

Transcription of Overlapping Sets of RNAs from the Genome of *Autographa californica* Nuclear Polyhedrosis Virus: A Novel Method for Mapping RNAs

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The insect baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) contains a double-stranded, supercoiled circular genome of 126 to 129 kilobase pairs in length. In cultured *Spodoptera frugiperda* insect cells the virus replications, and early and late phases of viral genome expression are discernible. We previously mapped 5 early and at least 32 late different viral polypeptides on the viral genome (H. Esche, H. Lübbert, B. Siegmann, and W. Doerfler, EMBO J. 1:1629-1633, 1982). However, at the same time we located 11 early and more than 90 late different size-classes of viral RNA on the AcNPV genome. Evidence for extensive RNA splicing in this virus system has not been adduced, although tiny splices cannot yet be ruled out (H. Lübbert and W. Doerfler, J. Virol. 50:497-506, 1984). The large number of AcNPV transcripts and the apparent lack of splicing have raised tantalizing questions about the mechanisms involved in the expression of AcNPV DNA and its regulation. It is also unknown how the widely differing numbers of RNAs and polypeptides can be correlated. For this reason, we have started to analyze in detail the map locations of some of the RNA size-classes in three different segments of the viral genome. For this purpose a novel method has been devised which will prove useful for the analyses of transcriptional patterns in complex viral genomes. The *EcoRI* fragments J, O through F, and Q, comprising viral DNA segments between 81.8 and 86.4, 32.6 and 41.0, and 88.2 and 89.7 map units, respectively, were investigated. Surprisingly, overlapping sets of viral RNAs of various lengths and with apparently common 3' termini in *EcoRI* fragments J (seven size-classes) and O through F (four size-classes) or with common 5' termini in *EcoRI* fragment Q (two size-classes) have been detected. At present, the functional significance of this mode of transcription is unknown. *EcoRI* fragment Q of AcNPV DNA encodes a 10,000-molecular-weight polypeptide which is expressed abundantly late after infection. The function of this protein has not yet been elucidated. The promoter and 5' part of the gene for the 10,000-molecular-weight polypeptide have been sequenced, and we have shown that at least two RNAs of different lengths are transcribed in this region and initiated at one site of three nucleotides. Studies on the expression of the AcNPV genome have revealed interesting properties not commonly found in other eucaryotic systems.

Due to several striking properties, baculoviruses have proved a very suitable tool for studies on the molecular biology of insect cell systems. The molecular biology of *Autographa californica* nuclear polyhedrosis virus (AcNPV) has been investigated in considerable detail in a number of laboratories. The genome of AcNPV is a double-stranded, supercoiled circular DNA molecule of some 126 to 129 kilobase pairs (11, 26). Restriction maps of this DNA have been published (11, 25, 26, 30, 36). Several baculovirus genomes have been shown to contain four or five areas of repetitive DNA (3, 12, 25) whose significance is unknown. There is evidence that certain AcNPV variants contain insertions of foreign elements (29). There is clear evidence for differential gene expression early and late after infection of insect cell cultures with AcNPV (9, 17, 18, 37, 44). It will be a major problem to unravel the mechanisms underlying the controlled expression of the viral genome. Late after infection, newly synthesized virions are first released by budding without cell lysis. In the last phase of the infection cycle, mature virions are occluded in the nucleus of the cell into polyhedral inclusion bodies which are predominantly made up of the AcNPV-encoded polypeptide polyhedrin (23,

43). This protein and a 10,000-dalton protein are abundantly expressed late in infected insect cells (35, 37, 38). The syntheses of these proteins are directed by highly efficient late promoters (1, 21, 34; A. Krebs, J. H. Kuzio, P. Faulkner, D. Z. Rohel, and E. B. Carstens, submitted for publication).

In previous work, translational maps of AcNPV polypeptides expressed early or late after infection have been established by in vitro translation of hybrid selected RNAs from AcNPV-infected insect cell cultures (1, 18, 25, 37, 43). Viral DNA fragments cloned in procaryotic vectors were used in some of these studies. Subsequently, 11 early and more than 90 late viral RNAs have been mapped on the viral genome by Northern blotting with the cloned viral DNA fragments as hybridization probes (25). By the application of several analytical procedures, evidence for extensive splicing of primary viral transcription products to mRNA could not be adduced (25). Although splices close to the ends of viral RNAs and splicing in minority populations of viral RNAs could not be ruled out, splicing of AcNPV-specific RNAs at levels comparable, e.g., to those of adenovirus RNAs in infected human cells did not occur. We have set out to establish detailed transcriptional maps for viral RNAs in distinct segments of the AcNPV genome.

In this report, viral RNAs in the fragments *EcoRI*-J, *EcoRI*-O and -F, and *EcoRI*-Q were precisely mapped. The

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latter fragment encodes the viral 10,000-dalton protein (p10). The expression pattern of this region and the structure of its promoter are, therefore, of particular interest. In fragments *EcoRI*-J and *EcoRI*-O and -F, overlapping sets of seven and four viral RNAs, respectively, of different lengths but with common 3' termini were found. In fragment *EcoRI*-Q at least two RNAs with a common 5' end were apparent. The promoter of the p10 gene was sequenced. The nucleotide sequence of this region proved to be rich in adenine plus thymine (A + T) residues, but otherwise its structure conformed to essential features known from many other eucaryotic systems.

MATERIALS AND METHODS

Cells and virus: infection of *Spodoptera frugiperda* cells with AcNPV. Cells of the *S. frugiperda* cell line (a gift of Keith Harrap, Oxford, England) were grown at 27°C in TC-100 medium (20) supplemented with 10% fetal calf serum and 0.26% tryptose phosphate broth. The cells were propagated in monolayers in 75-cm²-surface cell culture flasks and were subcultured two to three times weekly. *S. frugiperda* cells were also grown in suspension cultures in the same medium supplemented with 8% fetal calf serum at cell densities of ca. 4×10^5 cells per ml in spinner vessels thermostated at 27°C.

All virus inocula used in this work were derived from plaque isolate E of AcNPV (40). The DNA of this isolate has a restriction map (25, 26) slightly different from that of the isolate used in other laboratories (11, 30, 36). Logarithmically growing monolayer cultures of *S. frugiperda* cells (2×10^5 cells per cm²) were inoculated with extracellular AcNPV in growth medium at 25 PFU per cell. Adsorption proceeded for 1 h. Free extracellular virus was harvested 2 days after inoculation. Nuclear polyhedra were seen 1 to 2 days after infection. The extracellular virus was filter sterilized (45- μ m pore size; Millipore Corp.). In suspension cultures, cells were inoculated with extracellular virus at 1 PFU per cell. Infectious medium was harvested 48 to 72 h after infection and used exclusively as inoculum.

AcNPV plaque test. The method of Wood (47) was applied with minor modifications.

Preparation of the cloned AcNPV DNA fragments. Most of the *EcoRI* fragments of AcNPV DNA had been cloned in pBR322 DNA or in λ Charon 4a DNA (26) and were purified by routine methods.

Purification of AcNPV DNA. All virus preparations used were extracellular virus purified from the medium of infected cells. The medium was freed of cells by low-speed centrifugation, and the virions were subsequently pelleted by ultracentrifugation at $90,000 \times g$ for 1 h. The pellet was resuspended in 0.02 M Tris-hydrochloride (pH 7.5)–0.002 M EDTA and adjusted to 500 μ g of proteinase K per ml. After 45 min of incubation at 37°C, the solution was made 2% with sodium lauroyl sarcosinate, and incubation was continued for 10 min at 65°C, followed by 6 h at 37°C. Subsequently, the solution was adjusted with CsCl to a density of 1.62 g/ml, and 100 μ g of ethidium bromide per ml was added. This solution was centrifuged to equilibrium (SW60; 40,000 rpm, 42 h, 20°C). Two bands of viral DNA, the supercoiled circular and the relaxed bands, were isolated and freed of ethidium bromide by three extractions with isopropanol. CsCl and remainders of isopropanol were removed by dialysis against TE buffer (0.01 M Tris-hydrochloride [pH 7.5], 1 mM EDTA).

