# CONTRIBUTION OF MOLECULAR BIOLOGY TO THE IMPROVEMENT OF INSECT VIRUSES AS BIOLOGICAL CONTROL PRODUCTS

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ABSTRACT - Molecular biology has an important role in understanding biological properties of insect viruses used in biological control of insect pests. Molecular biology can also be used to improve or sustain the effectiveness of insect biocontrol products. There have been descriptions of 300 to 500 baculoviruses from various families of insects but mostly from Lepidoptera. Very few of these have been used as biocontrol products. The use of DNA restriction enzymes to characterize the genomes of baculoviruses has allowed for a precise method for identifying these different viruses. Once viruses have been identified, any contaminating virus can be detected in the preparations. These viruses may be improved by expanding the host range and virulence of baculoviruses by genetic recombination. The recombination events can be constructed and detected by molecular methodologies. Recombinant DNA techniques can also be used to modify baculoviruses in a way such that they produce bacterial or insect toxins. Such baculoviruses could kill target insects more quickly. Molecular biology methodologies are essential for quality analysis and detection of problems associated with production of insect viruses in which baculoviruses are generated with insertions of insect DNA. These transposons of DNA cause the mutant viruses to produce few polyhedra. Secondly, defective viral genomes are produced when baculoviruses are grown in continuous culture systems. Molecular biology is used to detect these and hopefully, can be used to correct these defects.

Index terms: baculoviruses, Lepidoptera, insect pests.

## CONTRIBUIÇÃO DA BIOLOGIA MOLECULAR PARA O MELHORAMENTO DE VÍRUS DE INSETOS COMO PRODUTOS DE CONTROLE BIOLÓGICO

RESUMO - A biologia molecular desempenha um importante papel na compreensão das propriedades biológicas dos vírus de insetos utilizados no controle biológico de insetos-praga. A biologia molecular pode, também, ser utilizada para melhorar ou manter a eficácia dos produtos utilizados no controle biológico de insetos. Existem descrições de 300 a 500 baculovírus de várias famílias de insetos mas a maioria pertencem a Lepidoptera. Poucos destes vírus têm sido utilizados como produtos de controle biológico. O uso de enzimas de restrição para caracterizar o genoma dos baculovírus tem permitido desenvolver um método preciso para a identificação destes diferentes vírus. Uma vez identificados, qualquer vírus contaminante pode ser detectado nas preparações. Estes vírus podem ser melhorados expandindo tanto sua gama de hospedeiros como sua virulência através de recombinação genética. Os eventos de recombinação podem ser gerados e detectados por metodologias moleculares. Técnicas de DNA recombinante podem também ser utilizadas para modificar os baculovírus de maneira que estes passem a produzir toxinas bacterianas ou outras toxinas que afetam insetos. Estes baculovírus poderiam matar seus insetos-alvo de maneira mais rápida. Métodos de biologia molecular são essenciais para a análise de qualidade e identificação de problemas associados com a produção dos vírus de insetos nos quais baculovírus são gerados com inserções de DNA do inseto. Estes transposons de DNA levam o vírus mutante a produzir poucos poliedros. Além disso, genomas virais defeituosos são gerados quando os baculovírus são produzidos em sistemas contínuos de cultura. Biologia Molecular é utilizada para detectar estes defeitos e possivelmente seja usada para corrigi-los.

Termos para indexação: baculovírus, Lepidoptera, insetos-praga.

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## INTRODUCTION

Baculoviruses are divided into 3 groups based upon their morphology: the occluded nuclear polyhedrosis viruses, the occluded granulosis viruses and the non-occluded baculoviruses. With the use of an electron microscope, this is about as far as one can go to identify baculoviruses of the hundreds isolated from lepidopterous pests (Fig. 1). It is even more difficult to determine whether a virus isolate is a mixture or a pure strain. Mixtures of viruses may replicate together in different hosts which would complicate bioassays used to determine host range and virulence.

