Termination and read-through proteins encoded by genome segment 9 of Colorado tick fever virus

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Genome segment 9 (Seg-9) of Colorado tick fever virus (CTFV) is 1884 bp long and contains a large open reading frame (ORF; 1845 nt in length overall), although a single in-frame stop codon (at nt 1052–1054) reduces the ORF coding capacity by approximately 40%. However, analyses of highly conserved RNA sequences in the vicinity of the stop codon indicate that it belongs to a class of ‘leaky terminators’. The third nucleotide positions in codons situated both before and after the stop codon, shows the highest variability, suggesting that both regions are translated during virus replication. This also suggests that the stop signal is functionally leaky, allowing read-through translation to occur. Indeed, both the truncated ‘termination’ protein and the full-length ‘read-through’ protein (VP9 and VP9*, respectively) were detected in CTFV-infected cells, in cells transfected with a plasmid expressing only Seg-9 protein products, and in the in vitro translation products from undenatured Seg-9 ssRNA. The ratios of full-length and truncated proteins generated suggest that read-through may be down-regulated by other viral proteins. Western blot analysis of infected cells and purified CTFV showed that VP9 is a structural component of the virion, while VP9* is a non-structural protein.

INTRODUCTION
Colorado tick fever virus (CTFV) is the prototype species of the genus Coltivirus, within the family Reoviridae (Mertens et al., 2000; Mertens, 2004). CTFV is the aetiologic agent of Colorado tick fever (CTF), a human arboviral disease that is widespread in the Rocky Mountain region of North America. Eyach virus (EYAV), a distinct species of Coltivirus, has been isolated in Europe (Rehse-Küpper et al., 1976; Chastel et al., 1984). The distribution of CTF matches that of its major vector, the tick Dermacentor andersoni; although, other Dermacentor species, Ixodes species, Haemaphysalis leporispalustris and Otobius lagophilus have also been reported to be involved in the virus cycle (Emmons, 1988).

The genomes of CTFV and EYAV are ~29,000 bp long and consist of 12 segments of dsRNA (Seg-1 to Seg-12) numbered in order of their decreasing size and increasing migration rate during agarose gel electrophoresis (Attoui et al., 2002b). Earlier studies have reported 55 to 86% aa identity between homologous CTFV and EYAV proteins, suggesting that these viruses have diverged from a common ancestor approximately 50 million years ago (Attoui et al., 2002b).

Each CTFV genome segment contains a unique open reading frame (ORF), which spans most of its length. The only exception is Seg-9 (1884 nt), which has a relatively short ORF (1014 nt) ending in an opal stop codon (UGA) at nt 1052–1054, with an 830 nt 3’ non-coding region (NCR). Sequence analysis of Seg-9 from EYAV also revealed a ‘short’ upstream ORF and a long 3’ NCR, with lengths identical to those of CTFV (Attoui et al., 1998, 2002b). These observations, together with comparisons to the genes of other viruses, suggest that ‘read-through’ may occur during the translation of CTFV and EYAV Seg-9.

We report the detection of both the full-length (VP9*) and the truncated translation product (VP9) from CTFV Seg-9, in CTFV-infected cells, in cells transfected with a plasmid expressing Seg-9 protein products, and after in vitro translation of undenatured Seg-9 ssRNA in rabbit reticulocyte lysates. To date, the coltiviruses are the only members of the family Reoviridae that have been shown to use a read-through mechanism during protein translation.

METHODS

Sequence analysis. The three codons upstream and downstream of the suspected leaky stop codon in Seg-9 of CTFV were manually aligned with those of other viruses in which read-through has been described. These include tobamoviruses, benyviruses, pomo-viruses, furoviruses, pecluviruses, tobraviruses, alphaviruses, arenaviruses, machlomoviruses, enamoviruses, carmoviruses, necroviruses, etc.
pancoviruses, retroviruses, luteoviruses, poleroviruses, clusterviruses, alloviruses, leviruses and cricket paralysis virus (family Dicistroviridae, genus Cripavirus).

