

Differential Factor Binding at the Promoter of Early Baculovirus Gene PE38 during Viral Infection: GATA Motif Is Recognized by an Insect Protein

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Received 23 January 1992/Accepted 3 March 1992

Regulatory elements interacting with DNA-binding proteins have been investigated in the promoter sequence of the early PE38 gene in the *Autographa californica* nuclear polyhedrosis virus (AcNPV). A GATA motif located 50 nucleotides upstream of the PE38 transcriptional start site is recognized differentially in the course of infection. As demonstrated by footprint and gel mobility shift assays, the GATA sequences TTATCT are protected by nuclear extracts from uninfected *Spodoptera frugiperda* cells and from *S. frugiperda* cells early postinfection (p.i.) but not by *S. frugiperda* cell extracts isolated 40 h p.i. We have compared the binding capacity of the insect GATA-like protein with that of the vertebrate GATA-1 factor identified as erythroid-specific factor. Our results indicate that a factor present in mouse erythroleukemia cells, presumably GATA-1, can bind to the insect GATA motif and vice versa. Evidence from transient expression studies suggests that the mutated GATA sequences do not influence PE38 promoter activity in cell culture.

The baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) is an insect DNA virus with the potential to encode about 100 genes. Viral gene expression is coordinately regulated and sequentially ordered in a cascade fashion (for reviews, see references 1, 6, and 10). To date, the molecular mechanism by which temporal regulation of AcNPV gene expression occurs is poorly understood. Since baculoviruses are used as highly efficient gene expression vectors as well as a model system to elucidate viral strategies in a nonvertebrate host, viral regulation of gene expression is of considerable interest.

At least two AcNPV genes, immediate early gene 1 (IE1) and immediate early gene N (IEN), are able to stimulate expression of other viral genes in a transient expression system (2, 17). *cis*-Acting regulatory elements in the promoter of the early 35K gene have been identified by analyzing recombinant viruses carrying a reporter gene under the control of the mutated 35K promoter (5). From this and other studies it has been suggested that the control elements for the early baculovirus promoters differ substantially from those for late promoters. The results of *in vitro* transcription experiments with the early transcribed baculovirus gene gp64 (20) support the notion that the baculovirus early promoters are recognized by the DNA-dependent RNA polymerase II of the host.

In an attempt to gain some understanding of how the early-to late-phase transition in viral gene expression is effected, we have initiated studies aimed at the identification of early viral regulatory genes. Recently, we described the PE38 gene, representing one of the major early transcripts. On the basis of data on the sequence, which includes a novel zinc finger motif (18), we postulate that PE38 might have a regulatory function (22). The objective of the study presented here was to detect the regulatory elements upstream of the PE38 gene interacting with DNA-binding proteins in response to viral infection. Our findings indicate differential

factor binding at the PE38 promoter when nuclear extracts of uninfected and infected (40 h postinfection [p.i.]) *Spodoptera frugiperda* cells are used. One of the sequence motifs protected by a factor(s) present in uninfected and early-phase infected *S. frugiperda* cells but missing in extracts of late-phase infected *S. frugiperda* cells includes a GATA motif.

The hexanucleotide A/T GATA T/C has been found to be recognized not only by a number of vertebrate proteins but also by invertebrate and fungal proteins, all containing highly conserved zinc finger motifs (7, 12, 23, 35, 36, 39). The best-characterized member of the GATA family is the erythroid-specific GATA-1 factor, formerly Eryf1 (8), GF-1 (26), or NF-E1 (42), which has been detected in human, mouse, and chicken erythroid cells (29). From our data, we conclude that an insect GATA-binding protein detectable in uninfected and early-phase infected *S. frugiperda* cells but not detectable during the very late phase of AcNPV infection interacts with a *cis*-acting element in the early transcribed PE38 promoter.

MATERIALS AND METHODS

Cells and viruses. *S. frugiperda* IPLB21 cells (41) were grown as monolayer cultures in TC100 medium supplemented with 10% fetal calf serum (14). Monolayers of *S. frugiperda* cells were inoculated with AcNPV plaque isolate E (38) at a multiplicity of 10 PFU per cell, as described earlier (22). Mouse erythroleukemia (MEL) cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum.

Plasmid constructions and oligonucleotides. Two PE38 promoter fragments and two oligonucleotides were fused to the prokaryotic chloramphenicol acetyltransferase (CAT) gene in pBLCAT3 (25): the *AccI*-*AccI* fragment of *BglI*-K (98.0 to 98.7 map units), including the transcriptional start site of PE38, was blunt ended by fill-in reaction with Klenow polymerase and was first cloned into the *SmaI* site of pBluescriptKS (+). The cloned *AccI*-*AccI* fragment was

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digested with *StyI* and blunt ended by *S1* nuclease, and the *BamHI* site in the polylinker was cleaved. The resulting fragment, comprising 207 nucleotides of the PE38 promoter, was inserted into the *BamHI* and blunt-ended *XhoI* sites of pBLCAT3 (pPE38-CAT207). The plasmid pPE38-CAT207 was digested with *PstI* and religated, thereby removing 98 nucleotides of the 5' part of the PE38 promoter (pPE38-CAT109).

Oligonucleotides were synthesized on an Applied Biosystems 381 A DNA synthesizer. Complementary pairs were annealed at 37°C for 25 min after being heated at 90°C for 10 min and used as competitors or probes. The oligonucleotides Ac-GATA, Ac-sGATA, MUT4, and GATA-1 are shown in Fig. 5 and 7, and the others are as follows:

Ad12MLP	5'AGGCATTTCCAGGTGGGGAAAAATGGTGGTGGCG3';
AcNPVSC	5'TCACCAACTCGTAAGCACAGTTTCGTTGTGAAGTG3';
BCF1	5'TTTGTCAATTGAATTGTTTCTTATCTCAAGGTG3';
I(ME53)	5'TCCTACGCATATACAATCTTATCTCTATAGAT3';
II(35K)	5'TGAGTGATCGTGTGTGTGTTATCTCTGGCAG3';
DX	5'TCAGGCGTGCAGCTATAAAGCAGGCACTCACCAACTCGTAGCAGACGTTTCGTTGTGAAGTGAACACGGGATAGCCTGGCATTCAATC3'
	CCGCACGTCGATATTTTCGTCGGTGAGTGGTTGAGCATCGTGTCAAGCAACACTTGACTTCACCTTGTGCCTATCGGACGGTAAAGTTAGAGCT

The pPE38-CATMUT plasmid was constructed by insertion of the ligated oligonucleotides DX and MUT4 into the *XhoI* and blunt-ended *BglIII* sites of pBLCAT3. The promoter constructs were all resequenced.

