Studies on the Control Region of the p10 Gene of the *Autographa californica* Nuclear Polyhedrosis Virus

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**SUMMARY**

5' deletion mutants of the *Autographa californica* nuclear polyhedrosis virus very late p10 gene promoter have been prepared and subjected to a transient expression assay in infected *Spodoptera frugiperda* cells. The control plasmid contained the chloramphenicol acetyltransferase (CAT) reporter gene under the control of the p10 promoter, which was included in a 230 bp sequence upstream from the p10 translation initiation codon. The control plasmid also contained a segment of the hrs enhancer downstream from the CAT gene. Promoter activity was unaffected by 5' deletion to position -77, which lies about 11 bp upstream from the p10 cap site. However, deletion of 12 more bp completely eliminated p10 promoter activity. Thus, the 5' border of the p10 promoter lies downstream from position -77, and the region between positions -77 and -65 contains an element that is important to promoter activity. This is the region that is conserved near the cap sites of late baculovirus genes. Our studies also show that transient expression of CAT under the control of the p10 promoter and hrs enhancer is higher when transfection occurs prior to infection by virus.

During the very late (δ) phase of infection of *Spodoptera frugiperda* cells by the *Autographa californica* nuclear polyhedrosis virus (AcNPV), two genes are expressed to a much greater extent than all others (Smith *et al.*, 1982, 1983; Rohel *et al.*, 1983). The first of these is the gene for the major occlusion body protein, polyhedrin. The other very late gene codes for an abundant late protein of M, 10000 (p10) whose function is unknown, and which is dispensable in viral replication (Vlak *et al.*, 1988). This gene has been mapped and sequenced (Kuzio *et al.*, 1984; Lübbert & Doerfler, 1984), and detailed mapping of the transcripts in the region of this gene has been performed (Rankin *et al.*, 1986; Liu *et al.*, 1986).

Possee & Howard (1987) have performed insertion and deletion mutagenesis on the polyhedrin gene 5' flanking region linked to the β-galactosidase reporter gene in a recombinant virus. They found that all of the 5' flanking region up to position -20 with respect to the cap site could be deleted with no effect on activity. By contrast, a deletion to position -7 destroyed 90% of expression. Other studies (Matsuura *et al.*, 1987) have shown that the entire polyhedrin leader region is required for full expression of a linked reporter gene. Together these results suggest that the size of the polyhedrin promoter is relatively small, and that it extends no further than 20 bp upstream from the cap site.

Weyer & Possee (1988) have begun an analysis of the sequence requirements for optimal expression of a chloramphenicol acetyltransferase (CAT) reporter gene under the control of the p10 promoter. They found that the entire leader region is required for full expression. Moreover, removal of the whole leader completely blocked expression. This result agrees well with the studies by Matsuura *et al.* (1987) on the polyhedrin leader region.

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Here we report further investigations on the p10 promoter. We have placed the CAT gene downstream from the p10 leader and 5' flanking region and have tested the effects of 5' deletions in this construct by transient assays in infected cells.

To locate the 5' border of the p10 control region, we performed 5' deletions on a plasmid designated pAEp10CAT(II), which has the bacterial CAT gene downstream from the p10 leader and 5' flanking region. Fig. 1 shows the strategy for this construction. We began with plasmid pAEp26p10CAT, which was constructed by resecting the large HindIII-BgII fragment of HindIII Q (87-5 to 89.0 map units) with Bal 31 nuclease, then removing overhangs with T4 DNA polymerase, blunt end-ligating into the SmaI site of pUC13 and inserting the CAT cartridge from plasmid pCM-4 (Pharmacia) into the adjacent BamHI site. The resulting plasmid contains the hr5 enhancer (Guarino & Summers, 1986; Liu et al., 1986), the p26 control and coding region (Liu et al., 1986), the p10 control region, and part of the p10 coding region. Sequencing (Sanger et al., 1977; Chen & Seeburg, 1985) showed that the CAT gene is inserted in phase just after codon 4 of the p10 gene.

In order to remove the p26 control region, pAEp26p10CAT was digested with XhoI and SalI, and the 1033 bp fragment was isolated. This fragment was inserted into the SalI site of plasmid pAE, which contains an AluI fragment bearing the majority of the hr5 enhancer, cloned into the SmaI site of pUC12. The result was plasmid pAEp10CAT(II), containing the CAT gene under the control of the p10 promoter, with the enhancer downstream from the CAT gene. Form II was distinguished from form I (with the enhancer upstream) by restriction digestion.

