The p95 Gene of *Bombyx mori* Nuclear Polyhedrosis Virus: Temporal Expression and Functional Properties

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As part of our effort to identify baculovirus proteins acting as transcriptional regulators, we have characterized a gene, p95, of *Bombyx mori* nuclear polyhedrosis virus (BmNPV) that encompasses an open reading frame for a putative 95-kDa polypeptide (P95). The N-terminal half of the conceptually translated P95 contains two zinc finger-type DNA-binding motifs, and its C terminus contains a proline-rich region reminiscent of transcriptional activation regions. Northern blot analysis indicates that two mRNA species, 3.5 and 1.7 kb in size, are transcribed from the p95 gene at different times postinfection. These two mRNA species are produced by differential polyadenylation site usage. While the longer transcript can encode the P95 protein, the shorter one may encode a prematurely terminated version of the P95 polypeptide produced by ribosome frameshifting occurring at heptanucleotide “slippage” sites located near the relevant polyadenylation site. Transcription of the p95 gene is initiated at a proximal site located 70 nucleotides upstream of the translation start codon of P95, a middle site located 170 nucleotides from the start codon, and a set of three closely spaced distal sites located 385, 390, and 409 nucleotides from the translation start codon. The middle and distant initiation sites are utilized before and after BmNPV DNA replication, while transcripts initiated at the proximal site occur largely during the late and very late stages of viral infection. Transient-expression assays indicate that P95 can stimulate gene expression driven by the promoter of its own gene and the promoter of the cytoplasmic actin gene of *B. mori*. The P95-mediated trans activation can be further augmented by BmIE1, an immediate-early gene product of BmNPV. In contrast to the case with the actin promoter, however, the promoter of the p95 gene can be trans activated by the product of its own gene only in the presence of BmIE1. Our data suggest that proteins P95 and BmIE1 of BmNPV and, by analogy, those of other baculoviruses may interact with each other and synergize to potentiate transcription.

Nuclear polyhedrosis viruses (NPVs) contain a covalently closed circular DNA genome that replicates in the nuclei of permissive lepidopteran cells (9). During an NPV infection, more than 100 viral genes are expressed in a cascade that can be divided into four phases: immediate early, delayed early, late, and very late. The expression of immediate-early viral genes is driven by host cellular factors, while delayed-early and late genes are transcribed in the presence of cellular factors and at least one immediate-early factor (17, 18, 20, 28, 31). A viral RNA polymerase resistant to α-amanitin is involved in the transition from early to late gene expression (5, 15, 55).

There is increasing evidence that transcriptional regulators of both host and viral origin play important roles in the expression of viral genes. Some of the viral regulators have been identified on the basis of their ability to trans activate expression from viral promoters in transient-expression assays. The products of the immediate-early genes, ie1, ie2, and ie0, have effects on the transcription of several early viral genes, and 18 viral genes were shown to affect expression of late or very late genes (10, 12, 17, 18, 29, 34). Moreover, other viral transcriptional regulators have been putatively identified on the basis of the presence of domains similar to those found in eukaryotic transcriptional regulators. For example, ME53 contains a zinc finger DNA-binding domain, while CG30 and PE38 contain both zinc finger DNA-binding and leucine zipper dimerization domains (27, 30, 35, 53). Finally, evidence has been provided suggesting that when transfected into insect cells, viral transcriptional regulators may also affect the transcriptional properties of host cell genes. Using the silkmoth cytoplasmic actin gene promoter (41, 42) as a model, we have previously shown that the product (BmIE1) of an immediately-early gene of *Bombyx mori* NPV (BmNPV) is able to stimulate the expression of a cellular gene in vitro (37).

As part of an ongoing effort to characterize baculovirus genes encoding transcription factors that regulate the expression of other viral genes (and maybe cellular genes as well), we have carried out an initial functional characterization of another baculovirus gene, p95. Transcriptional analysis indicates that the p95 gene, which contains an open reading frame (ORF) for a 95-kDa polypeptide (P95), gives rise to two transcripts, 1.7 and 3.5 kb in length. These two mRNAs are apparently produced by differential utilization of alternative polyadenylation sites. Of the two transcripts, only the longest can encompass the entire P95 ORF. The presence of heptanucleotide ribosome “slippage” motifs located directly adjacent to the polyadenylation site of the 1.7-kb transcript suggests that premature translation termination may occur by a frameshifting mechanism. The resultant shorter polypeptide would retain the DNA-binding domain of P95 but would lack its C-terminal proline-rich (putative activating) region. Transcripts from the p95 gene are initiated from three sets of transcription start sites exhibiting distinct temporal patterns of expression. The product of the p95 gene (protein P95 encoded by the 3.5-kb mRNA) can up-regulate the expression of genes placed under the control of the cytoplasmic actin promoter or the promoter of its own gene. However, while P95 can activate the actin gene
p95 ORF

