All subgenomic mRNAs of equine arteritis virus contain a common leader sequence

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ABSTRACT

During the replication of equine arteritis virus (EAV) six subgenomic mRNAs are synthesized (1). We present evidence that the viral mRNAs form a 3'-coterminal nested set and contain a common leader sequence of 208 nucleotides which is encoded by the 5'-end of the genome. The leader is joined to the bodies of mRNA 5 and 6 at positions defined by the sequence 5' UCAAC 3'. The part of the leader sequence flanking the UCAAC motif is very similar to the 5'-splice site of the *Tetrahymena* pre-rRNA. A possible internal guide sequence has been identified 43 nucleotides downstream of the leader sequence on the genome. Hybridization analysis shows that all EAV intracellular RNAs contain the leader sequence. These data imply that the viral subgenomic mRNAs are composed of leader and body sequences which are non-contiguous on the genome.

INTRODUCTION

In addition to genomic RNAs many animal viruses produce one or more subgenomic mRNA species to allow expression of viral genes in a regulated fashion. Currently, four mechanisms are known by which these mRNAs are generated: (i) Internal initiation by the RNA polymerase on the negative-stranded RNA e.g. in alphaviruses, paramyxoviruses and rhabdoviruses (2), (ii) discontinuous RNA synthesis involving either leader-primed transcription or co-transcriptional trans splicing e.g. in coronaviruses (3), (iii) recruitment of 5'-capped oligonucleotides from host mRNAs as primers for viral transcription e.g. in bunyaviruses and orthomyxoviruses (4-6) and (iv) cis-splicing utilizing the cellular pre-mRNA splicing apparatus e.g. in orthomyxoviruses (7-10).

On the basis of UV inactivation studies it has been postulated that mRNA synthesis in equine arteritis virus (EAV) also involves processing of a precursor RNA (1). EAV is a enveloped, positive-stranded RNA virus originally classified as a non-arthropod borne togavirus (11). The single-stranded, infectious RNA genome of EAV is approximately 13 kb in length (12) and viral replication involves the synthesis of six polyadenylated, subgenomic RNAs. Five virus-induced proteins have been identified in EAV infected cells (13), whereas three proteins are consistently present in virion preparations: the nucleocapsid protein (VP1) and two envelope proteins (VP2, VP3) (14). Pulse-chase experiments have demonstrated that the viral structural proteins are primary translation products rather than cleavage products of a polyprotein precursor (13). The translation product of the smallest viral subgenomic RNA (RNA 6) corresponds to the nucleocapsid protein (13). RNase T_1 fingerprinting of purified viral mRNAs revealed that these mRNAs possess common sequences (1,15,16).

In order to determine whether the synthesis of the subgenomic EAV mRNAs involves cleavage or splicing, the relationship between genomic and subgenomic EAV RNAs was studied using cDNA libraries synthesized on virion RNA and poly(A)-selected RNA from virus infected cells. Sequence and hybridization analysis revealed that all EAV mRNAs form a 3'-nested set and contain the same 5'-leader sequence.

MATERIALS AND METHODS

Sources of materials

Nucleotides, reverse transcriptase and most other enzymes were purchased from Pharmacia. Klenow polymerase, DNase I and proteinase K were from Boehringer. T_4 and *E. coli* DNA ligase were obtained from New England Biolabs, nuclease free BSA was from BRL. Human placental RNase inhibitor and radioactive nucleotides were supplied by Amersham.

Oligonucleotides

Oligonucleotides were synthesized using a Biosearch Model 8600 DNA synthesizer and purified by high performance liquid chromatography.
Oligonucleotide 32 (5' dAATGGACCGAAGACG 3'), oligonucleotide 63 (5' dACGTGATCGTCTTGAC 3'), oligonucleotide 64 (5' dTGGTTCCTGGGTGGC 3'), oligonucleotide 80 (5' dGCCGCGAACCACTAC 3') and oligonucleotide 78 (5' dAGTAAGAAAGGGAAC 3') all have a sequence complementary to the viral genome.

