

The Chilo iridescent virus DNA polymerase promoter contains an essential AAAAT motif

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The delayed-early DNA polymerase promoter of Chilo iridescent virus (CIV), officially known as *Invertebrate iridescent virus*, was fine mapped by constructing a series of increasing deletions and by introducing point mutations. The effects of these mutations were examined in a luciferase reporter gene system using *Bombyx mori* cells transfected with promoter constructs and infected with CIV. When the size of the upstream element was reduced from position –19 to –15, relative to the transcriptional start site, the luciferase activity was reduced to almost zero. Point mutations showed that each of the 5 nt (AAAAT) located between –19 and –15 were equally essential for promoter activity. Mutations at individual bases around the transcription initiation site showed that the promoter extended until position –2 upstream of the transcription start site. South-Western analysis showed that a protein of approximately 100 kDa interacted with the –19 nt promoter fragment in CIV-infected cells. This binding did not occur with a point mutant that lacked promoter activity. The AAAAT motif was also found in the DNA polymerase promoter region of other iridoviruses and in other putative CIV delayed-early genes.

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INTRODUCTION

Chilo iridescent virus (CIV), officially addressed as *Invertebrate iridovirus 6*, is the type member of the genus *Iridovirus*, in the family *Iridoviridae* (Fauquet *et al.*, 2005). Viruses within this family characteristically have linear double-stranded DNA that is both circularly permuted and terminally redundant (Darai *et al.*, 1983, 1985; Delius *et al.*, 1984; Goorha & Murti, 1982). Up to now, 12 complete sequences of iridovirus genomes have been determined (e.g. Williams *et al.*, 2005; Delhon *et al.*, 2006). One of these is the CIV genome, which has a size of 212 482 bp (Jakob *et al.*, 2001). The family *Iridoviridae* along with the families *Poxviridae* and *Asfarviridae* (Fauquet *et al.*, 2005) display complex replication and gene regulation strategies. For frog virus 3 (FV3), a vertebrate iridovirus belonging to the genus *Ranavirus*, it was shown that the initial round of DNA synthesis takes place in the host cell nucleus (Goorha *et al.*, 1978), while later in infection DNA concatemer formation and assembly into mature virions occur in the cytoplasm (Goorha, 1982).

Studies of infected cell-specific polypeptides provided a evidence for a temporally regulated pattern of gene expression dividing the CIV genes into three classes: immediate-early (IE or α), delayed-early (DE or β) and

late (L or γ) (Barray & Devauchelle, 1987) genes and demonstrated a regulation cascade with both positive and negative control mechanisms (D'Costa *et al.*, 2001, 2004). The transcription cascade was analysed in detail for Red Sea bream iridovirus, belonging to the genus *Megalocytivirus* (RSIV; Lua *et al.*, 2005). IE gene expression does, by definition, not require *de novo* protein synthesis. DE and L genes on the other hand do require some IE or DE gene products, respectively, to be transcribed. Transcription of IE genes occurs also in the presence of inhibitors of protein synthesis, like cycloheximide, indicating that these genes are transcribed by a host DNA-dependent RNA polymerase. Host RNA polymerase II is required for the synthesis of ranavirus IE RNAs (Goorha, 1981) and it is likely that this is also the case for CIV IE genes. Transcription of CIV IE genes also requires a virion-associated protein, since purified CIV DNA is not infectious by itself, as is also the case for ranavirus DNA (Willis & Granoff, 1985), but can be reactivated by adding viral protein in the form of UV-inactivated virions (Cerutti *et al.*, 1989).

Promoter studies have been performed for two IE genes of FV3 (ICR-169 and ICR-489; Willis, 1987; Beckman *et al.*, 1988), and for two early (ICP-18 and ICP-46) and one L (major capsid protein or MCP) gene of bohle iridovirus (Pallister *et al.*, 2005), which also belongs to the genus *Ranavirus*. Potential promoter regions were determined for

A supplementary table is available with the online version of this paper.

two CIV genes encoding the DNA polymerase [DNAPol; open reading frame (ORF) 037L] and the MCP (ORF 274L), respectively (Nalçacıoğlu *et al.*, 2003). Transcription of the early DNAPol gene is initiated 35 nt upstream of the translational start site and the L MCP transcripts are initiated 14 nt upstream of the AUG codon. Using a luciferase reporter gene assay, we showed for DNAPol that sequences between positions -27 and -6 relative to the transcriptional start site harboured promoter activity. For MCP this activity is located between positions -53 and -29 . Both the DNAPol and the MCP promoters were not active in the absence of virus infection, suggesting that their activity was dependent on a protein expressed earlier in the cascade, as expected for DE or L genes, or a protein associated with the virus particle.