\[
\begin{array}{cccccc}
\text{CiaI} & \text{EcoRV} & \text{SspI} & \text{StuI} & \text{SspI} & \text{CiaI} \\
49.94 & 50.9 & 51.8 & 52.5 & 52.7 & 54.95 \\
\end{array}
\]

FIG. 1. Location of the p95 gene in the BmNPV genome (ML1 isolate) and plasmids. pCG30/P39 (35, 36) was used as the starting material for all plasmids containing gene p95 or portions of it, as follows (Fig. 1): plasmid pLV contains a 0.62-kb SalI/EcoRV fragment (the N-terminal-coding region of the p95 gene) subcloned into the SalI and EcoRV sites of vector Bluescript SK+ (pBS). Plasmid pcCG30 (35, 36) contains the 3.5-kb mRNA gene (P95) has extensive sequence similarities with its counterpart in the BmNPV genome (ML1 isolate) and the HR3 enhancer (36); the HR3 enhancer (36); and the p95 gene, whose characterization is presented here, is shown in Fig. 1. Figure 1 also shows the various pCG30/P39 derivative plasmids used in this study.

Plasmid pCG30/P39 (35, 36) was used as the starting material for all plasmids containing gene p95 or portions of it, as described previously (23). For infection, cells were seeded in a six-well microtiter plate at a density of 10^5 per well and allowed to attach overnight. Cell monolayers were infected for 2 h at room temperature with BmNPV (ML1 isolate) at a multiplicity of infection of 20, washed with 1 ml of culture medium three times to remove the viral inoculum, and incubated at 28°C for the required periods of time.

Cells, virus, and infections. B. morr Bm5 cells were maintained at 28°C in complete IPL-41 medium containing 10% fetal calf serum as described previously (23). For infection, cells were seeded in a six-well microtiter plate at a density of 10^5 per well and allowed to attach overnight. Cell monolayers were infected for 2 h at room temperature with BmNPV (ML1 isolate) at a multiplicity of infection of 20, washed with 1 ml of culture medium three times to remove the viral inoculum, and incubated at 28°C for the required periods of time.

plasmid directly, it enhances transcription from its own promoter only in the presence of Bm1E1.

MATERIALS AND METHODS

RESULTS

The p95 gene. A map of a 6.6-kb CiaI fragment of the BmNPV (ML1 isolate) genome (plasmid pCG30/P39; map units 49.94 to 54.95) containing the previously characterized genes cg30, p95, and p15 (35, 36); the HR3 enhancer (36); and the p95 gene, whose characterization is presented here, is shown in Fig. 1. Figure 1 also shows the various pCG30/P39 derivative plasmids used in this study.

To clone the p95 gene promoter, a forward primer (FP) 5′-TCAGTGTCGAGTTTACGCCAGACATC (35, 36); the HR3 enhancer (36); and the p95 gene, whose characterization is presented here, is shown in Fig. 1. Figure 1 also shows the various pCG30/P39 derivative plasmids used in this study.

The nucleotide sequence of the 3.37-kb ClaI/StiI subfragment of pCG30/P39 (ML1 isolate; map units 49.94 to 52.53; plasmid pCS) is shown in Fig. 2. This fragment contains an ORF for a 95-kDa polypeptide. The protein encoded by the ORF (P95) has extensive sequence similarities with its counterparts in the T3 strain of BmNPV (98.1% identity) (reference 38 and data not shown) and Autographa californica NPV (AcNPV) (ORF83; 91% identity) (reference 4 and data not shown).

