APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1978, p. 411-421
0099-2240/78/0035-0411$02.00/0

Vol. 35, No. 2
Printed in U.S.A.

Restriction Endonuclease Analysis for the Identification of Baculovirus Pesticides

LOIS K. MILLER* AND KATHERINE P. DAWES

Department of Bacteriology and Biochemistry, University of Idaho, Moscow, Idaho 83843

Received for publication 7 July 1977

Gel electrophoresis of deoxyribonucleic acid (DNA) fragments generated by digesting the DNA genomes of nuclear polyhedrosis viruses (NPV) with restriction endonucleases provides DNA fragment patterns that may be used to identify different viruses of this group. Characteristic fragment patterns were obtained for three NPVs, which are important as biological pesticides (*Autographa californica* NPV, *Orgyia pseudotsugata* NPV, and *Heliothis zea* NPV). The DNA fragment patterns of the *A. californica* NPV genome did not change with passage through the alternate insect host, *Trichoplusia ni*. Heterogeneity in one preparation of *O. pseudotsugata* NPV was observed. The identification procedure is direct and precise. Applications of this procedure include quality control of commercial preparations of viral pesticides and screening for genetic alterations in the viruses.

The U.S. Environmental Protection Agency has registered two insect nuclear polyhedrosis viruses (NPVs) for commercial use as pesticides: the *Heliothis zea* baculovirus for control of the cotton bollworm and the tobacco budworm, and the *Orgyia pseudotsugata* baculovirus for control of the Douglas fir tussock moth. Several other NPVs, including the *Autographa californica* baculovirus which infects alfalfa and cabbage loopers, are candidates for Environmental Protection Agency registration as pesticides. The NPVs cause natural epizootic diseases in pest insect populations and have excellent potential as biological pesticides due to their specificity, environmental stability, rapid lethality in target hosts, and apparent safety for vertebrates, nontarget invertebrates, and plants (6-8).

To ensure the continued safe, responsible use of these insect viruses as pesticides, it will be necessary to monitor the quality of commercial virus preparations and detect genetic alterations in the viral genomes that might affect host range specificity or efficacy of the virus as a pesticide. A simple, yet precise, method of identification of viral pesticides is necessary for this purpose.

Since Summers and Anderson reported that NPVs contain large circular, supercoiled, double-stranded deoxyribonucleic acid (DNA) genomes (22), identification of these viruses by restriction endonuclease analysis seemed potentially possible. Restriction endonucleases (RENs) of the class II type recognize specific nucleotide sequences and cleave phosphodiester bonds at or very near the recognition site (for reviews, see 15 and 17). Specific fragments of DNA are produced by REN digestion of DNA, and these fragments may be separated on the basis of size by electrophoresis through agarose gels of appropriate concentrations (1, 20). The patterns of the fragments in the gels provide characteristic fingerprints of the DNA studied. Analysis of viral genomes by REN analysis has proven to be extremely useful in vertebrate virus and bacteriophage work (13-15, 17), including the recognition of recombinant viruses and minor alterations in the genotypes of closely related viruses (11, 12, 18).

Since RENs are now available commercially and since gel electrophoresis is a common analytical tool, REN analysis is a technique that might be easily and widely applied to NPV identification. Furthermore, the technique permits the analysis of the entire genome of the virus and not merely a few of roughly 100 genes contained on the viral genome. This provides considerable precision and the ability to recognize genotypic alterations in the viral genome that may not be recognizable phenotypically (3).

MATERIALS AND METHODS

Viruses. The *H. zea* baculovirus (heretofore referred to as the *H. zea* NPV) was provided by Carlo Ignoffo (U.S. Department of Agriculture-Agricultural Research Service, Columbia, Mo.) as a commercial pesticide preparation (Viron/H, International Minerals and Chemical Corp., Libertyville, Ill.). The *A. californica* baculovirus (*A. californica* NPV) was isolated from diseased larvae of either *A. californica* or *Trichoplusia ni*, provided by Marion Bell (U.S. Department of Agriculture-Agricultural Research Service, Phoenix, Ariz.). The *O. pseudotsugata* baculovirus (*O. pseudotsugata* NPV) was isolated from diseased *O. pseudotsugata* larvae collected during the

Kamloops, British Columbia, infestation of 1976 by Kurt Volker and R. W. Clausen, University of Idaho, Moscow, Idaho. Another preparation of the *O. pseudotsugata* NPV was isolated from diseased *O. pseudotsugata* NPV, which were infected with a virus preparation obtained from Mauro Martignoni (U.S. Department of Agriculture-Forest Service, Portland, Ore.). These two *O. pseudotsugata* NPV preparations are distinguished in the text as *O. pseudotsugata* NPV-K and *O. pseudotsugata* NPV-M, respectively. All the insect viruses except the *H. zea* NPV are the MEV variety, meaning that more than one nucleocapsid may be contained within a single envelope. The nucleocapsids of *H. zea* NPV are each singly enveloped (SEV).

Lambda bacteriophage was isolated from the temperature-sensitive lysogen cI857S7 *Escherichia coli* provided by Richard Roberts, Cold Spring Harbor, N.Y.

Isolation of occluded NPVs. *A. californica* and *O. pseudotsugata* NPVs were isolated from diseased larvae by homogenizing 0.5 to 1.5 g of larvae in 25 ml of distilled water for 1 min in a microanalytical mill (Tekmar A-10). The homogenate was filtered through glass wool and washed with 50 ml of 0.2% sodium dodecyl sulfate (SDS). The filtrate was centrifuged at 17,000 × *g* for 20 min. Pellets were suspended in 20 ml of distilled water, and 1 ml of 5 M NaCl was added. The suspension was centrifuged again at 17,000 × *g* for 20 min. Pellets were suspended in 10 ml of distilled water, layered onto 40% sucrose (wt/wt), and centrifuged at 17,000 × *g* for 2 h. Pellets were resuspended in distilled water and layered onto 35-ml linear gradients of 40 to 65% sucrose (wt/wt). Gradients were centrifuged at 96,000 × *g* for 3 h, and the band of occluded virus was collected, diluted with distilled water, and centrifuged at 17,000 × *g* for 20 min to pellet the purified occluded NPVs.

