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Identification of an Amino Acid Essential to the Normal Assembly of *Autographa californica* Nuclear Polyhedrosis Virus Polyhedra

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We compared the DNA sequence of the *Autographa californica* nuclear polyhedrosis virus polyhedrin gene with that of the polyhedrin gene from a morphology mutant called M5. A single point mutation was found at the *Bam*HI restriction site within the polyhedrin coding sequence. This point mutation caused a substitution of leucine for proline at amino acid 58 in the M5 polyhedrin. This point mutation was shown to be responsible for both the appearance of cubic polyhedra and the altered mobility of the polypeptide on sodium dodecyl sulfate-polyacrylamide gels by transferring the M5 polyhedrin gene to the wild-type virus by cotransfection. Recombinants were found which assembled cubic polyhedra in infected cells, had the *Bam*HI restriction site missing, and had an altered mobility of their polyhedrin polypeptide. Computer-predicted secondary-structure analysis indicated that the amino acid at position 58 could be critical to the proper folding of polyhedrin.

The polyhedrin gene of the subgroup A baculoviruses has attracted interest over the past few years, since its protein product is one of the most abundantly produced in virus-infected eucaryotic cells. Polyhedrin is of the utmost importance to the nuclear polyhedrosis viruses (NPVs), since it forms the occlusion body matrix in which mature infectious virus particles are found at late stages of infection. These occluded virus particles are responsible for the transmission of the virus in nature, indicating that polyhedrin is an essential gene product for virus infection in insects. This is not true for passage of NPVs in cell culture when nonoccluded (extracellular) virus particles are the infectious agents. Little is known of the occlusion process by which virions become embedded in crystallizing polyhedrin. The process occurs relatively late in the infectious cycle and correlates with a decrease in the amount of nonoccluded virus released from infected cells (20). Virus occlusion appears to be selected against during continued passage of some NPVs in cell culture, resulting in the production of few polyhedra per infected cell. There is some evidence that the lack of polyhedra in these variants correlates with an increase in titer of extracellular virus (15).

Genotypic alterations in an *Autographa californica* nuclear polyhedrosis virus (AcMNPV) polyhedron morphology mutant called M5 were described by Carstens (2). At least three regions of the genome of M5 are affected by mutation. Two of these sites include the insertion of 290 base pairs of DNA of almost identical sequence at 2.6 and 47 map units (E. B. Carotens, manuscript in preparation). The third mutation site manifests itself by the absence of a *Bam*HI restriction site normally present in the DNA of the wild type (wt) (2). These mutations are phenotypically associated with a variety of characteristics, including the replication of two size classes of M5 virion DNA, the assembly of cubic polyhedra in M5-infected cells, and the low numbers of virions occluded into the M5 polyhedra (1). To determine the functional relationship of some of these mutations to the morphogenesis of polyhedra, we have characterized the M5 polyhedrin gene.

The gene coding for AcMNPV polyhedrin has been mapped, and its nucleotide sequence has been determined (9; A. Krebs, E. B. Carstens, and J. H. Spencer, unpublished results). It consists of 732 bases, uninterrupted by introns, coding for 244 amino acids. We have confirmed the reading frame for the polyhedrin gene by direct amino acid sequencing of the first 20 N-terminal amino acids of wt and M5 polyhedrin. Both of the proteins had a proline residue as the N-terminal amino acid (unpublished results). The *Bam*HI site making up the left end of the *Bam*HI F fragment at 3.0 map units corresponds to the coding sequence of amino acids 56 to 58 of AcMNPV polyhedrin. Since this *Bam*HI site was missing in M5 DNA (2), we wanted to determine the extent of the mutation by sequencing the M5 polyhedrin gene region.