Preparation of nuclear extracts. Nuclear extracts were prepared from uninfected cells and at different times with AcNPV-infected *S. frugiperda* cells essentially as described by Parker and Topol (30). In contrast to the given protocol, the nuclear extract was not precipitated after nucleus lysis but was dialyzed directly against 40 mM KCl–10 mM Tris HCl (pH 8)–0.1 mM EDTA–1 mM dithiothreitol–10% glycerol for 4 h. After the dialyzed extracts were cleared by centrifugation, the aliquots were frozen in liquid N₂ and stored at –70°C. The protein concentration was usually 1 to 2 mg/ml.

DNase I footprinting. Published procedures (13, 19) were adapted. The DNA-binding reactions were carried out in a total volume of 50 µl, including 50 µg of crude nuclear extract and 5 µg of poly(dI)-poly(dC) in 5 mM Tris HCl (pH 8.0)–60 mM KCl–3 mM dithiothreitol–5% glycerol. The reaction mixtures were incubated at room temperature for 15 min prior to the addition of 0.5 to 1.5 ng of a 3'-end-labeled ³²P-DNA probe (2 × 10⁴ cpm Cerenkov). Binding was allowed to proceed at room temperature for 30 min, and then DNase I digestion was performed by adding an equal volume of 5 mM CaCl₂–10 mM MgCl₂ containing DNase I (0.2 Kunitz units). After incubation on ice for 90 s, the reaction was terminated by the addition of 50 µl of DNase I stop buffer (1% sodium dodecyl sulfate, 20 mM EDTA, 200 mM NaCl, 100 µg of tRNA per ml). The DNase I cleavage products were extracted twice with phenol-chloroform, ethanol precipitated, and analyzed by electrophoresis on a 6% polyacrylamide sequencing gel containing 7 M urea. In footprint competition assays, competitor oligonucleotides were added to the binding reaction before the extract was added.

Gel mobility shift assay and competition studies. The synthetic oligonucleotides to be tested for their interactions with proteins were 3' end labeled by fill-in reaction with Klenow polymerase and α-³²P-deoxynucleoside triphosphates. Cor-

responding to the DNA-binding conditions in the DNase I footprinting analysis, usually 2 µg of crude nuclear extract was incubated at room temperature for 20 min with 0.5 to 1.5 ng of a labeled oligonucleotide (1 × 10⁴ to 2 × 10⁴ cpm Cerenkov) in the presence of 1 µg of poly(dI)-poly(dC) in a final volume of 15 µl (9). The DNA-protein complexes were resolved by electrophoresis at 4°C on 5% (38:2, acrylamide-bisacrylamide) polyacrylamide gels in 25 mM Tris HCl–19 mM sodium borate–6.25 mM EDTA. The gels were dried and autoradiographed at –70°C, usually overnight. For competition experiments, the appropriate amounts of unlabeled, double-stranded oligonucleotide were added to the reaction mixtures. The reaction mixtures were allowed to preincubate for about 5 min at room temperature prior to

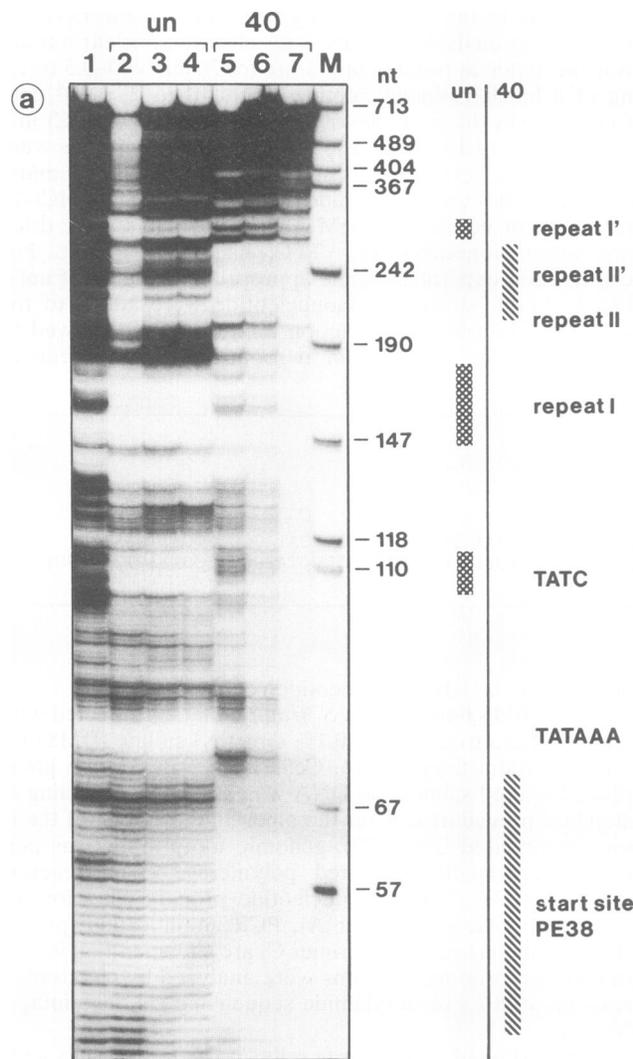
addition of the labeled nucleotide probe.

In vivo DMS footprinting. *S. frugiperda* cells infected with AcNPV were treated with 0.1% dimethyl sulfate (DMS) for 5 min at room temperature. Cells were washed with phosphate-buffered saline, and DNA was extracted according to standard procedures. After the piperidine cleavage of the in vivo-methylated DNA (27), genomic footprinting was performed by ligation-mediated polymerase chain reaction (PCR) (28, 31). The oligonucleotide primers used for the Sequenase reaction (primer A), PCR amplification (primer B), and labeling reaction (primer C) are shown in Fig. 9. The in vivo footprinting reactions were analyzed by electrophoresis on an 8% polyacrylamide sequencing gel containing 7 M urea.

Transfection of *S. frugiperda* cells. *S. frugiperda* cells were transfected with promoter-CAT gene constructs by the calcium phosphate precipitation technique according to Graham and van der Eb (16) with slight modifications. The transfection mixture included 10 to 15 µg of DNA and 180 mM CaCl₂ at final concentrations of 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.1), 140 mM NaCl, and 0.75 mM Na₂HPO₄. Before the DNA-Ca precipitates were added, cell cultures were incubated in Grace's medium and 10% fetal calf serum, which was replaced by TC100 medium and serum 18 h after transfection. Extracts were prepared 45 to 48 h after transfection, and CAT activity was determined as described earlier (15).