The development of insect cell lines in culture in which baculoviruses replicate permits the preparation of homogeneous and pure isolates of baculoviruses. At the same time, insect cell lines have advanced the use of molecular biology for baculoviruses. Hemolymph from larvae infected with wild-type virus can be diluted in culture medium and applied to monolayers of insect cells. Centers of infected cells or plaques that arise from a single virus infection event can be picked and grown in cells (Fig. 2). Viral DNA can be purified from individual plaques grown in cells, digested with restriction enzymes and electrophoresed in agarose gels (Maruniak et al. 1984). DNA differences in plaque isolates from wild type virus are detected at the genotypic level. These changes in migration of DNA fragments are caused by deletions or insertions of DNA or by point mutations in restriction enzyme recognition sites. The changes in DNA sizes and sites occurs in distinct regions on the genome.

Changes can be positioned on the viral genome by constructing a physical map of the viral DNA for a number of restriction enzymes. Different insect viruses have DNA physical maps that are easily distinguishable from each other by location of restriction sites (Brown et al. 1985). Differences in DNA migration using several enzymes shows very closely where plaque isolates are modified

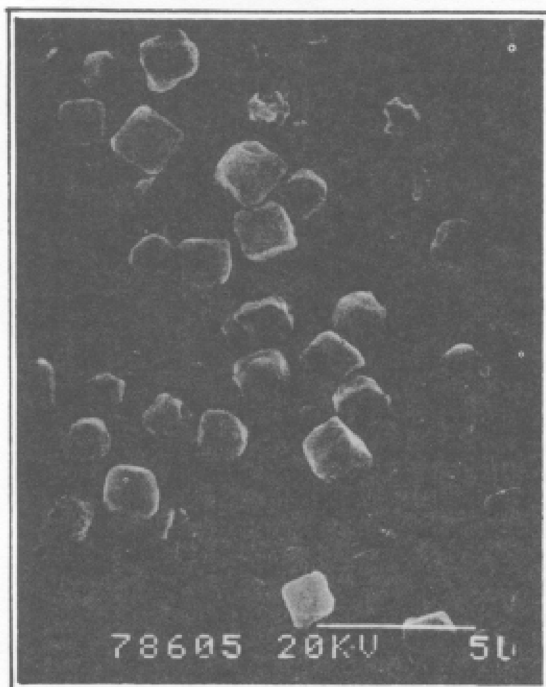


**FIG. 1. Scanning electron micrograph of *Anticarsia gemmatalis* polyhedra on a nitrocellulose filter. The bar indicates 5 micron. (Courtesy of Dr. Jackie Pendland).**

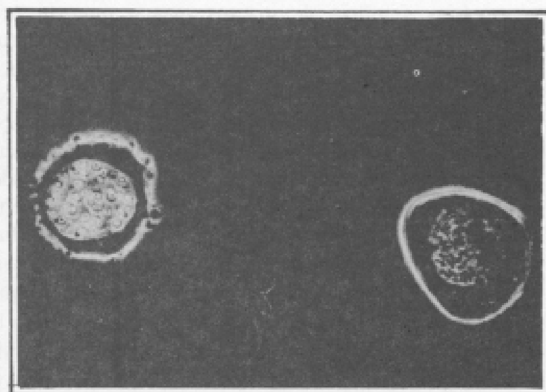

**FIG. 2. Light microscope view of TN368 cells infected with *Autographa californica* nuclear polyhedrosis virus. Many polyhedra can be seen in the nucleus at 200x magnification.**