The N terminus of P95 is enriched in hydrophobic residues. Also present near the N terminus (amino acids 141 to 265) is a partially duplicated sequence, FDXCX3CX2NXNXPXG (X_{19-40})GXHXVXEXCPXN (where X is any amino acid). The cysteine and histidine residues in this region are arranged in a manner reminiscent of CX2–4CX2 histidine residues in some regulatory proteins that bind DNA or RNA and interact with other proteins for regulation of gene expression (7, 21, 48). The C terminus of P95 contains a proline-rich region (amino acids 582 to 703). Proline-rich regions are diagnostic of transcription factor activation domains (8, 33, 40). The C terminus also has a high density of sequence similarity with the proline- and cysteine-rich region of the largest subunit of eukaryotic RNA polymerase II (references 2, 13, and 54 and data not shown), which can also serve as an activation domain (49). These structural features suggest that the P95 protein may function as a transcription factor.

polymerase (New England Biolabs) as previously described (35). For Northern hybridization analysis, 5 μg of nuclear acid from mock-infected or infected cells was resolved in an agarose gel containing methyl mercuric hydrate, transferred to a nylon membrane, and hybridized to each riboprobe as previously described (37).

Primer extension analysis. For primer extension, two primers were synthesized, P1 (5′-CGAGCCATTACGGATCAGATC-3′) (complement of nucleotides 419 to 397 [see Fig. 4]) and P2 (5′-GGTGGACACGGCAGGATGCGTG-3′) (complement of nucleotides 285 to 183 [see Fig. 4]). End-labeled primers were extended with reverse transcriptase following addition of 10 μg of nuclear acid from mock-infected or infected Bm5 cells as described previously (35).

Rapid amplification of cDNA ends (3′-RACE). To map the 3′ ends of p95 transcripts, total RNA isolated from Bm5 cells infected with BmNPV (for 6 or 24 h) was reverse transcribed by using an adapter/T7 primer, 5′-CTCTGCAGGTCAGCGAGATTCTCC-3′, and PCR amplified in two steps by using two nested gene-specific primers (both of them forward) in combination with the adapter primer 5′-CTCTGCAGGTCAGCGAGATTCTCC-3′. To map the polyadenylation site of the 3.5-kb mRNA, gene-specific primers FP2 and NP2 (see Fig. 2) were used. All amplifications were carried out at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 40 cycles (first amplifications) and 15 cycles (nested amplifications). PCR products were cloned into the Smal site of pBluescript and sequenced by using T3 and T7 primers.

Transfection and CAT assays. One million cells were transfected with 1 μg of plp95.cat, an equimolar amount of other test plasmids as indicated, and additional pBS DNA (to a total amount of 5 μg) in 1 ml of basal IPL-41 medium containing 30 μl of Lipofect (GibcoBRL). For transfection of infected cells, cells were first infected for 3 h and then transfected with 5 μg of plp95.cat following removal of the viral inoculum. All procedures for transfection, protein extraction and quantitation, and CAT assays were as described previously (37). All assays of gene activity were carried out at least three times, and all comparable results.

Nucleotide sequence accession number. The GenBank accession number for the p95 nucleotide sequence is U83330.
FIG. 2. The p95 gene. The nucleotide sequence of the 3.37-kb ClaI/StuI fragment is shown together with the deduced amino acid sequence for the P95 ORF. The arrows labelled FP and RP indicate the regions covered by the two primers used for the cloning of the p95 gene promoter. The arrows labelled FP1, NFP1, FP2, and NFP2 indicate the primers used in the 3'-RACE experiments. The underlined sequences represent the portion of the gene present in clone pLV whose insert was used as a probe for the hybridizations shown in Fig. 4. The stippled bar in the 3' UTR indicates the extent of the deletion of the HR3 enhancer in plasmid pCS. Cysteine and histidine residues present in the zinc finger-like domain are underlined and in boldface, proline residues in the proline-rich region are in boldface italic, and the EP repeats are underlined and in italic. Putative polyadenylation signals are double underlined, and the polyadenylation sites are indicated by vertical arrows. Putative heptanucleotide signals involved in ribosomal frameshifting are boxed, and a palindromic sequence that may function as a second signal for frameshifting is indicated by shaded boxes and inverted arrows. Translation termination codons that may result from frameshifting events are in boldface.

