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Demonstration of the protective effects of fluorescent proteins in baculoviruses exposed to ultraviolet light inactivation

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Abstract

Autographa californica multiple nucleopolyhedrovirus (AcMNPV) recombinants, namely AcRFP produced by fusion of the red fluorescent protein (RFP) gene with the polyhedrin gene, and a recombinant (pAcUW21-23GFP) carrying the green fluorescent protein (GFP) in its viral envelope, were evaluated for their resistance to inactivation by ultraviolet light. AcRFP recombinants produced incomplete polyhedra with low infectivity for *Trichoplusia ni* larvae, whereas AcuW21-23GFP produced normal polyhedra with high infectivity. Electron microscopy of AcRFP CL14 showed the incorporation of very few viral particles into polyhedrin matrix protein material. The LC_{50} for AcuW21-23GFP was 0.10 occlusion bodies/mm^2, whereas the LC_{50} values for several AcRFP recombinants ranged from 20 to 329 occlusion bodies/mm^2. When both the RFP and GFP recombinants were exposed to ultraviolet light (UV-B 280-320 nm), the results support the conclusion that these fluorescent proteins afford some protection against its damaging effects.

Keywords: AcMNPV; recombinants; electron microscopy, bioassays, insect cell culture

Abbreviation:

AcMNPV	*Autographa californica* multiple nucleopolyhedrovirus
BV	budded virus
CPE	cytopathogenic effect
ECV	extracellular virus
OB	occlusion body
ODV	occlusion derived virus
RFP	red fluorescent protein
GFP	green fluorescent protein
$TCID_{50}$	tissue culture infective dose at the 50 % level
UV-B	ultraviolet light of 280-320 nm

Introduction

Baculoviruses belong to the family Baculoviridae and are the viral agents of choice for the control of insect pests. Because they have some unique properties such as their virulence, specificity within the phylum Arthropoda, non-infectivity for beneficial insects and higher animals and their biodegradable nature, several of these insect viruses, called nucleopolyhedroviruses and granuloviruses, have been commercialized for insect control (Moscardi 1999). Most of the attention has been concentrated on the nucleopolyhedroviruses that infect Lepidopteran hosts. These baculoviruses have a biphasic replicative cycle in which two forms of the virus are produced in the infected cell, one that buds from the cytoplasmic membrane and is commonly referred to as budded virus or extracellular virus, and the other in which the viral particles are occluded into a protein matrix called an occlusion body (OB). The particles in occlusion bodies are called occluded derived virus (ODV) and initiate infection in the host following consumption of contaminated parts of the plant and their release in the alkaline midgut. The infection results in the production of budded virus that then disseminates to various target sites in the host. Thus, budded virus is responsible for spreading the infection within the host, whereas occlusion bodies, which are released from infected larvae through lysis, spread the virus in the environment between hosts.

One of the limitations of the use of baculoviruses as biological control agents is their loss of activity under field conditions due to inactivation by ultraviolet (UV) light, with most of the activity being lost within 24 h. Most of the studies to address this deficiency

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have centered on the use of microencapsulation and additives such as UV protectants (Ignoffo and Batzer 1971) and fluorescent brighteners (Shapiro and Vaughn 1995) to reduce or prevent inactivation by UV light. *In vitro* studies (Grasela *et al.* 2002) have demonstrated that cells in culture can serve as a protective device against inactivation by UV light, by either serving as a physical barrier or by some mechanism in which cells repair damaged DNA. In the present study, we employ a genetic approach by expressing fluorescent proteins in occlusion bodies and in the envelopes of ODV in order to determine if these compounds afford protection against the damaging effects of UV (UV-B 280-320 nm).

