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The promoter of the late p10 gene in the insect nuclear polyhedrosis virus *Autographa californica*: activation by viral gene products and sensitivity to DNA methylation

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In lepidopteran insect cells infected with the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV), two major late viral gene products are expressed: the polyhedrin, a 28 000 mol. wt. protein which makes up the mass of the nuclear inclusion bodies, and a 10 000 mol. wt. protein (p10) whose function is unknown. The nucleotide sequences of these strong promoters conform to those of other eukaryotic promoters and are rich in AT base pairs. We used the pSVO-CAT construct containing the prokaryotic gene chloramphenicol acetyl transferase (CAT) to study the function of the p10 gene promoter in insect and mammalian cells. Upon transfection of the pAcp10-CAT construct, which contained 402 bp of the p10 gene of AcNPV DNA in the *Hind*III site of pSVO-CAT, CAT activity was determined. The p10 gene promoter was inactive in human HeLa cells and in uninfected *Spodoptera frugiperda* insect cells. The same promoter was active, however, in AcNPV-infected *S. frugiperda* cells and exhibited optimal activity when cells were transfected 18 h after infection with the insect virus. This finding demonstrated directly that the p10 gene promoter required other viral gene products for its activity in insect cells. The nature of these products was unknown. The p10 gene promoter sequence contained one 5′-CCGG-3′ site 40 bp upstream from the cap site of the gene and two such sites 178 and 192 bp downstream from the ATG initiation codon of the gene. Since *Drosophila* DNA or *S. frugiperda* DNA contained no 5-methylcytosine or extremely small amounts of it, we were interested in determining the effect of site-specific methylations on the p10 gene insect virus promoter. Methylation at the 5′-CCGG-3′ sites led to a block of this promoter. These data showed that an AcNPV insect virus promoter could be rendered sensitive to site-specific methylations in *S. frugiperda* insect cells, albeit the DNA of these cells did not contain significant amounts of 5-mC.

Key words: baculovirus/insect virus promoter/site-specific promoter methylation/activation of late viral promoter/DNA transfection/CAT assay

Introduction

The insect baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) is a lepidopteran DNA virus with a double-stranded, supercoiled circular genome of some 126 – 129 kbp (Lübbert *et al.*, 1981; Cochran *et al.*, 1982). Two viral genes which are expressed abundantly late after AcNPV infection of insect cells (Adang and Miller, 1982; Smith *et al.*, 1982, 1983; Rohel *et al.*, 1983; Rohel and Faulkner, 1984; Lübbert and Doerfler, 1984b), have very strong promoters. One of the late genes encodes the polyhedrin polypeptide, the major constituent of the nuclear inclusion bodies (Vlak *et al.*, 1981), the other late gene encodes a 10 000 dalton protein (p10) of unknown function (Smith *et al.*, 1983; Rohel *et al.*, 1983; Lübbert and Doerfler, 1984b; Kuzio *et al.*, 1984). The nucleotide sequences of both promoters are rich in adenine and thymine (AT) nucleotides (Hooft van Iddekinge *et al.*, 1983; Lübbert and Doerfler, 1984b; Kuzio *et al.*, 1984).

The methylated nucleotide 5-methylcytidine (5-mC) in specific promoter sequences of eukaryotic genes can cause the long-term inactivation of these genes (Doerfler, 1983, 1984a, 1984b; Riggs and Jones, 1983; Taylor, 1984; Doerfler *et al.*, 1984). The promoters of viral (Kruczek and Doerfler, 1983; Simon *et al.*, 1983; Langner *et al.*, 1984) and non-viral (Busslinger *et al.*, 1983) genes were inactivated by the *in vitro* methylation of certain specific nucleotide sequences, e.g., of 5′-CCGG-3′ sequences. Methylations of the bodies of these genes at 5′-CCGG-3′ sequences. however, in general had no effect on gene expression (Busslinger *et al.*, 1983; Kruczek and Doerfler, 1983; Langner *et al.*, 1984). Adenovirus promoters were shown to be inactivated by *in vitro* methylation both after transfection into mammalian cells (Kruczek and Doerfler, 1983; Langner *et al.*, 1985) and after microinjection into *Xenopus laevis* oocytes (Langner *et al.*, 1984). The E2A gene of human adenovirus type 2 was not inactivated when its 5′-GGCC-3′ sequences were methylated (Vardimon *et al.*, 1982).

It is still unclear to what extent 5-mC could play a similar regulatory role in the gene expression of non-vertebrate eukaryotes, e.g., in insects. Most attempts to find 5-mC in *Drosophila* DNA (Smith *et al.*, 1982; Eick *et al.*, 1983) or in the DNA of *Spodoptera frugiperda* insect cells (T.Müller and W.Doerfler, unpublished results) have failed, but 5-mC has been reported to occur in *Drosophila* DNA in exceedingly small quantities (Achwal *et al.*, 1984). We have been interested to determine whether the promoter of the late AcNPV p10 gene is sensitive to sequence-specific DNA methylations.

We have previously used the chloramphenicol acetyl transferase (CAT) gene in the pSVO-CAT construct (Gorman *et al.*, 1982) to test the activities of viral promoters in the methylated or unmethylated state (Kruczek and Doerfler, 1983; Langner *et al.*, 1984, 1985). The promoter sequence of the p10 gene of AcNPV was now inserted into the *Hind*III site in front of the CAT gene in the pSVO-CAT construct. Activation of the p10 gene promoter in the pSVO-CAT construct required the presence of other viral gene products in AcNPV-infected insect cells. Furthermore, the activity of the p10 gene promoter was severely affected when the 5′-CCGG-3′ site in this promoter was methylated. These data demonstrate that methylation of specific promoter sequences in an insect virus can affect gene activity in insect cells, although 5-mC is not abundant in the DNA of these cells.