\begin{verbatim}
ATCGATAACGGAGCCGTCGTCGTCATGCTACATGCTACATGC
TGAAATCTCAGATGGACATGAAGCTAGATGCTGCTGCTGCT
TGCAAAATCGGAGCAGATGCTGACATGCTGACATGCTGAC
GTTCATCCGAGTCCGTCGTCGTCGTCGTCGTCGTCGTCGTC
TPCAGTGTCAGGACATGCTGACATGCTGACATGCTGACATGCT
GTTCATCCGAGTCCGTCGTCGTCGTCGTCGTCGTCGTCGTC
\end{verbatim}

\begin{verbatim}
TGGAAAATACATGGACATGAAGCTAGATGCTGCTGCTGCT
TGCAAAATCGGAGCAGATGCTGACATGCTGACATGCTGACATGCT
GTTCATCCGAGTCCGTCGTCGTCGTCGTCGTCGTCGTCGTC
GTTCATCCGAGTCCGTCGTCGTCGTCGTCGTCGTCGTCGTC
\end{verbatim}

\begin{verbatim}
VOL. 72, 1998 BmNPV p95 GENE
\end{verbatim}
Temporal expression of the \( p95 \) gene. A Northern hybridization was carried out to examine the temporal pattern of expression of the \( p95 \) gene by using riboprobes generated from a 620-bp fragment encompassing the zinc finger motif (subclone pLV [Fig. 1 and underlined region in Fig. 2]). As shown in Fig. 3, the antisense riboprobe detected two RNAs, 1.7 and 3.5 kb, in BmNPV-infected cells but not in mock-infected cells. The 1.7-kb transcript appeared at 9 h p.i. and accumulated to higher levels from 12 to 48 h p.i. The 3.5-kb RNA appeared at 12 h p.i. and increased at the later stages of viral infection to levels lower than those of the 1.7-kb RNA. The sense riboprobe did not hybridize to any transcripts (data not shown).

Primer extension analysis. The transcription start sites of the \( p95 \) gene were determined by primer extension with two primers, P1 and P2 (Fig. 4). As shown in Fig. 4, three extension products, a to c, were obtained with the P1 primer. The shortest product (c) was evident at 12 h p.i. and increased to a higher level at 24 h p.i. This start site was mapped at a T residue located 70 bp upstream of the P95 translation start codon (Fig. 4) and was contained within a TAAG motif, a motif similar to that proposed as a consensus motif for late baculovirus transcription start sites.

The longest and second-longest extension products were detectable as early as 6 h p.i. Because BmNPV DNA replication occurs after 6 h p.i. (35, 36), the transcripts originating from these two distant sites must represent delayed-early gene products. The intermediate start site (b) was mapped at 170 bp upstream of the P95 ORF. To map precisely the start site of the longest extension product (a), an upstream primer (P2) was also employed. As shown in Fig. 4, the longest extension product was composed of three closely spaced products, a1 to a3, which were mapped at sites located 385, 390, and 409 bp upstream of the P95 ORF. The a1 initiation site was located 15 bp beyond the end of the sequenced region in PCS DNA. The relevant extension product maps at a G residue (boldface) of a GTGGT motif in the sequence of the T3 isolate of BmNPV (38).

Based on the temporal specificities of the mapped transcripts, we conclude that \( p95 \) is transcribed from two sets of initiation sites, a and b, prior to DNA replication and gives rise to an abundant set of transcripts 1.7 kb long and that during the late stages of infection, transcription is switched to another, ORF-proximal initiation site that gives rise to a less prevalent 3.5-kb transcript. Of the two transcripts, only the latter encompasses a complete P95 ORF.

Determination of the polyadenylation sites of the \( p95 \) gene. The presence of mRNAs of different sizes arising from the \( p95 \) gene suggests the occurrence of a possible splicing variant or the utilization of alternative (and temporally regulated) poly-

FIG. 3. Temporal pattern of \( p95 \) gene transcription. Samples (5 \( \mu \)g) of total nucleic acid extracted from mock-infected cells (lane M) or cells infected with BmNPV for 3, 6, 9, 12, 18, 24, and 48 h p.i. (as indicated above each lane) were electrophoretically resolved and hybridized with the \( p95 \) antisense riboprobe generated from plasmid pLV DNA. The observed transcripts are marked by arrows, and their sizes, determined from the mobility of nonradioactive RNA standards run in a parallel lane of the same gel (0.24 to 9.5-kb RNA ladder; Gibco-BRL), are indicated.

