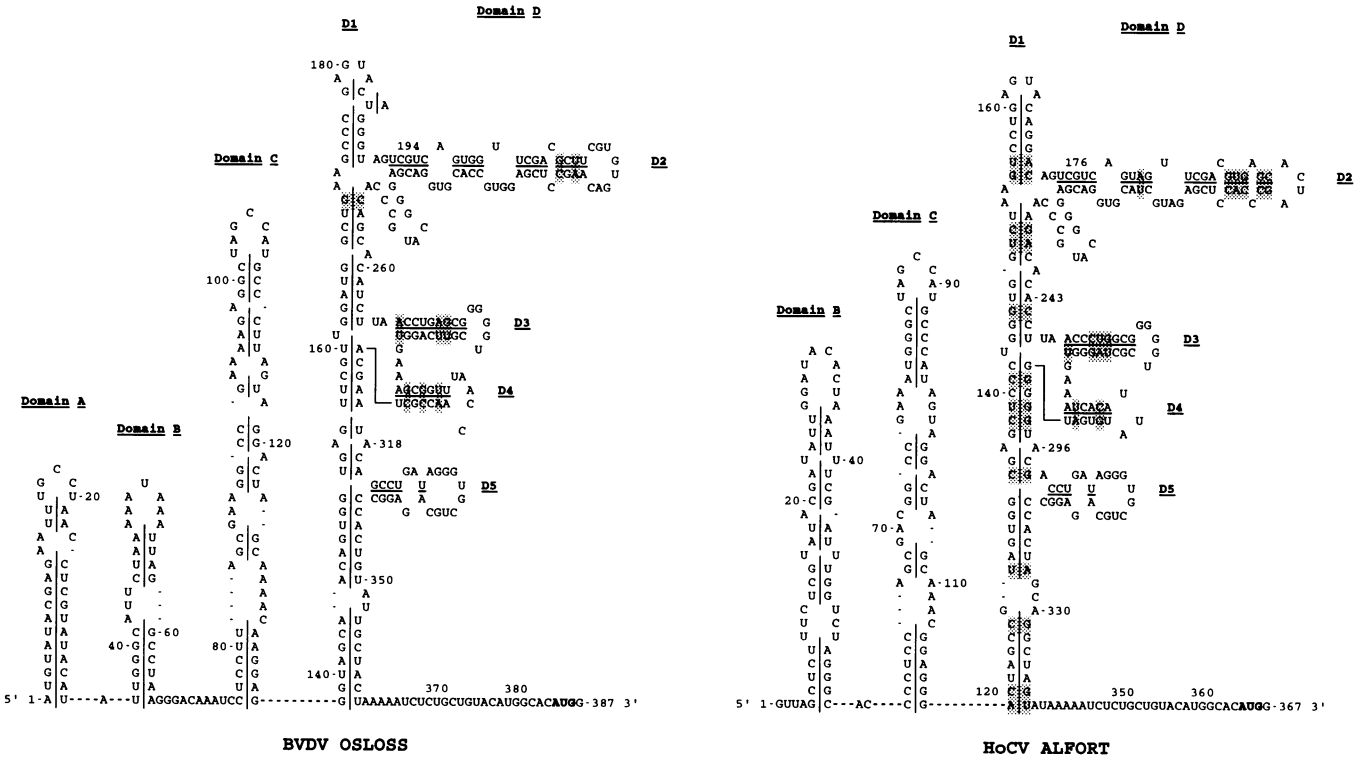


Figure 3. The consensus model of secondary structure for 5′-UTRs of five pestiviruses. The structure is composed of 4 domains: domain A to D. Domain D was divided into five subdomains as D1 to D5. Compared with SD-1 structure, the base pair substitutions contributing to the maintenance of the same structure of domain D in other four pestiviruses are shadowed. The bolded AUGs denote the polyprotein initiation codon.

