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Promoter analysis of the *Chilo* iridescent virus DNA polymerase and major capsid protein genes

Remziye Nalçacioğlu,[a,b] Hendrik Marks,[a] Just M. Vlak,[a] Zihni Demirbağ,[b] and Monique M. van Oers[a,*]

[a] *Laboratory of Virology, Wageningen University, Binnenhaven 11, 6709 PD Wageningen, The Netherlands*
[b] *Department of Biology, Faculty of Arts and Sciences, Karadeniz Technical University, 61080 Trabzon, Turkey*

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Abstract

The DNA polymerase (DNApol) and major capsid protein (MCP) genes were used as models to study promoter activity in *Chilo* iridescent virus (CIV). Infection of *Bombyx mori* SPC-BM-36 cells in the presence of inhibitors of DNA or protein synthesis showed that DNApol, as well as helicase, is an immediate-early gene and confirmed that the major capsid protein (MCP) is a late gene. Transcription of DNApol initiated 35 nt upstream and that of MCP 14 nt upstream of the translational start site. In a luciferase reporter gene assay both promoters were active only when cells were infected with CIV. For DNApol sequences between position −27 and −6, relative to the transcriptional start site, were essential for promoter activity. Furthermore, mutation of a G within the sequence TTGTTTT located just upstream of the DNApol transcription initiation site reduced the promoter activity by 25%. Sequences crucial for MCP promoter activity are located between positions −53 and −29.

Keywords: *Iridoviridae*; Iridovirus; *Chilo* iridescent virus; DNA polymerase; Major capsid protein; Helicase; Promoter studies; Luciferase

Introduction

The family *Iridoviridae* comprises four genera (van Regenmortel et al., 2000) of viruses infecting either vertebrates (Ranavirus, Lymphocystivirus) or invertebrates (Iridovirus, Chloriridovirus). *Chilo* iridescent virus (CIV), also called insect iridovirus 6, is the type species for the genus Iridovirus (Willis, 1990) and is a large icosahedral virus with a complex double-stranded DNA genome of 212,482 base pairs. Since iridoviruses cause lethal infections in important pest insect species, they have been considered as potential biological control agents (Kleespies et al., 1999; Hernandez et al., 2000).

The *Iridoviridae* along with *Poxviridae* and *Asfarviridae* are cytoplasmic DNA viruses (van Regenmortel et al., 2000) and, therefore, display complex replication and gene regulation strategies. Initially, it was assumed that iridoviruses replicated exclusively in the cytoplasm of host cells. However, UV irradiation and enucleation of cells prevented the replication of the vertebrate iridovirus Frog virus 3 (FV3), indicating that a funtional nucleus is required for iridovirus replication (Goorha et al., 1977). Electron microscopic studies showed that an initial round of viral DNA replication takes place in the cell nucleus (Goorha et al., 1978). In later stages of infection, concatamers of viral DNA are formed in the cytoplasm where also the assembly into mature virions occurs (Goorha, 1982).

The icosahedral virus particle of CIV comprises a capsid, an intermediate lipid layer, and the viral genome, which was found to be circularly permuted and terminally redundant (reviewed by Fischer et al., 1990). This genomic feature is also seen in other members of the family *Iridoviridae*, such as FV3 (Goorha and Murti, 1982), Lymphocystis disease virus (LCDV) (Darai et al., 1983), and Insect iridescent virus type 9 (Ward and Kalmakoff, 1991). Six origins of DNA replication have been reported for CIV (Fischer 1988a, 1988b; Handermann et al., 1992; Sonntag and Darai,

* Corresponding author. Fax: +31-317-484820.
E-mail address: monique.vanoers@wur.nl (M.M. van Oers).

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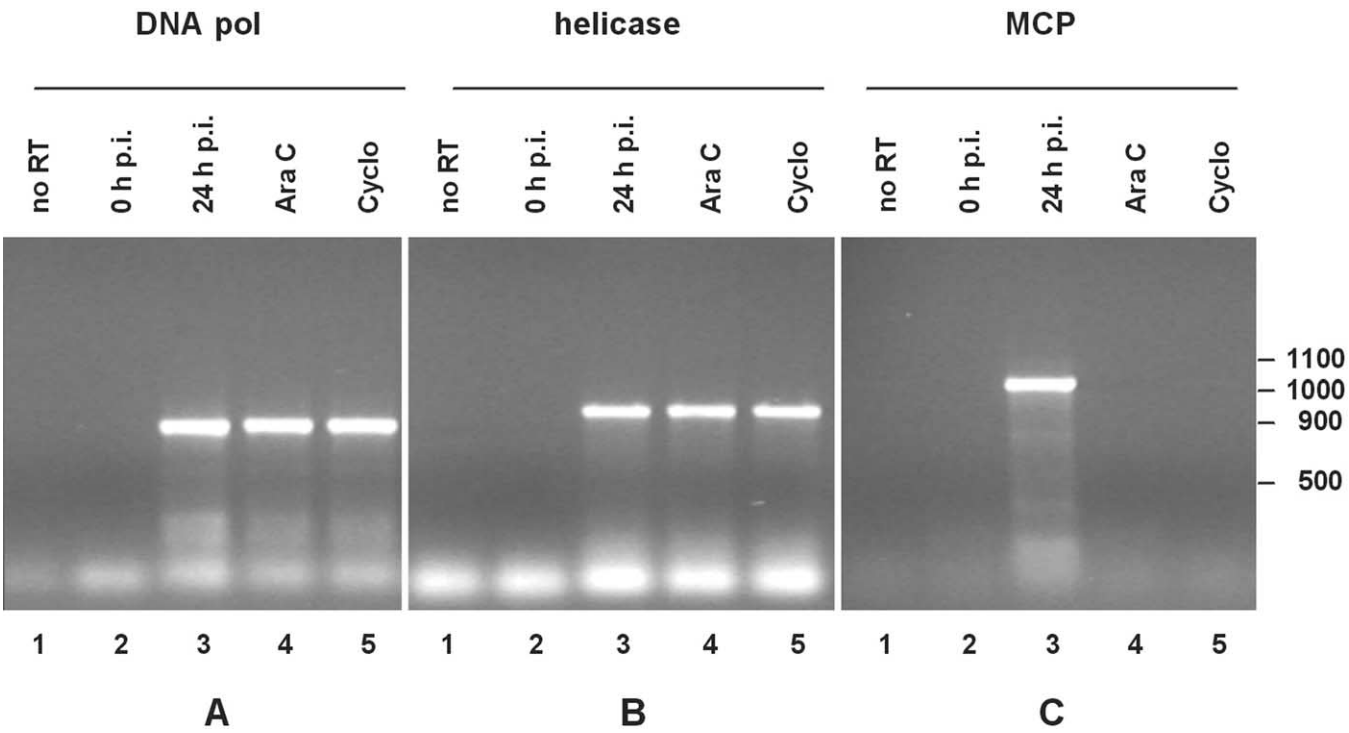