FIG. 4. Sites of \( p95 \) gene transcription initiation. (A) Primer extension analysis with primer P1 (shown in panel C) and 10 \( \mu \)g of nucleic acid obtained from mock-infected cells (lane 1) or cells infected with BmNPV for 6 h (lane 2), 12 h (lane 3), or 24 h (lane 4). Lanes G, A, T, and C show corresponding dideoxy nucleoside sequencing reactions of a standard DNA fragment. (B) Primer extension analysis with primer P2 (shown in panel C). The labelled primer was extended in the presence of 10 \( \mu \)g of nucleic acid from mock-infected cells (lane 1) or cells infected for 12 h (lane 2) or 24 h (lane 3). Sequencing lanes are as in panel A. (C) The first 420 bp of the insert of plasmid pCV containing the 5' flanking region, 5' UTR, and a portion of the ORF of the \( p95 \) gene is shown together with the locations of primers P1 and P2 used for the primer extension assays shown in panels A and B. The transcription start sites, a to c, are indicated above the sequence, and the sequence motif characteristic of late transcription start sites is in boldface.
adenylation sites in infected cells. Reverse transcription-PCR experimentation employing RNA of cells infected for 6 or 24 h with primers spanning most of the p95 gene (residues 641 to 2666 [Fig. 2]) failed to yield any products that might be generated from a spliced version of P95 mRNA (data not shown), suggesting that polyadenylation somewhere near the middle of the P95 ORF may be responsible for the appearance of the 1.7-kb transcript.

To examine this further, 3′-RACE was carried out with two nested forward primers located at the 3′ end of the zinc finger region (FP1 and NFP1) together with a polyadenylation anchor reverse primer. Two rounds of PCR resulted in the amplification of a 0.38-kb fragment from cDNA prepared from Bm5 cells at 24 h p.i. but not from cells at 6 h p.i. or uninfected cells (Fig. 5, left panel). The PCR fragment was subcloned and sequenced, and this resulted in the identification of a polyadenylation site at nucleotide 1572 (Fig. 2). This corresponds to the 3′ end of a 1.7-kb transcript initiated at the early start sites, a and b (Fig. 2 and 4), containing a poly(A) tail 150 to 200 nucleotides long. Our inability to detect the 3′ end of the 1.7 kb RNA by 3′-RACE at 6 h p.i. is probably due to the relatively low prevalence of this transcript in the total RNA population of the cells during the early stages of infection.

The polyadenylation site of the 3.5-kb transcripts was similarly determined by using two nested primers, FP2 and NFP2, located near the end of the P95 ORF. A 0.17-kb PCR fragment was amplified from cDNA prepared from Bm5 cells at 24 h p.i. (Fig. 5, right panel), cloned, and sequenced. This resulted in the determination of a polyadenylation site located 14 nucleotides downstream of the ORF stop codon (Fig. 2). Each of the two polyadenylation sites is found 12 or 13 nucleotides downstream of degenerate AATAAA motifs (underlined in Fig. 2) which, in combination with existing downstream GT-rich regions, may function as polyadenylation signals (45).

**Stimulation of actin gene expression by the p95 gene product.** The analysis of the amino acid sequences has suggested that the p95 gene may encode a transcription factor. On the other hand, the promoter of the silkmoth cytoplasmic actin gene is active in Bm5 cells transfected with plasmid pBmA.cat, in which the CAT ORF is driven by the actin promoter (25). To find out if P95 is capable of stimulating the expression of the actin gene promoter, we cotransfected Bm5 cells with pBmA.cat and any of the plasmids containing a partial P95 ORF (pCV and pCA) did not increase pBmA.cat-derived CAT activity. In contrast, cotransfection with the plasmid containing the complete p95 gene (pCS) stimulated CAT expression 29-fold. This result was highly reproducible upon multiple repeat experimentation. Therefore, it is clear that the p95 gene (under the control of the upstream sequences which lack the a1 initiation site [Fig. 4]) is expressed in transfected cells. Furthermore, the p95 gene product functions as a coactivator of the actin gene promoter in vitro.