RESULTS

Experimental design. Transcription of the PE38 promoter is detectable as early as 1 h p.i. and decreases in the very late phase of infection (22). Early transcription of PE38 is controlled by host factors and does not require viral protein synthesis for expression (22). Since the reduction of host protein synthesis at late times p.i. has been established (3), we addressed the question of a different regulation of PE38 during AcNPV infection by identifying the regulatory elements in the PE38 promoter. We carried out footprinting and gel mobility shift assays with nuclear extracts of uninfected



or AcNPV-infected *S. frugiperda* cells to determine the PE38 promoter regions which form DNA-protein complexes. After constructing various PE38 promoter fragments in front of a reporter gene, we tested the functional significance of at least one regulatory element that has protein-binding ability.

In vitro footprinting analysis of proteins binding to the PE38 promoter. In vitro DNase I footprinting of the 665bp *HincII*-*StyI* fragment, representing the coding strand, was carried out to examine the binding of nuclear proteins to sequences preceding the transcriptional start site of PE38. In the presence of crude nuclear extracts from uninfected or infected (40 h p.i.) *S. frugiperda* cells, a striking difference in factor binding is observed. As depicted in Fig. 1, two major protected regions are visible when nuclear extracts of infected (40 h p.i.) cells are used. One region includes the early transcriptional start site of PE38, and the other region is in the inverted repeats II and II', representing the symmetrical axis of the divergent promoter unit PE38-IEN (Fig. 1c). PE38 is transcribed in a direction opposite that of the previously characterized immediate-early gene IEN (2), and both genes are active at very early times after infection. When nuclear extracts of uninfected *S. frugiperda* cells were

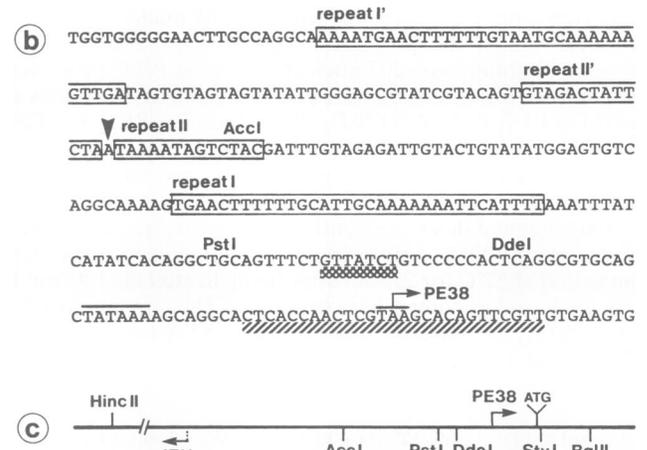


FIG. 1. In vitro DNase I footprinting of the PE38 promoter with nuclear extracts of uninfected or infected (40 h p.i.) *S. frugiperda* cells. (a) The coding strand was labeled by 3'-end filling of the *StyI* site. Prior to DNase I digestion, the labeled DNA was incubated with crude nuclear extracts of uninfected *S. frugiperda* cells (lanes un) or *S. frugiperda* cell extracts prepared 40 h p.i. (lanes 40). The control reaction mixtures containing naked DNA were digested with 0.045 U of DNase I (lane 1). For the DNA incubated with extract, 0.3 (lanes 2 and 5), 0.12 (lanes 3 and 6), and 0.06 (lanes 4 and 7) U of DNase I were used. Positions of the DNA size markers (lane M) are indicated on the right. Boxes: , protected regions with extracts of uninfected cells; , protected regions with cell extracts prepared 40 h p.i. (b) Nucleotide sequence of the PE38 promoter. A putative TATA signal is overlined, the transcriptional start site of PE38 is indicated by an arrow, the inverted repeats are boxed, and the protected sequences, deduced from the footprinting analyses, are shown. (c) Restriction map of the *HincII*-*BglII* (98.2 to 98.7 map units) fragment. The transcriptional start site of PE38 and the approximate location of the IEN start site are indicated by arrows. The translational start site of PE38 is shown.

analyzed, mainly three regions were protected. These regions are located in the inverted repeats I' and I and between nucleotides -50 and -56 relative to the transcriptional start (Fig. 1). Similar results were obtained with extracts from cells 3 h p.i. (data not shown) but not with extracts prepared 40 h p.i. (Fig. 1).

A comparison of the protected sequences with known protein-binding motifs revealed a hexanucleotide, AGA TAA, between nucleotides -50 and -55 that was previously identified as a binding site for the erythroid-specific factor GATA-1 (8, 26, 42). Recent studies have demonstrated that the GATA motif is recognized by a family of GATA proteins present not only in vertebrates (12, 23, 35, 36).

Insect GATA-like binding protein in the course of infection. We have investigated the binding of the insect GATA-like protein during the course of infection by performing gel mobility shift assays and in vitro footprinting analysis.

Crude nuclear extracts were prepared from uninfected or infected *S. frugiperda* cells at 3, 6, 12, 24, and 40 h p.i. When in vitro DNase I footprinting of the 665-bp *HincII*-*StyI* fragment was performed, protection of GTTATCT sequences was observed with extracts from uninfected *S. frugiperda* cells and from cells at 3, 6, and 12 h p.i. (see Fig. 4b).

The probe used in the mobility shift experiments was an oligonucleotide, designated Ac-GATA, containing the GATA motif of the PE38 promoter (see Fig. 5a). After incubation of

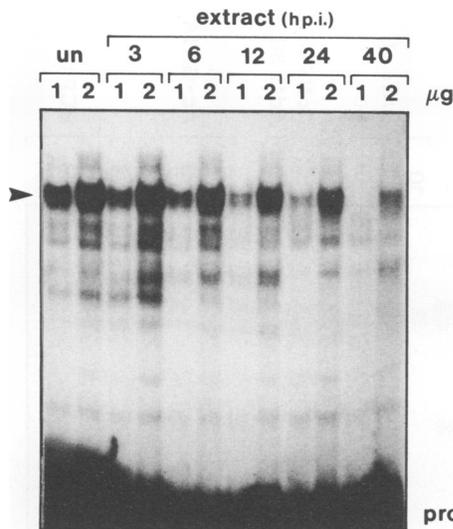


FIG. 2. Time course of protein binding to the insect GATA sequences. Band shift experiments were performed with 1 µg (lanes 1) or 2 µg (lanes 2) of nuclear extracts from *S. frugiperda* cells prepared at 3, 6, 12, 24, or 40 h p.i. or from uninfected cells (lanes un). The oligonucleotide Ac-GATA, shown in Fig. 5a, was 3' end labeled by fill-in reaction and used as probe. As nonspecific competitor, 1 µg of poly(dI)-poly(dC) was added to each reaction. The position of the major shifted complex is marked by an arrowhead.