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Virus growth and RNA isolation

The Bucyrus strain of EAV (17) was propagated in BHK-21 or Vero cells and the virus was concentrated as reported previously (1). Isolation of viral genomic RNA and EAV specific intracellular RNA was as described before (13,15).

cDNA synthesis and cloning

Four cDNA libraries were prepared using either viral genomic RNA or RNA from virus infected BHK-21 cells as a template. The first cDNA library was synthesized on poly(A)$^+$ RNA from virus infected cells using oligo(dT) as a primer. To prepare a second cDNA library, total cytoplasmic RNA from EAV infected cells was primed with the virus specific oligonucleotide 63. The other cDNA libraries were made from virion RNA, using oligo(dT) or random pentanucleotides as primer in the first strand cDNA reaction. The conditions for cDNA synthesis were adopted from Gubler and Hoffman (18). After homopolymer tailing (19), the double stranded cDNA was annealed to PstI cleaved, dG-tailed pUC9 DNA (Pharmacia). Transformation of *E. coli* strain JM109 or XL1-Blue (Stratagene) was according to Hanahan (20). Bacteria were grown in the presence of 50 μg/ml ampicillin on agar plates containing IPTG and X-gal. White colonies were used for further analysis.

Screening and analysis of recombinants

Recombinant DNA techniques were performed by standard methods (21), hybridization conditions were as described by Meinkoth and Wahl (22). The first cDNA library was screened by colony hybridization using radioactive genome fragments prepared by the incubation of alkali-treated virion RNA with T_4 kinase in the presence of [gamma-^{32}P]ATP. Virus specific clones were further analyzed by restriction enzyme mapping and dot spot hybridizations with ^{32}P-labelled oligo(dT). The other cDNA libraries were analyzed by using virus specific oligonucleotide or nick-translated probes in either colony or dot blot hybridizations. Positive clones were characterized in more detail by restriction enzyme analysis and Southern blotting.

DNA sequencing

DNA restriction fragments purified from agarose gels by binding to NA45 DEAE paper (Schleicher & Schuell) were ligated into M13mp18 and/or M13mp19. The ligation reactions were transformed into *E. coli* strain PC2495 (21). Single stranded DNA isolated from M13 subclones was sequenced using either Klenow polymerase and [α-^{32}P]dATP (23) or T_7 DNA polymerase (Pharmacia) and [α-^{35}S]dATP (24). As sequencing primers both the universal primer (Pharmacia) and the synthetic oligonucleotide primers were used. The sequencing reactions were resolved on 8 M urea, 6% polyacrylamide gels (25). Sequence data were assembled and analyzed using the computer programs designed by Staden (26).

Agarose formaldehyde gelelectrophoresis

Total cytoplasmic RNA extracted from EAV infected BHK-21 cells was denatured by treatment with formaldehyde and separated in a 1.75% agarose formaldehyde gel (22). After electrophoresis the gel was washed in 10 mM sodium phosphate buffer pH 7.0, dried on Whatmann 3 MM filterpaper and prehybridized for 30 min in 5×SSPE, 5×Denhardt, 0.05% SDS containing 100 μg/ml homomix I. A T_4 kinase labelled oligonucleotide probe was added to the prehybridization solution and incubation was continued for 16 h at the calculated melting temperature of the oligonucleotide minus 5°C (22). The gel was washed for 3 times 15 min in 5×SSPE, 0.1% SDS at the hybridization temperature prior to autoradiography at −70 °C using a hypersensitized film and an intensifying screen (27).

Primer extension

Oligonucleotides 63 and 80 were ^{32}P-labelled to a high specific activity (± 5.10^5 cpm/ng) using T_4 kinase. Subsequently, 10 ng of each oligonucleotide was annealed to 1 ug of total cytoplasmic RNA from mock and virus infected BHK-21 cells in the presence of 40 mM hydroxymethylmercury (total reaction volume 5 μl). The samples were incubated at room temperature for 10 min and hydroxymethylmercury was reduced by the addition of 2 μl 1.4 M 2-mercaptoethanol. To the samples 5 μl H_2O, 2 μl 1st strand cDNA buffer (500 mM Tris-HCl pH 8.3, 500 mM KCl, 80 mM $MgCl_2$), 4 μl dNTP mix (5 mM of each dATP, dCTP, dGTP and dTTP), 1 μl human placental RNase inhibitor and 1 μl AMV reverse transcriptase (17.5 U/μl) were added. The samples were incubated at 37 °C for 10 min followed by 1 h at 42°C. Reactions were terminated by the addition of 20 μl stop mix (80% formamide, 20 mM EDTA pH 8.0, bromophenol blue and xylene cyanol FF). After heating for 5 minutes at 96°C the samples were applied to a 8 M urea, 6% polyacrylamide gel. A sequence reaction was used as a size marker.