Restriction cleavage of DNA and separation of fragments by gel electrophoresis. DNA was restricted by following standard protocols, and the amounts of restriction endonucle-

ases required were pretested. Depending on size, fragments were separated by electrophoresis on 0.6 to 2% agarose gels, 4 to 10% polyacrylamide gels, or composite gels consisting of 0.7% agarose and 4.6% polyacrylamide (31). DNA fragments were eluted from low-melting-point agarose (Bio-Rad Laboratories) gels by excising the DNA fragment, adding five volumes of TE buffer, heating to 65°C, and extracting with phenol-chloroform (1:1). Polyacrylamide slices containing DNA fragments were mechanically pulverized, 2 volumes of TE buffer were added, and the mixture was kept at 45°C for several hours. Gel remnants were removed by centrifugation, and the DNA was recovered by ethanol precipitation.

Isolation of RNA from AcNPV-infected *S. frugiperda* cells. Several different methods were employed to extract RNA from infected cells. (i) Cytoplasmic RNA was prepared by the method of Villareal et al. (41). Infected cells were detached from the plastic surface and washed at 4°C with Tris-saline and with 10 mM Tris-hydrochloride (pH 7.5)–5 mM MgCl₂–10 mM NaCl–0.1% diethylpyrocarbonate. The cells were then resuspended in the same buffer containing 1% Nonidet P-40 and kept on ice for 7 min. Cytoplasmic and nuclear fractions were separated by low-speed centrifugation. The cytoplasmic fraction was diluted 1:2 with 0.1 M Tris-hydrochloride (pH 8.0)–0.3 M NaCl–0.015 M EDTA–0.1% diethylpyrocarbonate and adjusted to 1% sodium dodecyl sulfate. RNA was extracted three times with phenol-chloroform-isoamyl alcohol (25:24:1). The RNA was then ethanol precipitated.

Total intracellular RNA was extracted by one of the following procedures. (ii) Cells were mechanically detached and resuspended in 1 ml of 4 M guanidinium thiocyanate–0.5% sodium lauroyl sarcosinate–25 mM sodium citrate (pH 7.0)–0.1 M β -mercaptoethanol (10). The mixture was vortexed, and cellular debris was removed by low-speed centrifugation. The supernatant was mixed with 0.025 volume of 1 N acetic acid and 0.75 volume of ethanol and stored at –20°C for ca. 16 h. The RNA precipitate was then pelleted and resuspended in 0.5 volume of 7.5 M guanidine hydrochloride–25 mM sodium citrate (pH 7.0)–5 mM dithiothreitol and precipitated by adding 0.025 volume of 1 N acetic acid and ethanol. The solution was stored at –20°C for 3 h, and the RNA was pelleted. This latter procedure was repeated, and the RNA was finally redissolved in water and stored at –20°C. (iii) AcNPV-infected or uninfected *S. frugiperda* cells were treated with 1.5 ml of 3 M LiCl–6 M urea–10 mM sodium acetate (pH 5.3)–0.1% sodium dodecyl sulfate (13). Cells were homogenized, and the lysate was kept at 0°C for 16 h. The RNA precipitate was pelleted, redissolved in water, extracted once with phenol, twice with phenol-chloroform-isoamyl alcohol (25:24:1), and twice with chloroform-isoamyl alcohol (24:1), and ethanol precipitated.

Selection of polyadenylated RNA. The method of Aviv and Leder (4) was used.

DNA-RNA hybridization and treatment of hybrids with S1 nuclease. The procedure of Berk and Sharp (7) was applied. The DNA used for hybridization was reextracted with phenol-chloroform:isoamyl alcohol (25:24:1), mixed with the RNA to be analyzed, and ethanol precipitated. The precipitate was thoroughly dried and resuspended in 50 μ l of 80% formamide–40 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] (Sigma Chemical Co.) (pH 6.4)–0.4 M NaCl–1 mM EDTA (stored at –20°C). This solution was heated to 80°C for 10 min and then kept overnight at the temperature of hybridization (46 to 56°C). The optimal hybridization temperature was selected according to the guanine plus cytosine

content of each fragment of AcNPV DNA used. The base composition of each AcNPV DNA fragment used was determined after acid hydrolysis (49) by reverse-phase high-pressure liquid chromatography as described elsewhere (16).

At the end of the hybridization period, 0.5 ml of cold 0.28 M NaCl–0.03 M sodium acetate (pH 4.5)–2 mM ZnSO₄–5% glycerol–1 µg of denatured calf thymus DNA–100 U of S1 nuclease (Sigma) was added. The mixture was incubated at 20°C for 1 h. The S1 nuclease-resistant hybrids were extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated by adding isopropanol and using 20 µg of yeast tRNA as carrier. The precipitate was dissolved in water and used immediately for further analyses.

One- or two-dimensional electrophoresis of DNA-RNA hybrids on gels under neutral or alkaline conditions. The methods of Berk and Sharp (7) or of Favaloro et al. (19) were used. Neutral gels were poured in neutral electrophoresis buffer (0.04 M Tris-hydrochloride [pH 7.8], 5 mM sodium acetate, 1 mM EDTA) and alkaline gels were poured in 0.05 M NaCl–1 mM EDTA. After gelling, the gels were equilibrated in 30 mM NaOH–1 mM EDTA for several hours, and samples were electrophoresed in the same buffer. For two-dimensional electrophoresis experiments, gels were run in a Bethesda Research Laboratories model H4 electrophoresis chamber which had been modified to accommodate three slab gels (20 by 20 cm) stacked on top of each other. Gels were electrophoresed in one direction under neutral conditions at 30 V overnight. These gels were then denatured in 0.1 M NaOH for 45 min and subsequently in 30 mM NaOH–1 mM EDTA for 1.5 h. Denatured size markers were then filled into slots previously left empty, and electrophoresis was continued under alkaline conditions at 30 V overnight in the direction perpendicular to the one used previously. Samples were dissolved in water–10% glycerol–0.02% bromocresol green for neutral gels and in 0.1 N NaOH–10% glycerol–0.02% bromocresol green for alkaline gels.

Standard methods. Methods such as Southern transfer (39), DNA-DNA hybridization (46), nick translation of DNA fragments (33), and DNA sequencing (27, 28) have been described.

Specific labeling of the 5' or 3' termini of DNA fragments. DNA fragments were labeled at their 5' ends after treatment with calf intestine phosphatase (0.05 M Tris-hydrochloride [pH 8.0], 1 mM EDTA; 1 h, 37°C) using [γ -³²P]ATP and T4 polynucleotide kinase (32) as described (28). The 3' termini were labeled with the Klenow fragment (22) of *Escherichia coli* DNA polymerase I (24) by using the deoxynucleoside triphosphates (dNTPs), one of them α -³²P labeled. This method was described elsewhere (14).

Reverse transcripts of RNA and [α -³²P]dNTP labeling of cDNA. The method of Efstratiadis et al. (15) was followed. Limiting concentrations of dNTPs were used since short cDNA molecules had to be generated (100 to 600 nucleotides long). Total intracellular RNA (30 µg) from AcNPV-infected *S. frugiperda* cells was dissolved in 50 µl of 0.1 M Tris-hydrochloride (pH 8.3)–10 mM MgCl₂–0.14 M KCl–10 mM dithiothreitol–1 mM vanadyl-ribonucleoside complex–1 mM each of unlabeled dTTP and dCTP–100 µCi each of α -³²P-labeled dATP and dGTP–100 µg of oligodeoxythymidylate₁₂₋₁₈ primer per ml containing 100 U of reverse transcriptase (Bethesda Research Laboratories). The oligodeoxythymidylate primer was used to hybridize to the polyadenylate [poly(A)] tails of mRNA, to initiate reverse transcription at the 3' termini of mRNA. The solution was incubated at 42°C for 30 min. Soluble nucleotides were then removed by gel filtration over a Sephadex G50 column.