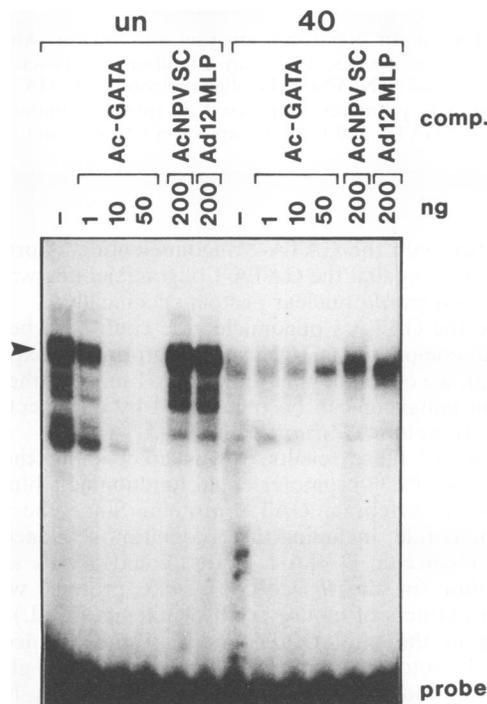


FIG. 3. Specific protein binding to the insect GATA sequences. Band shift analyses with nuclear extracts from uninfected *S. frugiperda* cells (lanes un) or from infected cells prepared 40 h p.i. (lanes 40). The 3'-end-labeled oligonucleotide Ac-GATA (~1 ng) was competed for by 1, 10, and 50 ng of unlabeled Ac-GATA competitor and by 200 ng each of the nonspecific competitors AcNPVSC and Ad12MLP. Lanes -, no further competitor added.

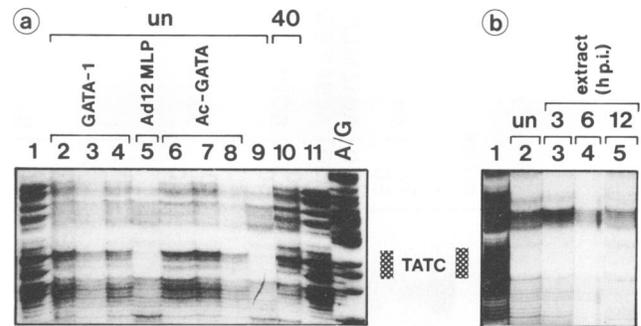


FIG. 4. In vitro DNase I footprinting of the insect GATA sequences. The promoter fragment and the extracts used are described in the legend to Fig. 1. (a) The amount of DNase I was 0.2 U per reaction mixture. Protein binding in uninfected *S. frugiperda* cell extracts was competed for by 500 (lane 6), 250 (lane 7), or 100 (lane 8) ng of the unlabeled oligonucleotide Ac-GATA; by 500 ng (lane 5) of the nonspecific oligonucleotide Ad12MLP; or by 500 (lane 2), 250 (lane 3), or 100 (lane 4) ng of the oligonucleotide GATA-1 shown in Fig. 5b. Lane 9, binding reactions without competitor. Lanes 1 and 11 include naked DNA digested with 0.0075 U of DNase I. Size markers (lane A/G) are Maxam and Gilbert A+G sequencing ladders of the *HincII-StyI* fragment. For comparison, the unprotected GATA sequences derived from binding reactions with extracts of infected (40 h p.i.) *S. frugiperda* cells are shown in lane 10. (b) The control reaction mixture containing naked DNA was digested with 0.015 U of DNase I (lane 1). For the DNA incubated with extract from uninfected *S. frugiperda* cells (lane 2) or from cells at 3 (lane 3), 6 (lane 4), or 12 (lane 5) h p.i., 0.06 U of DNase I was used.

the Ac-GATA oligonucleotide with extract from uninfected cells or from infected cells prepared 3 or 6 h p.i., one major DNA-protein complex whose intensity had decreased by 12 and 24 h p.i. and declined further at 40 h p.i. was visible. Additional minor shifted complexes were noted but were not investigated further. As demonstrated in Fig. 2, doubling the amount of extract resulted in a weak DNA-protein complex even at 40 h p.i. In order to challenge the specificity of these protein-DNA complexes, the binding was competed for by various synthetic oligonucleotides. In uninfected cell extracts, the binding was competed for by an excess of unlabeled Ac-GATA competitor but not by two nonspecific competitor oligonucleotides (Fig. 3). One of these nonspecific competitors, AcNPVSC, includes the transcriptional start site of PE38; the other, Ad12MLP, comprises a downstream part of the major late promoter of adenovirus type 12 DNA. In contrast, the weak complex produced by infected (40 h p.i.) cell extracts was nonspecific, since the Ac-GATA oligonucleotide did not compete and the intensity of the shifted complex was slightly increased by competition with the two nonspecific oligonucleotides AcNPVSC and Ad12MLP. Since we could not detect specific binding in *S. frugiperda* cell extracts prepared 40 h p.i., we conclude that the protein complex formed with the Ac-GATA oligonucleotide is quite different from extracts of uninfected and of early-phase infected *S. frugiperda* cells.

To further investigate the specificity of binding, DNase I footprint analyses were performed with crude nuclear extracts of uninfected *S. frugiperda* cells and specific or nonspecific competitors. As depicted in Fig. 4a, footprint competition with the Ac-GATA oligonucleotide led to unprotected GTTATCT sequences, but the nonspecific Ad12MLP oligonucleotide did not prevent protection of the GATA motif. Apart from the specific binding to the GATA

