Transcriptional regulation of the channel catfish virus genome direct repeat region

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Channel catfish virus (CCV), a member of the herpesvirus family, causes a severe haemorrhagic disease in juvenile channel catfish. In this report, we confirm that CCV gene expression is temporally regulated into immediate-early (IE), early and late phases, similar to that of other herpesviruses. The transcriptional regulation of the 14 genes within the direct repeat region of the CCV genome was determined by Northern hybridization analysis of RNA isolated from infected cells in the presence or absence of metabolic inhibitors. Two CCV genes within the direct repeat, ORFs 1 and 3, expressed IE transcripts. Early RNAs were encoded by ORFs 2–9 and 11–14. ORFs 4, 7 and 10–13 expressed late transcripts after the onset of viral DNA replication. A time-course study conducted without metabolic inhibitors confirmed that CCV direct repeat transcription is temporally regulated. The characterization of CCV transcription during cytolysis in vitro will provide a foundation for the analysis of CCV gene expression in tissues of acutely and latently infected catfish.

Introduction

Channel catfish virus (CCV) is a herpesvirus that causes a severe haemorrhagic disease in young channel catfish (*Ictalurus punctatus*) (Buck, 1990; Plumb, 1977). Fish with the disease swim erratically, eventually becoming lethargic, and often hang vertically in the water with their heads near the surface. Infected fish exhibit exophthalmia, haemorrhagic lesions at the base of fins, and haemorrhage and necrosis in the viscera, especially the liver and kidney. Most juvenile channel catfish die within 3 to 10 days of infection with CCV. Fish that survive the disease become latently infected (Gray et al., 1999), a hallmark of herpesvirus infection.

Little is known about the molecular biology of CCV or the virus replication cycle. The CCV genome (134–2 kb) contains two identical direct repeat regions (18–5 kb each) that flank the 97–1 kb unique region (Fig. 1) (Davison, 1992). Each direct repeat contains 14 open reading frames (ORFs), while the unique region contains 65 ORFs, for a total of 79 distinct ORFs in the genome (Davison, 1992). Although CCV is not closely related to any other known herpesvirus, some CCV genes share limited homology with salmonid herpesvirus genes (Davison, 1998).

Like other herpesviruses, CCV gene expression is divided into temporally regulated immediate-early (IE), early and late phases (Dixon & Farber, 1980; Silverstein et al., 1995, 1998; Huang & Hanson, 1998). Herpesvirus IE genes are transcribed immediately upon infection and usually encode viral transactivators that regulate the transcription of other viral genes (Everett, 1987). The early genes encode proteins that are generally involved in viral DNA synthesis, such as the DNA polymerase and thymidine kinase. Late genes are expressed after viral DNA synthesis begins and typically encode structural proteins, such as viral envelope glycoproteins.

An understanding of CCV gene expression is fundamental to determine the role of CCV genes in virus replication, pathogenesis and latency. The repeat regions of herpesvirus genomes often contain genes that encode regulatory proteins as well as gene products which are important in virus pathogenesis, including latency associated transcripts (Baxi et al., 1995; Mitchell et al., 1990; Priola & Stevens, 1991). In this study, we have identified the transcripts encoded by the CCV direct repeat region(s) and characterized the transcriptional regulation of the CCV direct repeat genes.
**Methods**

**Cell culture and virus propagation.** Channel catfish ovary cells (CCO) were maintained at 27 °C in Eagle’s minimum essential medium (EMEM) supplemented with penicillin (5000 U/ml), streptomycin (5000 U/ml) and 5% foetal bovine serum (FBS). CCV (Auburn-1 strain) was propagated in CCO cells. To generate CCV stocks, confluent monolayers were infected at an m.o.i. of 0-02 p.f.u. of CCV per cell for approximately 18–20 h. Infected cell pellets were resuspended in media and subjected to three freeze-thaw cycles. Cell debris was pelleted and the CCV supernatant was stored at −70 °C. Virus titre was determined by plaque assay on CCO cells.

**Protein synthesis inhibition.** CCO monolayers were incubated in methionine deficient media with or without cycloheximide (CH; 100 µg/ml) for 1 h. Cellular proteins were then labelled in vivo with 10 µCi/ml [35S]methionine. At the same time-point, cells were infected at an m.o.i. of 4 p.f.u. per cell (or mock-infected). At 1, 1.5, 3, and 6 h post-infection (p.i.), cells were harvested and washed in PBS. Cells were pelleted, resuspended in solubilization buffer (25 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% deoxycholate) and stored at −70 °C. Samples were sonicated on ice and cellular debris was pelleted. Supernatants were precipitated on ice with BSA and trichloroacetic acid (TCA) for 15 min. The precipitates were collected on Whatman GF/C glass-fibre discs, washed with ice cold 10% TCA and then with ethanol. The filters were dried and radioactivity was measured in a liquid scintillation counter. The radioactivity (c.p.m.) of samples that were not treated with CH represented 100% protein synthesis under each set of conditions.

