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Alternative Transcriptional Initiation as a Novel Mechanism for Regulating Expression of a Baculovirus *trans* Activator

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In this report, we show that the *Orgyia pseudotsugata* nuclear polyhedrosis virus p34 gene, which is homologous to the *Autographa californica* nuclear polyhedrosis virus PE-38 gene, is a *trans* activator. The predicted p34 protein contains a number of motifs that are similar to those found in other eukaryotic transcriptional *trans* activators, including a putative zinc finger DNA-binding domain, a glutamine-rich domain, and a leucine zipper. Northern (RNA) blot analysis showed that the p34 gene is expressed as a 1.1-kb mRNA from 1 to 48 h postinfection and as a 0.7-kb mRNA from 18 to 120 h postinfection. Mapping of these transcripts showed that they were 3′ coterminal but initiated at different 5′ start sites. The 1.1-kb transcript initiates at a baculovirus early gene motif (CACAGT) and encodes the entire p34 open reading frame (ORF). The 0.7-kb transcript initiates at a baculovirus late gene start site (GTAAG) internal to the p34 ORF. Western blot (immunoblot) analysis using p34 antisera showed that the 0.7-kb transcript is translated as an amino-terminally truncated 20-kDa form of the full length 34-kDa protein. Functional analysis indicated that the 34-kDa protein transcriptionally *trans* activates the IE-2 promoter whereas the 20-kDa protein does not. Therefore, p34 produces two functionally different proteins from the same ORF, using the novel mechanism of alternative transcriptional initiation.

The baculovirus *Orgyia pseudotsugata* nuclear polyhedrosis virus (Op*M*NPV) is a large DNA animal virus with a 130-kb double-stranded, circular genome. Baculoviruses have a complex viral life cycle that is believed to be regulated primarily at the level of gene transcription. Viral gene expression occurs in an ordered cascade, which can be temporally divided into early, late, and very late phases (for reviews, see Friesen and Miller [10] and Blissard and Rohrmann [4]). Viral early genes appear to be transcribed by host RNA polymerase II and expressed before viral DNA replication. Viral late genes are expressed after viral DNA replication, and an α-amanitin-resistant RNA polymerase is used for their transcription (11, 14, 17). For many early genes, transcription initiates at a conserved motif CAGT or CACAGT, whereas most late genes initiate at (A/G)TAAG.

The regulation of early and late gene expression involves both viral and cellular transcriptional *trans* activators that interact via mechanisms that are not presently well understood. Two Op*M*NPV *trans* activators, called IE-1 and IE-2, have been characterized and found to be homologous to the *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) IE-1 and IE-N genes, respectively (5, 15, 37, 38). The Op*M*NPV IE-1 gene encodes a 66-kDa protein that appears to be an acid domain *trans* activator similar to VP16 of herpes simplex virus type 1 (18, 37). IE-1 is the only known spliced baculovirus gene and is transcribed from early to very late times postinfection (p.i.). Western blot (immunoblot) analysis has also shown that IE-1 produces multiple gene products that are present at relatively high levels even at very late times p.i. In addition, a form of IE-1 has been shown to associate with purified budded virions (40). Analysis of both the Op*M*NPV and Ac*M*NPV IE-1 genes has shown that IE-1 is a multifunctional protein that is autoregulatory and, in addition, will *trans* activate both early and late viral promoters as well as repress early promoters (15, 18, 37).

The Op*M*NPV IE-2 gene is also expressed at early and late times p.i. but at levels significantly lower than those of the IE-1 gene and is barely detectable on Western blots by 48 h p.i. (38, 40). Like IE-1, IE-2 is a *trans* activator that has been shown to up regulate IE-1, p8.9, and its own promoter (38, 44). IE-2 may activate genes by directly binding DNA elements, as it contains an amino acid sequence that is homologous to a putative DNA-binding zinc finger motif ($Cx_2Cx_{11-27}CxHx_2Cx_2Cx_{6-16}Cx_2C$) found in a diverse array of proteins (1, 13, 23). Two Op*M*NPV genes have also been shown to have similar zinc finger genes, CG30 and the gene that we have named p34, which is homologous to the Ac*M*NPV PE-38 gene (3, 39, 41).

In this report, we describe the detailed analysis of the p34 gene and show that it is also a *trans* activator that up regulates the Op*M*NPV IE-2 gene in transient assays. In addition, we report that p34 produces a small 20-kDa protein at late times p.i. that is an amino-terminally truncated form of the full-length 34-kDa protein. The smaller protein is encoded by an mRNA that initiates from a late transcription start site located within the p34 open reading frame (ORF). p34 therefore appears to be a unique eukaryotic *trans* activator that produces multiple gene products from a single ORF by the novel mechanism of alternative transcriptional initiation.

MATERIALS AND METHODS

Cells and virus. *Lymantria dispar* LD652Y and *Spodoptera frugiperda* Sf9 cells were maintained in TC-100 media as described previously (36). Op*M*NPV was propagated in *L. dispar* cells as previously described (32). Time course studies of Op*M*NPV infection were analyzed by

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infecting LD652Y cells at a multiplicity of infection of 20, with the 0 h p.i. time point defined as the time after the virus was allowed to adsorb to the cells for 1 h.

Plasmid constructs and DNA sequencing. Plasmid clones of p34 were isolated from the *Hin*dIII-A restriction fragment of the cosmid Op47 (21). The IE-1 plasmid used in cotransfection was described previously (37). The IE-2 plasmid (IE2-E2.3) and the IE-2CAT reporter plasmid were constructed as previously described (38). The p34 gene constructs, Nhe-p34 and Nsi-p34, were subcloned from plasmid Sma-p34 (see Fig. 5a), using the convenient restriction endonuclease sites *Nhe*I and *Nsi*I to delete the upstream sequences of the p34 promoter. The Sma-p34Δ, Nhe-p34Δ, and Nsi-p34Δ constructs were constructed by deletion of *Hpa*I-*Kpn*I fragments from plasmids Sma-p34, Nhe-p34, and Nsi-p34, respectively.

RNA isolation and Northern (RNA) blotting. Total RNA from Op*M*NPV-infected LD652Y cells was prepared as previously described (37). Northern blots (5 μg of total RNA per lane in agarose gels containing 1.25% formaldehyde) were prepared as described by Thomas (42) and hybridized to single-stranded RNA probes at 60°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0), 5× Denhardt's solution (1× Denhardt's solution is 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, and 0.02% Ficoll 400), 0.1% sodium dodecyl sulfate (SDS), 100 μg of denatured salmon sperm DNA per ml, 100 μg of yeast RNA per ml, and 10% polyethylene glycol (molecular weight, 8,000). ^{32}P-labeled single-stranded RNA probes complementary to p34 mRNA were synthesized in vitro, using T3 or T7 RNA polymerase (34). After hybridization, the blots were washed twice in 0.1× SSC–0.1% SDS at 75°C and then exposed to Kodak XAR films with an intensifying screen. Total RNAs from the transfected cells were prepared as previously described (45).