H. zea NPVs were isolated by the same method, except that the initial steps involved suspending 50 g of Viron/H pesticide preparation in 150 ml of 0.2% SDS. The inert ingredients settled out, and the brown suspension was filtered through glass wool several times and rinsed with additional 0.2% SDS.

Isolation of NPV DNA. Purified occluded NPVs were disrupted with alkali (2). An equal volume of 0.1 M Na_2CO_3 was added to the NPVs, and the viruses were allowed to stand at room temperature for 1 h and then overnight at 5°C. Generally it was not necessary to purify the virions from occlusion body debris to obtain pure viral DNA, but isolation of *H. zea* NPV DNA required further purification of the nonoccluded virions after alkaline disruption. The solution of alkali-disrupted virus was layered onto 15-ml linear gradients of 25 to 65% sucrose (wt/wt). Gradients were centrifuged at 96,000 × *g* for 2 h, and the band of nonoccluded virus was collected, diluted with distilled water, and centrifuged at 96,000 × *g* for 20 min. The pellet was suspended in 0.01 M tris(hydroxymethyl)-aminomethane (Tris) and 0.001 M ethylenediaminetetraacetic acid (EDTA), pH 7.6.

SDS was added to the alkali-disrupted virus solution (or purified nonoccluded virions as described for *H. zea*) to a final concentration of 0.5%, and the solution was heated to 60°C for 15 min. The DNA was then separated from virion proteins by phenol extraction at room temperature. An equal volume of phenol, buffered with 0.01 M Tris and 0.001 M EDTA, pH 7.6, was added and mixed with the aqueous layer by gently shaking, and the layers were reformed by centrifugation. The aqueous layer was removed and again extracted with an equal volume of buffered phenol in the same fashion. The phenol layers were washed with an equal volume of 0.01 M Tris–0.001 M EDTA, pH 7.6, equilibrated with phenol. The aqueous layers were combined and dialyzed extensively in 1 liter of dialysis buffer (0.01 M Tris–0.001 M EDTA, pH 7.6) for at least 36 h with three changes of the dialysis buffer. It is worthwhile to start the DNA preparation with a high enough concentration of occluded NPVs so that further concentration of the DNA at this point can be avoided. The concentration of NPV DNA at this stage should be greater than 25 μg of DNA per ml. The number of occlusion bodies per milliliter needed to achieve this concentration of DNA per milliliter varies for each NPV, depending on the number of nucleocapsids per envelope and the number of enveloped virions per occlusion body. For *A. cal* NPV, *O. pseudotsugata* NPV, and *H. zea* NPV, the number of occlusion bodies per milliliter should be approximately 6×10^9, 1×10^9, and 2×10^{11} occlusion bodies per ml, respectively.

Phenol extraction effectively removes the vast majority of viral proteins, as judged by the ratio of optical density at 260 and 280 nm. The procedure preserves the structure of the DNA, as judged by equilibrium density centrifugation in CsCl in the presence of ethidium bromide (16). Approximately 25% of the DNA is recovered in supercoiled form if fresh or frozen viruses are used as starting material. The intact nature of the viral DNA was assessed in 0.2% agarose gels. A DNA band of high molecular weight ($70 \pm 15 \times 10^6$) was observed.

RENs. All the restriction enzymes used in this study are available from commercial sources. However, due to our extensive use of these enzymes, they were prepared in our laboratory from their bacterial sources. The bacterial strains and isolation procedures were obtained from Richard Roberts, Cold Spring Harbor, N.Y. The enzymes were tested for their purity by digestion of lambda bacteriophage DNA and subsequent electrophoresis of the DNA fragments as described below. Abbreviations of the enzymes follow recommended conventions and are: *Eco*RI, isolated from *E. coli* RY13; *Sal* I, isolated from *Streptomyces albus* G; *Hin*dIII, isolated from *Haemophilus influenza* R_d; *Bam*HI, isolated from *Bacillus amyloliquefaciens* H; *Hae* III, isolated from *Haemophilus aegyptius*; *Hha* I, isolated from *Haemophilus haemolyticus*; *Pst* I, isolated from *Providencia stuartii* 164; *Hae* III, isolated from *H. aegyptius*.

Digestion of NPV DNA with RENs. The NPV DNAs were digested with *Eco*RI in a solution of 0.01 M Tris (pH 7.6), 0.1 M $MgCl_2$, 0.1 M NaCl, and 1 mM dithiothreitol. Digestions of NPV DNA with the remaining RENs were carried out in 0.01 M Tris (pH 7.6), 0.1 M $MgCl_2$, 0.05 M $NaCl_2$, and 1 mM dithiothreitol. Incubations were generally for 3 h at 37°C. Sufficient enzyme was added for complete digestion within this time period, as judged by the digestion of lambda bacteriophage DNA.

Gel electrophoresis of DNA fragments. The DNA fragments produced by REN digestion of viral DNAs were fractionated by electrophoresis through 0.7% agarose (Sea Kem) gels. Vertical slab agarose gels were poured between two glass plates (20 by 20 cm) separated by strips of Plexiglas at the sides and held together by clamps. The bottom of the gel was plugged with an acrylamide gel. Slots were formed by inserting a Plexiglas comb at the top before agarose gel formation was complete. Similar apparatuses for slab gel electrophoresis are available commercially. The buffer used for electrophoresis (B buffer) was composed of 10.8 g of Tris base, 0.93 g of EDTA, sodium salt, and 5.5 g of boric acid in 1 liter of water, pH 8.3. The DNA fragments were loaded into the slots in B buffer plus 5% sucrose and tracer amounts of bromophenol blue. One microgram of DNA was loaded in a 50-μl, or less, volume. A constant current of approximately 10 mA was applied across the gel for approximately 16 h, until the bromophenol blue marker reached the acrylamide plug at the bottom of the gel.

Visualization of DNA fragments. The DNA fragments were stained by soaking the gel for 1 h in B buffer with 0.5 μg of ethidium bromide per ml (20). The ethidium bromide-stained fragments were visualized under ultraviolet light (UV), and photographs were taken under short-wave UV light using Tri-X film and a Polaroid MP-4 camera with an orange filter on the lens.

