Negative modulation of the chicken infectious anemia virus promoter by COUP-TF1 and an E box-like element at the transcription start site binding δEF1

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Expression of enhanced green fluorescent protein (EGFP) under control of the promoter-enhancer of chicken infectious anemia virus (CAV) is increased in an oestrogen receptor-enhanced cell line when treated with oestradiol and the promoter-enhancer binds unidentified proteins that recognize a consensus oestrogen response element (ERE). Co-transfection assays with the CAV promoter and the nuclear receptor chicken ovalbumin upstream promoter transcription factor 1 (COUP-TF1) showed that expression of EGFP was decreased by 50 to 60% in DF-1 and LMH cells. The CAV promoter that included sequences at and downstream of the transcription start point had less expression than a short promoter construct. Mutation of a putative E box at this site restored expression levels. Electromobility shift assays showed that the transcription regulator delta-EF1 (δEF1) binds to this E box region. These findings indicate that the CAV promoter activity can be affected directly or indirectly by COUP-TF1 and δEF1.

Chicken infectious anemia virus (CAV) is a small DNA virus of the family Circoviridae and has been classified as the sole member of the genus Gyrovirus (Pringle, 1999). CAV is present in virtually all commercial chicken operations (Yuasa, 1992; Yuasa et al., 1983). Clinical disease, characterized by anaemia, thymic and splenic atrophy and secondary infections including dermatitis (Schat, 2003), is limited to chickens less than 3 weeks of age. Older chickens may have decreased humoral immune responses causing poor vaccine responses, increased susceptibility to other infections (Adair, 2000) and decreased cell-mediated immune responses (Markowski-Grimsrud & Schat, 2003; McConnell et al., 1993). CAV infects T cell precursors in the thymic cortex and CD8+ splenic lymphocytes and haemocytoblasts in the bone marrow (Adair, 2000; Jurissen et al., 1992; Noteborn et al., 1994a). There is also evidence that CAV can be present in the reproductive system (Cardona et al., 2000a, b) and be vertically transmitted in antibody-positive or -negative hens (Miller et al., 2003).

Sequences in the 5′ non-transcribed region of the CAV genome are believed to be the sole promoter enhancer for CAV (Noteborn et al., 1994b; Phenix et al., 1994). This region has four or five direct repeat (DR) regions of 21 bases, with a 12 base insert between the first two (or three) and the last two DR. These regions are necessary for efficient viral transcription and replication (Noteborn et al., 1998, 1994b). The DR regions contain the sequence AGCTCA, which varies by one nucleotide from the oestrogen response element (ERE) half site, (A)GGTCA. Oestrogen receptor (ER), a ligand-induced nuclear receptor, binds as a homodimer to EREs in oestrogen-regulated genes with promoters that contain perfect or imperfect ERE half sites (Driscoll et al., 1998) arranged as palindromes or as widely spaced direct repeats (Aumais et al., 1996; Kato et al., 1995; Klinge et al., 1997; Krieg et al., 2001). Other ligand-induced nuclear receptors, such as thyroid receptor (TR), retinoic acid receptor, retinoid X receptor (RXR) and vitamin D receptor, bind elements resembling the ERE half-site arranged as DR regions with varying spacing (Carlberg, 1993; Quack et al., 2002), and may bind to a consensus ERE palindrome (Klinge et al., 1997). In addition, some orphan nuclear receptors without known ligands recognize similar elements. The most well-characterized is chicken ovalbumin upstream promoter transcription factor 1 (COUP-TF1) (Cooney et al., 1993; Park et al., 2003). Together, these nuclear receptors can bind a wide range of ERE-like motifs and often compete for binding to the same DNA sequence.