Materials and Methods

Baculoviruses and cell culture

AcMNPV-HPP (McIntosh *et al.* 1992), a clone of the wild-type AcMNPV, a red fluorescent protein (RFP) AcMNPV recombinant , a green fluorescent protein (GFP) AcMNPV recombinant, and pAcUW21-23GFP (Hong *et al.* 1997) were produced in the TN-CL1 cell line (McIntosh and Ignoffo 1989). The occlusion bodies were harvested and enumerated as previously described (Grasela and McIntosh 1998). AcMNPV- RFP recombinants (AcRFPCL1, AcRFPCL2 and AcRFPCL14) were randomly selected and purified several times by standard plaque purification and end point dilution methods (McIntosh *et al.* 1997: Lynn, 2003). TN-CL1 cells were inoculated with purified recombinants at a multiplicity of infection of 5 and incubated at 28° C for 5 days at which time OB were recovered and enumerated.

Construction of the red fluorescent protein (RFP) recombinant

The generation of the AcMNPV-RFP in which the RFP gene was fused to the polyhedrin gene was accomplished in two stages. In the first step a cloning technique based essentially on the report by Gritsun *et al*. (1997) with some minor modifications was employed. Oligonucleotide primers(1)5'-AAGTAAAGG AGTTTGCACCAGACGCACCTCTGTTCACTGG TCCGGCGTATAGGTCTTC CAAGAATGTTATC and (2) 5'-CGCACAGAATCT AGCGCTT AATAAATGTACTAATAACAA TGTATCGTGTTCTAAAGGA ACAGATGGTGGCG, designed to amplify the coding sequence of the RFP gene of the prokaryotic expression vector pDsRed (Clontech, BD Biosciences, www.clontech.com), were synthesized by the University of Missouri-Columbia DNA Core facility. The primers were designed such that during the PCR amplification process an amplicon would be generated with 55 bases (flanking region) at the 3'-end of the polyhedrin gene plus the first15 bases at the 5'-end of the RFP gene. The other flanking region consisted of 55 bases immediately downstream of the polyhedrin gene plus the last 15 bases of the 3' end of the RFP gene. The primers were also designed such that the termination codon of the polyhedrin gene and the initiation transcription site of the RFP gene were absent in the amplicon (Fig. 1). Ready-to-go PCR beads (Amersham Pharmacia Biotech, www.apbiotech.com) were used essentially as recommended by the manufacturer to amplify the RFP amplicon. Each PCR sample contained 10 pmol/µl of each primer, 1 µg/µl of pDsRed expression vector, 17 µl sterile MilliQ water, and 2 µl 10mM $MgCl_2$ in 25 µl total volume. Running conditions using an Omni Gene Hybaid thermal cycler were as follows: 95° C for 2 min, (1X); 95° C for 5 sec, 40° C for 1 sec, 72° C for 30 sec (40X); 72° C for 5 min (1X); held at 15° C. To verify if the amplification was successful 10 µl of the sample was run on a 1.5% Metaphor gel (BioWhittaker Molecular Applications, www.bioresearchonline.com/storefronts/biowhittaker.html) at 120 constant voltage for 1 h and stained with 1.0 µg of ethidium bromide in 250 ml TBE buffer. DNA bands containing the expected RFP gene with flanking regions were excised from the gel and extracted using GFX™ PCR DNA and Gel Band Purification kit (Amersham). This amplicon was then used in co-transfection experiments with wt AcMNPV DNA to generate AcMNPV-RFP recombinants. DNA was then extracted from semi-purified (containing wt AcMNPV) RFP recombinants and the complete fusion gene (polyhedrin + RFP) was then amplified from this mixture by long DNA template PCR under the following conditions: 95° C, 10 sec; 58° C, 30 sec; 72° C, 3 min 10X; then 95° C, 10 sec; 58° C, 30 sec; 72° C, 3 min, plus a 30 sec increment 20X; 15° C, hold. The upstream and downstream primers, designed to amplify a 1465-bp polyhedrin -RFP gene fusion amplicon were: 5'-TAACCATCTCGCAA ATAAATAAGTA TTTTACTG TTTTCG and 5'-AATTGT CTGTAAATCAACAACGC ACAGAATCTAG CGCT, respectively. These two primer sequences were designed

