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A Baculovirus Gene with a Novel Transcription Pattern Encodes a Polypeptide with a Zinc Finger and a Leucine Zipper

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An *Autographa californica* nuclear polyhedrosis virus gene encoding a 30-kilodalton polypeptide with two different sequence motifs characteristic of DNA-binding proteins was identified immediately downstream of the major capsid protein gene (vp39). The gene, CG30, was characterized by sequencing, transcriptional mapping, in vitro translation of hybrid-selected RNA, and comparison of the derived polypeptide sequence with published data bases. The initial ATG of the 792-base-pair CG30 open reading frame is two nucleotides downstream of the vp39 terminal TAA codon. Early transcripts of CG30 initiate within the vp39 coding sequence. At late times, bicistronic transcripts initiate from the vp39 promoter, continue through CG30, and terminate at the same site as the early transcripts. In vitro translation of hybrid-selected early CG30 RNA yields a polypeptide of 30 kilodaltons. The predicted CG30 polypeptide sequence has characteristics of a eucaryotic transcriptional activator and is novel in having two potential DNA-binding domains. A stretch of acidic residues bridges a zinc finger at the amino terminus and a leucine zipper with a flanking basic region at the carboxyl terminus.

The baculovirus *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) is a double-stranded DNA virus with a 128-kilobase-pair covalently closed, circular genome. Ac*M*NPV genes are expressed in a temporally regulated program consisting of at least three phases of gene expression: (i) an early phase prior to DNA replication, (ii) a late phase including viral DNA replication and the formation of budded extracellular virus, and (iii) a very late phase dominated by high-level expression of the p10 and polyhedrin genes, culminating in the production of polyhedral occlusion bodies.

Only a fragmented picture of Ac*M*NPV gene organization is available, but it is clear that early, late, and very late genes are interspersed throughout the Ac*M*NPV genome, and it is likely that this interspersion plays a regulatory role in viral gene expression (8). Although very few Ac*M*NPV genes contain introns (4, 29), complex transcriptional patterns are frequently observed that consist of overlapping transcripts of different temporal classes that may transverse one or more genes in the sense or antisense direction (9, 16, 37). Such transcriptional patterns may reflect intricate mechanisms of baculovirus gene regulation (8, 9, 37).

Much of the temporal regulation of Ac*M*NPV gene expression is mediated at the transcriptional level. Steady-state levels of transcripts of different temporal gene classes exhibit striking and characteristic changes during infection (7, 9, 29, 33, 46). Evidence for the involvement of both *cis*- and *trans*-acting viral factors in Ac*M*NPV transcriptional regulation is mounting. Cycloheximide blocks late and very late viral gene transcription but increases and temporally extends the transcription of early genes (41). Thus, synthesis of early gene products appears to be required for late and very late transcription as well as the turnoff of early transcription (5, 8, 41, 47). Early viral promoters have properties which resemble those of eucaryotic promoters, and a putative upstream regulatory consensus sequence has been identified (36, 47). Transient expression assays have identified a gene IE-1 which operates in *trans* to activate transcription from a variety of early genes (13, 15) and *cis*-acting enhancer elements which respond to IE-1 (14).

Late and very late promoters appear to differ significantly from conventional eucaryotic promoters and early viral promoters. Late and very late transcripts initiate within an invariant TAAG sequence (2, 39, 42, 46, 50). The TAAG TATT sequence at the transcriptional start point of polyhedrin RNA is the predominant determinant of polyhedrin promoter activity (40; B. G. Ooi, C. Rankin, and L. K. Miller, submitted for publication), suggesting a novel promoter recognition and initiation mechanism. A virus-induced α-amanitin-resistant RNA polymerase activity has been implicated in the expression of late and very late baculovirus genes (10, 12).

In this study, we described the unique arrangement and transcriptional pattern of two Ac*M*NPV genes: the major capsid protein gene (vp39) and a gene encoding a novel polypeptide, CG30. At early times postinfection (p.i.), CG30 is transcribed from an initiation site within vp39. At late times, CG30 is transcribed from the vp39 initiation sites as the second gene of a bicistronic message. The predicted polypeptide sequence of CG30 contains two different nucleic acid-binding motifs, a zinc finger and a leucine zipper with a flanking basic region.

MATERIALS AND METHODS

Virus and cells. Ac*M*NPV L-1 (27) was propagated in *Spodoptera frugiperda* IPLB-SF-21 (SF21) cells (48) grown in TC100 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum, 0.6 μg of amphotericin B per ml, 0.06 mg of penicillin G per ml, 0.27 mg of streptomycin sulfate per ml, and 0.26% tryptose broth. For time courses, monolayers of cells (10^7 cells per 100-mm plate or 10^6 cells per 35-mm plate) were inoculated with second-passage Ac*M*NPV at a multiplicity of 20 PFU per cell for 1 h at room temperature. Time zero was defined as the time when the inoculum was removed and incubation at 27°C was initiated. To block protein synthesis, we used cycloheximide at 100 μg/ml throughout the time course beginning with a 30-min pretreatment prior to inoculation.

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To block DNA replication, aphidicolin (5 μg/ml) was added to the medium after the adsorption period and maintained throughout the time course (41).

Sequencing strategy. The Ac*M*NPV *Hin*dIII C-*Xho*I A fragment, 53.5 to 58.9 map units (m.u.), was cloned into Bluescript KS(+) and KS(−) cloning vectors (Stratagene, San Diego, Calif.). Exonuclease III (17) and mung bean nuclease were used to generate a series of overlapping deletions in the cloned DNA fragment. Single-stranded deletion clones representing both DNA strands downstream of the capsid protein-coding sequence were sequenced by the dideoxyribonucleotide-chain termination method (43) with a modified T7 DNA polymerase and a Sequenase kit purchased from United States Biochemical Corp. (Cleveland, Ohio). The deduced protein sequence of the downstream open reading frame (ORF) was compared with the NBRF data base, release 10.0, and translated sequences from GenBank, release 55, using the Lipman-Pearson FASTP algorithm (28) as implemented by the International Biotechnologies, Inc.-Pustell Cyborg software. Amino acid matches were scored with the PAM250 matrix (44).