Viral DNA was isolated from purified extracellular virions as previously described (18). The *Eco*RI fragments of M5 DNA were cloned into the plasmid pBR322 and host *E. coli* HB101 cells by using standard techniques (5). Some M5 viral DNA, including an *Xho*I-*Bam*HI fragment carrying the entire polyhedrin gene, was subcloned into the plasmid vectors pUC8 and pUC9 (19) by using *E. coli* JM83 cells as the host (13). The *Xho*I-*Bam*HI M5 fragment (map position 2.6 to 4.7) was digested with *Hin*dIII, *Hpa*II, *Hin*cII, or *Kpn*I, and the various restriction fragments were cloned into the appropriately digested M13mp8 or M13mp9 vectors (12, 14). The inserts were sequenced by the dideoxy chain termination method (16), and the nucleotide sequence data from each gel reading were compiled and analyzed with a computer program described previously (17). The results confirmed the sequence previously published for the AcMNPV variant E2 polyhedrin gene (9), although four minor sequence variations from this published sequence were seen in the noncoding upstream sequence of M5 (Fig. 1). None of these changes occurred within the coding region of the polyhedrin gene, but rather represented deletions of adenine residues at three points (−1, −121, and −135) and the substitution of thymine

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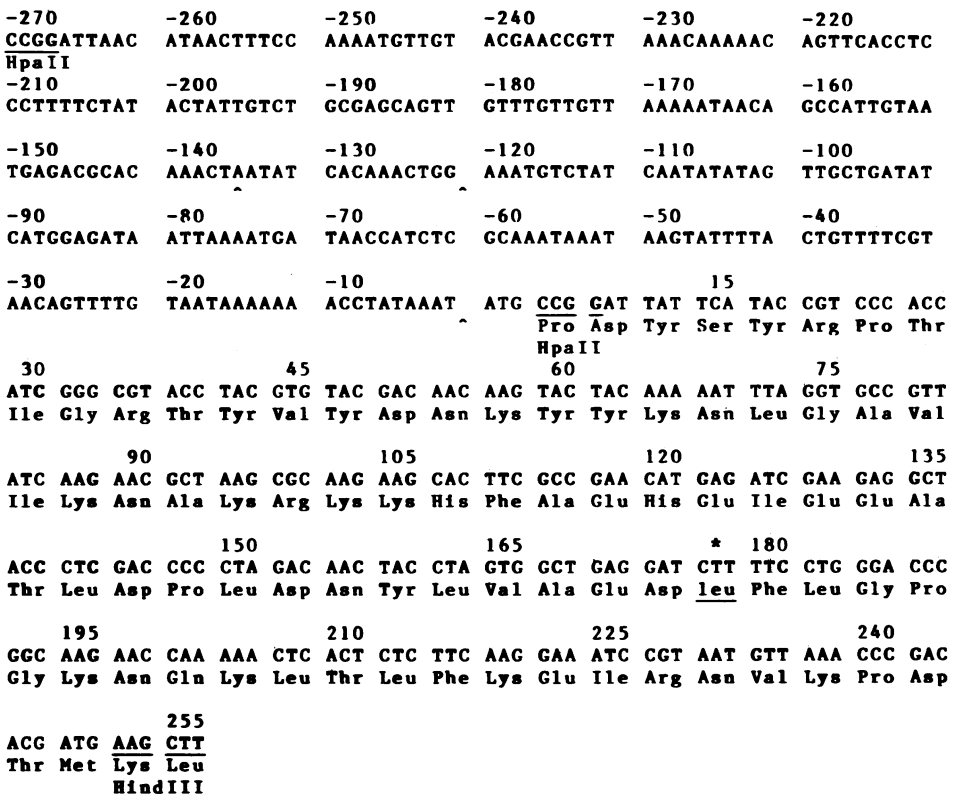

FIG. 1. Sequence of M5 DNA upstream of the *Hin*dIII site at 3.3 map units. The sequence of M5 DNA including 270 bases of noncoding upstream DNA and 255 bases of the coding region of the polyhedrin gene is shown. The sequence of our wt strain (HR3) was identical to this sequence, with the exception of the mutation at position 176 (*), which substituted a T for C, thereby eliminating the *Bam*HI recognition site in M5. Three point mutations representing the deletion of adenine residues in HR3 compared with the E2 strain (12) are also indicated (^).

for guanine at position −195 in the upstream noncoding region. The M5 sequence matched the sequence of the wt (HR3) sequence previously determined (Krebs et al., unpublished data). The sequence of M5 DNA in the vicinity of the *Bam*HI site revealed a single base substitution, changing the wt sequence of 5′-GGATCC-3′ to 5′-GGATCT-3′ in M5 (Fig. 1). This transition of cytosine to thymine did not change the reading frame of the M5 coding sequence, but did result in a conversion of amino acid 58 from proline in wt to leucine in M5. No other mutations were found within the M5 polyhedrin coding region (data not shown).