Fig. 1. RT–PCR for DNApol (A), helicase (B), and MCP (C) transcripts. Total RNA was obtained from CIV-infected cells and amplified by PCR (lanes 1) or by RT–PCR at 0 h p.i. (lanes 2), 24 h p.i. (lanes 3), and after 24 h in the presence of Ara-C (lanes 4) or cycloheximide (lanes 5) using primers specific for each transcript.

1992); recently its complete genome sequence has been published (Jakob et al., 2001).

Previous studies on infected cell-specific polypeptides provided evidence for a temporal cascade subdividing the CIV mRNAs into three temporal classes: immediate-early (IE or α), delayed-early (DE, β), and late (L, γ) (Barray and Devauchelle, 1987). The immediate-early class contained 38 transcripts synthesized in the absence of de novo protein synthesis. The delayed-early class contained 34 transcripts formed in the presence of DNA synthesis inhibitors, but not protein synthesis inhibitors, and the late class with 65 transcripts contained the full array of viral RNA formed only in the absence of inhibitors (D'Costa et al., 2001). The precise coding function of all these transcripts, however, was not clear from these studies.

Although much is known about the morphology, composition, and genome structure of CIV and some other members of the genus Iridovirus (reviewed by Fischer et al., 1990; Ward and Kalmakoff, 1991; Williams, 1998), there is hardly any information on the requirement for functional promoter regions in the CIV genome. Knowledge on promoter function is crucial for the understanding of iridovirus gene regulation. Promoter studies in the *Iridoviridae* family have been performed for only two immediate-early genes of FV3 (ICR-169 and ICR-489; Willis, 1987; Beckman et al., 1988), of which only one showed a detailed mutational analysis. In the present study the transcription initiation sites were determined for an immediate-early and a late CIV gene, encoding DNA polymerase (DNApol; ORF 037L) and the major capsid protein (MCP; ORF 274L), respectively, by 5′ RACE analysis. Sequences essential for promoter activity were determined by deletion mutagenesis in combination with a luciferase reporter system.

Results

The transcriptional classes of the DNA pol, helicase, and MCP genes

To select genes belonging to both the early and the late temporal classes of transcription for promoter studies, we examined the expression of DNApol and helicase (ORF 161L) (both presumed early) and MCP (late) at the transcriptional level. To that aim, SPC-BM-36 cells were infected with CIV in the presence or absence of cycloheximide, which inhibits de novo polypeptide synthesis, and Ara-C, an inhibitor of DNA replication. Total cellular RNA was extracted from cells 24 h postinfection (p.i.) and analyzed for the presence of DNApol, helicase and MCP transcripts by reverse transcription–polymerase chain reaction (RT–PCR) (Fig. 1). DNApol transcripts were observed at this time point (Fig. 1A, lane 3) and DNApol transcription was not affected by the presence of inhibitors of DNA (Fig. 1A, lane 4) or protein synthesis (lane 5). Similar results were obtained for helicase (Fig. 1B). Because viral DNA replication is not required for the expression of these two CIV genes, they belong by definition to an early gene class,

Table 1
Oligonucleotides used for RT-PCR and 5′RACE

Primer name	Sequences (5′-3′)
DNAPOL F	CAAGGAACAAGAACATATAAG
DNAPOL R	CACCTCTTTGTCCCATTTTAGG
DNAPOL SP1	CTCCCGGAGATGTTTGAAC
DNAPOL SP2	CACAGGCTTTAAATCTCCTA
DNAPOL SP3	GAACTTTAGGCTGTCCGC
MCP F	CTTCTGGTTTCATCGATATCG
MCP R	CCAAGTGCTCCGCCGGAA
MCP SP1	CCACCTAATTCTACCATTGG
MCP SP2	GGAATTCTCACTCTTAGCC
MCP SP3	GCAGACCATTCGCTTCCA
HEL F	CTACAACATCCGCTGCTAAC
HEL R	GGAATGGAAAACCAGACAATGTG

as expected. We classified the DNApol and helicase genes as immediate-early (IE) genes, because the onset of transcription did not require de novo protein synthesis. In the presence of Ara-C or cycloheximide, MCP expression was inhibited (Fig. 1C, lanes 4 and 5), indicating that MCP transcription requires de novo protein synthesis and is initiated after the onset of DNA replication. MCP is therefore designated as a late gene, in accordance with earlier results by D'Costa et al. (2001), who analyzed CIV transcription in IPRI-CF-124T cells by Northern blot analysis. As a control to show that no traces of viral DNA were amplified in this experiment, the RT step was omitted from the RT–PCR (lanes 1). CIV DNApol, helicase, and MCP transcripts most likely do not have polyA tails, because they could not be amplified when an oligod T primer was used in the RT step (not shown).