Transcriptional mapping. Monolayers of Ac*M*NPV-infected SF21 cells were harvested at various times p.i., and total cell RNA was isolated by the guanidinium isothiocyanate method (3). The 5′ and 3′ ends of the transcripts were mapped by S1 nuclease protection analysis (49). Probes were generated by cleaving an appropriate recombinant plasmid at a *Bal*I (56.5 m.u., Fig. 1B) restriction site unique to the Ac*M*NPV sequence contained in the plasmid. The fragments were then radiolabeled either at the 5′ end with T4 polynucleotide kinase and 50 μCi of [γ-^{32}P]ATP (3,000 Ci/mM; Dupont-NEN Research Products, Boston, Mass.) or at the 3′ end with T4 DNA polymerase and 50 μCi of [α-^{32}P]dCTP (3,000 Ci/mM; Dupont, NEN Research Products). The 3′-end-labeled probe extended to the *Sal*I site at 55.3 m.u., while the 5′-end-labeled probe extended past the *Eco*RV site at 58.0 m.u. to a *Pvu*II site in the vector. The appropriate fragments were purified on agarose gels to yield probes labeled exclusively at either the 3′ or 5′ end of the Ac*M*NPV sequence. Hybridizations were conducted at 49°C in 80% formamide–40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 6.4)–0.4 M NaCl–1 mM EDTA. Nuclease-resistant fragments were analyzed on 6% polyacrylamide–7 M urea sequencing gels.

For primer extension analysis, a 16-base-pair (bp) oligonucleotide primer (5′-CGTACGTCGGGTATTC-3′) complementary to a region downstream from the 5′ end (Fig. 1A, single overline) was synthesized and radiolabeled at its 5′ end with T4 polynucleotide kinase and 50 μCi of [γ-^{32}P]ATP (3,000 Ci/mM). The primer was annealed to total-cell RNA isolated from uninfected cells, infected cells isolated at 6 h p.i., and cycloheximide-treated infected cells isolated at 12 h p.i. The primer was extended with cloned Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) in the presence of 0.5 mM deoxynucleoside triphosphates. A sequencing ladder was generated by using the labeled oligonucleotide to prime a dideoxyribonucleotide-chain termination sequencing reaction from a single-stranded DNA spanning the region.

Northern (RNA) blot analysis was performed with 5μg of poly(A)$^+$ RNA per lane (21). RNA was denatured by glyoxalation (34), fractionated on 1% agarose gels, and transferred to Zeta-Probe nylon membranes (Bio-Rad Laboratories, Richmond, Calif.). The blot was probed with a 236-bp strand-specific DNA probe synthesized from a single-stranded DNA template containing the Ac*M*NPV sequence between nucleotide 950 and the *Bal*I site at nucleotide 714 (Fig. 1a; Fig. 2 top, bar a) by using Sequenase and 50 μCi of [α-^{32}P]dCTP (3,000 Ci/mM).

Hybrid selection and in vitro translation. CG30-specific RNA was selected from total RNA isolated from cycloheximide-treated cells harvested at 12 h p.i. by hybridization to 30 μg of cloned DNA complementary to CG30 transcripts that was immobilized on nitrocellulose filters (6, 9). Immobilized DNA consisted of double-stranded DNA, a Bluescript KS(+) plasmid containing DNA from the middle of the CG30 ORF (Fig. 1a, nucleotides 714 to 950; Fig. 2 top, bar a), and single-stranded DNA, phage rescued from Bluescript KS(+) containing DNA complementary to the CG30 leader (Fig. 1a, upstream of nucleotide 375; Fig. 2 top, bar b). Hybridizations were conducted for 12 h at 42°C in 50% formamide–10 mM PIPES (pH 6.4)–0.4 M NaCl–1 mM EDTA containing 3.0 mg of RNA from Ac*M*NPV-infected cells. In addition, an RNA template was synthesized from a Bluescript KS(−) plasmid carrying the cloned gene by using T3 RNA polymerase as described by the manufacturer (Stratagene). Briefly, 1 μg of plasmid DNA cleaved downstream of the ORF was transcribed by using 10 U of T3 RNA polymerase at 37°C for 30 min. The 25-μl reaction mixture contained 40 mM Tris hydrochloride (pH 8), 8 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl, 30 mM dithiothreitol, 0.4 mM each ribonucleoside triphosphate, and 25 U of RNasin (Promega Biotec, Madison, Wis.). Prior to translation, the DNA template was removed by digestion with RNase-free DNase I (Bethesda Research Laboratories). RNA hybrid selected from total infected-cell RNA and RNA synthesized from cloned Ac*M*NPV templates was translated in a rabbit reticulocyte system (Promega Biotec) in the presence of 50

FIG. 1. Nucleotide sequence, derived peptide sequence, restriction map, sequencing strategy, and ORF distribution of the *Dra*I-*Sca*I (56.0 to 57.1 m.u.) subfragment of the Ac*M*NPV *Hin*dIII C-*Xho*I A (53.5 to 58.9 m.u.) restriction fragment. (A) Nucleotide sequence of the *Dra*I-*Sca*I subfragment. The predicted amino acid sequence of GC30 (nucleotides 401 to 1193) and the carboxyl terminus of vp39 (nucleotides 1 to 396) are printed below the nucleotide sequence. The *Bal*I site (nucleotide 714) used for radiolabeling probes for S1 nuclease protection assays is indicated by an arrowhead with an asterisk. The nucleotides complementary to the oligonucleotide primer used to map the 5′ end of the RNA are singly overlined. The approximate 3′ ends determined by S1 nuclease protection assays at nucleotides 1093 and 1293 are marked with closed circles. Potential polyadenylation signals are underlined. The 5′ end is indicated by an arrow at nucleotide 159. Sequences corresponding to conserved sequences observed in other early Ac*M*NPV genes are indicated by double overlines. (B) Map of the *Hin*dIII C-*Xho*I A restriction fragment with selected restriction sites indicated (H, *Hin*dIII; E, *Eco*RI; S, *Sal*I; B, *Bal*I; M, *Mst*I; N, *Nar*I; V, *Eco*RV; P, *Pvu*II; X, *Xho*I). The relative positions of the vp39 and CG30 ORFs are shown by arrows above the restriction map. A restriction map of the sequenced *Dra*I-*Sca*I (56.0 to 57.1 m.u.) region is shown in expanded form, below, with the location of the CG30 ORF depicted as an open arrow. The locations of the leucine zipper, the zinc finger, the acidic region, and the basic region are indicated above the representation of the CG30 ORF. (C) Sequencing strategy for the region. Arrows indicate the location and direction of sequencing from exonuclease III deletion clones. (D) ORF distribution within the three possible reading frames of top (1, 2, and 3) or bottom (1′, 2′, and 3′) DNA strands. Termination codons are represented by vertical lines. The CG30 ORF is translated in reading frame 3′, while the carboxyl terminus of the vp39 coding sequence is translated in frame 1′. kb, Kilobase pairs.

