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Binding of Cellular Proteins to the Leader RNA of Equine Arteritis Virus

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Abstract. The genome of equine arteritis virus (EAV) produces a 3′ coterminal-nested set of six subgenomic (sg) viral RNAs during virus replication cycle, and each set possesses a common leader sequence of 206 nucleotides (nt) in length derived from the 5′ end of the viral genome. Given the presence of the leader region within both genomic and sg mRNAs, it is likely to contain cis-acting signals that may interact with cellular or viral proteins for RNA synthesis. Gel mobility shift assays indicated that proteins in Vero cell cytoplasmic extracts formed complexes with the positive (+) and negative (−) strands of the EAV leader RNA. Several cell proteins with molecular masses ranging from 74 to 31 kDa and 58 to 32 kDa were detected in UV-induced cross-linking assays with the EAV leader RNA (+) and (−) strands, respectively. In both cases, intense bands were observed at the 58–52 kDa molecular weight markers. Results from competition gel mobility shift assays using overlapping cold RNA probes spanning the leader RNA (+) strand indicated that nt 140–206 are not necessary for binding to cell proteins.

Key words: Cell protein binding, equine arteritis virus (EAV), leader region, RNA probes

Equine arteritis virus (EAV), the aetiologic agent of equine viral arteritis, is the prototype member of the *Arteriviridae* family. Together with the *Coronavirus* and *Torovirus* genera of the *Coronaviridae* family, the arteriviruses form the order *Nidovirales* [1]. The EAV genome is a positive (+), single-stranded, 5′-capped and 3′-polyadenylated RNA molecule of 12.7 kb in length (1). It contains, in the direction 5′-3′, two large open reading frames (ORFs) 1a and 1b, which encode the viral replicase, and seven smaller ORFs designated 2a, 2b and 3 to 7 which express the other EAV proteins [1]. Each ORF is preceded by the sequence motif 5′-UCAAC-3′, termed the leader-body junction site [1] or the transcription regulating sequence (TRS) [2]. The TRS is involved in the synthesis of a 3′ coterminal nested set of six subgenomic (sg) mRNAs through their fusion, likely by a discontinuous minus-strand transcription mechanism, with a common leader sequence of 206 nucleotides (nt) identical to and derived from the extreme 5′terminus of the viral genome [2].

Evidence supporting the involvement of host proteins during many stages of the replication cycles of viruses has been reported in a large number of virus systems [3,4]. Given the presence of the leader region within both EAV genomic and sg mRNAs, it is likely to contain cis-acting signals that may interact with cellular or viral proteins for RNA synthesis and/or protein translation. In fact, MA104 cell proteins have been shown to bind with the 3′ noncoding region RNA negative (−) strand of several arteriviruses, including EAV [5]. Here, we report that cell proteins form complexes not only with the (−) strand but also with the (+) strand of the EAV leader RNA. The molecular weights (MW) of binding proteins in the RNA–protein complexes were also determined. Finally, we show that

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nucleotides (nt) 140–206 of the leader RNA (+) strand were dispensable for binding to cell proteins.

To this end, Vero cells were mock- or infected at a multiplicity of infection of 0.1 with EAV [6]. Cytoplasmic extracts (CE) from both uninfected and virus-infected cells were collected at 40 h post-infection accordingly to published procedures [5,7]. CEs were aliquoted and stored at −80 °C until used (within a month). PCR amplifications of the sense and antisense EAV leader region (206 nt) from a parental plasmid (pUC18-EAV4B8; Archambault, unpublished) were carried out using appropriate primers that were designed on the basis of the 5′ end of the genome sequence of EAV [6] (GenBank accession # AF001259). The primer pairs used [(+) sense CAAGGTACC*TAATACGACTCACTATA* GCTCGAAGTGTGTATGGTG CC and (−)sense CAACTGCAGTAGAGATCGACGACGATGG CA, and (+) sense CAAGGTACC*TAATACGAC TCACTATAGTA*GAGATCGACGACGATGGC A and (−) sense CAACTGCAGCTCGAAGTGTG TATGGTGCC to obtain the leader sense and antisense sequences, respectively] contained the T7 RNA polymerase promoter (in italics) for the generation of *in vitro* RNA transcripts, and appropriate selected enzyme restriction sites (GGTACC: Kpn I, and CTGCAG: Pst I) for cDNA cloning purposes in order to generate plasmids pEAVL(+) and pEAVL(−).