RESULTS

Characteristic REN fragment patterns of NPV DNA. Digestion of *H. zea* NPV DNA, *O. pseudotsugata* NPV DNA, and *A. californica* NPV DNA with a REN isolated from *S. albus* G (*Sal* I) results in specific fragmentation of the viral genomes as determined by fractionation of the fragments through 0.7% agarose slab gels (Fig. 1). The fragment patterns observed are extremely reproducible and are characteristic of the viral DNA studied. The coelectrophoresis of *Sal* I-digested lambda bacteriophage DNA provides standards for estimating the molecular weights of the fragments produced from NPV digestion and assurance that the *Sal* I enzyme is functioning properly (see below).

Digestion of these same viral DNAs with a REN isolated from *H. influenzae* D (*Hin*dIII) also results in specific fragmentation of the viral genomes as judged by gel electrophoresis of the DNA fragments (Fig. 2). The *Hin*dIII fragment patterns of the viral DNAs differ from the *Sal*

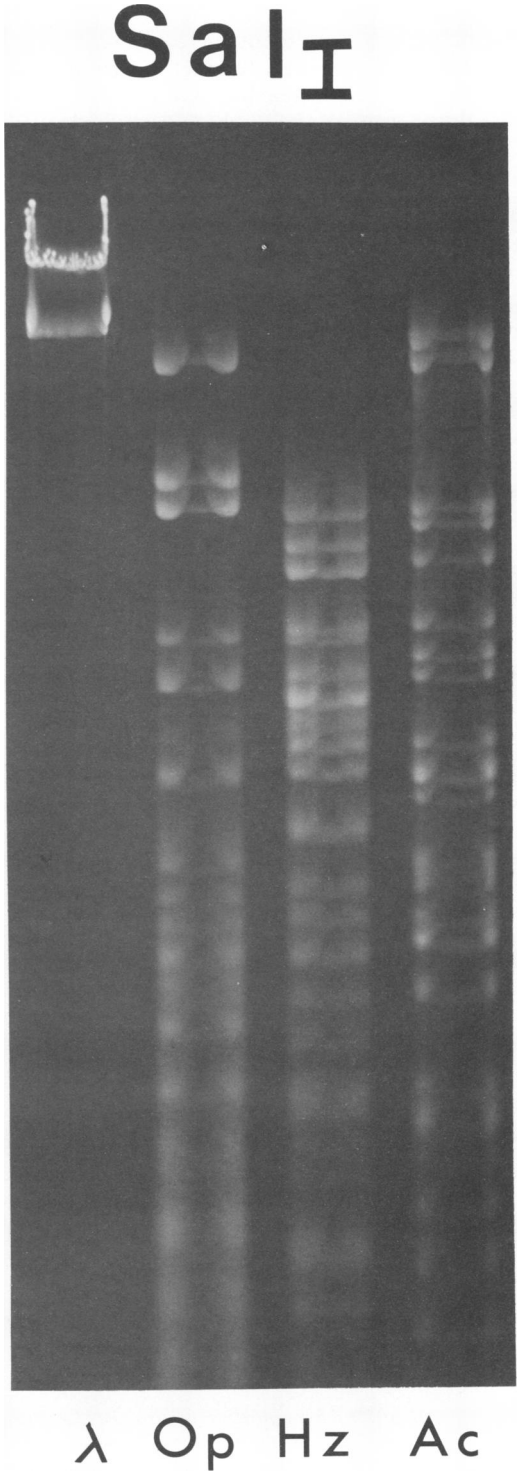


FIG. 1. *Sal I fragmentation of viral DNAs. Purified viral DNA isolated from O. pseudotsugata NPV-K (Op), H. zea NPV (Hz), A. californica NPV (Ac), and lambda bacteriophage (λ) were digested with the Sal I REN (see text). The resulting DNA fragments were fractionated on the basis of size by electrophoresis through 0.7% agarose in B buffer. After electrophoresis, the DNA fragments were stained with ethidium bromide by immersing the gels for 1 h in B buffer containing 0.5 μg of ethidium bromide per ml. The fragment patterns were photographed under UV illumination as described in Materials and Methods.*

I fragment patterns as expected, since *Sal* I and *Hin*dIII recognize different nucleotide sequences in DNAs. The *Hin*dIII fragment patterns are also extremely reproducible and are characteristic fingerprints of the viral DNA studied.

Several different RENs have been tested in our laboratory for their ability to produce fragment patterns that may be used for viral identification. All the RENs tested thus far (*Sal* I, *Eco*RI, *Hin*dIII, *Bam*HI, *Pst* I, *Hha* I, and *Hae* III) produce specific fragments. The most useful RENs for identification purposes are those enzymes recognizing hexanucleotide sequences in the DNA (*Sal* I, *Eco*RI, *Hin*dIII, *Bam*HI, and *Pst* I) rather than tetranucleotide sequences (*Hha* I and *Hae* III). The tetranucleotide-recognizing enzymes produce more fragments than can be easily fractionated and, therefore, give less distinctive patterns for each NPV DNA (data not shown).