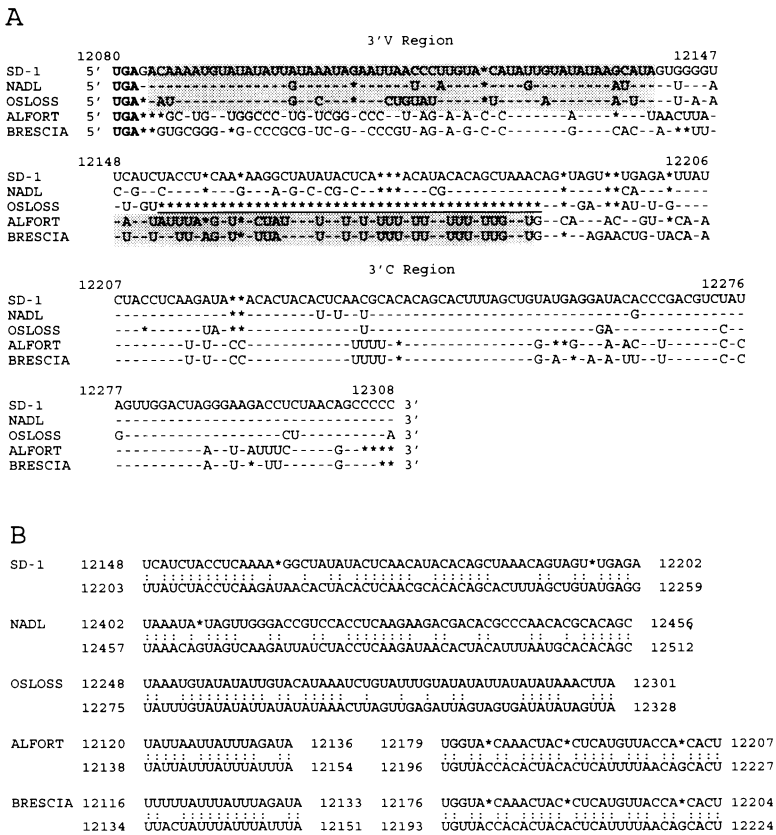


Figure 4. Primary structure of 3′-UTRs of five pestiviruses. **(A)** Comparison of nucleotide sequence of the 3′-UTR. 3′V region and 3′C region denote the variable and conserved region, respectively. The nucleotide sequence shown in the top line is for SD-1 and the coordinates of the nucleotides for SD-1 are given above the sequence. Hyphens indicate the identity with SD-1 sequence. Asterisks indicate the deletions compared with one another. The polyprotein stop codon UGAs are bolded. A 41 base deletion in Osloss are underlined. The AU rich stretches are shadowed. **(B)** Imperfect repeat sequences in the 3′-UTRs of five pestiviruses. The coordinates of the first and last nucleotides of the repeat sequences are given. The identity of nucleotide between the repeats are indicated by colon. Asterisks denote the deletions of nucleotides.

The translation initiation of pestivirus RNA is not satisfactorily accommodated by the scanning model proposed by Kozak (42). According to this model, ribosomes and associated factors initially bind to the capped 5′ end of mRNA and scan the RNA molecule to reach the authentic initiator AUGs which is usually the first AUG and is in favorable sequence context, and the secondary structure upstream the authentic AUG dramatically decrease the translation initiation efficiency (43, 44). Therefore, it is tempting to speculate that, similar to the translational strategy of picornavirus and HCV, an internal translational initiation mechanism could be predicted in pestivirus based on the following considerations. (I) It has been proposed that pestivirus RNA lacks a cap structure at the 5′ end of the genome (4), suggesting a possible cap-independent translation initiation mechanism. (II) Pestivirus has a relatively long 5′-UTR in which multiple cryptic AUGs and several small ORFs are present upstream of the authentic AUG and it is unlikely that before reaching the authentic initiation site, which is in a weak context, the scanning ribosome could bypass 6 to 8 upstream AUGs, some of which have an equal or more favorable context for efficient initiation. (III) Since a conserved and stable secondary structure was predicted in the 5′-UTR of pestivirus, it seems unlikely that the ribosome could traverse such a great length of secondary structure by binding at the 5′ end of the genome and scanning to reach the authentic translational initiation site. It has also been reported that secondary structure rather than merely a linear nucleotide sequence functions as IRES in picornavirus (33, 45) and HCV (16). Nucleotide sequence comparison of the 5′-UTR between pestiviruses and HCV revealed that the IRES identified within the 5′-UTR of HCV corresponds to the sequence from nucleotides 133 to 371 in pestiviruses (16, 40), where the conserved secondary structure domain D (nucleotides 139 to 361) is located. Therefore, domain D may be the candidate for IRES in pestivirus.