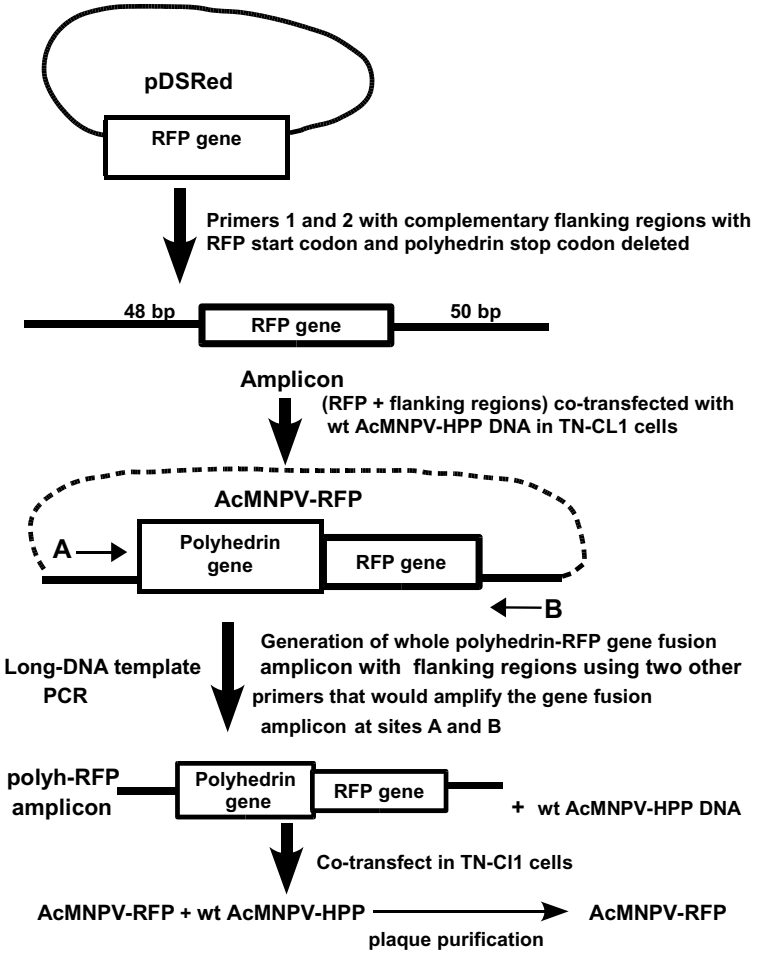

Figure 1. Schematic diagram illustrating the construction of the AcMNPV recombinant (AcRFP) from the fusion of the RFP gene with the polyhedrin gene and co-transfection of amplicon with wt AcMNPV-HPP.

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such that during co-transfection of the PCR-amplified RFP amplicon and wild type AcMNPV DNA, the termination codon of the polyhedrin gene from the wild type viral DNA and initiation codon of the RFP gene would be deleted while the initiation site from the polyhedrin gene and the termination signal from the RFP coding sequence would be retained during allelic recombination. This would generate a continuous coding sequence containing both the polyhedrin and RFP genes for incorporation into the viral genome by allelic recombination that would express a complete, functional polyhedrin-RFP fusion protein. These molecular modifications permit a continuous read-through transcription of the fused polyhedrin and RFP gene sequences. Ready-to-go PCR beads were used as previously described.