Secondary structure of Seg-9 of CTFV was predicted using the MFOLD program (www.bioinfo.rpi.edu/applications/mfold/).

Sequences of Seg-9 of CTFV-FI (accession no. AF000720), CTFV-69V28 (AF007182), CTFV-R1575 (AF007183), CTFV-S6-14-03 (AF007184) and EYAV-Gr (AF282475) were aligned using CLUSTAL W program (Thompson et al., 1994). Nucleic acid identity of 221 nt sequence spanning the stop codon was calculated using the p-distance algorithm implemented in the MEGA2 package (Kumar et al., 2001).

The amino acid sequences of Seg-9 of CTFV and EYAV were compared with protein sequences in protein databases using the protein–protein BLAST program (www.ncbi.nlm.nih.gov/blast).

Cloning of CTFV Seg-9 sequence (nt 41–1054) into pGEX-4T-2. Seg-9 of CTFV was previously cloned as a full-length product (Attoui et al., 2002b). A schematic description of the organization of this segment is shown in Fig. 1. The sequence between the ATG and the in-frame stop codon (nt 41–1054) of Seg-9 (encoding the termination protein, VP9) was PCR amplified using virus-specific primers (underlined) tailed with EcoRI and NotI restriction enzyme sites (bold) [VP9-ExpS: 5’-GGGCTGGCAAGCCACGTT-CCGGGAGCTGCATGTGTCAGAGG-3’ (position 41–62); cleavable by EcoRI; VP9-ExpR: 5’-GTCAGTCTGACATGCTGCCCGCTTA-TCAACACGACCCCTTTGT-3’ (position 1054–1034) cleavable by NotI], to facilitate cloning into pGEXT-4T-2 vector (Amersham Biosciences). The fusion protein was recovered from the bacterial lysate were recovered using the Bugbuster protein purification reagent (Novagen). The soluble fraction from glutathione elution and the solubilized protein from inclusion bodies were subjected separately to SDS-PAGE.

Following electrophoresis, the soluble fraction of the fusion protein was electro-blotted onto nitrocellulose membrane, as described earlier (Mohd Jaafar et al., 2003). A 1 : 500 dilution of anti-CTFV mouse immune ascitic fluid (obtained from the American type culture collection) was used as primary antibody to assay the immunoreactivity of the expressed protein.

Seven-week old female BALB/c mice were injected intraperitoneally with 50 μg soluble fraction GST–VP9 protein in the adjuvant Montanide ISA50V (Seppic) on two occasions with a 3-week interval. Three weeks after the last injection, an aliquot of blood was recovered from the caudal vein and tested by Western blot for the presence of anti-VP9 antibodies, using CTFV-infected Vero cells recovered at 5 days post-infection (p.i.), as a target. Pristane was injected for the induction of ascitic fluid, which was recovered 2 weeks later.

Cell cultures, virus propagation and purification. Vero, BHK-21 and L-929 cells were grown as monolayers in Eagle’s minimum essential medium (EMEM) supplemented with 10% fetal bovine serum, penicillin (100 U ml⁻¹) and streptomycin (100 μg ml⁻¹) at 37 °C under 5 % CO₂.

The CTFV-FI strain was obtained from the American Type Culture Collection (reference N-7180). CTFV was propagated in Vero cells and the supernatant was recovered and titrated as described earlier (Karabatsos et al., 1987). Vero cells were infected with 5 p.f.u. per cell in 225 cm² flasks and incubated for 96 h. Infected cells from 20 culture flasks were recovered and pelleted by centrifugation at 2000 g, and lysed with 10 ml 18 M M₂ water, followed by addition of Tween 20 to a final concentration of 0.2 %. The resulting lystate was homogenized in a Potter homogenizer for 15 passages and mixed vigorously with an equal volume of Vertrel XF (Dupont), which is an ozone friendly solvent (substituting Freon 113). Following centrifugation at 10000 g, the resulting supernatant was recovered and treated again with Vertrel XF, followed by another round of centrifugation at 10000 g. The aqueous phase was layered on top of a discontinuous gradient (adapted from Mertens et al., 1987) in 0.2 M Tris/HCl pH 8 made up of 1.5 ml 55 % CaCl₂, 3.5 ml 30 % CaCl₁ and 1 ml 30 % (w/v) sucrose, followed by centrifugation using an SW41 rotor at 220000 g at 10 °C for 2 h. The virus material was recovered from the interface between the two CaCl₂ layers, diluted in 0.2 M Tris/HCl buffer pH 8, and centrifuged at 100000 g for 1 h. The virus pellet was suspended in 100 μl of 10 mM Tris/HCl pH 8, and processed for negative staining electron microscopy, using uranyl acetate.