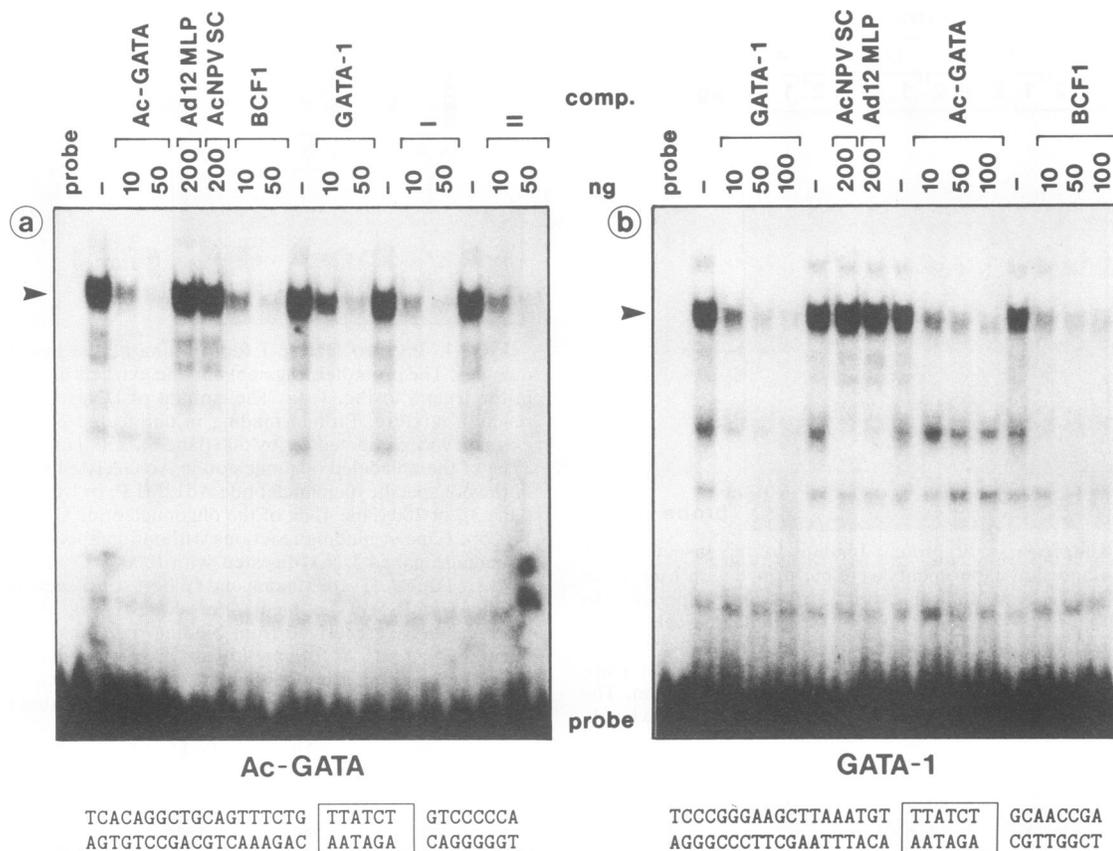


FIG. 5. Interaction of the insect GATA-like protein with the vertebrate GATA-1 motif. Band shift analysis with nuclear extracts from uninfected *S. frugiperda* cells and the oligonucleotides Ac-GATA (a) or GATA-1 (b) as probes. The assay conditions are described in the legends to Fig. 2 and 3. Nonspecific competitors were the oligonucleotides Ad12MLP and AcNPVSC. The oligonucleotides Ac-GATA, BCF1, GATA-1, I (GATA motif of the ME53 promoter), and II (GATA motif of the 35K promoter) were used as specific competitors. The arrowheads indicate the major shifted complexes. The nucleotide sequences of Ac-GATA and GATA-1 are shown below, and the GATA consensus sequences are boxed.

sequences, these results indicate that the region close to the GATA motif cannot be recognized by other factors in uninfected extracts when the GATA binding protein is competed out.

Binding of the insect GATA-like protein and recognition of the Ac-GATA motif are not restricted to insect cells. From the DNA-binding data, we conclude that a host-specific nuclear protein binds to the Ac-GATA oligonucleotide in a sequence-specific manner during the early phase of infection. The question arose whether the specific binding is mediated by the hexamer sequence AGATAA, as has been shown for GATA-binding proteins of other species. Therefore, we performed competition experiments using oligonucleotides of the same length as Ac-GATA, including the GATA-1 motif of the chicken β -globin enhancer (8), designated GATA-1, or of the *Bombyx* chorion factor I (35), termed BCF1. These three oligonucleotides share the minimal consensus sequence AGATAA without any additional sequence similarity. As demonstrated in Fig. 5a, the Ac-GATA probe was shifted by extracts from uninfected cells, and the formation of this complex could be competed for by the GATA-1 and BCF1 oligonucleotides. The results of additional experiments indicated that the ratio of molar excess for each specific competitor (Ac-GATA, GATA-1, and BCF1) was nearly the same. The converse experiment was

performed with the GATA-1 oligonucleotide as probe (Fig. 5b), confirming that the GATA-1 oligonucleotide was able to bind *S. frugiperda* nuclear protein specifically.

Since the GATA-1 oligonucleotide could also be used as specific competitor in DNase I footprinting experiments (Fig. 4a), we conclude that the GATA-1 motif in the chicken β -globin enhancer can be recognized by an insect GATA-binding protein in *S. frugiperda* cells.

In light of these results, we asked whether the GATA motif of the PE38 promoter could function as a binding site also for a vertebrate GATA protein. Since the GATA-1 oligonucleotide, including the recognition sequences of the erythroid-specific GATA-1 factor, could act as a specific competitor for the insect GATA-like protein, we tested nuclear extracts of mouse erythroleukemia (MEL) cells for binding to the viral GATA motif. The existence of the GATA-1 factor interacting with the human β -globin enhancer in induced and uninduced MEL cells has been demonstrated recently (42). For gel mobility shift assays, we used two oligonucleotides as probes, GATA-1 or Ac-sGATA, which is eight nucleotides shorter than Ac-GATA (Fig. 7a). Each oligonucleotide, the vertebrate GATA-1 or the invertebrate Ac-sGATA, formed two major complexes with extracts from uninduced MEL cells that migrated at approximately the same position (Fig. 6a and b). Each of