The IE1 protein of BmNPV (BmIE1) was previously shown to be also capable of stimulating the expression of several tested genes in vitro, including gene 39K of AcNPV, gene p30 of BmNPV, and the cytoplasmic actin gene of Bombyx (22, 35, 37). To investigate whether P95 and BmIE1 function synergistically on the actin gene promoter, we cotransfected Bm5 cells either with pBmA.cat and pCS DNA or with these two plasmids and pBmIE1, an expression vector for BmIE1 (37). As shown in Fig. 6, the relative CAT activity obtained from cells transfected with pBmA.cat in the presence of BmIE1 was 61 times higher than that in its absence (relative activity of 1). Most importantly, the relative CAT activity obtained from cells cotransfected with pBmA.cat, pBmIE1, and pCS was consistently higher (127-fold over the activity obtained from pBmA.cat alone in the example shown in Fig. 6) than that obtained following cotransfection with either pBmA.cat and pBmIE1 (61-fold) or pBmA.cat and pCS (29-fold). These data indicate that BmIE1 is able to further augment the P95-mediated stimulation of the actin gene promoter.

**Expression of p95 is regulated by BmIE1 and P95.** To study the regulation of p95 further, a portion of the p95 gene upstream sequences encompassing the b and c, but not the a, initiation sites (nucleotides 36 to 394 [Fig. 4]) was inserted into pBl cat, the promoterless reporter containing the CAT ORF and the 3′ UTR of the silkmoth actin gene, to yield vector pP95.cat. To test whether the cloned sequences encompass a (probably minimal) promoter element that functions in the context of an infection, pP95.cat DNA was transfected into the BmNPV-infected Bm5 cells. As shown in Fig. 7A, CAT activity was detectable in infected but not in mock-infected cells, indi-
The observed absence of a detectable effect of P95 on p95 gene promoter activity in the absence of BmIE1 can be explained in two, mutually nonexclusive, ways. Either BmIE1 and P95 act synergistically on the p95 gene promoter to maximize the rate at which the latter is transcribed, or the level of expression of the p95 gene (yielding P95) in the absence of BmIE1 is too low to cause a detectable increase in CAT activity. Indirect evidence in support of the latter possibility was provided by a final set of cotransfection experiments in which the abilities of two different versions of the p95 gene (pCS and pCS.Dhr3) to trans activate the actin gene promoter were tested. As shown in Fig. 7C, removal of the HR3 enhancer from the p95 gene (plasmid pCS.Dhr3) resulted in a pronounced (eightfold) reduction of the P95-mediated increase in actin promoter activity.

**DISCUSSION**

We have characterized a gene of BmNPV, p95, encoding a 95-kDa transcription factor (P95) and possibly a second protein that lacks the C-terminal half of P95. Transcription of the p95 gene occurs during the early and late stages of viral infection and is up-regulated, at least in transfection assays, by the product of an immediate-early gene of BmNPV, BmIE1. The p95 gene is transcribed at low levels in transfected Bm5 cells, and its transcription is apparently augmented by the HR3 enhancer located at its 3' UTR. The complete protein encoded by the p95 gene can stimulate the expression of the silkmoth cytoplasmic actin gene as well as that of its own gene in vitro.

Two RNA transcripts, 1.7 and 3.5 kb, are detected by using as a probe a fragment containing the N-terminal zinc finger domain of P95. The 3.5-kb mRNA can encode the complete P95 polypeptide, which consists of 835 amino acids. The 1.7-kb RNA does not have such a coding capacity. Because no other ORFs reading in the same orientation as P95 exist in the genome of BmNPV upstream of gene p95 (for at least 6.0 kb upstream of the sequenced region [38]) and no other ORFs of any significant length are present in the first 1.7 kb of the p95 gene, we considered the possibility that the 1.7-kb transcript encodes an alternative polypeptide containing the zinc finger motif (the hybridization probe that detects the presence of the 1.7-kb RNA contains this domain) and only a limited number of additional amino acids (discussed below).

Although the Northern hybridizations failed to detect the 1.7- and 3.5-kb transcripts at 6 h p.i., the more sensitive primer extension method revealed that p95 is transcribed before viral DNA replication, which in BmNPV-infected Bm5 cells occurs some time between 6 and 9 h.p.i. The transcription of p95 during the early stages of infection is also supported by the results of the transient-expression assays (transfections), which showed that the product of the p95 gene (plasmid pCS) is able to stimulate actin-CAT gene expression (Fig. 6).