Detection of Seg-9 translation products in infected cells or in purified virus by Western blot and immunofluorescence. Vero, BHK-21 and L-929 cells in 75 cm² flasks were infected with CTFV, as described above. Cells were harvested at 18 and 96 h p.i. and pelleted, then dissolved at 100 °C for 10 min in 100 μl protein denaturation buffer (160 mM Tris/HCl pH 6.8, 4 mM EDTA, 3-6 % SDS, 60 mM DTT, 0-2 % β-mercaptoethanol, 0-8 % methionine). Virus suspensions, purified by CaCl₂ gradient centrifugation, were dissolved in the same buffer.

![Fig. 1. Schematic representation of the organization of CTFV Seg-9. The ORFs of Seg-9: nt 1–40, 5’ non-coding region; nt 1847–1884, 3’ non-coding region; nt 41–1054, ORF encoding the termination protein designated VP9 (38 kDa); nt 41–1846, ORF encoding the read-through protein designated VP9’ (65 kDa) and the leaky stop codon is boxed.](image-url)
Twenty microtites of each protein solution (infected cells or purified virus) were loaded in polyacrylamide minigels and run in a Bio-Rad Miniprotein II (Bio-Rad). Proteins in the gel were electroblotted onto nitrocellulose membranes. Membranes were blocked in 5% skimmed milk in PBS for 1 h and incubated overnight with anti-VP9 antibodies diluted at 1:500 in 5% milk in PBS. Membranes were washed with TBST (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20) and incubated with horseradish peroxidase conjugated anti-mouse antibody at 1:500 (Jackson Immunoresearch) in 5% milk in PBS. Peroxidase activity was detected using 4-chloro-1-naphthol substrate according to manufacturer’s instructions (Sigma).

Vero cells were grown in flat bottom culture tubes containing 12 mm glass coverslips (Corning) and infected as described above. At 48 h p.i., the cells were fixed in cold methanol (–20°C).

Fixed cells were washed six times with PBS and then incubated at 37°C for 1 h with a 1:500 dilution in PBS of anti-CTFV ascitic fluid to detect intracellular CTFV proteins. Another set of infected cells were incubated with anti-recombinant VP9 ascitic fluid.

Cells were washed with PBS followed by incubation at 37°C for 1 h with a fluorescein isothiocyanate-conjugated anti-mouse antibody and processed for indirect immunofluorescence.

Cloning of Seg-9 in pcDNA vector, transfection of L-929 cells and identification of the translation products. The longest ORF of Seg-9 between nt 41 and 1846 (encoding putative read-through protein, VP9* (positions 41–68) and Exp9pcdr 5'-AACCATGTGGATATCTGCAGCCTAACC-3' (positions 1840–1813). The amplicon was gel purified using GeneClean kit and ligated into the pcDNA vector. pcDNA-VP9 recombinant vector was transfected into XL-Blue bacteria (Stratagene). Recombinant pcDNA-VP9 vector was purified and 1 μg was transfected by electroporation into L-929 cells, using an Easyjet-plus electroporator and the Optimix eukaryotic cell electroporation kit (Equibio), as directed by the manufacturer. Following electroporation, cells were transfected into 6-well plates and incubated with EMEM supplemented with 10% fetal bovine serum. Geneticin (450 μg ml–1) was added to the cells after 24 h. Following 90 min post-transfection, the cells were recovered and lysed in protein denaturation buffer. The lysate was run on a 10% polyacrylamide gel, and transferred onto a nitrocellulose membrane. The presence of VP9 was detected by Western blot using anti-VP9 antibodies as described above.