Primary structure feature of 3′-UTR of pestivirus genome

The pestiviruses also have a relatively long 3′-UTR, 188 nucleotides for BVDV Osloss (6) and approximately 228 nucleotides for the other four strains (2, 5, 7, 8). The complete 3′ terminal nucleotide sequences of BVDV NADL, BVDV SD-1 and HoCV Brescia have been reported to be established (4, 5, 8). It is not known whether the 3′-UTRs of BVDV Osloss and HoCV Alfort cover the complete nucleotide sequence of the 3′ end of their genomes. However, when these sequences are aligned with BVDV NADL and SD-1, BVDV Osloss genome ends at the same position as BVDV NADL and SD-1, HoCV Alfort genome ends 4 bases upstream the 3′ end of BVDV NADL and SD-1 genome. Therefore, it is reasonable to assume that the reported 3′-UTR sequence of those viruses cover almost, if not completely, the authentic nucleotide sequence of 3′ end of their genomes (Fig. 4). Comparison of the nucleotide sequence among those 5 strains of pestivirus led to the identification of a variable region (designated as 3′V region) and a conserved region

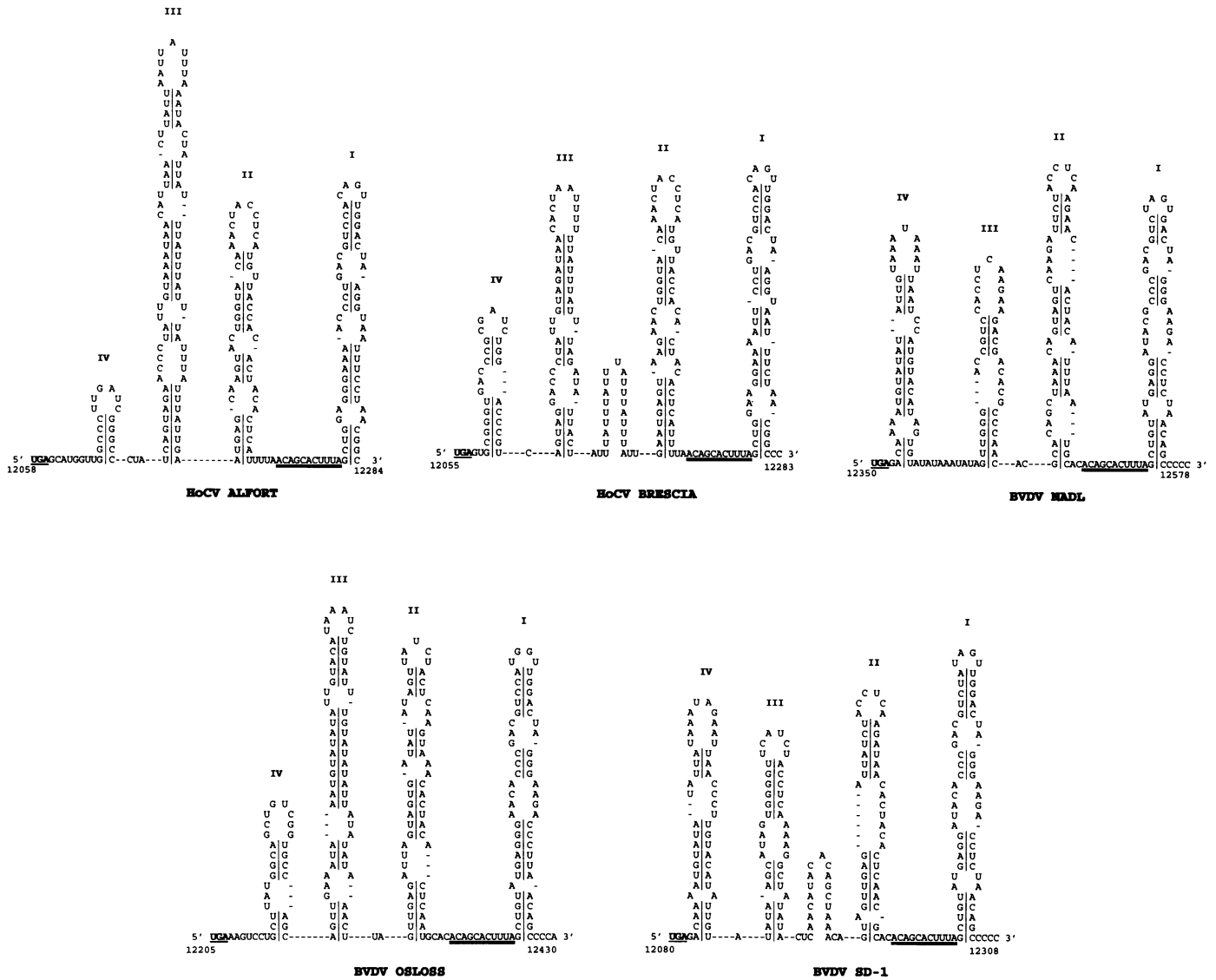


Figure 5. Consensus model of secondary structure for 3′-UTRs of five pestiviruses. Four stem-loop structures are designated as stem-loop I to IV in 3′ to 5′ direction. The polyprotein stop codon UGAs are underlined. The conserved linear sequence between stem-loop I and II are double underlined.

(designated as 3′C region) within the 3′-UTR of pestiviruses. The 3′V region contains 127 nucleotides, starting at the UGA stop codon of the polyprotein at position 12080 and ending at nucleotides 12206. The nucleotide sequence variation in this region is particularly remarkable between BVDV group and HoCV group. The most obvious difference in this region is the presence of a 41 base deletion in BVDV Osloss, which is responsible for the size variation of 3′-UTR between Osloss