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Physical Maps of *Autographa californica* and *Rachiplusia ou* Nuclear Polyhedrosis Virus Recombinants

MAX D. SUMMERS,* GALE E. SMITH, JOHN D. KNELL, AND JOHN P. BURAND

Department of Entomology, Texas A&M University, College Station, Texas 77843

TN-368 cells were infected simultaneously with the closely related *Autographa californica* (Ac*M*NPV) and *Rachiplusia ou* (Ro*M*NPV) nuclear polyhedrosis viruses. Progeny viral isolates were plaque purified, and their DNAs were analyzed with restriction endonucleases. Of 100 randomly cloned plaques, 7 were Ac*M*NPV and Ro*M*NPV recombinants, 5 were Ro*M*NPV, and 88 were Ac*M*NPV. The recombinants contained DNA sequences derived from both parental genomes. By comparing the restriction cleavage patterns of parental and recombinant DNAs, the crossover sites were mapped. A single double crossover was detected in each of the seven recombinant genomes. In addition, six of the seven recombinants revealed a crossover site mapping between 78 and 89% of the genome. The structural polypeptides of the seven recombinants and two parental viruses were analyzed by polyacrylamide gel electrophoresis, and their polyhedrins were identified by tryptic peptide mapping. An analysis of the segregation of three enveloped nucleocapsid proteins and of the polyhedrins among the recombinants located the DNA sequences coding for Ac*M*NPV structural polypeptides with molecular weights of 37,000 (a capsid polypeptide), 56,000, and 90,000 and the Ro*M*NPV structural polypeptides with molecular weights of 36,000 (a capsid polypeptide), 56,000, and 91,000. The Ac*M*NPV and Ro*M*NPV polypeptides of molecular weights 37,000 and 36,000, respectively, mapped within 78 to 89% or 1 to 29%, the polypeptides of molecular weights 55,000 and 56,000 mapped within 78 to 29%, and the polypeptides of molecular weights 90,000 and 91,000 mapped within 19 to 56% of the genome. The region of the parental DNAs that codes for polyhedrin was located within 70 to 89% of the genome.

The baculovirus *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) contains a relatively complex genome that can code for as many as 50 to 75 proteins. Twenty-five to thirty polypeptides have been detected in purified viruses by analytical sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (7, 14, 16). It is likely that most of the Ac*M*NPV structural proteins are coded for by the viral genome; however, direct evidence that these are viral gene products has not been published. Polyhedrin (12) is a major baculovirus protein with a molecular weight of 25,000 to 30,000 (14). In the nucleus of infected cells it assembles to form a crystalline polyhedron containing enveloped nucleocapsids. Recent data (4; J. M. Vlak, personal communication) provide evidence that polyhedrin is a baculovirus gene product. However, the location on the Ac*M*NPV genome of the gene that codes for polyhedrin or the location of other Ac*M*NPV structural polypeptide genes is not known.

Restriction endonuclease fragment patterns and physical maps of Ac*M*NPV and *Rachiplusia ou* (Ro*M*NPV) DNAs have shown that the two genomes are closely related (7, 9). The structural polypeptides of Ac*M*NPV and Ro*M*NPV are also similar (14) when compared by SDS-polyacrylamide gel electrophoresis. However, there are some useful exceptions. (i) A major capsid polypeptide and at least two minor viral structural polypeptides of Ac*M*NPV and Ro*M*NPV differ in size and electrophoretic mobility (9, 14); (ii) the polyhedrin proteins are different as determined by tryptic peptide mapping (9, 13). By constructing restriction endonuclease maps for both viral DNAs (8, 9), we determined that, of 60 DNA restriction sites mapped, about 25 are different. In cloned preparations of the Ro*M*NPV wild-type isolate, we have also been able to detect putative recombinants of Ac*M*NPV and Ro*M*NPV (9). An analysis of these isolates revealed a segregation of Ac*M*NPV and Ro*M*NPV restriction endonuclease enzyme cleavage sites and structural polypeptides. The parental types of the putative Ac*M*NPV and Ro*M*NPV recombinants could not be determined; therefore, we decided to conduct a study to produce recombinants with known parental types. Physical maps of the crossover points in

AcMNPV and RoMNPV recombinants produced from mixed infection with known parental types could then be constructed as described for adenovirus recombinants by Williams et al. (19). This approach has been successfully applied to mapping gene products and mutations of herpesvirus (1, 5, 11), an animal virus with a genome comparable in size to those of baculoviruses.

In this report we show that stable AcMNPV and RoMNPV recombinants were isolated from doubly infected cells. We mapped the DNA crossover sites in recombinant genomes and measured the approximate recombination frequency. AcMNPV and RoMNPV polyhedrin and several other structural polypeptides of the viral recombinants were shown to segregate in the recombinant viruses, which is evidence that these structural polypeptides were viral gene products. In addition, we determined the approximate location of AcMNPV and RoMNPV DNA templates that specify for polyhedrin and three other structural polypeptides.

MATERIALS AND METHODS

Cells and virus. For these studies a continuous cell line of *Trichoplusia ni*, TN-368, was maintained as described by Volkman and Summers (17) and used for the production of AcMNPV, RoMNPV, and viral recombinants. Cells in log phase were infected, and extracellular virus and polyhedra were purified as described previously (14, 18).

The E2 and S1 variants of AcMNPV, for which we have constructed DNA restriction maps (8), were used in this study. AcMNPV E2 and S1 were plaque purified from a wild-type isolate of AcMNPV obtained from Pat Vail (United States Department of Agriculture, Science and Education Administration, Fresno, Calif.) (7). RoMNPV was obtained from the Cornell Experiment Station (Geneva, N.Y.) (9). Two viral isolates used in this study, AR7 and AR8, were plaque purified from a wild-type isolate of RoMNPV, originally obtained from James Harper (Auburn University). AR7 and AR8 are naturally occurring recombinants of AcMNPV and RoMNPV (9). We have included AR7 and AR8 in this report only as a comparison to recombinants produced from known parental genomes. These two naturally occurring recombinants were not used to map the location of gene products.