In vitro synthesis of [^{32}P]-labeled or cold competitor RNA transcripts from *Pst 1*-linearized plasmid clones was performed with T7 RNA polymerase [5]. RNA–protein binding reactions were determined by gel mobility shift electrophoresis (GMSE) assay [5]. To this end, CE (10 μg of total protein), 5×10^4 cpm (counts per minute) of one of the [^{32}P]-labeled EAV RNA probes, and 1 μg of poly(I)-poly(C) or 10 μg of yeast tRNA (to reduce non-specific binding) to a final volume of 10 μl were incubated for 30 min at room temperature in binding buffer [5]. For competition assays, unlabeled RNA (8 μg) and CE proteins were incubated for 15 min prior to addition of the relevant [^{32}P]-labeled RNA probe. In all cases, the RNA–protein complexes were resolved through polyacrylamide gel electrophoresis (PAGE) [5] using a 6% nondenaturing polyacrylamide gel. To determine the molecular masses of binding proteins to RNA, the UV-induced cross-linking (UV-ICL) assays were conducted by incubating 20 μg of CE proteins with one of the radiolabeled RNA probes (1×10^5 cpm), 5 μg yeast tRNA and 20 μl of binding buffer to a final volume of 30 μl for 30 min at room temperature [5]. For competition UV-ICL assays, unlabeled competitor EAV leader RNA or non-competitor yeast tRNA was added to the CE in binding buffer for 15 min at room temperature prior to addition of the relevant [^{32}p]-labeled RNA probe. The reaction mixtures were irradiated (254 nm; UV Stratalinker 2400, Stratagene) for 10 min on ice and then treated with 3 μg RNAse A and 0.5 U RNAse T1 for 30 min at room temperature to digest unprotected RNA. In some cases, the reaction mixtures were treated with 50% methanol-acetone in attempts to achieve better resolution of the binding proteins [5]. In all cases, the protein complexes were separated by 10% SDS-PAGE.

By using GMSE assays (Fig. 1a), both (+) and (−) strands of the EAV leader RNA were found to bind to polypeptides from uninfected Vero cells (lane 2), as determined by the formation of RNA–protein complexes. Complete inhibition of the formation of detectable complexes by using homologous unlabeled RNA (+) or (−) strand prior to the addition of the [^{32}P]-labeled RNA probes was observed (lane 6). No RNA-protein complexes were detected with the addition of neither bovine serum albumin (BSA) (lane 7), or proteinase K-digested CE from uninfected Vero cells (lane 4) to the [^{32}P]-labeled RNA probes. In contrast, no inhibition of RNA–protein complex formation was detected by using of yeast tRNA (lane 5), and, in the case of the leader RNA (+) strand, poly(I)-poly(C) (lane 5′) that were used as negative competition controls. Combined together, these results indicated that the observed cell protein-RNA interactions are specific. Because similar RNA-protein interactions (as judged by similar electrophoresis mobilities) were obtained with CE from EAV-infected cells (lane 3, Fig. 1a), a feature also reported in other nidoviruses [5,8], further experiments were conducted only with the CE from uninfected Vero cells.

As shown in Fig. 1b (lane +), the leader RNA (+) strand bound host proteins with MW ranging from 31 to 74 kDa, with the p54 being the most abundant protein. The p54 and the protein bands observed at the 54–52 kDa MW marker obtained from precipitation of the RNA–protein complexes