and other pestiviruses. The high heterogenicity of the nucleotide sequence in this region indicated that this region may not have a direct critical function for the virus replication. It may function as an auxiliary element for the maintenance of functional structure for other regions. The 3′C region is composed of 102 nucleotides at the 3′ terminal portion of the 3′-UTR. This region was particularly conserved among BVDV group and HoCV group. The high conservation of the nucleotide sequence in this region implied that some important signals for viral RNA replication may be harbored in this region.

An AU-rich stretch as reported in BVDV NADL (2) and HoCV Brescia (8) was also found in BVDV Osloss, BVDV SD-1 and HoCV Alfort (Fig. 4). However, the locations of the AU-rich stretch in BVDV group and HoCV group are different (Fig. 4). Repeat sequence searching revealed that there is no perfect, relatively large repeat sequence within the 3′-UTR of pestivirus although a 8 nucleotide repeat has been identified in BVDV NADL (2). However, as reported in BVDV NADL (2), one stretch of imperfect repeat sequence containing about 56 nucleotides with a 35 bases identity was also identified in BVDV Osloss and SD-1 (Fig. 4). In addition, two stretches of imperfect repeat sequence, one containing 18 nucleotides with a 14 base identity and the other consisting of 32 nucleotides with a 25 base identity, were identified in both HoCV Alfort and Brescia (Fig. 4). The nucleotide sequences present in the repeats are not conserved between different viruses, and the repeat stretches are also located in the different positions for different viruses. The repeat sequences present in the 3′-UTR of flavivirus was

suggested to be viral replication signal (46). The function of the AU-rich stretch and imperfect repeat sequence identified in the 3′-UTR of pestiviruses remains to be determined.