Translation of CTFV Seg-9 dsRNA and mRNA in vitro. dsRNA was extracted from purified virus using a guanidinium isothiocyanate reagent (one component RNA NOW; Biogentex) as recommended by the manufacturer. Individual segments of dsRNA were fractionated by electrophoresis on a 10% polyacrylamide slab gel as described earlier (Attoui et al., 1998). The polyacrylamide gel was stained with ethidium bromide and Seg-9 RNA band was excised, chopped and incubated overnight in 2 ml TEN buffer (10 mM Tris/HCl pH 8, 1 mM EDTA, 100 mM NaCl) with gentle agitation. RNA was precipitated from TEN by adding 10 volumes of butanol, washed successively in 100% and 75% ethanol and air dried. The pellet was dissolved in 100 μl RNase-free water and the dsRNA was purified using RNAid kit (BIO 101).

dsRNA solution was lyophilized, and dried RNA was denatured in DMSO at a concentration of 2 μg ml–1 and heated at 50°C for 15 min as described by McCrae & Joklik (1978). The denatured RNA was translated using a rabbit reticulocyte lysate (RRL; Promega).

pcDNA-VP9 vector was linearized with Pmel enzyme and used for in vitro transcription using the MEGAscript T7 RNA polymerase kit as recommended by the manufacturer (Ambion). Vector DNA was removed by treatment with 125 U DNase I (Invitrogen) for 30 min at 37°C and the ssRNA purified using the MEGAgclear RNA purification kit (Ambion). RNA concentration was adjusted to 1 μg ml–1 and used for the translation in RRL.

The translation reaction volume was 50 μl and it contained 1 μl of either DMSO-denatured Seg-9 dsRNA (2 μg) or the in vitro transcribed ssRNA (1 μg) of Seg-9, 1 μl of amino acid mixture lacking methionine as supplied by the manufacturer, 2 μl [35S]methionine [final 20 μCi (740 kBq)] and 35 μl RRL. The reaction was carried out at 30°C for 90 min. Following addition of an equal volume of denaturation buffer, 20 μl of the translation product was heated to 96°C for 5 min and analysed on polyacrylamide minigel. The gel was dried and autoradiographed.

RESULTS

Sequence analysis

The CTFV and EYAV Seg-9 stop codon, at nt 1052–1054, is flanked by a downstream ‘CGG’ codon (encoding arginine). The ‘leaky’ stop codons of the tobravirus, pleuivirus, furovirus and pomovirus (Beier & Grimm, 2001) have the same configuration (Table 1). Similarly, the alphavirus leaky stop codon is ‘UGA’, flanked by a downstream cytosine that is essential for read-through (Strauss & Strauss, 1994); the same configuration is found in Seg-9 of CTFV and EYAV.

Sequence analysis showed that Seg-9 sequences from four CTFV strains (CTFV-FI, CTFV-69V28, CTFV-R1575 and CTFV-86-14-03) are 99-9% identical to each other and 75% identical to Seg-9 of EYAV (with 83% aa identity between the deduced CTFV and EYAV protein sequences). The UGA stop codon at position 1052–1054 and the downstream CGG codon were present in each of these virus strains. A database search for other proteins with similarities to the read-through translation product VP9’ of CTFV identified eukaryotic ‘transcription initiation factor IIB’ (TFIIB), showing 21% identity and 43% similarity with a 105 aa sequence (aa 39–144) in VP9’. TFIIB plays a major role in the activation of transcription of eukaryotic genes.

The predicted secondary structure of CTFV Seg-9 mRNA was analysed using the MFOLD program. Thirty-six stable secondary structures were generated, the most stable of which is shown in Fig. 2. A hairpin structure, downstream of the leaky stop codon, is present in all 36 possible structures, suggesting that it might play a role in translational read-through.