**CCV DNA synthesis inhibition.** CCO cells were infected with 4 p.f.u. CCV per cell or mock-infected (in the presence or absence) of cytosine β-d-arabinofuranoside (Ara-C; 50 or 100 µg/ml). At 1 and 6 h p.i., cells were removed and washed in PBS. Total cell DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega). DNA from each sample (6 µg) was denatured in 0.25 M NaOH, 0.5 M NaCl, diluted in 0.1 x SSC (1 x SSC = 150 mM NaCl, 15 mM sodium citrate), 0.125 M NaOH; and applied to a positively charged nylon membrane (Boehringer Mannheim). The resulting dot blot was neutralized in 0.5 M Tris–HCl (pH 7.4) and cross-linked by exposure to ultraviolet light. Prior to hybridization, the blot was denatured in 0.2 M Tris–HCl (pH 7.5) and incubated in prehybridization solution (10% dextran sulfate, 1% SDS, 1 M NaCl). A digoxigenin (DIG)-labelled CCV double-stranded DNA probe (ORF 59, nucleotide 78703 to 79740 on the CCV genome) was denatured and added directly to the prehybridization solution for hybridization at 65 °C for 16–18 h. Alkaline phosphatase-conjugated anti-DIG antibody and the chemiluminescent substrate disodium 3-(4-methoxyspiro (1,2-dioxetane-3,2′-(5′-chboro)tricyclo[3.3.1]dec-4-yl)phenyl) phosphate (CSPD; Boehringer Mannheim) were used for detection of the probe by exposure to film. The relative signal from each dot was measured densitometrically and used to calculate the relative percentage of DNA synthesis in each sample.

**RNA isolation and Northern hybridization.** CCO cells were infected with 4 p.f.u. of CCV per cell. For CCV IE RNA, the cells were incubated in 100 µg/ml CH for 30 min prior to infection as well as during infection. CCV IE RNA was isolated at 1 and 1.5 h p.i. For CCV early RNA, cells were incubated in 100 µg/ml Ara-C during infection and total RNA was isolated at 6 h p.i. For late CCV RNA, cells were incubated in media without inhibitors and total RNA was isolated at 6 h p.i. RNA was also isolated from CCO cells in the absence of CCV infection (mock-infected). Total cell RNA was isolated from the cells using the RNAzol B reagent (Tel Test I), an adaptation of the guanidinium thiocyanate method (Chomczynski & Sacchi, 1987). RNA was quantified by absorbance measurements and stored in DEPC-treated water at −70 °C until used.

For Northern hybridization analysis RNA (10 µg) samples were treated with dimethyl sulfoxide and deionized glyoxal. Samples (7 µg) were fractionated by electrophoresis in a 1:2% agarose gel containing 10 mM sodium phosphate (pH 7), 10 mM iodoacetic acid and 1µg/ml ethidium bromide. Ribosomal RNA (28S and 18S) was visualized under ultraviolet light to ensure equivalent amounts of RNA in each lane. The RNA was transferred to a positively charged nylon membrane (Boehringer Mannheim). Blots were prehybridized in 10% dextran sulfate, 1% SDS, 1 M NaCl. Portions of CCV genes were amplified by PCR and then labelled with DIG. The CCV-specific double-stranded DNA probes were added directly to the prehybridization solution for hybridization at 65 °C for 16–18 h. CCV transcripts were detected by chemiluminescence and film exposure employing alkaline phosphatase-conjugated anti-DIG antibody and CSPD.
Results

Inhibition of CCV protein and DNA synthesis by metabolic inhibitors

The efficiency of CH and Ara-C in CCV-infected CCO cells was evaluated to ensure that metabolic inhibitors that are commonly used with mammalian cells also perform effectively in fish cells. Total cellular protein was labelled \textit{in vivo} with \textsuperscript{[\textsuperscript{35}S]}methionine. Cell lysates were prepared from CCV-infected (1, 1–5, 3 and 6 h p.i.) and mock-infected CCO cells in the presence and absence of CH (100 \textmu g/ml). Total protein was precipitated with TCA. For each sample set, total protein isolated in the absence of CH represented 100\% protein synthesis. Protein synthesis in the presence of CH was calculated as a percentage of total protein synthesis. In mock-infected CCO cells CH inhibited protein synthesis by 99–3\%. In CCO cells infected with CCV for 1, 1–5, 3 and 6 h, CH inhibited protein synthesis by 97–5, 98–4, 99–9 and 99–9\%, respectively.