To demonstrate that this transition in DNA sequence correlated to the phenotypic alteration in the polyhedron morphology, the recombinant plasmids pM5XB3.25, containing the M5 *Xho*I-*Bam*HI fragment cloned into the *Sal*I-*Bam*HI site of pUC8, or pM5EcoI, containing the M5 *Eco*RI I fragment, were used to cotransfect *Spodoptera frugiperda* cells with purified wt DNA (4, 8). Control cultures were transfected with wt DNA alone or wt DNA plus plasmids containing the M5 *Eco*RI C fragment or the M5 *Eco*RI CI fragments (manuscript in preparation). Direct plaque isolates or extracellular virus from liquid overlay transfections were screened by plaque assay for the presence of recombinant virus assembling cubic polyhedra. The frequency of these cubic polyhedra producing recombinants was low, approximately 0.1% when pM5XB3.25 was used. Little difference was seen when linear rather than superhelical plasmids were used, nor did varying the concentration of wt viral DNA or plasmid DNA have much effect on the recombination frequency. We do not know why our recombination frequency appears to be much lower than that published by other groups, although it may be related to the size of the fragment used to rescue, since recombination frequencies up to 2.5% were observed when pM5EcoI was used. The integrity of the viral or the plasmid DNA or both may also play an important role in this process. In any case, recombinants assembling cubic polyhedra were found only in cotransfection of wt DNA plus pM5XB3.25, or wt DNA plus pM5EcoI. No plaques which genotypically expressed cubic polyhedra were found in any of the control transfections. Occasionally, plaques containing a few cells with cubic polyhedra were seen following transfection with wt DNA. However, upon plaque purification, the phenotype expressed by these aberrant plaque isolates was always wt. Recombinant plaque isolates were further plaque purified three or four times, and then viral DNA was isolated and analyzed by restriction enzyme digestion. A representative gel is shown in Fig. 2.

The genomes of all the M5 recombinants examined were identical to wt DNA in all aspects of their restriction patterns except for the absence of the *Bam*HI site at 3.0 map units. As expected, they did not show the presence of an insert in the *Sst*I G region, as did M5. This established a strong correlation between the mutation in nucleotide 172 of the polyhedrin gene and the expression of cubic polyhedra. There was no evidence of submolar restriction fragments in the digestions of the recombinant virus DNA, indicating that these viruses directing the synthesis of cubic polyhedra did not replicate two different size classes of viral DNA. This

result suggested that the mutation in the polyhedrin gene was probably not involved in this aspect of the M5 phenotype.

A second phenotypic effect of the M5 mutation within the polyhedrin gene was the increased mobility of the polyhedrin polypeptide on sodium dodecyl sulfate (SDS)-polyacrylamide gels (1). Polyhedra purified from recombinant infected *S. frugiperda* cells and analyzed on SDS-polyacrylamide gels also expressed this characteristic, indicating that the point mutation also affected the mobility of the recombinant protein in SDS-polyacrylamide gels (Fig. 3). Since we have sequenced the entire coding region of the M5 polyhedrin gene and have found that the only change from wt DNA in M5 DNA is the single base change at the *Bam*HI site, this result provided additional evidence that the single amino acid change in M5 polyhedrin was sufficient to cause severe phenotypic alterations of this protein. De Jong et al. showed that single amino acid changes dramatically alter the mobility of mammalian α-crystallin A chains in SDS-polyacrylamide gels (7). They suggested that proline may affect the conformation of SDS-protein complex, and that this in turn affects the mobility of the complex on the gels. This may also provide an explanation for the altered peptide pattern observed when the M5 polyhedrin was digested with V8 protease (1).

It was previously reported that M5-infected cells occluded few virus particles into the cubic polyhedra (1). To determine whether the mutation in the M5 polyhedrin gene also affected virion occlusion, thin sections of wt-, M5-, and recombinant-infected *S. frugiperda* cells were examined by electron microscopy. Some typical examples of the different types of polyhedra are shown in Fig. 4. The results obtained the AcM5poly1 were variable, since some cubic polyhedra were free of occluded virus, whereas others did contain bundles of virions (Fig. 4C). Therefore, the significance of the mutation in the M5 polyhedrin gene on the occlusion of virus particles was not clear.