A.

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1 AC TTG CAA ATC GAC ACG GAG GAA TTG AGG TTT AGA AAT TGC GCC ACG TGT ATA ATT GAC GAA ACG GGT CTG GTC GCG TCT GTG CCC GAC
    Leu Gln Ile Asp Thr Glu Glu Leu Arg Phe Arg Asn Cys Ala Thr Cys Ile Ile Asp Glu Thr Gly Leu Val Ala Ser Val Pro Asp

90 GGC CCC GAG TTG TAC AAC CCG ATA AGA AGC AGT GAC ATT ATG AGA AGT CAA CCC AAT CGT TTG CAA ATT AGA AAC GTT TTG AAA TTT GAA
   Gly Pro Glu Leu Tyr Asn Pro Ile Arg Ser Ser Asp Ile Met Arg Ser Gln Pro Asn Arg Leu Gln Ile Arg Asn Val Leu Lys Phe Glu

180 GGC GAC ACA CGT GAG CTG GAC AGA ACG CTT AGC GGA TAC GAA GAA TAC CCG ACG TAC GTT CCG CTG TTT TTG GGA TAC CAA ATA ATC AAT
    Gly Asp Thr Arg Glu Leu Asp Arg Thr Leu Ser Gly Tyr Glu Glu Tyr Pro Thr Tyr Val Pro Leu Phe Leu Gly Tyr Gln Ile Ile Asn

270 TCA GAA AAC AAC TTT TTG CGC AAC GAC TTT ATA CCA AGA GCA AAT CCT AAC GCT ACT CTG GGC GGC GGC GCA GTG GCA GGT CCT GCG CCT
    Ser Glu Asn Asn Phe Leu Arg Asn Asp Phe Ile Pro Arg Ala Asn Pro Asn Ala Thr Leu Gly Gly Gly Ala Val Ala Gly Pro Ala Pro

360 GGT GTT GCA GGC GAA GCA GGT GGA GGA ATA GCC GTC TAA AA ATG GAG TTT GTT AAA TTG CAA TGC AAC ATT TGT TTT TCG GTT GCA GAA
    Gly Val Ala Gly Glu Ala Gly Gly Gly Ile Ala Val  *
  1                                                  Met Glu Phe Val Lys Leu Gln Cys Asn Ile Cys Phe Ser Val Ala Glu

449 ATT AAA AAT TAT TTT CTG CAA CCA ATA GAC AGA TTG ACT ATA ATA CCC GTA TTA GAA CTA GAC ACG TGC AAA CAT CAA TTA TGC TCA ATG
 17 Ile Lys Asn Tyr Phe Leu Gln Pro Ile Asp Arg Leu Thr Ile Ile Pro Val Leu Glu Leu Asp Thr Cys Lys His Gln Leu Cys Ser Met

539 TGT ATA CGC AAG ATT AGA AAA CGA AAA AAA GTA CCA TGT CCC TTG TGT AGG GTT GAA AGT TTG CAT TTC AAC GTT TAC AGC GTA AAC CGA
 47 Cys Ile Arg Lys Ile Arg Lys Arg Lys Lys Val Pro Cys Pro Leu Cys Arg Val Glu Ser Leu His Phe Asn Val Tyr Ser Val Asn Arg
                                                                                                                    *

629 AAC GTT GTG GAT GTG ATT AAA TGC AGC GCG TCG AGT GTC GCA CAA TGG AAT AAA ATA AAC GCT AAT TTT GAT GCG GCC TCT TTG GCC AGT
 77 Asn Val Val Asp Val Ile Lys Cys Ser Ala Ser Ser Val Ala Gln Trp Asn Lys Ile Asn Ala Asn Phe Asp Ala Ala Ser Leu Ala Ser

719 GTG TTG TTT GAA AAA AGT CTG TTG GAC GAC GCA GAA GAC AAC AAC GCA AAT GCC GAC GAC ACT ATG CTG TCT GAA GCA CAA GCG ATA CTA
107 Val Leu Phe Glu Lys Ser Leu Leu Asp Asp Ala Glu Asp Asn Asn Ala Asn Ala Asp Asp Thr Met Leu Ser Glu Ala Gln Ala Ile Leu

809 AAA AAA CTA CAG GTA GAC ATT GCC GAA CAA ACG CAG CTC AAC ATT AAA CAA CAA TTA GAT TTA GAC AAA TTA CAA CAA ACA AGC GTT TCT
137 Lys Lys Leu Gln Val Asp Ile Ala Glu Gln Thr Gln Leu Asn Ile Lys Gln Gln Leu Asp Leu Asp Lys Leu Gln Gln Thr Ser Val Ser

899 ATG CAA GAA AAG TTA GAC AAA ATT AAA AGC GAC TAC AAC AAC ATG CAT AAA TCT TTT AAA GAA TTG CAA CTG AAA CGA ATT ACA ACT GAA
167 Met Gln Glu Lys Leu Asp Lys Ile Lys Ser Asp Tyr Asn Asn Met His Lys Ser Phe Lys Glu Leu Gln Leu Lys Arg Ile Thr Thr Glu

989 AAG GCG CTA AAA TCG CTC AAT GAC GAT TAC GCA AAA CTT GCG TCT AAA AAC GCC AAA TTG AGT AGC GAA AAT AAG GTT TTA TCA AAT AAA
197 Lys Ala Leu Lys Ser Leu Asn Asp Asp Tyr Ala Lys Leu Ala Ser Lys Asn Ala Lys Leu Ser Ser Glu Asn Lys Val Leu Ser Asn Lys

1079 AAT ATT GAA TTG ATT AAA CAC AAA AAT TTA TTA CAA AAC GAG TAC ACA ACA TTA CAA TCA TAT AAA TGT ATA ACC AAC GCC ACT ATT ACC
 227 Asn Ile Glu Leu Ile Lys His Lys Asn Leu Leu Gln Asn Glu Tyr Thr Thr Leu Gln Ser Tyr Lys Cys Ile Thr Asn Ala Thr Ile Thr