The inhibition of viral DNA synthesis by Ara-C was measured in CCV-infected (and mock-infected) CCO cells by DNA hybridization (Fig. 2). A DNA probe specific for CCV ORF 59 was used to detect CCV DNA. No specific signal was detected in mock-infected CCO DNA samples. Only the input level of CCV DNA was detected in total cell DNA isolated at 1 h p.i. from CCV-infected cells in the absence of Ara-C. At 6 h p.i. a 2-2-fold increase in the amount of CCV DNA was detected in the absence of Ara-C, indicating that viral DNA replication had occurred. In the presence of 50 µg/ml Ara-C the synthesis of CCV DNA was inhibited by 50\%, and in the presence of 100 µg/ml Ara-C the synthesis of CCV DNA was inhibited by 90\%.

Since CH (100 µg/ml) efficiently blocked protein synthesis in the CCV-infected CCO cells, this inhibitor was used in subsequent experiments to identify CCV IE RNA. Ara-C (100 µg/ml), which effectively blocked CCV DNA synthesis in CCO cells, was used in experiments to define CCV early RNA.

Transcriptional regulation of CCV genes

Northern analysis of CCV RNA indicated that ORFs 1 and 3 were the only IE genes within the CCV direct repeat region (Fig. 3; Table 1). At both 1 and 1–5 h p.i., transcription of the 3–0 kb ORF 1 transcript (IE 1) and the 1–6 kb ORF 3 transcript (IE 3) was not inhibited by CH. The 3–0 kb IE 1 transcript remained at high levels at 6 h p.i. However, the abundance of the 1–6 kb IE 3 transcript was significantly reduced at 6 h p.i.

Several ORFs in the direct repeat region of the CCV genome were expressed in the early class of CCV transcription (Table 1). These early viral transcripts were not blocked by Ara-C, but were inhibited by CH. In addition to the 1–6 kb IE transcript, ORF 3 was also expressed as a 2–9 kb early transcript (Fig. 3). Representative Northern analyses showing additional early transcripts encoded by ORFs 2, 8 and 12 are shown in Fig. 4. CH decreased or blocked transcription of early RNAs at 1 h p.i. However, by 1–5 h p.i. this inhibition was no longer detectable for some transcripts (Fig. 4, ORF 8). This apparent ‘leakage’ around the CH block has been previously reported (Silverstein \textit{et al.}, 1998).

Late CCV transcripts were expressed after the onset of viral DNA replication, which begins in CCV-infected CCO cells at
### Table 1. Transcriptional regulation of the CCV direct repeat ORFs

<table>
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<tr>
<th>ORF</th>
<th>ORF size</th>
<th>Probe location*</th>
<th>Transcript size</th>
<th>Transcriptional stage</th>
<th>Putative function†</th>
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<td>Protein kinase</td>
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</table>

* Numbers indicate nucleotide locations on the CCV genome (Davison, 1992).
† Putative function of gene products based on sequence data (Davison, 1992).
‡ A 2·3 kb early transcript was seen on some films.

**Fig. 4.** Northern blot hybridization analyses of CCV early and late genes. Total RNA was isolated from CCV-infected and mock-infected CCO cells and analysed as described for Fig. 3. The 2·8 and 2·4 kb late and 1·9 kb early transcripts encoded by ORF 12 are not shown. Upon longer exposure of the film, the 1·4 and 1·0 kb ORF 12 transcripts were detected at 1·5 h.p.i. in the absence of CH, but were not detected in the presence of CH.
approximately 3 h p.i. (Huang & Hanson, 1998). Synthesis of these late viral RNAs was inhibited by Ara-C at 6 h p.i. CCV DNA direct repeat ORFs 4, 7, 10, 11 and 12 were each expressed as late transcripts (Fig. 4; Table 1). The results also indicated that ORF 59, the putative major surface glycoprotein, is a CCV late gene (Fig. 4).

**Time-course of CCV gene transcription.**

The progression of CCV transcription in infected CCO cells was examined by Northern blot hybridization analysis employing specific CCV gene probes (Table 1). Total RNA was isolated from infected cells at hourly intervals from 1 to 6 h p.i. The time-course of transcripts encoded by ORFs 1, 2, 3, 5, 14 and 59 is shown in Fig. 5. The 3–0 kb IE 1 and 1–6 kb IE 3 transcripts were readily detected as early as 1 h p.i. The IE 1 transcript was highly expressed throughout the 6 h infection. The IE 3 transcript was detected at 1 to 4 h p.i., but was more difficult to detect at 5 and 6 h p.i. This result is consistent with a previous finding that the ORF 3 IE transcript has a short half-life (Silverstein *et al.*, 1998). The CCV early transcripts (2–9 kb ORF 2, 2–9 kb ORF 3, 1–4 kb ORF 5 and 1–4 kb ORF 14 transcripts) were detected in relatively high abundance from 2 to 6 h p.i. In general, early transcription peaked around 3 to 4 h p.i. The 1–2 kb ORF 59 late transcript was not readily detected until 4 h p.i., after the onset of viral DNA synthesis.