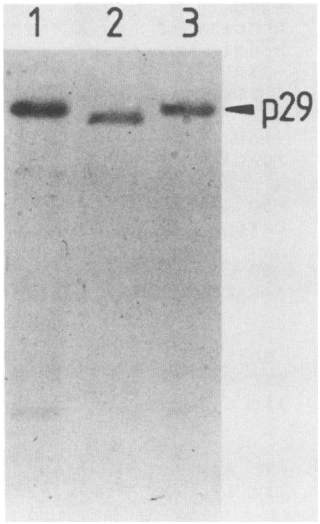

FIG. 3. Comparison of purified polyhedrins. Polyhedra were purified from wt- and AcM5poly1-infected cells. Following solubilization and boiling in SDS sample buffer, portions were subjected to electrophoresis through 12% SDS-polyacrylamide gels. The arrowhead indicates the position of the wt polyhedrin polypeptide (p29) in lanes 1 and 3. The AcM5poly1 polypeptide migrated faster, with a size of approximately 28 kilodaltons (lane 2). This corresponded to the altered mobility of the M5 polyhedrin polypeptide (2).

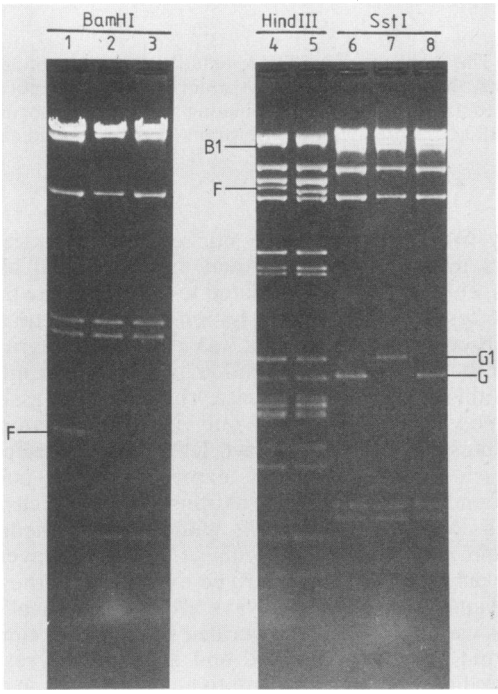

FIG. 2. Restriction endonuclease analysis of WT, M5, and AcM5poly1 recombinant viral DNA. Purified viral DNA from wt (lanes 1 and 6), M5 (lanes 2, 4, and 7), and AcM5poly1 (lanes 3, 5, and 8), a recombinant of wt virus, was digested with *Hin*dIII, *Bam*HI, and *Sst*I. The resulting fragments were analyzed by agarose gel electrophoresis. The *Hin*dIII and *Sst*I fragment patterns of AcM5poly1 were identical with those of wt, while the *Bam*HI pattern was the same as M5, indicating that the *Bam*HI site at 3.0 map units was altered in the recombinant. There was no indication of an insertion in the *Hin*dIII F fragment or the *Sst*I G fragment in AcM5poly1, suggesting that these mutations were not necessary for the morphological expression of cubic polyhedra.

A comparison of the amino acid sequence of AcMNPV polyhedrin derived from the DNA sequences with three other NPV polyhedrins derived from amino acid sequencing (11) indicated that 70% of the amino acids in these sequences were identical. Since the DNA sequence of the *Bombyx mori* (BmNPV) polyhedrin gene has now been published (10), it was possible to compare with confidence the amino acid sequences of AcMNPV and BmNPV polyhedrin with that of M5 polyhedrin. The alpha-helix, beta-sheet, and random-coil conformational parameters, based on these predicted amino acid sequences, were generated and plotted by using a computer program modified to run on an Apple II microcomputer (6). Figure 5 shows the computer-predicted secondary structure of these three polyhedrins. The amino acid sequences of the AcMNPV and BmMNPV polyhedrins vary by 14%, but their predicted secondary structures were very similar. We have also looked at similar predictions based on amino acid sequences of other polyhedrins (11), and they all generate very similar models (results not shown). These models suggest that the region preceding amino acid 58 potentially forms an alpha-helix structure and a beta-pleated sheet structure prior to a region of coils including a turn introduced by the proline at position 58. In M5 polyhedrin, the leucine at position 58 allows the extension of the alpha-helix, a reduction by two amino acids of the length of the random coil, and no turn until the proline at amino acid 62.