Transcription initiation sites of DNApol and MCP

Based on the RT–PCR results described above, DNApol and MCP were chosen as examples of an immediate-early and a late CIV gene, respectively. The transcription start sites for DNApol and MCP were identified by 5′ RACE analysis using total RNA extracted from SPC-BM-36 cells at 24 h p.i. with CIV. 5′ RACE was performed using three specific primers (SP1, SP2, and SP3) for each gene (Table 1). Amplified 5′ ends of cDNA fragments were cloned and four DNApol clones and three MCP clones were sequenced. The four clones obtained for DNApol showed that the transcription initiation site (+1) is either at the C located 35 nt upstream of the translation initiation site (+36, Fig. 2A) or at one of the three T's 5′ proximal to this cytosine. Because the first strand cDNA was tailed with a dA tail we cannot discriminate between these positions. The three clones obtained for the MCP 5′ RACE analysis indicated that the transcription initiated at the A located 14 nt (+1) upstream of the translational start codon (Fig. 2B).

Promoter analysis

To determine the DNA sequences responsible for transcriptional activation of the DNApol and MCP genes, a series of mutants with progressive 5′ deletions in the region upstream of the translational start were constructed and fused to a firefly luciferase reporter gene. To prevent loss of promoter activity in case the promoter region would extend over the ATG, a small 5′ part of the open reading frame of DNApol or MCP was included as well (Fig. 3). The resulting plasmids were transfected to SPC-BM-36 cells together with a control plasmid containing *Renilla* luciferase under control of the baculovirus IE-1 promoter (Jarvis et al., 1996) to be able to correct for variations in transfection efficiency. The transfected cells were either mock-infected or infected with CIV 18 h posttransfection and harvested 6 h later when transfected with DNApol constructs or at 24 h p.i. when analyzing MCP constructs. Transfections were done in triplicate and cell extracts were assayed for firefly and *Renilla* luciferase activity.

When the transfected cells were mock-infected no expression from either the DNA pol or the MCP promoter was observed (not shown), indicating that both promoters were not active in the absence of virus infection. When cells were infected with CIV after transfection high levels of luciferase activity were observed for the DNApol and MCP constructs starting at −247 or −268 respectively (PC1s) (Figs. 3 and 4). For DNApol, which has a translation start at position +36 (see earlier discussion), constructs starting between −247 and −6 relative to the transcriptional start site were tested (Fig. 4A). Luciferase levels were slightly reduced (to 70−80%) when the length of the upstream region was reduced from 86 to 43 nt and remained at that level when further shortened to 27 nt. Luciferase activity was almost zero with 6 uptream nt remaining and comparable to that observed for the presumed negative control starting at position +23. These results indicate that a major determinant for promoter activity is located approximately between positions −27 and −6 with probably some additional stimulating activity between position −86 and −43. The sequence in the −27 to −6 region is predominantly AT-rich, but starts with CAGCC (see Fig. 2A). This region might be involved in binding an as-yet-unknown viral transcription factor, present in the virus particle.

For MCP, the luciferase activity was unaltered when the length of the upstream sequence was reduced from position −268 to −73. A strong reduction was seen when the fragment started at position −29, to very low activities (10%) with 10 nt remaining. The presumed negative control for MCP, starting at position +9, had no activity. The results obtained indicate that the main sequences determining MCP promoter activity are located between 53 and 29 nt upstream of the transcription start (Fig. 2B). The MCP upstream region is also an AT-rich area.

Mutational analysis of a possible immediate-early promoter motif

The DNApol gene of CIV contains a 7-bp sequence (TTGTTTT) immediately upstream of the transcription start site (positions −7 to −1; see also Fig. 1). This motif was

