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Overlapping Sets of Viral RNAs Reflect the Array of Polypeptides in the *Eco*RI J and N Fragments (Map Positions 81.2 to 85.0) of the *Autographa californica* Nuclear Polyhedrosis Virus Genome

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In several parts of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) genome, nested sets of overlapping RNAs with common 3′ or 5′ termini have been recognized. In the present report, the pattern of viral transcription and the arrangement of viral gene products in the region of 81.2 to 85.0 map units were investigated. In this segment of the AcNPV genome, at least nine size classes of viral RNA were identified which ranged in size from 1.3 kilobases (kb) to 4.6 kb and exhibited common 3′ termini. The detailed restriction map and the nucleotide sequence of this part of the AcNPV genome were determined. Computer analyses revealed several open reading frames (ORFs) on the rightward-transcribed strand with potential TATA and CAAT signals preceding many of the potential ORFs and the 5′ termini of some of the mapped RNAs. The leftward-transcribed strand was devoid of major ORFs. The presumptive polypeptides encoded by the larger ORFs ranged in size from 11.3 to 55.6 kilodaltons (kDa). The amino acid sequence of the presumptive polypeptide encoded by ORF3, a 33.6-kDa molecule, exhibited an unusual, clustered 16-fold repeat of the dipeptide arginine-serine in a protein that showed an overall preponderance of basic amino acids. The results of in vitro translation experiments with hybrid-selected RNAs homologous to internal subfragments of the 81.2- to 85.0-map-unit region yielded polypeptides of approximately 28, 34 to 36, and 48 to 50 kDa, which were close in size to the lengths of the major ORFs derived from the nucleotide sequence. The localizations of individual size classes of RNAs in the 81.2- to 85.0-map-unit region of the viral genome were determined precisely at the 3′ and 5′ termini by S1 protection analyses. Within a sequence of eight nucleotides, all RNAs had the same 3′ terminus, which lay close to multiple polyadenylation signals. The initiation sites of the nine different RNA size classes were precisely mapped. As the cap sites of the smaller RNAs (<1.8 kb) were determined by S1 protection analyses, a multitude of RNA initiation sites became apparent. It was also shown that the different RNA size classes in the 81.2- to 85.0-map-unit region were detectable as early as 2 h and at least until 36 to 48 h after infection. In unselected cytoplasmic RNA, the size classes of viral RNAs specific for the *Eco*RI J fragment were detectable early as well as late after infection, although at early times the larger RNAs were detectable in smaller amounts. However, when the polyadenylated RNA molecules were analyzed, the higher-molecular-weight size classes (≥3.6 kb) were not observed early after infection.

The transcriptional program of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) genome is characterized by the absence of extensive, perhaps of any, RNA splicing and by the generation of nested sets of multiple overlapping RNA molecules in several segments of the viral genome (8, 16, 17, 23). It has been suggested that this unusual arrangement of viral RNA molecules may compensate for the lack of RNA splicing in this 126- to 129-kilobase-pair (kbp) viral genome (16). Viral transcripts of increasing lengths which encompass different parts of a viral DNA segment may allow the flexible expression of a variety of polypeptides in a large genome that apparently lacks a penchant for RNA splicing. Determination of the nucleotide sequence in the viral DNA segments under consideration and a detailed mapping of the viral transcripts are preconditions to further investigation of the mechanism of transcription in the AcNPV genome. There is evidence that in some of the segments of the AcNPV genome, the different transcripts may respond to a temporal regulation of the expression program (8, 26).

In the present study, we investigated the organization of genes in the *Eco*RI N and J fragments of AcNPV DNA. The occurrence of overlapping sets of RNAs has been described for this segment of the AcNPV genome (17). The nucleotide sequence of 4,889 bp in the segment of map units 81.2 and 85.0 was determined and analyzed for open reading frames (ORFs) and relevant genetic signals. Moreover, the amino acid sequences of the corresponding polypeptides were derived from the DNA sequence. Nine different size classes of RNAs were mapped precisely relative to the DNA sequence, and the actual translation products were determined by in vitro translation of AcNPV-specific RNAs selected on subclones of the *Eco*RI J fragment. Additional size classes of RNAs occur at low abundance. Lastly, evidence for a temporal control of the different RNA size-classes in the *Eco*RI N and J fragments could be adduced. *Eco*RI-N- and -J-specific RNAs were first detected 2 h postinfection.

MATERIALS AND METHODS

Many of the techniques applied in this study have been described previously, and only brief references will be given to these methods.

Cells, viruses, and virus-infected cells. A continuous cell line of *Spodoptera frugiperda* cells was maintained in monolayer cultures with TC-100 medium (9) supplemented with 10% fetal calf serum. The AcNPV stocks used in our laboratory, derived from a single plaque isolate, and methods of virus propagation and purification were as described

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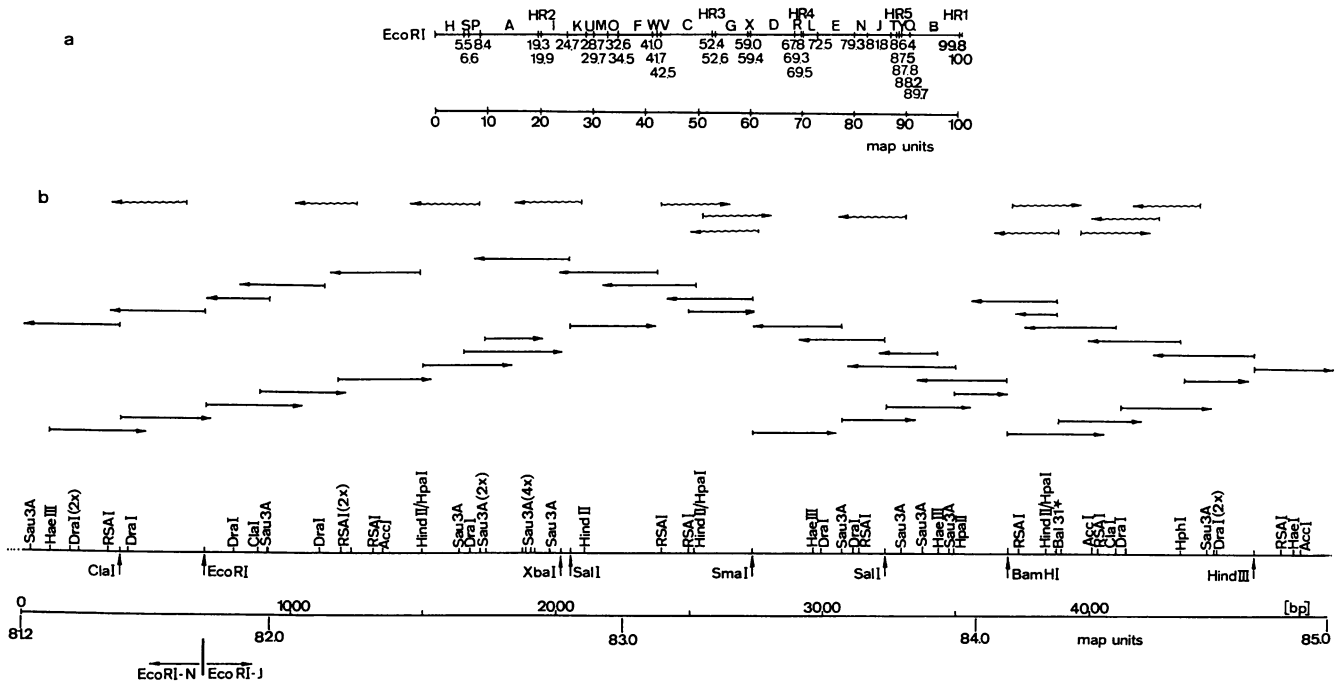

FIG. 1. (a) *Eco*RI restriction map of the AcNPV genome (16). The numbers refer to map units on a scale from 1 to 100. Conventionally, the AcNPV map, though circular in reality, is linearized (31). (b) Detailed restriction map and sequencing strategy of the 81.2- to 85.0-map-unit segment of the viral genome. Only restriction sites which were relevant for the sequencing strategy are indicated. Nucleotide numbers refer to the determined sequence (Fig. 2). Segments designated by straight arrows were sequenced as M13 subclones with a commercial M13 primer. Segments marked by wavy arrows were sequenced by using synthetic oligonucleotide primers and the *Eco*RI J fragment clone. The nucleotide sequence of each subsegment was determined at least twice.

previously (30). For the isolation of AcNPV-specific RNAs at various times after infection, confluent monolayers of *S. frugiperda* cells were diluted 1:4 and were infected 6 h later with about 50 to 100 PFU of extracellular AcNPV.