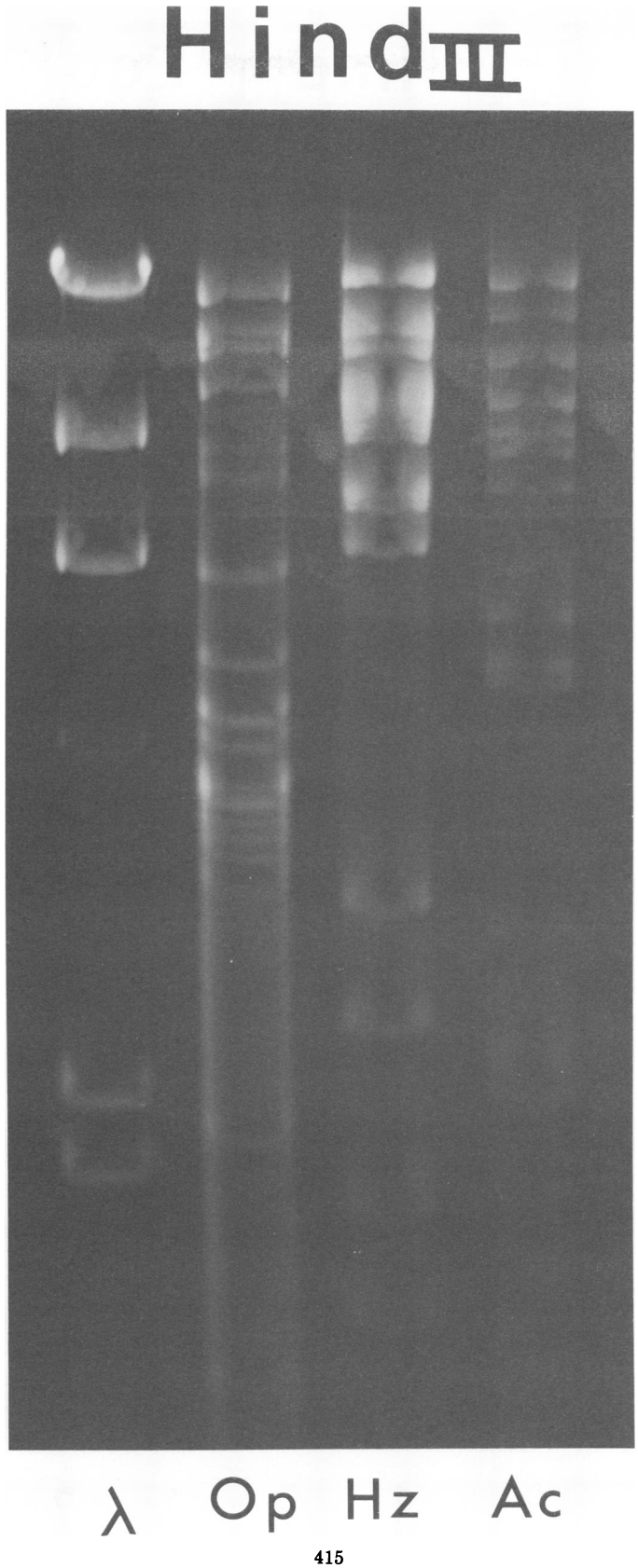
Lack of host effects on REN fragment patterns. Since the *A. californica* NPV replicates in both *A. californica* and *T. ni* (24), it was possible to use this virus to determine if an alternate insect host affects the fragment patterns of the *A. californica* NPV DNA. *A. californica* NPV was isolated from both *A. californica* and *T. ni* larvae, and the DNAs from both virus preparations were digested with *Sal* I, *Hin*dIII, and *Eco*RI (Fig. 3). The *A. californica* NPV DNA fragment patterns were unchanged by passage through the alternate host, *T. ni*. The fuzziness of the fragment pattern observed for NPV DNA from *T. ni* is a result of the increased volume of the more dilute *T. ni*-derived NPV DNA sample loaded on the gels so that the intensity of the staining of the two DNA preparations was equivalent.

Homogeneity of NPV preparations and size of NPV DNA genomes. The relative homogeneity of the NPV preparations studied is reflected in the number and size of the fragments produced by RENs. Gross heterogeneity of the virus preparations would result in the production of a vast number of fragments with a cumulative molecular weight much larger than the molecular weight estimates of the viral DNA derived by independent methods. Although there is some argument in the literature concerning the exact size of NPV DNA genomes, most size estimates indicate a genome size between 50×10^6 and 85×10^6 daltons. Exact values may depend on the viral genome studied. The overall molecular weights of the viral DNAs may be calculated from REN patterns by summation of the molecular weights of the DNA fragments. The molecular weights of the fragments are determined by comparing their mobilities in agarose gels with standard DNAs of known molecular weight such as λ*Sal* I and λ*Hin*dIII. A sufficient range of standard fragment sizes is necessary for proper analysis throughout the gel.

In Table 1, rough estimates of the sizes of the major *Sal* I fragments for *O. pseudotsugata* NPV DNA, *H. zea* NPV DNA, and *A. californica* NPV DNA (Fig. 1) are listed. More exact size estimates require variations in the agarose gel concentration and detailed analyses of different REN patterns (L. K. Miller and K. P. Dawes, manuscript in preparation). Summation of the molecular weights of the fragments, taking into account the variation in intensity of the fragment bands indicating comigration of more than one fragment, yields rough molecular weight estimates of the viral DNA genomes—$64 \pm 8 \times 10^6$, $62 \pm 7 \times 10^6$, and $72 \pm 8 \times 10^6$ daltons for the *O. pseudotsugata*, *H. zea*, and *A. californica* NPV DNA genomes, respectively. Ambiguity in these values largely reflects the need for variation of agarose gel density and the number of small fragments too faint and diffuse for complete analysis. The molecular weight estimates correspond well with estimates from independent methods of analysis; the general homogeneity of the NPV preparations studied is indicated. Bands of very weak intensity suggest the presence of low levels of heterogeneity or contamination as indicated below.

Detection of heterogeneity in NPV preparations. The fragment patterns for the *O. pseudotsugata* NPV in Fig. 1 and 2 were obtained from DNA isolated from NPV-diseased *O. pseudotsugata* larvae collected during an infestation of *O. pseudotsugata* in Kamloops, British Columbia. We obtained another sample of *O. pseudotsugata* NPV from Mauro Martignoni and passed this virus in our laboratory through *O. pseudotsugata* larvae. The fragment patterns of the two viral DNA preparations were compared (Fig. 4). The same basic fragment patterns were obtained with both viruses, but the *O. pseudotsugata* NPV derived from the Martignoni stock (NPV-M) revealed additional

FIG. 2. *HindIII fragmentation of viral DNAs. Purified viral DNA isolated from O. pseudotsugata NPV-K (Op), H. zea NPV (Hz), A. californica NPV (Ac), and lambda bacteriophage (λ) were digested with the HindIII REN (see text). The resulting DNA fragments were fractionated on the basis of size by electrophoresis through a 0.7% agarose in B buffer. After electrophoresis, the DNA fragments were stained with ethidium bromide by immersing the gels for 1 h in B buffer containing 0.5 μg of ethidium bromide per ml. The fragment patterns were photographed under UV illumination as described in Materials and Methods.*