Complementary sequences between the 5′ and 3′ terminus of the genome have been described in a number of single stranded RNA viruses (9, 46). When the 3′ terminal nucleotide sequence was compared with the complementary sequence at the 5′ terminus, no significantly homologous sequence was found in pestiviruses although a 17 nucleotide sequence in the 3′-UTR was reported to be complementary to the sequence in the 5′-UTR in HoCV Brescia (8).

Predicted secondary structure of 3′-UTR of pestivirus genome

The secondary structure of the 3′-UTR from the 5 pestiviruses was predicted separately. No consensus structure was found when DNASIS was used to predict the secondary structure of 3′-UTR. However, when FOLD was used, the predicted secondary structure for five pestiviruses was very similar to one another. The consensus model contains a series of 4 stem-loop structures, (designated as stem-loop I, II, III and VI) (Fig. 5). The optimal ΔG values of the structure are −48.7 Kcal/mol, −44.1 Kcal/mol, −56.3 Kcal/mol, −46.6 Kcal/mol and −42 Kcal/mol for SD-1, NADL, Osloss, Alfort and Brescia, respectively. Stem-loop I is located at the 3′ portion of the 3′C region and conserved among all the 5 pestiviruses. It should be noted that the intervening sequence ACAGCACUUUA between stem-loop I and II was conserved in the five pestiviruses (Fig. 5). Stem-loop II was composed of part of the 3′V region and part of the 3′C region of the primary structure and its structural detail was relatively conserved. Both stem-loop III and VI are located in the 3′V region and were less conserved in terms of the length of the stem and structural configuration, particularly between BVDV group and HoCV group.

Interactions between viral template RNA and the proteins of the replication complexes are presumed to be mediated by a signal sequence present in the viral RNA template (9, 33, 47). An RNA hairpin at the extreme 5′ end of the poliovirus genome was important for efficient viral RNA replication (48). A 3′ terminal secondary structure has been described in flaviviruses (47, 49, 50), a number of positive stranded RNA plant viruses (9, 51) and RNA bacteriophages (52). For flaviviruses (47, 50) and brome mosaic virus (BMV) (53, 54), such structures have been implicated in initiation of minus-strand RNA synthesis. Secondary structure prediction for the 5′ and 3′-UTR of pestivirus genomes resulted in the identification of a stable and conserved stem-loop structure at extreme 5′ (Fig. 2, domain A) and 3′ end (Fig. 5, stem-loop I) of the viral RNA genome. Whether these structures function as the recognition signals for viral plus and minus strand RNA replication remains to be determined. In other experiments, a 100: 1 asymmetric synthesis of plus and minus strand RNA of BVDV during the virus replication cycles was observed (Deng and Brock, unpublished data). A formidable task for future work is to determine whether two different RNA replication complexes or two different recognition signals with various efficiencies on the RNA templates are responsible for the regulated synthesis of plus and minus strand viral RNA of pestiviruses.

Recently pestivirus was reclassified into family Flaviviridae (30, 55, 56). However, the features of the primary and secondary structure of 5′-UTR of pestivirus resemble that of picornavirus and HCV rather than flaviviruses. In addition, the predicted cap-independent translation mechanism of pestivirus is totally different from that of a number of the family Flaviviridae. Therefore, the suggested establishment of a separate family for classification of pestiviruses may be desirable (7, 8, 57).

ACKNOWLEDGMENTS

Salaries and research support were provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University. Journal article no. 252−92. This work was supported in part by Cooperative Research Agreement 88-34116-3653 from the U.S. department of Agricultural Science and Education Administration. The authors would like to thank Sylva Riblet and Jerry Meitzler for excellent technical assistance, and Fengmin Tian for typing of the manuscript.