Results

Experimental design and purpose of experiments

The functional requirements of the strong promoter of the late

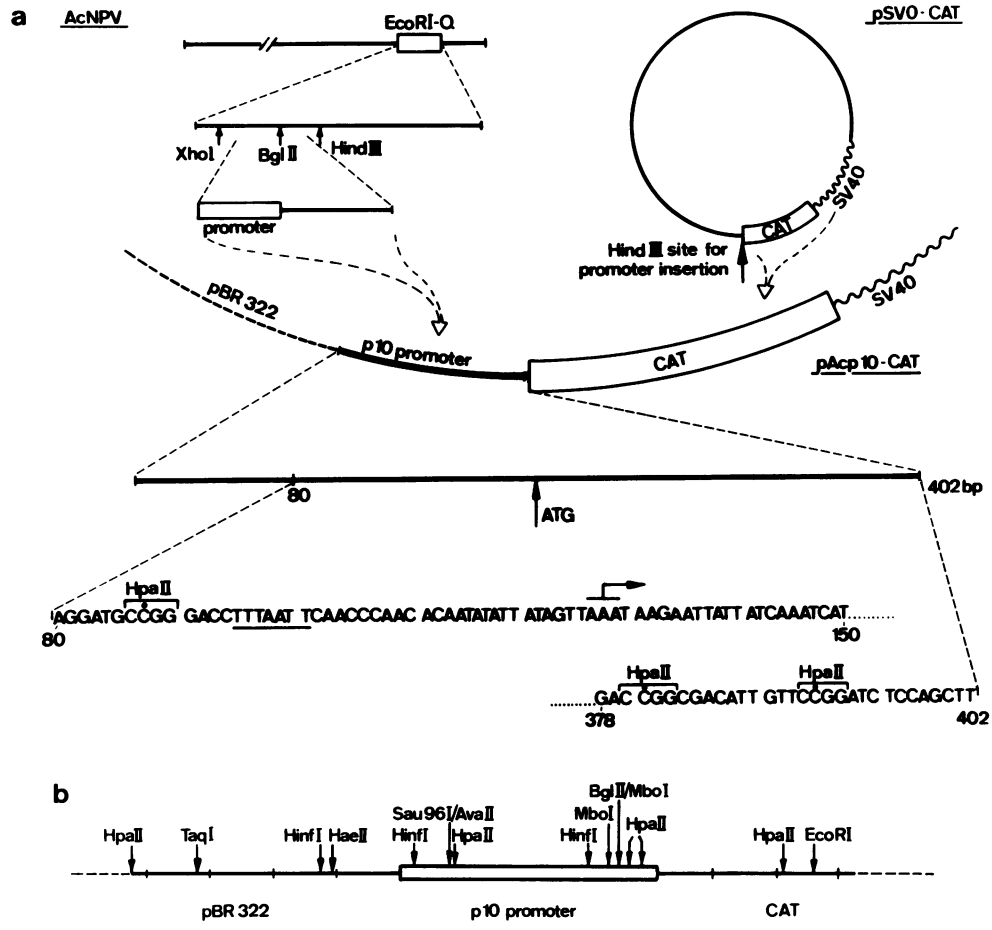

Fig. 1. The pAcp10-CAT construct. The promoter of the p10 gene of AcNPV (Lübbert and Doerfler, 1984b) was inserted into the *Hind*III site of the pSVO-CAT construct using *Hind*III linkers (Gorman *et al.*, 1982). **(a)** Construction plan. Maps of the *Eco*RI-Q fragment of the AcNPV genome (top left), and of the pSVO-CAT plasmid DNA were presented. Part of the promoter sequence with the only 5'-CCGG-3' (*Hpa*II) site and the cap site (AAA↓) were shown. Part of the coding sequence of the p10 gene (ATG, *Hpa*II sites) is shown. Part of the promoter nucleotide sequence was reconfirmed using conventional methods for sequence determination (Maxam and Gilbert, 1977, 1980). **(b)** Restriction map of the construct. Strategic restriction sites for mapping and sequencing were indicated. The DNA was cut with restriction endonucleases as indicated, and fragments were separated by electrophoresis on a 1.5% agarose gel and visualized by photography using a u.v. illuminator and a Polaroid MP4 Land camera.

p10 gene of AcNPV DNA in insect cells were studied. The promoter was inserted by standard procedures into the pSVO-CAT construct (Gorman *et al.*, 1982). Promoter activities were tested in mammalian, as well as in uninfected and AcNPV-infected insect cells. Since the p10 gene was abundantly expressed late in AcNPV-infected cells, it was expected that other viral functions were required for its expression. Moreover, the presence of only one 5'-CCGG-3' site 40 nucleotides upstream from the cap site permitted us to determine the effect of site-specific DNA methylation on promoter activity in insect cells. There were two additional 5'-CCGG-3' sites 178 and 192 bp downstream from the ATG initiation codon of the p10 gene. Studies on the effects of site-specific DNA methylations on an AcNPV promoter in an insect cell system were of particular interest, since it was still questionable whether insect DNA contained 5-mC.