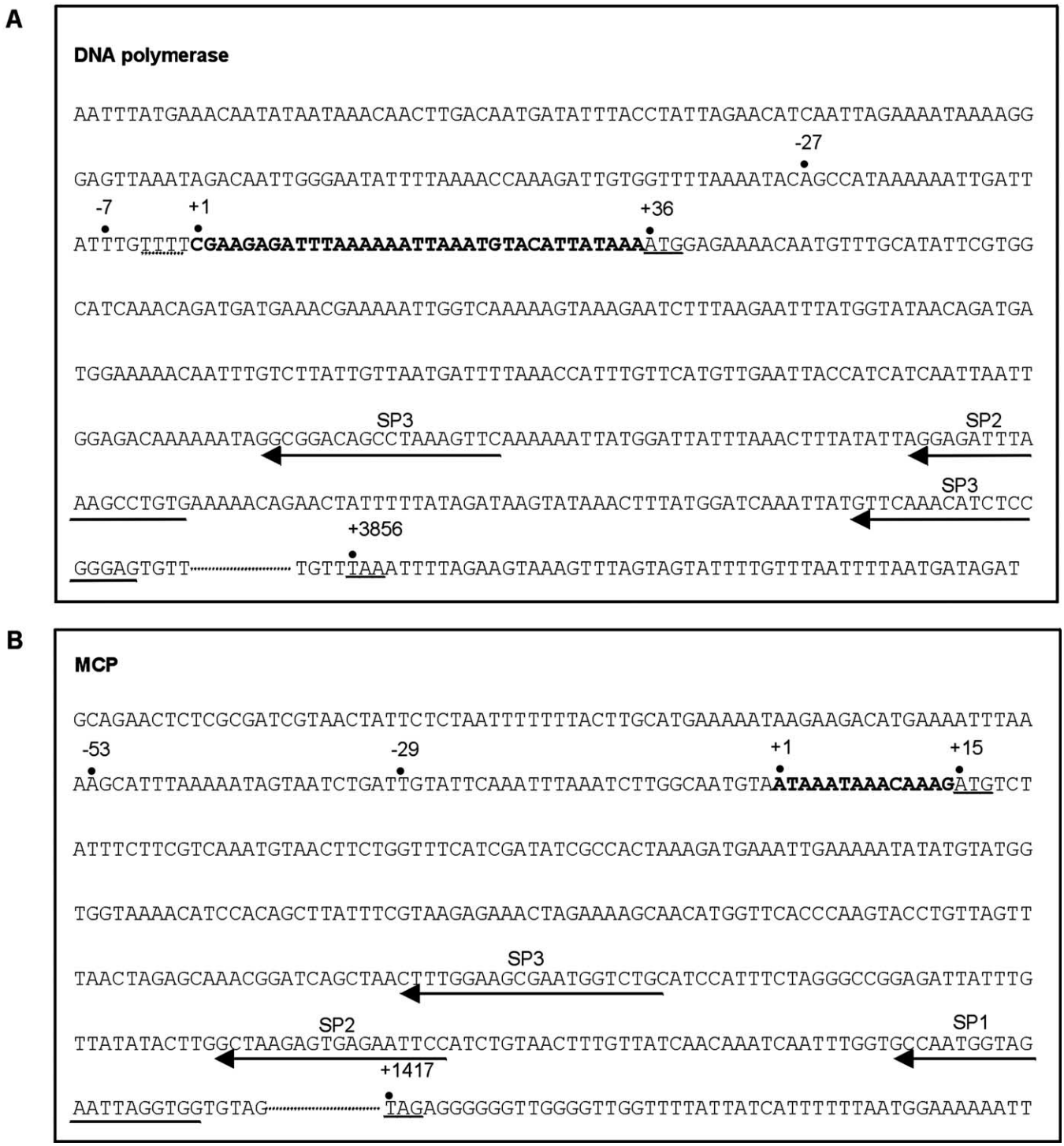

Fig. 2. Determination of the 5′ ends of the DNApol (A) and MCP (B) transcripts by 5′ RACE analysis. The start site of transcription is position +1. Sequences printed in bold show the 5′ untranslated regions (5′ UTR). Primers used for 5′ RACE analysis are indicated as SP1, SP2, and SP3.

also found in the upstream region of helicase and other possibly early genes, such as ORF107L encoding a DNA-dependent RNA polymerase subunit (Jakob et al., 2001). To identify whether this sequence could be an important motif for early gene activity in CIV, this sequence was modified in the upstream sequence of DNApol by changing the G (−5) into a C in the 247-nt promoter construct (PC1). This mutation reduced the amount of luciferase synthesized by approximately 25% compared to the level found with the wild-type 247-nt construct, indicating that this sequence might be part of the promoter but is not crucial for promoter activity. This is in accordance with the deletion analysis (Fig. 4) showing that the promoter of DNApol extends upstream of position −6.