Primer extension, S1 nuclease mapping, and RNase protection assays. Primer extension assays were performed as described previously (37). Two 17-base oligonucleotides, 5′-AACTCGCTGAGCAACCG-3′ and 5′-TGCCTCTTCCTC AGTGG-3′, were used as primers for mapping the 5′ ends of the p34 early and late mRNAs, respectively. S1 nuclease mapping assays were also used to map the 5′ and 3′ ends of p34 transcripts as previously described (37). For RNase protection assays, a *Pvu*II-*Pst*I fragment from the IE-2CAT construct was cloned into pBS(+) at the *Hin*cII and *Pst*I sites. After digestion with *Ava*II, a ^{32}P-labeled RNA probe was generated by using T7 RNA polymerase (34). RNase protection assays were performed by using an Ambion RPA II RNase protection assay kit. Sequencing reactions were performed with double-stranded plasmid DNA templates (43).

Expression of p34 in *Escherichia coli* and production of anti-p34 antisera. An *Xho*I DNA fragment containing the C-terminal 142 amino acids of the p34 ORF was cloned into the pGEX-2T expression vector at the *Bam*HI site (35). A glutathione *S*-transferase–p34 fusion protein was expressed in *E. coli* DH5α cells by induction with isopropyl-β-D-thiogalactopyranoside (IPTG), and inclusion bodies containing the fusion protein were further purified as described previously (22). Rabbit polyclonal antisera were produced against the purified fusion protein by using standard techniques (34). The rabbit anti-p34 polyclonal antisera were used directly without further purification.

Western blotting and immunological detection of p34-related proteins. Op*M*NPV-infected or plasmid-transfected LD652Y or Sf9 cells for analysis by Western blotting were harvested by scraping off monolayers with a rubber policeman and were collected by low-speed centrifugation (3000 rpm for 5 min). The cell pellet was washed once with 1 ml of phosphate-buffered saline (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl [pH 7.5]), resuspended in 100 μl of protein sample buffer (0.125 M Tris-Cl [pH 6.8], 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue), and sheared with a 25-gauge syringe. Proteins were boiled and subjected to SDS–10 or 12% polyacrylamide gel electrophoresis (20) and electrophoretically transferred onto nitrocellulose (Amersham) or Immobilon (Millipore) membranes, using an LKB Novablot semidry transfer apparatus according to standard techniques (16). Normally, total cell protein from 2.5×10^4 to 5.0×10^4 cells was analyzed per sample. The Western blots were incubated with monoclonal IE-2 antibodies (40) or polyclonal p34 antisera at a 1:1,000 dilution. Antigen-antibody complexes were detected by using a peroxidase-conjugated secondary antibody (1:5,000 dilution; Jackson Laboratories) and visualized with the enhanced chemiluminescence system (Amersham).

Transient assays. Cells were transfected with plasmid DNA as described previously (37). Transfected cells were harvested, and cell extracts were prepared for analysis of chloramphenicol acetyltransferase (CAT) activity by the method of Neuman et al. (28). Normally, total cell protein from 1.0×10^5 to 2.0×10^5 transfected cells was assayed for CAT activity. The amounts of plasmids used in different transient assays are specified in the figure legends.

RESULTS

The p34 ORF was previously identified 225 bp downstream from the Op*M*NPV enhancer element, and comparison with genes encoding other baculovirus proteins revealed that it was homologous (37% identity) to the PE-38 gene of Ac*M*NPV (19, 39). The p34 ORF codes for a protein predicted to be 307 amino acids in length with a molecular mass of 34,691 Da. The predicted amino acid sequence contains a number of motifs that are found in many eukaryotic transcription factors. These include a basic domain, a zinc finger motif, an acid domain, a glutamine-rich domain, and a leucine zipper (Fig. 1). As has been suggested by Krappa and Knebel-Mörsdorf (19) for the homologous PE-38 gene, these motifs strongly suggest that this protein is a transcription factor and probably regulates baculovirus gene expression. We were therefore interested in further characterizing this Op*M*NPV gene and determining its function in Op*M*NPV-infected cells.

Transcriptional mapping analysis of the p34 gene. Northern blot analysis was used to initially characterize the temporal expression of p34 transcripts (Fig. 2). A 291-bp single-stranded RNA probe complementary to p34 mRNAs from the *Kpn*I-*Pst*I DNA fragment internal to the p34 ORF (probe B; Fig. 3a) was hybridized to total RNA extracted from Op*M*NPV-infected LD652Y cells at various times p.i. The RNA probe detected two p34 transcripts, a 1.1-kb transcript expressed from 1 to 48 h p.i. and a late 0.7-kb transcript expressed from 18 to 120 h p.i. This was an unexpected result, as the smaller 0.7-kb transcript is not large enough to contain the complete 921-bp p34 ORF. To precisely map and determine the origin of the 1.1- and 0.7-kb transcripts, S1 nuclease protection and primer extension assays were performed.

The map locations of the 5′ ends of the two p34 transcripts were initially determined by S1 nuclease protection assays using a 5′-end-labeled 1.8-kb *Pst*I-*Xho*I DNA probe (Fig. 3a

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  1 .....................................................MSS  3

  4 RYSPYRIRNVGERRRVQERLLSEFNSRPVTAVAAVCAVCLETYCVQSNNI  53

 54 IDFLMPSECTHLFCYKCVLNMYKNAMNVPRAAVSCPMCNKKVGTWQAFFP 103

104 NSVVSCKFIKKTGDRTPACLQFMLALKTIQDRYMATEEEAETEPSFVIKN 153

154 LQAQLDAAQKEARDLQESMERQKQAHNVAWNSSCEQVTALQTTLADMQAQ 203

204 LDRSEALSSTLAEHNRAANVQIDSLRRAVQRLEAAQSAPVSVNVEFNDNA 253

254 RQNTNLHERFRSYVYSTVSDMMIEDSIKSLQSHVFGAACLPCSVNVEINF 301

302 PFDE 307
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FIG. 1. Amino acid sequence and predicted domains of p34. The amino acids are presented as single letters; the sequence was obtained from Theilmann and Stewart (39). The basic domain is shown as boxed boldface letters. The conserved cysteine and histidine residues in the zinc finger domain are boxed, as is the first methionine residue in the p34 late mRNA. The short stretch of five glutamic acid residues is indicated (−). The glutamine-rich domain is shown by the shaded box, and the leucine zipper is shown by boldface letters. The underlined amino acids indicate the location of the 5′ initiation site of the p34 late transcript.