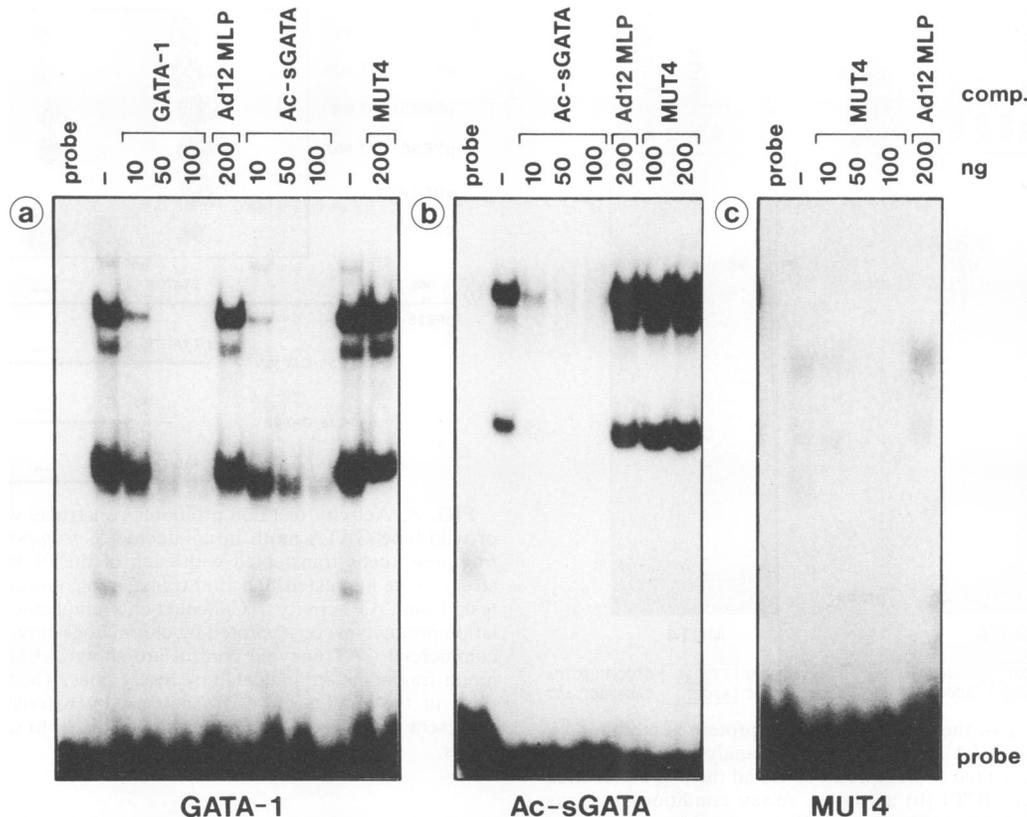


FIG. 6. Protein binding to the insect GATA motif in MEL cells. Band shift analysis with crude nuclear extracts of uninduced MEL cells and the oligonucleotides GATA-1 (a), Ac-sGATA (b), or MUT4 (c) as probes. The nucleotide sequences of Ac-sGATA and MUT4 are shown in Fig. 7. The assay conditions and competitors are comparable to those described in the legends to Fig. 2, 3, and 5 except that 2 μ g of nuclear extract from MEL cells was used.

these complexes was competed for by GATA-1 or Ac-sGATA but not by the nonspecific oligonucleotide Ad12MLP (Fig. 6a and b). These results therefore suggest that the erythroid-specific GATA-1 factor binds specifically to a GATA motif in the baculovirus promoter PE38.

In contrast, in nuclear extracts of *Drosophila* KC cells or HeLa cells, no specific complexes which corresponded to the GATA motif were formed (data not shown). Several groups have analyzed the level of GATA gene expression in a variety of cell culture lines indicating the presence of nonerythroid GATA-binding proteins, i.e., in HeLa cells (24, 44). However, GATA-binding proteins have so far not been shown to be expressed in cultured *Drosophila* cells.

Mutations in the GATA motif abolish specific binding of insect GATA-like protein and GATA-1 factor. Mobility shift assays were carried out with two oligonucleotides, Ac-sGATA and MUT4, containing the authentic Ac-GATA motif of the PE38 promoter and the mutated form, respectively, to strengthen the notion that an insect GATA-like protein binds to the hexanucleotide TTATCT. The oligonucleotide MUT4 differs from Ac-sGATA in that four nucleotides in the GATA recognition sequence have been altered (Fig. 7b). Using the oligonucleotide Ac-sGATA as a probe and nuclear extracts from uninfected *S. frugiperda* cells, we demonstrated that the oligonucleotide MUT4 could not compete for the binding of the GATA-like protein (Fig. 7a). The converse experiment, in which the oligonucleotide MUT4 was used as probe, resulted in a slowly migrating

complex which could be competed for by the unlabeled oligonucleotide MUT4 but not by Ac-sGATA (Fig. 7b). These results indicate that the complex formed with the oligonucleotide MUT4 is not due to binding of the GATA-like protein. In extracts from MEL cells, no complex with the oligonucleotide MUT4 was observed (Fig. 6c).

Functional significance of the GATA motif in the PE38 promoter. Since the results of the DNA-binding experiments described above suggest that an insect GATA-like protein specifically binds to the GATA motif in the PE38 promoter, we asked whether the recognition of this motif has functional significance for the activation of PE38 transcription in uninfected *S. frugiperda* cells. Therefore, regions of the PE38 promoter including the GATA motif or the mutated form were placed upstream of the CAT gene, and the activities of these constructs were evaluated by transient expression in *S. frugiperda* cells.

The scheme in Fig. 8b outlines the structural features of the constructs pPE38-CAT207 and pPE38-CAT109, both containing the GATA motif coupled to promoter sequences of different lengths. In uninfected *S. frugiperda* cells, it turned out that the longer construct, pPE38-CAT207, was as efficiently expressed as pPE38-CAT109, indicating the absence of activating sequences in the segment between -67 and -165. Since the mutated GATA sequences (MUT4) have not been shown to specifically bind to the insect GATA-like protein (Fig. 7b), we replaced the GATA motif in the pPE38-CAT109 construct by the mutated form. How-

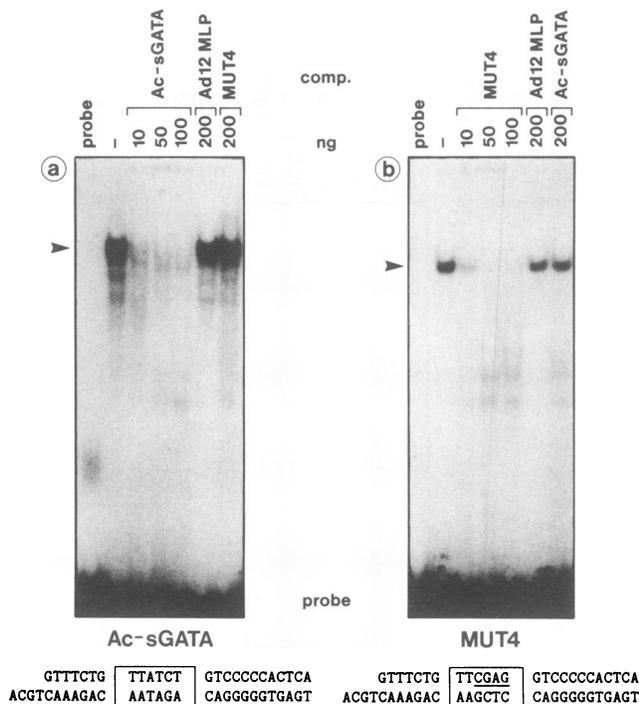


FIG. 7. Binding of the insect GATA-like protein is inhibited by mutation of the GATA motif. Band shift analysis with nuclear extracts from uninfected *S. frugiperda* cells and the oligonucleotide Ac-sGATA (a) or MUT4 (b) as probe. Assay conditions were as described in the legends to Fig. 2 and 3. The nucleotide sequences of Ac-sGATA (a) and MUT4 (b) are shown below. The GATA consensus sequences are boxed, and the mutated nucleotides are underlined.