Hind III
λ
Op
Hz
Ac

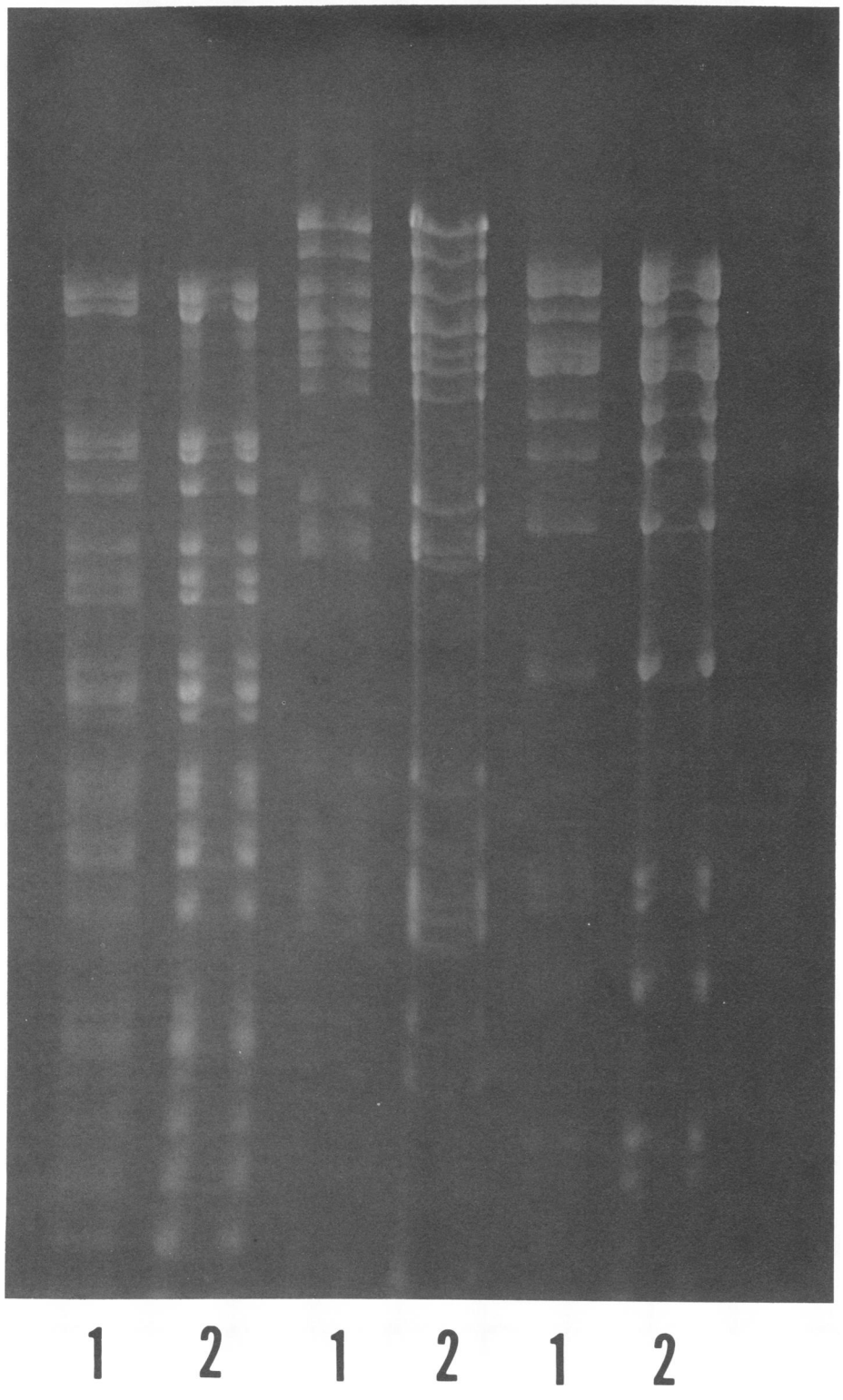

Sal I
Hind III
Eco R I
1 2 1 2 1 2

TABLE 1. *Molecular weight estimates of Sal I fragments of NPV DNA and total viral DNA size*[a]

Sal I fragment sizes × 10^{-6} daltons		
O. pseudotsugata NPV DNA	*H. zea* NPV DNA	*A. californica* NPV DNA
7.5–8.4	3.9–4.3	8.5–10
4.8–5.0	3.7–3.9	7.5–8.5
4.5–4.8	3.5–3.7×	4.4–5.1
3.3	3.1–3.2	4.1–4.4
3.0×	2.9	3.7–4.0
2.7	2.8×	3.3–3.4
2.5×	2.7	3.1–3.2
2.2	2.6	3.0–3.1
2.1	2.5×	2.6
2.05	2.4×	2.4×
1.9	2.2	2.3
1.8	2.15	2.05
1.65	1.9	2.0
1.60	1.8	1.9
1.56	1.7×	1.8
1.52	1.5	1.7×
1.4×	1.4	1.5
1.2×	1.3×	1.3
1.1×	<1 ~9 fragments	1.2×
<1 ~12 fragments		1.0
	~62 ± 7 total	<1 ~8 fragments
~64 ± 8 total		
		~72 ± 8 total

[a] Estimations of the molecular weights of each *Sal* I fragment were made by comparing the mobility of the fragments in 0.7% gels (see Fig. 1) relative to the mobility of λDNA fragments of known size including λ*Sal* I fragments and λ*Hin*dIII fragments (see Fig. 2). Mobility is generally linearly related to the log of the molecular weight of the fragment for fragments 10×10^6 or less in these gels. An "×" next to the fragment size indicates the fragment has an increased intensity of staining, suggesting that two or three fragments are of such similar size that they comigrate in the gel. Summation of the fragment sizes, taking into account comigration of fragments, provides an estimate of the total molecular weight of each viral genome.

bands of much weaker intensity than the bands corresponding with the NPV derived from the Kamloops infestation (NPV-K). The weaker intensity of these bands indicates a lower concentration of these fragments in the DNA preparation. The possibility that the weaker-intensity bands were a result of incomplete digestion of the *O. pseudotsugata* NPV-M DNA was investigated. Excess enzyme and longer incubation times had no effect on the NPV-M patterns; the persistent presence of weaker-intensity bands in the NPV-M preparation only, suggested heterogeneity of the DNA in this preparation.