Viral DNA and cloned viral DNA fragments. Viral DNA was isolated from purified extracellular virions as previously described (16, 30). The cloned viral DNA fragments (15) were propagated in *Escherichia coli* strain HB101/λ and were purified by standard procedures.

Subcloning of viral DNA fragments from the *Eco*RI J and *Eco*RI N fragments. The map locations of the AcNPV DNA fragments were apparent from the scheme in Fig. 1a as well as the locations of subfragments that were used in nucleotide sequence studies (Fig. 1b). Subfragments were recloned by standard procedures into suitable sites in the multiple cloning region of the phage M13 derivatives mp8, mp9, mp10, mp11, mp18, or mp19 (20, 21) or in pUC18. Standard protocols were used for restriction fragment isolation (6).

Preparation and labeling of fragments I to VI (see Fig. 7). (i) Fragment I. The *Eco*RI N fragment was cleaved with *Cla*I, and the linearized plasmid was then labeled at its 5′ termini by [γ-^{32}P]ATP (80 to 150 μCi) and polynucleotide kinase after dephosphorylation of the termini by calf intestine phosphatase. The DNA was then cleaved with *Bam*HI and the ~700-bp *Bam*HI-*Cla*I fragment was isolated by polyacrylamide gel electrophoresis.

(ii) Fragment II. Conditions were similar to those described for fragment I, except that the *Eco*RI-N clone was first cut with *Eco*RI, labeled, and then cleaved with *Cla*I. The 309-bp *Cla*I-*Eco*RI fragment was purified as described above.

(iii) Fragment III. The *Eco*RI-*Sal*I subclone in plasmid pUC18 was cleaved with *Mlu*I, and the 5′ termini of the linearized plasmid were labeled as described above. After being cut with *Cla*I, the 726-bp *Cla*I-*Mlu*I fragment was purified.

(iv) Fragment IV. The *Eco*RI-*Sal*I fragment clone was cut with *Sal*I, labeled at the 5′ termini, and then cleaved with *Hpa*I. The 541-bp *Hpa*I fragment was used for S1 protection analysis.

(v) Fragment V. The *Eco*RI J fragment clone was cut with *Nco*I and labeled at the 5′ termini, and the DNA was then cleaved with *Hpa*I. The 362-bp *Hpa*I-*Nco*I fragment was used.

(vi) Fragment VI. The *Sma*I-*Bam*HI subclone in plasmid pUC18 was cut with *Sal*I and again the 5′ termini were labeled as described above, and the DNA was finally cleaved with *Sma*I. The 510-bp *Sma*I-*Sal*I fragment was used for S1 protection analysis.

Determination of nucleotide sequences. The dideoxynucleotide chain termination method was used employing the Klenow fragment of *E. coli* DNA polymerase (12), an M13-specific primer oligodeoxyribonucleotide, [α-^{35}S] deoxyribonucleoside triphosphates, and, alternatingly, one of the dideoxyribonucleoside triphosphates (27). In some of the experiments, synthetic oligonucleotide primers were produced in a DNA synthesizer (model 381A; Applied Biosystems) and used for nucleotide sequence determinations.

Analyses of nucleotide sequences by computer programs. The following programs of the University of Wisconsin Genetics Computer Group were used: MAP, FRAMES, FIND, SEQED, PUBLISH, and CHOUFAS (5). All analyses were performed on a VAX11/750 computer.

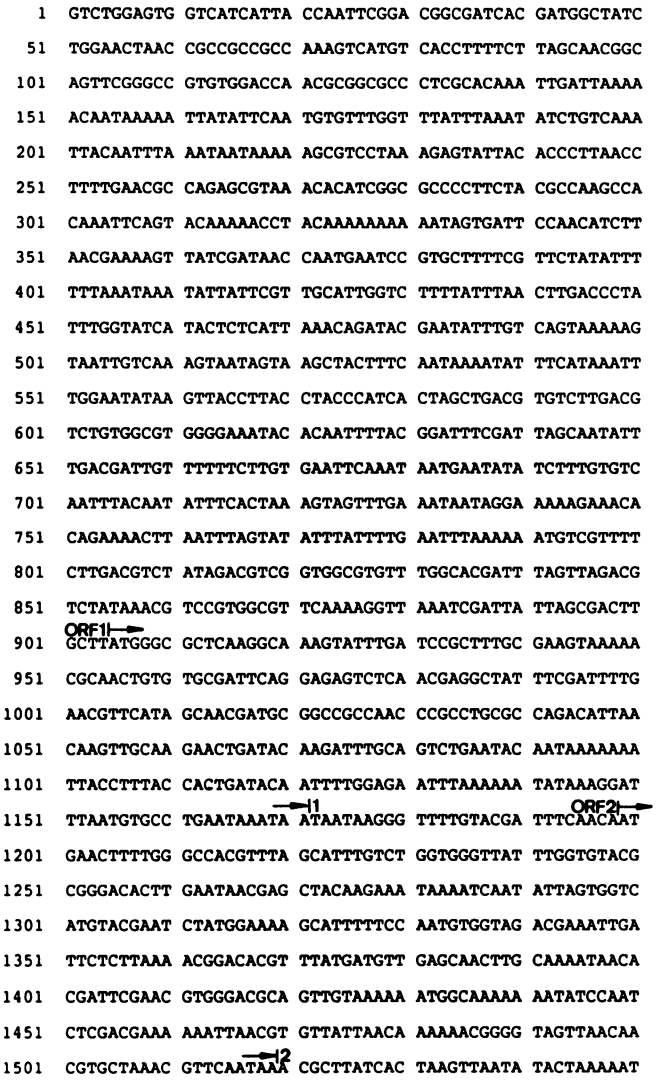

FIG. 2. Nucleotide sequence of the entire 81.2- to 85.0-map-unit fragment of AcNPV DNA. The sequence of the 5′→3′ DNA strand (left to right) is presented. Initiation (|→) and termination (→|) codons of presumptive ORFs are indicated. The polyadenylation signals are underlined; the termination points are indicated (★).