Early p95 gene transcription is initiated at two sets of sites (a1 to a3 and b [Fig. 4]), but transcription from the b site is barely detectable at 12 h p.i., suggesting that initiation from this site may be inhibited after viral DNA replication. At the late and very late stages of infection, the p95 gene is predominantly transcribed from the c initiation site within a TAAG motif (a consensus sequence for initiation of late or very late genes). Although the three initiation sites discussed here have been assigned indirectly to the two p95 gene transcripts based on their temporal patterns of expression (sites a and b for the 1.7-kb RNA and site c for the 3.5-kb RNA), this type of analysis cannot exclude the possibilities that both RNAs are...
initiated from all three initiation sites and that the transition from the early to the late phase of infection is also accompanied by changes in transcriptional termination, polyadenylation, or splicing of the p95 gene transcripts.

In view of the fact that the 1.7-kb transcript is more abundant than the 3.5-kb RNA but does not include the complete P95 ORF, we undertook a transcription mapping study to determine the sequences encompassed by each RNA. Our mapping experiments (Fig. 5) have shown that the two transcripts are produced as a result of differential usage of two polyadenylation sites, a proximal one occurring downstream of the zinc finger region and a distal site located downstream of the P95 ORF stop codon (Fig. 2). The alternative possibility, that the 1.7-kb RNA represents a spliced version of the 3.5-kb transcript, has been excluded by our inability to detect reverse transcription-PCR products other than those predicted to originate from unspliced P95 RNA in a comprehensive PCR-based detection assay that employed primers spanning the p95 gene almost in its entirety (data not shown).

Because the proximal polyadenylation site for the 1.7-kb mRNA occurs in the middle of the ORF, the question arises as to whether translation termination can occur on this mRNA. We have noticed that the region between the zinc finger domain and the proximal polyadenylation site is occupied by several A/T-rich sequences that resemble the heptanucleotide sequence motifs previously reported to act as −1 or +1 ribosomal frameshifting signals in other viral mRNAs (Fig. 2) (1, 16, 19, 24). An additional sequence that may form a stem-loop structure and function as a second signal for frameshifting is also present 50 to 80 nucleotides upstream from the polyadenylation site (Fig. 2). Ribosomal frameshifting events (+1 or −1) occurring in infected cells at some of these sites could result in premature translation termination at any one of several termination codons that exist in both shifted frames prior to the polyadenylation signal (Fig. 2). These would result in the generation of a truncated polypeptide of approximately 45 kDa (hereafter termed P95/45).

Thus, our data suggest that two proteins may be produced from the p95 gene, a less abundant protein, P95, containing the zinc finger region and the C-terminal proline-rich trans-activation domain, and a more prevalent polypeptide, P95/45, that contains the zinc finger region but lacks the C-terminal activation domain. As discussed below, the 95-kDa protein can act as a transcriptional activator in transfected Bm5 cells. Whether the putative P95/45 indeed exists and, if so, whether it has a transcriptional role in infected cells has yet to be established.

All major features of the conceptual P95 protein are conserved between BmNPV and AcNPV. However, a divergent region was observed in its C terminus (amino acids 663 to 677), especially in the domain containing the EP repeats. There are three, four, and seven EP repeats in the P95 of the BmNPV MLI isolate (this study), the BmNPV T3 isolate (38), and the AcNPV S6 isolate (4), respectively. EP repeat motifs have been described previously for the ryanodine receptor of rabbit skeletal muscle sarcoplasmic reticulum and E. coli TonB protein and are probably involved in protein-protein interactions or ion binding (11, 32). The relevance of the observed differences in the EP regions of the different P95 proteins is unclear. P95 also contains two additional motifs of potential importance: two partially repeated sequences, each of which includes a zinc finger motif (CCHR) at its N terminus, and a proline-rich region that overlaps the region of the EP repeats at its C terminus. CCHR motifs have been identified in retroviral nucleocapsid proteins, in proteins from several plant viruses, and in a human protein that can bind single-stranded DNA or RNA (6, 21, 22, 26, 43, 46, 51). The proline-rich region, on the other hand, has a high degree of sequence identity with the C-terminal heptad repeats of the largest subunit of eukaryotic DNA-dependent RNA polymerase II (2, 13, 54). Thus, a transactivation domain may be present in the C terminus of P95. Whether P95 is able to bind to specific nucleic acid sequences or interact with other proteins or both has not been investigated.