DISCUSSION

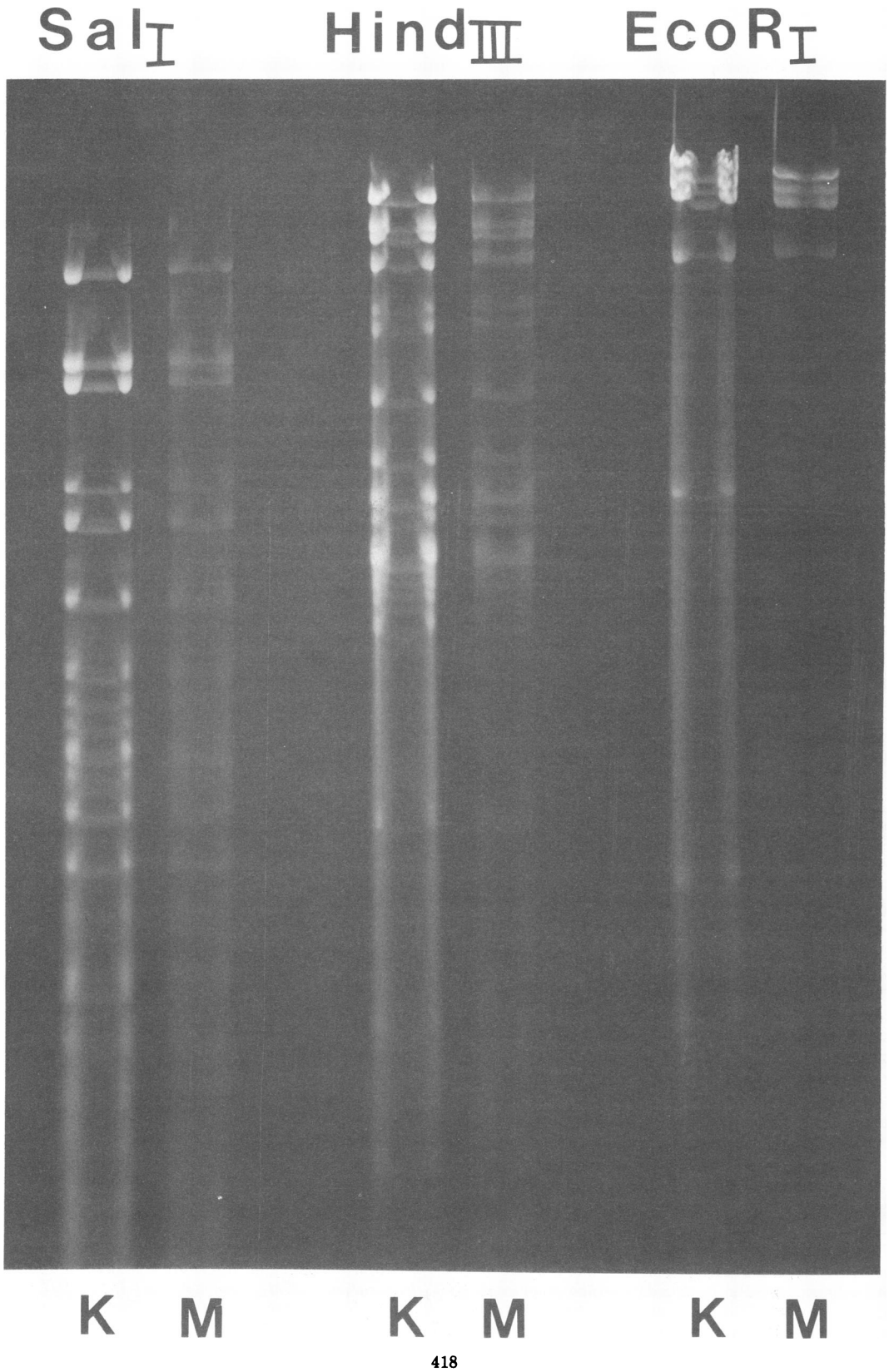
The digestion of NPV DNA with RENs results in the production of specific DNA fragments. Fractionation of the fragments on the basis of size by electrophoresis through agarose gels results in DNA fragment patterns characteristic of the NPV DNA studied and may be used as fingerprints for identifying these viral pesticides.

The reproducibility and ease of obtaining REN fragment patterns of NPV DNAs suggest a broad applicability of this identification technique to a variety of pesticide-related problems, including quality control of commercial virus preparations. That a commercial virus preparation, field-collected virus, and laboratory-produced virus were all amenable to identification by this technique adds additional confidence in the broad applicability of the technique.

The possibility that the host insect may affect the fragment patterns of the viral DNAs was considered. From theoretical evaluations of the data, it is unlikely that the Lepidopteran hosts of the NPVs studied modify the viral DNA in a manner that significantly affects REN recognition and cleavage of the NPV DNA genomes. The size of NPV DNA genomes is between 55 $\times 10^6$ and 80 $\times 10^6$ daltons, as judged by analysis of DNA fragment sizes (see Table 1 and Miller and Dawes, manuscript in preparation), by DNA reassociation kinetic analyses (9), by sedimentation velocity analysis (22), and by electron microscopy (19). The probability of finding a specific hexanucleotide sequence in a DNA of this size (roughly 9×10^4 nucleotide base pairs), assuming random distribution of the four nucleotides, is $9 \times 10^4 \times (1/4)^6$. Thus, on the assumption of random base distribution, one would expect a REN, which recognizes a specific hexanucleotide sequence, to generate roughly 20 DNA fragments upon digestion of an NPV DNA. This is generally observed with *Sal* I, *Hin*dIII, and *Eco*RI digestion of the NPV DNAs tested. Obviously some variation is expected owing to the nonrandom distribution of nucleotides in DNA under evolutionary pressure. But clearly there is no evidence, from the frequency of REN cleavage of NPV DNA, for extensive modification systems operating in the NPV-infected Lepidopteran hosts.

However, for empirical data concerning host insect effects on NPV DNA fragment patterns, we utilized the fact that the *A. californica* NPV infects both *A. californica* and *T. ni*. The *Sal* I, *Hin*dIII, and *Eco*RI fragment patterns of *A. californica* NPV were exactly the same whether

FIG. 3. *REN fragment patterns of A. californica NPV passaged through different host insects. A. californica NPV was propagated by passage through larvae of either A. californica (2) or the alternate host, T. ni (1). The A. californica NPV DNA was purified from each NPV preparation and digested with Sal I, HindIII, or EcoRI RENs. The REN fragment patterns were compared by co-electrophoresis through a 0.7% agarose slab gel followed by ethidium bromide staining as described in the text.*


Sal I
Hind III
EcoR I
K M
K M
K M

the virus was passaged through *A. californica* or through *T. ni* hosts. To extrapolate the finding to more diverse alternate host systems would be risky, but a safeguard is to obtain fragment patterns using several different RENs. The possibility that an alternate host would modify a wide spectrum of hexanucleotide sequences is minimal.