Characteristics of the pAcp10-CAT construct

The promoter insert in the pAcp10-CAT construct was analyzed both by restriction cleavage and by sequence determination (Lübbert and Doerfler, 1984b). The scheme in Figure 1a outlines the origin and some of the structural features of this construct. The *Hga*I subfragment from the *Xho*I-*Hind*III fragment in the *Eco*RI-Q segment of AcNPV DNA (Figure 1a) was cloned into the *Hind*III site of pSVO-CAT with *Hind*III linkers. The results of the restriction analyses (Figure 1b) and nucleotide sequence determinations (Figure 1a) established the schemes shown in Figure 1a. The sequence shown (Lübbert and Doerfler, 1984b) was reconfirmed for part of the promoter in the pAcp10-CAT construct by nucleotide sequence determination both from the proximal and distal ends of the promoter insert. A total of 290 and 150 nucleotides was determined, respectively.

Transfection of the pAcp10-CAT construct into mammalian and insect cells

The pAcp10-CAT construct was inactive after transfection into human HeLa cells or into uninfected *S. frugiperda* insect cells (Figures 2, 3). However, the pAcp10-CAT gene construct was expressed in *S. frugiperda* insect cells when they had been infected with AcNPV prior to transfection. The scheme presented in Figure 2a describes the design of experiments in which *S. frugiperda* cells were infected with AcNPV at a multiplicity of 10 p.f.u./cell and were subsequently transfected with 20 μg of pAcp10-CAT DNA at 1, 6, 12 and 18 h after infection. An uninfected culture was also transfected. As a negative control, AcNPV-infected cells were transfected with pSVO-CAT DNA at 18 h after infection. At 45 h after the last (18 h p.i.) transfection experiment, all cultures were harvested, cell extracts were prepared and assayed for CAT activity. The results obtained

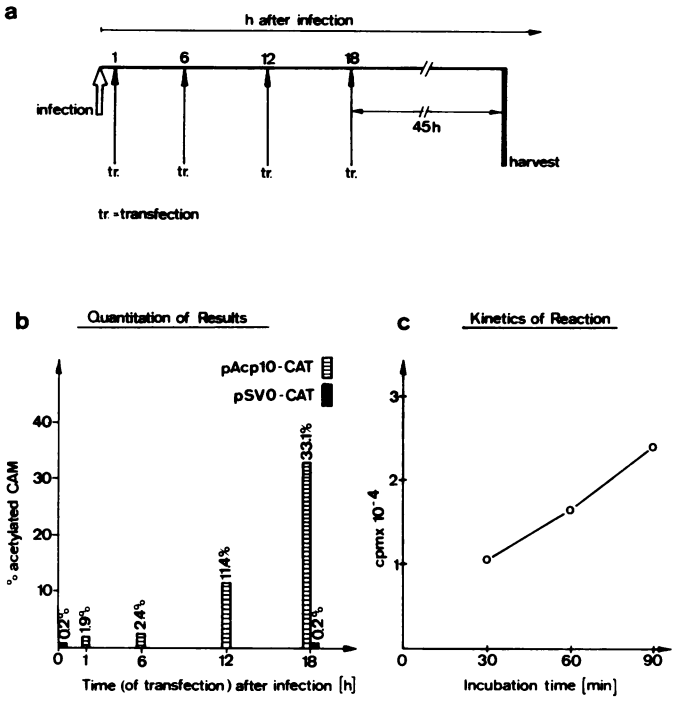

Fig. 2. Design of transfection experiments. Transfection of AcNPV-infected *S. frugiperda* insect cells with the pAcp10-CAT construct. The experimental design **(a)** and the results of a series of experiments **(b)** were shown in which AcNPV-infected *S. frugiperda* cells were transfected at different times after infection. Most of the experimental details were described in the text. The results of the transfection experiment with pSVO-CAT in *S. frugiperda* cells 18 h after infection with AcNPV were also presented. Part **(c)** depicted the time curve of a typical CAT reaction.

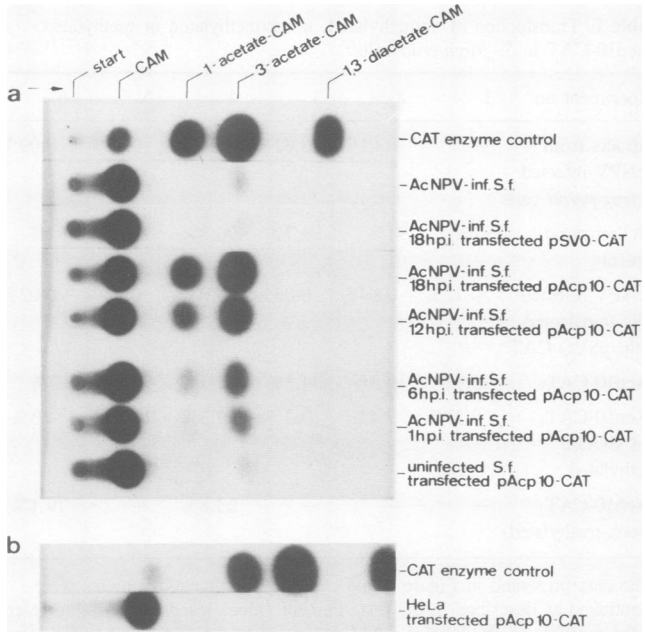

Fig. 3. The pAcp10-CAT construct is expressed in AcNPV-infected cells, but not in uninfected insect cells or human HeLa cells. *S. frugiperda* cells were infected with AcNPV as described. At time periods after infection as indicated, the cells were transfected with the pAcp10-CAT construct. At 45 h after the 18 h infection experiments, extracts of cells were prepared and incubated with ^{14}C-labeled chloramphenicol (CAM). CAM and its acetylation products were separated by chromatography on silica gel plates and visualized by autoradiography. The quantitation of the data shown here has been presented in part in Figure 2b. The results of a CAT enzyme control, in which commercial enzyme was used, and the results of experiments with extracts from non-transfected cells or with AcNPV-infected *S. frugiperda* cells transfected with the pSVO-CAT construct were shown, as well as the results of an experiment in which human HeLa cells were transfected with pAcp10-CAT.