and b). At 1 h p.i., only a 536-nucleotide (nt) fragment was detectable, while at 36 h p.i., a 170-nt fragment was also observed. This finding is consistent with the Northern blot analysis, in which a single message is detected at 1 h p.i. and two messages are detected at 36 h p.i. The 536-nt S1 nuclease-protected fragment approximately maps the 5′ end of the early mRNA to an early gene motif (CACAGT) 35 bp upstream from the start of the p34 ORF. However, the 170-nt protected fragment detected at 36 h p.i. maps the 5′ end of the late transcript to a late gene motif (GTAAG) 327 bp downstream from the start codon of the p34 ORF. Surprisingly, we were unable to detect S1 nuclease-protected fragments at 72 h p.i., when only the 0.7-kb transcript is observed on Northern blots. Similar results were obtained with the 3′ S1 nuclease mapping and primer extension experiments; a possible explanation for this result is discussed below.

The 3′ ends of the p34 early and late transcripts were also mapped by S1 nuclease protection assays. A 3′-end-labeled

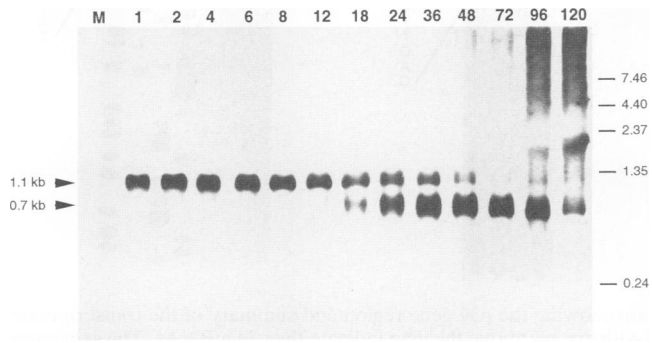


FIG. 2. Northern blot analysis of p34 mRNAs from Op*M*NPV-infected LD652Y cells at various times p.i. Total RNA (5 μg per lane) hybridized to a single-stranded RNA probe generated from the *Kpn*I-*Pst*I restriction fragment from the p34 ORF (probe B; Fig. 3a). Numbers above the lanes indicate hours p.i. M, mock-infected cells. Numbers on the left indicate sizes of the two p34 transcripts; numbers on the right represent the sizes (in kilobases) of marker RNAs.

*Pvu*II-*Xho*I DNA fragment (0.8 kb) was used, and a single protected fragment of 379 nt was detected at 1 and 36 h p.i. (Fig. 3a and c). This maps the 3′ ends of early and late p34 mRNAs to a position 65 nt downstream from the end of the p34 ORF and 13 bp downstream from a consensus polyadenylation signal (AATAAA; Fig. 3a). The two transcripts would therefore be 1,027 and 661 bp in the absence of a poly(A) sequence, which agree quite closely with the sizes determined by Northern blot analysis.

To confirm the S1 nuclease protection assays and to determine whether splicing was occurring, the 5′ ends of the 1.1- and 0.7-kb transcripts were also mapped by primer extension assays. Two 17-base oligonucleotides complementary to sequences 118 bp downstream from the early start site and 101 bp downstream from the late gene consensus start site were used (Fig. 3a, d, and e). The results showed that at 1 and 36 h p.i., the 5′ end of p34 early mRNA mapped to the first A of the conserved early transcription start motif CACAGT. The 5′ end of p34 late mRNA mapped to the first A of the conserved baculovirus late transcription start site motif GTAAG and was detectable only at 36 h p.i., not at 1 h p.i. The primer extension assays therefore confirm the S1 nuclease protection results and, in addition, show that the two different transcription start sites for the early and late mRNAs are not due to splicing.

As mentioned above, the S1 nuclease protection and primer extension assays should be able to detect the late 0.7-kb mRNA at 72 h p.i. as well as at 36 h p.i., but as shown in Fig. 3, we were unable to detect any protected or extended fragments at 72 h p.i. To confirm that the S1 probes were homologous to the very late (72 h p.i.) mRNA, several contiguous single-stranded RNA probes that included the sequences of the S1 nuclease and primer extension probes were hybridized to Northern blots. Probes beyond the 5′ and 3′ mapped ends of the p34 late transcript failed to detect the 0.7-kb mRNA (probes A and F; Fig. 3a), but all probes within the mapped region (probes B to E; Fig. 3a) detected a 0.7-kb transcript at late times p.i., identical to the result shown in Fig. 2. These results confirmed that the S1 nuclease and primer extension probes were homologous to the 0.7-kb mRNA detected at late times p.i. Interference with the mapping of RNA transcripts by opposite-strand transcription of baculovirus late genes has been clearly demonstrated by Ooi and Miller (29) and Lu and Carstens (24). To determine whether this could be occurring in our assays, we probed a Northern blot with a sense single-stranded RNA probe from the p34 ORF that would hybridize to transcripts from the antisense strand (probe G; Fig. 3a). This probe detected high levels of transcripts from the antisense strand at very late times p.i. (Fig. 3f). We therefore conclude that the inability to detect the late 0.7-kb transcript at 72 h p.i. by S1 nuclease protection and primer extension assays is likely due to high levels of RNA transcripts produced from the complementary strand at very late times p.i. These RNA transcripts would compete with the labeled DNA probes by forming more stable RNA-RNA hybrids with the p34 late mRNA.