Due to the common occurrence of seroconversion at the time of sexual maturation in specific-pathogen-free flocks (Miller et al., 2001) and the presence of viral DNA in male and female gonads (Cardona et al., 2000b) and in freshly laid fertilized eggs (Miller et al., 2003), we hypothesized that CAV gene regulation is influenced by the regulation of
the reproductive system. Previous experiments found a significant increase in CAV promoter-driven expression in the ER enhanced cell line, LMH/2A, when treated with oestrogen (Miller et al., 2005), and that the ERE-like sequences from the CAV promoter could compete for binding to unidentified proteins recognizing a consensus ERE. This suggested that members of the nuclear receptor superfamily provide a mechanism to regulate CAV activity when low viral genome copy numbers are present (Miller et al., 2005). In addition, comparison of expression from a short (pEGFP-SE) and long (pEGFP-LE) CAV promoter construct, found less expression from pEGFP-LE that was largely due to decreased mRNA transcription (Miller et al., 2005). Sequences at the transcription start point (TSP) present in pEGFP-LE were found to have a potential E box-like element similar to site-binding δ-crystalline enhancer binding protein (δEF1) near the TSP in the Epstein–Barr virus BZLF promoter (Kraus et al., 2003).

The transcriptional regulator δEF1 is most commonly associated with repression of target genes (van Grunsven et al., 2001) as well as being part of an oestrogen signalling cascade that activates the chicken ovalbumin gene (Chamberlain & Sanders, 1999; Dillner & Sanders, 2002a, b). It is an important regulator of gene expression during embryonic development and is remarkably conserved between species, with homologues ZEB1 and Zfh-1a in humans and Drosophila, respectively (Akai & Storey, 2003; Comijn et al., 2001; Miyoshi et al., 2004; Postigo, 2003).

To examine whether TR and COUP-TF1 can modulate CAV promoter expression, we used co-transfection experiments of CAV promoter constructs with expression vectors for TR and COUP-TF1. Additional CAV promoter constructs were developed to determine the presence of repressor elements in the long promoter. DNA–protein binding assays were used to determine if δEF1 binds to sequences at the promoter TSP.

A spontaneously immortalized chicken fibroctic cell line, DF-1, was obtained from the ATCC (Rockville, MD) and grown in Dulbecco’s modified eagle media with 10% fetal bovine serum (FBS), 1.5 g NaHCO₃ l⁻¹, and 1% antibiotic-antimyotic mix (Invitrogen). LMH, the chicken hepatocyte cell line induced by chemical mutagenesis using diethylaminoethylamine, was also obtained from the ATCC. This cell line was maintained in Waymouth’s MB752/1 medium (Invitrogen) supplemented with 10% FBS, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹ and grown on 0.1% gelatin-coated flasks. All cells were grown at 37 °C with 5% CO₂.

The COUP-TF1 and TR expression vectors pRS-COUP-TF1 (Cooney et al., 1991, 1992) and pEX-cTRα (Forman & Samuels, 1991) were kindly provided by Ming-Jer Tsai (Baylor College of Medicine) and Herbert H. Samuels (NYU), respectively. Two separate clones were tested for each vector and an empty vector containing the Rous sarcoma virus (RSV) promoter pRc-RSV (Invitrogen) was used to co-transfect negative controls. Construction of the reporter plasmids containing the CAV short and long promoters, pEGFP-SE and pEGFP-LE, has been previously described by Miller et al. (2005). Deletion mutants were designed with 63 or 71 nucleotide (nt) deletions from the 3’ end of the long promoter and identified as pEGFP-LE-63 and pEGFP-LE-71, respectively (Fig. 1). In addition, the