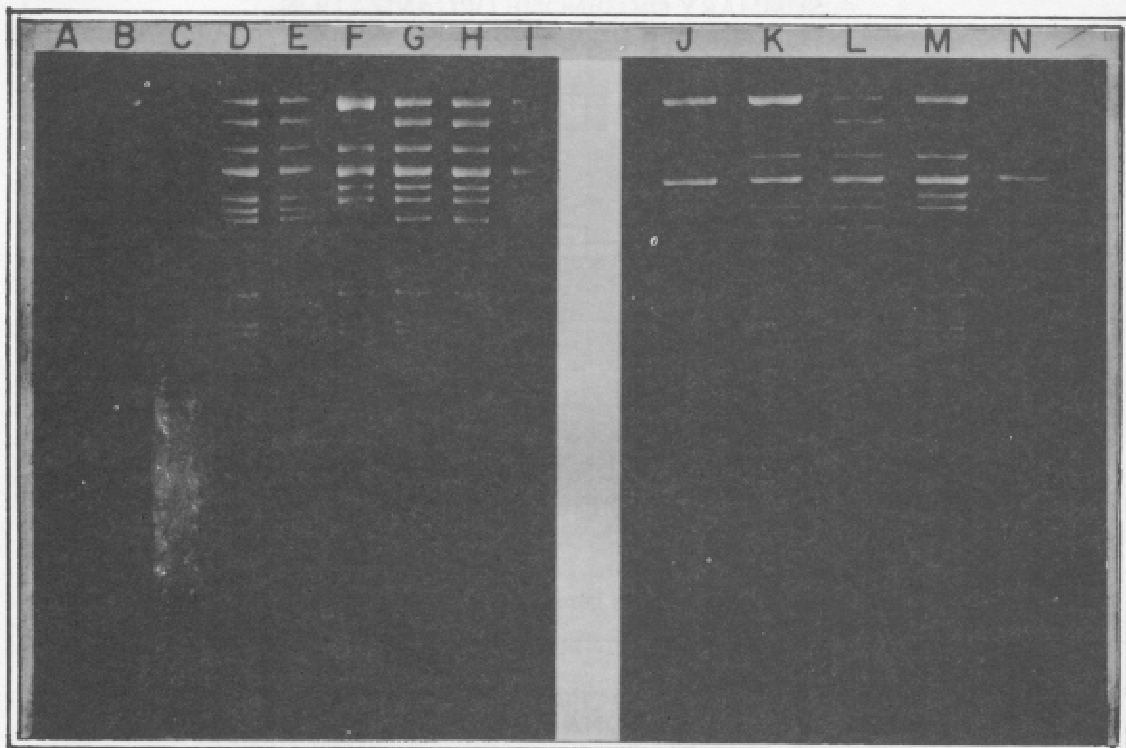


**FIG. 3. DNA restriction enzyme profiles of *Autographa californica* nuclear polyhedrosis virus plaque purified isolates. Lambda DNA markers are cut with BamH1 lane A and EcoR1, BAMH1 in lane A and B. A wild-type preparation is in lane C. The plaque isolates are lanes D-N. This is an ethidum bromide stained 0.75% agarose gel electrophoresis of the HindIII digested DNAs.**

relative to each other (Fig. 3). Summers and colleagues (1980) showed physical map differences between the closely related baculoviruses *Autographa californica* NPV and *Rachiplusia ou* NPV. Recombination events between these viruses were located on the DNA map. This information also was used in experiments to initially locate the gene coding for polyhedrin.

Another method for comparing the relatedness or similarities of baculoviruses is by DNA-DNA hybridization. To do this, DNA restriction fragments are electrophoresed and transferred to filter papers (Southern 1975). A second virus or viral DNA fragment is radioactively labeled and hybridized to the immobilized DNA on the filters. Not all fragments hybridize, only the highly homologous ones (Smith & Summers 1982, Jewell & Miller 1980, Brown et al. 1985).

Fig. 4 shows a summary of comparing DNAs of *Spodoptera exempta* nuclear polyhedrosis virus (SeNPV), *S. frugiperda* NPV (SfNPV), and *Autographa californica* NPV (AcNPV). The top line represents the genome of SeNPV. Regions where variation occur are the shaded boxes. As expected, the regions that hybridize between these viruses is highly conserved, consequently the variant regions do not overlap with these. The AcNPV genome is shown on the bottom line where it hybridizes to SfNPV and where it