In the present study, the promoter region of the CIV DNAPol gene was analysed in detail by generating a further series of deletion mutants and introducing point mutations in the core promoter region. Transcriptional analysis classified DNAPol as a DE gene and mutagenesis indicated that nucleotides from positions -19 to -15 (AAAAT) are equally important for transcriptional activity. The interaction of viral proteins with the -19 promoter element was studied.

METHODS

Cells and virus. *Bombyx mori* SPC-BM-36 cells were obtained from the German Collection of Micro-organisms and Cell Cultures (DSMZ) and grown in Grace's insect medium supplemented with 10% fetal bovine serum (Invitrogen) at 27 °C. CIV (invertebrate iridescent virus 6) was kindly supplied by C. Joel Funk (USDA-ARS Western Cotton Research Laboratory, Phoenix, USA). The virus was propagated in larvae of the wax moth, *Galleria mellonella*, purified as described by Marina *et al.* (1999) and quantified by using UV spectroscopy.

Transcript analysis in the presence of inhibitors. SPC-BM-36 cells were infected with 5 µg CIV particles ml⁻¹ as described by D'Costa *et al.* (2001). Appropriate cultures were pretreated 1 h before infection with cycloheximide or AraC (both from Sigma) at final concentrations of 200 and 100 µg ml⁻¹, respectively, to inhibit either protein or DNA synthesis. Total RNA was isolated from cells at 0 or 24 h post-infection (p.i.) using Trizol (Invitrogen) according to the manufacturer's instructions. For RT-PCR analysis, 2 µg total RNA from CIV-infected cells was reverse transcribed using 10 U Superscript II reverse transcriptase (Invitrogen), 10 U RNAsin (Promega) and a specific reverse primer for DNAPol (DNAPol R) in a total reaction volume of 20 µl. The cDNAs obtained were amplified by PCR using specific forward and reverse primers (DNAPol F and DNAPol R; see Supplementary Table S1 available in JGV Online). PCR products were analysed in a 1.5% agarose gel stained with EtBr.

Control reactions in which the RT step was omitted were also performed. To verify the activity of cycloheximide we also performed an RT-PCR for the L MCP transcripts (data not shown).

Promoter constructs with deletions and point mutations. Upstream sequences for DNAPol starting at positions -19 and -15 were amplified by PCR from the promoter construct, DNAPol PC-247 (Nalçacıoğlu *et al.*, 2003), a derivative of pSP-Luc⁺ (Promega) containing a DNAPol upstream element of 247 bp, its 5'-untranslated

(UTR) region and the first 9 nt of the DNAPol ORF fused to a luciferase reporter gene. The forward PCR primers (DNAPol F series) introduced *Bgl*II restriction sites (see Supplementary Table S1). At the 3' end a primer (Luc R *Nar*I) was used that annealed downstream of the *Nar*I site in the luciferase ORF (Fig. 1a). In this way, the size of the PCR products was increased to facilitate their cloning. The resulting DNA fragments were cloned between the *Bgl*II and *Nar*I sites of pSP-Luc⁺, thereby generating constructs PC-19 and PC-15 (see Fig. 1a). The deletion constructs PC-86 and PC-27, starting at -86 and -27 upstream of the transcription initiation site, respectively, have been made in a similar way (Nalçacıoğlu *et al.*, 2003).

Point mutations in the DNAPol upstream region were introduced by a two-step PCR amplification. In the first step for each construct, the forward primer DNAPol F-247 and a construct specific DNAPol R mut primer were used to introduce the mutation (see Supplementary Table S1). The original promoter construct, PC-247, was used as a template for this mutagenesis. The resulting DNA products were column purified from the gel (Roche) and used as 5' mutagenic primers in the second PCR together with a 3' reverse primer annealing to the luciferase ORF (primer Luc R *Nar*I). The products of the second PCR were digested with *Bgl*II and *Nar*I, cloned into pSP-Luc⁺ and verified by automated sequencing (Baseclear). In this way, a series of plasmid constructs were obtained as outlined in Supplementary Table S1 and Fig. 1(b). Construct PC-19 was used as a template for the mutant -19 (PC mut-19), in which the A at -18 was converted into a C. For this amplification, the forward primer DNAPol mut-19 and the reverse Luc R *Nar*I primer were used (Supplementary Table S1, Fig. 1b).