Detection of multiple p34 polypeptides in infected and transfected cells. The transcriptional mapping of the p34 gene demonstrated that it is differentially expressed as two mRNAs at early and late times p.i. The 0.7-kb transcript sequence contains only a single ORF which is in the same reading frame as p34. This finding suggests that the 0.7-kb mRNA must code for a truncated form of p34. A start codon is located 49 nt downstream from the late transcriptional start site; a protein produced from this ORF would be 182

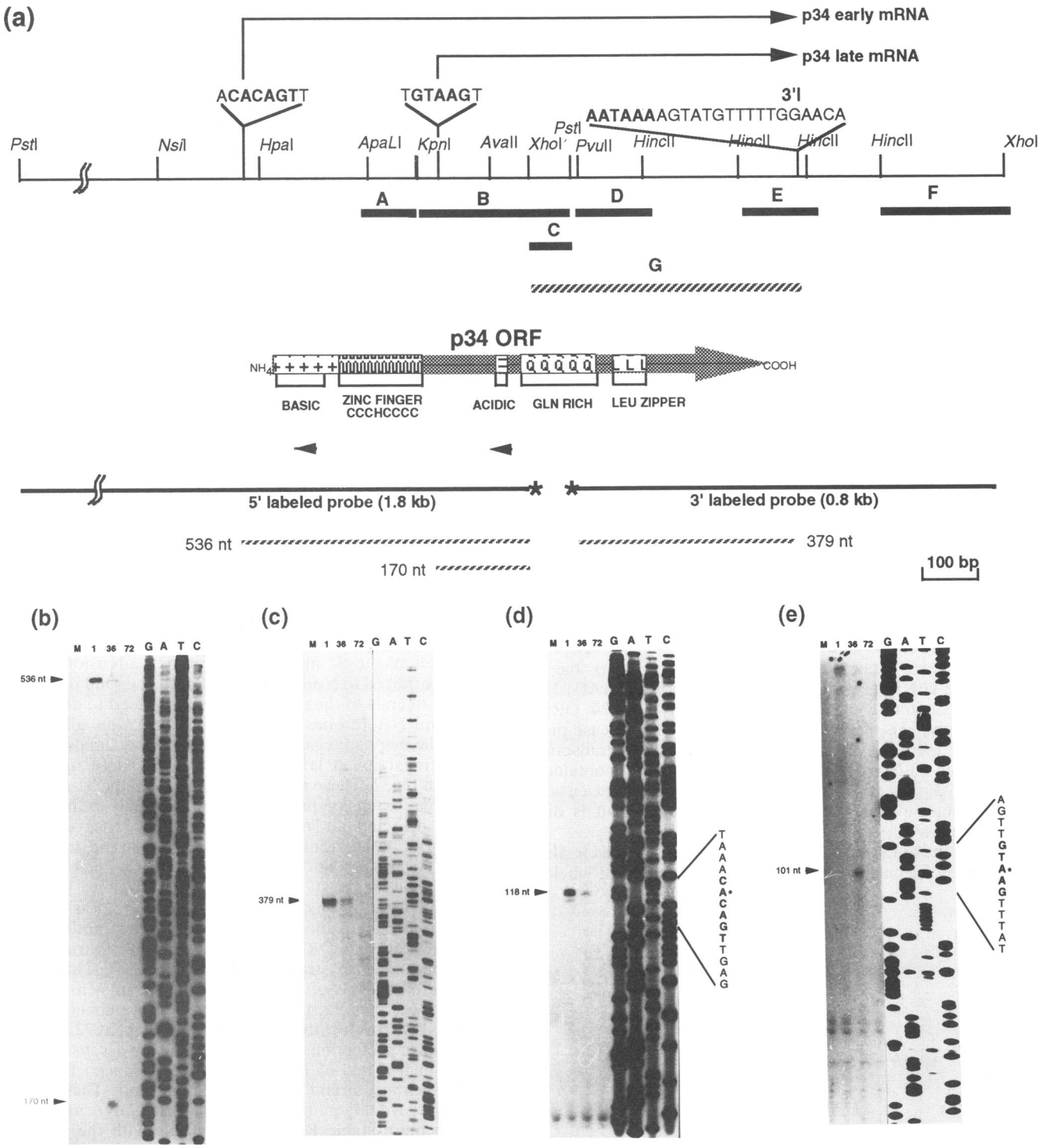
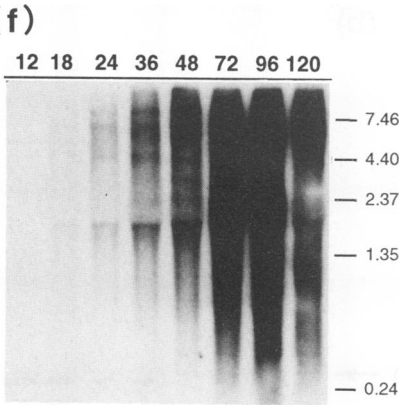


FIG. 3. Transcriptional mapping of the p34 gene. (a) Schematic diagram showing the p34 gene region and summary of the transcriptional mapping results. The p34 ORF is indicated by a large arrow; the thin lines with arrows above the map indicate the p34 mRNAs. The sequences around the early and late 5′ initiation and 3′ termination (3′ |) sites are also shown. The thick lines below the restriction map represent the locations of single-stranded RNA probes used for Northern blot analyses of p34. The black lines (A to F) represent probes homologous to p34 mRNAs, and the hatched line represent the probe used to detect antisense mRNAs. Locations of the predicted p34 protein domains are shown below the p34 ORF. The end-labeled probes used in 5′ and 3′ S1 nuclease protection assays are indicated by the lines with asterisks. The hatched lines indicate the protected fragments. The oligonucleotide primers used for primer extension analysis of the 1.1-kb early and 0.7-kb late transcripts are represented by the two small arrows. (b) S1 nuclease protection analysis of the 5′ initiation sites of the p34 early and late transcripts, using a 1.8-kb *Xho*I-*Pst*I probe. (c) S1 nuclease protection analysis of the 3′ termination site, using a 0.8-kb *Pvu*II-*Xho*I probe. (d and e) Primer extension analysis of the 1.1- and 0.7-kb transcripts, respectively. Numbers above the lanes indicate hours p.i., and



amino acids long and have a predicted molecular mass of 20,468 Da. To investigate the possibility that a truncated form of p34 was produced in infected cells, a polyclonal antiserum against an *E. coli*-expressed recombinant glutathione *S*-transferase–p34 fusion protein was raised. A Western blot of Op*M*NPV-infected cells at various times p.i. detected a 34-kDa protein at 1 h p.i. that reached maximum steady-state levels at 8 to 24 h p.i. but remained detectable up to 120 h p.i. (Fig. 4a). As predicted from the transcriptional mapping, at late times p.i., small proteins were also detected by the p34 antiserum. Two proteins (20 and 21.5 kDa) appeared from 24 to 120 h p.i., while the smallest protein (18 kDa) was detected from 48 to 120 h p.i. The p34 antiserum was produced against a glutathione *S*-transferase–p34 fusion protein and therefore contained antibodies to glutathione *S*-transferase and p34. To confirm that all proteins detected on the Western blot were p34 specific, we performed a control Western blot analysis with antisera raised only against glutathione *S*-transferase (Fig. 4b). The results showed that the 18-kDa protein detected in Fig. 4a was due to a cross-reaction between an 18-kDa viral late protein and glutathione *S*-transferase antibodies. The 34-, 21.5-, and 20-kDa proteins did not react and are therefore specific to the p34 antiserum and are the products of the p34 ORF.