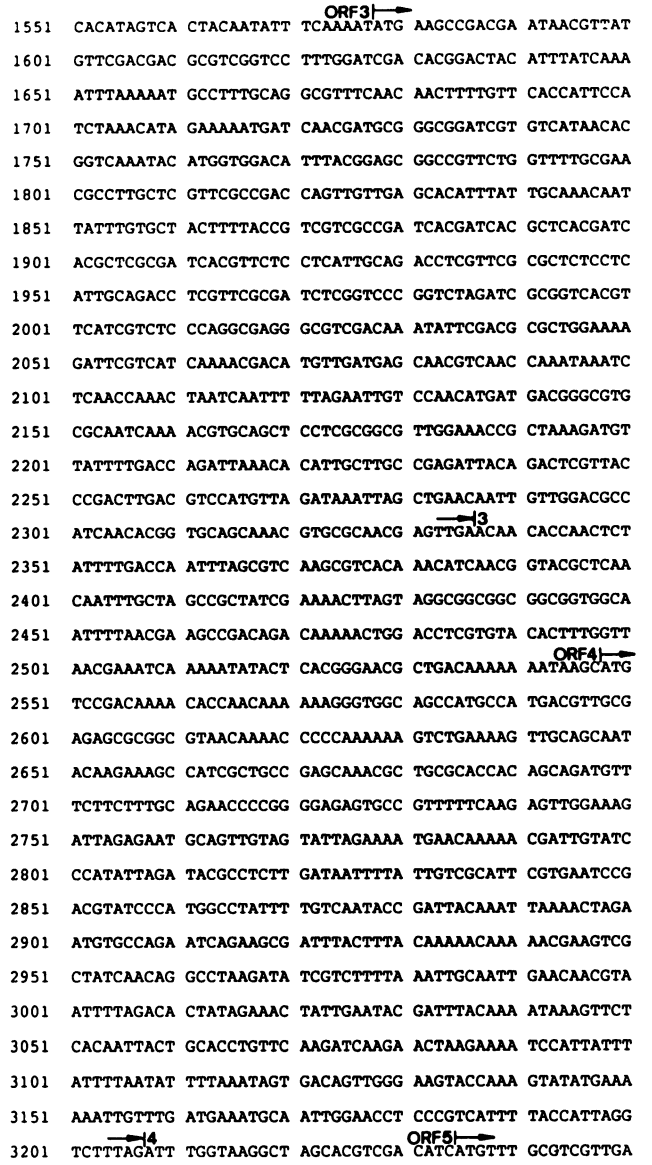

FIG. 2—*Continued.*

Isolation of cytoplasmic RNAs at different times after infection of *S. frugiperda* cells with AcNPV. *S. frugiperda* cells growing in monolayer cultures were infected with extracellular AcNPV at multiplicities of about 50 to 100 PFU per cell. At the indicated times after infection (see Fig. 6 to 9), the cytoplasmic RNA was isolated by the hot phenol method (29). Polyadenylated [poly(A)$^+$] RNA sequences were selected on poly(dT) fixed to Sepharose (2). The poly(A)$^+$ RNA was eluted in 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA–0.05% sodium dodecyl sulfate from poly(dT)-Sepharose, ethanol precipitated, and stored at −20°C for further use. Eventually, the RNA was suspended in sample buffer [20 mM 3-(*N*-morpholino)propanesulfonic acid, 50% formamide, 1% Ficoll, 2.2 M formaldehyde, 0.2% bromophenol blue] and analyzed by electrophoresis on 0.6 to 0.8% agarose gels containing 2.2 M formaldehyde (28). RNA was heat denatured for 10 min at 60°C before electrophoresis.

RNA transfer experiments and S1 protection analyses of RNAs. Upon completion of electrophoresis, RNA was transferred from the gels to nitrocellulose filters as described (1, 28). AcNPV-specific RNAs were visualized by hybridization to AcNPV DNA or to cloned subfragments of viral DNA followed by autoradiography. Hybridization probes were ^{32}P labeled by nick translation (25).

The 3′ and 5′ termini of RNA molecules transcribed from the *Eco*RI N and J fragments were precisely mapped by S1 protection analyses by previously published protocols (3, 16, 17). Suitable restriction endonuclease subfragments in the *Eco*RI-N or -J region were terminally labeled and used for S1 protection analyses (see Fig. 6 and 7 below). The principle of the terminal-labeling procedure will be described here for one example (fragment III, cf. Fig. 7A and above). The *Eco*RI-*Sal*I pUC18 subclone of the *Eco*RI J fragment was linearized by *Mlu*I cleavage, and the 5′ termini were dephosphorylated with calf intestine phosphatase and sub-

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3251  CCTCAGAGCA AAAGCTGTTA TTAAAAAAAT ATAAATTTAA CAATTATGTG
3301  AAAACGATCG AGTTGAGTCA AGCGCAGTTG GCTCATTGGC GTTCAAACAA
3351  AGATATTCAG CCAAAACCTT TGGATCGTGC AGAAATTTTA CGTGTCGAAA
3401  AGGCCACCAG GGGACAAAGC AAAAATGAGC TGTGGACGCT ATTGCGTTTG
3451  GATCGCAACA CAGCGTCTGC ATCGTCCAAC TCGTCCGGCA ACATGTTACA
3501  ACGACCAGCG CTTTTGTTTG GAAACGCGCA AGAAAGTCAC GTCAAAGAAA
3551  CCAACGGCAT CATGTTAGAC CACATGCGCG AAATCATAGA AAGTAAAATT
3601  ATGAGCGCGG TCGTTGAAAC GGTTTTGGAT TGCGGCATGT TCTTTAGCCC
3651  CTTGGGTTTG CACGCCGCTT CGCCCGATGC GTATTTTTCT CTCGCCGACG
3701  GAACGTGGAT CCCAGTGGAA ATAAAATGTC CGTACAATTA CCGAGACACG
3751  ACCGTGGAGC AGATGCGTGT CGAGTTGGGG AACGGCAATC GCAAGTATCG
3801  CGTGAAACAC ACCGCGCTGT TGGTTAACAA GAAAGGCACG CCCCAGTTCG
3851  AAATGGTCAA AACGGATGCG CATTACAAGC AAATGCAACG GCAGATGTAT
3901  GTGATGAACG CGCCTATGGG CTTTTACGTG GTCAAATTCA AACAAAATTT
3951  GGTGGTGGTT TCTGTGCCGC GCGACGAAAC GTTCTGCAAC AAAGAACTGT
4001  CTACGGAAAA CAACGCGTAC GTGGCGTTTG CCGTGGAAAA CTCCAACTGC
4051  GCGCGCTACC AATGCGCCGA CAAGCGACGG CTTTCATTCA AAACGCACAG
4101  CTGCAATCAC AACTATAGTG GTCAAGAAAT CGATGCTATG GTCGATCGCG
4151  GAATATATTT AGATTATGGA CATTTAAAAT GTGCGTACTG TGATTTTAGC
4201  TCAGACAGTC GGGAAACGTG CGATTCTGTT TTAAAACGCG AGCACACCAA
4251  CTGCAAAAGT TTTAACTTGA AACATAAAAA CTTTGACAAT CCTACATACT
4301  TTGATTATGT TAAAAGATTG CAAAGTTTGC TAAAGAGTCA CCACTTTAGA
4351  AACGACGCTA AAACACTTGC CTATTTTGGT TACTATTTAA CTCATACAGG
4401  AACCCTGAAG ACCTTTTGCT GCGGATCGCA AAACTCGTCG CCCACCAAAC
4451  ACGATCATTT AAACGACTGT GTATATTATT TGGAAATAAA ATAAACCTTT
4501  ATATTATATA TAATTCTTTT ATTTATACAT TTGTTTATAC AATTTTATTT
4551  ACGACAAATA TTGACTCGTT GTTCAGAAAG TTTAATAAGC TTGTCAATTT
4601  CTTCGGCTTG CAAAGGGCTG CCAACGCGTT CGTTTTGAAT GCGCGTAATC
4651  CGGTTTACGG TATTGTTGGC GCGAACAATA AACTCCTCAA CTGGCAAATT
4701  AACATTTTGT TTGCGTACTC ATTGTGCACT GCGGCCAGGT TTTGTAGAAT
4751  GTTTTCGGGA AAAATGGCAA TTCTATTAAA TTTGACATGT TTTTGATTGT
4801  ATACATAGTT TTGATATTCT TCCAGCGTAG GATATTTGTT TAAACTCTTG
4851  ACGCATTCAA TGTACAATTT GTGCAGTGAC AAAATTCTG
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FIG. 2—*Continued.*

sequently ^{32}P labeled with polynucleotide kinase using 80 to 150 μCi of [γ-^{32}P]ATP (specific activity, 5,000 μCi/mmol; Amersham Corp.). The reaction was carried out in 70 mM Tris hydrochloride (pH 7.5)–10 mM $MgCl_2$–5 mM dithiothreitol–0.1 mM KCl for 90 min at 37°C. The DNA was then cut with *Cla*I, and the *Cla*I-*Mlu*I fragment was isolated on and reextracted from a 6% polyacrylamide gel. In other instances, the 3′ termini were labeled by using Klenow polymerase and the [α-^{32}P]deoxyribonucleoside triphosphates. Cytoplasmic RNA was isolated from *S. frugiperda* cells 24 h after infection with AcNPV, and the poly(A)$^+$ RNA was isolated by standard methods (2). About 8 μg of poly(A)$^+$ RNA was annealed with 20,000 cpm of ^{32}P-5′-labeled DNA fragment in 40 mM PIPES [piperazine-*N*-*N*′-bis(2-ethanesulfonic acid)], pH 6.4–1 mM EDTA–0.4 M NaCl–80% formamide at 42 to 48°C for 6 to 10 h. Subsequently, the hybrids were treated with 150 U of S1 nuclease (32) at room temperature for 30 min. In control experiments, 10 μg of yeast tRNA (Sigma Chemical Corp.) was used instead of cellular and viral RNA. The S1-resistant fragments were resolved by electrophoresis on 6% polyacrylamide gels in 6 M urea (18). As marker DNA, pSV2 or pBR322 DNA was cut with *Hpa*II, and the fragments were terminally ^{32}P labeled, denatured, and coelectrophoresed. After electrophoresis, the gels were dried and autoradiographed on Kodak XAR-5 film.