1169 ACA AAT GTT ACA ATA AAT GTA GAT TAA TTAACTGTTTACATAACATTCTACTTTAATGTAATAATATTCTTCAATTTCTTGGGTTTTAGAATTAAAATTAACTCTATATA
 257 Thr Asn Val Thr Ile Asn Val Asp  *

1279 TATGTCTCTTGTTTATTTTCACACCTTCTTCAATATCGTGATGCCAATTATTTGTGTCGCGCCATCGGCTATCGTTTA
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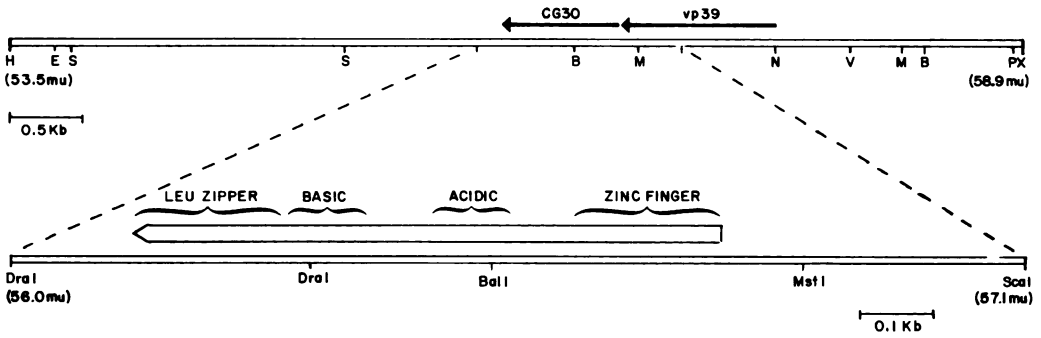
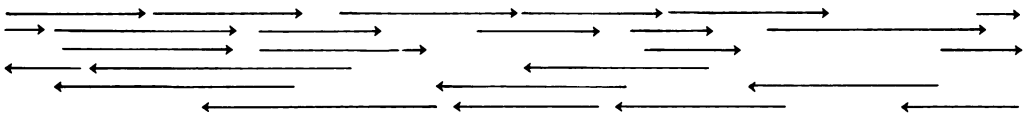
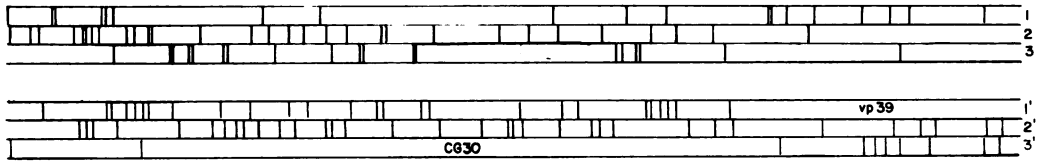
B.



C.



D.