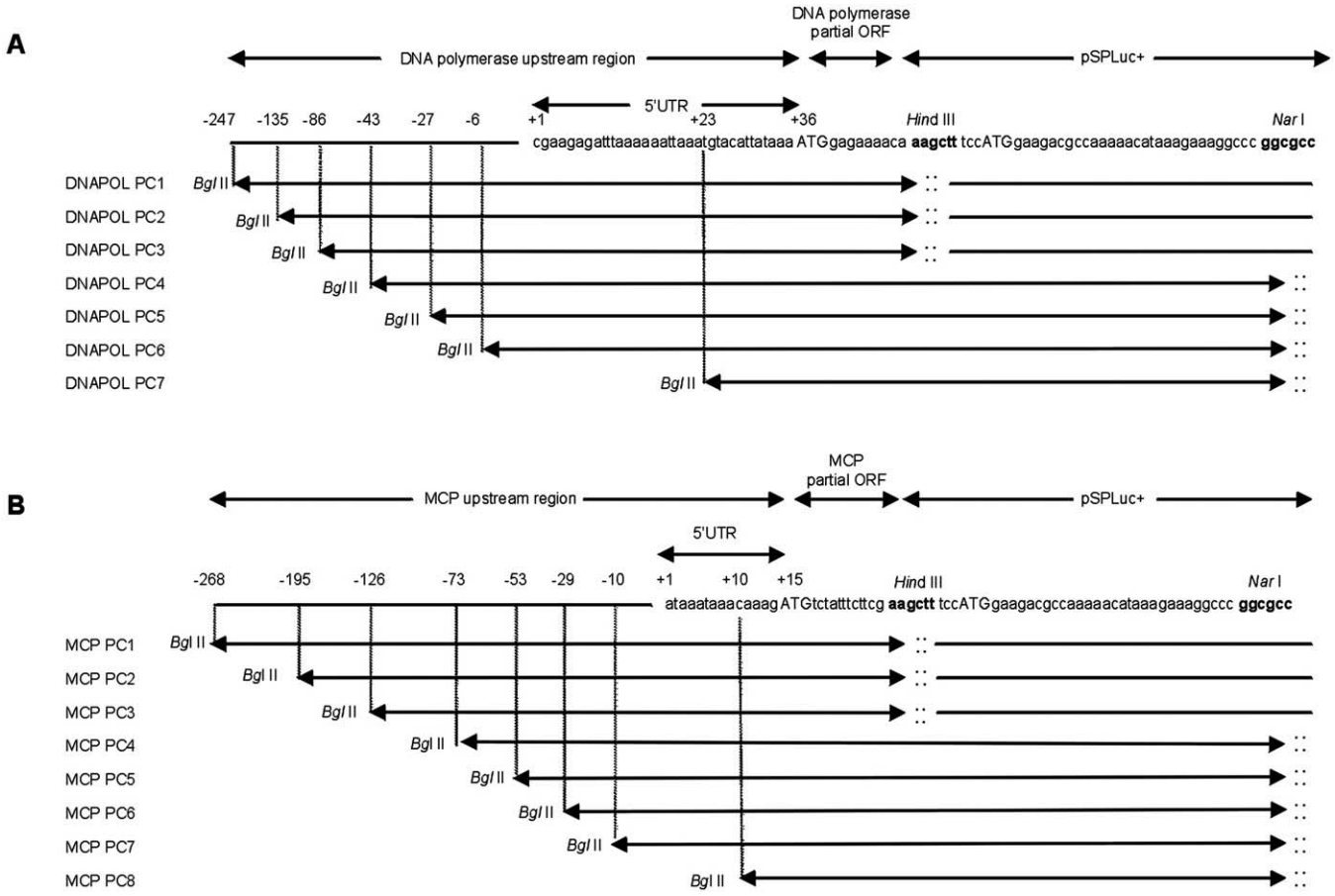

Fig. 3. Schematic representation of luciferase reporter gene constructs. DNA fragments decreasing in length and located upstream of the open reading frames for DNA polymerase (A) and MCP (B) were fused to a luciferase reporter gene. The sequences fused to luciferase included the original CIV translational start site and nine additional nucleotides derived from DNApol or an additional 12 nt from the MCP ORF, followed by a *Hin*dIII site.

Computer analysis of promoter regions

In order to find common motifs in regions upstream of CIV open reading frames as determined by Jakob et al. (2001), we have performed computational analyses of the 80 nt upstream of the putative translational start codons in order to find sequence motifs of 4 to 6 nt that are present with higher frequency in these areas than in the rest of the genome following the method described by Marks et al. (2003). When this computational method was applied to a baculovirus genome, the early (CAGT) and late (TAAG) promoter motives were found with ratio's of 1.6 and 4.6, respectively (Marks et al., 2003). In the CIV analysis, AT-rich motifs were found to be enriched in upstream regions, especially the 4-mer motifs TAAA and ATAA, which were present approximately two times more often in upstream areas than in the rest of the genome. TAAA is seen three times in the DNApol upstream region, at −56, −35, and −22 (see Fig. 2A), the latter of which is in fact ATAAA, which combines both 4-mer motifs. Deletions that included these motifs resulted in a drop in promoter activity (see Fig. 4). Also in the late MCP gene promoter TAAA motifs are present and in the upstream region of the helicase gene this motif is seen repeatedly.

Discussion

This is the first study to identify promoter sequences in an insect iridovirus and the first late promoter to be analyzed in the family *Iridoviridae* as a whole. Based on RT–PCR results, the promoter areas of DNApol and MCP were analyzed in more detail, as models for an immediate early and a late gene, respectively. CIV promoter constructs for both the DNApol and the MCP gene were only active in the presence of CIV infection. Similar results were found for Frog virus 3 (FV3, genus *Ranavirus*) (Willis and Granoff, 1985), when examining activity of the immediate early FV3 ICR-169 promoter in a transfection assay using chloramphenicol acetyltransferase (CAT) as reporter. CAT synthesis was observed only when cells were subsequently infected with FV3. Additional experiments showed that the virus particle, but not the viral DNA was needed to activate the FV3 promoters. A similar way of activation appears to be true for CIV, because purified viral DNA was not infectious unless complemented with UV-irradiated virus particles (Cerutti et al., 1989). The exact component of the virus particle responsible for early gene activation is not known.

The dependence on components of the virus particle for early gene transcription is shared by irido-, pox-, and asfar-