REFERENCES

1. Renard, A., Schmetz, D., Guiot, C., Brown-Shimmer, S., Dagenais, L., Pastoret, P. P., Dina, D. and Martial, J. A. (1987a) Ann. Rech. Vet., **18**, 121−125.
2. Collett, M. S., Larson, R., Gold, C., Strick, D., Anderson, D. K. and Purchio, A. F. (1988a) Virology, **165**, 191−199.
3. Collett, M. S., Larson, R., Belzer, S. K. and Retzel, E. (1988b) Virology, **65**, 200−208.
4. Brock, K. V., Deng, R. and Riblet, S. (1992) J. Virol. Methods, **38**, 39−46.
5. Deng, R. and Brock, K. V. (1992) Virology, **191**, 867−879.
6. Renard, A., Dian, D. and Martial, J. A. (1987b) European Patent Application, No. 0208672.
7. Meyers, G., Rümenapf, T. and Thiel, H.-J. (1989) Virology, **171**, 555−567.
8. Moormann, R. J. M., Warmerdam, P. A. M. and Meer, B. V. D. (1990) Virology, **177**, 184−198.
9. Strauss, E. G. and Strauss, J. H. (1983) Curr. Top. Microbiol. Immunol., 105, 1−98.
10. Jang, S. K., Krausslich, H. -G., Nicklin, M. J. H., Duke, G. M., Palemenberg, A. C. and Wimmer, E. (1988) J. Virol., **62**, 2636−2643.
11. Pelletier, J. and Sonenberg, N. (1988) Nature (London), **334**, 320−325.
12. Jackson, R. J. (1988) Nature (London), **334**, 292−293.
13. Jang. S. K., Davies, S. V., Kaufman, R. J. and Wimmer, E. (1989) J. Virol., **63**, 1651−1660.
14. Iizuka, N., Yonekawa, H. and Nomoto, A. (1991) J. Virol., **65**, 4867−4873.
15. Borman, A. and Jackson, R. J. (1992) Virology, **188**, 685−696.
16. Tsukiyama-Kohara, K., Iizuka, N., Kohara, M. and Nomoto, A. (1992) J. Virol., **66**, 1476−1483.
17. Macejak, D. G. and Sarnow, P. (1991) Nature (London), **353**, 90−94.
18. Racaniello, V. R. and Meriam, C. (1986) Virology, **155**, 498−507.
19. Pilipenko, E., Blinov, V. M., Romanova, L.I., Sinyakov, A.N., Maslova, S.V. and Agol, V.I. (1989) Virology, **168**, 201−209.
20. Lipton, H. L., Calenoff, M., Bandyopadhyay, P., Miller, S. D., Canto, M. C. D., Gerety, S. and Jensen, K. (1991) J. Virol., **65**, 4370−4377.
21. Macadam, A. J., Pollard, S. R., Ferguson, G., Dunn, G., Skuce, R., Almond, J. W. and Minor, P. D. (1991) Virology, **181**, 451−458.
22. Collett, M. S. (1992) Comp. Immun. Microbiol. infect. Dis., 15, 145−154.
23. Lipman, D. J. and Pearson, W. R. (1985) Science, **227**, 1435−1441.
24. Zuker, M. and Stiegler, P. (1981) Nucleic Acid Res., **9**, 133−148.
25. Pelletier, J., Flynn, M. E., Kaplan, G., Racaniello, V. R. and Sonenberg, N. (1988) J. Virol., **62**, 4486−4492.
26. Belsham, G. J. (1992) EMBO J., **11**, 1105−1110.
27. Purchio, A. F., Larson, R., Torborg, L. L. and Collett, M. S. (1984) J. Virol., **52**, 973−975.
28. Kuge, S., Kawamura, N. and Nomoto, A. (1989) J. Virol., **63**, 1069−1075.
29. Kozak, M. (1986) Cell, **44**, 283−292.
30. Collett, M. S., Moennig, V. and Horzinek, M. C. (1989) J. Gen. Virol., **70**, 253−266.
31. Hagenbüchle, O., Santer, M. and Steitz, J. A. (1978) Cell, **13**, 551−563.
32. Kozak, M. (1983) Microbiol. Rev., **47**, 1−45.
33. Agol, V. I. (1991) Adv. Virus Res., **40**, 103−180.
34. Pestova, T. V., Hellen, C. U. T. and Wimmer, E. (1991) J. Virol., **65**, 6194−6204.