Fragment selection of AcNPV RNA and in vitro translation in a reticulocyte lysate system. Standard protocols were followed (7, 22). Subfragments of the *Eco*RI J fragment of AcNPV DNA were used for RNA selection by standard methods (24). The reticulocyte lysate system was purchased from Bethesda Research Laboratories, Inc., or from Amersham Corp. ^3H-labeled polypeptides were resolved on 12.5% polyacrylamide gels containing sodium dodecyl sulfate (14). Commercially available ^{14}C-labeled marker proteins (14.3, 30, 46, and 69 kilodaltons [kDa] Amersham) were used as size markers. Fluorographs were prepared by established methods (4).

RESULTS

Restriction map and nucleotide sequence analyses of parts of the *Eco*RI N and J fragments of AcNPV DNA. The AcNPV isolate used in our laboratory was derived from a single plaque isolate (30) that was selected from a virus stock provided by M. D. Summers. The restriction map of DNA from this AcNPV isolate differed slightly from that of DNA used in many other laboratories. The *Eco*RI N and J fragments in our DNA preparations corresponded to map positions 79.3 to 86.4 or to the *Eco*RI E and H fragments in the conventional AcNPV map (31), respectively.

The scheme in Fig. 1a presents the *Eco*RI restriction map of AcNPV DNA (15, 16), and Fig. 1b shows a detailed restriction map of the *Eco*RI-N and -J regions of the AcNPV genome. This map was obtained by standard restriction enzyme cleavage procedures. The map was determined in detail for the 81.2- to 85.0-map-unit fragment across the *Eco*RI N-J fragment border, since these were the DNA segments from which the nested set of overlapping RNA molecules was transcribed (17). The nucleotide sequence for the segment of AcNPV DNA shown in Fig. 1b was determined by using M13 subclones or synthetic oligonucleotide primers in the standard dideoxy-chain-termination procedure (27). Individual subclones used in the sequencing experiments are indicated by straight arrows (Fig. 1b). Oligonucleotide-primer-initiated sequences are indicated by wavy arrows. The sequence of 4,889 nucleotides (nt) and the locations of the initiation and termination sites of the major ORFs are shown in Fig. 2. The printout format of this sequence was based on the program SEQED.

Results of computer analyses of the nucleotide sequence from map units 81.2 to 85.0 of the AcNPV genome. The scheme in Fig. 3 reproduced all ORFs on the 5′→3′ and 3′→5′ strands in the nucleotide sequence that the computer program FRAMES had detected. It was apparent that at least in this section the AcNPV genome did not contain major ORFs on the leftward-transcribed strand, with the possible exception of two relatively short ORFs. ORFs were found in all three reading frames on the rightward-transcribed strand, and there was no overlap between them (Fig. 3 and 4). The common 3′ ends of these RNAs were determined by S1 protection analysis to nt 4490 or to a position 8 bp downstream (see below). The individual 5′ ends were also assigned (see Fig. 7). RNA molecules of lower abundance were designated by weaker lines. Map locations of the ORFs were compared with the exact posi-

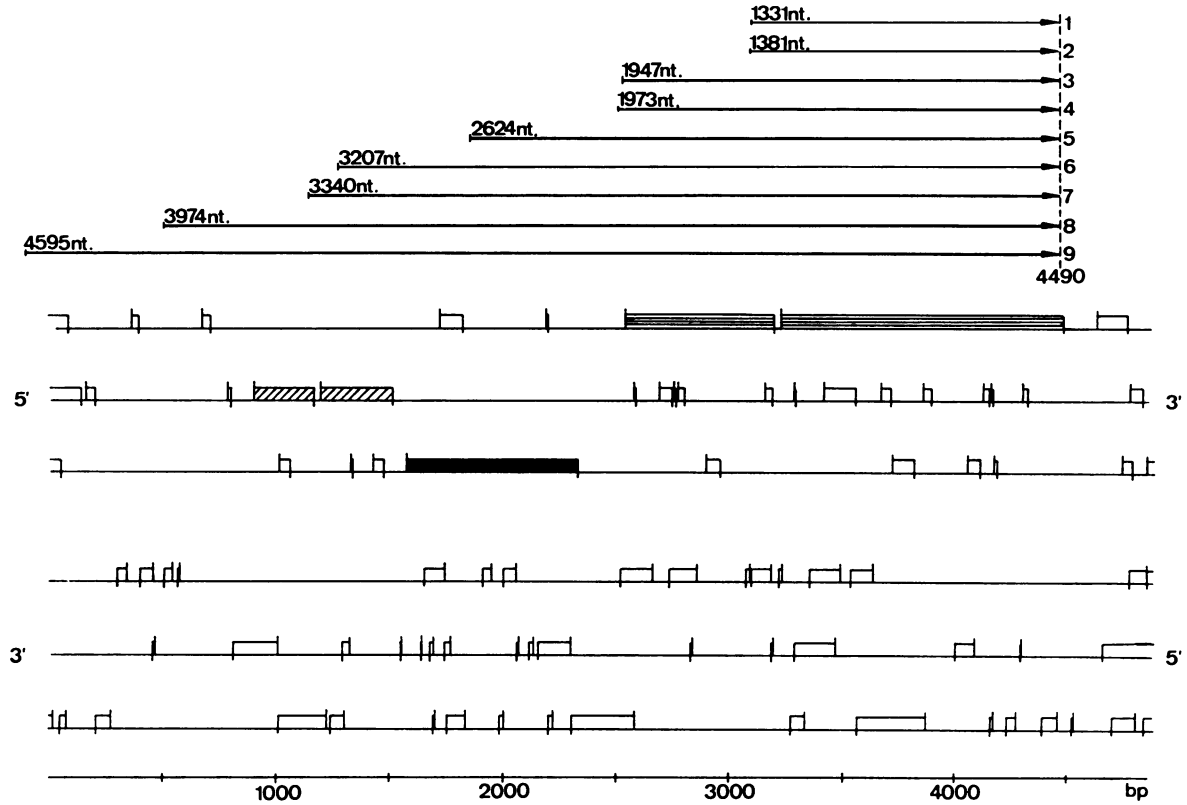

FIG. 3. Map locations of RNA size classes 1 to 9 and of ORFs in the 81.2- to 85.0-map-unit segment of AcNPV DNA. Arrows 1 to 9 designate RNA size classes encoded by this segment. The lengths of each size class, determined as described for Fig. 6 and 7, are indicated in number of nucleotides. The positions of the common 3′ termini were located to nt 4490 and to a position 8 nt further downstream (cf. Fig. 6 and 7) relative to the sequence shown in Fig. 2 (nucleotide scale, 0 to 4,500). The three possible reading frames in the 5′→3′ and 3′→5′ directions are shown. In the 5′→3′ direction, ORFs of significant lengths are depicted as follows: ▤ (reading frame 1), ▨ (reading frame 2), and ▬ (reading frame 3). In this and the following figures, the less-abundant RNA molecules are indicated by weaker horizontal lines.

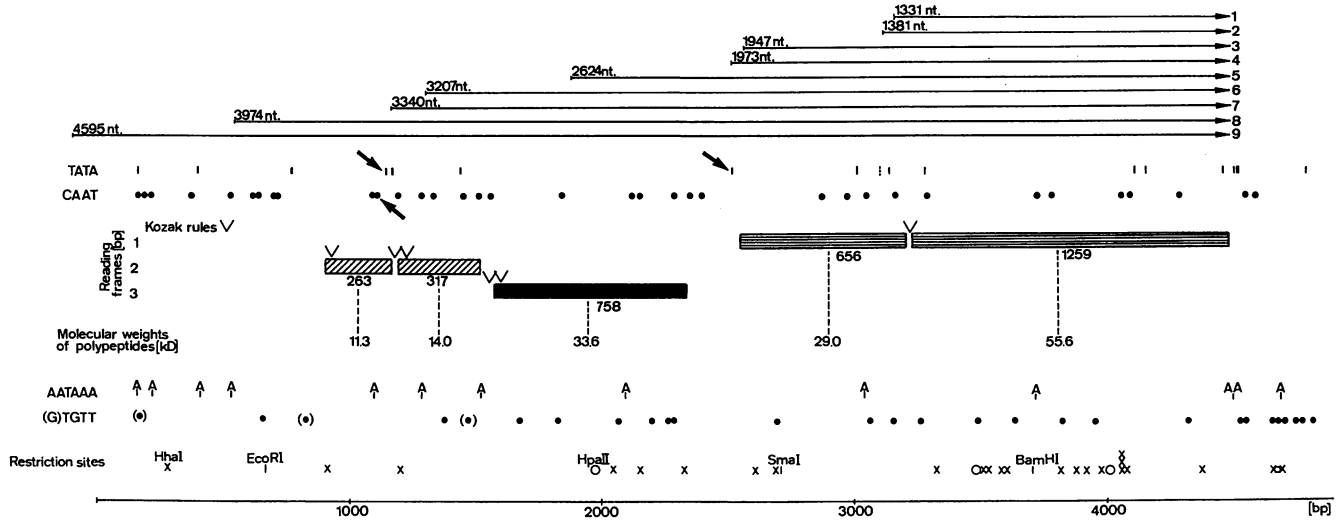

FIG. 4. Genetic signals in the 81.2- to 85.0-map-unit segment of the AcNPV genome. Most of the signals are explained in the scheme itself or in the text. The nine major size classes of RNAs (1 to 9) are indicated by arrows, and their lengths are given in number of nucleotides. The ORFs in frames 1 to 3 were marked exactly as in Fig. 3. Some of the important restriction sites are also included (cf. Fig. 1B).