The temporal appearance of the 34-kDa protein is very similar to that of the early 1.1-kb mRNA detected in the Northern blot analysis, and this protein has a size corresponding to the full-length p34 ORF. Therefore, we concluded that the 34-kDa protein detected at early times p.i. by the p34 antiserum is encoded by the early 1.1-kb mRNA. The 20- and 21.5-kDa proteins correspond approximately in size and in temporal expression to the 20,468-Da protein that was predicted to be encoded by the 0.7-kb late p34 transcript. These immunoblots therefore support the transcription data and indicate that p34 is producing an amino-terminally truncated protein at late times p.i.

To further confirm the origin of the early and late gene products of p34, we performed transient assays with plasmid constructs to express the p34 proteins. Two plasmids were constructed, one containing the entire p34 gene (Sma-p34; Fig. 5a) and a second construct that deleted the amino-terminal region of the p34 ORF and placed the p34 late mRNA coding sequence under the control of the p34 early promoter (Sma-p34Δ; Fig. 5a), since late gene promoters are not active in uninfected cells. Sma-p34Δ increases the untranslated leading sequence of the late mRNA by 28 nt, but no additional start codons are introduced. Both of these constructs were transfected into Sf9 cells, and the transiently expressed proteins were compared with p34 proteins produced in virus-infected cell extracts by Western blotting (Fig. 5b). Transfection with Sma-p34 produced a 34-kDa protein identical in size to the p34 protein detected at the early times of Op*M*NPV infection (8 h). The late gene construct Sma-p34Δ produced two proteins with the same sizes, 20 and 21.5 kDa, as those in Op*M*NPV-infected cells at late times p.i. (48 h). In addition, one minor protein band that was not detected in the initial time course analysis was observed between the 20- and 21.5-kDa proteins in both the infected and transfected cells (Fig. 4a). As indicated above, the predicted molecular mass of the 182-amino-acid protein from the p34 late mRNA was 20 kDa. It is possible that the two slightly larger late proteins are posttranslationally modified forms of the 20-kDa product. Our combined transcriptional and Western blot data therefore provide evidence that the p34 early mRNA encodes a 34-kDa protein from the full-length p34 ORF, while the p34 late mRNA uses the same ORF to produce an amino-terminally truncated protein or proteins. For simplicity, we will refer to the three observed small late protein bands as the 20-kDa p34 protein.

Regulation of p34 by IE-1 and enhancer elements. We have previously shown that an Op*M*NPV enhancer consisting of approximately 12 tandemly repeated 66-bp elements is located 225 bp upstream of the p34 ORF (Fig. 5a) (39). To investigate whether the enhancer sequences influenced p34 regulation, transient assays were performed to examine p34 expression from plasmid constructs with or without the enhancer. The level of p34 gene expression was examined by Western blot analysis using p34 antisera. The results in Fig. 5c demonstrated that the enhancerless construct Nsi-p34 did not produce detectable levels of p34, whereas the enhancer-containing plasmid Sma-p34 produced easily detectable levels of p34. It should be noted that on long exposure, low levels of p34 could be detected in the Nsi-p34-transfected cells (data not shown). The Op*M*NPV enhancer element has been shown activate *cis*-linked genes to a much greater extent when *trans* activated by IE-1 (39). To determine whether IE-1 would cause a further stimulation of p34 expression, the plasmid constructs with or without upstream enhancer sequences were cotransfected with IE-1 (Fig. 5c). Interestingly, the *trans* activation of the Nsi-p34 construct by IE-1 increased the amount of p34 to levels almost equivalent to those obtained with Sma-p34 transfected in the absence of IE-1. *trans* activation of Sma-p34 by IE-1, however, caused significantly higher levels of expression (approximately fivefold). These results indicate that IE-1 can directly *trans* activate the p34 promoter in the absence of the

M refers to mock-infected cells. (f) Northern blot analysis of p34 antisense RNAs transcribed at late times p.i. Labeling is the same as in Fig. 2. Total RNA (5 μg per lane) was hybridized to a single-stranded RNA probe (probe G; Fig. 3a) and exposed to film for 2 h. In panels d and e, the sequencing ladders (GATC) were generated by using the same labeled oligonucleotides as used in the primer extension assays, whereas in panels b and c, plasmid and M13 mp18 sequences, respectively, were used as size markers. Numbers and arrows on the left indicate the size of the primer extended and S1 nuclease-protected fragments; sequences on the right in panels d and e represent the regions surrounding the major transcription start sites (*).

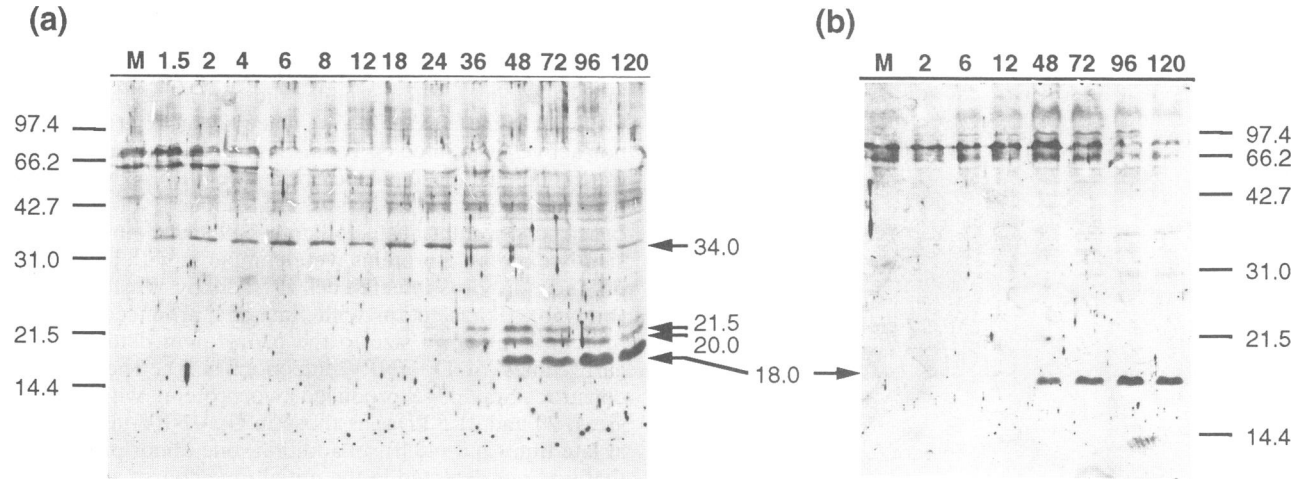


FIG. 4. Western blot analysis of p34 expression in Op*M*NPV-infected LD652Y cells. (a) Total protein from virus-infected cells at various times p.i. was analyzed by Western blotting with p34 antisera to detect p34-related proteins. (b) Western blot of various infected cell extracts analyzed with glutathione *S*-transferase antisera. Numbers above the lanes indicate hours p.i. M, mock-infected cells. Numbers on the right and left represent the sizes (in kilodaltons) of the marker proteins. The positions and sizes of the p34-related proteins are indicated by arrows and numbers between the two blots.

enhancer sequences, but much greater activation is obtained if the enhancer is present. Moreover, these results show that the enhancer can also cause a significant increase in p34 expression in the absence of IE-1.