Isolation of DNA from OB

The Puregene DNA extraction kit (Gentra, Systems Inc., http://mbbnet.umn.edu/company_folder/gsi.html) and isolation methodology with modifications (McIntosh et al. unpublished) were used to isolate DNA from OB as follows. Occlusion derived virus was recovered from approximately 10^7 OB by alkali treatment as previously described (McIntosh and Ignoffo 1988). The sample was then incubated in a water bath at 60° C for 15 min and cooled to room temperature. Next, proteinase K was added to give a final concentration of 200 µg/ml and incubated for 2 h in a 37° C water bath followed by cooling at room temperature. Protein precipitation solution (1.7 ml) was added and vortexed at high speed for 20 sec, and spun at 10,00 x g for 3 min in a BHG Hermle Z230M table-top centrifuge. The supernatant was poured into a clean 15 ml glass tube containing 2 ml isopropanol and inverted 50X and centrifugated at 10,000 x g for 1 min. The supernatant was then poured off and 2 ml of 70% ethanol was added to wash the DNA pellet. The ethanol was poured off after centrifugation at 10,000 x g for 3 min and allowed to air dry for 15 min. About 175 µl of DNA hydration solution was added to the sample and allowed to stand overnight at room temperature. DNA was quantified using a Gene Quant II (Amersham Pharmacia Biotech, Piscataway, NJ).

Verification of the recombinant RFP by determination of the polyhedrin-RFP fusion gene junctions

It was necessary after the cloning and isolation of the recombinant baculovirus (AcRFPCL14) to verify its identity by actual DNA sequence, as was expected to be generated by the above designed oligonucleotide primers, at the upstream-junction between the 3'-end of the polyhedrin gene and 5'-end of the RFP gene as well as the downstream-junction between the 3'-end of the RFP gene and the immediate down-stream flanking region of the polyhedrin gene.

To sequence these two junctions, DNA from ca. 10^7 OB/ml of recombinant virus was extracted employing the above procedure. The following primers were designed to amplify the two junctions: (1) 5'- ATTCTCCTTGA AGTTT CCCTG and 5'-CTCGTGCCCATTGACCGTTCC for the up-stream junction, and (2) 5'-TAACAAGC CACAACGAAGACT and 5'-ACGCACAGAATCTAGCGCTTA for the down-stream junction. Both primer synthesis and DNA sequencing was conducted by the University of Missouri-Columbia DNA Core facility. The fidelity of the predicted DNA sequences of the two above described junctions were confirmed by performing DNA sequencing twice, thus verifying the successful generation of the recombinant. Based on the size of the polyhedrin-RFP fusion gene plus genomic DNA generated by Bam HI digestion of AcRFP CL14 DNA, a predicted fragment of approximately 2611 bp was observed in the gel profile of the recombinant DNA but was absent in the wt AcMNPV DNA profile. Bam HI would typically cut the wild type AcMNPV DNA at a site 200 bp into the polyhedrin gene and at another site further downstream resulting in a 1932 bp restriction fragment, as well as at other sites. With the insertion of the RFP gene at the 3'-end of the polyhedrin gene and subsequent Bam HI digestion of the recombinant DNA, this same fragment was extended to 2611 bp by the addition of the 677 bp RFP gene.

Co-transfection of insect cells

TN-CL1 cells grown in ExCell 401 (JHR Biosciences, www.jrhbio.com) plus10% heat inactivated (56° C/30 min) fetal bovine serum (Summit Biotechnology, www.biobank.co.kr/maker/sss/summit.shtml) were seeded into 24 well plates at 1 x 10^5 cells/ml/well and incubated for several hours, or overnight at 28° C. DNA derived from the occlusion virus of AcMNPV-HPP was used at a concentration ranging from 125 ng/5 ml to 500 ng/5ml, whereas the RFP amplicon or the polyhedrin-RFP amplicon was used at 3x the concentration of Ac-HPP DNA. Prior to co-transfection, the medium was removed and the cells gently washed with fresh 0.5 ml of ExCell 401 without fetal bovine serum and the medium removed and fresh 0.5 ml serum free medium added. To each sample tube containing Ac-HPP- DNA and amplicon DNA, 3 µl of transfection reagent (Mirus TransIT®-LT-2, PanVera, Corp., www.invitrogen.com/) was added and brought up to 50 µl total volume with sterile water and the samples were incubated at 28° C for 15 min. The contents of each tube were then added in a drop-wise manner to each well containing cells, mixed gently by rocking and incubated at 28° C for 5 hr. At the end of this period, 1 ml of serum-free medium containing 50 units/ml penicillin, 50 µg/ml streptomycin, and 60 µg/ml gentamycin (stock10 µg/ml) were added to each well and cells incubated for 5-7 days or until a cytopathogenic effect (CPE) was observed. Cells that were successfully co-transfected were readily identifiable under UV light by their red fluorescence employing an Olympus CK2 microscope with a BH2-RFC attachment.