ever, transfection of pPE38-CATMUT demonstrates that the level of CAT expression does not change significantly in comparison with the level of pPE38-CAT109 (Fig. 8a). Even when the GATA motif and sequences up to -40 are lacking (pPE38-CAT82), the PE38 promoter is still active (Fig. 8). Therefore, the specific binding to the mutated GATA oligonucleotide (MUT4), which is probably not mediated by a GATA factor, presumably does not contribute to promoter activity in this test system. From the results of these experiments, binding of the GATA-like factor does not appear to be responsible for promoter activation in uninfected *S. frugiperda* cells.

In vivo footprinting analysis of the insect GATA-like binding protein in the PE38 promoter. Transient expression of the PE38 promoter constructs does not necessarily demonstrate the relevance of the GATA motif for the expression of the PE38 gene in vivo. We therefore examined the in vivo DNA-protein interaction at the GATA sequences by the method of in vivo DMS footprinting by ligation-mediated PCR (28). After treatment of infected *S. frugiperda* cells with 0.1% DMS at 3 or 46 h p.i., the methylated guanine residues were cleaved with piperidine (27). The footprinting of the PE38 coding strand containing the GATA sequence was expected to reveal decrease or enhancement (4) of the G residue in the GTTATCT sequence, which was shown to be protected in vitro (Fig. 1). The pattern of the unprotected G residues is identical in early-phase (3 h p.i.) and late-phase (46 h p.i.) infected *S. frugiperda* cells, with one exception (Fig. 9). The G residue close to the GATA sequences is

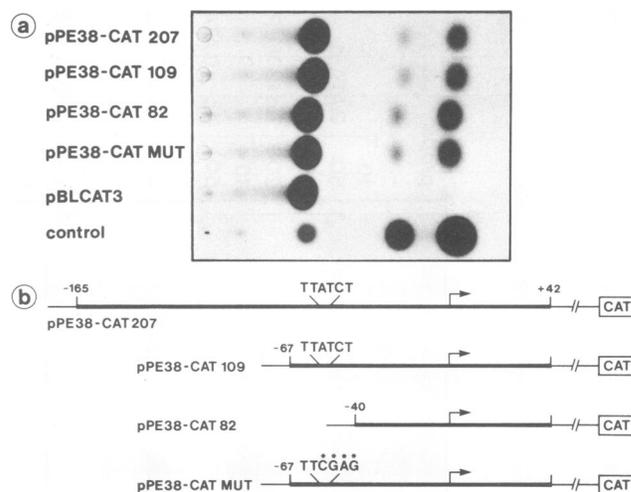


FIG. 8. Activity of PE38 promoter constructs with the mutated or wild-type GATA motif in uninfected *S. frugiperda* cells. (a) *S. frugiperda* cells transfected with each of the PE38 promoter constructs were harvested 40 h after transfection, and cell extracts were tested for CAT activity. ^{14}C -labeled chloramphenicol and its acetylation products were separated by chromatography. The results of a commercial CAT enzyme control are shown. (b) Lengths of promoter fragments are indicated by heavy lines. The mutated nucleotides in the GATA motif are marked by asterisks. The arrows represent the transcriptional start site of PE38, to which numbering refers.

enhanced in early-phase infected *S. frugiperda* cells but not in cells at 46 h p.i. This enhanced DMS reactivity might be due to DNA-protein contacts close to the G residue during the early phase of infection. The protection of the GATA motif in vivo has been shown previously by Tsai et al. (40). Their results from DMS footprinting by ligation-mediated PCR suggest the protection of internal and external G residues of the GATA consensus in the GATA-1 promoter.

Presence of GATA motifs in other early baculovirus promoters. It is expected that early viral promoters include sequence elements recognized by a variety of host factors. Since we identified one of these host recognition sequences in the PE38 promoter, we analyzed other baculovirus promoters for the presence of the GATA motif. A computer search revealed that A/T GATA T/C sequences are detectable in those parts of the AcNPV genome that have already been sequenced. However, GATA motifs at similar positions and in the same orientation as in the PE38 promoter are present only in some of the early promoters, as shown in Fig. 10. To investigate whether these GATA sequences are able to bind the GATA-like protein, we used two oligonucleotides as specific competitors for the GATA-binding motif of the PE38 promoter. One oligonucleotide includes the GATA motif of the ME53 promoter (I in Fig. 5a), and the other contains the GATA motif of the 35K promoter (II in Fig. 5a). The ME53 promoter (21) and the 35K promoter (11) are transcribed during the very early phase of infection without previous viral protein synthesis. As shown by mobility shift assays (Fig. 5a), both oligonucleotides specifically compete for binding to the Ac-sGATA probe, indicating that the GATA motif in the 35K and ME53 promoters can be recognized also by the insect GATA-binding protein. The functional significance of the GATA motif in these promoters has yet to be determined.