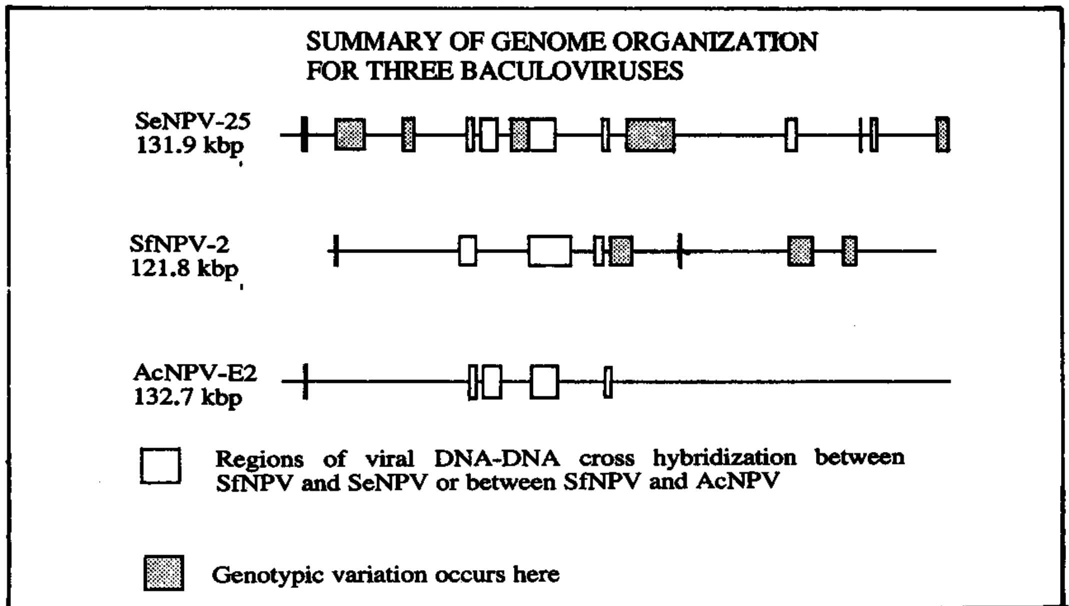


**FIG. 4. Summary of genome organization for three baculoviruses. The horizontal lines are the linear presentation of the circular DNA for each virus. The shaded boxes are where genotypic variation is located in *Spodoptera exempta* NPV or *S. frugiperda* NPV (Brown and colleagues, 1985; Maruniak and colleagues, 1984). The open boxes represent where DNA hybridization occurs when genomic DNA is labeled and hybridized to the other virus (Maruniak unpublished; Modified from Brown and colleagues, 1984; 1987).**

differs in hybridization from SeNPV (Browr et al. 1987).

This provides a general physical definition of the genomic differences among these baculoviruses. This approach goes beyond the morphological characterization so that we can now look at genus-species or strain comparisons of these baculoviruses to others. Additionally, this information can be used for interpreting results of experiments involving genetic manipulation.

These two viruses SeNPV and SfNPV have a different host range both in cell culture and in larvae. Both viruses replicate in *S. frugiperda* cells and larvae. Experiments were done to recombine these viruses in this permissive cell line. The progeny virus was plaqued purified in *Trichoplusia ni* cells in which only the SeNPV parental replicates. The recombinant progeny were detected by restriction profile changes occurring in the DNA. These new DNA profiles look mostly like the SeNPV parental virus.

These changes can be mapped on the SeNPV genome map and compared to the regions of variation and homology between the viruses (Fig. 5). Regions where recombination occur overlap both variant DNA and homologous DNA. The recombinant progeny were tested for changes in virulence to the *Spodoptera* and *Trichoplusia* cells. The changes in virulence were no more than a log titer different which is within the standard deviation. Therefore, although recombination

appears to occur in several regions, it is not significantly affecting virulence (Maruniak 1989).

Early in the history of insect cell culture, Hink & Strauss (1976), and Potter et al. (1976) found that upon serial passage of baculoviruses, induction of plaque size variants occurred. Plaques with many polyhedra (MP) and few polyhedra (FP) per cell were observed. Not only were fewer polyhedra found, but these were less virulent to larvae. It was not until the use of molecular techniques were applied to these phenotypic variants that the genetic changes were