To produce AcMNPV S1 and RoMNPV R1 for mixed infection, each virus was grown in TN-368 cells. Infected TN-368 cells, containing virus polyhedra, were collected at 48 h postinfection and mixed with the diet of *T. ni* larvae. After 3 to 5 days, infected larvae were collected and polyhedra were purified by banding at a density of 1.25 g/ml in sucrose gradients (density range, 1.18 to 1.29 g/ml) as described by Smith and Summers (7).

Mixed infection with AcMNPV and RoMNPV. Purified AcMNPV S1 and RoMNPV R1 polyhedra were disrupted with an alkaline solution of 0.1 M Na_2CO_3, 0.01 M EDTA, and 0.17 M NaCl (pH 10.9), and the liberated virus (alkali-liberated virus) was purified by banding at a density of 1.21 to 1.25 g/ml in sucrose gradients (14). TN-368 cells were infected with a mixture of AcMNPV S1 and RoMNPV R1 alkali-liberated virus at a multiplicity of 500 PFU per cell for each virus. Infected cells were gently rocked for 1 h at 27°C, and the inoculum was removed. The cell monolayers were washed with culture medium without fetal bovine serum and then with culture medium containing sufficient antiserum (1:50 dilution, vol/vol) to AcMNPV alkali-liberated virus to neutralize any AcMNPV or RoMNPV not washed from the cells or remaining in the medium. Volkman et al. (18) demonstrated that antiserum to AcMNPV alkali-liberated virus will neutralize the AcMNPV and RoMNPV alkali-liberated virus (i.e., the parental virus) but not AcMNPV or RoMNPV extracellular virus (i.e., progeny virus) released into the culture medium. The infected cells were incubated at 27°C in TNM-FH medium (15) containing AcMNPV alkali-liberated virus antiserum at a final concentration of 1:50 (vol/vol). The extracellular virus was collected at 24 h postinfection, and the viral progeny were plaque purified three times as described (7) by using a modification of the polyhedral plaque assay.

Screening for viral recombinants. The DNAs from each plaque-purified isolate obtained from the mixed infection with AcMNPV S1 and RoMNPV R1 were radiolabeled with $^{32}P_i$ and the viral ^{32}P-labeled DNAs were purified as follows. Multiwell plates (Falcon 3040) were seeded with 2×10^5 TN-368 cells per well and infected with each viral isolate for 1 h at 27°C. The virus was removed, and 100 μCi of $^{32}P_i$ (500 Ci/mmol, New England Nuclear Corp.) in 200 μl of phosphate-free medium (7) was added to each well. The plates were incubated 48 h at 27°C. To disrupt the cells, the medium was removed and a solution of 1 mg of protease K per ml in 0.5 ml of 0.01 M Tris and 0.01 M EDTA (pH 7.5) was added to the infected cells. The plates were incubated at 37°C for 2 h and the polyhedra, which had been released by cellular disruption, were pelleted by centrifugation at 10,000 × *g* for 15 min. To release the virus, the pellet of polyhedra was suspended in a solution of 0.1 M $NaCO_3$, 0.17 M NaCl, and 0.01 M EDTA (pH 10.9) and incubated at 37°C for 5 min. The alkali-liberated virus was pelleted by centrifugation at 100,000 × *g* for 30 min, and the pellet was suspended in a solution of 200 μl of 0.01 M Tris, 0.01 M EDTA (pH 7.5), 1% SDS, and 0.2 mg of protease K per ml. This solution was incubated for 2 h at 37°C. The viral ^{32}P-labeled DNA was extracted once with phenol (saturated with 0.01 M Tris–0.001 M EDTA [pH 7.5]) and once with chloroform-isoamyl alcohol (24:1). Each sample was adjusted to a final concentration of 0.15 M with NaCl, 3 volumes of ethanol were added, and the viral ^{32}P-labeled DNA was precipitated overnight at −20°C. The ethanol precipitate was centrifuged at 10,000 × *g* for 20 min and washed twice with 70% ethanol, and the viral ^{32}P-labeled DNA was lyophilized to remove residual alcohol.

To screen for AcMNPV and RoMNPV recombinants, viral ^{32}P-labeled DNA samples from each plaque-purified isolate were restricted with *Eco*RI, and the restriction enzyme digests were electrophoresed in 0.75% agarose gels. The gel slabs were dried

on filter paper and autoradiographed as described previously (8). Isolates with *Eco*RI restriction patterns different than those of the parental viruses, Ac*M*NPV and Ro*M*NPV, were considered putative recombinants. Each putative recombinant was plaque purified twice before further analysis.

In vitro labeling and purification of DNA restriction fragments. Ro*M*NPV DNA was restricted with *Eco*RI, and the fragments were labeled intact to a specific activity of 1×10^7 to 2×10^7 cpm/μg of DNA by a modification of the nick-translation procedure of Rigby et al. (6). The reaction mixture (100 μl) contained 0.05 M Tris, 0.005 M $MgCl_2$, 0.001 M 2-mercaptoethanol (pH 7.8), 5 μg of bovine serum albumin, 0.001 M each dCTP, dGTP, dTTP, and [α-^{32}P]dATP (300 to 400 Ci/mmol), 1.0 μg of Ro*M*NPV R1 *Eco*RI fragments, and 2 U of *Escherichia coli* DNA polymerase I (New England Biolabs). After incubation at 14°C for 4 to 6 h, the reaction was stopped by adding EDTA to 0.01 M and 100 μg of protease K per ml. The viral ^{32}P-labeled DNA was extracted with phenol, and the unincorporated label was removed by precipitating the DNA three times with 70% ethanol.

Ro*M*NPV R1 ^{32}P-labeled *Eco*RI fragments were suspended in agarose gel electrophoresis buffer (0.04 M Tris, 0.005 M sodium acetate, 0.001 M EDTA [pH 7.8]) and electrophoresed in a 0.6% agarose gel slab for 60 h at 75 V in the presence of ethidium bromide, and the UV-visible bands were cut from the gel. To check the purity of the DNA fragments, 200 to 500 cpm of each band was electrophoresed in agarose, dried, and autoradiographed as described above.