RESULTS

Novel method to map AcNPV-specific RNAs on the viral genome: experimental design. The results of experiments to map more than 90 different AcNPV-specific RNAs on the viral genome revealed sets of overlapping RNA molecules at the same region of the AcNPV genome. Evidence for extensive splicing of viral RNA, which could have explained the peculiar transcription patterns, could not be adduced (25). To elucidate the transcription patterns of viral RNA, a new method was developed that allowed us to determine map positions of viral RNA very precisely. The method was based on S1 nuclease digestion of DNA-RNA hybrids (7) and on the analysis of such hybrids by two-dimensional electrophoresis on agarose gels (19). The technique was applied to mapping AcNPV-specific RNAs in fragment *EcoRI*-J (81.8 to 86.4 map units) and fragments *EcoRI*-O and -F (32.6 to 41.0 map units) of the AcNPV genome (Fig. 1). Transcripts of fragment *EcoRI*-Q (88.2 to 89.7 map units) were mapped by conventional S1 nuclease analyses as described by Berk and Sharp (7).

The principle of the new mapping technique is outlined in Fig. 2 and exemplified for RNA populations derived from fragment *EcoRI*-J. Multiple overlapping RNAs can be recognized and mapped by this procedure. Within a given fragment of DNA, one or several restriction sites were selected which are traversed by unspliced RNA. The DNA was cut with the particular restriction endonuclease or endonucleases and hybridized with RNA. The predominant populations of hybrids were those between one DNA fragment (Fig. 2, fragment a, b, c, or d) and the corresponding RNA. The trimolecular reaction involving two adjacent DNA fragments and the RNA molecule spanning the restriction site also occurred, although less frequently. After treatment with S1 nuclease, the hybrids were analyzed by two-dimensional electrophoresis on agarose gels. In the neutral direction (N) (Fig. 2), fragments a, b, c, d, a + b, or c + d were separated according to size. In the alkaline direction (A), the RNA was degraded, and fragments a + b or c + d separated into fragments a, b, c, and d according to their (reduced) sizes in alkali. They were delayed in the N direction and were found outside the diagonal line (Fig. 2). In this way it was possible to position RNA molecules precisely relative to one or several restriction sites on the viral genome. In the analysis described for fragment *EcoRI*-J, two restriction sites (*Sma*I and *Bam*HI) were chosen to subdivide the relatively large fragment. In principle though, one subdividing restriction site would be sufficient.

AcNPV DNA was cut either with *Sma*I or *Bam*I, which both have sites within fragment *EcoRI*-J (Fig. 2). Each of these cut DNA preparations, or a mixture of both was denatured and hybridized with RNA from AcNPV-infected cells, and the reaction products were treated with S1 nuclease. S1 nuclease removed DNA and RNA stretches without homologies. The *Sma*I and *Bam*HI restriction sites, which had been previously opened, were now spanned in some of the hybrids by RNA molecules with sequence homologies to either fragment. Since a trimolecular reaction was required to generate these complex hybrids, they were not abundant. Bimolecular hybrids containing only one DNA fragment (Fig. 2, fragment a, b, c, or d) occurred more frequently. The S1 nuclease-treated hybrid molecules were next analyzed by two-dimensional electrophoresis on agarose slab gels, and all DNA fragments derived from fragment *EcoRI*-J were visualized by Southern blotting and probing with the ³²P-labeled fragment *EcoRI*-J. In the first (neutral) direction the trimo-

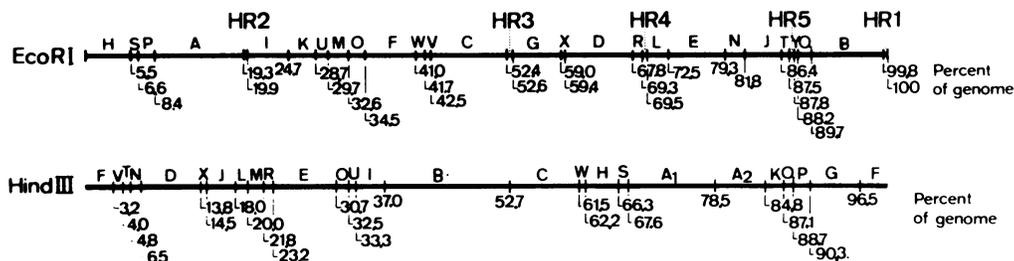


FIG. 1. Physical maps (adapted from reference 25, Fig. 1) of the (126 to 129-kilobase-pair) genome of AcNPV plaque isolate E. The circular map was linearized for presentation between fragments *EcoRI*-H and -B as suggested (43). The positions of the repeated regions HR1 and HR5 are indicated (12). Similar repeat regions were also found in plaque isolate E (25).

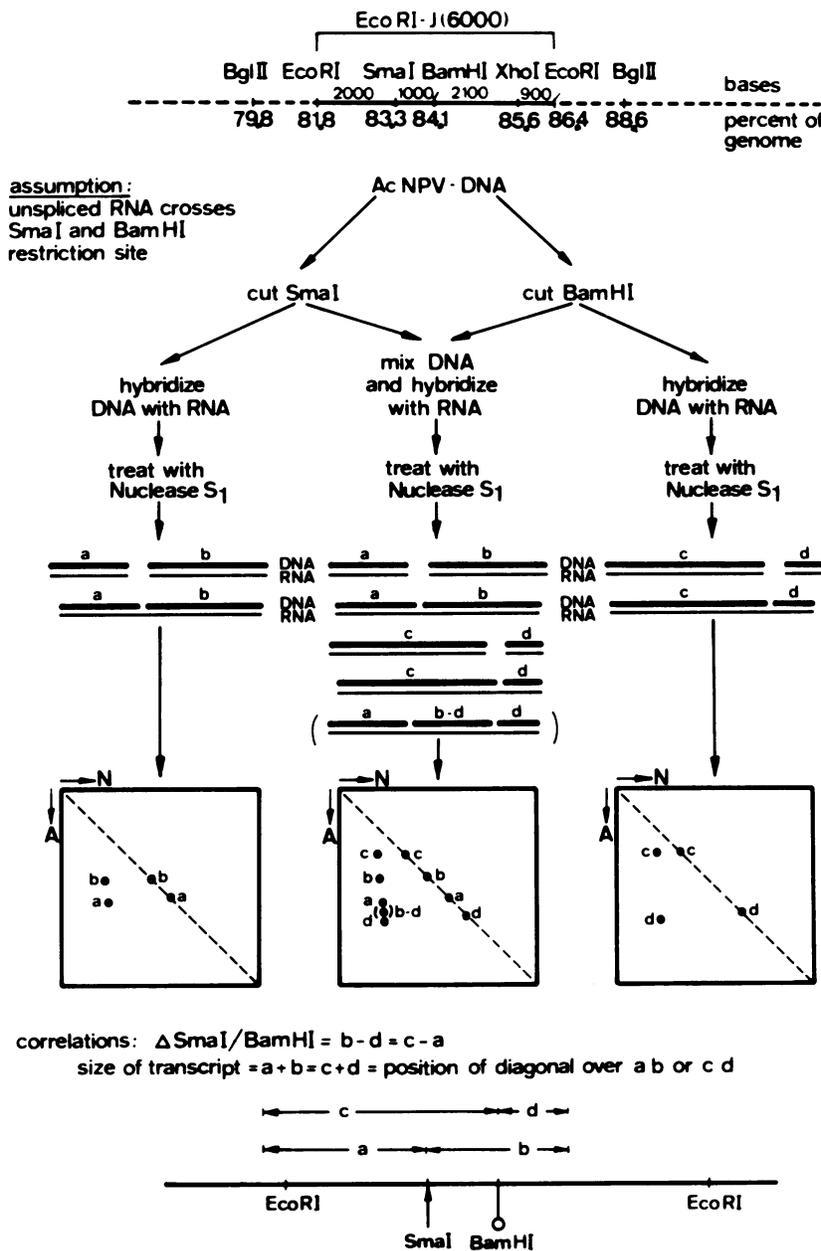


FIG. 2. Illustration of the method used to map overlapping transcripts of fragment *EcoRI*-J of AcNPV DNA. The expected results have been shown for one hypothetical unspliced RNA which traverses the *SmaI* and *BamHI* restriction sites in fragment *EcoRI*-J. The length of this RNA could transcend beyond the limits of fragment *EcoRI*-J. Details are given in the text.