Transfections and luciferase assays. SPC-BM-36 cells were seeded at a density of 1.5×10^6 cells per 35 mm tissue culture dish in Grace's supplemented medium without serum (Invitrogen). The cells were transfected using Cellfectin (Invitrogen) with 2 µg DNAPol plasmid constructs and 2 µg control plasmid to normalize for variations in the efficiency of transfection. This control plasmid, pIC-IE-1 (Nalçacıoğlu *et al.*, 2003), contains the *Autographa californica* nucleopolyhedrovirus IE1 promoter (Jarvis *et al.*, 1996) upstream of the *Renilla* luciferase reporter gene in the pRL-nul vector (Promega). Cells were infected with CIV at a concentration of 5 µg ml⁻¹ 18 h post-transfection and further incubated at 27 °C. Cells were harvested 6 h p.i. with CIV. Transfections were carried out in triplicate, and firefly and *Renilla* luciferase activities were measured in cell extracts using the Dual luciferase reporter assay system (Promega) following the manufacturer's instructions.

Preparation of nuclear extracts. Nuclear extracts were prepared based on a protocol by Blissard *et al.* (1992). A shake flask culture of 250 ml containing 3×10^6 SPC-BM-36 cells ml⁻¹ in Grace's insect medium was either mock-infected or infected with CIV at a concentration of 5 µg culture ml⁻¹. At 8 h p.i., cells were pelleted at 2000 r.p.m. in an SS34 rotor and resuspended in five packed cell pellet volumes of buffer A (10 mM Tris/HCl pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.2 mM Pefabloc C) and incubated for 10 min on ice. Cells were collected by centrifugation as before and resuspended in two packed cell pellet volumes of buffer A and lysed by 10 slow strokes in a Dounce homogenizer. At this step, a sample was checked microscopically for cell lysis using trypan blue. The homogenate was centrifuged for 10 min at 2500 r.p.m. in an SS34 rotor to pellet the nuclei. The pellet was resuspended in an equal volume (1 nuclear volume) of buffer C [20 mM Tris/HCl pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol (v/v), 0.5 mM DTT, 0.2 mM Pefabloc C] by repetitive pipetting (10 ×) and homogenized with 15 slow strokes in a Dounce homogenizer. The resulting homogenate was stirred slowly for 30 min at 4 °C. Nuclear membranes were removed from the extract by centrifugation at 14 000 r.p.m. for 30 min at 4 °C in a benchtop centrifuge. The