Our transient-expression assays have shown that P95 can stimulate the expression of the silkworm actin gene promoter in transfected Bm5 cells. This result demonstrates that the specific p95 gene construct used in this assay (clone pCS) is transcriptionally active in these cells in the absence of other viral gene products and also suggests that P95 (assuming that P95/45 is not produced in transfected cells) acts as a transcription factor.

In transfected cells, P95 and BmIE1 mediate an increase in the activity of the actin gene promoter independently of each other (Fig. 6). The observed superactivation of the actin gene promoter upon cotransfection with p95 and ic1 (Fig. 6) may be due to an additive effect of the two transcription factors on the actin promoter. This possibility takes into consideration the increase in the level of P95 expression mediated by BmIE1 (Fig. 7B) and is supported by the observation that removal of the HR3 enhancer from the 3′ UTR of the p95 gene (plasmid pCS.Δhr3; Fig. 1 and 2), a manipulation that presumably causes down-regulation of the p95 gene promoter or destabilization of P95 nuclear RNA, results in a significant reduction in the P95-mediated increase in actin promoter activity (Fig. 7C). Alternatively, BmIE1 and P95 could interact with each other and increase the rate of transcription from the actin promoter cooperatively. The C terminus of BmIE1 contains an Src homology 3 (SH3) domain (36a), a domain that is present in all Src-type protein tyrosine kinases and is known to mediate protein-protein interactions (39, 44, 47, 52), and P95 contains three sets of PXXP sequences in the proline-rich region (amino acids 670 to 673, 676 to 679, and 681 to 684 [Fig. 2]). Because proteins with SH3 regions can interact with PXXP domains (reference 3 and references therein), it is possible that P95 and BmIE1 may form protein-protein complexes and act cooperatively on the actin promoter.

Despite the fact that the minimal promoter of the p95 gene that lacks the a1 start site is active in cells cotransfected with pBmA.cat and pCS (shown indirectly by the stimulation of the actin gene promoter [Fig. 6]), its activity was not detectable in cells transfected with pP95.cat (Fig. 7A). This may be due to the fact that this minimal promoter lacks the HR3 enhancer that is present in the intact p95 gene (in plasmid pCS) and therefore is transcribed at a lower level than the p95 promoter found in the context of the pCS plasmid. Surprisingly, however, plasmid pP95.cat was found to be inactive even upon cotransfection with plasmid pCS, which expresses P95, but it became active in the presence of IE1 (Fig. 7B). The obvious discrepancy, relative to the results obtained with the actin gene promoter, may be due to a differential effect of the P95 protein on the transcriptional activities of the two promoter elements (actin and p95), e.g., a lower affinity for a p95 promoter binding site. Thus, P95 could stimulate the activity of its own gene promoter but only when present at levels higher than those required for activation of the actin gene promoter. The alternative and more attractive possibility, however, is that P95 can modulate the expression of its own gene only in the presence of BmIE1 and that BmIE1 recruits P95 to the promoter of its own gene via protein-protein interactions. Further studies are necessary to distinguish between these two possibilities and to establish the relevance of our findings to the strategies that baculoviruses employ in order to propagate themselves in the
cells of their hosts. A third possibility, that P95 encodes a DNA stabilization protein that results in increased stability of transcribed DNA, and thus increased actin gene promoter-driven CAT activity, in cells cotransfected with the p95 gene has been excluded on the basis of our inability to detect any significant differences in transcribed DNA content among cells transfected with the different plasmid constructs 2 to 3 days post-transfection (data not shown).

At present, we have no information concerning the actual presence of the putative P95/45 protein in infected cells. The cotransfections with plasmids containing the 5′ portions of the p95 gene (even construct pCA, which encompasses the proximal polyadenylation site [Fig. 1]) did not yield any relevant information. This is not surprising, however, because premature translation termination is probably dependent on the availability of virally encoded factors that are absent from transfected cells. Should P95/45 be proven to be present in infected cells, questions related to its functional relevance will need to be addressed. It is possible that P95/45 could bind to DNA target sites and act as a repressor of gene expression. Alternatively, however, it is also possible that P95/45 functions in infected cells as a gene regulator different from P95 by recruiting viral coactivators that are different than those interacting with P95.

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