lecular DNA-RNA hybrids migrated considerably more slowly than the shorter bimolecular hybrids. After alkali degradation of the RNA components, the remaining DNA fragments derived from tri- and bimolecular hybrids migrated equally fast in the second (alkaline) direction. Thus, the DNA fragments from trimolecular hybrid molecules, in which the RNA components spanned a restriction site, would migrate below the diagonal lines on two-dimensional gels (Fig. 2, broken lines). DNA fragments derived from bimolecular complexes migrated on the diagonal line on two-dimensional gels, establishing an internal size scale for easy reference. It was apparent from the scheme at the bottom of Fig. 2 that the fragment sizes generated by the procedure described had to be related by the simple equation $b - d = c - a$ or $a + b = c + d$. The experiment, in which the *Sma*I- and *Bam*HI-generated DNA fragments were mixed before hybridization with RNA and S1 nuclease treatment, accommodated all fragments to be analyzed on one two-dimensional gel. It was obvious from the experimental design described that with fragment *Eco*RI-J as hybridization probe, RNA molecules with homologies to that fragment could be mapped in this way. The use of fragments which had been pregenerated by *Sma*I or *Bam*HI treatment, i.e., two different restriction endonucleases, allowed us to determine the lengths of DNA stretches protected by RNA hybridization on both sides of either restriction site.

The method outlined above offers the advantage that overlapping RNA molecules with homologies to a certain DNA segment can be analyzed irrespective of size and can be unequivocally mapped on a single gel. Signals due to one RNA can be distinguished from those due to other RNAs. The polarity of the RNA molecules analyzed can simultaneously be determined by using restriction fragments as probes which had been specifically 32 P-labeled at their 3' or 5' terminus (see below). The procedure described has been devised and is best suited for the mapping of unspliced RNAs, as is the case with the majority of AcNPV-specific RNAs (25). This procedure should, however, be generally applicable.

Mapping RNA populations on fragment *Eco*RI-J of AcNPV DNA. The new technique was first applied to the mapping of RNA molecules transcribed from fragment *Eco*RI-J and from neighboring fragments of AcNPV DNA. A mixture of 1 μ g each of AcNPV DNA cleaved with *Bam*HI or *Sma*I was used in hybridization experiments with 20 μ g of total RNA from AcNPV-infected cells isolated at 24 h postinfection. The hybrids were treated with S1 nuclease, and fragments were separated by two-dimensional gel electrophoresis at neutral pH in one direction and alkaline pH in the other direction. Hybrids were visualized by blotting and hybridization to the 32 P-labeled cloned fragment *Eco*RI-J. The results and details of the experiment are shown in Fig. 3a. There are four (a through d) and three (e through g) sets of fragments arrayed above one another and lying outside the diagonal line. The smallest of these fragments are not abundant and are difficult to detect on this gel but are better detected on other gels (data not shown). Fragments of plasmid pBR322, which had been cleaved with various restriction endonucleases and electrophoresed on the gel in the second direction at alkaline pH (Fig. 3a), served as markers. With either *Bam*HI- or *Sma*I-cut AcNPV DNA, the same procedure was repeated, and only two signals appeared below the diagonal, as shown in Fig. 2 (data not shown).

The fragment sizes in bp determined in the mapping experiment shown in Fig. 3 have been tabulated in Fig. 4a. RNA size-classes a through g correspond to those derived

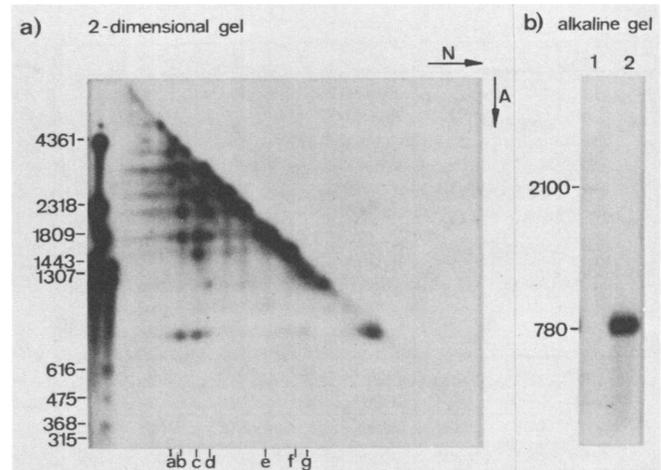


FIG. 3. Analyses of transcripts from fragment *Eco*RI-J of AcNPV DNA. (a) AcNPV DNA (1 μ g each) was cut with *Bam*HI or *Sma*I, and fragments were mixed and hybridized to 20 μ g of total RNA isolated from AcNPV-infected *S. frugiperda* cells. The S1 nuclease-resistant hybrids were separated by two-dimensional electrophoresis on a 2% agarose gel at neutral or alkaline pH in the N or A direction, respectively. Hybrids were transferred to nitrocellulose paper (39) and hybridized with fragment *Eco*RI-J previously 32 P-labeled by nick translation (33). Letters a through g refer to hybrid signals underneath the diagonal line (cf. Fig. 2). In two lanes on the left, alkali-denatured size markers were included when the gel was electrophoresed in the alkaline (A) direction. The first lane contained fragments *Eco*RI and *Bgl*II, and the second lane contained the fragments *Taq*I of pBR322 DNA. The sizes of the marker fragments are indicated in the left margin in nucleotides. (b) The 2,100-bp segment of fragment *Eco*RI-J between the *Bam*HI and *Xho*I restriction sites (Fig. 2) was 3' labeled with 32 P at the *Bam*HI cleavage site and hybridized to 70 μ g of RNA isolated from AcNPV-infected cells (lane 2) or with 70 μ g of yeast tRNA (lane 1). Hybrids were treated with S1 nuclease and separated on neutral or alkaline 2% agarose gels. Only the alkaline gel is shown here. The neutral gel contained the same size bands. Numbers refer to the fragment sizes in nucleotides.

from the data presented in Fig. 3. These data confirm the previously made prediction that the sum of the *Bam*HI-generated hybrid fragments equals the sum of the *Sma*I-generated fragments after S1 nuclease treatment and the values agree with the corresponding positions on the diagonal. The RNA sizes (Fig. 4a, RNA, bottom line) represent data published earlier which had been obtained by regular Northern blot analyses of AcNPV RNAs with homologies to fragment *Eco*RI-J (25). These molecular weights are slightly greater than the ones determined in the present experiment, because RNA populations analyzed by Northern blotting also include the lengths of the poly(A) tails of mRNA.