Transfer of DNA fragments to nitrocellulose and blot hybridization. *Eco*RI fragments of Ac*M*NPV E2 DNA were fractionated in a 0.6% agarose slab gel (20 by 20 cm). Ten micrograms of DNA was loaded into a continuous sample chamber and electrophoresed. The DNAs were then denatured by placing the slab gel into 0.5 N NaOH–1.5 M NaCl at room temperature for 4 h. Subsequently, the gels were neutralized in 1.0 M Tris–1.5 M NaCl (pH 7.5) for 2 h. The denatured DNA fragments were then transferred to nitrocellulose filter sheets (Schleicher and Schuell BA85) by the method of Southern (10) with 10× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The nitrocellulose filter sheets were rinsed in 2× SSC, dried at 80°C under vacuum for 2 h, and cut into strips 0.5 cm wide; each strip contained about 0.25 μg of transferred Ac*M*NPV E2 *Eco*RI fragments. The strips were preincubated (for 4 h at 65°C) in Denhardt solution (2) in 2× SSC containing 100 μg of heat-denatured calf thymus DNA per ml. Purified ^{32}P-labeled *Eco*RI restriction fragments of Ro*M*NPV were denatured at 100°C for 10 min and then hybridized to the preincubated strips of transferred Ac*M*NPV E2 *Eco*RI fragments. Hybridization was done in 0.5 M NaCl–0.1 M sodium phosphate–0.05 M EDTA (pH 7.0)–1.0% SDS (hybridization buffer) with 100 μg of heat-denatured calf thymus DNA per ml. Strips were incubated for 24 h at 66°C with viral ^{32}P-labeled DNA fragments in 2 ml of hybridization buffer. The filters were washed twice with 0.5× hybridization buffer (30 min at 65°C), twice with 2× SSC (15 min at 65°C), and then dried and autoradiographed.

Restriction enzyme and agarose gel electrophoresis. The restriction endonucleases *Sac*I, *Kpn*I, *Xho*I, *Bam*HI, and *Eco*RI were purchased from New England Biolabs. The conditions for enzyme digestion and gel electrophoresis were described (8).

Polyacrylamide gel electrophoresis. To analyze the viral structural polypeptides, purified virus was disrupted in 1.0% SDS–5.0% 2-mercaptoethanol at 100°C for 3 min. Samples of 30 μg of viral protein were electrophoresed in 11% polyacrylamide vertical gel slabs in the presence of 0.1% SDS as described by Laemmli (3) as modified for baculovirus preparations (13).

Tryptic peptide mapping of polyhedrin. Polyhedrin proteins from Ac*M*NPV, Ro*M*NPV, and each viral recombinant were purified, digested with trypsin, and prepared for two-dimensional tryptic peptide mapping as described previously (9, 13). The positions of the hydrolysates were detected with 1.0% ninhydrin–0.5% cadmium acetate.

RESULTS

Mixed Infection in vitro with Ac*M*NPV and Ro*M*NPV. Nondefective plaque-purified isolates of Ac*M*NPV and Ro*M*NPV (S1 and R1, respectively) were used as parental viruses to produce viral recombinants by mixed infection in TN-368 cells. To eliminate or minimize the amount of parental virus contaminating progeny virus preparations, neutralizing antisera specific for parental virus was added after infection. Progeny extracellular Ac*M*NPV is not neutralized by alkali-liberated virus antisera (18). TN-368 cells were infected with the parental viruses at a high and equal multiplicity to ensure that all the cells would be infected with both Ac*M*NPV and Ro*M*NPV.

To screen for recombinants, 100 plaques of the extracellular virus were obtained and the *Eco*RI restriction fragment patterns of each viral DNA were analyzed as described above. Of 100 progeny viral DNAs, 87 had the same *Eco*RI restriction pattern as Ac*M*NPV S1 DNA, 5 were the same as Ro*M*NPV R1 DNA, and 8 isolates had *Eco*RI restriction patterns that were different from either of the parental viruses and were considered putative recombinants. Of the eight putative recombinants, seven were shown to be true Ac*M*NPV and Ro*M*NPV recombinants. One of the putative recombinants had a slightly altered Ac*M*NPV *Eco*RI restriction pattern (not shown) and was partially defective for producing polyhedra in both TN-368 cells and *T. ni* larvae.

Hybridization of Ro*M*NPV ^{32}P-labeled DNA to Ac*M*NPV DNA. Ro*M*NPV R1 DNA was cleaved with *Eco*RI and radiolabeled in vitro, and each of the viral ^{32}P-labeled DNA restriction fragments was purified by agarose gel electrophoresis. Each viral ^{32}P-labeled DNA band was hybridized to filter strips containing transferred Ac*M*NPV E2 *Eco*RI restriction

bands as described above. An autoradiogram of RoMNPV ^{32}P-labeled *Eco*RI fragments (Fig. 1A) shows that some of the extracted fragments were contaminated with one or more lower-molecular-weight fragments. This is especially evident with larger RoMNPV fragments as they are the most difficult to resolve. Further purification was not needed as the hybridization pattern of ^{32}P-labeled RoMNPV fragments to transferred AcMNPV *Eco*RI fragments was interpreted without difficulty.

RoMNPV ^{32}P-labeled DNA formed stable hybrids to all of the transferred AcMNPV *Eco*RI fragments (Fig. 1B). The stoichiometric binding of RoMNPV ^{32}P-labeled DNA to AcMNPV *Eco*RI fragments is similar to what was obtained for hybridization to homologous DNA (not shown, but compare Fig. 1B with the AcMNPV *Eco*RI pattern in Fig. 2). Thus, extensive DNA homology exists between RoMNPV and all of the *Eco*RI fragments of AcMNPV DNA. The hybridization of RoMNPV ^{32}P-labeled DNA fragments to transferred AcMNPV fragments (Fig. 1B and Table 1) substantially confirms the AcMNPV and RoMNPV DNA linkage map (see Fig. 5A). The only results which would not have been predicted from the DNA linkage map (see Fig. 5A) were the hybridization of RoMNPV *Eco*RI-B and -Q fragments to AcMNPV *Eco*RI-P and -BC, respectively. Changes, if any, in the *Eco*RI linkage map of AcMNPV and RoMNPV that might be needed would be only minor and would not significantly affect the mapping of recombinant crossover sites in this report.