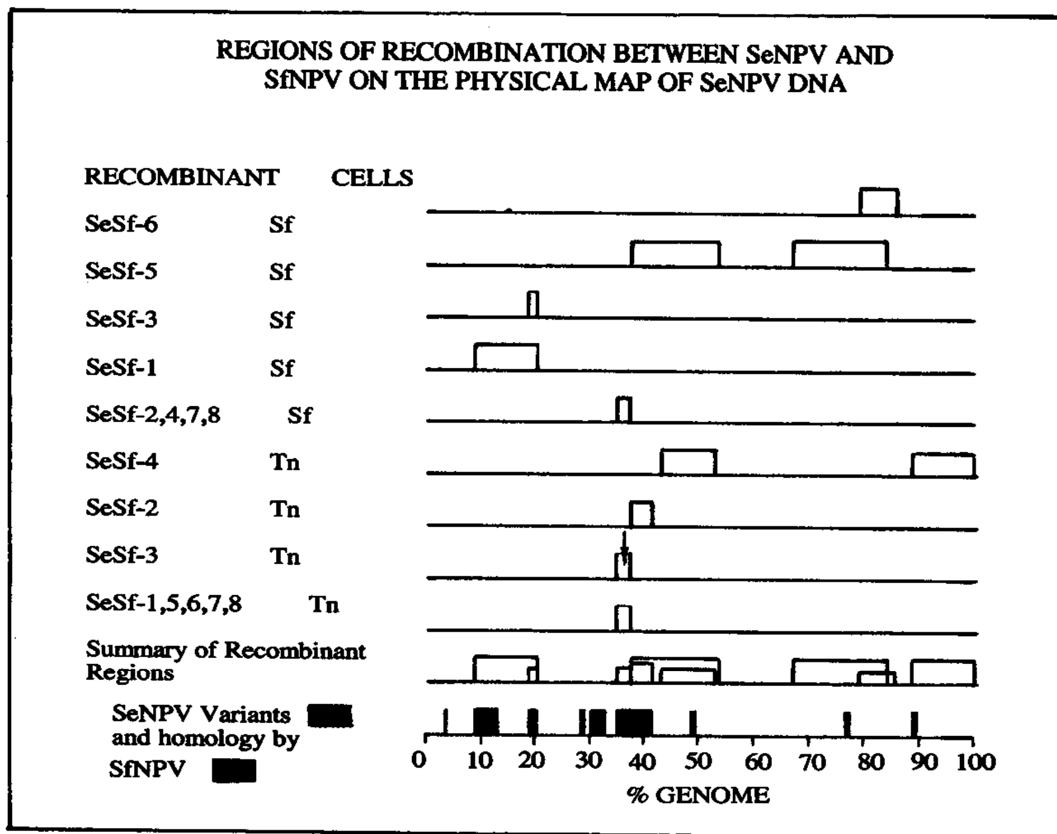


FIG. 5. Regions of recombination between SeNPV and SfNPV on the physical map of SeNPV DNA. Recombination between SeNPV and SfNPV was done in IPLB-Sf21 cells. The progeny virus was plaqued in either TN368 (Tn) or IPLB-Sf21 (Sf) cells. Plaque isolates with different EcoR1 or HindIII DNA profiles were designated, SeSf1-8 Tn or Sf depending upon the cell line in which they were plaqued. The open boxes were approximated by comparing the EcoR1 and HindIII DNA fragments that migrated differently from the SeNPV-25 parental virus. The partially shaded boxes indicate SeNPV genotypic variaton (modified from Brown and colleagues, 1985). The dark boxes indicate DNA-DNA homology between SeNPV and SfNPV (Maruniak, unpublished; modified from Brown and colleagues, 1984; 1987).

understood. This was first shown by Miller & Miller (1982), then by Fraser et al. (1983).

From wild-type and few polyhedra plaque variants, viral DNAs were digested with a restriction enzyme, electrophoresed and the DNA fragments were transferred to filters. The DNA filters were hybridized with a cloned wild-type (pW+) fragment labeled with radioactive phosphorus (Fraser et al. 1983). Changes in migration of DNA fragments of the variants showed that they were related by hybridization. These plaque variants were analyzed by fine mapping. This showed DNA of various sizes inserted into these cloned fragments. One of these cloned DNA fragments with inserted DNA was labeled and hybridized to itself or to TN-368 cell line DNA digested with various enzymes. These results indicate that the inserted DNA in few polyhedra mutants was from insect host cell DNA. This predicts that because few polyhedra mutants are rapidly generated in cell culture, the large-scale production of baculoviruses in insect cells will be difficult without additional modifications in the cell lines or in the viral DNA where cellular DNA inserts.

A presentation by Kool et al. (1990), at the International Congress of Virology in Berlin, indicated that other problems will occur when baculoviruses are produced in cell cultures. They reported that defective viruses with 43% of the DNA deleted are generated rapidly. These defective viruses replicate faster than wild-type, but they need some wild-type virus to grow.