ORF1: MetGlyAlaGlnGlyLysValPheAspProLeuCysGluValLysThrGlnLeuCysAla
IleGlnGluSerLeuAsnGluAlaIleSerIleLeuAsnValHisSerAsnAspAlaAla
AlaAsnProProAlaProAspIleAsnLysLeuGlnGluLeuIleGlnAspLeuGlnSer
GluTyrAsnLysLysIleThrPheThrThrAspThrIleLeuGluAsnLeuLysAsnIle
LysAspLeuMetCysLeuAsnLys

ORF2: MetAsnPheTrpAlaThrPheSerIleCysLeuValGlyTyrLeuValTyrAlaGlyHis
LeuAsnAsnGluLeuGlnGluIleLysSerIleLeuValValMetTyrGluSerMetGlu
LysHisPheSerAsnValValAspGluIleAspSerLeuLysThrAspThrPheMetMet
LeuSerAsnLeuGlnAsnAsnThrIleArgThrTrpAspAlaValValLysAsnGlyLys
LysIleSerAsnLeuAspGluLysIleAsnValLeuLeuThrLysAsnGlyValValAsn
AsnValLeuAsnValGln

ORF3: MetLysProThrAsnAsnValMetPheAspAspAlaSerValLeuTrpIleAspThrAsp
TyrIleTyrGlnAsnLeuLysMetProLeuGlnAlaPheGlnGlnLeuLeuPheThrIle
ProSerLysHisArgLysMetIleAsnAspAlaGlyGlySerCysHisAsnThrValLys
TyrMetValAspIleTyrGlyAlaAlaValLeuValLeuArgThrProCysSerPheAla
AspGlnLeuLeuSerThrPheIleAlaAsnAsnTyrLeuCysTyrPheTyrArgArgArg
ArgSerArgSerArgSerArgSerArgSerArgSerArgSerProHisCysArgProArg
SerArgSerProHisCysArgProArgSerArgSerArgSerArgSerArgSerArgSer
ArgSerSerSerProArgArgGlyArgArgGlnIlePheAspAlaLeuGluLysIleArg
HisGlnAsnAspMetLeuMetSerAsnValAsnGlnIleAsnLeuAsnGlnThrAsnGln
PheLeuGluLeuSerAsnMetMetThrGlyValArgAsnGlnAsnValGlnLeuLeuAla
AlaLeuGluThrAlaLysAspValIleLeuThrArgLeuAsnThrLeuLeuAlaGluIle
ThrAspSerLeuProAspLeuThrSerMetLeuAspLysLeuAlaGluGlnLeuLeuAsp
AlaIleAsnThrValGlnGlnThrCysAlaThrSer

ORF4: MetSerAspLysThrProThrLysLysGlyGlySerHisAlaMetThrLeuArgGluArg
GlyValThrLysProProLysLysSerGluLysLeuGlnGlnTyrLysLysAlaIleAla
AlaGluGlnThrLeuArgThrThrAlaAspValSerSerLeuGlnAsnProGlyGluSer
AlaValPheGlnGluLeuGluArgLeuGluAsnAlaValValValLeuGluAsnGluGln
LysArgLeuTyrProIleLeuAspThrProLeuAspAsnPheIleValAlaPheValAsn
ProThrTyrProMetAlaTyrPheValAsnThrAspTyrLysLeuLysLeuGluCysAla
ArgIleArgSerAspLeuLeuTyrLysAsnLysAsnGluValAlaIleAsnArgProLys
IleSerSerPheLysLeuGlnLeuAsnAsnValIleLeuAspThrIleGluThrIleGlu
TyrAspLeuGlnAsnLysValLeuThrIleThrAlaProValGlnAspGlnGluLeuArg
LysSerIleIleTyrPheAsnIleLeuAsnSerAspSerTrpGluValProLysTyrMet
LysLysLeuPheAspGluMetGlnLeuGluProProValIleLeuProLeuGlyLeu

ORF5: MetPheAlaSerLeuThrSerGluGlnLysLeuLeuLeuLysLysTyrLysPheAsnAsn
TyrValLysThrIleGluLeuSerGlnAlaGlnLeuAlaHisTrpArgSerAsnLysAsp
IleGlnProLysProLeuAspArgAlaGluIleLeuArgValGluLysAlaThrArgGly
GlnSerLysAsnGluLeuTrpThrLeuLeuArgLeuAspArgAsnThrAlaSerAlaSer
SerAsnSerSerGlyAsnMetLeuGlnArgProAlaLeuLeuPheGlyAsnAlaGlnGlu
SerHisValLysGluThrAsnGlyIleMetLeuAspHisMetArgGluIleIleGluSer
LysIleMetSerAlaValValGluThrValLeuAspCysGlyMetPhePheSerProLeu
GlyLeuHisAlaAlaSerProAspAlaTyrPheSerLeuAlaAspGlyThrTrpIlePro
ValGluIleLysCysProTyrAsnTyrArgAspThrThrValGluGlnMetArgValGlu
LeuGlyAsnGlyAsnArgLysTyrArgValLysHisThrAlaLeuLeuValAsnLysLys
GlyThrProGlnPheGluMetValLysThrAspAlaHisTyrLysGlnMetGlnArgGln
MetTyrValMetAsnAlaProMetGlyPheTyrValValLysPheLysGlnAsnLeuVal
ValValSerValProArgAspGluThrPheCysAsnLysGluLeuSerThrGluAsnAsn
AlaTyrValAlaPheAlaValGluAsnSerAsnCysAlaArgTyrGlnCysAlaAspLys
ArgArgLeuSerPheLysThrHisSerCysAsnHisAsnTyrSerGlyGlnGluIleAsp
AlaMetValAspArgGlyIleTyrLeuAspTyrGlyHisLeuLysCysAlaTyrCysAsp
PheSerSerAspSerArgGluThrCysAspSerValLeuLysArgGluHisThrAsnCys
LysSerPheAsnLeuLysHisLysAsnPheAspAsnProThrTyrPheAspTyrValLys
ArgLeuGlnSerLeuLeuLysSerHisHisPheArgAsnAspAlaLysThrLeuAlaTyr
PheGlyTyrTyrLeuThrHisThrGlyThrLeuLysThrPheCysCysGlySerGlnAsn
SerSerProThrLysHisAspHisLeuAsnAspCysValTyrTyrLeuGluIleLys

FIG. 5. Amino acid sequences of the presumptive polypeptides encoded by ORFs 1 to 5. These sequences were derived from the nucleotide sequence presented in Fig. 2 by the computer programs MAP and PUBLISH. The ORF numbers correspond to those in Fig. 3 and 4. The striking repeat of Arg-Ser sequences in ORF3 is underlined.

tions of the nine major RNA size classes (see Fig. 3, 4, 6, and 7). The positions of ORFs and RNAs relative to each other were consistent with the possibility that the selective translation of individual size classes of RNAs would provide a different and unique set of AcNPV-specific polypeptides in infected cells. Such a mechanism would introduce variability into the expression pattern, although it remained unknown which factors influenced the selection for the translation of individual RNA size classes.