Protein expression and detection of translation products of Seg-9 in CTFV-infected cells or in purified virus by Western blot and immunofluorescence

Most of the expressed GST–VP9 fusion protein was present as inclusion bodies and only a fraction was soluble in the bacteria (Fig. 3). The inclusion bodies were solubilized in 0-3% Sarkosyl, which denatured the GST active sites, hence the fusion protein failed to bind to glutathione resin.
Table 1. The virus genera, including Coltivirus, in which read-through occurs and which have a CGG codon following their leaky stop codon

Conserved codons downstream of the stop codon are bold.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>−3</th>
<th>−2</th>
<th>−1</th>
<th>Stop</th>
<th>+1</th>
<th>+2</th>
<th>+3</th>
</tr>
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<tr>
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<td>Colorado tick fever virus</td>
<td>GGC</td>
<td>UGC</td>
<td>UGU</td>
<td>UGA</td>
<td>CGG</td>
<td>UGU</td>
<td>UGG</td>
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<tr>
<td></td>
<td>Eyach virus</td>
<td>GGU</td>
<td>UGU</td>
<td>UGU</td>
<td>UGA</td>
<td>CGG</td>
<td>UGU</td>
<td>UGG</td>
</tr>
<tr>
<td>Furovirus</td>
<td>Chinese wheat mosaic virus</td>
<td>UUC</td>
<td>GAC</td>
<td>AAA</td>
<td>UGA</td>
<td>CGG</td>
<td>UGU</td>
<td>GGG</td>
</tr>
<tr>
<td></td>
<td>Soil-borne wheat mosaic virus</td>
<td>CUU</td>
<td>ACU</td>
<td>AAA</td>
<td>UGA</td>
<td>CGG</td>
<td>UUU</td>
<td>CGG</td>
</tr>
<tr>
<td>Pecluvirus</td>
<td>Peanut clump virus</td>
<td>CAG</td>
<td>ACC</td>
<td>AAA</td>
<td>UGA</td>
<td>CGG</td>
<td>UUU</td>
<td>GGG</td>
</tr>
<tr>
<td>Pomovirus</td>
<td>Beet soil-borne virus</td>
<td>AGU</td>
<td>ACU</td>
<td>AAA</td>
<td>UAA</td>
<td>CGG</td>
<td>UGU</td>
<td>GGG</td>
</tr>
<tr>
<td></td>
<td>Beet virus Q</td>
<td>UCU</td>
<td>GUU</td>
<td>CAA</td>
<td>UAA</td>
<td>CGG</td>
<td>UGU</td>
<td>GGG</td>
</tr>
<tr>
<td></td>
<td>Broad bean necrosis virus</td>
<td>GGU</td>
<td>CCU</td>
<td>AAA</td>
<td>UGA</td>
<td>CGG</td>
<td>UGU</td>
<td>GGG</td>
</tr>
<tr>
<td>Tobravirus</td>
<td>Pea early browning virus</td>
<td>GCU</td>
<td>AUG</td>
<td>AAA</td>
<td>UGA</td>
<td>CGG</td>
<td>UGU</td>
<td>GGG</td>
</tr>
<tr>
<td></td>
<td>Pepper ringspot virus</td>
<td>GCU</td>
<td>GCC</td>
<td>UUA</td>
<td>UGA</td>
<td>CGG</td>
<td>UGU</td>
<td>CGG</td>
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<td></td>
<td>Tobacco Rattle virus</td>
<td>ACC</td>
<td>GUC</td>
<td>UUA</td>
<td>UGA</td>
<td>CGG</td>
<td>UUU</td>
<td>CGG</td>
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</tbody>
</table>

However, the naturally soluble fraction of the fusion protein was purified by glutathione affinity chromatography. Both solubilized fusion protein from inclusion bodies and naturally soluble fusion protein were analysed by SDS-PAGE (Fig. 3). The expressed GST–VP9 reacted with anti-CTFV antibodies in a Western blot assay (data not shown).