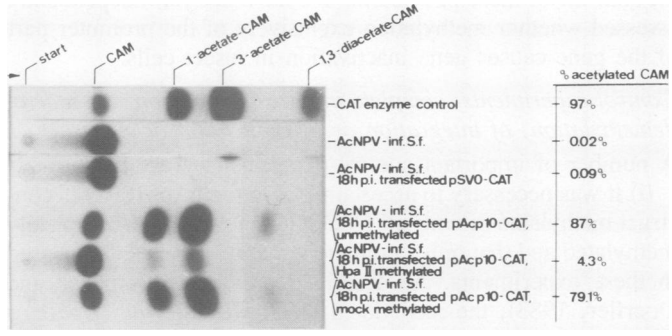

Fig. 4. Inactivation of the pAcp10-CAT construct by methylation of the 5′-CCGG-3′ site in the promoter of the p10 gene construct. Experimental details have been described in the text. An autoradiogram of a silica gel chromatogram has been shown. The quantitation of the data presented here was also included in Table I (experiment 6).

(Figures 2b, 3) were quantitated by assessing the ^{14}C activities in acetylated forms of chloramphenicol which were separated by t.l.c. (Materials and methods). The results were plotted as percent values of ^{14}C-labeled chloramphenicol converted to acetylated forms. In control experiments, ^{14}C-labeled chloramphenicol was incubated with purified CAT which converted ~97% of the chloramphenicol added to acetylated forms (Figures 3, 4, Table I). A 60 min incubation period was chosen for the CAT assay, since this time was in the linear range of the reaction (Figure 2c). When the time after infection, at which cells were transfected, was extended beyond 18 h, the yields of acetylated chloramphenicol started to decrease. The results presented for the 1, 6 and 12 h time points (Figure 2b) did not change significantly when the cells were harvested 24 h after the actual transfection for each time point. For all subsequent experiments, AcNPV-infected *S. frugiperda* insect cells were transfected at 18 h after infection. The data presented indicated that early and/or late viral gene functions were required to activate the p10 gene promoter. This transfection system might offer a tool to investigate the nature of these supplementary functions. The p10 gene promoter was inactive in uninfected insect cells or in mammlian cells.

Methylation of 5′-CCGG-3′ sites in the p10 gene promoter affects the activity of the pAcp10-CAT construct in infected insect cells

The *in vitro* methylation of the 5′-CCGG-3′ site located 40 nucleotides upstream from the cap site of the p10 gene promoter in the pAcp10-CAT construct leads to the inactivation or a very marked decrease in the activity of this promoter in AcNPV-infected *S. frugiperda* cells (Figure 4, Table I). The insert in the pAcp10-CAT construct also contained two additional 5′-CCGG-3′ sites which were located 178 bp and 192 bp downstream from the ATG initiation codon inside the p10 gene. Methylation of these sites might contribute to the shut-off of the p10 gene promoter, although this possibility has not been tested yet. There was some variation in the extent to which the activity of the pAcp10-CAT construct was decreased by methylation of the 5′-CCGG-3′ sites (Table I). Nevertheless, it was clear that in AcNPV-infected *S. frugiperda* insect cells the activity of the baculovirus promoter of the p10 gene was sensitive to site-specific DNA methylation. It was also shown that the activity of the pAcp10-CAT construct was not affected by mock-methylations (Table I).

Table I. Transfection of unmethylated, mock-methylated or methylated pAcp10-CAT in *S. frugiperda* cells[a]

Experiment no.	1	2	3	4	5	6
Extracts from AcNPV-infected *S. frugiperda* cells	–	0.01%	0.02%	0.1%	0.13%	0.09%
CAT enzyme control	97%	97%	98.7%	96.8%	93.2%	97%
AcNPV-infected cells transfected with: pSVO-CAT	0.12%	0.04%	0.03%	0.08%	0.04%	0.02%
pAcp10-CAT	36.9%	19.3%	34.5%	15.1%	79.8%	87%
pAcp10-CAT, 5′-CCGG-3′ methylated	0.27%	9.4%	10.2%	9.3%	10.4%	4.3%
pAcp10-CAT (mock-methylated)	–	–	–	23.8%	–	79.1%

[a]The data presented in Figure 4 and results of similar experiments were quantitated as described in the text. Percent values listed in the Table refer to the portions of ^{14}C-labeled CAM converted to the acetylated forms. The results in experiment 6 are those shown in Figure 4. Experiments 1 – 5 describe similar experiments which were performed to test the reproducibility of the system. The mock-methylated construct was incubated with *Hpa*II DNA methyltransferase in the absence of SAM to demonstrate that this treatment did not affect the activity of this construct.

Methylations of 5′-CCGG-3′ sites in the 3′-located main parts of genes do not have noticeable effects on gene expression, as documented for the pSV2-CAT construct in mammalian cells (Kruczek and Doerfler, 1983) and for the E2A gene of adenovirus type 2 DNA in amphibian cells (Langner *et al.*, 1984). *In vitro* methylations of 5′-CCGG-3′ sites which are located upstream from the TATA signal in adenovirus promoters led to the shut-off of these promoters. We have not yet independently assessed whether methylation exclusively of the promoter part of the gene causes gene inactivation in insect cells.

Control experiments: complete in vitro methylation, absence of demethylation, of integration or of replication of construct

A number of important control experiments were performed.