Functional analysis of p34. As discussed above, the various motifs in the p34 amino acid sequence strongly suggest that this protein is a transcription factor. To determine whether p34 was capable of up regulating other viral genes, we performed a number of transient assays with various Op*M*NPV genes and found, as shown below, that p34 up regulated the IE-2 promoter. A reporter gene construct containing the CAT gene under the control of the Op*M*NPV IE-2 promoter (IE-2CAT) (38) was transfected into Sf9 cells with the p34 plasmid Sma-p34 (Fig. 6a). The results indicated that IE-2CAT expression was stimulated approximately twofold when this construct was cotransfected with p34. These transfections have been repeated more than 15 times and are highly reproducible. The IE-2CAT construct has a high basal-level activity, and therefore a twofold increase represents a significant increase in the actual levels of CAT produced.

To determine whether the amino-terminally truncated 20-kDa form of p34 also positively activated IE-2CAT expression, we cotransfected IE-2CAT with Sma-p34Δ. The results showed that Sma-p34Δ caused no significant increase in IE-2CAT expression, which indicates that the 20-kDa p34 protein is not a functional *trans* activator of the IE-2 early promoter (Fig. 6a). These results are consistent with the model that the amino terminus of p34 contains a DNA-binding domain that is required for directing the protein to the IE-2CAT promoter.

To confirm the results showing that p34 *trans* activated the IE-2 promoter, we cotransfected the complete IE-2 gene with p34 and analyzed the level of IE-2 expression by Western blot analysis using monoclonal antibodies specific to IE-2 (Fig. 6b). Two p34 plasmids were used: Nsi-p34, which contains the promoter sequences downstream of the enhancer element, and Nhe-p34, which contains the promoter sequences and a single 66-bp repeat of the enhancer element that is sufficient to stimulate expression of *cis*-linked genes (Fig. 5a) (39). Western blot analysis shows that in the absence of p34, IE-2 is barely detectable. Cotransfection with either Nsi-p34 or Nhe-p34 results in an increased level of IE-2 expression. The higher level of stimulation observed with plasmid Nhe-p34 is most likely due to the higher level of p34 expression when the enhancer is linked in *cis* to the gene (Fig. 5c). This result confirms the CAT assay data and shows that p34 is a *trans* activator that up regulates IE-2 gene expression.

trans activation of IE-2 by p34 could be occurring by a number of mechanisms, but the predicted structure of this protein strongly suggested that it is a transcription factor. To determine whether p34 up regulates IE-2 expression directly at the level of transcription, we transfected the IE-2CAT reporter construct with or without Nhe-p34 and analyzed RNA levels by RNase protection assays. We used a ^{32}P-labeled 371-nt RNA probe that covered the 5′ end of the IE-2CAT reporter gene, including the conserved baculovirus early transcription start motif CACAGT. As shown in Fig. 6c, a single 176-nt RNA-protected fragment is detected, which maps the initiation site of the IE-2CAT mRNAs expressed in the transfected cells to the expected CACAGT motif. In addition, the level of IE-2CAT transcription is approximately twofold higher when this construct is cotransfected with Nhe-p34. This result shows that p34 up regulates the levels of IE-2CAT RNA and therefore suggests that p34 is a transcriptional *trans* activator.

DISCUSSION

In this study, we present a detailed analysis of the Op*M*NPV p34 gene and show that it is a *trans* activator that will up regulate IE-2 gene expression. Transcriptional analysis of the p34 gene showed that it is expressed as 1.1- and 0.7-kb 3′-coterminal mRNAs that code for a full-length 34-kDa protein and a 20-kDa amino-terminally truncated protein, respectively. The expression of these proteins was confirmed by Western blot analysis of Op*M*NPV-infected and plasmid-transfected cells to show that p34 produced 34- and 20-kDa proteins at early and late times p.i., respectively