Restriction analysis of AcMNPV and RoMNPV recombinants. The recombinant DNA restriction enzyme fragment patterns were not affected by passage of the viruses in vivo (data not shown). Therefore, for analysis of viral DNAs and virus structural proteins the AcMNPV and RoMNPV recombinants, AR1, AR13, AR22, AR36, AR61, AR67, and AR74

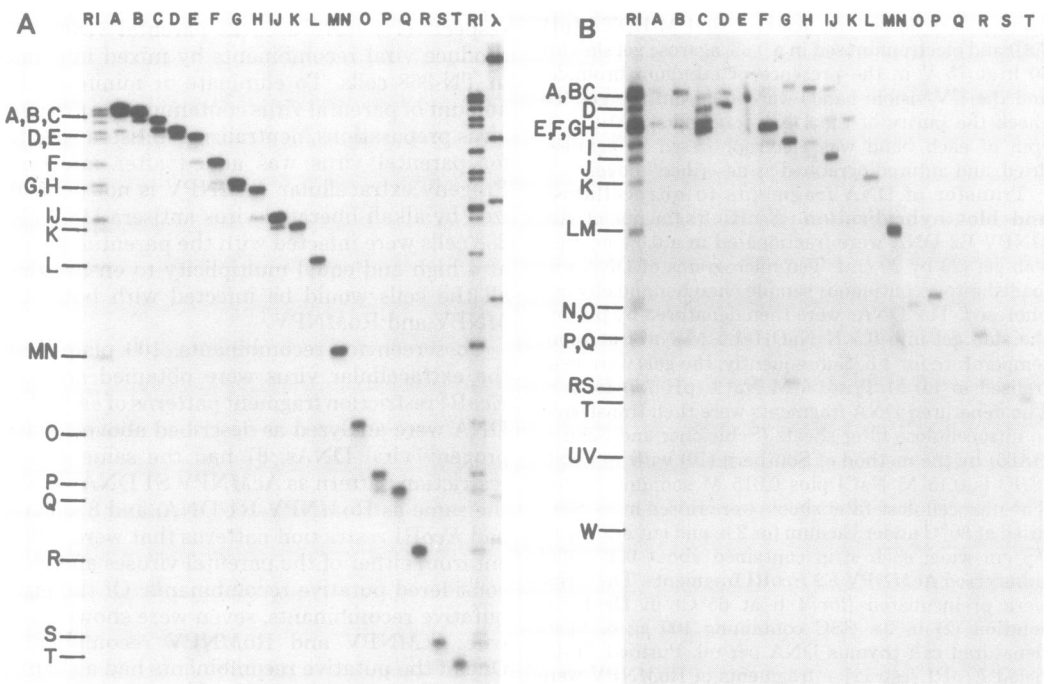

FIG. 1. *The isolation and hybridization of in vitro-labeled RoMNPV ^{32}P-labeled DNA restriction fragments to AcMNPV filters. (A) RoMNPV DNA was cleaved with EcoRI, and the restriction fragments were labeled with ^{32}P as described in the text. Lambda DNA was cleaved with HindIII and labeled in a similar manner. RoMNPV ^{32}P-labeled EcoRI fragments were electrophoresed in 0.6% agarose, each band was cut from the gel, and amounts of 200 to 300 cpm of each EcoRI fragment (A through T) were electrophoresed in 0.6% agarose adjacent to unfractionated RoMNPV ^{32}P-labeled EcoRI fragments (R1) and ^{32}P-labeled lambda HindIII fragments (λ). RoMNPV EcoRI fragments are lettered as described previously (9). (B) Isolated RoMNPV ^{32}P-labeled DNA fragments EcoRI-A through -T were hybridized to filter strips containing transferred EcoRI fragments of AcMNPV. An unfractionated preparation of RoMNPV ^{32}P-labeled DNA (R1) was also hybridized to a strip of transferred AcMNPV EcoRI bands A through W, which have been lettered as described previously (8). After 24 h at 68°C, each strip was washed and then autoradiographed at −70°C for 2 days, using a Dupont Lightning Plus intensifying screen.*

were grown in *T. ni* larvae. *Eco*RI, *Kpn*I, and *Bam*HI restriction fragments of the recombinant viral DNAs (Fig. 2, 3, and 4, respectively) were electrophoresed in 0.6% agarose gels adjacent to the endonuclease fragments of Ac*M*NPV S1 and Ro*M*NPV R1. The method used to map the recombination sites between Ac*M*NPV and Ro*M*NPV in the recombinant viral genomes has been described by Williams et al. (19). To apply this procedure, we first identified the Ac*M*NPV and Ro*M*NPV restriction fragments which had different mobilities on agarose gels so that the parental origin of these fragments in each of the recombinant DNA restriction enzyme patterns could be determined. Only those fragments which were the result of an additional restriction site on one parental genome as compared to the other parental genome were accepted as suitable markers. Restriction fragments of one parental genome which were the result of a loss of a restriction site relative to the other were not accepted, as their presence in recombinant genomes could have been the result of mutation within the endonuclease recognition sequence instead of as a result of a true recombination event. The restriction fragments of Ac*M*NPV and Ro*M*NPV that fit the above criteria have been indicated by an asterisk in Fig. 2, 3, and 4. These marker restriction fragments allow the location of 21 restriction cleavage sites along the parental genomes (Fig. 5A) which can be identified as Ac*M*NPV or Ro*M*NPV in recombinant