In vivo and in vitro bioassays

All bioassays were conducted by topical application of virus preparation (based on OB counts or dilutions of the original OB suspension) to individual wells containing a semi-synthetic diet (Ignoffo 1965). Twenty-five one day old *T. ni* larvae/dose were exposed in triplicate and the percent mortality was recorded at 7 days following incubation at 30° C. LC_{50} values were calculated by probit analysis as previously described (Kariuki and McIntosh 1999). TN-CL1 was used as the indicator cell line for the quantification of viral titers ($TCID_{50}$/ml) of extracellular virus or budded virus by a previously described method (Grasela and McIntosh 1998).

For mortality studies, each AcRFP recombinant as well as Ac-HPP was produced in a T-75 cm^2 flask of TN-CL1 cells at an multiplicity of infection of 5 and cell pellets were recovered at the end of the incubation period by centrifugation at 3,000 x g for 30

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min in a Beckman Model TJ-6 table top centrifuge (Beckman Coulter, Inc. www.beckman.com). Each cell pellet was standardized by re-suspension in 10 ml of ultra-pure water and sonicated through several cycles at 50 watts in a Sonifier Cell Disruptor, Model W185 (Heat Systems-Ultrasonics Inc. Plainview, N.Y.). This 10 ml volume represented the original suspension from which serial dilutions were made to perform mortality studies.

Exposure to Ultraviolet Light (UV-B)

Samples were exposed by placing 2 ml of the OB suspensions in wells of a 12 well plate (Corning, Costar, www.corning.com/). Half of the wells were covered with aluminum foil (shielded) and the other half were not shielded. Samples were exposed for various periods of time to ultraviolet light, UV-B (280-320 nm), in the chamber of a Suntest CPS system (Atlas Electric Devises Co., Chicago, IL). The dose rate was 3600 KJ/m^{2}x2h. Following exposure, samples were bioassayed against *T. ni* larvae and mortalities and LC_{50} values calculated. Based on half-life values of baculoviruses, 1 min in the Suntest CPS chamber is equivalent to 20 min of exposure to outdoor sunlight. The half-life of baculoviruses tested was found to be 4.8 min in the Suntest CPS chamber (C. Ignoffo, personal communications).

Electron Microscopy

Samples were prepared for transmission electron microscopy as previously described (Kariuki and McIntosh 1999) and were processed by the Electron Microscopy Core Facility at the University of Missouri, Columbia.

Results

The results of infectivity studies of the AcMNPV recombinants, AcRFPCL1, AcRFPCL2, AcRFPCL14 and pAcUW21-23GFP, as well as a clone (Ac-HPP) of the wild-type (wt) AcMNPV, are presented in Table 1. The highest $TCID_{50}$ titers were recorded for AcRFPCL2 (1.15 x 10^8), AcRFPCL14 (9.49 x 10^7) and Ac-HPP (1.15 x 10^8). AcRFPCL1 and pAcUW21-23GFP gave lower $TCID_{50}$ titers of 3.30 x 10^6 and 1.15 x 10^6 respectively.

When examined under UV light, AcRFPCL14 infected TN-CL1 cells gave bright red fluorescence that was distinct in nature compared with a more diffused fluorescence for pAcUW21-23GFP (Figure 2). The other AcRFP recombinants displayed a fluorescence similar to that of AcRFPCL14.