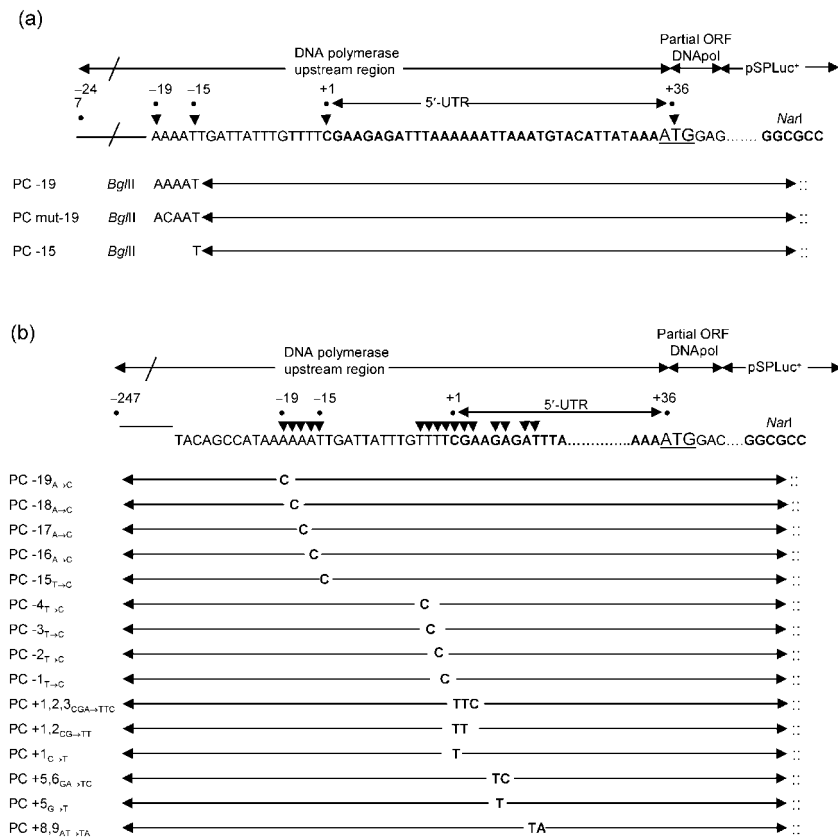


Fig. 1. Schematic representation of DNAPol promoter deletion and point mutations. Upstream DNA fragments decreasing in length (a) and with mutations at different points (b) were fused to a luciferase reporter gene. The sequences fused to luciferase included the original CIV translational start site and nine additional nucleotides derived from the DNAPol ORF. The positions of the point mutations are given by the letters corresponding to the bases that were changed; the size of the DNA fragment included in the constructs is illustrated by the arrowed lines.

supernatant was dialysed against 70 volumes of buffer D [20 mM Tris/HCl pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20% glycerol (v/v), 0.5 mM DTT, 10 mM 2-mercaptoethanol] for approximately 3 h at 4 °C. After dialysis, the solution was centrifuged for 15 min at 14 000 r.p.m. in a benchtop centrifuge and aliquots of 50 µl were frozen in liquid nitrogen and transferred to -80 °C for storage. The protein concentration was determined by the method of Bradford (1976).

South-Western blot analysis. Nuclear extracts (50 µg) prepared as described above were subjected to electrophoresis in a 0.1% SDS-12% polyacrylamide gel. Separated proteins were transferred to PVDF membrane (Millipore). Blotted proteins were completely denatured in 6 M guanidinium hydrochloride solution prepared in PBS and then stepwise renatured by serially diluting the guanidinium chloride solution. Membranes were further incubated overnight in PBS solution containing 5% fat-free milk and then washed in PBS solution containing 0.5% fat-free milk. Protein-DNA-binding reactions were performed in 0.1 M maleic acid, 0.15 M NaCl, pH 7.5 with 1% blocking powder (Roche) containing DIG-labelled -19 and mutant -19 (mut-19) probes overnight at 4 °C. These probes were prepared by digesting the plasmids DNAPol PC-19 and PC mut-19 with *Bgl*III and *Nar*I, followed by purification of the resulting 113 bp fragments from agarose gel. Terminal transferase was used to label the DNA fragments (100 ng) at the 3' ends with DIG-11-ddUTP according to the DNA Gel Shift kit protocol (Roche). For competition assays, the membranes were incubated with labelled -19 or mut-19 fragments. After incubation with the probes, the membranes were washed at room temperature for 30 min with PBS containing 0.5% milk and 0.3% Tween 20. Membranes were further incubated for 1 h at room temperature with anti-DIG (1:20 000)

diluted in 0.5% milk in PBS and then washed three times for 10 min with PBS/Tween 20 equilibrated with detection buffer (Roche). Chemiluminescent detection was performed with the substrate CSPD [disodium-3-(4-methoxyphosphoryl)-1,2-dioxetane-3,2'-(5'-chloro)tricyclo {3.3.1.1^{3,7}} decan-4-yl] phenyl phosphate; Roche Diagnostics].

RESULTS

Classification of the DNAPol promoter

Previous studies suggested that the DNAPol gene was controlled by an IE promoter (Nałçacioğlu *et al.*, 2003). If this were true, the CIV DNAPol gene would behave differently from its homologues in other large DNA viruses including vaccinia and baculoviruses. To elucidate this point, the transcriptional analysis based on RT-PCR was repeated using fresh batches of protein and DNA synthesis inhibitors, and fresh RNA samples. Controls without an RT step were negative for all RNA samples (data not shown). In our previous paper this latter control was not performed and the PCR product observed in the presence of cycloheximide may have arisen from amplification of residual CIV DNA in the RNA sample. To verify that cycloheximide functioned properly we also checked that the L MCP transcript was not formed in the presence of this inhibitor (data not shown). The experiments performed showed that DNAPol was easily detected when no inhibitors were present or in the presence of AraC, an

inhibitor of DNA synthesis. DNAPol transcription was however inhibited by cycloheximide (Fig. 2). This new study classifies the DNAPol gene as a DE gene, since protein expression is needed for its expression. This brings the regulation of the CIV DNAPol in line with the expression pattern of RSIV DNAPol (Lua *et al.*, 2005) as well as that of other large DNA viruses.