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Fig. 1. (a) Schematic diagram and sequence of the downstream region of the CAV promoter from the TATA box at −31 (boxed) to +88 relative to the TSP (at +1), indicated by the arrow. The ATG codon at +27 is circled. An E box-like sequence at the TSP is indicated by bold type and underlining. (b) EGFP expression in DF-1 (solid bars) and LMH (hatched bars) cells transfected with pEGFP-SE, pEGFP-LE-71, pEGFP-LE-63, pEGFP-LE-63ΔTSP and pEGFP-LE. Mutation of nucleotides in the E box (pEGFP-LE-63ΔTSP), but not the deletions at the 3' end of pEGFP-LE, restored the level of EGFP expression to that of pEGFP-SE. Bars indicate means ± SD. Columns with different letters are significantly different by two-tailed Student’s t test (P<0.05).
construct pEGFP-LE-63ΔTSP was made with a 3 nt substitution in the E box-like sequence of pEGFP-LE-63 (Fig. 1b). These new promoters were generated by PCR using a forward primer, 5′-GTTACTATCTCACCATTCTA-3′ (nucleotides 13–35), and the following reverse primers: 5′-ACCGCCTTGCATACCTACTGCT3′ (nucleotides 328–350) for pEGFP-LE-63 and 5′-TGCGTATACTACTGCGACC-3′ (nucleotides 322–342) for pEGFP-LE-71. The construct with the mutated E box at the TSP, pEGFP-LE-63ΔTSP, was generated using the reverse primer 5′-ACCGCCTTGCGTATCTGC-3′ (nucleotides 328–350); the substituted nucleotides are indicated by lower case bold letters. PCR products were cloned into the pCR2.1 TOPO vector (Invitrogen) and subcloned into pEGFP-1 expression vector (BD Biosciences) using the EcoRI site. All constructs were verified by sequencing (Biotechnology Resource Center at Cornell University). The plasmids pEGFP-N1 (BD Biosciences), with EGFP expression under control of the immediate–early cytomegalovirus (CMV) promoter, and the promoterless pEGFP-1 were used as the positive and negative controls, respectively.

Transactivation assays were performed as described previously (Miller et al., 2005). Briefly, cells were transfected using Lipofectamine Plus or Lipofectamine 2000 (Invitrogen) and 0.3 μg EGFP reporter plasmid, with 0.2 μg pRS-COUP-TF1, pEX-cTR1 or the negative control pRc-RSV in each well of six-well plates. Cells were harvested in 200 μl reporter lysis buffer (Promega), frozen at −80 °C for one freeze–thaw cycle, vortexed for 15 s and centrifuged at 25 000 g for 5 min. Supernatant EGFP was measured using a TD 360 fluorometer (Turner BioSystems) that was calibrated with recombinant GFP (BD Biosciences). All experiments were run in triplicate wells and repeated at least three times. Samples were normalized for transfection efficiency by dividing by the positive control vector; results reported are the mean percentage of CMV-driven expression. Statistical comparisons were made using the two-tailed Student’s t-test and differences were considered significant if the P value was <0.05.

Co-transfection assays with both pEGFP-SE and pEGFP-LE and the expression vectors for COUP-TF1 resulted in a significant reduction in EGFP expression (Fig. 2). Co-transfection with the expression vector for TR did not alter expression (data not shown), which is not surprising since TR is often active as a heterodimer with RXR (Berrodin et al., 1992; Hallenbeck et al., 1992). The similar responses for both pEGFP-SE and pEGFP-LE suggest that the regulation is through the common elements in the short form. It is not yet clear if this is a direct or secondary response due to other COUP-TF1 target genes. Further assays to determine protein–DNA binding using a proven chicken COUP-TF1 antibody will be needed to address this question. Transfection assays to define the difference between the short and long versions of the CAV promoter further found that in DF-1 and LMH cells, pEGFP-LE-63ΔTSP had restored expression to that of the short promoter construct pEGFP-SE but not the deletion mutants pEGFP-63 or pEGFP-71 (Fig. 1b).

Electrophoretic mobility shift assays were used to detect DNA–protein interactions at the TSP using nuclear protein extracts from LMH or DF-1 cells and oligonucleotide probes spanning the E box-like element. Oligonucleotides (Integrated DNA technologies) were as follows: forward,
5'-gcAGTACGGAAGCGGCTCCCGGT-3' and reverse, 5'-tccACCCGGACCGCCTTGCGTATACCTACT-3'. Lower case letters indicate nucleotides that are 5' overhangs included for more efficient end-labelling. Oligonucleotides were annealed and labelled with [γ-32P]ATP using a 5'-end labelling kit (Amersham).