Western blot analyses of infected cells were performed using the anti-GST–VP9 antibodies. Major and minor protein bands, at 36 and 65 kDa, respectively, were detected in Vero, BHK-21 and L-929 cells infected with CTFV (Fig. 4a, b, c). We consider it likely that these proteins represent VP9 and VP9′, which were generated by translation termination or read-through of the in-frame stop codon in Seg-9, respectively. No bands were detected in lysates from uninfected cells (Fig. 4e).

Although both bands (VP9 and VP9′) were observed early in infection in Vero, BHK-21 and L-929 cells (up to 18 h p.i.), the larger protein band (65 kDa, VP9′) was not detected late-on (at 96 h p.i.) after extensive CPE (cytopathic effect) had occurred, which starts at ~30 h p.i.

Fig. 2. Secondary structure of the ssRNA of Seg-9 as predicted by MFOLD program. (a) The most stable structure (dG = −625 kcal mol⁻¹) predicted by MFOLD program. Circled region representing the suggested hairpin structure downstream of the UGA stop (position 1052–1054) is shown in detail in (b). NNNNNNNNNN, refers to stem–loop structures, which are not described.
The negative staining of virus particles purified by a CsCl density-gradient centrifugation showed a mixture of intact virus particles and core-like particles (Fig. 5). A 36 kDa protein band was detected in Western blots of purified CTFV virus particles, demonstrating that VP9 is a structural component of the virion. However, the full-length VP9 generated by read-through was not detected in these assays, indicating that it is not a virion structural protein (Fig. 4f).

Indirect immuno-fluorescence analysis using anti-CTFV and anti-GST–VP9 antibodies showed that the translation products of Seg-9 are abundant in CTFV-infected Vero cells and are located in the cytoplasm (Fig. 6). However, visible fluorescence was also detected in the nucleolus.

**Seg-9 translation products in L-929 cells transfected with pcDNA-VP9**

L-929 cells were electroporated with the pcDNA-VP9 vector (containing the full-length Seg-9 ORF) and selected with geneticin, generating over 100 recombinant cell clones. Western blot analysis of cell lysates from the pooled clones (using anti-VP9 antibodies) detected translation products at both 36 (VP9) and 65 kDa (VP9'). No bands were detected in lysates of untransfected cells (Fig. 4e). Although VP9' is only a minor translation product in CTFV-infected Vero, BHK-21 and L-929 cells (see above), the 36 and 65 kDa protein bands were present in similar amounts in

**Fig. 3.** SDS-PAGE of GST–VP9 fusion protein from bacteria. Coomassie stained gel showing the GST–VP9 from the solubilized inclusion bodies (IB) and from glutathione-affinity purified soluble fraction (SF). The purity of GST–VP9 from the soluble fraction is higher than the GST–VP9 from inclusion bodies.

**Fig. 4.** Western blot analysis of CTFV-infected cells, cells expressing cloned Seg-9 under CMV promoter control and purified virus. (a) CTFV-infected Vero cells (E, cells recovered early at 18 h p.i.; L, cells recovered late at 96 h p.i.), (b) CTFV-infected BHK-21 cells, (c) CTFV-infected L-929 cells, (d) L-929 cells transfected with pcDNA-VP9, (e) negative control of Western blot showing non-infected/transfected Vero, BHK-21 and L929 cells tested with anti-recombinant VP9 antibodies, (f) proteins of virus purified by CsCl gradient centrifugation, tested with anti-recombinant VP9 antibodies and showing a single reactivity (F1 has a higher virus protein content than F2).
pcDNA-VP9 transfected L-929 cells (Fig. 4d). This suggests that other CTFV-specific products generated during virus infection and replication, can down-regulate read-through (this might explain the absence of VP9 late in CTFV infection).