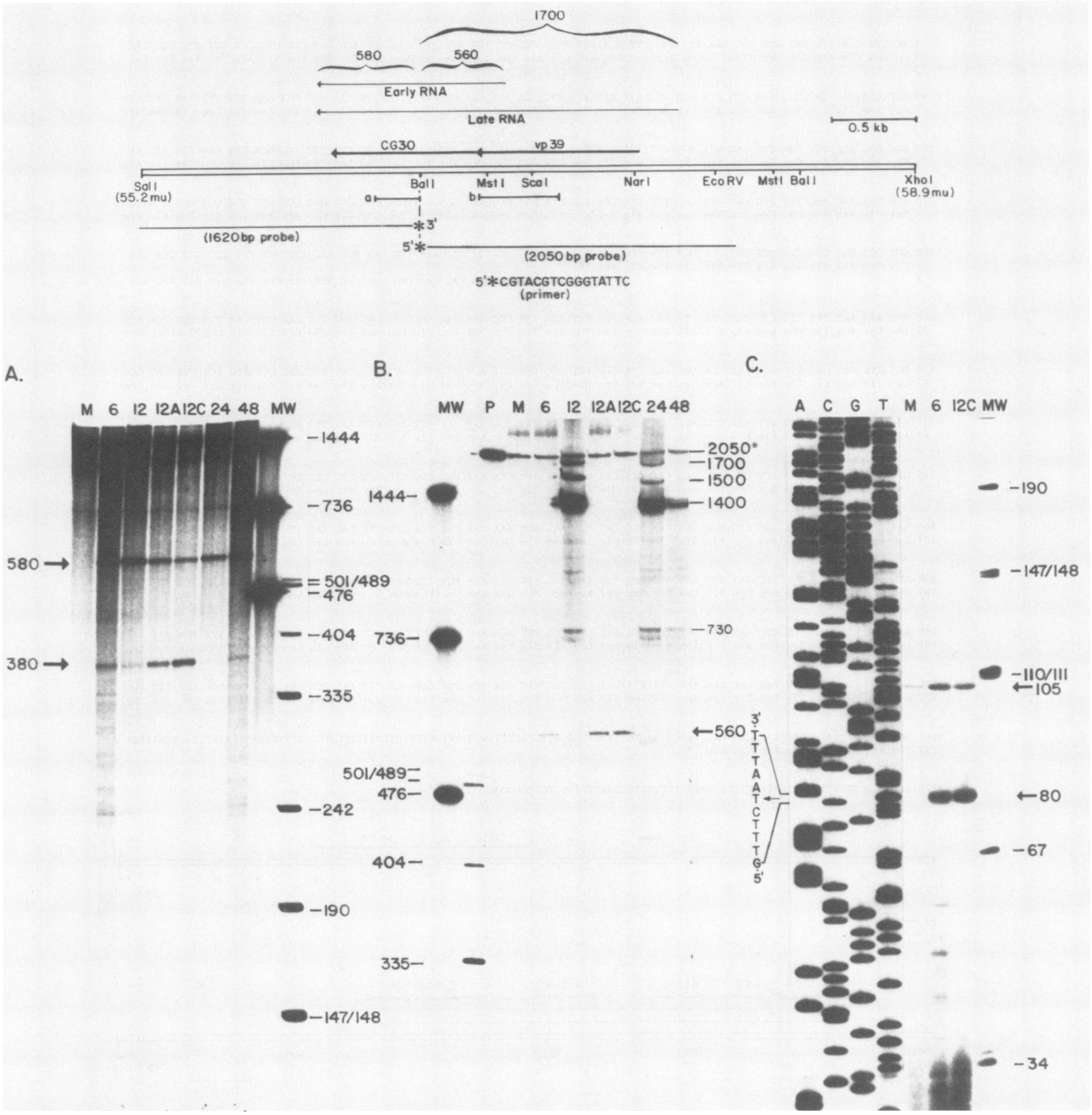


FIG. 2. Mapping the 3′ and 5′ termini of CG30 RNA. The diagram at the top of the figure shows the locations of the S1 probes and the oligonucleotide used for primer extensions. The labeled ends are indicated by asterisks. Early and late RNAs defined by S1 nuclease protection are shown above the restriction map. The bars below the restriction map indicate the locations of the sequences used for probing Northern blots (a) and for hybrid selection of RNA for in vitro translation (a and b). kb, Kilobase pairs. (A) Results of an S1 nuclease protection assay with a 1,620-nucleotide probe 3′ end labeled at the *Ball* site (Fig. 1A, nucleotide 714). Total RNA isolated from mock-infected cells (lane M), from Ac*M*NPV-infected cells at 6, 12, 24, and 48 h p.i. (lanes 6, 12, 24, and 48), and from Ac*M*NPV-infected cells at 12 h p.i. in the presence of aphidicolin (lane 12A) or cycloheximide (lane 12C) was hybridized to the probe and treated with S1 nuclease. The nuclease-resistant hybrids were analyzed on a sequencing gel. The sizes of the molecular weight standards (lane MW) in nucleotides are shown to the right of the panel. Protected fragments of approximately 580 and 380 nucleotides are indicated by arrows to the left of the panel. (B) Results of an S1 nuclease protection assay with a 2,050-nucleotide probe containing the Ac*M*NPV *Ball*-*Eco*RV fragment and a portion of the Bluescript KS(−) plasmid 5′ end labeled at the *Ball* site (Fig. 1A, nucleotide 714). Total RNA as described for panel A was hybridized to the 5′-end-labeled probe, treated with S1 nuclease, and analyzed on a sequencing gel. The time points are indicated at the top of the panel, with the 2,050-bp probe shown in lane P. The sizes of molecular weight markers (lane MW) are indicated to the left of the panel in nucleotides. The probe and major protected fragments of 730, 1,400, 1,500, and 1,700 nucleotides observed with RNA isolated at 12, 24, and 48 h p.i. are

μCi of [^{35}S]methionine (1,200 Ci/mM; Dupont, NEN Research Products) at 30°C for 1 h (38). Proteins from infected and uninfected cells incubated for 1.5 h in methionine-deficient growth medium were radiolabeled by incubating cell monolayers (10^6 cells per 35-mm plate) in methionine-deficient growth medium containing 200 μCi of [^{35}S]methionine per ml. After 1-h incubations, cells were collected at 3, 6, 12, and 30 h p.i., washed with phosphate-buffered saline (27), and lysed in 1% Nonidet P-40–50 mM Tris hydrochloride (pH 8.0)–150 mM NaCl. Pulse-labeled proteins from infected cells and radiolabeled in vitro translation products were analyzed by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (24) followed by fluorography.

RESULTS

Identification and sequencing of early gene in *Hin*dIII C-*Eco*RI G region of Ac*M*NPV restriction map. We recently located the gene for the Ac*M*NPV major capsid protein (vp39) within the *Hin*dIII C-*Xho*I A (53.5 to 58.9 m.u.) restriction fragment of the virus (46). A probe 3′ end labeled at the *Mst*I site (at 56.8 m.u. within the vp39 coding region [Fig. 1B]) used to determine the 3′ termini of vp39-specific RNAs by S1 nuclease protection analysis revealed protected fragments in RNA samples isolated from infected cells at 6 h p.i. and from 12-h-infected cells treated with inhibitors (aphidicolin and cycloheximide) which blocked late transcription from the vp39 initiation sites. These results suggested that an early transcript was initiating upstream of the *Mst*I site within the vp39 coding sequence. To explore this possibility, we first sequenced the region downstream of the vp39 coding sequence (56.9 to 57.6 m.u.) to identify any significant downstream ORFs (Fig. 1A and C).

Translation of this DNA sequence by computer revealed a major ORF, CG30, of 872 nucleotides starting within the vp39 coding sequence (Fig. 1A, nucleotide 320) but in a different reading frame (Fig. 1D). The two ORFs overlap, but the first methionine codon of CG30 is located 2 bp downstream from the TAA termination codon of vp39. The polypeptide sequence consists of 264 amino acids with a predicted molecular weight of 30,000. We designated this gene CG30 because it is located within the *Hin*dIII C and *Eco*RI G region of the restriction map and encodes a 30-kilodalton (kDa) polypeptide. The CG30 sequences are entered in the GenBank data base as accession M26529.

A search of the NBRF and GenBank data bases with the derived polypeptide sequence revealed some correspondence between the carboxyl terminus of the Ac*M*NPV polypeptide and α-helical regions of myosin and tropomyosin protein sequences (22, 32). Closer visual inspection of the predicted amino acid sequence revealed two motifs that have been observed in eucaryotic regulatory proteins: a zinc finger (1, 35) and a leucine zipper (26), the latter corresponding to the region of similarity to myosin α-helices.

Determination of RNA termini by S1 nuclease protection. To determine the 3′ termini of the CG30 transcripts, we hybridized a 1,620-bp DNA fragment exclusively labeled at the 3′ terminus at the *Bal*I site (Fig. 1A, nucleotide 714) and extending downstream of the CG30 ORF (Fig. 2) to total RNA isolated from uninfected cells and from infected cells with and without inhibitors at various times p.i. S1 nuclease analysis revealed protected DNA fragments of approximately 580 and 380 nucleotides for all RNAs except those from mock-infected cells (Fig. 2A). The 380-nucleotide fragment was most prominent in RNA samples isolated from infected cells without inhibitors at 6 h p.i. and at 12 h p.i. from aphidicolin- and cycloheximide-treated cells (Fig. 2A, lanes 6, 12A, and 12C) but was also observed as a faint band at 12, 24, and 48 h p.i. (Fig. 2A, lanes 12, 24, and 48). The 3′ termini reflected by the 380- and 580-nucleotide fragments correspond approximately to nucleotides 1093 and 1293, respectively (Fig. 1A, closed circles). There are putative polyadenylation signals located at nucleotides 1073 and 1180 (Fig. 1A, underlined). The terminus reflected by the 380-nucleotide protected fragment interrupts the CG30 ORF and would result in a polypeptide of 26 kDa or less.