RESULTS

EAV-specific intracellular RNAs form a 3'-coterminal nested set

cDNA libraries were synthesized on poly(A)-selected RNA from EAV infected cells (library I) and EAV genomic RNA (library II) using oligo(dT) as a primer. The insert size of several virus specific recombinants was determined. Four oligo(dT)-positive clones from library I (008, 026, 027 and 045) and one oligo(dT)-positive clone from library II (106) were selected for further analysis. All cDNA clones from library I displayed a similar restriction pattern, clones with longer inserts showing additional fragments at their 5'-termini (Figure 1). Clone 106 perfectly fits the restriction enzyme map obtained for the cDNA clones from

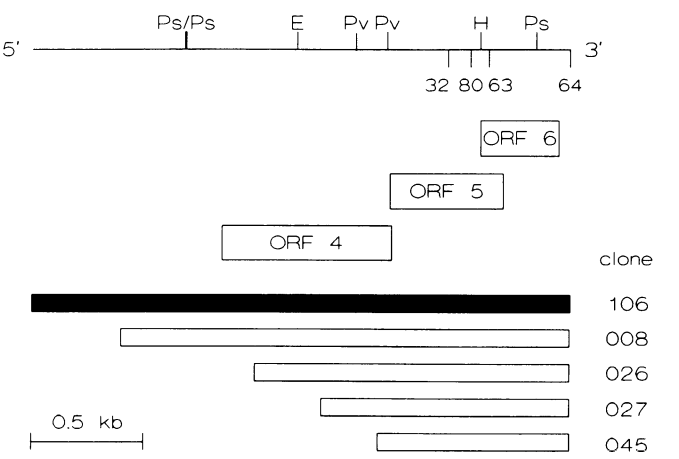


Figure 1. Physical map of 5 oligo(dT) positive EAV cDNA clones. The subgenomic cDNA clones are represented by white bars, the genomic cDNA clone is represented by a black bar. E, EcoRI; H, HindIII; Ps, PstI; Pv, PvuII. The position of oligonucleotides 32, 63, 64 and 80 is indicated below the restriction map. ORF 4, 5 and 6 denote the three open reading frames.