(i) It was necessary to ascertain that in the pAcp10-CAT construct methylated *in vitro* all 5′-CCGG-3′ sites were completely methylated and that only completely methylated DNA was used in these experiments. As described previously (Kruczek and Doerfler, 1983), the methylated DNA was cut with *Hpa*II or *Msp*I, the fragments were separated by electrophoresis on 1.2% agarose gels, transferred to nitrocellulose filters (Southern, 1975) and hybridized with ^{32}P-labeled pAcp10-CAT DNA. Complete methylation of all 5′-CCGG-3′ sites in the construct was shown by sensitivity of the DNA to *Msp*I and refractoriness to *Hpa*II (data not shown, cf. Figure 5, lanes 8,9). The *in vitro* methylated pAcp10-CAT DNA was also resistant to *Hpa*II cleavage, when the construct was previously cut with *Eco*RI. Thus, resistance to *Hpa*II cleavage could not have been due to structural constraints on the plasmid.

We could also show that the pAcp10-CAT construct in the total nuclear DNA re-extracted from *S. frugiperda* insect cells 48 h after transfection had remained refractory to *Hpa*II and sensitive to *Msp*I cleavage as was apparent from blot hybridization experiments (Figure 5, lanes 8 – 10). Thus, there was no evidence that during the 48 h period of the transfection experiment, the *in vitro* methylated pAcp10-CAT construct had become demethylated.

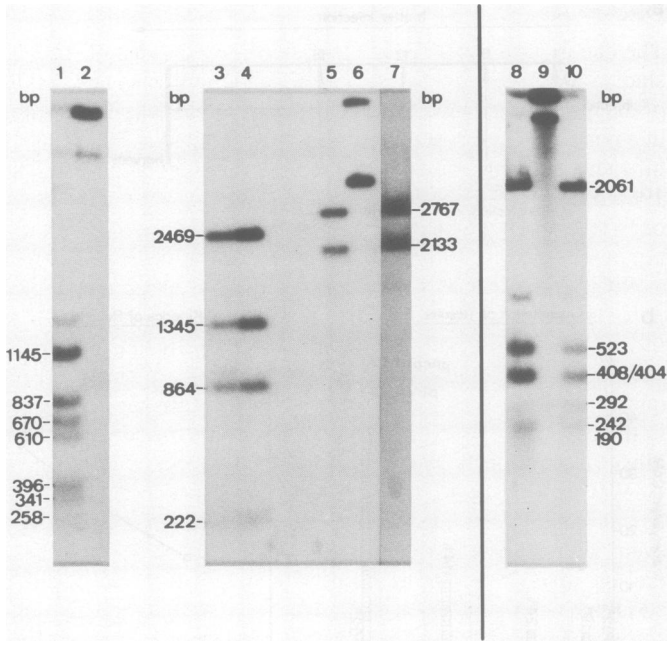

Fig. 5. The pAcp10-CAT construct DNA, which was transfected into AcNPV-infected *S. frugiperda* insect cells was not demethylated or integrated, nor did it replicate. The intranuclear DNA from AcNPV-infected and subsequently pAcp10-CAT transfected *S. frugiperda* insect cells (designated as 'cellular DNA' below) was isolated 48 h after transfection and cleaved with various restriction endonucleases. As internal controls, pAcp10-CAT plasmid DNA was cut with the same enzymes. Fragments were separated on 1.5% agarose gels, transferred to nitrocellulose filters and hybridized to pAcp10-CAT which was ^{32}P-labeled by nick-translation. Autoradiograms of the filters after hybridization were presented. Fragment sizes in base pairs were indicated. pAcp10-CAT construct DNA cut with *Dpn*I **(1)**, *Ava*II **(4)**, uncut **(6)**, *Eco*RI **(7)**, or *Hpa*II **(10)**. 'Cellular DNA' (see above) cut with *Mbo*I **(2)**, *Ava*II **(3)**, *Eco*RI **(5)**, *Msp*I **(8)** or *Hpa*II **(9)**.

(ii) The same experiment and a similar analysis, in which the intracellular DNA extracted from *S. frugiperda* insect cells 48 h after transfection with pAcp10-CAT DNA was cleaved with *Eco*RI (Figure 5, lanes 5 and 7) or *Ava*II (Figure 5, lanes 3 and 4), failed to provide evidence for integration of the construct DNA into insect cell DNA at specific sites. All pAcp10-CAT fragments from cellular DNA co-migrated with those from marker construct DNA which had not been transfected but had been cut with the same enzymes and had been co-electrophoresed on the same gel. Hence, there was no reason to assume that the transfected CAT gene could have come under the control of an insect cell promoter.

(iii) Lastly, we could rule out the possibility that the pAcp10-CAT construct had replicated in *S. frugiperda* insect cells after transfection. The construct had originally been propagated on an adenine methylation-proficient (dam$^+$) *Escherichia coli* strain, hence all 5′-GATC-3′ sequences were adenine methylated. Had the construct replicated in *S. frugiperda* cells during the time of the experiment, the construct DNA would have become unmethylated, and hence refractory to the restriction endonuclease *Dpn*I which cuts only the sequence 5′-G^{m}ATC-3′ (Lacks and Greenberg, 1975). DNA extracted from *S. frugiperda* cells 48 h after the transfection with the pAcp10-CAT construct contained this construct in a *Mbo*I-resistant form as shown by blot hybridization (Figure 5, lane 2). Thus, the 5′-G^{m}ACT-3′ sites had not become demethylated, i.e., the construct DNA had not replicated in the insect host cell. We concluded that, within a 48 h period, the transfected pAcp10-CAT construct was not demethylated, integrated at specific sites or replicated in *S. frugiperda* insect cells.