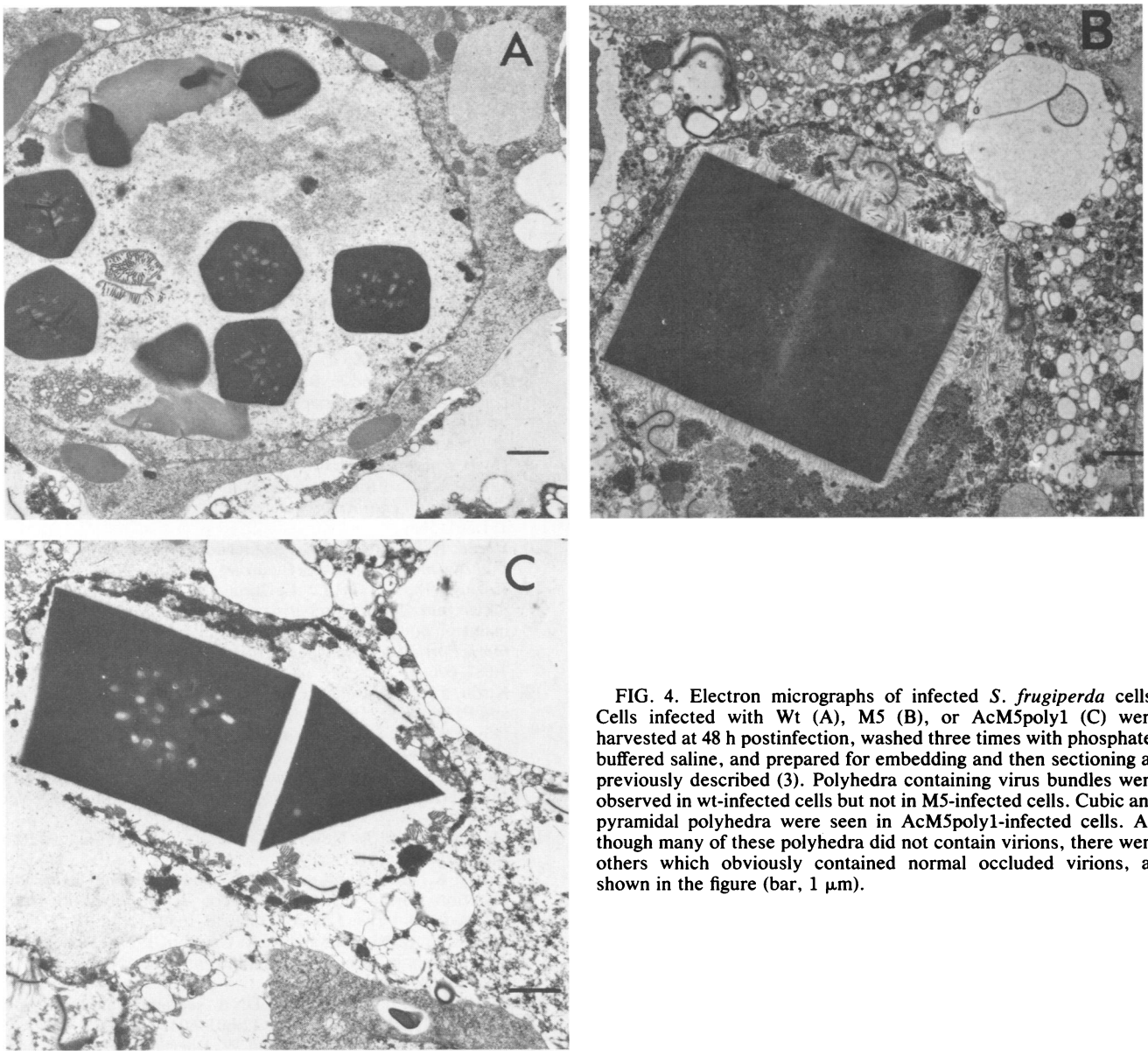

FIG. 4. Electron micrographs of infected *S. frugiperda* cells. Cells infected with Wt (A), M5 (B), or AcM5poly1 (C) were harvested at 48 h postinfection, washed three times with phosphate-buffered saline, and prepared for embedding and then sectioning as previously described (3). Polyhedra containing virus bundles were observed in wt-infected cells but not in M5-infected cells. Cubic and pyramidal polyhedra were seen in AcM5poly1-infected cells. Although many of these polyhedra did not contain virions, there were others which obviously contained normal occluded virions, as shown in the figure (bar, 1 μm).