The results of the experiment described in the legend to Fig. 3b permitted us to determine the polarity of the RNA molecules analyzed. Fragment *Eco*RI-J cloned in the *Eco*RI site of pBR322 DNA was cut with *Bam*HI, and the 3' or 5' end at this site was 32 P-labeled as described (see above). Subsequently, the fragment was cleaved further with *Xho*I and separated from the plasmid by *Eco*RI cleavage. The 3' or 5' 32 P-labeled *Eco*RI-*Bam*HI (3,000-base-pairs [bp]) and *Bam*HI-*Xho*I (2,100-bp) fragments (cf. map in Fig. 2) were purified by gel electrophoresis on agarose gels and hybridized at 55°C for 16 h with 70 μ g of total cellular RNA which had been prepared by the lithium chloride-urea method. The S1 nuclease-resistant hybrids were analyzed by neutral and alkaline gel electrophoresis, and the gels were dried and

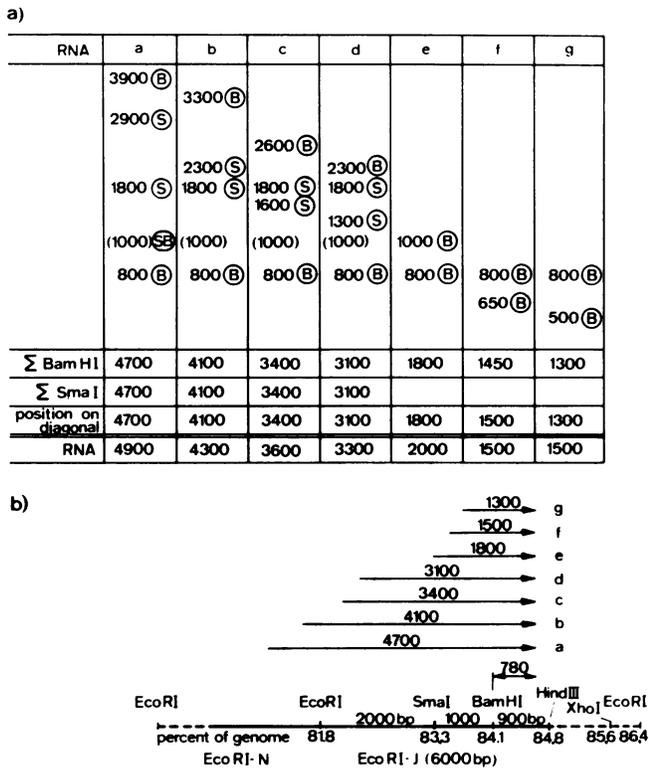


FIG. 4. Map of the RNA populations transcribed from fragment *EcoRI-J*. (a) Size determination in nucleotides of RNA-specific signals appearing underneath the diagonal line in Fig. 3a. The DNA restriction fragments were rejoined by hybridization with RNAs spanning these restriction sites. The symbols (S, *SmaI*; B, *BamHI*; or SB, both enzymes) indicate enzymes used to generate hybrids of the indicated sizes. As in Fig. 3, individual RNA populations corresponding to hybrid signals in Fig. 3 were designated a through g. As described in the text, the total lengths of the two hybrids generated after *BamHI* cleavage equal the total lengths of the hybrids generated after *SmaI* cleavage and thus represent the length of the transcribed region. The total lengths (Σ *BamHI* or Σ *SmaI*) are shown in the lower part of the table and agree well with the corresponding positions on the diagonal over the individual signals, and these lengths are also included. Sizes of RNA classes determined by Northern blotting and reported earlier (25) were designated by RNA. The lengths of these RNA classes are augmented by the poly(A) tails. (b) Diagram of positions and orientations of transcripts mapped in fragment *EcoRI-J*. Their sizes and positions were derived from the two-dimensional gel shown in Fig. 3a and interpreted in Fig. 4a. Orientations of transcripts were determined in the experiments shown in Fig. 3b and 5.

autoradiographed. When the 3' 32 P-labeled *BamHI-XhoI* fragment (2,100 bp) was used in the hybridization experiments, a 780-nucleotide fragment was protected (Fig. 3b). This fragment was not detectable when the 5' 32 P-labeled 2,100-bp fragment or yeast tRNA was used as a control. The very weak 2,100-bp band (Fig. 3b) was due to renatured DNA fragments.

When the 5' 32 P-labeled 3,000-bp *EcoRI-BamHI* fragment was used for hybridization, AcNPV RNA protected 2,600- and 2,300-nucleotide fragments and a few weak bands. Thus, the 5' to 3' polarity of all RNA molecules had to be from left to right (Fig. 4), and all RNA populations appeared to have a common poly(A) addition site. It is apparent from the map in Fig. 4b that only the left part of the *EcoRI* fragment was transcriptionally mapped.

The polarity of the mapped transcripts was confirmed by a second experimental approach. RNA was isolated from AcNPV-infected cells and was reverse transcribed to cDNA as described, using the $[\alpha\text{-}^{32}\text{P}]\text{dNTPs}$. The cDNA fragments originating from the 3' termini of viral RNA had average lengths of between 100 and 600 nucleotides as revealed by electrophoresis on 10% polyacrylamide-8.3 M urea gels. The cDNA was then hybridized to the cloned fragment *EcoRI-J*, which had been previously cut with *EcoRI*, *BamHI*, and *HindIII* (Fig. 5a), *EcoRI*, *BamHI*, and *XhoI* (Fig. 5b), or *EcoRI*, *BamHI*, and *SmaI* (Fig. 5c). These DNA fragments were separated by electrophoresis on agarose gels and transferred by Southern blotting (39) to nitrocellulose filters. The ^{32}P cDNA, which was mainly derived from the 3' termini of the RNA molecules, hybridized to the corresponding region of fragment *EcoRI-J*, i.e., the 900-bp *BamHI-HindIII* fragment (Fig. 5a), the 2,100-bp *BamHI-XhoI* fragment (Fig. 5b), and the 3,000-bp *BamHI-EcoRI* fragment (Fig. 5c) (cf. map in Fig. 2). These results confirm the localization of a common 3' end of the viral RNA molecules with homologies to fragment *EcoRI-J* to the right of the *BamHI* site and consequently establish the direction of transcription from left to right. As a note of caution, we should like to add that we cannot rule out that in reverse transcription some bias is exerted, preferring some RNA molecules over others, although there is no evidence for that bias.

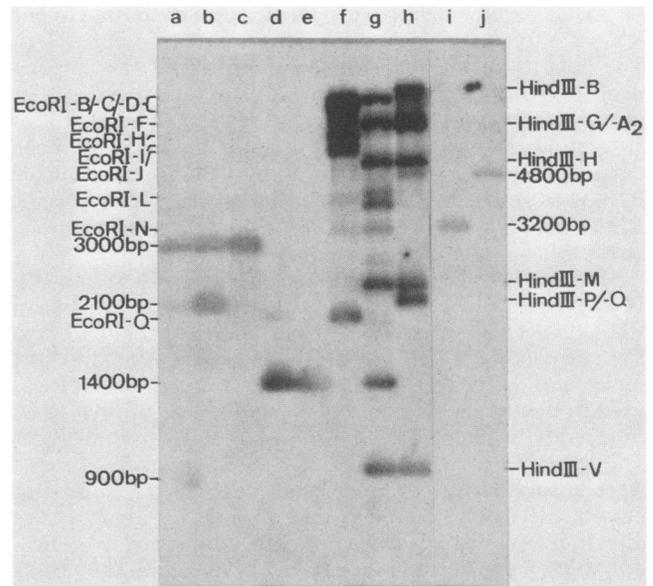


FIG. 5. Hybridization of AcNPV DNA fragments with a ^{32}P cDNA probe: mapping of the 3' termini of AcNPV-specific RNAs. The fragment *EcoRI-J* cloned in pBR322 (0.15 μg) was cut with the restriction enzymes *EcoRI*, *BamHI*, and *HindIII* (track a), *EcoRI*, *BamHI*, and *XhoI* (track b) or *EcoRI*, *BamHI*, and *SmaI* (track c). The clone containing fragment *EcoRI-Q* (0.15 μg) was cut with *EcoRI* and *HindIII* (track d) or *EcoRI*, *BglII*, and *HindIII* (track e), or 0.15 μg of the cloned fragment *EcoRI-F* was cut with *EcoRI* and *HindIII* (track i) or *EcoRI*, *BglII* and *HindIII* (track j). AcNPV DNA (0.5 μg) was cut with *EcoRI* (track f), *EcoRI* and *HindIII* (track g), or *HindIII* (track h). DNA fragments were separated on a 1% agarose gel, transferred to nitrocellulose filters (39), and hybridized with a ^{32}P cDNA probe complementary to the 3' ends of RNAs from AcNPV-infected cells (isolated 24 h postinfection). Fragments were visualized by autoradiography. The positions of the *EcoRI* and *HindIII* fragments of AcNPV DNA and the lengths of subfragments in bp are indicated in the margins.