It is also advisable to obtain identifying fragment patterns by using several different RENs to increase the precision of the identification technique. This is intuitively obvious by considering that the largest fragment produced by *Sal* I digestion of *A. californica* NPV, for instance, is approximately 9×10^6 daltons (Table 1). This size is equivalent to approximately 15 genes. The smaller *Sal* I fragments of *A. californica* NPV (less than 1×10^6 daltons) are roughly equivalent to one gene. By using several different RENs, different segments of the genome are analyzed in varying detail. To detect minor alterations in the genetic makeup of the NPVs, this increase in precision will be necessary.

The use of RENs having hexanucleotide sequences is clearly sufficient for the basic identification of NPVs. The number of fragments produced by these enzymes is small enough for readily distinguishing differences in fragment patterns. RENs with tetranucleotide sequences produce too many fragments for easy comparisons, but these enzymes will eventually be useful in the fine structure analysis of the NPV DNA genomes.

Rough estimations of the molecular weights of the *O. pseudotsugata* NPV, *H. zea* NPV, and *A. californica* NPV DNA genomes have been calculated by comparing the mobility of REN-produced NPV DNA fragments with DNA fragments of known molecular weight (Table 1). The main purpose of providing these estimates is to indicate that the NPV preparations are relatively homogeneous. Had the NPV preparations been grossly heterogeneous, the fragment patterns would be so complex (exhibit a huge number of fragments) that REN analysis would fail as an effective identification technique (although it would clearly demonstrate heterogeneity). That REN digestion yields useful fingerprints of uncloned viral DNAs which reflect a molecular weight of a single NPV DNA genome adds considerable strength to the applicability of the technique for studying field-collected, uncloned virus preparations. It is important to note that we are not suggesting that the virus preparations we have studied are completely homogeneous. It is also important to note that the molecular weight estimates calculated in Table 1 are approximate.

The ability to detect heterogeneity in a NPV preparation by using REN analysis has been demonstrated with one preparation of the *O. pseudotsugata* NPV. The basis for this heterogeneity is unknown presently. There are three likely possibilities: (i) there is a contaminating virus species in the *O. pseudotsugata* NPV-M preparation; (ii) there is a low level of a genetic variant of the *O. pseudotsugata* NPV present in the virus preparation; (iii) there are defective virus particles present in the *O. pseudotsugata* NPV-M preparation. Defective virus particles of several mammalian viruses are frequently generated by high-multiplicity passage of virus in its host (5, 23, 26). It is actually rather surprising that the laboratory-prepared virus showed heterogeneity rather than the field-isolated virus.

A comparison of the REN identification technique with immunochemical techniques reveals different advantages for the two approaches. Although the development of immunochemical techniques for NPV identification has been slow, owing to the presence of an occlusion body surrounding the virions, the techniques are being improved continuously (for a review, see 4). With careful purification of virion proteins and antisera production, the immunochemical techniques provide a basis for identification of NPVs. However, identification by protein serology is based on only a few, easily isolated, virion proteins. The size of the NPV genomes indicates the presence of roughly 100 different viral genes. REN analysis screens the entire NPV genome and does not rely on the expression of the genes or the easy purification of the viral gene products. To extend protein serological techniques to identifying viruses passaged through alternate hosts, it is necessary to determine which virion antigens are host specified and which are virus specified. In the one system we have studied, there is no detectable effect of the host on NPV DNA fragment patterns. With regard to sensi-

FIG. 4. *Detection of heterogeneity in one preparation of O. pseudotsugata NPV. DNA purified from O. pseudotsugata NPV isolated from diseased O. pseudotsugata larvae collected during the Kamloops infestation in British Columbia (NPV-K) was compared with DNA purified from another stock of O. pseudotsugata NPV passaged through laboratory-reared O. pseudotsugata larvae (NPV-M) by REN analysis, using Sal I, HindIII, and EcoRI enzymes. After digestion of the DNA with the different RENs, the fragments were electrophoresed through 0.7% agarose gels, and fragment patterns were visualized by ethidium bromide staining and UV illumination. Higher concentrations of the NPV-K DNA were used than NPV-M DNA in an attempt to determine if the heterogeneity observed in the NPV-M preparation could be detected in the NPV-K preparation.*

tivity in detecting minor quantities of specified impurities or the NPVs themselves, protein immunological techniques will excell the REN identification technique, since REN analysis requires 1 μg of viral DNA per fragment pattern. However, REN analysis will detect major impurities without a preconceived notion of what type of impurity is involved. REN analysis will also reveal genetic variations in the NPV genomes that may be undetectable by serological techniques. For the comprehensive monitoring of quality of commercial pesticide preparations, both REN analysis and protein serology will be necessary. For the mere identification of NPVs, in the same or alternate host, REN analysis is the more precise of the two techniques.

The precision of REN analysis has been documented in mammalian virus systems. Among the many applications of REN to viral genetic analysis are: (i) the identification of recombinant viruses (12, 18); (ii) the detection of evolutionary changes during serial passage of viruses (5, 10, 25); (iii) the detection of genotypic mutations that have no known phenotypic effect (11); and (iv) the identification of closely related virus strains (13, 21). The application of REN analysis to viral pesticides is expected to facilitate identification of different viruses and to detect genetic alterations that may arise through continued use as pesticide agents.

ACKNOWLEDGMENTS

We thank M. R. Bell, USDA-ARS, Phoenix, Ariz., for supplying *A. californica* NPV-infected insect larvae, H. W. Smith, Department of Entomology, University of Idaho, for supplying the *O. pseudotsugata* NPV-K-infected larvae, C. Ignoffo, USDA-ARS, Columbia, Mo., for supplying Viron/H, and M. Martignoni for a sample of *O. pseudotsugata* NPV from which we prepared the *O. pseudotsugata* NPV-M stock. We also thank Richard Roberts, Cold Spring Harbor Laboratory, N.Y., for bacterial strains and procedures for REN purification.