Discussion

The baculovirus AcNPV, at the molecular level one of the best studied DNA insect viruses, is well suited to investigate details of the mechanisms involved in the expression of the viral genome in a non-vertebrate host. The viral RNA produced in productively infected cells is probably not spliced (Lübbert and Doerfler, 1984a), and overlapping, nested sets of RNAs with common 3′ or 5′ termini are generated in various parts of the genome (Lübbert and Doerfler, 1984b). In the present study, we have started to elucidate functional properties of the p10 gene promoter which governs the expression of one of the major late gene products of AcNPV in productively infected insect cells.

This viral promoter contains ~70% AT base pairs and requires for its activity – at least after transfection into insect cells in the pAcp10-CAT construct – the presence of one or several other viral gene products. It is presently unknown whether these AcNPV products are acting directly on the p10 gene promoter or indirectly *via* the mediation of cellular gene products. In any event, the experimental system described here may offer a suitable tool to investigate the nature and genomic location of these additional viral functions.

Site-specific DNA methylation as a modulator of gene activity has been considered a phylogenetically late addition to the repertoire of mechanisms regulating the expression of genes in higher eukaryotes, such as vertebrates. The available evidence indicates that the introduction of methyl groups at specific promoter and 5′ sequences serves to inactivate a gene for a long time, perhaps permanently (for reviews, cf. Doerfler, 1983, 1984a, 1984b). It is questionable whether 5-mC would play a similarly prominent regulatory role in *Drosophila* whose DNA may contain 5-mC in exceedingly small amounts (Achwal *et al.*, 1984), if at all. Similarly, we have shown that the DNA of *S. frugiperda* host cells used in this study did not contain amounts of 5-mC or N^6-mA detectable by reverse-phase h.p.l.c. (T.Müller and W.Doerfler, unpublished data). It was, therefore, of considerable interest to investigate whether methylation of the 5′-CCGG-3′ site in the promoter sequence of a late AcNPV gene would lead to the inactivation of this promoter in insect cells. The possibility of elucidating that problem by using the pAcp10-CAT construct prompted the series of experiments reported here. The results described imply that 5-mC is recognized as a regulatory signal even in insect cells. The results do not prove that the same signal does in fact play a regulatory role in arthropods, but our data attest to the concept that 5-mC can be generally recognized in the regulation of eukaryotic genes.

The juxtaposition of results on the activation of the p10 gene promoter in AcNPV-infected insect cells by specific viral functions, and on the inactivation of this promoter by site-specific methylation was not necessarily meant to imply that the two events were mechanistically linked, although this possibility could not be ruled out. It was rather that we had to devise a system in which the inactivation of a previously active promoter could be demonstrated, hence a system had to be sought in which the p10 promoter was actually expressing.

It is unknown whether methylation of one 5′-CCGG-3′ site at a location 40 bp upstream from the cap site of the p10 gene was sufficient for gene inactivation or whether the two sites downstream from the ATG initiation codon would also play a role. There is evidence that the methylation of 5′-CCGG-3′ sites in the 3′- and coding regions of eukaryotic genes have no effect on gene activity, at least in mammalian cells (Kruczek and Doerfler, 1983; Langner *et al.*, 1985). The scheme shown in Figure 6 summarizes *in vitro* methylation data gleaned from the adenovirus and baculovirus systems and the γ-globin gene. It appears that the inhibitory function of methylation at 5′-CCGG-3′ sites is exerted mainly when this signal is introduced in positions upstream from the TATA or cap sites of a viral promoter. It is unknown whether these results can be extrapolated to other eukaryotic systems. However, results reported by Busslinger *et al.* (1983) for the γ-globin gene are consistent with this interpretation.

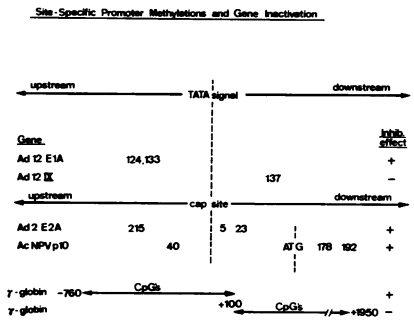

Fig. 6. Summary of experimental results on specific promoter methylations. The promoter regions of various viral genes and of the γ-globin genes are presented schematically. Some of the important features of promoters have been indicated. The numbers refer to the distances in nucleotides for individual 5′-CCGG-3′ sequences from the TATA signal, the cap site, or the ATG initiation codon as indicated for individual promoters. These 5′-CCGG-3′ sites had been *in vitro* methylated. In the γ-globin gene many cytidine residues were methylated. 'Inhibitory effect + or –' indicates whether such *in vitro* methylations affected or did not affect gene activity. Some of the data schematically summarized were presented previously: Ad12, E1A, Ad12 IX (Kruczek and Doerfler, 1983), Ad2 E2A (Langner *et al.*, 1984), γ-globin gene (Busslinger *et al.*, 1983).

Further work will be directed towards fixing the pAcp10-CAT construct in the methylated or unmethylated form in the genome of the insect host cell and then to assess the activity of the p10 gene. We also seek to investigate what other AcNPV functions are required for the activity of the p10 gene promoter.

Materials and methods

Cells and virus

Origin and culture conditions of the lepidopteran cell line *S. frugiperda* have been described earlier (Tjia *et al.*, 1979, 1983). HeLa cells were grown as monolayer cultures in the Dulbecco modification of Eagle medium (Bablanian *et al.*, 1965) supplemented with 10% fetal bovine serum.

The insect baculovirus AcNPV was propagated on monolayer or suspension cultures of *S. frugiperda* cells (Lübbert and Doerfler, 1984a). For the inoculation of *S. frugiperda* cells, extracellular virus, i.e., medium from infected cells, was used.