The 5′ termini were examined with a 2,050-bp probe containing 1,820 bp of viral DNA sequences, including the entire vp39 gene, 5′ end labeled exclusively at the same *Bal*I site used to locate the 3′ ends of the RNAs (Fig. 1A, nucleotide 714; Fig. 2). RNA isolated at 6 h p.i. and at 12 h p.i. from aphidicolin- and cycloheximide-treated cells (Fig. 2B, lanes 6, 12A, and 12C) protected a fragment of approximately 560 nucleotides, reflecting an early transcript initiating from within the vp39 coding sequence and extending through the CG30 ORF (see also primer extension data below). Strong protected fragments of approximately 1,400, 1,500, and 1,700 nucleotides were observed with RNA samples isolated 12, 24, and 48 h p.i. (Fig. 2B, lanes 12, 24, and 48). These protected fragments correspond to the upstream transcriptional start sites of the vp39 gene and reflect the extension of these late transcripts through CG30 as previously reported (46). An approximately 730-nucleotide fragment observed at 12, 24, and 48 h p.i. corresponds to an A+T-rich region and thus may be an artifact of the technique caused by breathing of the RNA-DNA hybrids and their subsequent digestion by S1 nuclease. Analysis of 5′ ends by primer extension with RNA isolated at these times showed no evidence of transcripts initiating at this site (data not shown). However, a splice junction at this site has not been ruled out.

Primer extension analysis of 5′ ends of early CG30 RNA. To determine the precise 5′ initiation site of the early RNA, a synthetic oligonucleotide complementary to a region downstream from the 5′ initiation site suggested by the protected fragment from S1 nuclease analysis (Fig. 1A, single overline; Fig. 2) was 5′ end labeled, annealed to RNA, and extended with reverse transcriptase. The extension products were analyzed on sequencing gels beside a sequencing ladder of the region (Fig. 2C). Two extension products were observed. An 80-nucleotide extension product was observed only in

indicated to the right of the panel. The probe is indicated by an asterisk. A major protected fragment of 560 nucleotides is marked with an arrow to the right of the panel. (C) Results of a primer extension analysis. The location of the nucleotide sequence complementary to the oligonucleotide primer is indicated by a double overline in Fig. 1A. Lanes A, C, G, and T contain the indicated dideoxyribonucleotide sequencing reactions with the 5′-end-labeled probe to prime synthesis from an appropriate cloned DNA template. Lanes M, 6, and 12C contain primer extension reactions with the 5′-end-labeled probe to prime reverse transcriptase reactions from RNA isolated from mock-infected cells. Ac*M*NPV-infected cells 6 h p.i., and cycloheximide-treated Ac*M*NPV-infected cells 12 h p.i. respectively. The sizes of molecular weight standards (lane MW) in nucleotides are shown to the right of the panel. Extension products of approximately 105 and 80 nucleotides are noted by arrows to the right of the panel. The sequence of the complementary DNA strand at the 5′ terminus of the RNA is printed to the left of the sequencing ladder. The nucleotide corresponding to the 5′ start site is indicated by an arrow.

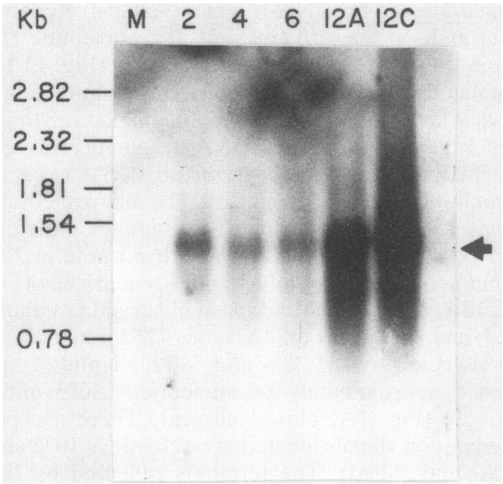


FIG. 3. Northern blot analysis of CG30. Lanes containing RNA from mock-infected cells (lane M), infected cells 2, 4, and 6 h p.i. (lanes 2, 4, and 6), and aphidicolin (lane 12A)- and cycloheximide (lane 12C)-treated cells 12 h p.i. are indicated at the top of the panel. Molecular weights are indicated to the left of the panel in kilobase pairs (Kb). Glyoxalated RNA samples were fractionated on a 1% agarose gel, blotted onto a nylon membrane, and probed with a strand-specific DNA probe (Fig. 2 top, bar a) spanning nucleotides 714 to 950 (Fig. 1A). An approximately 1.3-nucleotide RNA species identified by the probe is indicated by an arrow to the right of the panel.

Ac*M*NPV-infected cells (Fig. 2C, lanes 6 and 12C) and corresponds to the T on the complementary strand marked by an arrow (Fig. 2C) and to an A at nucleotide 159 on the coding strand (Fig. 1A, arrow). These data are consistent with the expected initiation site reflected by the 560-nucleotide protected fragment in the S1 nuclease protection assay. An extension product of approximately 105 nucleotides was seen with RNA from mock-infected cells as well as with RNA from infected cells (Fig. 2C, lanes M, 6, and 12C) and thus may represent a product from a cellular RNA containing a region complementary to the oligonucleotide primer. There is no consensus TATA box near the CG30 early initiation site, although a ATTAAT sequence located 30 nucleotides upstream of the initiation site may serve a similar function (Fig. 1A). The sequence AAATTAG at the transcriptional initiation site of CG30 is identical to that of ETL, another early Ac*M*NPV gene (5). The sequences ATCGTTTGC and CACGTGTAT located 256 and 357 nucleotides upstream from the initial CG30 ATG (double overlines, Fig. 1A) are homologous to sequences observed upstream of the ATG in other early Ac*M*NPV genes with reported consensus sequences A(A/T)CGTGTR (36) and (a/c)tcGTGTn(c/t) (47).