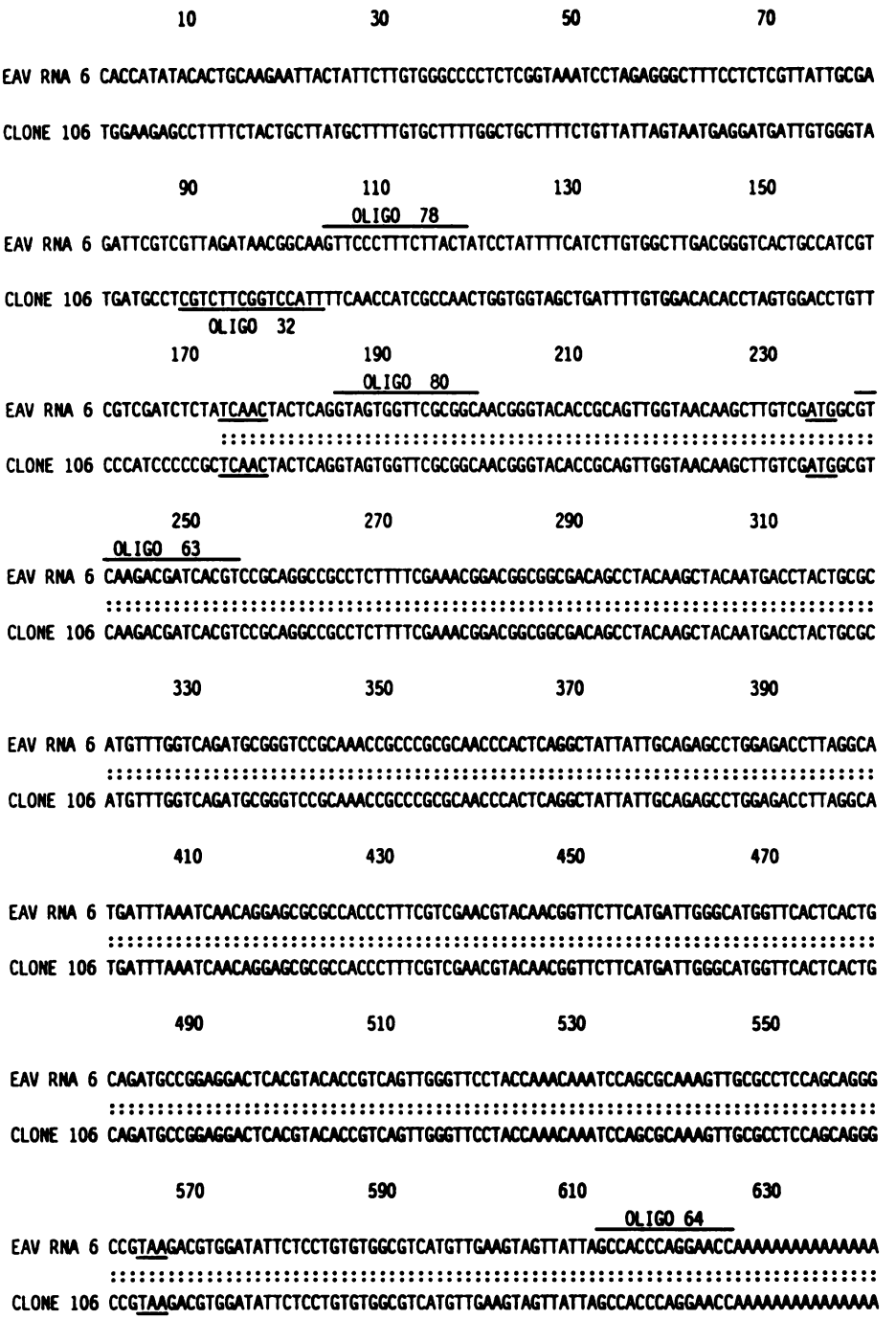


Figure 2. Sequence of the 3'-terminus of viral genome. The sequence of cDNA clone 106 is aligned with the consensus sequence of mRNA 6 derived from specific cDNA clones 015 and 033. The position of oligonucleotides 32, 63, 64, 78 and 80 is specified. The initiation and termination codon of ORF 6 and the 5' TCAAC 3'motif are underlined. The sequence of cDNA clones 008, 026, 027 and 045 is identical to the sequence of clone 106.

EAV specific intracellular RNAs (Figure 1). Sequence analysis of the cDNA clones from both libraries revealed the presence of three open reading frames (ORF 4, 5 and 6) with coding capacities of 330, 486 and 765 nucleotides respectively (Figure 1). The nucleotide sequence of ORF 6 is shown in Figure 2. Details on the nucleotide sequence of ORF 4 and 5 will be presented elsewhere (Chirnside et al., in preparation).

On the basis of the sequence information a number of oligonucleotides (for positions see Figure 1 and 2) were synthesized. Hybridization experiments, performed on total RNA from virus infected cells, showed that oligo 64 which covers the extreme 3'-end of the consensus sequence as well as oligo 80 which is complementary to the region just upstream of ORF 6 bind to all EAV intracellular RNAs (Figure 3). The same result was obtained using kinased oligo 63 which binds just downstream of the initiation codon of ORF 6 (data not shown). These findings illustrate that the EAV subgenomic RNAs form a 3'-nested set which is situated at the 3'-end of the genome.

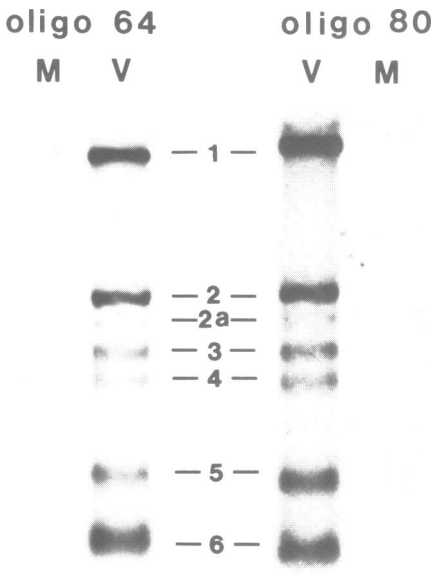
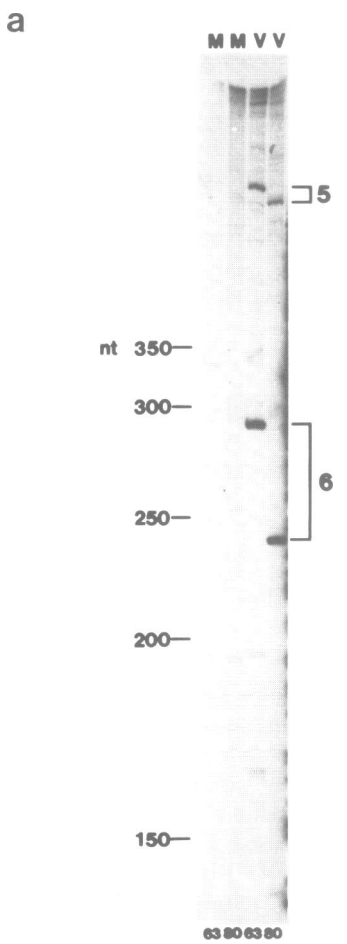