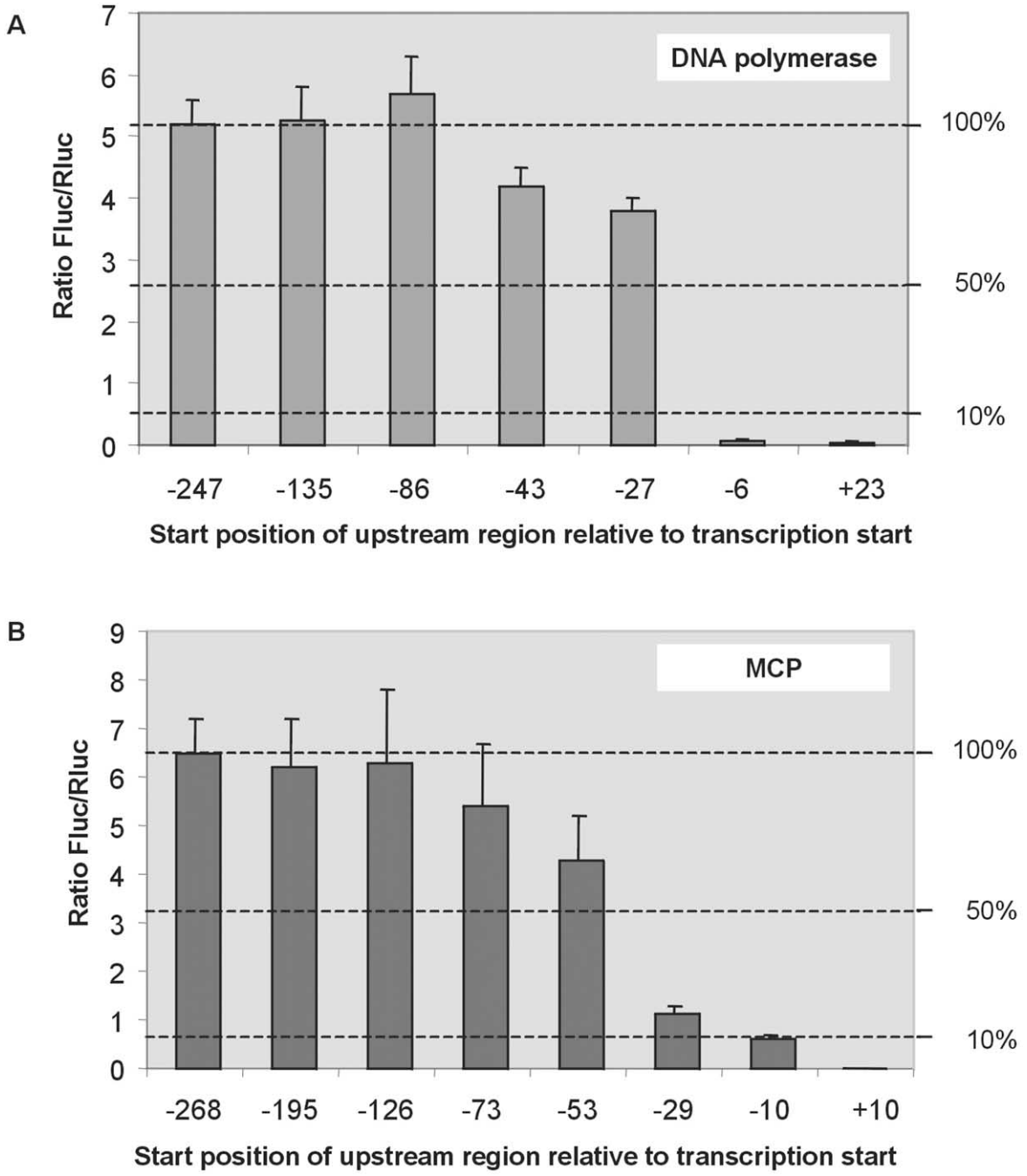

Fig. 4. Promoter analysis of CIV DNApol and MCP genes. SPC-Bm-36 cells were transfected with the various reporter gene constructs for DNApol (A) and MCP (B) followed by infection with CIV. Firefly luciferase activities were normalized based on the activity of *Renilla* luciferase, which was expressed from a control plasmid containing the baculovirus IE-1 promoter. The luciferase levels are given relative to the level obtained with the longest constructs, which was set at 100%.

viruses. In vaccinia virus, for instance, the polypeptide RAP94 is required for early transcription. RAP94 is a late gene and may be a virion-associated early transcription factor (Ahn et al., 1994). In addition, the vaccinia virus early transcription factor (VETF) is required, which is also expressed late in infection and binds to early promoters as well as to the viral RNA polymerase (reviewed by Moss, 1996). A gene product that could perform similar functions in CIV might be encoded by ORF O22L, which is homologous to an early transcription factor of the vertebrate iridovirus Lymphocystis disease virus (LCDV-1, genus *Lymphocystisvirus*) (Jakob et al., 2001). Iridovirus FV3 requires the host nucleus and hence host RNA polymerase II for the onset of transcription (Goorha et al., 1977, 1978), whereas pox- and asfarviruses use a viral RNA polymerase for early gene expression in the cytoplasm (Baroudy and Moss, 1982), which is present in the virions. African Swine Fever virus does, however, require a functional nucleus for its

replication (Garcia-Beato et al., 1992). In CIV and LCDV-1 homologs of RNA polymerase subunits have been found that may be involved in the cytoplasmic transcription in later stages of infection (Tidona et al., 1997; Jakob et al., 2001). In contrast to pox- and asfarvirus mRNAs, most iridovirus mRNAs are not polyadenylated (reviewed by Williams, 1998). This is apparently also the case for the CIV DNApol, helicase, and MCP transcripts, as we were unable to amplify these transcripts in RT–PCR using oligo dT primers.

Thus far, detailed iridovirus transcriptional analysis had only been performed for two immediate early FV3 genes, with no mutual conservation of sequence motifs (Willis, 1987; Beckman et al. 1988). A 23-bp region upstream of a TATA-like motif was identified that regulated transcription of the major immediate-early gene ICR-169. When the TATA-like motif (TATTTTA) in the upstream region was deleted, 16–50% of promoter activity remained. This motif most likely serves to position the RNA polymerase on the ICR-169 promoter prior to transcription initiation. Whether the TTGTTTT motive, found in several CIV early genes, including DNApol and helicase, has a similar function needs to be analyzed in more detail. Its location immediately upstream of the transcription initiation site of DNApol might direct to such a function. Mutagenesis of only one nucleotide (G→C) is, however, insufficient to eliminate promoter activity entirely.