Translation of CTFV Seg-9 dsRNA and mRNA in vitro

Purified Seg-9 dsRNA from CTFV (denatured with DMSO) and Seg-9 ssRNA (transcribed in vitro from linearized pcDNA-VP9) were translated in vitro in RRL. The VP9 ‘termination’ protein (36 kDa) was detected in both cases by autoradiography (Fig. 7a, b). However, the VP9 ‘read-through’ protein (65 kDa) was only detected (as a faint band) in translation products from the in vitro transcribed ssRNA (Fig. 7b, c).

DISCUSSION

The genome segments of members of the family Reoviridae are predominantly mono-cistronic, with unique large ORFs that span most of each mRNA (Mertens et al., 2000). However, individual reoviruses (like the members of other virus families) have also developed a variety of strategies to efficiently organize and maximize the RNA sequences, which can be used to express different viral proteins within the limited size of the viral genome. Several mechanisms exist that result in the translation of more than one protein product from a single genome segment. These include multiple (in-phase) translation start codons, as seen in segment M3 of mammalian orthoreovirus (MRV) (encoding μNS and μNSC products) (Mertens et al., 2000), and segment 10 of bluetongue virus (encoding NS3 and NS3a proteins) (French et al., 1989).

Alternatively, some genome segments encode up to three different translation products from overlapping but out of phase ORFs. Examples include segment S1 of MRV, which encodes sigma-1 and sigma-1NS, while segment S1 in Nelson bay reovirus and Avian reovirus encode P10, P17 and sigma-C. Non-overlapping ORFs also exist in segment 7 of aquareovirus C, which encodes NS16 and NS31 from two distinct non-overlapping ORFs (Attoui et al., 2002a), and in a number of different segments of the fijiviruses (plant reoviruses). Coding information concerning the genome segments and proteins of the dsRNA viruses is summarized at www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/ (Mertens, 2004).

Other translational strategies that allow RNA viruses to expand the genetic information of their relatively small genomes include negative-strand ORFs, translational frameshift and suppression of non-sense codons or ‘read-through’. However, these last three strategies have never previously been reported for the reoviruses. Translational frameshift and read-through are processes that can circumvent stop codons, thus providing a regulatory mechanism for gene expression by allowing the differential production of more than one polypeptide from a single gene. In mammalian cells, read-through results in the synthesis of proteins and viruses containing selenocysteine usually in C-terminal extended regulatory or structural proteins (Chernoff et al., 1996; Beier & Grimm, 2001).

The termination of peptide synthesis during translation of eukaryotic mRNAs is predominately directed by eukaryotic peptide-chain release factors (eRFs), in response to the termination codons UAA, UAG and UGA (Inge-Vechtomov et al., 2003). This results in the binding of eRF1 to the ribosome (in presence of eRF3), and triggers peptide-chain release. The suppression or read-through of UGA stop codons has been reported to be more efficient than UAG and UAA. Suppression of UGA results in the incorporation of arginine, cysteine or tryptophan by ribosomes, while...
Read-through in segment 9 of Colorado tick fever virus

Fig. 7. Translation product of Seg-9 (dsRNA and ssRNA) of CTFV in rabbit reticulocyte lysate. (a) In vitro translation products from DMSO-denatured CTFV Seg-9 dsRNA, showing one protein band at approximately 36 kDa. (b) Translation products from Seg-9 undenatured ssRNA (prepared by in vitro transcription using T7 RNA polymerase) showing a band at 36 kDa and another fainter band at approximately 65 kDa, (c) negative print of image (b). M, Size marker labelled in kDa.

suppression of UAA and UAG results in the incorporation of glutamine (Beier & Grimm, 2001).

Read-through stop codons were first described in the RNA of enterobacteria phage Qβ (family Leviridae, genus Allolevivirus) by Hofstetter and colleagues (1974). Subsequently, read-through has been described in a number of animal, plant and bacterial virus families (as well as in eukaryotic cells) including Leviridae, Luteoviridae, Retroviridae, Tombusviridae and Dicistroviridae (in particular cricket paralysis virus belonging to genus Cripavirus) (Chernoff et al., 1996; Beier & Grimm, 2001; Harrell et al., 2002). Read-through was shown to depend on the presence of natural suppressor tRNAs and codon context effects, including both the primary nucleotide sequence and the secondary structure of the RNA in the vicinity of the leaky stop codon (Alam et al., 1999; Beier & Grimm, 2001).