The data thus collected demonstrate that fragment *EcoRI*-J of AcNPV DNA can code for at least seven RNA molecules of different lengths with an apparent common 3' terminus. This common 3' terminus is located ca. 780 nucleotides to the right of the *Bam*HI site (Fig. 4b). These RNA populations range in size from 1,300 to 4,700 nucleotides and overlap each other (Fig. 4b). The two longest populations of RNA molecules extend into the neighboring fragment *EcoRI*-N and had been found there earlier by Northern blot analysis (25). These RNA molecules were also apparent when a blot similar to the one shown in Fig. 3a was hybridized to the ³²P-labeled fragment *EcoRI*-N (data not shown). The functional significance of this arrangement of transcription products is not known. It has been shown by Northern blot analyses that additional RNA populations are harbored by this fragment and may be less abundant, hence difficult to detect by the technique employed (25). Are there seven independent sites of initiation of transcription, or do the seven different populations of RNA molecules have a common leader too short to be detected by the procedure employed? This problem has not yet been resolved.

Mapping RNA populations on fragments *EcoRI*-O and -F of AcNPV DNA. The results of conventional Southern blot analyses revealed that fragments *EcoRI*-O and -F (Fig. 1) did not have detectable homologies (data not shown). The *EcoRI* site at 34.5% of the genome was used to map viral RNAs in a manner similar to the one described above. One microgram of AcNPV DNA was cut with *EcoRI* and hybridized to 20 μg of RNA from AcNPV-infected cells at 50°C for 16 h. The RNA was extracted 24 h after infection. The hybrids were treated with S1 nuclease, and the fragments were separated by two-dimensional gel electrophoresis at neutral pH in one direction and alkaline pH in the second direction, followed by blotting and hybridization to the ³²P-labeled fragment *EcoRI*-O or -F or a mixture of both fragments. The results of an analysis with both fragments *EcoRI*-O and -F as hybridization probes are presented in Fig. 6a. Four pairs of signals are detectable in positions a, b, c, and d underneath the diagonal line. Molecular size markers were used as described in the legend to Fig. 3. In each set of signal pairs (a, b, c, d) one fragment is ca. 600 nucleotides long. This signal lights up when ³²P-labeled fragment *EcoRI*-F is used as hybridization probe. Fragments 2,400, 2,200, 1,600, and 1,200 nucleotides long hybridized to fragment *EcoRI*-O exclusively (data not shown). The sizes of fragments in positions a, b, c, and d (Fig. 6a) with homologies to fragments *EcoRI*-F and -O add up to more than 3,000, 2,800, 2,200, and 1,800 nucleotides, respectively (Fig. 6b). These fragment sizes also correspond to signals on the diagonal located precisely above signals a through d (Fig. 6a). RNAs 3,600, 2,900, 2,300, and 1,800 nucleotides long have been observed previously by conventional Northern blot analyses with fragment *EcoRI*-O as probe (25). The RNA more than 3,000 nucleotides long may extend beyond fragment *EcoRI*-O, which is only 2,400 nucleotides long. Minor size differences between published RNA sizes and fragment lengths determined in the experiment shown in Fig. 6 can be accounted for by the poly(A) tails, which were removed by S1 nuclease treatment. The arrangement of four different RNA size-classes on fragments *EcoRI*-O and -F of the AcNPV genome is shown in Fig. 6b. The [³²P]cDNA preparation originating on the 3' termini of viral RNA molecules (see above) did not hybridize to fragment *EcoRI*-O, but annealed predominantly to the left end of fragment *EcoRI*-F delineated by the *EcoRI* and a *Hind*III site (Fig. 5, tracks i and j; map in Fig. 6b). These latter data indicate that the 3'

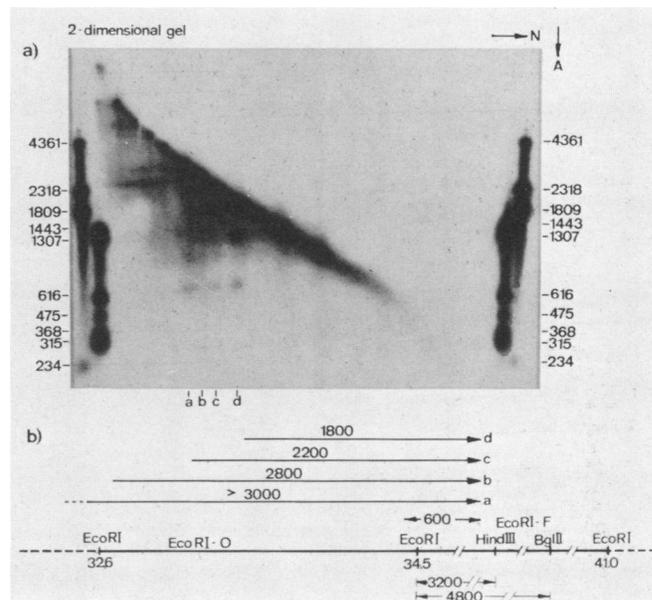


FIG. 6. Mapping of transcripts in fragments *EcoRI*-O and -F of AcNPV DNA. (a) AcNPV DNA (1 μg) was cut with *EcoRI* and hybridized to 20 μg of RNA isolated from *S. frugiperda* cells 24 h after infection with AcNPV. After S1 nuclease digestion, resistant hybrids were separated by two-dimensional electrophoresis on 1.5% neutral (N) and alkaline (A) agarose gels, transferred to nitrocellulose filters, and hybridized with the cloned fragments *EcoRI*-O and -F, which had previously been labeled with ³²P by nick translation. In the second direction at alkaline pH, the *EcoRI* and *Bgl*II fragments of pBR322 DNA were coelectrophoresed as size markers in the right and left external lanes, and the *Taq*I fragments of pBR322 DNA were coelectrophoresed as size markers in the two lanes adjacent to the external ones. Size markers were alkali denatured before electrophoresis. Fragment sizes were designated in bases. Signals of four hybrid populations designated a, b, c, and d underneath the diagonal line are apparent. Their sizes are related to each other, as explained in the legends to Fig. 2 and 4. (b) Schematic presentation of positions and orientations of transcripts mapped in fragments *EcoRI*-O and -F according to results derived from experiments shown in Fig. 5 and 6a. For details, see the text.

ends of the RNA molecules are located in fragment *EcoRI*-F. Thus, the 5' to 3' orientation of this class of RNA molecules is directed from left to right on the viral genome (Fig. 6b).

Mapping RNA populations on fragment *EcoRI*-Q of AcNPV DNA. Fragment *EcoRI*-Q encodes the 10,000-dalton protein which is very abundantly expressed late after infection of *S. frugiperda* cells with AcNPV (1, 35, 37, 38). It was therefore interesting to analyze patterns of expression of this viral DNA segment. Conventional Northern blotting analyses had mainly revealed a 2.6- and a 0.76-kilobase (kb) band of viral RNA which hybridized to the cloned fragment *EcoRI*-Q (25).