The data presented in Fig. 4 demonstrate that the nucleotide sequences preceding many of the ORFs carry TATA-like signals and presumptive CAAT boxes. In particular, the TATA and CAAT boxes in front of the sites of initiation of the 3,340- and 1,947-nt RNAs were located at the expected distances from the actual cap sites (TATA and CAAT signals designated by arrows [Fig. 4]). The nucleotide sequences at the 5′ ends around the ATG initiation triplets of some of the ORFs conform to the Kozak rules (13). Enhancer consensus sequences (11) have not been detected. Perhaps the enhancing signals would be located more proximally or more distally, possibly in one of the neighboring homologous regions (10) (cf. Fig. 1a), although these homologous regions are very distant. The nucleotide sequence reveals several potential polyadenylation signals. Three of these sites, which lie close to the common 3′ end of the RNAs, may be of functional significance. These polyadenylation signals are followed in their 3′ vicinity by several TGTT-containing sequences which had been previously identified downstream from polyadenylation sites (19). Moreover, the scheme in Fig. 4 lists the number of nucleotides in each sequence comprising an ORF and the molecular weights (in kilodaltons) of the presumptive polypeptides encoded within these ORFs. It is worth recalling that in an earlier study, 34- (major component), 48-, and 28-kDa (minor components) polypeptides were observed after in vitro translation of RNA molecules from AcNPV-infected cells which had been hybrid selected on the cloned *Eco*RI J fragment of the AcNPV genome (16). Similar in vitro translation results were obtained with RNAs hybrid selected on subfragments of the *Eco*RI J fragment (see below).

Amino acid sequences of the presumptive proteins in ORFs 1 to 5. The amino acid sequences of the five ORFs in the 81.2- to 85.0-map-unit region of the AcNPV genome are reproduced in Fig. 5. These ORFs comprised potential polypeptides of 11.3, 14.0, 29.0, 33.6, and 55.6 kDa. A potentially very interesting polypeptide was that in ORF3. Its sequence exhibited a 16-fold repeat of the sequence Arg-Ser in a polypeptide in which the basic amino acids predominated. Due to differences in codon usage, these repeats in the amino acid sequence were not reflected in the nucleotide sequence. A symmetry was recognized in the arrangement ". . .Arg-Arg-Arg-Ser-. . .-Arg-Ser-Ser-Ser. . ." at the flanks of this repeat. The Arg-Ser repeat was subdivided into two segments which were separated by two repeats of the sequence Pro-His-Cys-Arg-Pro. The actual occurrence of any of the theoretically possible polypeptides in AcNPV-infected *S. frugiperda* cells remains as yet unproven.

Determination of the 3′ termini of the RNA molecules by S1 protection analysis. The 461-nt *Cla*I-*Hin*dIII subfragment (Fig. 6) was labeled at the 3′ termini by filling in [α-^{32}P]deoxyribonucleotides by using the Klenow fragment (12) of the DNA polymerase. This fragment was used for S1 protection analyses of the 3′ termini of the different RNA size classes. The DNA fragment protected by hybridization to RNA from AcNPV-infected *S. frugiperda* cells against S1 nuclease cleavage had a length of 360 nt (Fig. 6). By analyzing the sequence data presented in Fig. 2, the 3′ termini of the RNA molecules were placed at nt 4490. A second major fragment (368 nt) was also observed, demonstrating that a second 3′ end of at least some of the RNA molecules was located at nt 4498. There may have been a third minor band of an intermediate length. It was concluded that within a sequence of 8 nt all nine RNA size classes terminated at the same site.

Mapping of the individual 5′ termini of RNA molecules by S1 protection analyses. The scheme in Fig. 7A shows the map locations and labeling sites (designated by stars) of six restriction subfragments (I to VI) in the *Eco*RI-N-J regions of AcNPV DNA. These subfragments were used to assess the individual 5′ start sites of the nine major RNA size classes. Map locations of these RNAs are also indicated. The exact protocols for preparing and labeling the designated fragments are described in Materials and Methods. The autoradiograms (panels I to V, Fig. 7B) present the results of S1 nuclease protection analyses in which the poly(A)$^+$ cytoplasmic RNA from *S. frugiperda* cells was

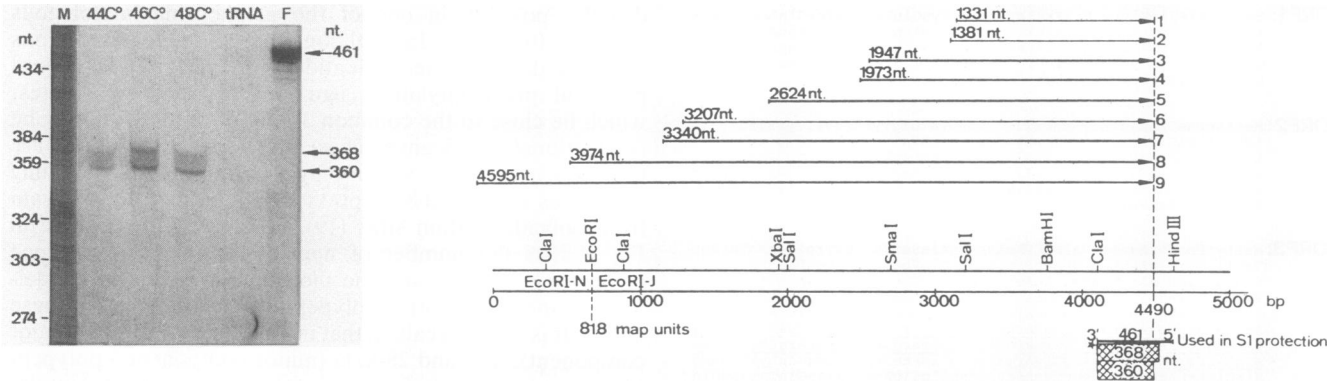

FIG. 6. Determination of the precise map location of the 3′ termini for the nine major RNA size classes on the AcNPV genome by S1 nuclease protection analysis. *S. frugiperda* cells growing in monolayer culture were infected with 50 to 100 PFU of extracellular AcNPV. At 24 h after infection, the cytoplasmic RNA was isolated, and 50 μg of RNA was annealed at the indicated temperatures to the 461-bp *Cla*I-*Hin*dIII fragment which had been labeled as described in Materials and Methods. The hybrids were treated with 150 U of S1 nuclease at 20°C under conditions detailed elsewhere (16, 17). The protected DNA fragments were resolved on a 6% polyacrylamide gel in 8 M urea (18). The marker (M) lane represents the T track of a M13mp19 nucleotide sequence. In lane tRNA, 10 μg of tRNA was used in the annealing experiment. In this negative control, no protection was apparent. In lane F the double-stranded *Cla*I-*Hin*dIII fragment was treated with S1 nuclease and coelectrophoresed. The restriction map on the right presents relevant features of the DNA segment and indicates the locations and lengths of the nine major RNA size classes, as well as the position of the DNA fragment used in S1 protection analysis experiments. The protected 360- and 368-nt fragments are indicated by cross-hatched bars.

isolated at 24 h postinfection with AcNPV and was hybridized to the DNA fragments (I to VI, Fig. 7A) which had been ^{32}P labeled at their 5′ termini. Hybrids were treated with Sl nuclease, and resistant DNA fragments were resolved, after alkali treatment, by electrophoresis on denaturing 6% polyacrylamide gels (18). The sizes of the protected DNA fragments were 470 nt for fragment I, 158 nt for fragment II, 460 and 327 nt for fragment III (the 327-nt fragment had variable intensity), 160 nt for fragment IV, 343 and 317 nt for fragment V, and 120 and 70 nt for fragment VI (Fig. 7). The 368- and 360-nt fragments protected by all RNAs at the 3′ termini in the *Cla*I-*Hin*dIII fragment (cf. Fig. 6) are also shown in Fig. 7A. The S1 nuclease protection data were evaluated by including DNA fragment controls, the results of annealing experiments in which yeast tRNA was used as negative control (I to V, Fig. 7B), and marker DNA fragments for precise size determinations. It was also apparent that in addition to the fragment sizes indicated (I to VI), the total lengths of the DNA fragments (Fig. 7A) were also protected with fragments II to VI, because of the presence of the overlapping RNA molecules. Furthermore, we observed a multitude of protected minor subfragments as one progressed from fragments II to VI in the protection experiments. These results suggested the existence of multiple, less-well-defined start sites. This increase in the number of start sites may be due to the occurrence of numerous additional, minor start sites of transcription in the region of the EcoRI J fragment.

The fragment lengths presented place the sites of transcription initiation for RNA size classes 1 through 9 at nt −105 (this part of the DNA segment was not sequenced), 516, 1150, 1283, 1866, 2517, 2543, 3109, and 3159, respectively. These numbers refer to the nucleotide sequence shown in Fig. 2. The locations of the 5′ termini of the nine RNA size classes shown schematically in Fig. 3, 4, 6, and 7 conform to these determinations. The lengths of the different size classes are 4,595, 3,974, 3,340, 3,207, 2,624 1,973, 1,947, 1,381, and 1,331 nt.