Figure 3. Hybridization analysis of total cytoplasmic RNA from mock (M) and virus (V) infected BHK-21 cells. The RNAs were separated in a 1.75% agarose formaldehyde gel and the dried gel was incubated with kinased oligonucleotides 64 and 80. The EAV specific intracellular RNAs are numbered according to Van Berlo et al. (15).



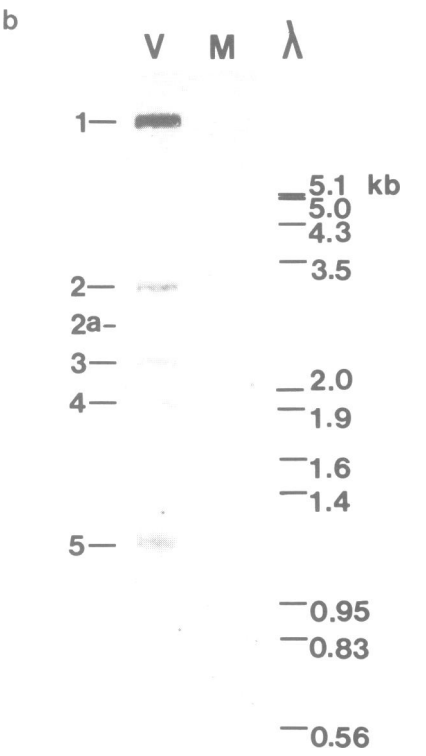


Figure 4. Determination of the 5'-end of RNA 6. (a) Primer extension on RNA from mock (M) and virus (V) infected BHK-21 cells. The elongation products were analyzed on a 8 M urea, 6% polyacrylamide gel. The primers used are pointed out below the gel. Samples from sequence reactions were included to yield the size ladder shown on the left. The extension products of RNA 5 and 6 are indicated separately. The extension products of RNA 5 were not within the separating range of the sequence gel. (b) Hybridization of oligonucleotide 32 to total cytoplasmic RNA from mock (M) and EAV (V) infected cells. The RNAs were resolved on a 1.5% agarose formaldehyde gel. A EcoRI/HindIII digest of bacteriophage lambda DNA, filled in with Klenow polymerase in the presence of [α-^{32}P]dATP, was used as a size marker. The numbers at the right indicate the sizes and positions of the resulting fragments.

Identification of a common leader sequence

To map the 5'-end of RNA 6 on the genome a primer extension experiment was carried out. End-labelled oligonucleotides 63 and 80 were annealed to RNA from mock and EAV infected cells and extended by reverse transcriptase. The elongation products were analyzed by polyacrylamide gel electrophoresis (Figure 4a). Both oligonucleotide primers yielded 2 discrete fragments when RNA from virus infected cells was used as a template. We interpret the lower bands to be primer extension products of RNA 6 and the upper bands to originate from RNA 5. On the basis of the lengths of the elongation products (290 nucleotides for oligo 63 and 235 nucleotides for oligo 80) the 5'-end of RNA 6 maps to position 661 relative to the 3'-end of the genome. The size difference between the RNA 6 extension products reflects the distance between oligo 63 and 80 on the genome.

To analyze whether the sequence at the 5'-end of RNA 6 is identical to the aligned genome sequence, oligonucleotide 32 (for position see Figure 1) was hybridized to RNA from EAV infected cells (Figure 4b). Remarkably, oligo 32 failed to bind to RNA 6, although the 5'-end of RNA 6 clearly extends beyond the position of oligo 32 on the genome. The most plausible

explanation is that the smallest viral subgenomic RNA emerges from the joining of noncontiguous RNA segments.