The cloned fragment *EcoRI*-Q was cleaved at the *Hind*III site (map in Fig. 7), and the 3' or 5' termini at this site were ³²P labeled as described. Subsequently, the DNA was cut with *EcoRI*, and the selectively labeled 600-bp *EcoRI*-*Hind*III fragment (map in Fig. 7) was isolated by gel electrophoresis and hybridized to 20 μg of RNA isolated 18 h after the infection of *S. frugiperda* cells with AcNPV. The hybrids were treated with S1 nuclease and analyzed on neutral or alkaline gels. On either type of gel the fragments migrated in equivalent positions. This result further supported the notion that extensive splicing of AcNPV-specific RNA from that region could not have taken place.

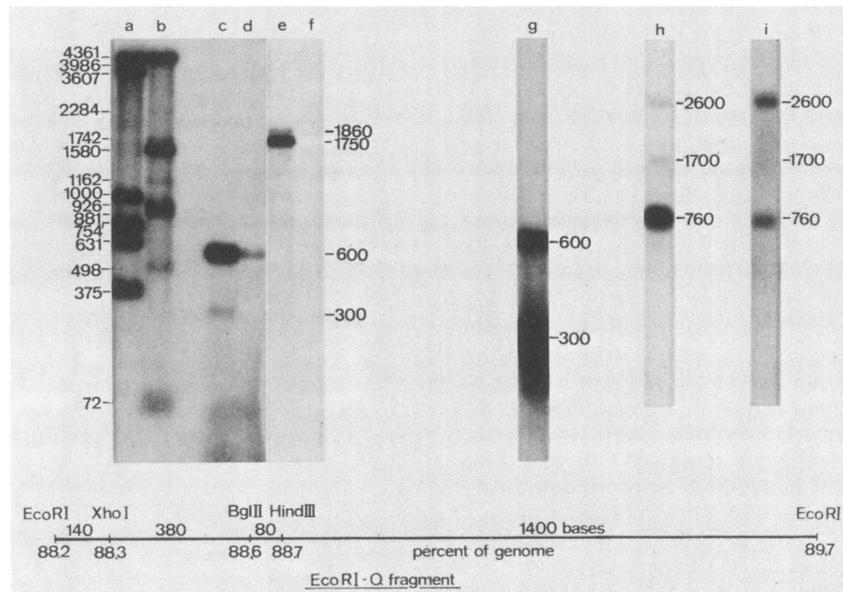


FIG. 7. Mapping of RNAs transcribed from fragment *EcoRI-Q* of AcNPV DNA. The 600-bp segment of fragment *EcoRI-Q* to the left of the *HindIII* site was 5' labeled at the *HindIII* site and hybridized to 20 μ g of RNA isolated 18 h after infection of *S. frugiperda* cells with AcNPV (track c). Hybrids were treated with S1 nuclease and separated on 2% neutral or alkaline agarose gels. Similar experiments were performed with the same DNA fragment and 20 μ g of an RNA preparation isolated 43 h after infection (track g). In a control experiment, 20 μ g of yeast tRNA was used (track d). The 1,860-bp subfragment of fragment *EcoRI-Q* to the right of the *XhoI* site was labeled at the 5' terminus of the *EcoRI* site and hybridized with 20 μ g of RNA isolated 18 h after infection (track e) or with 20 μ g of yeast tRNA (track f). Plasmid pBR322 DNA was first linearized with *EcoRI* and labeled at the 3' and 5' ends with 32 P. As size markers, portions of the plasmid DNA were cut with *BamHI*, *HinfI*, or *PstI* (track a) or *BglII*, *AvaI*, or *DdeI* (track b). After electrophoresis, the gels were dried and autoradiographed. Only results obtained with alkaline gels are shown. Comparable patterns were observed with neutral gels. In tracks h and i Northern blots are shown on which 3 μ g of total cellular RNA isolated 24 h after infection were electrophoresed. These blots were hybridized with the 32 P-labeled 600-bp *EcoRI-HindIII* subfragment (track h) or the 1,400-bp *HindIII-EcoRI* subfragment (track i) of fragment *EcoRI-Q* (map). Numbers indicate fragment, hybrid, or RNA sizes in nucleotides.

When the 600-bp *EcoRI-HindIII* fragment 5' labeled at the *HindIII* site was used in this hybridization experiment, an S1 nuclease-resistant fragment of ca. 300 bp in length was generated (Fig. 7, track c). A similar experiment was performed with RNA isolated 43 h after infection. In this case a much more intense 300-bp band was observed, indicating that this RNA was made in much higher abundance very late after infection (Fig. 7, track g). A similar increase in abundance late after infection has only been observed for the 1,400-nucleotide RNA and the 760-nucleotide RNA (34, 35). The control experiment, in which yeast tRNA was used for hybridization, revealed a 600-bp fragment (Fig. 7, track d) which was due to renatured DNA. Size markers were also shown (Fig. 7, tracks a and b). The 600-bp fragment which was 3' labeled at the *HindIII* site was not protected by viral RNA and did not yield any bands.

In a second type of experiment, the RNA from AcNPV-infected cells was reverse transcribed with [α - 32 P]dNTPs and oligodeoxythymidylate as primer such that the cDNA was initiated at the 3'-terminal poly(A) tails of mRNAs. The cDNA generated mainly from the 3' ends of the RNA molecules hybridized to the 1,400-bp *HindIII-EcoRI* fragment (Fig. 5, tracks d, e, and g; maps in Fig. 7 and 8). This result further supported the interpretation that the 3' ends of the *EcoRI-Q*-specific RNAs were located toward the right of the *HindIII* site in this fragment. Recently published data suggested that the transcribed region of the 0.76-kb RNA coding for the p10 protein contained the *HindIII* site and that transcription terminated to the right of this site (1).

Lastly, fragment *EcoRI-Q* was excised with *EcoRI*, the 5' termini were 32 P labeled, and the fragment was subsequently cut with *XhoI* (Fig. 7 and 8). The 1,860-bp *XhoI-EcoRI* fragment was isolated by gel electrophoresis and hybridized with 20 μ g of RNA from AcNPV-infected (18 h) *S. frugiperda* cells. The hybrids were treated with S1 nuclease. A hybrid of 1,750 bp in length was detectable together with an 1,860-bp fragment due to renatured DNA (Fig. 7, track e). The 1,750-bp hybrid was not apparent upon hybridization with tRNA from yeast (Fig. 7, track f). Thus, apart from the 760-nucleotide RNA in fragment *EcoRI-Q*, a second RNA of at least 1,750 nucleotides was located which extended into fragment *EcoRI-B* adjacent to the right of fragment *EcoRI-Q*. On conventional Northern blots, both fragments *EcoRI-B* and *EcoRI-Q* hybridized to a 2.6-kb RNA (25). Thus, the 1.75-kb hybrid possibly corresponds to part of the 2.6-kb RNA molecule. This assignment, however, is not yet definitive, and it is likely that the 2.6-kb transcript exceeds the bounds of fragment *EcoRI-Q*. From the data presented so far, it was predicted that the 0.76- and 2.6-kb RNAs both crossed the *HindIII* site (Fig. 8) and might be initiated at close to the same site. It was shown experimentally that on a regular Northern blot of RNA from AcNPV-infected cells the 600-bp *EcoRI-HindIII* fragment hybridized to 2,600-, 1,700-, and 760-nucleotide RNAs (Fig. 7, track h). The 1,400-bp *HindIII-EcoRI* fragment also hybridized with 2,600- and 760-nucleotide RNAs (Fig. 7, track i). The exact location of the 1,700-nucleotide RNA has not yet been determined.