The coding region of Seg-9 of CTFV was shown to contain a large open reading frame (nt 41–1846), which includes an in-frame stop codon at position 1052–1054 (Attoui et al., 1998; 2002b). Several factors indicate that effective read-through of this stop codon can occur during translation of Seg-9 mRNA. Sequence analysis showed that the stop codon is followed by a CGG codon (encoding arginine), a configuration similar to that of leaky stop codons previously reported for the tobaviruses, pecluviruses, furoviruses and pomoviruses (Table 1). A cytosine residue is also found after the alphavirus leaky stop codon (Strauss & Strauss, 1994).

Comparison of Seg-9 from different CTFV strains (CTFV-Fi, CTFV-69V28, CTFV-R1575 and CTFV-S6-14-03) has shown that the sequence around the leaky stop codon is conserved.

In addition comparisons between Seg-9 from CTFV and EYAV showed an identical in-frame stop codon. Sequence variation detected in Seg-9 is mainly a function of the third base position in codons situated both before and after the stop codon (Attoui et al., 2002b). This indicates that the proteins encoded by both regions of Seg-9 are subject to functional constraints and consequently to genetic selective pressure. This is possible if both regions are translated into functionally significant proteins during virus replication.

Mouse hyper-immune ascitic fluid (prepared against the GST–VP9 protein expressed in bacteria) was used to identify both read-through (VP9′) and termination proteins (VP9) in Vero, BHK-21 and L-929 cells infected with CTFV, which were harvested before extensive CPE had occurred, although the ratio of VP9′ to VP9 was <1. This conclusively demonstrates the occurrence of read-through during translation of Seg-9 mRNAs in naturally infected cells. The ascitic fluid also identified VP9, but not the longer read-through protein, VP9′, in purified CTFV virions, demonstrating that VP9 is a structural component of the virus particle, while indicating that VP9′ is ‘non-structural’. However, VP9′ was synthesized in much larger relative amounts in L-929 cells transfected with pcDNA containing a cloned full-length copy of Seg-9 (VP9′ to VP9 ratio of ≥1). These observations suggest that the frequency of read-through may be down-regulated in the presence of the CTFV proteins, possibly leading to the reduced synthesis of VP9′ observed during the later stages of the virus replication cycle. The relatively efficient read-through observed in the transfected cells suggests that VP9 and VP9′ are not themselves responsible for this regulation.

VP9 (but not VP9′) was detected in the in vitro translation products generated in a RRL from DMSO-denatured CTFV Seg-9 dsRNA. However, translation of undenatured Seg-9 ssRNA (transcribed in vitro) generated both VP9 and a faint product corresponding to VP9′ (at 65 kDa), suggesting that read-through is dependent on features of the Seg-9 ssRNA secondary structure that are lost during denaturation in DMSO. Secondary structure has previously been reported to be essential for regulation of read-through during the translation of mRNAs from some other viruses (Wills et al., 1991). In particular, the predicted stable hairpin structure, situated downstream of the CTFV Seg-9 UGA stop codon (Fig. 2), might represent a functional signal for read-through.

The significance of the similarities detected between the amino acid sequence encoded by CTFV Seg-9 (beyond the stop codon) and TFIIB, which plays a major role in the activation of the transcription of eukaryotic genes, is unclear. However, the presence of fluorescence (caused by binding of VP9-specific antibodies) in the nucleolus of infected cells suggests that the translation products of Seg-9 transit to the nucleus and might play a role in the regulation of host cell transcription.

Finally, the significance of the read-through phenomenon...
and its mechanistic involvement in replication of CTFV is still to be defined in future work. The occurrence of Seg-9 read-through during coltivirus replication remains (to date) the only documented case of this mechanism that involves a member of the family Reoviridae.

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