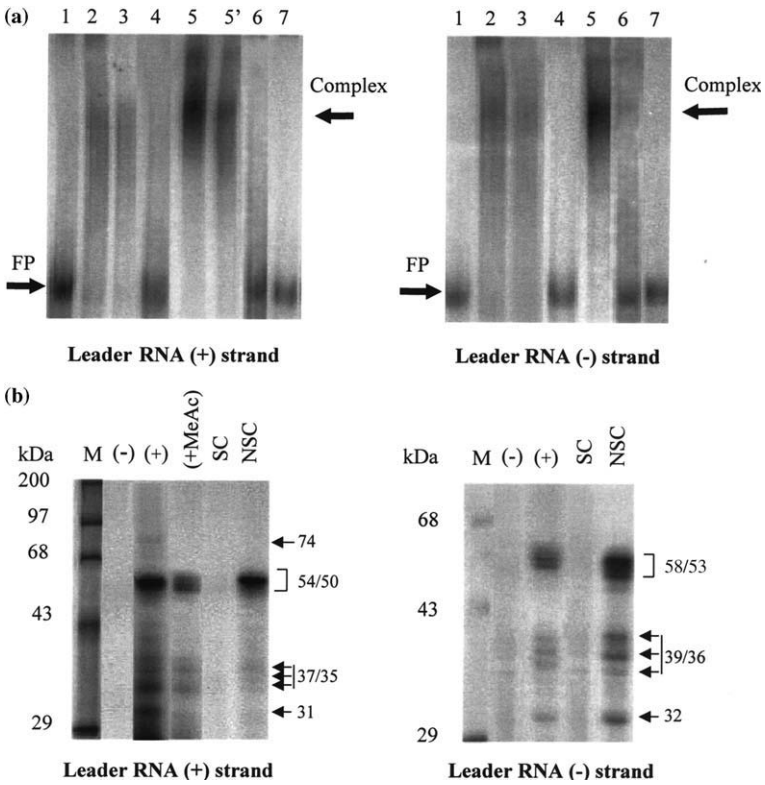

Fig. 1. Analysis of EAV leader RNA–protein interactions. (a) Specific binding of Vero cell proteins to the positive (+) and negative (−) strands of the leader RNA in gel mobility shift electrophoresis assays. Lane 1: free [^{32}P]-labeled RNA probe (FP); lane 2: addition of cytoplasmic extract (CE) from uninfected Vero cells; lane 3: addition of CE from EAV-infected Vero cells (40 h pi); lane 4: addition of 10 μg of proteinase K-digested CE from uninfected Vero cells to the [^{32}P]-labeled RNA probe (control); lanes 5 and 5′ refer to non-specific competition with yeast tRNA or with poly(I)-poly(C), respectively; lane 6: specific competition with EAV leader unlabeled RNA transcript; lane 7: addition of BSA to the [^{32}P]-labeled RNA probe (control). (b) Detection of proteins in Vero CE that bound to the leader RNA (+) and (−) strands by UV-induced cross-linking assays. Lane (−): free [^{32}P]-labeled RNA probe (FP) with no CE; lane (+): CE added; lane (+ Me Ac): CE added, followed by methanol-acetone treatment; lane SC: specific competition with EAV leader unlabeled RNA transcripts; lane NSC: nonspecific competition with yeast tRNA. Lane M: Molecular weight standard. Arrows indicate the major detected bands.

with 50% methanol-acetone (lane + MeAc) were believed to represent various isoforms of the PTB protein [5]. However, attempts to detect PTB by using immunoprecipitation assays [5] failed (not shown). Nevertheless, the binding of cell proteins to the EAV leader RNA (+) strand was found to be specific by incubating the CE with unlabeled homologous leader RNA (specific competitor, lane SC) or tRNA (non-specific competitor, lane NSC prior to addition of the [^{32}P]-labeled RNA probe. Binding of host proteins with MW ranging from 32 to 58 kDa to the (−) strand of the EAV leader RNA was also observed. These proteins included two clusters of distinct bands at the 58/53, and 39/36 kDa MW markers, as well as another protein of 32 kDa. Hwang and Brinton [5] identified distinct MA104 cell proteins of 103, 86, 55, 36, and 30 kDa to bind to the EAV antisense leader RNA. The proteins of 103 and 86 kDa detected by these authors were not observed in our study. Although it is not known whether the clustered proteins observed here are distinct proteins or isoforms of the same protein, they are similar in size to the p55, p36, and p30 detected by Hwang and Brinton [5]. However, these authors were not able to unequivocally identify these to any known proteins, including PTB (p55) and the p35/38 heterogeneous La antigen, which both have been reported to bind RNA sequences of a large number of RNA viruses, including that of the