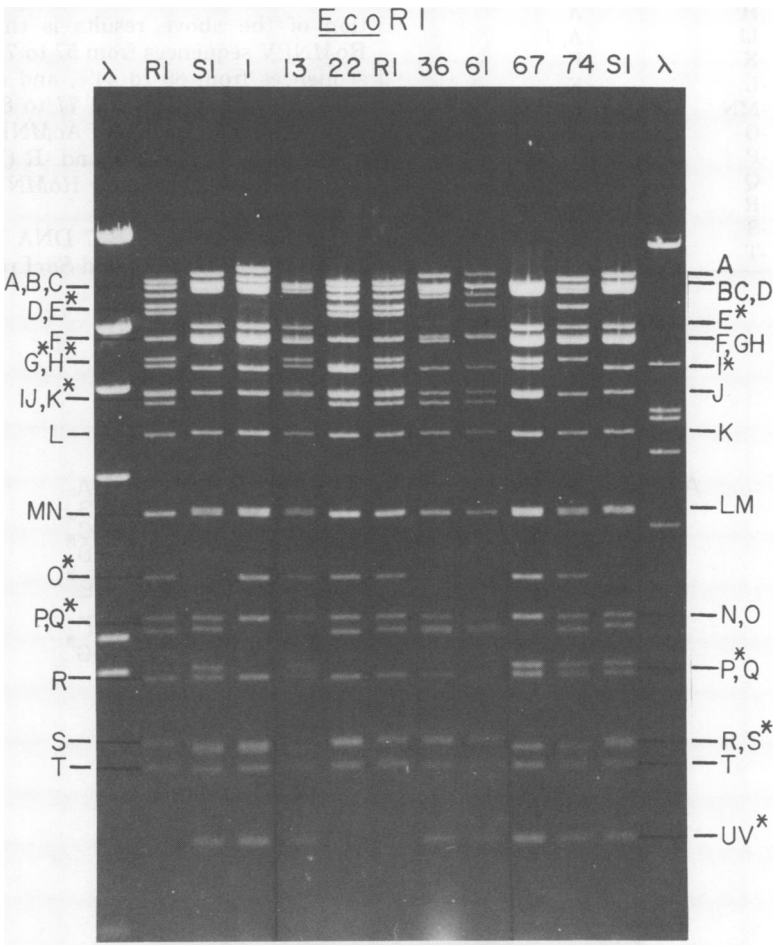

FIG. 2. *RoMNPV DNA (R1), AcMNPV DNA (S1), and AcMNPV and RoMNPV recombinant DNAs (AR1, AR13, AR22, AR36, AR61, AR67, and AR74) were cleaved with EcoRI and electrophoresed in 0.6% agarose. RoMNPV DNA fragments are lettered on the left and AcMNPV DNA fragments are lettered on the right as described previously (8, 9). A HindIII digest of lambda DNA in the first slot (λ) and an EcoRI digest of lambda DNA in the last slot (λ) were included as molecular-weight standards. Approximately 0.7 μg of DNA was electrophoresed in each slot. The UV fluorescent bands were photographed as described in the text. Any minor, submolar bands in this figure and Fig. 3, 4, and 5 were a result of incomplete digestion.*

genomes. A detailed explanation of mapping of crossover sites will be given for one of the recombinants, AR13.

Mapping recombination sites. AR13 DNA was cleaved with *Eco*RI, *Kpn*I, *Bam*HI, and *Sac*I. The AR13 DNA restriction bands that could be identified as Ro*M*NPV fragments were *Eco*RI-G, -H, and -O; and *Kpn*I-C and -E (Fig. 2 and 3). All of the identifying Ac*M*NPV restriction fragments (Fig. 2, 3, and 4) were also found in AR13 restriction digests except for Ac*M*NPV *Eco*RI-I. A *Sac*I digest of AR13 and the other recombinants was also done. *Sac*I digests will not be shown, as the positions on the genomes that this enzyme can define (about 31 and 57%) are similar to those obtained with *Kpn*I (see Fig. 5A). However, *Sac*I digests did help to confirm results obtained from the other enzyme digests. AR13 DNA had restriction bands migrating with Ac*M*NPV *Sac*I-AB, -C, -D, -F, -G, -H, and -I; and Ro*M*NPV *Sac*I-E. The simplist interpretation of the above results is that AR13 has Ro*M*NPV sequences from 57 to 77%, Ac*M*NPV sequences from 89 to 44%, and two crossover sites from 44 to 57% and 77 to 89% (Fig. 5B). Note that the absence of Ac*M*NPV restriction fragments *Eco*RI-A, -O, and -R (Fig. 2) is consistent with AR13 having Ro*M*NPV sequences from 57 to 77%.

The recombinant AR67 DNA had the same *Eco*RI, *Kpn*I, *Bam*HI, and *Sac*I restriction pat-

TABLE 1. *Hybridization of in vitro-labeled RoMNPV DNA fragments to AcMNPV DNA fragments*

Ro*M*NPV ^{32}P-labeled DNA probe	Homologous Ac*M*NPV *Eco*RI fragments
*Eco*RI-A	BC, F, UV
-B	BC, P
-C	E, GH
-D	D
-E	BC
-F	GH
-G	L, R
-H	A
-IJ	A, J
-K	E
-L	K
-MN	LN
-O	A, O
-P	N
-Q	BC
-R	Q
-S	S
-T	T