Finally, deletions were performed (Guo & Wu, 1983) by opening the plasmid with HindIII, resecting with exonuclease III and nuclease S1, and removing remaining single-stranded overhangs with T4 DNA polymerase. The 5'-deleted promoters were cut out of the plasmid with SacI, size-selected by low melting point agarose gel electrophoresis, blunt-ended with T4 DNA polymerase, and inserted into the Smal site of pUC12. The result was a set of plasmids [5'-d-pAEp10CAT(II)] with variable deletions in the 5' flanking region of the p10 gene, having the CAT gene under the control of the p10 promoter. The exact extents of deletion in selected plasmids were confirmed by sequencing according to the Sanger dideoxynucleotide method (Sanger et al., 1977; Chen & Seeburg, 1985).

We included the enhancer in this construct because our experience has been that such constructs give considerably higher CAT activity than constructs lacking the enhancer. Fig. 2 shows CAT assays that reinforce this point. Note the much higher CAT activity with the two plasmids containing the enhancer [Ep10CAT(I) and Ep10CAT(II), with enhancers upstream and downstream, respectively, from the CAT gene] and the plasmid lacking the enhancer (p10CAT). Note also the generally higher CAT expression when cells were transfected with plasmid first, then infected with virus, rather than vice-versa. For this reason, all our subsequent assays were done by transfecting 4 h pre-infection. These results involving timing seem to conflict with those of Weyer & Possee (1988), which showed that 4 h (p.i.) post-infection was the optimal time for transfection with p10CAT plasmids. However, these investigators did not try transfecting before infection, nor did their plasmids contain the viral enhancer. Thus, there may not be a conflict. In fact, as Fig. 2 shows, our plasmid lacking the enhancer (p10CAT) gave higher CAT activity when transfected 4 h p.i. than when transfected 4 h pre-infection.

Fig. 3 summarizes the data from four duplicate experiments in which several 5'-deleted plasmids were tested for CAT expression. The numbers (−230, −203, etc.) denote the position of the 5' end of the p10 flanking sequence relative to the p10 translation initiation codon (ATG). The parent plasmid DNA (−230) is not deleted and extends up to the XhoI site. The activity of this plasmid in each experiment is taken as 1-0, and the activities of the deleted plasmids are compared to it. It is apparent that no significant loss of activity occurred upon deletion to position −77, but complete loss of activity occurred upon deleting 12 additional bp. This places the 5' border of the p10 promoter somewhere between positions −77 and −65, and indicates that an important part of the promoter lies between these two positions. As indicated by the boxes in Fig. 3, this is the location of the 12 bp sequence that is highly conserved near the cap sites of a variety of late baculovirus genes (Rohrmann, 1986).
Fig. 1. Construction of plasmid pAEp10CAT(II) and deletion of p10 5' flanking region. The steps in the construction are detailed in the text. Note that the orientation of pAE is opposite to that of pAEp10CAT(II), and that the site labelled Ss in pAE is the same as the one labelled Sac in pAEp10CAT(II). Unlabelled restriction sites are either EcoRI sites in the enhancer (in pAEp26p10CAT) or unused sites in the multiple cloning site of the vector. Restriction enzymes and other enzymes were obtained from Promega Biotec, IBI, Bethesda Research Laboratories and New England Biolabs and were used according to their manufacturers' recommendations. Abbreviations: Ss, SrtI; E, EcoRI; hr., homology region 5; Sal, Sall; Xho, Xhol; B, BamHI; En, enhancer; Sac, SacI; Sma, SmaI.
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Fig. 2. Effect of enhancer and time of transfection on CAT expression under control of the p10 promoter. *S. frugiperda* (IPLB-SF21) cells (Vaughn et al., 1977) were transfected either 4 h pre-infection (−4) or 4 h post-infection (+4) with the following plasmids. p10CAT was made in the same way as pAEp10CAT (fig. 1), but using pUC12 instead of pAE, so it lacks the enhancer. Ep10CAT(I) has the same enhancer segment as pAEp10CAT, but it is inserted just upstream from the p10 promoter. This was accomplished by inserting the 1033 bp segment of pAEp26p10CAT into pAE in the opposite orientation from that in Ep10CAT(II). Ep10CAT(II) is the same as pAEp10CAT(II), having the enhancer segment just downstream from the CAT gene. The control cells were transfected with Ep10CAT(II) but were not infected by virus (AcNPV L-1 extracellular virus; Lee & Miller, 1984). Transfection was performed using a modified calcium phosphate precipitation technique (Graham & Van der Eb, 1973; Gorman et al., 1982). Plasmid DNAs were prepared from transformed *Escherichia coli* JM103 or JM107 cells (Birnboim & Doly, 1979). After RNase treatment and phenol extraction, the DNAs were precipitated with 0·8 M-NaCl and 6·5% polyethylene glycol, then the DNA pellets were dissolved in 1·8 g/ml CsCl and 60 to 80 μl of ethidium bromide (10 mg/ml) was added. These solutions were underlaid beneath 1·8 g/ml CsCl and centrifuged in a Beckman type 80Ti rotor at 65000 r.p.m. for 6 h (Garger et al., 1983). The bands containing superhelical DNA were removed, extracted with 1-butanol and dialysed against 10 mM-Tris–HCl pH 7·5, 1 mM-EDTA. Cultured *S. frugiperda* cells were grown to 0·4 × 10⁶ to 0·6 × 10⁶/ml, transferred to 100 mm diam. plates, allowed to attach for 1 h at room temperature, and then transfected with 20 μg plasmid DNA (including pUC12 DNA used as carrier). The cells were fed with medium containing 12% foetal bovine serum for 4 h, then infected with extracellular virus at an m.o.i. of 20 p.f.u./cell. These cells were then incubated 48 h before harvesting for CAT assay. When cells were infected before transfection, the medium above was replaced with standard medium (10% foetal bovine serum) 4 h after transfection, and the cells were incubated for a total of 48 h before harvesting. CAT assays were performed as follows. Cells were harvested by rinsing, transferring to plastic centrifuge tubes, washing three times with ice-cold phosphate-buffered saline (Lee & Miller, 1978), and resuspending in 4 mM-Tris–HCl pH 7·4, 1 mM-EDTA, 1 mM-EGTA, 0·15 M-NaCl for 10 min at room temperature. Finally, the cell pellets were resuspended in 100 μl of 0·25 mM-Tris–HCl pH 7·8 and heated at 65°C for 10 min. Cell extracts were prepared (Guarino & Summers, 1986) and assayed for CAT activity (Gorman et al., 1982).