This region of polyhedrin (amino acids 54 to 73) is highly conserved through many different NPVs (11), suggesting that it represents an important domain for the proper conformation of the paracrystal structure of polyhedra. The analysis of other mutations within the polyhedrin gene should indicate other domains critical to the proper functioning of the AcMNPV polyhedrin. This information is important for future experiments in altering this protein to increase the efficacy of baculoviruses by, for example, increasing the number of virions which become occluded in the polyhedra.

In summary, we have shown that the M5 polyhedrin gene contains a single point mutation resulting in the substitution of leucine for proline at amino acid position 58. This appears to be sufficient for the drastic morphological change in the M5 polyhedra. This was demonstrated by transfer of the M5 polyhedrin gene to wt virus by cotransfection of wt DNA and a recombinant plasmid carrying the M5 polyhedrin gene. All recombinants were missing the *Bam*HI site at 3.0 map units, indicating that they were true recombinants and not spontaneous mutants. The polyhedra synthesized by these recombinant viruses were morphologically identical to the cubic polyhedra seen in M5 infected cells. The polyhedrin polypeptide synthesized in AcM5poly1-infected cells also migrated with increased mobility over the wt polyhedrin on SDS-polyacrylamide gels, demonstrating that the point mutation dramatically alters the physical characteristics of this protein.

In the wild insect virus population, a mutation such as the one present in the M5 polyhedrin gene would be selected against, since the mutation also affects the ability of the newly enveloped virions to be occluded at late times in the nucleus. Since AcM5poly recombinants assembled polyhedra with both few and many occluded virions, it is probable that factors in addition to the crystal-packing forces are responsible for the lack of occluded virus in M5-infected cells. We are currently investigating the other regions of the

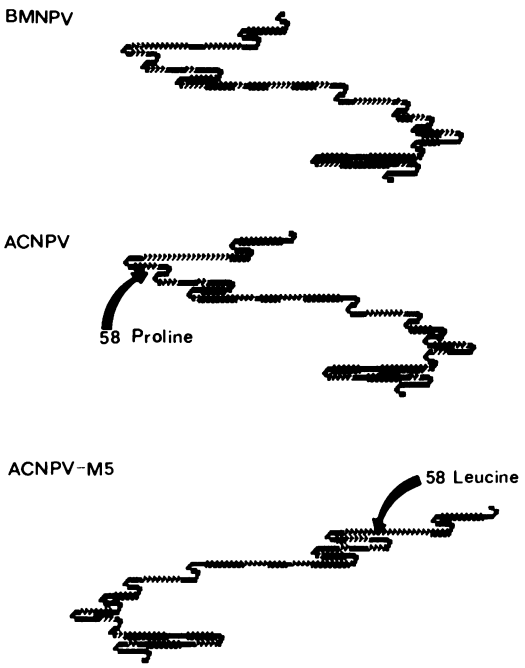

FIG. 5. Predicted secondary structure of polyhedrin. The amino acid sequences of BmNPV, AcMNPV, and M5 polyhedrin were derived from the DNA sequence (9, 10). These amino acid sequences were then analyzed by a computer program (6) which predicted the secondary structure of these proteins based on the rules established by Chou and Fasman. The plots show the predicted locations of alpha-coil (〰), beta-sheet (▰) and random-coil (▬) regions of the polypeptides running from the N terminus down to the C terminus. The plots of BmNPV and AcNPV polyhedrins were very similar, even though their primary amino acid sequences vary by 14%. In contrast, the single amino acid difference at position 58 in the M5 polyhedrin sequence resulted in a substantial change in the predicted secondary structure.

M5 genome which carry mutations to determine their effect not only on virion occlusion but also on DNA replication.

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