**Discussion**

This study confirms that CCV transcription is temporally regulated in a manner similar to that of other herpesviruses. CCV IE transcripts encoded by ORFs 1 and 3, early transcripts encoded by ORFs 2–9 and 11–14, and late transcripts encoded by ORFs 4, 7 and 10–13 were identified in experiments employing metabolic inhibitors. The temporal regulation of CCV direct repeat gene expression was further established by a time-course analysis of CCV transcription in infections performed in the absence of metabolic inhibitors.

Metabolic inhibitors that are commonly used in studying the transcriptional regulation of mammalian herpesviruses were effective in CCV-infected CCO cells. Ara-C inhibited 90% of CCV DNA synthesis in these cells and effectively blocked the production of late CCV transcripts. In addition, CH blocked greater than 97% of protein synthesis in these cells for up to 6 h p.i. However, our analysis, as well as that of Silverstein *et al.* (1998), suggests that the CH block ‘leaks’ over time to allow transcription of some CCV genes that are not IE. We defined CCV IE transcripts as those which could be readily detected at 1 h p.i. and were not inhibited by CH.

Only two CCV direct repeat region genes were transcribed in the IE stage. In addition to the 1–6 kb IE 3 transcript described previously (Silverstein *et al.*, 1998), we also identified a 3 kb IE 1 transcript. Computer analysis of ORFs 1 and 3 and their putative peptide sequences using the FastA and TFastA (Pearson & Lipman, 1988) programs found no sequence homology between the two genes, and no significant homology to any known DNA or protein sequences. ORF 1 encodes a protein with a predicted molecular mass of 93–5 kDa and an isoelectric point (pI) of 9.97. The protein encoded by ORF 3 has a predicted molecular mass of 32.3 kDa and a pl of 4.26, and a predicted helix–turn–helix motif, a potential DNA-binding site, near the amino terminus (amino acids 8–27). The potential DNA-binding site within the ORF 3 protein supports the possibility that this protein may be involved in regulation of viral transcription, similar to other herpesvirus IE proteins.

Most of the genes within the CCV direct repeat region were expressed during the early stage of transcription (Table 1). Among these early genes, ORF 5 encodes the CCV thymidine kinase (Hanson & Thune, 1993), and ORF 14 encodes a putative protein kinase. Two of the genes encode putative zinc-binding proteins (ORFs 9 and 12) and three
encode potential membrane proteins (ORFs 6, 7 and 8). The study indicates that ORF 12 encodes early and late transcripts, since they were not detected at 1–0 h p.i. and are inhibited by CH block (Fig. 4). This result conflicts with a previous finding indicating that ORF 12 is an IE gene (Huang & Hanson, 1998). In the latter study, IE RNA was isolated following CH block for 8 h, beyond the time when the ‘leak’ in the CH block appears to begin.

Only one gene in the direct repeat region, ORF 10, was exclusively transcribed during the late stage. In addition to this late gene, ORFs 4, 7, 11 and 12 encoded both late and early transcripts. ORFs 7 and 10 encode putative membrane proteins and may be structural virion peptides. ORFs 11 and 12 are related to ORF 9 and encode putative zinc-binding proteins. The function of the protein encoded by ORF 4 is unknown.

All ORFs within the CCV direct repeat region have at least one potential polyadenylation site near their 3′ ends, or share such a site with a downstream ORF suggesting 3′-coterminal transcription (Fig. 1; Davison, 1992). This study demonstrates that several CCV direct repeat genes encode multiple RNA species some of which are apparently transcribed as 3′-coterminal families. The results confirm that two sets of genes, ORFs 5 and 6 as well as ORFs 8 and 9, encode bicistronic transcripts, as previously reported (Silverstein, 1995, 1998). ORFs 2 and 3 also appear to encode a 2.9-kb bicistronic transcript. Transcription of ORFs 10, 11, 12 and 13 is particularly complex, encoding multiple early and late polycistronic RNAs (Table 1). Further analysis including mapping of the 5′ and 3′ ends of individual transcripts will be required to confirm these polycistronic RNA species.

CCV infection of channel catfish provides a useful natural model in which to study the molecular mechanisms involved in herpesvirus pathogenesis and latency. CCV DNA can be detected in tissues of acutely and latently infected catfish (Gray et al., 1998, 1999). Studies are in progress to characterize CCV gene expression in acutely infected fish and to identify latency associated transcripts in tissues of latently infected fish. The characterization of CCV transcription provides a foundation for the further investigation of the molecular basis of CCV pathogenesis and latency.

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References


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