In a study related to the previous two, several baculoviruses were passed in *Spodoptera* cells to establish persistent viral infections. Infectious virus was recovered up to cell passage 100 with SfNPV and over passage 53 with SeNPV. It is interesting that these viruses also killed larvae when injected into the hemocoel. When the DNA from SeNPV persistent infections was analyzed, inserted DNA was found in EcoR1-F fragment. By hybridization, this DNA was determined to be repetitive DNA that inserted into the viral genome. In contrast, persistent infections with SfNPV showed deletions of several kilobases of viral DNA similar to that, but not as great in size as that shown by Kool et al. (1990).

All of these previous reports on persistent infections, defective viruses, insertions of viral or cellular DNA, and genomic variation indicate that further research on the basic molecular biology of baculoviruses is very important in solving in vitro production of biological pesticides.

This raises the question of whether changes are occurring in biological pesticides produced in insect larvae. Fraser et al. (1983), not only showed production of few polyhedra mutants in cell culture but also from infectious hemolymph of larvae. This provided an impetus to study field isolates of the *Anticarsia* viral pesticide.

An early isolate, 1977, of *Anticarsia* NPV was analyzed for genotypic variation by plaquing and restriction enzyme analysis. A total of 50 plaques were obtained, but only 6 distinct genotypes for HindIII and Pst1 were detected (Maruniak, Place, Gowan, Zanotto, Moscardi, unpublished). The AgNPV-2 profile was found in 40% of the 50 plaques (Maruniak et al. 1986). A 1985, *Anticarsia* baculovirus produced by F. Moscardi for large scale application in Brazil (Moscardi 1989), was analyzed for genetic variation. This time, of 50 plaques, 12 unique genotypes were obtained by HindIII and Pst1 restriction analysis of the viral DNA (Maruniak, Zanotto, Moscardi, Garcia, unpublished). This is twice as many as the 1977 preparation. It is also interesting to note that none of the 1985 genotypes appeared to be like the 1977 plaque isolates.

Currently, the DNA variant regions of the AgNPV plaque isolates are being mapped in detail. The entire genome of AgNPV has been cloned for the HindIII fragments into the pGEM (Promega, Inc.) plasmid. This has allowed for us to determine where the polyhedrin gene is located by hybridization of cloned fragments to AcNPV DNA containing

the polyhedrin gene. A fine strucuture map of the HindIII-G fragment which contains the AgNPV polyhedrin gene has been constructed (Zanotto 1990). The fine map showed which fragments could be subcloned so that sequencing of the polyhedrin gene would be faster. Double stranded DNA sequencing was done with oligonucleotide primers and labeled nucleotides (Zanotto 1990). With information on the DNA sequence of the AgNPV polyhedrin, this important biological pesticide can now be genetically altered to maintain stability or for other important biological characteristics, such as faster killing activity.

Researchers are attempting to find ways to kill insects more quickly with baculoviruses. One way, by Martens et al. (1990), is by putting the *Bacillus thuringiensis* toxin gene behind the polyhedrin promoter of AcNPV. The recombinant AcNPV-Bt produced toxin crystals in insect cells. These crystals were able to kill larvae not susceptible to AcNPV infection. Since polyhedra were not produced, the virus is not stable in the environment and cannot be compared to wild type virus for quickness of killing larvae. Therefore, they are putting the Bt gene behind another viral promoter, p10. Weyer et al. (1990), have constructed baculoviruses with two p10 promoters that could be used for this purpose.

Other novel approaches to improving viral pesticides can be seen in the work by O'Reilly & Miller (1989). They identified a baculovirus gene, uridine 5'diphosphate glucuronosyl transferase which interferes with larval molting. Manipulation of this gene may affect how quickly baculoviruses kill insects. However, serial passage of the virus such as would be done in biopesticide production results in mutants of this enzyme. Another interesting possible improvement by Hammock et al. (1990), involves expressing insect juvenile hormone esterase in a baculovirus vector. Upon infection with this recombinant virus, insects stop feeding earlier than in wild type virus, thus reducing feeding damage. Finally, Derksen & Granados (1988), have identified a viral protein that enhances the infectivity of baculoviruses by rapid disruption of the peritrophic membrane of larvae. Manipulation of this protein may enhance the infectivity of baculoviruses.

In summary, applied and basic biology of insects and baculoviruses have interfaced to a great extent in the last ten years. A number of questions on the biology of the infection processes of baculoviruses are being investigated and in some cases resolved using molecular and recombinant DNA techniques.

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