By using the conventional sequencing technique of

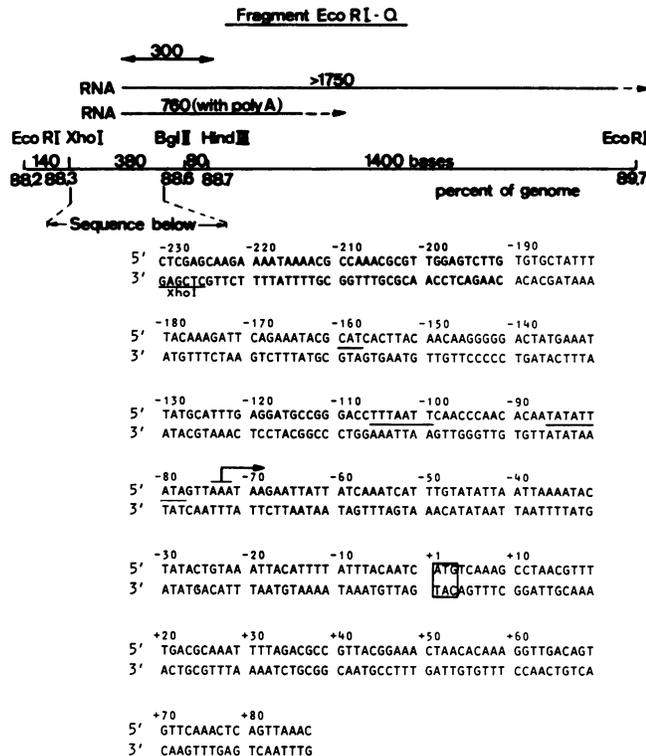


FIG. 8. Positions and orientations of transcripts mapped in fragment *EcoRI-Q* and the nucleotide sequence of part of this fragment. The positions and orientations of RNAs mapped in fragment *EcoRI-Q* were derived from the experiment shown in Fig. 7. Starting from the *XhoI* site, a nucleotide sequence of 318 bp to the right was determined several times. The *XhoI* site, possible TATA (Goldberg-Hogness) signals, and a presumptive CAT signal are underlined. The site of initiation and the direction of transcription of the mapped 0.76- and 2.6-kb RNAs are marked (arrow). An initiation signal for translation (ATG) is designated by a box, and the A in this triplet represents nucleotide +1.

Maxam and Gilbert (27, 28), the nucleotide sequence around the initiation site of the 2.6- and 0.76-kb RNAs was determined. This nucleotide sequence, starting at the *XhoI* site and extending 318 bp to the right, is shown in Fig. 8. The *EcoRI-BglII* subfragment of the *EcoRI* fragment was 5' labeled at the *BglII* site and hybridized to RNA from AcNPV-infected cells. The hybrids were treated with S1 nuclease, and the reaction products were coelectrophoresed on the sequencing gels. There were three bands corresponding to nucleotides -74, -73, and -72 (data not shown), indicating that the 0.76- and 2.6-kb RNAs were initiated at this AAA sequence. Several eucaryotic transcripts start at such a sequence (8). Possible TATATTATA or TTTAATT motifs start at -86 or -106, respectively, and may serve the function of a Goldberg-Hogness signal. There is also a CAT signal at position -160. Similar signals in equivalent positions have been described in promoters of herpes viruses (45). The sequenced stretch of the p10 protein gene contains >60% A + T nucleotides. The 10,000-dalton protein initiates at the ATG triplet in position +1, since that ATG starts an open reading frame. In comparison, it is interesting to note that the promoter region of the polyhedrin gene is also rich in A + T nucleotides (Krebs et al., submitted), and transcription also starts at an AAA sequence (21).

DISCUSSION

The expression of the AcNPV genome in productively infected insect (*S. frugiperda*) cells seems to pose a number of very interesting problems. In previous work we mapped a large number of RNAs on the viral genome and have been unable to find evidence for splicing of viral RNA (25). Minor splices could not be rigorously ruled out. The data presented in this report have demonstrated that at least in the segments of the AcNPV genome that have been investigated, overlapping classes of RNA exist. These RNA molecules had their 3' or 5' ends at the same or closely the same sites on the viral genome. The functional meaning of overlapping sets of viral RNAs with common 3' or 5' termini is unknown. It is tempting to speculate that such a pattern of transcription generates variety in the expression of viral DNA that is provided by RNA splicing in other viral genomes or in the

At present, we can offer but not distinguish between a number of possible explanations for this remarkable pattern of expression of the AcNPV genome. The overlapping RNA molecules with common 3' termini found in fragments *EcoRI-J* and *EcoRI-O* and -F might indeed have different initiation sites. Alternatively, they might have a very short common leader sequence spliced on to all RNA molecules. Furthermore, these RNA molecules might have been generated by processing of RNA molecules starting at the 5' terminus. The different-length 5' ends of transcripts might be involved in the regulation of translation or of the processing of translation products. The overlapping RNAs in fragment *EcoRI-Q* start at a common 5' site, and they appear to be truncated at different lengths. Data published previously (25) have revealed these multiple sets of RNAs by conventional RNA blotting. Not all of these RNA bands were, however, of equal intensity. Are these peculiar expression patterns dependent on time after infection (35) or on culture conditions of the cells? The data available so far do not answer these questions.

We mapped viral RNAs more precisely in three segments of viral DNA comprising map units 32.6 to 41.0, 81.8 to 86.4, and 88.2 to 89.7. Based on the method described by Berk and Sharp (7), a novel technique was developed to map sets of RNAs relative to certain restriction sites on the viral genome. This technique offers distinct advantages over previously published procedures and will be generally applicable. In each of the viral DNA segments mapped, the direction of RNA transcription was from left to right, relative to the AcNPV genome linearized at fragment *EcoRI-H*, as suggested by Vlak and Smith (42). There is preliminary evidence that in several other segments of the AcNPV genome the direction of transcription is also from left to right (unpublished data).

Judging from the transcriptional map of AcNPV DNA (17, 18, 25, 37, 43, 44), in vivo labeling experiments (9, 48), and earlier mapping results (25), there appear to be more viral RNA species than corresponding classes of AcNPV-encoded proteins. In fragment *EcoRI-J* three proteins could be mapped by in vitro translation of hybrid-selected RNA (25). Similarly, in fragment *EcoRI-O* only one protein was mapped by this procedure (18). The same protein was mapped in fragment *EcoRI-F*. There is, of course, no reason to assume that by the method of in vitro translation of hybrid-selected RNAs, all RNAs detectable by blotting could be translated with equal efficiency or that all the polypeptides that are actually produced in infected cells can be detected by this method.

At least two RNAs were located in fragment *EcoRI-Q*. The 0.76-kb RNA is very likely to encode the 10-kilodalton protein (34, 38). It is unknown what proteins the 2.6-kb RNA encodes. With fragments *EcoRI-Q* and *EcoRI-B*, RNAs were selected that could be translated into 52,000- and 25,000-dalton proteins (18). Is the 2.6-kb RNA related to these polypeptides? Both the 0.76- and the 2.6-kb RNAs are synthesized in large amounts late in the infection of insect cells with AcNPV.

Overlapping transcription products as described here were also observed in herpes simplex virus- and vaccinia virus-infected cells (2, 5, 45). These systems also showed a minimum of spliced RNAs. Some of the overlapping RNAs in fragments *EcoRI-J* or *EcoRI-O* and -F differ in lengths by several hundred or even thousand nucleotides; hence they may be derived from promoters which are positioned far apart. It is not clear at the moment whether these overlapping RNAs represent messengers for a single polypeptide or for different polypeptides. There are several examples of transcriptional complexity in eucaryotic systems in that a single gene can be transcribed from alternative promoters (for a recent review see reference 6). Clearly, this problem has not yet been resolved in the AcNPV system. However, the present results explicitly raise that question.

RNAs for the 10,000-dalton protein and for polyhedrin are synthesized in abundant amounts late after infection (1, 34, 38). Presumably, these genes have strong promoters. The promoter region of the 10,000-dalton protein is surprisingly rich (ca. 60%) in A + T residues. The sequence data for the polyhedrin gene region of AcNPV DNA also demonstrate a promoter rich in A + T residues (21; Krebs et al., submitted).

In future work we are planning to sequence one or two of the viral DNA segments studied and to precisely map the sites of initiation of transcription for each class of RNA molecules. It appears that the baculovirus genome may offer yet another interesting example of a highly specialized pattern of transcription.

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