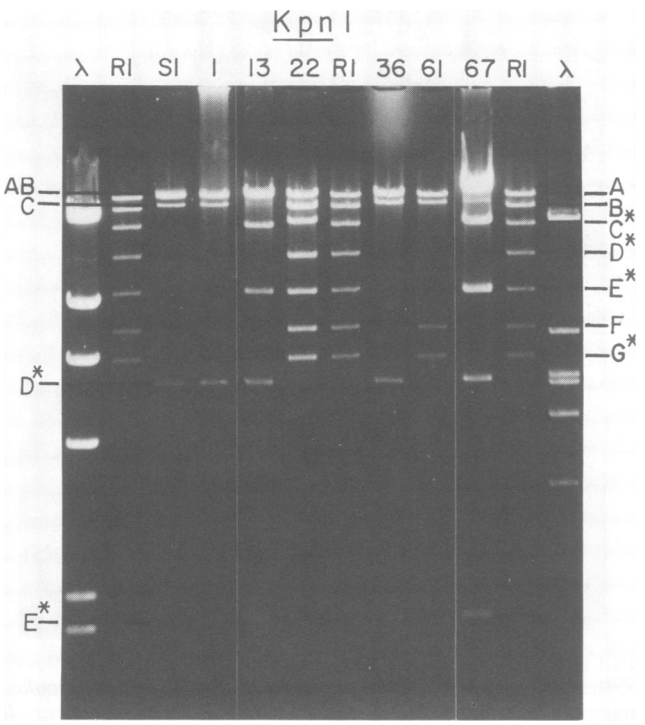

FIG. 3. *RoMNPV DNA (R1), AcMNPV DNA (S1), and recombinant DNAs (AR1, AR13, AR22, AR36, AR61, and AR67) were cleaved with KpnI and electrophoresed in 0.6% agarose. The lettering of RoMNPV (right) and AcMNPV (left) restriction fragments, lambda DNA standards (λ), and photography were as described in Fig. 2. The KpnI restriction pattern of AR74 (not shown) is the same as that of AcMNPV (S1).*

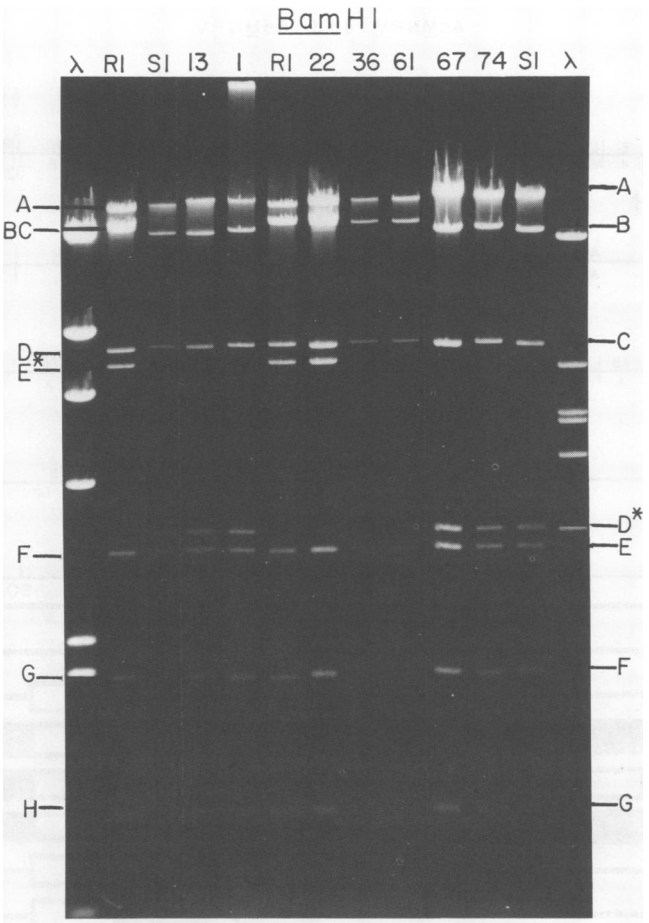

FIG. 4. *RoMNPV DNA (R1), AcMNPV DNA (S1), and recombinant DNAs (AR1, AR13, AR22, AR36, AR61, AR67, and AR74) were cleaved with BamHI and electrophoresed in 0.6% agarose. The lettering of RoMNPV (left) and AcMNPV (right) restriction fragments, lambda DNA standards (λ), and photography were as described in Fig. 2.*

terns as AR13. AR67 is included in this study because the *Xho*I restriction bands of AR13 and AR67 DNAs (not shown) were not identical and the results indicated that AR67 had a different crossover site between 77 and 89% than did AR13. The recombination maps of AR1, AR22, AR36, AR61, AR67, and AR74 were determined in a similar manner and are shown in Fig. 5B.

Structural polypeptides of recombinant viruses. Four Ac*M*NPV structural polypeptides (with molecular weights of 30,000 [polyhedrin], 37,000, 56,000, and 90,000) can be distinguished from four Ro*M*NPV polypeptides (with molecular weight of 30,000 [polyhedrin], 36,000, 55,000, and 91,000). Ac*M*NPV-Ro*M*NPV polyhedrins with a molecular weight of 30,000 can be distinguished by two-dimensional tryptic peptide mapping (9, 13). The identity of these four structural polypeptides in recombinant viruses (Table 2) was determined by comparing the virus structural polypeptides in an SDS-polyacrylamide gel (Fig. 6). The identity of each polyhedrin was determined by two-dimensional electrophoresis of polyhedrin tryptic peptides on thin-layer cellulose sheets (not shown).

Mapping of Ac*M*NPV and Ro*M*NPV polypeptides. Correlating the segregation of polypeptides specified by the recombinant viruses with the DNA sequence arrangements of the recombinant viruses was done to map the location of the DNA templates specifying Ac*M*NPV and Ro*M*NPV polypeptides. The approximate location of the sequences that specify for polyhedrin proteins can be determined from the following information. Both AR1 and AR22 recombinants had Ac*M*NPV polyhedrin. The only Ac*M*NPV sequences that AR1 and AR22 have in common are from about 70 to 89%, (Fig. 5B); thus the polyhedrin gene should be specified within this region of the genome. The location