Infection of S. frugiperda cells with AcNPV

S. frugiperda cells, which were eventually transfected with plasmid constructs, were AcNPV-infected as 50–60% confluent monolayer cultures. AcNPV inocula were allowed to adsorb for 1–2 h. Subsequently, the cells were washed and maintained in medium (Gardiner and Stockdale, 1977) containing 10% fetal calf serum. At various times after inoculations (cf. Figures 2, 3, 4), infected complexes or uninfected control cells were transfected (see below).

Propagation and purification of bacterial plasmids

The pBR322-cloned *Eco*RI-Q fragment of AcNPV DNA (Lübbert *et al.*, 1981), the pSVO-CAT (Gorman *et al.*, 1982), and the pAcp10-CAT constructs were propagated on *E. coli* strain HB101/λ, and purified by equilibrium sedimentation in CsCl-ethidium bromide density gradients (Clewell and Helinski, 1972).

Construction of the pAcp10-CAT plasmid

The *Eco*RI-Q fragment of AcNPV DNA was cut with *Hga*I (Figure 1a). The fragments generated were treated with 100 units of S1 nuclease (Lübbert and Doerfler, 1984a) to remove protruding termini and were subsequently phenolized and precipitated with ethanol. *Hind*III linkers (Bethesda Research Laboratories) were ligated onto the blunt ends thus generated and subsequently cut with *Hind*III. The *Hga*I fragment containing the p10 promoter (Figure 1a) was purified by electrophoresis on a 5% polyacrylamide gel and identified by its size deduced from

the known sequence (Lübbert and Doerfler, 1984b). The fragment was excised, eluted from the gel (Deuring and Doerfler, 1983), and was finally ligated into the *Hind*III site of pSVO-CAT (Gorman *et al.*, 1982) which had been cut with *Hind*III and pre-treated with calf intestine phosphatase (2.8 units/μg). DNA was then transfected into HB101/λ cells. The correct plasmid construct was identified by restriction analyses using *Bgl*II, *Hin*fI, *Hpa*II and *Sau*96I (Figure 1b) and was designated pAcp10-CAT to indicate that it contained the p10 gene promoter of AcNPV DNA.

Control of the nucleotide sequence of the p10 promoter insert in pAcp10-CAT

Parts of the promoter insert were re-sequenced to ascertain the promoter structure. To determine the proximal promoter sequence, the DNA was *Ava*II cut (Figure 1b). The 3′ termini generated were ^{32}P-labeled using the Klenow fragment of DNA polymerase I and [α-^{32}P]dNTPs, and the DNA was then cut with *Taq*I. The 394-bp fragment containing this segment was purified by electrophoresis on a 5% polyacrylamide gel. The nucleotide sequence was determined by the Maxam-Gilbert procedure (Maxam and Gilbert, 1977, 1980). Similarly, the distal promoter sequence was determined upon *Sau*96I cleavage and 5′ labeling of the construct using polynucleotide kinase and [γ-^{32}P]ATP followed by *Eco*RI cleavage (Figure 1b) and isolation of the 567-bp fragment as described above.

In vitro methylation of the pAcp10-CAT construct

The purified pAcp10-CAT construct was *in vitro* methylated using the *Hpa*II DNA methyltransferase (4 units/μg of DNA) (Biolabs, Beverly, MA) following a protocol previously published (Kruczek and Doerfler, 1983). There are one 5′-CCGG-3′ (*Hpa*II) site 40 nucleotides upstream from the cap site of this gene and two such sites 178 and 192 bp downstream from the ATG codon within the coding sequence of the p10 gene (Figure 1a, and Lübbert and Doerfler, 1984b). The promoter lacks *Hha*I sites. It had to be ascertained that the plasmid construct was completely methylated (Vardimon *et al.*, 1982; Kruczek and Doerfler, 1983; Langner *et al.*, 1984). As a test, *in vitro* methylated DNA was cleaved with *Msp*I or *Hpa*II (Waalwijk and Flavell, 1978), the fragments were separated by electrophoresis on a 1.2% agarose gel, blotted (Southern, 1975), and hybridized to pAcp10-CAT DNA ^{32}P-labeled by nick-translation (Rigby *et al.*, 1977). In control experiments, the pAcp10-CAT plasmid was mock-methylated with *Hpa*II DNA methyltransferase under standard conditions, except that the methyl donor S-adenosylmethionine (SAM) was omitted.

Assays for absence of intracellular replication, integration or demethylation

At 48 h after the transfection of *S. frugiperda* cells, the total nuclear DNA was isolated (Sutter *et al.*, 1978). The DNA was then incubated with *Dpn*I (Lacks and Greenburg, 1977) to check for replication of the construct in *S. frugiperda* cells. Unreplicated DNA should have been sensitive to *Dpn*I cleavage at 5′-G^{m}ATC-3′ sequences since the construct had been grown on an adenine methylation-proficient host (HB101/λ) and would have maintained its methylated pattern in mammalian cells, unless it had replicated. The DNA was also incubated with *Mbo*I. Unreplicated DNA which had remained adenine methylated in the sequence 5′-G^{m}ATC-3′, should prove refractory to the action of this enzyme. Alternatively, the DNA was cut with *Eco*RI, *Ava*II or with *Hpa*II or *Msp*I. Subsequently, DNA fragments were separated by electrophoresis on 1.5% agarose gels, transferred to nitrocellulose filters (Southern, 1975), and hybridized to ^{32}P-labeled pAcp10-CAT DNA followed by autoradiography.