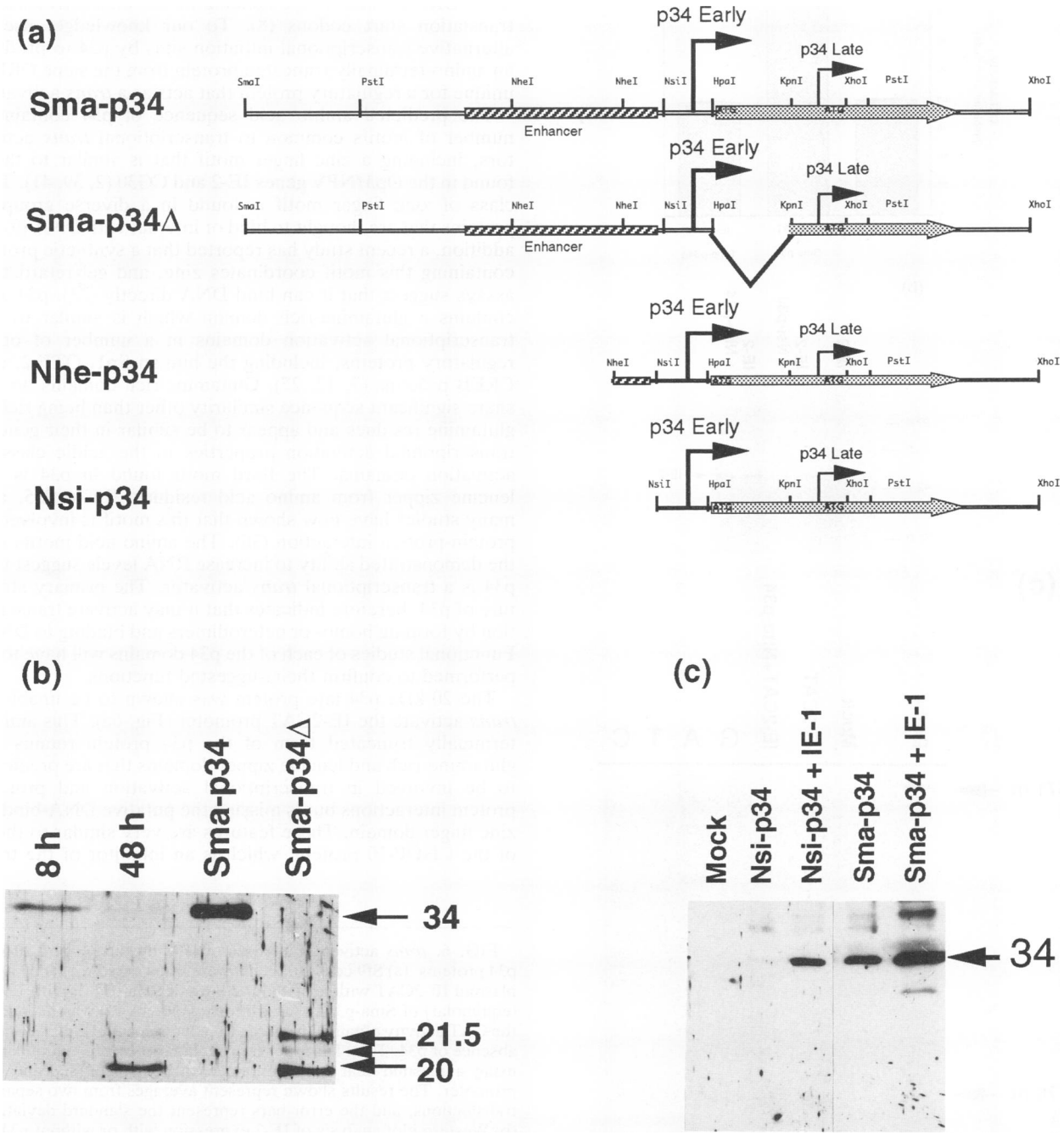
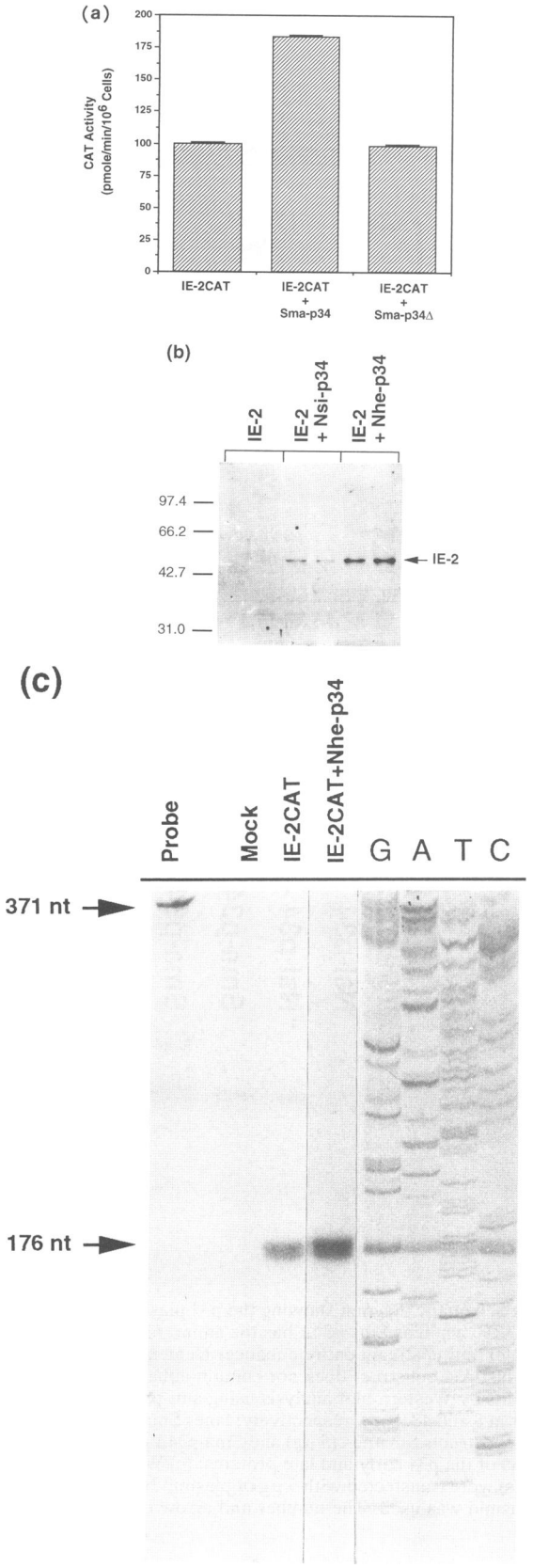


FIG. 5. Western blot analysis of p34-related proteins in transfected cells. (a) Schematic diagram showing the p34 plasmid constructs used in the transfections. Sma-p34, Nhe-p34, and Nsi-p34 all contain the entire p34 ORF, whereas Sma-p34Δ has the amino-terminal region of the p34 ORF deleted. The Sma-p34 and Sma-p34Δ constructs contain the 5′ promoter and upstream entire enhancer element (39). The Nhe-p34 construct contains a single copy of the 66-bp repeat of the enhancer, and the Nsi-p34 construct does not contain enhancer sequences. (b) Comparison of infected cells and transiently expressed p34 early and late proteins by Western blot analysis using anti-p34 antisera. Lanes 8 h and 48 h represent the viral proteins from the Op*M*NPV-infected LD652Y cells at 8 and 48 h p.i., respectively; lanes Sma-p34 and Sma-p34Δ represent the proteins from Sf9 cells (10^6 cells) transfected with the p34 plasmid constructs Sma-p34 (5 μg) and Sma-p34Δ (5 μg), respectively. Numbers and arrows on the right indicate the sizes (in kilodaltons) and positions of the p34 early and late proteins. (c) Western blot analysis of p34 gene expression activated by the enhancer and by IE-1. Sf9 cells (10^6 cells) were transfected with 5 μg of plasmid Nsi-p34 and an equal molar amount of plasmid Sma-p34. In the cotransfections, 5 μg of the IE-1 plasmid was used. The number and arrow on the right indicate the size (in kilodaltons) and position of the 34-kDa p34 early protein.



(Fig. 4). Recently, it has become clear that many eukaryotic transcriptional regulators produce multiple proteins from the same ORF by alternative splicing or the alternative use of translation start codons (8). To our knowledge, use of alternative transcriptional initiation sites by p34 to produce an amino-terminally truncated protein from the same ORF is unique for a regulatory protein that acts as a *trans* activator.