Northern blot analysis. To affirm the size and temporal regulation of CG30 transcripts, poly(A)-selected RNA from uninfected cells and infected cells with or without inhibitors at various times p.i. was analyzed on Northern blots with a single-strand radiolabeled probe complementary to the central portion of the ORF (Fig. 1A, nucleotides 714 to 950; Fig. 2 top, bar a). A 1.3-nucleotide RNA was observed at 2, 4, and 6 h p.i. (Fig. 3, lane 2) and was also observed at 12 h in the presence of aphidicolin and cycloheximide (Fig. 3, lanes 4, 6, 12A, and 12C). We could not determine whether this transcript was present at 12 h or at later times in the absence of inhibitors because the probe hybridized strongly to the late vp39 gene transcripts initiating upstream, which obscured the signal from the smaller CG30 transcripts (data not shown). Compared with vp39 transcripts, this RNA is present in low abundance. A 1.3-nucleotide RNA species is consistent with the approximately 1,140-nucleotide RNA predicted from the distal 3′ end and the 5′ end determined by S1 nuclease and primer extension analyses, with the addition of a poly(A) tail.

Hybrid selection and in vitro translation of RNA corresponding to CG30 ORF. To demonstrate that a protein was encoded by this ORF, we translated CG30-specific RNA in a cell-free protein synthesis system and analyzed the products by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. RNA for translation was selected from total RNA isolated from cycloheximide-treated infected cells 12 h p.i. with two different DNA fragments: a single-stranded DNA complementary to the CG30 leader and a double-stranded fragment complementary to a central region of the ORF,

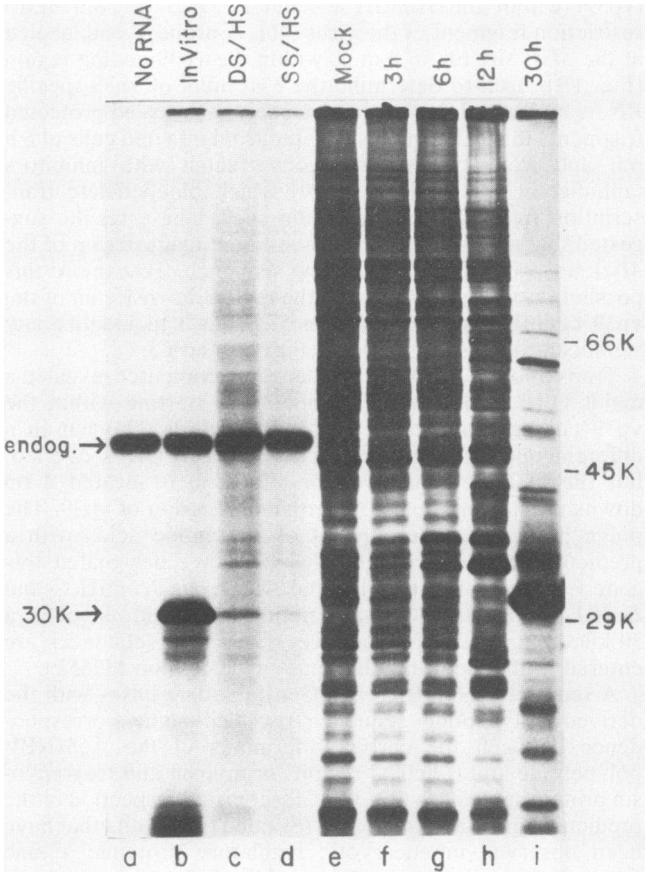


FIG. 4. In vitro translation of CG30-specific RNA. An autoradiogram of a sodium dodecyl sulfate-polyacrylamide gel is presented. Lanes a to d contain the in vitro translation products from a control containing no RNA, an RNA synthesized from cloned DNA with T3 RNA polymerase (In Vitro), and hybrid-selected RNA isolated from cycloheximide-treated Ac*M*NPV-infected cells at 12 h p.i. with a double-stranded DNA (DS/HS) or a single-stranded DNA (SS/HS) to select RNA as described in Materials and Methods. Lanes e to i contain pulse-labeled cell lysates from mock-infected cells and Ac*M*NPV-infected cells at various times p.i. as indicated at the top of the autoradiogram. Molecular masses are indicated to the right in kilodaltons (K). An endogenous globin transcript (endog.) and 30-kDa polypeptides translated from the synthetic RNA template and both hybrid-selected RNA samples are indicated by arrows to the left of the panel.