To test this hypothesis cDNA library I was examined for sequences diverging from the genomic sequence. A number of oligo 32 negative but oligo 63 positive clones with insert sizes ranging from 500 to 700 nucleotides were identified (Figure 5). Sequence analysis of the inserts of clones 033 and 015 revealed that both clones deviated from the genome consensus sequence at the same position (Figure 2). Since these clones are presumably derived from RNA 6, the deviating sequence could represent the leader of RNA 6. Direct evidence was obtained by hybridization of EAV intracellular RNAs to oligonucleotide 78 which is complementary to the deviating sequence (for position see Figure 2). As is shown in Figure 6 all EAV specific RNAs possess a common leader sequence. The band that is visible in the mock lane is believed to result from aspecific binding of oligo 78 to the large ribosomal RNA. The same set of leader-positive RNAs were observed after hybridization with a radioactive labelled restriction fragment derived from the leader sequence of clone 033 (data not shown). Subsequently, the oligo(dT)-primed cDNA library and a newly constructed oligo(63)-primed cDNA library were probed with oligonucleotide 32 and 78. A number of double positive transformants were identified (Figure 5). On the basis of length and hybridization properties, these cDNA clones are thought to be produced from RNA 5. Sequence analysis of clone 038 showed that the leader sequence is present at the 5′-end of the insert.

Comparison of the sequence of the oligo 78$^+$ clones with the corresponding genomic sequence indicates identical junction sites between the leader and body sequences of RNA 5 and 6. In both mRNAs the divergence sites are marked by the sequence 5′ TCAAC 3′ (Figure 7a). The same sequence motif is retained at the putative junction sites for the synthesis of the larger subgenomic RNAs (Chirnside *et al.*, in preparation). By combining the results of the primer extension experiment and the position of the junction site of RNA 6, the leader is calculated to be 208 nucleotides long.

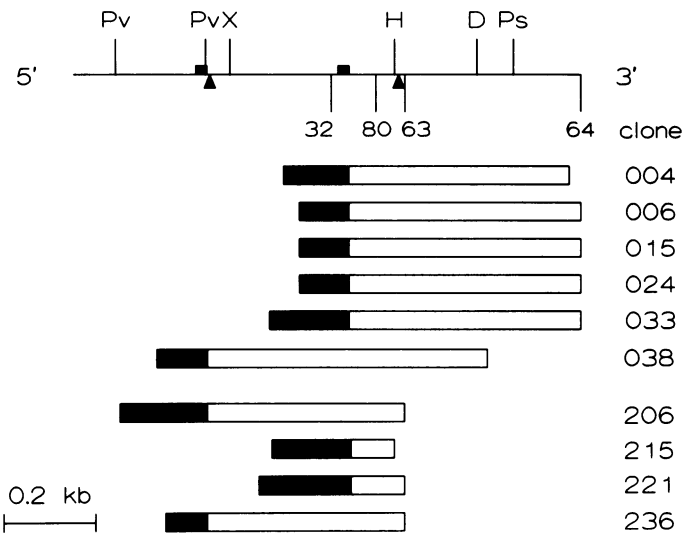


Figure 5. Overview of virus specific clones diverging from the genome consensus sequence. A physical map of the 3′-end of the genome is shown at the top of the diagram. D, DraI; H, HindIII; Ps, PstI; Pv, PvuII; X, XbaI. The position of oligonucleotides 32, 63, 64 and 80 is indicated below the restriction map. The filled rectangles represent the conserved 5′ TCAAC 3′ motif. The black triangles indicate the translation initiation codons of ORF 5 and 6. The deviating cDNA clones are represented by bars of which the black part depicts the leader sequence. The clones 004 to 038 are from the oligo(dT)-primed subgenomic cDNA library, the clones 206 to 236 represent oligo(63)-primed cDNA clones.

Localization of the leader sequence on the genome

A random-primed genomic cDNA library was screened by colony hybridization with leader specific oligonucleotide 78. Of 86 virus specific clones tested only clone 586, having an insert of ± 1400 bp, hybridized to oligo 78. Sequence analysis of part of the insert showed that its 5′-end encompasses the leader sequence including the conserved TCAAC motif (Figure 7b). To map clone 586 on the EAV genome, restriction fragments from the inserts of clone 106 (located at the 3′-end of the genome) and 586 were purified and used to probe the random-primed genomic cDNA library. The inserts of positive transformants were analyzed by restriction enzyme digestion, nick-translated and used to search for overlapping cDNA clones extending towards both directions of the viral genomic RNA. This 'genomic RNA walking' resulted in a continuous map of approximately 13 kb (Figure 8). Clone 586 is located at the 5′-end of this restriction map.

DISCUSSION

Our experiments have yielded three results that refer to the structure of arteriviral mRNAs.