35. Pilipenko, E. V., Gmyl, A. P., Maslova, S. V., Svitkin, Y. V., Sinyakov, A. N. and Agol, V. I. (1992) Cell, **68**, 119–131.
36. Duke, G. M., Hoffman, M. A. and Palmenberg, A. C. (1992) J. Virol., **66**, 1602–1609.
37. Choo, Q. -L., Richman, K. H., Han, J. H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coit, D., Medina-Selby, A., Barr, P. J., Weiner, A. J., Bradley, D. W., Kuo, G. and Houghton, M. (1991) Proc. Natl. Acad. Sci. USA, **88**, 2451–2455.
38. Kato, N., Hijikata, M., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., Sugimura, T. and Shimotohno, K. (1990) Proc. Natl. Acad. Sci. USA, **87**, 9524–9528.
39. Inchauspe, G., Zebedee, S., Lee, D. -H., Sugitani, M., Nasoff, M. and Prince, A. M. (1991) Proc. Natl. Acad. Sci. USA, **88**, 10292–10296.
40. Han, J. H., Shyamala, V., Richman, K. H., Brauer, M. J., Irvine, B., Urdea, M. S., Tekamp-Olson, P., Kuo, G., Choo, Q. -L. and Houghton, M. (1991) Proc. Natl. Acad. Sci. USA, **88**, 1711–1715.
41. Brinton, M. A. and Dispoto, J. H. (1988) Virology, **162**, 290–299.
42. Kozak, M. (1989a) J. Cell Biol., **108**, 229–241.
43. Pelletier, J. and Sonenberg, N. (1985) Cell, **40**, 515–526.
44. Kozak, M. (1989b) Mol. Cell. Biol., **9**, 5134–5142.
45. Haller, A. A. and Semler, B. L. (1992) J. Virol., **66**, 5075–5086.
46. Rice, C. M., Lenches, E. M., Eddy, S. R., Shin, S. J., Sheets, R. L. and Strauss, J. H. (1985) Science, **229**, 726–733.
47. Brinton, M. A., Fernandez, A. V. and Dispoto, J. H. (1986) Virology, **153**, 113–121.
48. Racaniello, V. R. and Meriam, C. (1986) Virology, **155**, 498–507.
49. Wengler, G. and Castle, E. (1986) J. gen. Virol., **67**, 1183–1188.
50. Hahn, C. S., Hahn, Y. S., Rice, C. M., Lee, E., Dalgarno, L., Strauss, E. G. and Strauss, J. H. (1987) J. Mol. Biol., **198**, 33–41.
51. Hall, T. C. (1979) Int. Rev. Cytol., **60**, 1–26.
52. Zinder, N. (1975) RNA Phages. Cold Spring Harbor Press, Cold Spring Harbor.
53. Dreher, T. W. and Hall, T. C. (1988) J. Mol. Biol., **201**, 31–40.
54. Miller, W. A., Bujarski, J. J., Dreher, T. W. and Hall, T. C. (1986) J. Mol. Biol., **187**, 537–546.
55. Horzinek, M. C. (1991) Arch. Virol. (Suppl.), **3**, 55–56.
56. Francki, R. I. B., Fauquet, C. M., Knudson, D. L. and Brown, F. (1991) Arch. Virol. (Suppl.), **2**, 1–450.
57. Thiel, H. -J., Stark, R., Weiland, E., Rümenapf, T. and Meyers, G. (1991) J. Virol., **65**, 4705–4712.