Mutational analysis of the DNAPol promoter

Previous results revealed that sequences between -27 and -6 relative to the transcription initiation site were essential for promoter activity of the CIV DNAPol gene, by linking the DNAPol promoter region to a luciferase gene copy (Nalçacıoğlu *et al.*, 2003). For further mapping of the promoter region of this gene, sequences starting at -19 or -15 were fused to a firefly luciferase reporter gene and tested in a transient expression assay. These constructs included the 5'-UTR sequences as well as the first 9 nt of the ORF of DNAPol to prevent loss of promoter activity, in case the promoter region extends over the ATG (Fig. 1a). The luciferase reporter was expressed efficiently when it was linked to the promoter fragment starting at -19 (Fig. 3a; PC-19) and the level of expression was comparable to that observed for the constructs starting at -27 (PC-27). This level was about 25 % lower than that in the -247 and -86 constructs (PC-247 and PC-86), in agreement with previous results (see Nalçacıoğlu *et al.*, 2003). Expression was drastically reduced (over 80 %) when the length of the

upstream region was reduced from -19 to -15 nt (PC-15; Fig. 3a). This result showed that the core promoter starts before position -15 .

To confirm the importance of three adenine residues located between -19 and -15 for promoter activity (see Fig. 1a), these adenines were modified by changing the As one by one into Cs in a 247 nt long promoter construct (PC-247). In that way, the plasmids PC-18_{A→C}, PC-17_{A→C} and PC-16_{A→C} were constructed (Fig. 1b). These mutations reduced the amount of luciferase synthesized by approximately 80–90 % compared with the level found with the wild-type 247 nt construct (Fig. 3b). This result confirmed the deletion studies (Figs 1a, 3a) and showed that each of these three As is required for promoter activity.

To determine whether the core promoter starts at the A at -19 and includes the T at -15 , these nucleotides were individually changed into Cs in the 247 nt promoter construct. Transfection results of the PC-19_{A→C} and PC-15_{T→C} constructs showed that the A at position -19 is the starting base essential for promoter activity and that the T at -15 is also crucial (Figs 1b, 3b).

To determine the 3' end of the promoter, four bases upstream and six bases downstream of the transcriptional start site (position $+1$) were mutated individually or in small groups, resulting in 10 new mutant constructs (PC-4_{T→C}, PC-3_{T→C}, PC-2_{T→C}, PC-1_{T→C}, PC + 1,2,3_{CGA→TTC}, PC + 1,2_{CG→TT}, PC + 1_{C→T}, PC + 5,6_{GA→TC}, PC + 5_{G→T} and PC + 8,9_{AT→TA}; see Fig. 1b). Mutations at each of the three bases from -4 to -2 relative to the transcription initiation site reduced promoter activity by 25–40 % (Fig. 3b). Mutating the base just before the transcription initiation site (PC-1_{T→C}) did not affect promoter activity. Changing single bases downstream of the transcription initiation site did not reduce promoter activity either (Fig. 3b). These results showed that the DNAPol promoter did not extend beyond the T at position -2 . The bases from -4 to -2 played a less prominent role in promoter activity than the AAAAT motif located from -19 to -15 . The role of the individual nucleotides between positions -14 and -5 has not been analysed further in this study.

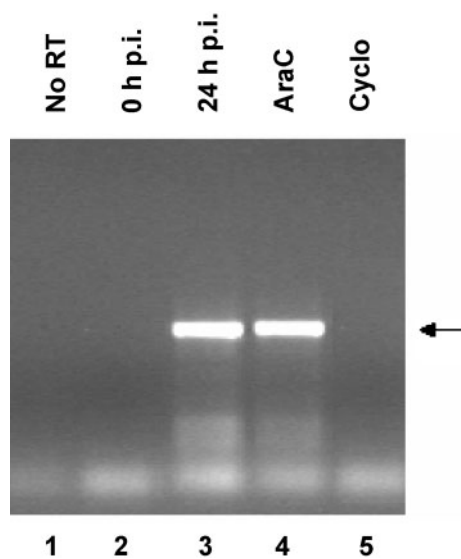


Fig. 2. Determination of the temporal class of DNAPol transcripts. Total RNA was obtained from CIV-infected cells and amplified by PCR (lane 1) or by RT-PCR at 0 h p.i. (lane 2), 24 h p.i. (lane 3) and after 24 h in the presence of AraC (lane 4) or cycloheximide (Cyclo; lane 5) using primers specific for DNAPol transcripts.