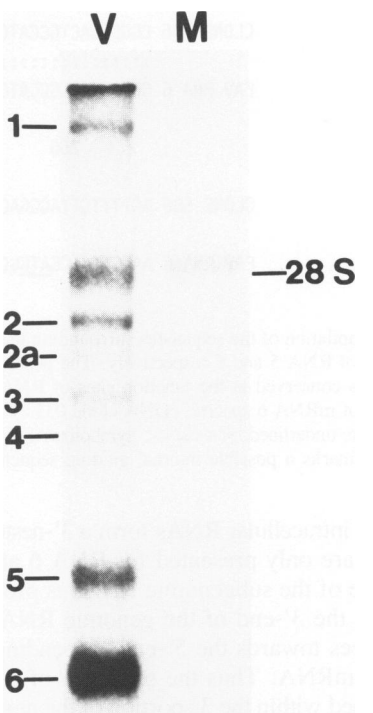


Figure 6. Hybridization analysis of total cytoplasmic RNA from mock (M) and virus (V) infected BHK-21 cells. The RNAs were separated in a 1.75% agarose formaldehyde gel and the dried gel was incubated with end-labelled oligonucleotide 78. The position of the large ribosomal RNA (28 S) is indicated.

a

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genome 5/6    CCCATCCCCCGC TCAAC TACTCAGGTAGTGGTT -35 nt- ORF 6
                           ||||||||||||||||||
                           ||||||||||||||||||
EAV RNA 6     CGTCGATCTCTA TCAAC TACTCAGGTAGTGGTT -35 nt- ORF 6
              ||||||||||||
              ||||||||||||
EAV RNA 5     CGTCGATCTCTA TCAAC CTTGTGAGGTTGGGGG -15 nt- ORF 5
                           ||||||||||||||||||
                           ||||||||||||||||||
genome 4/5    ACGGCAGCAAAG TCAAC CTTGTGAGGTTGGGGG -15 nt- ORF 5
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b

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                10                  30                  50                  70

CLONE 586 TGCCATATACGGCTCACCACCATATACACTGCAAGAATTACTATTCTTGTGGGCCCCTCTCGGTAAATCCTAGAGGGCTT
                          ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EAV RNA 6                 CACCATATACACTGCAAGAATTACTATTCTTGTGGGCCCCTCTCGGTAAATCCTAGAGGGCTT

                90                  110                 130                 150
                                                    OLIGO 78
CLONE 586 TCCTCTCGTTATTGCGAGATTCGTCGTTAGATAACGGCAAGTTCCCTTTCTTACTATCCTATTTTCATCTTGTGGCTTGA
          ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
EAV RNA 6 TCCTCTCGTTATTGCGAGATTCGTCGTTAGATAACGGCAAGTTCCCTTTCTTACTATCCTATTTTCATCTTGTGGCTTGA

                170                 190                 210                 230
                                 <<<<<<                                      >>>>>>>>>
CLONE 586 CGGGTCACTGCCATCGTCGTCGATCTCTATCAACTACCCTTGCGACTATGGCAACCTTCTCCGCTACTGGATTTGGAGGG
          ::::::::::::::::::::::::::::::::::::
EAV RNA 6 CGGGTCACTGCCATCGTCGTCGATCTCTATCAACTACTCAGGTAGTGGTTCGCGGCAACGGGTACACCGCAGTTGGTAAC

                250

CLONE 586 AGTTTTGTTAGGGACTGGTC

EAV RNA 6 AAGCTTGTCGATGGCGTCAA
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Figure 7. (a) Compilation of the sequences surrounding the divergence sites. The sequences entitled genome 4/5 and 5/6 represent the genomic sequences enclosing the junction sites of RNA 5 and 6 respectively. The position of ORF 5 and 6 relative to the junction sites of RNA 5 and 6 is indicated. A stretch of 5 nucleotides (5′ TCAAC 3′) is conserved at the junction sites of RNA 5 and 6. (b) Sequence of the 5′-terminus of the viral genome. The sequence of cDNA clones 586 is aligned with that of mRNA 6 specific cDNA clone 033. The part of the leader sequence that binds oligo 78 is indicated. The 5′ TCAAC 3′motifs and the initiating codon of ORF 6 are underlined. <<<<<< symbolizes a region with sequence homology to the 5′-splice site of the self-splicing pre-rRNA of Tetrahymena thermophila, >>>>>>>>> marks a possible internal guiding sequence.