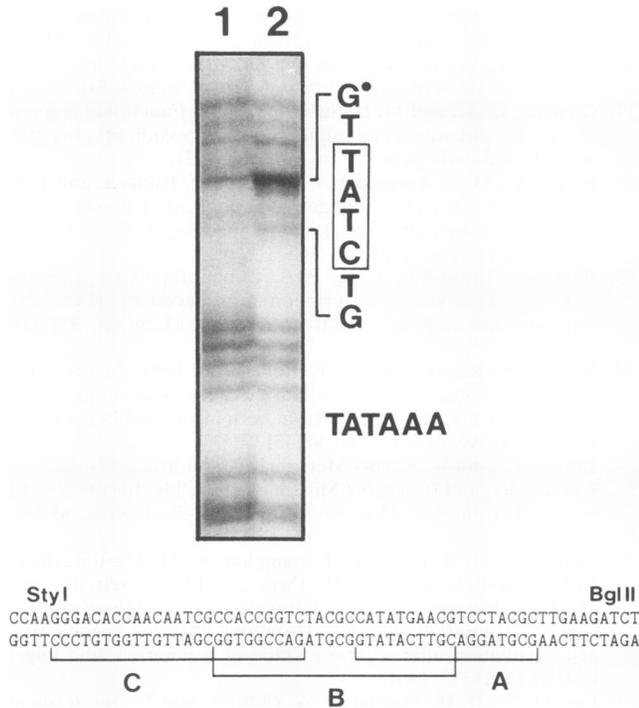


FIG. 9. In vivo DMS footprinting of the GATA sequences (in the PE38 promoter) in AcNPV-infected *S. frugiperda* cells. The coding strand was visualized by ligation-mediated PCR footprinting; the set of oligonucleotide primers used is shown below. DMS-treated DNA from infected *S. frugiperda* cells at 46 h p.i. (lane 1) is compared with DMS-treated DNA at 3 h p.i. (lane 2). Enhancement of the guanine residue in the GATA sequences is indicated (-). The gel was autoradiographed; the exposure was for 50 min at room temperature.

DISCUSSION

An understanding of early AcNPV gene regulation requires knowledge of the host protein factors involved and the promoter sequence elements upon which they act. We have chosen the promoter of the early transcribed PE38 gene to investigate DNA-protein interactions. Our results in DNase I footprint assays (Fig. 1) have shown that several proteins present in extracts of uninfected *S. frugiperda* cells can bind to the PE38 promoter region comprising 200 nucleotides upstream of the transcriptional start site. The corresponding DNA-binding sequences apparently fail to be recognized by factors in extracts prepared from *S. frugiperda* cells at 40 h p.i. This failure might be related to the reduced transcriptional activity of PE38 in the late phase of infection (22). One of the sequence elements recognized by the host factors includes the binding motif GATA, raising the possibility that this region of DNA mediates binding of a member of the GATA-binding protein family. The results of in vitro DNase I and in vivo DMS footprinting experiments reported here indicate the interaction of the PE38 promoter with an insect GATA-like protein, which we designate *S. frugiperda* nuclear protein 1 (SfNP-1). To date, no host transcription factor which might regulate baculovirus promoters has been identified. Because of the results of time course experiments on DNA-protein interactions, we postulate that protein binding to the GATA motif changes after viral DNA replication (~12 h p.i.) and is not detectable at 40 h p.i. These data suggest that SfNP-1 concentration probably decreases in the course of viral infection. However, we cannot exclude

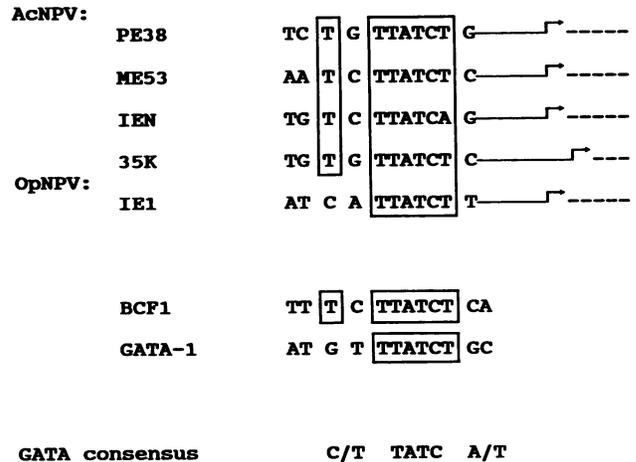


FIG. 10. Localization of GATA motifs of other early baculovirus promoters. GATA consensus sequences and a common flanking T residue are boxed. The transcriptional start sites are indicated by arrows. IE1, immediate-early 1 from *Orygia pseudotsugata* nuclear polyhedrosis virus (OpNPV) (37).

the possibility that cellular or viral factors might have displaced SfNP-1 late during infection.

The specific binding of the GATA-like protein is inhibited by mutation of the AGATAA sequence in the PE38 promoter to CTCGAA. No stable complex of the mutated GATA sequences is formed with SfNP-1 in *S. frugiperda* cells or with the factor in MEL cells, which presumably represents GATA-1. The same mutation in the chicken β -globin enhancer has previously been shown to prohibit binding of the GATA-1 factor (8). We also tested PE38 promoter CAT constructs lacking the GATA sequences or containing the mutated or wild-type GATA motif. Since there was no dramatic difference in CAT expression, we conclude that prevention of SfNP-1 binding does not notably affect promoter activation. If SfNP-1 did not contribute to basal promoter activity in cell culture, one could speculate that SfNP-1 binding served as a target for modulating transcription in the course of infection and in different tissues of the infected host organism. The mutated GATA sequences in the chicken β -globin enhancer likewise exhibited a rather slight effect on transient expression (8). Nevertheless, the functional significance of GATA motifs in the transcription of globin and nonglobin erythroid-expressed genes has been demonstrated by several mutagenesis studies of promoters or enhancers (26, 32-34, 43).

Members of the GATA protein family identified in vertebrates as well as invertebrates share highly related zinc finger domains and bind to similar if not identical sequences in the transcriptional regulatory region of a number of different genes. Therefore, it is tempting to speculate that DNA recognition by the GATA transcription factors is highly conserved among different species, although their functions could be unrelated. Our findings indicate the presence in *S. frugiperda* cells of an insect GATA-like protein which recognizes the AcNPV GATA motif, the GATA-binding motif of silkworm chorion genes, and the vertebrate GATA sequences (GATA-1). Likewise, the vertebrate presumptive GATA-1 factor from uninduced MEL cells can bind to the insect motif. These results are consistent with the observations of Skeiky and Iatrou (35) on silkworm chorion promoter binding factors. The vertebrate

GATA-1 motif was shown to compete for the *Bombyx* chorion factor 1 (BCF1) complex, and the BCF1 binding sequence seems to form a stable complex with GATA-1 from induced K562 cells, a human erythroleukemia cell line.

Our data begin to provide the basis for an understanding of how temporal regulation of the PE38 promoter occurs by demonstrating the differential binding of an insect GATA-like factor in vitro and probably in vivo.

ACKNOWLEDGMENTS

We thank Matthias Henze for providing MEL cells, Ruth Jüttermann for nuclear extracts of HeLa cells, Harald Saumweber for nuclei of *Drosophila* KC cells, Irmgard Hölker for synthesis of oligodeoxynucleotides, Gerti Meyer zu Altschildesche for medium preparation, and Petra Böhm for expert editorial work. We also thank Christiane Zock for helpful discussions.

This research was supported by grant SFB274-TP5 from the Deutsche Forschungsgemeinschaft.

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