South-Western blot analysis

To study whether viral proteins bind to the -19 promoter fragment South-Western analysis was performed.

Nuclear extracts were prepared from either mock- or CIV-infected *Bombyx mori* cells. The proteins in these extracts were separated by SDS-PAGE and electroblotted onto PVDF membranes. When the membranes were incubated with a -19 promoter fragment end-labelled with DIG-11-ddUTP, a protein was detected with an estimated size of 100 kDa (Fig. 4a). This protein–DNA interaction was not observed with nuclear extracts of mock-infected cells (Fig. 4a), indicating that a viral protein or a virus-induced protein interacted with the DNAPol promoter, in line with the finding that DNAPol is a DE gene.

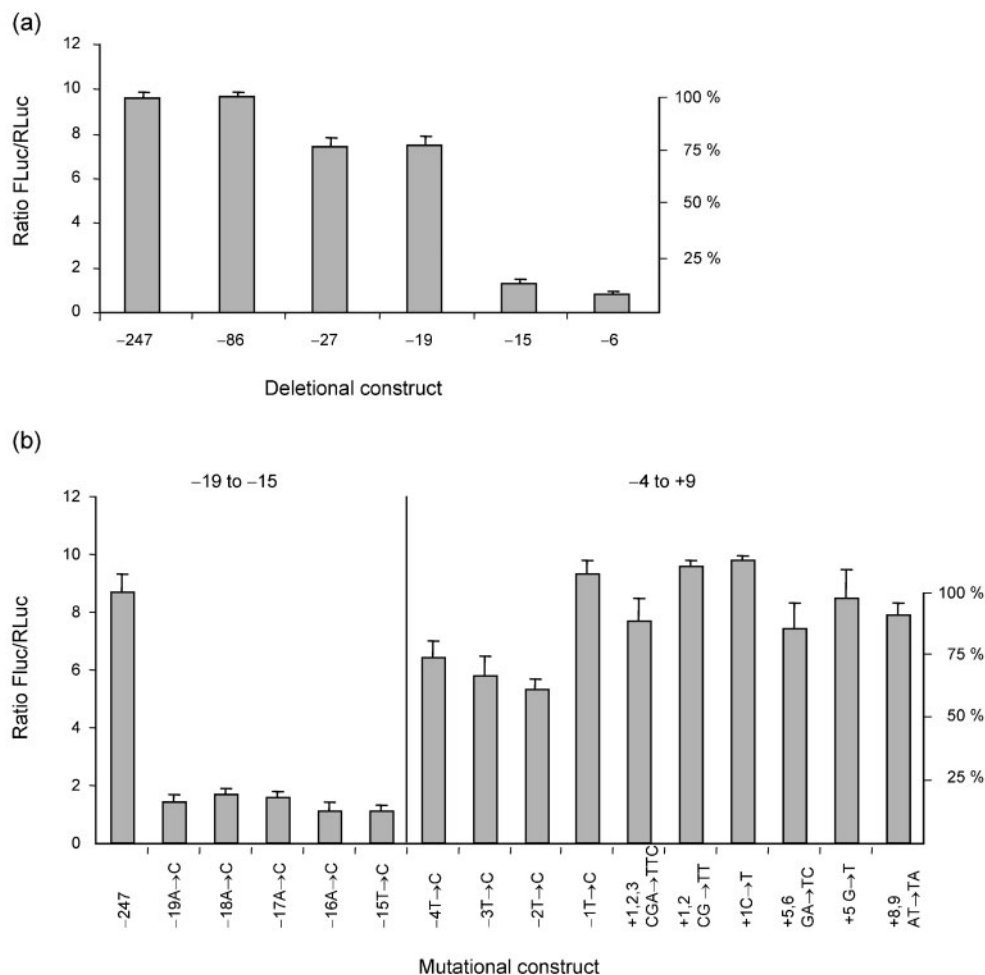


Fig. 3. Promoter analysis of the CIV DNAPol gene. SPC-BM-36 cells were transfected with constructs containing deletion (a) or point mutations (b) of the DNAPol promoter fused to a luciferase reporter gene. After transfection, cells were infected with CIV. Firefly luciferase activities were normalized based on the activity of *Renilla* luciferase, which was expressed from a cotransfected control plasmid containing the baculovirus IE-1 promoter. The relative proportion gives the ratio firefly/*Renilla* luciferase (FLuc/RLuc) versus the ratio obtained for the positive -247 control construct, which is set at 100%.

In order to show that this interaction was specifically directed against the CIV DNAPol promoter and not to any DNA fragment, a mutated -19 fragment was used as a probe, in which the A at -18 was changed into a C. In this case no protein interaction was observed (Fig. 4b). A similar result was obtained with the -15 promoter fragment as the probe (data not shown). The unlabelled -19 probe was able to compete with the labelled -19 probe for binding in a competition assay (Fig. 4c), while the mutated -19 probe did not compete with the -19 probe (Fig. 4d).