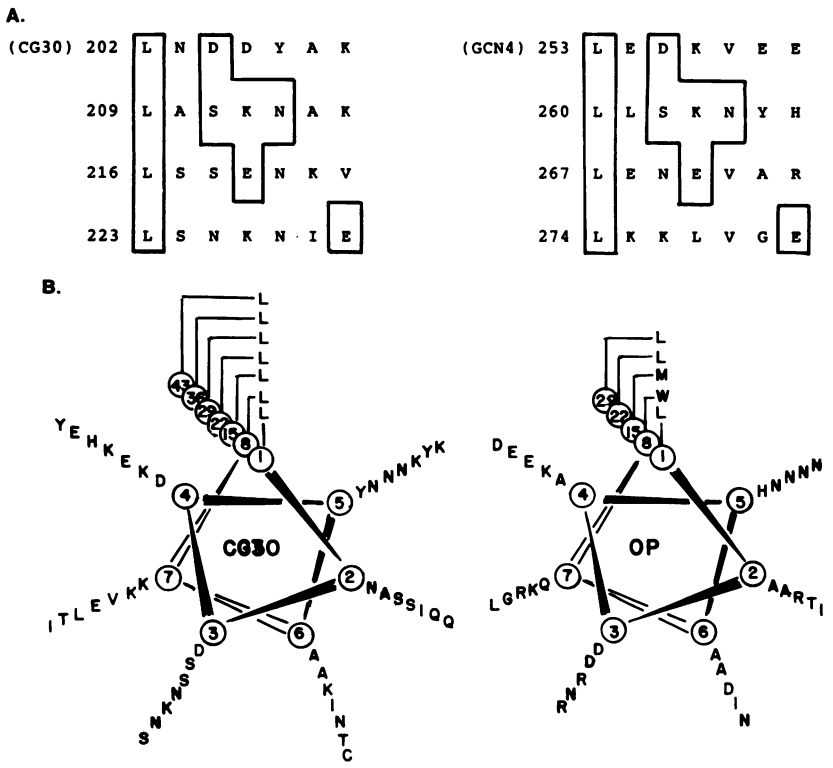


FIG. 5. CG30 leucine zipper. (A) Comparison of the leucine zippers in the CG30 and the CGN4 polypeptide sequences. Amino acid residues are represented by the standard single-letter code. The first four of the seven leucine repeat units of CG30 between amino acid residues 202 and 229 are presented on the left, and the four leucine repeat units of GCN4 between amino acid residues 253 and 280 are presented on the right. Identical amino acid residues are boxed. (B) α-Helical wheel plots of the entire CG30 leucine zipper (amino acids 202 to 252) and the Op*M*NPV counterpart (OP) (amino acids 202 to 236). The amino acid residues are arranged to indicate their relative positions when they are in an α-helical conformation. The linear order of the residues in the polypeptide is indicated by circled numbers (spoke positions 1 to 7). The amino acid residues are arranged around the spokes in sequential order beginning with the first leucine, amino acid residue 202, in the first position on spoke 1 and ending with isoleucine, residue 252 for CG30, and leucine, residue 236 for Op*M*NPV, in the last position on spoke 7. The leucine repeats in the zipper motif are numbered on spoke 1 beginning with residue 202.

previously used to probe Northern blots (Fig. 1A, nucleotides 714 to 950; Fig. 2 top, bar a). In addition, T3 RNA polymerase was used to transcribe RNA from a cloned DNA template containing the ORF.

The major translation product of the T3 polymerase-synthesized RNA was a polypeptide of approximately 30 kDa (Fig. 4, lane b). Translation of RNA hybrid selected by both single- and double-stranded DNA also yielded products of approximately 30 kDa (Fig. 4, lanes c and d) which comigrated with the product of the synthesized RNA. Virus-induced polypeptides comigrating with the in vitro translation products were observed in pulse-labeled infected-cell lysates as early as 3 h p.i. (Fig. 4, compare lanes e and f).

Minor polypeptide bands of less than 30 kDa were also observed in the in vitro translation reactions. These could reflect the degradation of the input RNA. In addition, three bands ranging from approximately 33 to 37 kDa were observed in both in vitro translations employing selected RNA from infected cells (Fig. 4, lanes c and d) but not in the reaction utilizing a synthetic RNA (Fig. 4, lane b). These are most likely artifacts of the selection procedure since the early CG30 transcript is of relatively low abundance, while prominent polypeptide bands of similar molecular weight are observed in infected cells (Fig. 4, lanes g and h).

DISCUSSION

We identified a baculovirus gene that encodes a polypeptide with features characteristic of a transcriptional regulatory protein. The derived polypeptide sequence of CG30 has two nucleic acid-binding motifs, a zinc finger ($C\text{-}X_2\text{-}C\text{-}X_{11}\text{-}C\text{-}X_2\text{-}C$, amino acids 44 to 62 of Fig. 1A [1, 35]), and a leucine zipper with a flanking basic region (25, 26). CG30 also contains a very acidic region between these two DNA-binding motifs (Fig. 1B). The acidic region (pI 4.44) spans amino acids 100 to 196, with an exceptionally acidic stretch (pI 3.13) between amino acids 100 and 131 (Fig. 1A). Acidic regions mediate transcriptional activation by polypeptides with either zinc fingers or leucine zippers (18, 30, 31, 45). CG30 is unique, to our knowledge, in having both a zinc finger and a leucine zipper motif. The presence of two separate nucleic acid-binding motifs in the same polypeptide raises the interesting possibility that CG30 recognizes and activates transcription from more than one DNA recognition sequence in the manner of chimeric lexA-GCN4 proteins (19). Alternatively, the CG30 leucine zipper might function as a dimerization domain to orient the zinc finger with respect to the DNA (23). The leucine zipper of CG30 (Fig. 1A, amino acids 202 to 250) consists of seven leucine heptameric repeats, which is two repeats longer than the longest zipper reported by Landschulz et al. (26). The first four repeats resemble the leucine zipper of the yeast transcriptional activator GCN4 (Fig. 5A). We are currently developing assays to monitor CG30 DNA-binding and transcriptional activation properties.

Transcripts of CG30 are present at both early and late times p.i. Early CG30 transcripts initiate within the coding

region of the late gene vp39 as early as 2 h p.i., are present at least until 6 h p.i., and are synthesized in the presence of inhibitors of DNA and protein synthesis. At late times, CG30 is also transcribed from the vp39 promoter as the second gene of an abundant bicistronic message. This may ensure that high levels of CG30-specific RNA are produced at late times in infection. However, it is not known whether the CG30 ORF of this bicistronic message is translated in Ac*M*NPV-infected cells. If CG30 is a transcriptional regulator, this type of transcription would make CG30 a prime candidate in the regulation of hyperexpressed genes at late times in infection. We also noted that the vp39 and CG30 ORFs overlap (Fig. 1D) and that if a low level of translational frameshifting occurred during the translation of the carboxyl terminus of the capsid protein, a capsid-CG30 fusion protein would contain DNA-binding sites. The possibility is particularly intriguing in light of recent reports of other eucaryotic viruses expressing low levels of capsid–nucleic acid-binding protein fusions, some by translational frameshifting mechanisms (11, 20).

We have noticed that the analogous region of the *Orgyia pseudotsugata* nuclear polyhedrosis virus (Op*M*NPV) genome (2) encodes a 249-amino-acid polypeptide which is 50% identical to the CG30 polypeptide and has similar sequence motifs. Both polypeptides are basic, although CG30 contains 11% lysine residues, whereas the Op*M*NPV counterpart contains 11% arginine. Like CG30, this ORF is carried as part of a bicistronic message with p39, but the first methionine codon is 10 rather than 2 nucleotides from the p39 termination codon. The location of the zinc finger in the Op*M*NPV polypeptide is identical to that of CG30, but is has three additional amino acid residues in the central region, resulting in the pattern $C\text{-}X_2\text{-}C\text{-}X_{14}\text{-}C\text{-}X_2\text{-}C$. An additional pair of upstream cysteine residues (Fig. 1A, amino acid residues 8 to 11, $C\text{-}X_2\text{-}C$) is also conserved in the Op*M*NPV polypeptide. Amino acid residues 202 to 236 at the carboxyl terminus are 51% identical to the equivalent region of CG30 and resemble a leucine zipper except that one leucine is replaced by methionine, as occurs in human L-*myc* (26), and another leucine is replaced by tryptophan (Fig. 5B).

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