The predicted amino acid sequence of p34 contains a number of motifs common to transcriptional *trans* activators, including a zinc finger motif that is similar to those found in the Op*M*NPV genes IE-2 and CG30 (2, 39, 41). This class of zinc finger motif is found in a diverse group of proteins that are thought to bind or interact with DNA (9). In addition, a recent study has reported that a synthetic protein containing this motif coordinates zinc, and gel retardation assays suggest that it can bind DNA directly (23). p34 also contains a glutamine-rich domain which is similar to the transcriptional activation domains in a number of other regulatory proteins, including the human Sp1, OTF-2, and CREB proteins (7, 12, 27). Glutamine-rich domains do not share significant sequence similarity other than being rich in glutamine residues and appear to be similar in their general transcriptional activation properties to the acidic class of activation domains. The third motif found in p34 is the leucine zipper from amino acid residues 214 to 235, and many studies have now shown that this motif is involved in protein-protein interaction (30). The amino acid motifs and the demonstrated ability to increase RNA levels suggest that p34 is a transcriptional *trans* activator. The primary structure of p34 therefore indicates that it may activate transcription by forming homo- or heterodimers and binding to DNA. Functional studies of each of the p34 domains will have to be performed to confirm their suggested functions.

The 20-kDa p34 late protein was shown to be unable to *trans* activate the IE-2CAT promoter (Fig. 6a). This amino-terminally truncated form of the p34 protein retains the glutamine-rich and leucine zipper domains that are predicted to be involved in transcriptional activation and protein-protein interactions but is missing the putative DNA-binding zinc finger domain. These features are very similar to those of the CHOP-10 protein, which is an inhibitor of the tran-

FIG. 6. *trans* activation analysis of IE-2 by the 34- and 20-kDa p34 proteins. (a) Sf9 cells (10^6 cells) were transfected with 0.05 μg of plasmid IE-2CAT with or without 2.0 μg of Sma-p34 or with 1.9 μg (equimolar) of Sma-p34Δ. An arbitrary value of 1.0 was defined as the CAT activity obtained from the transfection with IE-2CAT in the absence of p34. Total DNA per transfection was made equimolar by using a plasmid that only contained the enhancer and the p34 promoter. The results shown represent averages from two separate transfections, and the error bars represent the standard deviation. (b) Western blot analysis of IE-2 expression with or without p34. A plasmid containing the complete IE-2 gene (0.1 μg) with its −275 promoter (38) was cotransfected into Sf9 cells with or without 1.0 μg of each p34 plasmid (Nsi-p34 and Nhe-p34). The protein samples from the transfected cells were resolved on SDS–10% polyacrylamide gels, and IE-2 was detected with IE-2 monoclonal antibodies. Each transfection was performed in duplicate. The arrow on the right indicates the IE-2 protein band, and numbers on the left show the locations and sizes (in kilodaltons) of marker proteins. (c) RNase protection analysis of IE-2CAT expression *trans* activated by p34. Sf9 cells (10^6 cells) were transfected with 1 μg of IE-2CAT plasmid with or without 10 μg of the Nhe-p34 construct. Sizes of the full-length probe and protected RNA fragment are shown on the left. The sequencing ladder (GATC) was generated by using pUC19 sequences as a size marker.

scription factors C/EBP and LAP (33). CHOP-10 is homologous to C/EBP and retains a functional leucine zipper but has an inactive DNA-binding domain. It was shown that CHOP-10 inhibits the DNA binding of C/EBP and LAP by forming heterodimers that cannot bind DNA. It is possible that the 20-kDa p34 protein can form heterodimers with the same proteins as the functional 34-kDa protein, thus forming nonfunctional complexes and inhibiting the activities of the full-length p34 protein. Our initial experiments to test this hypothesis have indicated that the 20-kDa p34 late protein is capable of a small but consistent inhibition of the activity of the 34-kDa protein (data not shown). Therefore, at late times p.i. when the molar amounts of the 20-kDa protein are much higher than amounts of the 34-kDa protein (Fig. 4), significant inhibition of the p34 *trans* activator may occur. This may be important because even though the 1.1-kb transcript is not detected by 72 h p.i., the 34-kDa protein remains detectable up to 120 h p.i. This finding suggests that the 34-kDa protein is stable and that inhibition of its early promoter activation function could be essential.

Recent results from our laboratory have shown that p34 will also *trans* activate a second Op*M*NPV early gene called p8.9. p8.9 is a small gene that is upstream and adjacent to the IE-2 gene but is transcribed in the opposite direction (39, 44). Interestingly, IE-2 also up regulates both the IE-2 and p8.9 promoters (38, 44), which suggests that IE-2 and p34 simultaneously *trans* activate this bidirectional promoter.

Passarelli and Miller (31) have recently shown that the Ac*M*NPV gene IE-N, which is the homolog of IE-2, significantly increases expression from late gene promoters. In addition, it was shown that PE-38, the Ac*M*NPV homolog of p34, augmented, but is not required for, late gene activation. Our experiments have shown that p34 *trans* activates the IE-2 promoter, which therefore suggests the PE-38 augmentation of late genes observed by Passarelli and Miller (31) is due to activation of IE-N. Recently Lu and Carstens have also shown that PE-38 will *trans* activate the p143 promoter, increasing expression levels in transient assays approximately fourfold (25). It is unknown whether a 20-kDa p34 homolog is expressed in Ac*M*NPV-infected cells, but comparison of p34 and PE-38 amino acid and nucleic acid sequences indicates that the late transcript start site is conserved (TGTAAGTTTA), which suggests that a similar transcript would be produced in Ac*M*NPV-infected cells. Previous transcriptional analysis of the homologous Ac*M*NPV PE-38 gene did not detect any small transcripts similar to the p34 late transcript (19).

It has already been shown that the baculovirus *trans* activator IE-1 produces multiple protein products by alternative splicing (6, 18, 37). This study shows that the *trans* activator p34 uses a different mechanism, alternative transcriptional initiation, to produce different proteins from the same ORF. It is possible that this mechanism is utilized by other baculovirus genes to increase the repertoire of proteins that can be produced from a single ORF. Another possible example of this is the Ac*M*NPV p143 gene, which has an early transcript that initiates internally to the full-length p143 ORF (26). If this transcript is translated, it would produce an amino-terminally truncated protein that contains the helicase motifs of this putative helicase protein. As more baculovirus genes are characterized, it will be interesting to determine how frequently this novel mechanism is utilized.

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