Transfection of cells with pAcp10-CAT or pSVO-CAT DNA and CAT assay

Published procedures (Graham and van der Eb, 1973; Gorman *et al.*, 1982) were used as detailed previously (Kruczek and Doerfler, 1983). The conversion of ^{14}C-labeled chloramphenicol (CAM) to acetylated products was measured by incubating CAM with extracts of cells prepared 48 h after transfection followed by CAM extraction with acetyl acetate, t.l.c. using silica gel sheets (Macherey-Nagel, Düren, FRG) and autoradiography. For quantitation, radioactive spots were excised from the chromatogram, and the ^{14}C-activity in each spot was determined by scintillation counting. As a positive control, ^{14}C-labeled CAM was incubated with commercial CAT enzyme (PL Biochemicals, Milwaukee, WI), and the conversion of CAM to its acetylation products was determined.

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References

Achwal,C.W., Ganguly,P. and Chandra,H.S. (1984) *EMBO J.*, **3**, 263-266.
Adang,M.J. and Miller,L.K. (1982) *J. Virol.*, **44**, 782-793.
Bablanian,R., Eggers,H.J. and Tamm,I. (1965) *Virology*, **26**, 100-113.
Busslinger,M., Hurst,J. and Flavell,R.A. (1983) *Cell*, **34**, 197-206.
Clewell,D.B. and Helinski,D.R. (1972) *J. Bacteriol.*, **110**, 1135-1146.
Cochran,M.A., Carstens,E.B., Eaton,B.T. and Faulkner,P. (1982) *J. Virol.*, **41**, 940-946.
Deuring,R. and Doerfler,W. (1983) *Gene*, **26**, 283-289.
Doerfler,W. (1983) *Annu. Rev. Biochem.*, **52**, 93-124.
Doerfler,W. (1984a) *Adv. Viral Oncol.*, **4**, 217-247.
Doerfler,W. (1984b) *Curr. Top. Microbiol. Immunol.*, **108**, 79-98.
Doerfler,W., Langner,K.-D., Kruczek,I., Vardimon,L. and Renz,D. (1984) in Razin,A., Riggs,A.D. and Cedar,H. (eds.), *DNA Methylation*, Springer Verlag, NY, pp. 221-247.
Eick,D., Fritz,H.-J. and Doerfler,W. (1983) *Anal. Biochem.*, **135**, 165-171.
Gardiner,G.R. and Stockdale,H. (1975) *J. Invert. Pathol.*, **25**, 363-370.
Gorman,C.M., Moffat,L.F. and Howard,B.H. (1982) *Mol. Cell. Biol.*, **2**, 1044-1051.
Graham,F.L. and van der Eb,A.J. (1973) *Virology*, **54**, 536-539.
Hooft van Iddekinge,B.J.L., Smith,G.E. and Summers,M.D. (1983) *Virology*, **131**, 561-565.
Kruczek,I. and Doerfler,W. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 7586-7590.
Kuzio,J., Rohel,D.Z., Curry,C.J., Krebs,A., Carstens,E.B. and Faulkner,P. (1984) *Virology*, **139**, 414-418.
Lacks,S. and Greenberg,B. (1975) *J. Biol. Chem.*, **250**, 4060-4066.
Langner,K.-D., Vardimon,L., Renz,D. and Doerfler,W. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 2950-2954.
Langner,K.-D., Weyer,U. and Doerfler,W. (1985) submitted.
Lübbert,H. and Doerfler,W. (1984a) *J. Virol.*, **50**, 497-506.
Lübbert,H. and Doerfler,W. (1984b) *J. Virol.*, **52**, 255-265.
Lübbert,H., Kruczek,I., Tjia,S.T. and Doerfler,W. (1981) *Gene*, **16**, 343-345.
Maxam,A.M. and Gilbert,W. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 560-564.
Maxam,A.M. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499-560.
Rigby,P.W.J., Dieckmann,M., Rhodes,C. and Berg,P. (1977) *J. Mol. Biol.*, **113**, 237-251.
Riggs,A.D. and Jones,P.A. (1983) *Adv. Cancer Res.*, **40**, 1-30.
Rohel,D.Z. and Faulkner,P. (1984) *J. Virol.*, **50**, 739-747.
Rohel,D.Z., Cochran,M.A. and Faulkner,P. (1983) *Virology*, **124**, 357-365.
Simon,D., Stuhlmann,H., Jähner,D., Wagner,H., Werner,E. and Jaenisch,R. (1983) *Nature*, **304**, 275-277.
Smith,G.E., Vlak,J.M. and Summers,M.D. (1982) *J. Virol.*, **44**, 199-208.
Smith,G.E., Vlak,J.M. and Summers,M.D. (1983) *J. Virol.*, **45**, 215-225.
Southern,E.M. (1975) *J. Mol. Biol.*, **98**, 503-517.
Sutter,D., Westphal,M. and Doerfler,W. (1978) *Cell*, **14**, 569-585.
Taylor,J.H. (1984) *DNA Methylation and Cellular Differentiation*, published by Springer Verlag, Wien/NY.
Tjia,S.T., Carstens,E.B. and Doerfler,W. (1979) *Virology*, **99**, 399-409.
Tjia,S.T., Meyer zu Altenschildesche,G. and Doerfler,W. (1983) *Virology*, **125**, 107-117.
Vardimon,L., Günthert,U. and Doerfler,W. (1982) *Mol. Cell. Biol.*, **2**, 1574-1580.
Vlak,J.M., Smith,G.E. and Summers,M.D. (1981) *J. Virol.*, **40**, 762-771.
Waalwijk,C. and Flavell,R.A. (1978) *Nucleic Acids Res.*, **5**, 3231-3236.

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