DISCUSSION

We have previously reported that the CIV DNAPol gene has a 35 nt region as 5'-UTR and that sequences within 27 nt relative to the transcriptional start site (at $+1$) were

important for promoter activity (Nalçacioğlu *et al.*, 2003). The study presented here reveals that a small region of 19 bp (AAAATTGATTATTTGTTTT), located between -19 and -2 relative to the mRNA start site, is responsible for promoter activity of the DNAPol gene. Mutations in the AAAAT motif in this region have a major effect on promoter activity, showing that this motif is an essential part of the core promoter structure. Mutations at the downstream side have less effect (-4 , -3 or -2). The role of individual nucleotides positioned at -14 to -5 was not analysed in this study.

The DNAPol promoter as a whole does not show a common structure with other CIV genes. In a similar way, the critical promoter sequence of the ICR 169 IE promoter of FV3 (ATATCTCACAGGGGAATTGAAAC) is also not conserved in other FV3 genes (Willis, 1987). The critical AAAAT motif, on the other hand, was found in the 100 nt

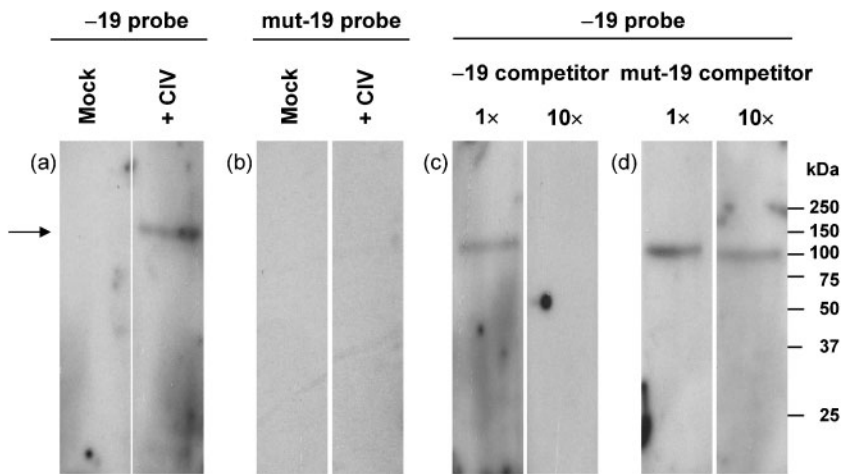


Fig. 4. Interaction of the DNAPol core promoter with nuclear proteins of mock- and CIV-infected SPC-BM-36 cells. Nuclear extracts were subjected to 12% SDS-PAGE and electroblotted onto PVDF membrane. The blots were probed with -19 (a) or mutant -19 (b) promoter fragments, which were end-labelled with DIG-11-ddUTP. The arrow indicates an approximately 100 kDa protein, which binds to the -19 promoter fragment. Competition assays were performed on blots probed with labelled -19 probe in the presence of 1- or 10-fold excess of unlabelled -19 (c) or mutant -19 (d) fragments as competitor.