(i) The EAV intracellular RNAs form a 3′-nested set. Although sequence data are only presented for RNA 6 and part of RNA 5, the structure of the subgenomic RNAs is probably such they all incorporate the 3′-end of the genomic RNA but extend for various distances towards the 5′-end, depending on the size of the respective mRNA. Thus the sequence of each subgenomic RNA is contained within the 3′-portion of the next larger mRNA. The 5′-end of each mRNA therefore comprises a sequence, which does not overlap the sequence of the smaller subgenomic RNAs. The oligonucleotide fingerprints of individual mRNAs are consistent with this model (15,16).

(ii) A common leader of approximately 208 nucleotides is fused to the bodies of the EAV subgenomic RNAs. For mRNA 5 and 6 the junction sites are preceded by the sequence 5′ UCAAC 3′. Although all intracellular EAV RNAs hybridize to the leader probe and additional UCAAC boxes are located at genome positions estimated to accommodate the junction sites for mRNA 2 to 4 (data not shown), the position of the leader sequence within

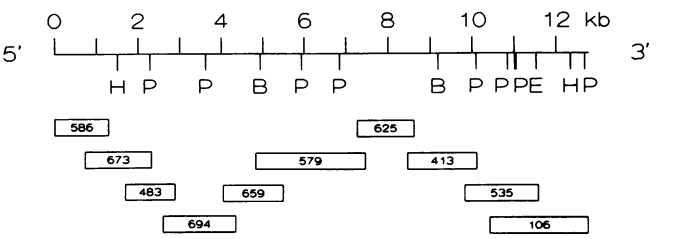

Figure 8. Physical map of the EAV genome. B, BamHI; E, EcoRI; H,HindIII; P, PstI. cDNA clones covering a continuous stretch of approximately 13 kb are aligned relative to the restriction map.

these subgenomic RNAs remains to be determined. The absence of primer extension products mapping to the junction sites of RNA 5 and 6 suggests that the joining of leader and body

sequences may be an efficient and specific process. There is no detectable heterogeneity at the junction sites or in the length of the leader as deduced from the primer extension result and sequence analysis of several subgenomic cDNA clones.

(iii) The leader sequence of the EAV mRNAs is encoded by the extreme 5'-end of the genome. Although the exact 5'-terminus of the genome has not been determined, the random-primed cDNA library contained no clones extending beyond the 5'-end of clone 586. A 5' UCAAC 3' sequence is preserved just downstream of the leader sequence on the genome (Figure 7b).

Although the structure of the EAV mRNAs resembles that of the coronaviral mRNAs (3), UV inactivation studies revealed a interesting difference between the transcription strategy of both viruses. The subgenomic RNAs of coronaviruses are transcribed independently (28,29), whereas synthesis of the EAV mRNAs involves the processing of a genome-sized precursor RNA (1).

Our current working hypothesis is that the EAV subgenomic RNAs are generated by splicing of genomic RNA at multiple sites defined by the sequence 5' UCAAC 3'. The efficiency of splicing at different sites could account for the observed differences in molarity between the subgenomic RNAs. Alternative splicing has shown to be an effective mechanism to control gene expression from complex transcriptional units (30,31). Since the sequence requirements for pre-mRNA splicing (32) are not fulfilled, conventional splicing (33,34) is unlikely. The sequences at the junction sites are also incompatible with the splice sites involved in tRNA splicing and (self-)splicing of RNAs containing group II introns (35). Remarkably, the sequence at the 3'-end of the leader 5' UCGAUCUCUA 3' strongly resembles the 5'-splice site of the self-splicing pre-rRNA of *Tetrahymena* (36–38) whereas a possible internal guide sequence 5' UUUGGAGGG 3' has been identified 43 nucleotides downstream of the leader sequence on the genome (Figure 7b). Completing the sequence analysis of the viral genome will allow the full identification of a possible group I intron.

Recently, it has been shown that the coronavirus mRNAs are transcribed from negative-stranded subgenomic RNAs (39,40). We can not exclude that the EAV mRNAs are also transcribed from subgenomic negative-stranded RNAs since data on the template for mRNA synthesis is not yet available. If subgenomic negative-stranded RNAs are involved in the mRNA synthesis of EAV the conclusions of the UV inactivation studies need to be reconsidered e.g. processing of a negative in stead of a positive-stranded template as suggested by Van Berlo *et al.* (1).

Studies to identify the template for mRNA synthesis are in progress. The cloning of the complete EAV genome permits the construction of an infectious cDNA clone to study arteriviral mRNA synthesis in more detail. The presence of a nested set of subgenomic mRNAs with a common leader sequence clearly illustrates that the arteriviruses differ from the togaviruses and certainly need reclassification in the future.

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