The outcome of the 5′ RACE and the luciferase experiments was supported by computer analyses (Table 3) showing the relative enrichment of several AT-rich motifs in the promoter regions of CIV genes. It is difficult, though, to discriminate specific AT-rich viral motifs from the more general TATA-box sequences. In order to confirm the general importance of these motifs for CIV promoter activity more gene promoters need to be analyzed in depth using site-directed mutagenesis in combination with luciferase reporter gene assays. Such studies are also needed to reveal what discriminates early from late CIV promoter motifs. Interesting to note is that pox-and asfarvirus promoter motifs are also AT-rich (Moss, 1996; Rodríguez et al., 1996, Garcia-Escudero and Viñuela, 2000). In early poxvirus genes, transcription initiation starts with a purine 12–17 nt downstream of a consensus core region (A)6TG(A)5A/TA conserved among Poxviridae (reviewed by Moss, 1996). Late promoters in vaccinia virus, TAAA (intermediate) and TAAAT (late), are present just upstream of the ATG and in fact overlap with the ATG. The late MCP promoter sequence of CIV appears to be located further upstream compared to late genes in vaccinia virus. In our promoter deletion analysis, though, we included the original ATG and the first few coding nucleotides of DNApol and MCP in the luciferase constructs in case the promoter would extend beyond the translational start site.

The present studies show that CIV DNApol and MCP genes have short 5′ UTRs of 35 and 14 nt, respectively, and that sequences essential for promoter activity are located between position −27 and −6 nt for DNApol and between −53 and −29 for MCP, relative to the transcriptional start sites. The mutational analysis of the TTGTTTT motif in the DNA polymerase gene indicates that the 3′ promoter limit may extend at least toward the transcriptional start site, but further studies are needed to set the 3′ limits of both promoters. Possible AT-based motifs have been detected in these regions. To identify more general patterns for CIV, and possibly other iridoviruses, early and late gene promoter activity of more genes need to be analyzed as documented in this article combined with site-directed mutagenesis of putative motifs. In addition, efforts should focus on identifying transactivators for CIV early and late promoters.

Materials and methods

Cells and viruses

Bombyx mori SPC-BM-36 cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and grown in monolayer cultures at 27°C in supplemented Grace's insect medium containing 10% fetal bovine serum (Invitrogen). *Chilo* iridescent virus type-6 (CIV) was a gift from C. Joel Funk (USDA-ARS Western Cotton Research Laboratory). SPC-BM-36 cells were infected with 5 μg/ml of CIV particles as described by D'Costa et al. (2001).

RNA isolation and RT–PCR analysis of MCP and DNA polymerase mRNAs

SPC-BM-36 cells were infected with CIV as described above. Appropriate cultures were pretreated 1 h before infection with cycloheximide or Ara-C (both from Sigma) at final concentrations of 200 and 100 μg/ml, respectively, to inhibit either protein or DNA synthesis. These inhibitors were maintained at the above levels throughout the infection. Total RNA was isolated from cells at 0 h p.i. or at 24 h p.i. using Trizol (Invitrogen) according to the manufacturer's instructions. For RT–PCR analysis, 2 μg of total RNA from CIV infected SPC-BM-36 cells was reverse transcribed using 10 units of Superscript II reverse transcriptase (Invitrogen); 10 units of RNAsin; and 250 nM of specific reverse primers for DNApol, helicase, and MCP mRNAs (DNAPOL R, HEL R, and MCP R; see Table 1) in a total reaction volume of 20 μl. The cDNA's obtained were amplified by PCR using specific forward and reverse primers (DNAPOL F and DNAPOL R, HEL F and HEL R, and MCP F and MCP R; see Table 1). PCR was performed in a final volume of 50 μl containing 400 nM of each primer, 0.2 mM of each dNTP in 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 9.0 at 25°C), 50 mM KCl, 0.1% Triton X-100, and 0.5 units of *Taq* DNA polymerase (Promega). PCR products were analyzed in a 1.5% agarose gel stained with EtBr. Two controls were performed, in which the RNA was used for PCR directly while omitting the RT step or in which cDNA obtained from CIV infected cells at 0 h p.i. was analyzed.

Table 2
Oligonucleotides used for the preparation of the various promoter constructs[a]