In vitro translation of viral RNAs hybrid selected on subfragments of the *Eco*RI J fragment of AcNPV DNA. Previous in vitro translation analyses of viral RNAs were performed with RNAs hybrid selected on the entire *Eco*RI J fragment (16). It was conceivable that some of the RNAs selected in this way might have originated in neighboring fragments. Moreover, by using subfragments of the EcoRI J fragment for hybrid selection experiments, the analysis might be improved in sensitivity. Cytoplasmic RNA was isolated 24 h after infection of *S. frugiperda* cells with AcNPV. AcNPV-specific RNAs were hybrid selected either on the *Eco*RI J fragment or on the *Eco*RI-*Sma*I or *Sma*I-*Bam*HI subfragment. The locations of these subfragments were apparent from the maps in Fig. 6 and 7. The selected RNAs were in vitro translated in a reticulocyte cell-free lysate by standard procedures. The in vitro-synthesized polypeptides were labeled with [^{35}S]methionine or with a mixture of ^{3}H-labeled amino acids and then analyzed by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels. When the entire *Eco*RI J fragment was used for RNA selection, polypeptides of 48- to 50-, 34- to 36-, and 28-kDa were observed. With the *Eco*RI-*Sma*I fragment, RNAs encoding a 34- to 36-kDa polypeptide and a 28-kDa polypeptide were selected, and the 48- to 50-kDa protein was hardly detectable with this RNA population. Upon RNA selection on the *Sma*I-*Bam*HI fragment, the three translation products listed above were again obtained (Fig. 8).

These results confirmed earlier findings and indicated which of the available ORFs were actually utilized. The sizes of the polypeptides synthesized in vitro under the control of hybrid-selected RNAs were commensurate with the lengths of some of the ORFs read off the nucleotide sequence, i.e., the presumptive proteins with molecular weights of 55,600, 33,600, and 29,000 (Fig. 4 and 5). Polypeptides of 11.3 or 14.0 kDa were not seen in in vitro translation experiments.

Time course of viral RNA synthesis in the *Eco*RI J and N fragments of AcNPV DNA. We next investigated the times after infection the different size classes of AcNPV-specific RNAs encoded by the *Eco*RI J and N fragments could be detected in the cytoplasm of AcNPV-infected cells. Cytoplasmic RNA was prepared from AcNPV-infected *S. frugiperda* cells at 2, 6, 12, 24, 36, and 48 h after infection

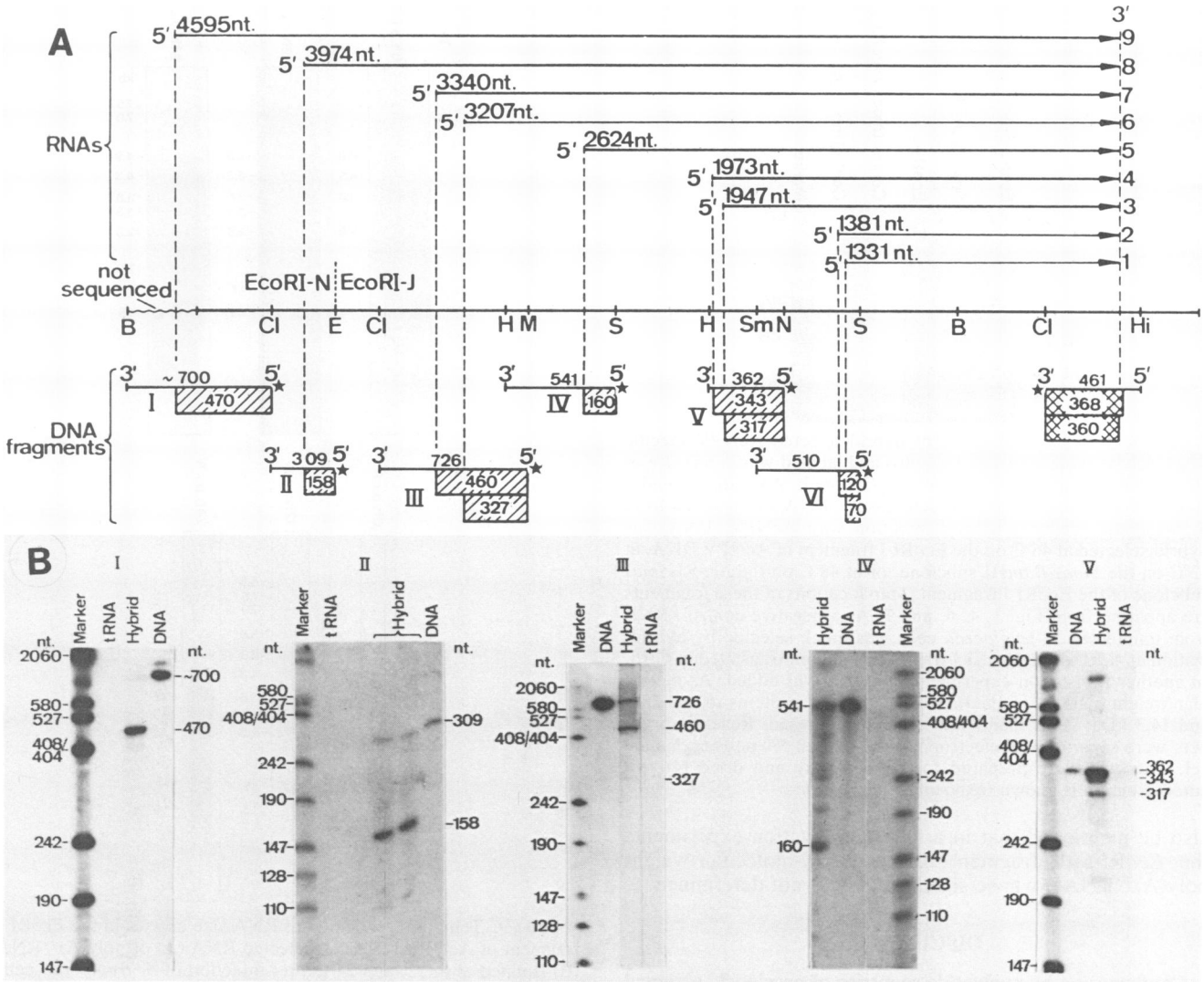

FIG. 7. Mapping of the 5′ termini of nine different RNA size classes in the region of the *Eco*RI N and J fragments of AcNPV DNA. Experimental conditions were as described in the text. (A) Schematic description of the mapping experiments. In the center of the scheme, the relevant restriction sites (as explained in the graph) are indicated. These DNA fragments (DNA and bracketed fragments I to VI) were all ^{32}P labeled at the 5′ termini (★) (see Materials and Methods). The subfragments designated by hatched boxes and identified by nucleotide lengths were protected by RNA annealing in the S1 nuclease protection experiments (see Fig. 7B). These subfragment sizes were derived from autoradiograms I to V (Fig. 7B). The subfragments preserved in the S1 cleavage experiment (Fig. 6) were also included as cross-hatched boxes. In the upper part of the scheme (RNAs and bracket), the nine major RNA size classes are schematically shown with their lengths and the precise map locations as derived from the results presented here. Abbreviations: Cl, *Cla*I; E, *Eco*RI; H, *Hpa*I; M, *Mlu*I; S, *Sal*I; Sm, *Sma*I; N, *Nco*I; B, *Bam*HI; Hi, *Hin*dIII. (B) Analyses of DNA fragments I to V protected against S1 nuclease cleavage by AcNPV RNA from infected *S. frugiperda* cells. The derivation of DNA fragments I to V was as described in Materials and Methods. A series of autoradiograms is shown. The sizes (in number of nucleotides) of the protected fragments are indicated. In each experiment a denatured DNA size marker, a DNA fragment control, and the results of mock hybridizations to yeast tRNA are included.

and was analyzed by gel electrophoresis and RNA blot hybridization by using the ^{32}P-labeled *Eco*RI J fragment as the hybridization probe. Both the unselected and the poly(A)$^+$ RNA populations were analyzed for the occurrence of the different size classes at different times after infection. The data presented in Fig. 9A demonstrate that in the unselected RNA, most of the different size classes were represented both early and late after infection, although the amounts of the larger viral RNAs detectable early were rather low. The significance of the high-molecular-weight size classes (8.8, 7.6, and 7.0 kb) late after infection was not known. In contrast, in the poly(A)$^+$ RNA populations, the larger RNA size classes failed to be represented as early as 6 h after infection, whereas they could be detected at 12 and 24 h after infection (Fig. 9B). These data suggest that temporal regulation in the synthesis of the *Eco*RI-J-specific RNA size classes occurred also at the posttranscriptional level.