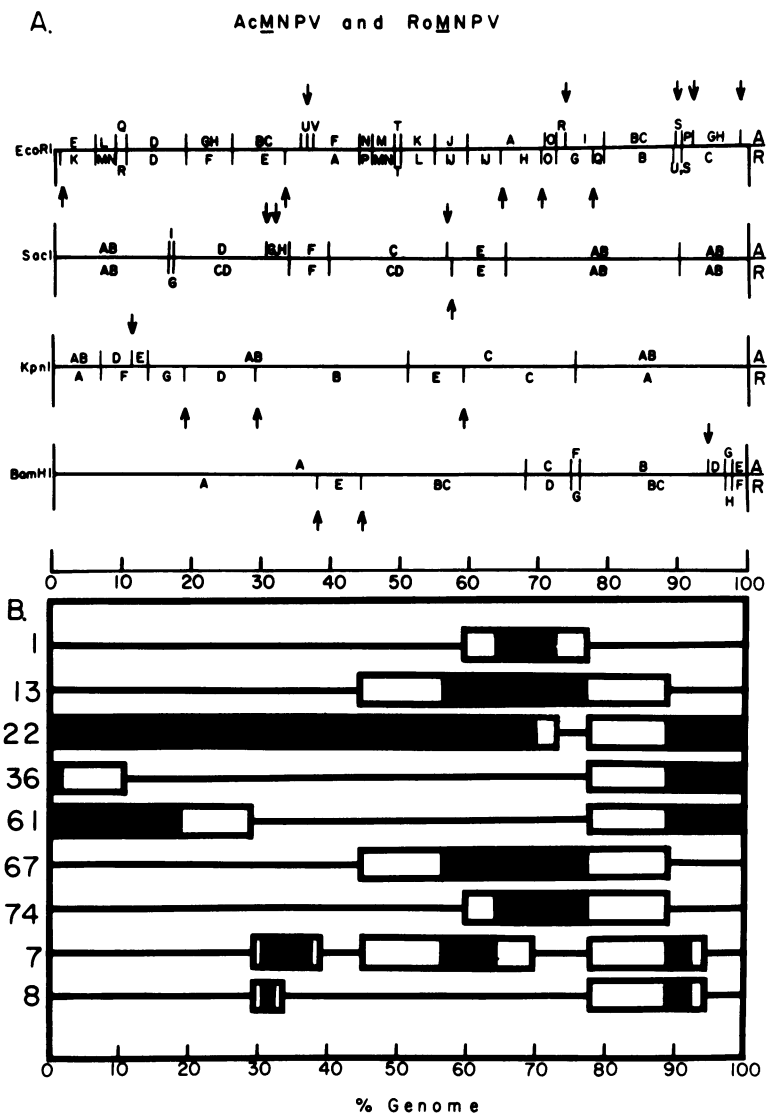

FIG. 5. *(A) The EcoRI, SacI, KpnI, and BamHI restriction maps of AcMNPV (A) and RoMNPV (R) are lettered and aligned as described previously (9). Those restriction sites used to identify AcMNPV DNA (↓) and RoMNPV DNA (↑) sequences in recombinant genomes were determined as described in the text. (B) AcMNPV and RoMNPV DNA sequence arrangements in recombinant genomes. The thin and thick horizontal lines represent AcMNPV and RoMNPV sequences, respectively. The open rectangles represent the boundaries within which crossovers occurred. The position of AcMNPV and RoMNPV sequences in AR7 and AR8 (two naturally occurring recombinants) cannot be determined with absolute certainty (see text) and are included in this figure for comparison only.*

of polyhedrin gene from 70 to 89% is consistent with the fact that AR36 and AR61 have Ac*M*NPV polyhedrin and that AR13, AR67, and AR74 have Ro*M*NPV polyhedrin (Fig. 5B). A comparison of AR22 *Eco*RI restriction fragments to Ro*M*NPV bands (Fig. 2) shows that AR22 has all of the Ro*M*NPV bands except *Eco*RI-G and -Q, and none of the Ac*M*NPV bands except *Eco*RI-I. Although the recombination maps do not define the crossover sites in AR22 precisely enough to locate the polyhedrin gene in Ac*M*NPV *Eco*RI-I and Ro*M*NPV *Eco*RI-G or -Q, we have additional data (in preparation) to support this hypothesis. Ac*M*NPV and Ro*M*NPV polypeptides (with molecular weights of 37,000 and 36,000, respectively) mapped from 78 to 89% or 1 to 29%, those of molecular weights 55,000 and 56,000 mapped

from 78 to 29%, and those of molecular weights 90,000 and 91,000 mapped from 19 to 56%. The segregation of these proteins in two natural recombinants, AR7 and AR8 (Table 2), is consistent with the above results.

DISCUSSION

We have shown in this report that baculovirus recombinants can be produced by mixed infection with Ac*M*NPV and Ro*M*NPV. The frequency of recombination was difficult to measure as only those recombinants in which crossovers occurred in regions affecting the *Eco*RI restriction patterns in recombinant DNAs would have been detected. The observation that 7 of 100 isolates from doubly infected cells were recombinants suggests that the frequency was a minimum of 7%.

TABLE 2. *AcMNPV and RoMNPV structural polypeptides of recombinant viruses*[a]

Recombinants	36K(R) or 37K(A)	55K(R) or 56K(A)	90K(A) or 91K(R)	Polyhedrin
1	A	A	A	A
13	A	A	A	R
22	R	R	R	A
36	A	R	A	A
61	R	R	A	A
67	A	A	A	R
74	A	A	A	R
7	R	R	R	A
8	R	A	A	A

[a] Polypeptides identified as Ac*M*NPV are labeled as A; those of Ro*M*NPV are labeled as R; K, thousand.