Our results are in excellent agreement with previous work (Possec & Howard, 1987; C. Rankin et al., personal communication) on the promoter for the other very late gene, the polyhedrin gene. Possec & Howard (1987) showed that deletion to within 20 bp of the polyhedrin cap site had no effect on promoter activity, whereas deletion of an additional 13 bp reduced
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Fig. 3. Nucleotide sequences and relative CAT expression levels of 5' deletion mutants of pAEpl0CAT(II). Partial sequences are presented for the 5' flanking regions of nine plasmids whose designations (-230 to -63) indicate how much of the 5' flanking region remains, relative to the first base of the p10 translation initiation codon, along with results of four independent duplicate CAT assays (numbered 1 to 4) on cells transfected with these plasmids. Transfections at 4 h pre-infection and CAT assays were performed as described in the legend of Fig. 2. The averages of each set of four experiments are listed at the far right. The 12 bp consensus sequence around the p10 cap site is boxed.

promoter activity by 90%. Deleting through the cap site destroyed all promoter activity. C. Rankin et al. (personal communication) performed linker scanning mutation of the polyhedrin control region and found that mutations that interrupted the sequence TAAGTATT around the cap site drastically reduced expression from the polyhedrin promoter.

We have not shown directly that transcription in our transient assays begins at the correct cap site. Nevertheless, this point has already been dealt with in studies by Knebel & Doerfler (1987) and by Weyer & Possee (1988), using similar plasmids. In both these cases, transcription of the CAT gene under the control of the p10 promoter started at or near the correct cap site in transient assays. Although this does not demonstrate correct initiation on our plasmids, it is very unlikely that small deletions near the correct cap site could completely block transcription from a remote, incorrect cap site.

The conserved regions around the cap sites of the two very late genes therefore seem to be crucial to the activities of the promoters for these two genes. Other work (Matsuura et al., 1987; Weyer & Possee, 1988; C. Rankin et al., personal communication) has shown that the leader regions of these two genes are also important for full expression of linked genes, although no short sequence within either leader has been shown to be absolutely indispensable.

The picture emerging from all these investigations is that the promoters for the very late genes of this virus do not at all resemble the promoters recognized by eukaryotic RNA polymerase II. This accords with our previous finding (Grula et al., 1981; Fuchs et al., 1983) that late viral gene expression is insensitive to α-amanitin and is therefore directed by an enzyme distinct from host RNA polymerase II.

The very late genes are clearly among those transcribed by this virus-induced polymerase, and their promoters include the 'Rohrmann box,' whose sequence is TATAAGNACTT (Rohrmann, 1986). Part of this sequence is strikingly similar to the promoter recognized by yeast mitochondrial RNA polymerase, TATAAGTA, which suggests a similarity between the virus-
induced RNA polymerase and the yeast mitochondrial polymerase. Other work in our laboratory (C. L. Yang & R. F. Weaver, unpublished) shows a cross-reactivity between an antibody directed against T7 RNA polymerase and two subunits of the virus-induced polymerase. Since T7 polymerase and yeast mitochondrial RNA polymerase share considerable sequence similarity (Masters et al., 1987), this also suggests a relationship between the virus-induced RNA polymerase and a mitochondrial polymerase.

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REFERENCES


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