In some of the RNA transfer experiments, the poly(A)$^+$ cytoplasmic RNA isolated 16 to 24 h postinfection of *S. frugiperda* cells with AcNPV was hybridized to the ^{32}P-labeled *Eco*RI-*Sal*I fragment (Fig. 7A). As expected, this fragment hybridized predominantly to the 4.9, 4.3, 3.6, 3.3, and 2.8-kb RNAs (data not shown, cf. Fig. 9). It should

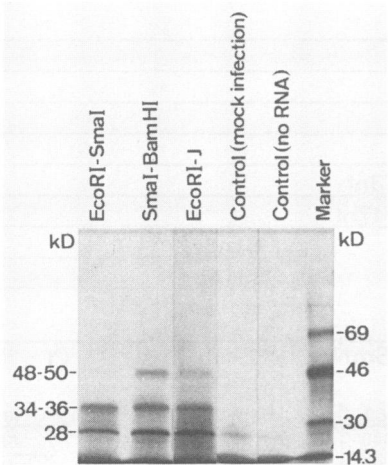

FIG. 8. In vitro translation of hybrid-selected AcNPV-specific RNA. RNAs were translated in vitro and labeled with a mixture of ^{3}H-labeled amino acids (92 to 156 Ci/mmol; Amersham) under standard conditions. The total cytoplasmic RNA was isolated from *S. frugiperda* cells 24 h after infection with AcNPV. RNA was hybrid selected at 46°C on the *Eco*RI J fragment of AcNPV DNA, at 52°C on the *Sma*I-*Bam*HI subclone, or at 48°C on the *Eco*RI-*Sma*I subclone of the EcoRI J fragment. Map locations of these fragments are apparent from Fig. 1, 4, 6, and 7. As a negative control, RNA from uninfected *S. frugiperda* cells was mock selected by hybridization at 46°C to the *Eco*RI J fragment and then translated in vitro. In another translation experiment, no RNA was added. As molecular weight markers, a mixture of ^{14}C-labeled proteins (69, 46, 30, and 14.3 kDa; Amersham) was coelectrophoresed. Reaction products were separated by electrophoresis on a 12.5% polyacrylamide gel. The gels were prepared for fluorography and dried (4). An autoradiogram is shown (exposure time, 24 h).

also be mentioned that in a few hybridization experiments the *Eco*RI-*Sal*I fragment revealed low-molecular-weight poly(A)$^+$ RNAs, whose significance was not determined.

DISCUSSION

Confirmation by nucleotide sequence of previously assigned transcriptional arrangement in the *Eco*RI J and N fragments of AcNPV DNA. By nucleotide sequence determination and S1 protection analyses, the 3′ and 5′ termini of the nine major size classes of AcNPV-specific RNAs have been precisely mapped relative to the *Eco*RI J and N fragments (Fig. 2 to 4, 6, and 7). The results have refined the previously assigned map positions of these RNAs (17). The 3′ ends of the nested sets of RNAs correspond to nt 4490 and 4498 of the sequence determined. There are two polyadenylation sites close to and one following the common 3′ terminus of the nine major RNA size classes. The 5′ ends of the individual RNA size classes are placed at nt −105, 516, 1150, 1283, 1866, 2517, 2543, 3109, and 3159 in the determined sequence (Fig. 2 and 7). In this way the map locations of these RNAs have been unequivocally determined.

As mentioned above (see also Fig. 7), there is evidence that several additional RNA size classes, apart from the nine major classes mapped precisely, are transcribed from the *Eco*RI J fragment of AcNPV DNA in AcNPV-infected *S. frugiperda* cells. This finding might indicate that the mechanism of transcription initiation was not very precise but rather allowed initiations at quite a number of sites which were remarkably rich in A · T nucleotide pairs. The nine determined sites (Fig. 7) were the predominant ones.

Sequential array of polypeptides in the *Eco*RI J fragment. On the rightward-transcribed strand of the linearized

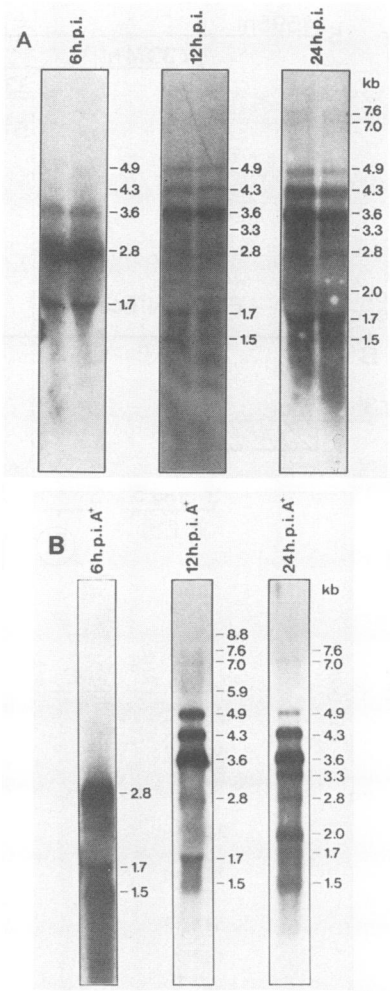

FIG. 9. Temporal regulation of RNA size classes in the *Eco*RI J fragment of AcNPV DNA. Unselected RNA (A) or poly(A)$^+$ RNA (B) isolated at 6, 12, and 24 h after infection of *S. frugiperda* cells with AcNPV was analyzed by electrophoresis on 0.6 to 0.8% agarose gels, by RNA transfer to nitrocellulose filters, and by hybridization to the ^{32}P-labeled *Eco*RI J fragment. Autoradiograms of these experiments are shown. Experimental details were as described in the text. In panel B, the 6-h autoradiogram was exposed about three times longer than the 12- or 24-h autoradiogram.

AcNPV genome (31), five ORFs were recognized which were located in different reading frames (Fig. 2 to 4). The corresponding polypeptides ranged in size from 11.3 to 55.6 kDa and were serially arranged in this part of the viral genome without any overlap (Fig. 4). It is not yet proven that all of these polypeptides were synthesized in AcNPV-infected *S. frugiperda* cells. The arrangement of ORFs was suggestive in that it would allow for the stepwise sequential expression of certain combinations of viral polypeptides, depending on which size classes of RNAs from this region were predominantly synthesized and chosen for translation. In this way the viral expression schedule might be adapted to the specific needs in the viral replication cycle. It was proposed earlier (17) that the flexibility introduced by this mode of viral gene expression might be a substitute for the versatility afforded by the splicing mechanism that other viral systems, but not AcNPV, had perfected. There may be additional, more-complicated explanations for the occurrence of multiple RNA molecules in many segments of the

AcNPV genome. Further investigations are required to understand the true meaning of this transcriptional program. Moreover, AcNPV infection might somehow interfere with cellular RNA splicing and shut off cellular gene expression, since AcNPV gene expression was apparently rendered independent of the splicing mechanism.

Among the presumptive ORFs, a potentially interesting polypeptide is the 33.6-kDa protein which contains a 16-fold repeat of the Arg-Ser dipeptide sequence. It will be challenging to investigate whether this protein is actually synthesized in AcNPV-infected insect cells. Since a 34- to 36-kDa protein has also been found by in vitro translation of RNA selected on the *Eco*RI J fragment or on some of its subfragments, it is likely that this polypeptide is a viral gene product.

In vitro translation of hybrid-selected RNAs. The following polypeptides have been synthesized in vitro by translating AcNPV-specific RNAs selected on the *Eco*RI-*Sma*I and *Sma*I-*Bam*HI subfragments of the *Eco*RI J fragment: a 48- to 50-kDa polypeptide, a 34- to 36-kDa polypeptide, and a 28-kDa polypeptide (Fig. 8). It should be noted that the major ORFs, as derived from the nucleotide sequence data, agree quite well with the results of these and previous (16) in vitro translation experiments. Such experiments in reticulocyte lysates have often yielded reliable results and reflect, to some degree, the patterns of polypeptides synthesized in virus-infected cells. However, this method does not assure the congruence of translation patterns obtained in vivo with those obtained in vitro.

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