upstream of the putative translational start codons of several other putative CIV DE genes, such as two exonucleases (012L and 244L), a topoisomerase II (045L), an endonuclease homologue (369L), a helicase (161L), a ligase (205R), the largest subunit of DNA-dependent RNA polymerase (176R) and the five small subunits (107L, 343L, 349L, 428L and 454R), two subunits of ribonucleoside diphosphate reductase (085L and 376L), thymidylate synthase (225R), thymidylate kinase (251L), nucleoside triphosphatase (022L), thioredoxin (453L) and two possible apoptosis inhibitors (157L and 193R), as derived from the CIV genome sequence (Jakob *et al.* 2001). Many homologues of the genes listed here also have an AAAAT motif in close proximity of their start codon in the other completely sequenced invertebrate iridescent virus [invertebrate iridescent virus 3 (IIV-3) or *Aedes taeniorhynchus* iridescent virus] (Delhon *et al.*, 2006).

A striking resemblance was also found when the region upstream of the DNAPol ORF was compared to the corresponding region in 12 completely sequenced iridovirus genomes. Eight of these viral genomes showed a similar AAAAT motif in the DNAPol upstream region including IIV-3. The three sequenced ranavirus genomes shared the related TAAAT motif in their DNAPol promoter regions. Whether this points towards a conserved regulation of DE promoter activity in iridoviruses needs to be established. The distance of these motifs to the start of the DNAPol ORF varied between 44 and 105 nt. The DNAPol gene of lymphocystis disease virus 1 is the only one lacking such a motif within reasonable distance of the ORF.

The temporal expression of three classes of CIV mRNA molecules during the course of infection (D'Costa *et al.*, 2001) suggests that both *cis*-acting DNA sequences and *trans*-acting regulatory factors interact at specific times post-infection to initiate transcription of the appropriate mRNAs. IE genes do not require *de novo* protein synthesis for their expression. It has previously been shown that purified CIV DNA is not able to start infection unless complemented with UV-irradiated virus particles (Cerutti

et al., 1989), suggesting that for IE gene expression in CIV a virion-associated protein is required. DE transcripts require at least one earlier gene product for their expression. In this study, we found a protein of approximately 100 kDa, which interacted with the active site of the DNAPol promoter and may represent a transactivator protein. This protein is considered virus-specific or virus-induced because in mock-infected control cells no binding was observed. Nuclear extracts prepared at different time points post-infection and in the presence or absence of protein synthesis inhibitors may help to determine the timing of expression of this 100 kDa protein in the expression process.

Although a single DNA-binding protein was observed for the DE DNAPol promoter, its expression may require more transactivating proteins. This could be the case if these proteins form a multimeric complex with non-identical subunits in which only the 100 kDa protein has affinity for DNA. This kind of indirect interaction of proteins with DNA has been observed for instance in phage T4 systems where two phage-specified proteins bind DNA only if the T4 DNAPol is also present (Huang & Buchanan, 1974). In addition, DNA-binding proteins that require specific ions or cofactors for their binding to DNA or proteins, which bind outside the region used for the probe and may modulate the level of expression, will be missed by this approach. Such a protein is expected for the region between positions -86 and -47, removal of which has a negative effect on promoter activity (Nalçacıoğlu *et al.*, 2003) and may be identified in a similar way with nucleotide stretches of this region as a probe.

Until now there have only been two reports about DNA-binding proteins in the family *Iridoviridae*. Twelve virus-induced DNA-binding proteins were found in FV3-infected cells by DNA affinity chromatography (Goorha, 1981). These proteins have molecular masses ranging from 14 to 119 kDa. The DNA-binding protein interacting with the DNAPol promoter in CIV-infected nuclear extracts has a molecular mass of approximately 100 kDa. In the CIV

genome sequence, a total of 468 ORFs (of which 230 are non-overlapping) have been identified (Jakob *et al.*, 2001) and molecular masses around 100 kDa are predicted for 022L, 045L, 050L, 085L, 176R, 179R, 184R, 261R, 295R, 396L and 428L ORFs. When these gene candidates were examined for their possible roles as a transactivator of the DNAPol promoter, we noted that 045L (DNA topoisomerase II), 176R and 428L (the large and a small subunit of DNA-dependent RNA polymerase) have predicted functions associated with DNA. Expressing these candidate genes in an heterologous system followed by testing the ability of the purified proteins to specifically bind to the -19 probe can be used to further address this point.

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