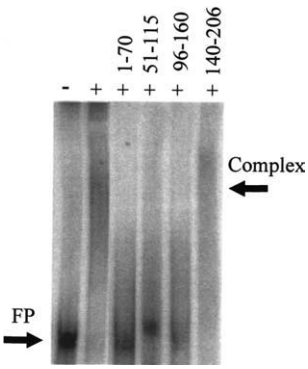

Fig. 2. Localization of the protein-binding regions of the positive (+) strand of the EAV leader RNA in competitive gel mobility shift electrophoresis assays. Competition assays were conducted by using overlapping unlabeled RNA transcripts spanning the leader RNA (+) strand. Lane -: free [^{32}P]-labeled RNA probe (FP) with no cytoplasmic extract (CE); lane +: CE added. The numbers refer to nucleotides of the leader region contained in the cold RNA transcripts.

coronavirus Mouse hepatitis virus, a nidovirus as EAV [4,9].

In order to delineate the region in the (+) strand of EAV leader RNA that was involved in interactions with cell proteins, overlapping deletion mutants of the leader RNA (+), strand were generated by inversed PCR [10] from plasmid pEAVL(+) using appropriate primers. These mutants spanned nucleic acids 1–70, 51–115, 96–160, and 140–206 of the EAV leader RNA (+) strand, respectively. Localization of the cell protein-binding regions of the leader RNA (+) strand was then achieved in competition GMSE assays in which the overlapping cold RNA transcripts derived from the plasmid mutants were allowed to react with the Vero cell CE prior to addition of the [^{32}P]-labelled probe representing the full-length (+) strand of the EAV leader RNA. Thus, competition from any of these cold RNAs would be indicative of the presence of protein-binding regions within the EAV RNA. The results indicated that only nt 140–206 did not compete with the [^{32}P]-labeled RNA transcript (Fig. 2), and, therefore, appeared to be not necessary for binding to cell proteins. In contrast, competition was obtained with cold RNAs derived from all other plasmid mutants. These results argue for the presence of multiple protein binding sites within the EAV leader genomic RNA, a characteristic already observed in other (+) strand RNA viruses [8,11]. Interestingly, the binding regions identified here in the (+) strand of the EAV leader RNA contain several RNA secondary structures [6,12]. These structures are recognized to play important roles in the replication cycle of several (+) strand RNA viruses [4,5,13]. Nevertheless, it is noteworthy that the RNA stem-loop structure containing the leader TRS element [12], and for which there is a proven significance in EAV RNA synthesis, was not covered by the RNA probes used in our study.

A large number of cellular factors are believed to participate in the replication of DNA and RNA viruses in their host cells [3,4]. For instance, several cellular factors were reported to bind both the 5′-end (+) and 3′-end (−) strands of MHV [8,14]. In fact, PTB binds to the (+) strand of the leader RNA of MHV, where it serves as a regulator of viral transcription (15). Here, several cellular factors, the nature of which has yet to be determined, bound to both the (+) and (−) strands of the EAV leader RNA. Although cell proteins were previously described to bind to the (−) strand of the EAV leader RNA [5], and presumably would have a role in the synthesis of (+) RNA transcripts from (−) RNA templates [2,16], it is the first demonstration of cell protein interactions with the (+) strand of the EAV leader RNA. Although the significance of the protein-leader RNA interactions has yet to be determined, the importance of the EAV nonstructural protein 1 (nsp1) for the synthesis of viral RNAs [17], and the recently reported interaction of nsp1 with the cellular transcription factor p100 [18] argue for the involvement of cellular factors in the synthesis of viral RNAs in EAV. Moreover, cell protein binding with the (+) strand of the EAV leader RNA might be important in protein translation from the viral mRNAs [19]. Further experiments are needed to identify and elucidate the role of the cell factors described here in the biogenesis of EAV.

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