Primer name	Sequences (5′-3′)	Position
DNAPOL Luc F1	GG**AGATCT**CGTGAAGGCAAATGATGA	−247/−230
DNAPOL Luc F2	GG**AGATCT**CAACTTGACAATGATATTTAC	−135/−115
DNAPOL Luc F3	GG**AGATCT**GGGAGTTAAATAGAGACAATTG	−86/−65
DNAPOL Luc F4	GG**AGATCT**GTGGTTTTAAAATACAGCC	−43/−25
DNAPOL Luc F5	GG**AGATCT**GCCATAAAAAATTGATTATTTG	−27/−6
DNAPOL Luc F6	GG**AGATCT**GTTTTCGAAGAGATTTAAAAAA	−6/+17
DNAPOL Luc F7	GG**AGATCT**GTACATTATAAAATGGAGAAAAC	+23/+46
DNAPOL Luc R	GG**AAGCTT**TGTTTTCTCCATTTTATAATG	+48/+27
MCP Luc F1	GG**AGATCT**CAATACATAACAATCTTTCAT	−268/−248
MCP Luc F2	GG**AGATCT**GTCTAGACTTAAAATGTC	−195/−178
MCP Luc F3	GG**AGATCT**GAACTCTCGCGATCGTAAC	−126/−108
MCP Luc F4	GG**AGATCT**GAAGACATGAAAATTTAAAAGC	−73/−52
MCP Luc F5	GG**AGATCT**GCATTTAAAAATAGTAATCTG	−53/−33
MCP Luc F6	GG**AGATCT**GTATTCAAATTTAAATCTTGGC	−29/−8
MCP Luc F7	GG**AGATCT**GGCAATGTAATAAATAAACAAAG	-10/+14
MCP Luc F8	GG**AGATCT**CAAAGATGTCTATTTCTTCG	+10/+29
MCP Luc R	GG**AAGCTT**CGAAGAAATAGACATCTTTG	+29/+10
Luc *Nar*I R	GGAATGGCGCCGGGCCTTTCTTTATG	85/62 (Luc)
3′ Mutagenesis primer	CTCTTCGAAAAGAAATAATC	+7/−13

[a] Restriction sites were added to the gene specific DNAPOL and MCP primers to assist cloning into the reporter vector pSP-Luc$^+$; *Bgl*II, printed in bold, *Hind*III, bold and underlined. The Luc *Nar*I primer annealed to the pSP-Luc$^+$ vector downstream of the *Nar*I site.

5′ RACE analysis

To determine the length of the 5′ untranslated region for the mRNAs of DNApol and MCP we performed 5′ RACE (Rapid Amplification of cDNA Ends) analysis using a 5′ RACE kit (Roche) following the procedure supplied by the manufacturer with a set of three specific primers. First-strand cDNA was synthesized from 2 μg total RNA isolated at 24 h p.i. using gene-specific primers (DNAPOL SP1 and MCP SP1; see Table 1). The first-strand cDNA was then isolated and dA tailed. This was followed by two consecutive nested PCRs with specific primers (see Table 1). MCP SP2 and DNAPOL SP2 primers were used for the first PCR with an oligo dT anchor primer (Roche). SP3 primers were used for the second PCR in combination with a PCR anchor primer (Roche). The amplified fragments were cloned into pGEMT-Easy (Promega) and analyzed by automated sequencing (Baseclear, Leiden, NL).

Preparation of promoter constructs

Upstream sequences for DNApol, starting at positions −247, −135, or −86, and for MCP, starting at −268, −195, and −136, were amplified by PCR from CIV DNA. The primers used at the 5′ end of the sequences to be cloned introduced *Bgl*II restriction sites. The primer at the 3′ end annealed from position +48 for DNApol and from +29 for MCP relative to the transcriptional start and introduced a *Hind*III restriction site (Fig. 3). The amplified DNA fragments were digested with *Bgl*II and *Hind*III, and cloned in-frame with a luciferase reporter gene in the vector pSP-Luc$^+$ (Promega). In this way the plasmids DNApol PC1, PC2, and PC3 and the plasmids MCP PC1, PC2, and PC3 were generated. The other promoter constructs for DNApol (−43, −27, −6, and +23) and MCP (−73, −53, −29, −10, and +10) were amplified by PCR from the PC1 promoter constructs. The primers directed against the 5′ end had *Bgl*II restriction sites as described above. At the 3′ end primers were used annealing distal to the *Nar*I site in the luciferase ORF of the pSPLuc$^+$ vector (Fig. 3). In this way the sizes of the generated fragments were increased to simplify their cloning. The resulting DNA fragments were cloned between the *Bgl*II and *Nar*I sites of pSP-Luc$^+$, thereby generating DNApol PC4–PC7 and MCP PC4–PC8. The primers used for preparation of these promoter constructs are listed in Table 2.

Mutagenesis of the G into a C within the TTGTTTT sequence in the DNApol upstream region was performed in a two-step PCR. In the first step, the 5′-forward primer DNAPOL luc F1 and a 3′-mutagenesis primer (see Table 2) were used to amplify viral DNA. The resulting DNA product was purified from gel and used as 5′ mutagenic primer in the second PCR together with a second 3′-reverse primer (DNApol luc R, see Table 2). The product of the second PCR was digested with *Bgl*II and *Hind*III, cloned in pSP-Luc$^+$, and verified by automated sequencing.

Transfections and luciferase assays

SPC-BM-36 cells were seeded the day before transfection at a density of 1.5×10^6 cells/35-mm tissue culture dish in Grace's supplemented medium without serum. The cells were transfected using cellfectin (Invitrogen) with 2 μg test plasmid DNA and 2 μg of the control plasmid pIC-IE1 to normalize for the efficiency of transfection. The pIC-IE-1 plasmid was obtained from M.C.W. van Hulten

(Wageningen University). This plasmid was generated by cloning the *Autographa californica* nucleopolyhedrovirus immediate early 1 (IE1) promoter together with the hr5 region (Jarvis et al., 1996) upstream of the *Renilla* luciferase reporter gene in the pRL-nul vector (Promega). At 18 h after transfection, cells were infected with CIV at a concentration of 5 μg/ml and further incubated at 27°C. Cells were harvested 6 h p.i. for DNApol and 24 h p.i. for MCP promoter analysis. Firefly and *Renilla* luciferase activities were measured in cell extracts using the Dual luciferase reporter assay system (Promega) following the manufacturer's instructions.

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