The limitations of the mapping procedure used in this study to locate Ac*M*NPV and Ro*M*NPV DNA sequences coding for structural proteins were governed by three factors, the number and distribution of enzyme cleavage sites available to locate crossover sites, the distribution of crossovers in recombinant genomes, and the number of recombinants compared. The fact that Ac*M*NPV and Ro*M*NPV have many cleavage sites in common, about 35 of 60 that have been mapped (9), has made the accurate placement of crossovers difficult. This is especially true when recombination has occurred where there are no unique Ac*M*NPV or Ro*M*NPV DNA restriction cleavages, e.g., from 77 to 89% and from 44 to 55%. The distribution of

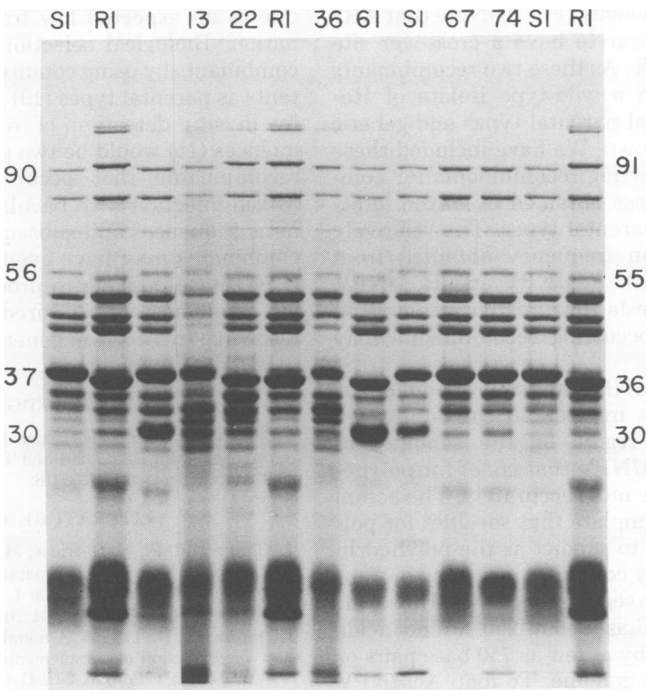

FIG. 6. *RoMNPV (R1), AcMNPV (S1), and AcMNPV-RoMNPV recombinants (AR1, AR13, AR22, AR36, AR61, AR67, and AR74) were purified from virus polyhedra by disruption in dilute alkali and sucrose density centrifugation (alkali-liberated virus). About 30 μg of each virus preparation was electrophoresed in 11% polyacrylamide and stained with Coomassie brilliant blue. AcMNPV polypeptides compared in this study are labeled in kilodaltons on the left, and RoMNPV polypeptides are labeled on the right. The 30,000-molecular-weight polypeptide, which has the same mobility as AcMNPV or RoMNPV polyhedrin, may be present as a contaminant in some or all of the virus preparations. Note the increased staining intensity of this band in AR1 and AR66 as compared to the 30,000-molecular-weight band in AcMNPV (S1).*

recombination sites in viable recombinant genomes (Fig. 5) does not appear to be random. All but one of the Ac*M*NPV and Ro*M*NPV recombinants, AR1, had a crossover between 77 and 89%. Whether we have inadvertantly selected for recombinants with crossovers in this region or this reflects something of the nature of Ac*M*NPV and Ro*M*NPV recombination is not known.

All Ac*M*NPV and Ro*M*NPV recombinant genomes contained only one detectable double crossover. It is possible that some of the recombinants have additional crossovers that occurred between marker restriction sites and have not been detected. Assuming that the rate of recombination is equal in all parts of the genome, it is easy to predict if 7% of the recombinants contained only one double crossover, then (7/100 × 7/100) × 100 or 0.49% of the viral progeny should have two double crossovers. Thus, only a few of the progeny viruses from doubly infected cells would be predicted to have had more than one double crossover. It is interesting that two naturally occurring recombinants, AR7 and AR8, described previously by Smith and Summers (9), apparently have participated in supernumary crossover events (Fig. 5). Note that AR7 and AR8 also appear to have a crossover site between 77 and 89%. As these two recombinants were isolates from a wild-type isolate of Ro*M*NPV, the original parental types and genetic history are not known. We have included these two naturally occurring recombinants for comparison only to those obtained by mixed infection with known parental types. The relatively high recombination frequency obtained from mixed infection with Ac*M*NPV and Ro*M*NPV in this study suggests that further attempts to isolate naturally occurring recombinants may prove successful.

Of the four Ac*M*NPV and four homologous Ro*M*NPV proteins mapped in this study, we have located the region on the genomes of Ac*M*NPV and Ro*M*NPV that codes for polyhedrin (70 to 89%) the most accurately. The actual size of the DNA template that specifies for polyhedrin is difficult to predict as the polyhedrin structural gene may contain intervening or nonpolyhedrin message segments of DNA. However, polyhedrin which has about 250 amino acids could be coded for by as few as 750 base pairs or less than 1% of the genome. To map Ac*M*NPV polyhedrin or other Ac*M*NPV gene products more precisely than presented here, either other approaches will need to be developed to locate the templates specifying viral gene products or the present approach will need to be improved. We feel that two such improvements would be (i) to map Ac*M*NPV and Ro*M*NPV with additional restriction endonucleases so that crossover sites could be more accurately measured and (ii) to search for other baculoviruses that would be capable of recombining with Ac*M*NPV which have few or no restriction sites in common with Ac*M*NPV and which have gene products that can easily be identified. Such viruses would be expected to have only partial DNA sequence homology with Ac*M*NPV. We are presently surveying the DNA homology among baculoviruses to select for those that are less closely related to Ac*M*NPV and Ro*M*NPV.

The aim of this paper is to show that baculovirus recombinants can be obtained from doubly infected cells without the use of biological selection for recombinants, and that certain viral proteins can be mapped on baculovirus genomes by this approach. The method used is applicable to the selection of recombinants where mixed infection would be expected to yield a relatively high frequency of viable recombinant progeny, such as in this study. However, attempts to find viral recombinants from mixed infection with Ac*M*NPV and other baculoviruses by analyzing the DNA restriction patterns of randomly picked plaques would likely be of limited use due to an expected low frequency of recombination. Biological selection of baculovirus recombinants by using conditional lethal viral mutants as parental types (19) or physical selection by in situ detection of recombined viral sequences (15) would be two approaches to detect recombinants that occur at low frequency. Mixed infection with baculoviruses or other genetic sequences and subsequent isolation of recombinants may have even wider applications. It might be possible to produce hybrid baculovirus genomes with desired properties without resorting to in vitro genetic engineering with specific DNA fragments.

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