This research was supported by Public Health Service grant AI-01567 from the National Institute of Environmental Health Sciences. The paper was published with the approval of the Idaho Agricultural Experiment Station as Research Paper no. 7751.

ADDENDUM IN PROOF

The *O. pseudotsugata* NPV-M preparation, provided by M. Martignoni, was an industrial product (NPI-72-L/1-B/2). Dr. Martignoni has informed us that the additional fragments we observe for this virus preparation seem to coincide with fragments from the *O. pseudotsugata* NPV-SEV DNA and are thus indicative of weak contamination of this virus in the NPV-MEV preparation. This supports our conclusion that REN analysis will be useful in quality control of commercial virus preparations.

LITERATURE CITED

1. **Aaij, C., and P. Borst.** 1972. The gel electrophoresis of DNA. Biochim. Biophys. Acta **269**:192-200.
2. **Bergold, G. H.** 1947. Die Isolierung des Polyedervirus und die Natur der Polyeder. Z. Naturforsch. Teil B **2**:122-143.
3. **Brockman, W. W., T. N. H. Lee, and D. Nathans.** 1973. The evolution of new species of viral DNA during serial passage of simian virus 40 at high multiplicity. Virology **54**:384-397.
4. **Di Capua, R. A., and P. W. Norton.** 1976. Immunochemical characterization of the baculoviruses present status, p. 317-330. *In* E. Kurstak and K. Maramorosch (ed.), Invertebrate tissue culture: applications in medicine, biology, and agriculture. Academic Press, New York.
5. **Frenkel, N., R. J. Jacob, R. W. Honess, G. S. Hayward, H. Lockes, and B. Roizman.** 1975. Anatomy of herpes simplex virus DNA. III. Characterization of defective DNA molecules and biological properties of virus particles containing them. J. Virol. **16**:153-167.
6. **Ignoffo, C. M.** 1968. Viruses—living insecticides, p. 129-167. *In* K. Maramorosch (ed.), Current topics in microbiology and immunology. Springer-Verlag, New York.
7. **Ignoffo, C. M.** 1973. Development of a viral insecticide: concept to commercialization. Exp. Parasitol. **33**:380-406.
8. **Ignoffo, C. M.** 1973. Effects of entomopathogens on vertebrates. Ann. N.Y. Acad. Sci. **217**:141-164.
9. **Kelly, D. C.** 1977. The DNA contained by nuclear polyhedrosis viruses isolated from four *Spodoptera* sp. (Lepidoptera; Noctuidae): genome size and homology assessed by DNA reassociation kinetics. Virology **76**:468-471.
10. **Khoury, G., G. C. Fareed, K. Berry, and M. A. Martin.** 1974. Characterization of a rearrangement in viral DNA: mapping of the circular simian virus 40-like DNA containing a triplication of a specific one-third of the viral genome. J. Mol. Biol. **87**:289-301.
11. **Miller, L. K., B. E. Cooke, and M. Fried.** 1976. Fate of mismatched base-pair regions in polyoma heteroduplex DNA during infection of mouse cells. Proc. Natl. Acad. Sci. U.S.A. **73**:3073-3077.
12. **Miller, L. K., and M. Fried.** 1976. Construction of infectious polyoma hybrid genomes *in vitro*. Nature (London) **259**:598-601.
13. **Miller, L. K., and M. Fried.** 1976. Construction of the genetic map of the polyoma genome. J. Virol. **18**:824-832.
14. **Miller, L. K., and R. L. Sinsheimer.** 1974. Nature of ØX174 linear DNA from a DNA ligase-defective host. J. Virol. **14**:1503-1514.
15. **Nathans, D., and H. O. Smith.** 1975. Restriction endonucleases in the analysis and restructuring of DNA molecules. Annu. Rev. Biochem. **44**:273-293.
16. **Radloff, R., W. Bauer, and J. Vinograd.** 1967. A dye-buoyant density method for the detection and isolation of closed circular DNA in HeLa cells. Proc. Natl. Acad. Sci. U.S.A. **57**:1514-1521.
17. **Roberts, R. J.** 1976. Restriction endonucleases. Crit. Rev. Biochem. **3**:123.
18. **Sambrook, J., J. Williams, P. A. Sharp, and T. Grodzicker.** 1975. Physical mapping of temperature-sensitive mutations of adenoviruses. J. Mol. Biol. **97**:369-390.
19. **Scharnhorst, D. W., K. L. Saving, S. B. Vuturo, P. H. Cooke, and R. F. Weaver.** 1977. Structural studies on the polyhedral inclusion bodies, virions, and DNA of the nuclear polyhedrosis virus of the cotton bollworm, *Heliothis zea*. J. Virol. **21**:292-300.
20. **Sharp, P. A., B. Sugden, and J. Sambrook.** 1973. Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using analytical agarose-

ethidium bromide electrophoresis. Biochemistry **12**:3055–3063.

21. **Skare, J., W. P. Summers, and W. C. Summers.** 1974. Structure and function of herpesvirus genomes. I. Comparison of five HSV-1 and two HSV-2 strains by cleavage of their DNA with *Eco* R I restriction endonuclease. J. Virol. **15**:726–732.
22. **Summers, M. D., and D. L. Anderson.** 1973. Characterization of nuclear polyhedrosis virus DNAs. J. Virol. **12**:1336–1346.
23. **Thorne, H. V.** 1968. Detection of size heterogeneity in the supercoiled fraction of polyoma virus DNA. J. Mol. Biol. **35**:215–226.
24. **Vail, P. V., D. L. Jay, and D. K. Hunter.** 1973. Infectivity of a nuclear polyhedrosis virus from the alfalfa looper, *Autographa californica*, after passage through alternate hosts. J. Invert. Pathol. **21**:16–20.
25. **Wagner, M., J. Skare, and W. C. Summers.** 1974. Analysis of DNA of defective herpes simplex type I by restriction endonuclease cleavage and nucleic acid hybridization. Cold Spring Harbor Symp. Quant. Biol. **39**:683–686.
26. **Yoshiike, K.** 1968. Studies on DNA from low density particles of SV40. I. Heterogeneous defective virions produced by successive undiluted passages. Virology **34**:391–401.