Nuclear proteins were extracted as described previously (Miller et al., 2005). Briefly, cell cultures grown in six-well plates were washed once with cold wash buffer (1× PBS, 1% FBS, 10 μM leupeptin), incubated on ice with 100 μl buffer A [10 mM HEPES pH 7.8, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM PMSF, 10 μM leupeptin, 1 mM dithiothreitol (DTT)] for 30 min and scraped from the plates. The nuclei were pelleted, resuspended in 70 μl buffer C (25%, v/v, glycerol, 20 mM HEPES pH 7.8, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM PMSF, 10 μM leupeptin, 1 mM DTT) and incubated on ice for 60 min, with stirring. Debris was pelleted and the supernatant was assayed for protein concentration with a Coomassie kit (Pierce Biotechnology). Protein (10 μg) was incubated with poly(dI-dC) (Sigma) in binding buffer (50 mM Tris/HCl pH 7.6, 50 mM KCl, 10% v/v, glycerol, 20 mM ZnSO4, 0.5 mM DTT) for 15 min at room temperature, before addition of the labelled probe (100,000–200,000 c.p.m.). For supershift/blocking assays, 2 μl anti-chicken–δEF1 antibody (a kind gift from Michel Samuels, University of Minnesota) (Chamberlain & Sanders, 1999) or antibody to SIP1 (Santa Cruz Biotechnology), another transcription regulator, were added to the protein/blocking buffer mix and preincubated for 15 min at 37 °C then 15 min at room temperature before addition of the labelled probe. Protein–DNA complexes were resolved on 5% polyacrylamide gels with 0.5 × Tris/borate/EDTA running buffer at 150 V for 2 h, and visualized by autoradiography. Images were processed with Adobe Photoshop.

Incubation of nuclear proteins from LMH cells with the TSP probe resulted in two specific bands, and addition of anti-δEF1 antibody, previously shown to block DNA–protein binding (Chamberlain & Sanders, 1999; Kraus et al., 2003), resulted in loss of band 1, a diminished band 2 and faster migration of band 3 (Fig. 3a, lanes 3 and 4). Incubation of nuclear proteins from DF-1 cells with the TSP probe resulted in three specific bands and anti-δEF1 antibody led to the loss of band 1 and diminishing of bands 2 and 3 (Fig. 3b, lanes 3 and 4). Addition of anti-SIP1 did not result in changes for either cell type (Fig. 3a, b, lane 5). Protein binding in band 1 was completely blocked, indicating that this band consisted of only δEF1 whereas band 2 most likely contained δEF1 as part of a larger multi-protein complex, since this band was diminished by the blocking antibody. This is not surprising given that δEF1 regulates target genes by competition for binding sites with other E box binding proteins, as well as through recruitment of, and competition for, other cofactors (Furusawa et al., 1999; Postigo & Dean, 1997, 1999; Postigo et al., 1997, 1999; Sekido et al., 1994).

Fig. 3. Nuclear extracts from LMH (a) or DF-1 (b) cells were incubated with [γ-32P]ATP-labelled oligonucleotides from the CAV promoter. Blocking antibodies against δEF1 or SIP1 were added as indicated by + or – signs. C indicates controls, without nuclear proteins. Protein–DNA complexes (described in the text) are labelled with numbers to the left of the gels.
data from transfection assays using the pEGFP-LE-63ΔTSP promoter construct (Fig. 1) as well as the binding assays suggest that the E box sequence and δEF1 play a role in regulation of viral transcription. More work is needed to further define this role and to identify other proteins binding at this site.

CAV is a very simple virus that encodes only three viral proteins and must rely on cellular factors for transcription control. Transfection assays from previous work (Miller et al., 2005) showed that expression from the CAV promoter can be modulated by ER in ER-enhanced cell lines. The current study found that expression from the CAV promoter was decreased on co-transfection with COUP-TF1 and the nucleotide sequence at the TSP binds to δEF1 and other unidentified proteins. A common feature of the nuclear receptors and δEF1 is their key role in cell type and temporal regulation of gene expression. These cellular transcription regulators would be effective in controlling viral gene expression when the virus is present in low copy numbers, while high copy numbers could dilute regulating factors and escape this control.

Acknowledgements

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References


Repression of chicken anemia virus transcription