All of the AcRFP recombinants gave significantly higher LC_{50} values (low infectivity) than pAcUW21-23GFP (LC_{50} was 0.1 OB/mm^2) (Table 1). *T. ni* larvae that were infected with AcRFP recombinants typically showed a pink to red color under ordinary light as depicted in Figure 3. It was considered that low infectivity of AcRFP recombinants might be due to an error in the enumeration of OB counts since counts were based on fluorescent particles which might not be OB, or incompletely formed OB. Consequently, mortality studies based on serial dilutions of standardized sonicated cell pellet suspensions and not on OB counts were performed. Results of such studies are presented in Table 2 and confirm the low infectivity of the recombinants. At the 10^{-2} dilution, AcRFPCL1, AcRFPCL2 and AcRFPCL14 gave mortalities of 4%, 16% and 46.6% respectively, whereas at a dilution of 10^{-4} Ac-HPP gave a mortality of 92%.

AcRFPCL14 was selected for further electron microscopy studies and exposure to UV light inactivation. To determine whether or not OB were incompletely formed and thus occluded fewer viral particles, transmission electron microscopy studies were conducted on AcRFPCL14. Figure 4 illustrates the incomplete assembly of the polyhedrin protein matrix that appears as grayish material with few viral particles occluded (arrow). Many viral particles can be seen outside these irregular shaped bodies as well as illustrated in Figure 5. Figure 6 is a higher magnification of Figure 4 showing virions at the edge of the polyhedrin matrix.

The results of UV-B studies are presented in Table 3 and show that both AcRFPCL14 and AcGFP recombinants afforded protection to the ODV when exposed to the detrimental effects of UV light. Although the infectivity of the AcRFPCL14 recombinant is much lower than the Ac-HPP, nevertheless the former afforded better protection against UV-B (33.7 fold = 78.6/2.33) as compared with Ac-HPP. The pAcUW21-23GFP recombinant gave the best protection against UV-B compared with the AcRFPCL14 (3-fold) recombinant and Ac-HPP (102-fold). Even prolonged exposure of pAcUW21-23GFP OB for 4 h to UV-B did not result in extensive inactivation of the virus giving a non-shielded to shielded ratio of 6.5. A non-shielded to shielded ratio of <= 1 would indicate no inactivation by UV-B.

Discussion

The recombinants AcRFPCL1, AcRFPCL2 and AcRFPCL14 represent different clonal isolates selected randomly following co-transfection. The differences in infectivity results between these recombinants as represented in Table 1 may be a reflection of inaccurate fluorescent OB counts, since the particles were irregular in shape and size, and attempts were made to count only those

Table 1. *In vitro* and *in vivo* infectivity of AcMNPV recombinants and AcMNPV-HPP[a] a clone of the wild type virus.

Virus	TCID50/ml + SEM	LC50 (OB/mm2) + SEM
AcRFPCL1	3.30 x 106 + 0.001	63.00 + 10.72
AcRFPCL2	1.15 x 108 + 0.088	328.75 + 49.3
AcRFPCL14	9.49 x 107 + 0.001	20.10 + 3.24
pAcUW21-23GFP	1.15 x 106 + 0.001	0.10 + 0.02
AcMNPV-HPP	1.15 x 108 + 0.097	0.095 + 0.02

[a]TN-CL1 cells were used for the generation of $TCID_{50}$ values and 24 h old *Trichoplusia ni* larvae for conducting bioassays. Twenty-four wells per dilution were employed in 96 well plates for viral titrations and 75 larvae per dilution for infectivity studies.

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particles that approximated the size of normal occlusion bodies. Alternatively the low number of viral particles incorporated into the OB-like protein matrix material may account for differences in observed infectivity and is supported by the observed mortality studies (Table 2) which were not based on OB counts. It was not possible to determine from these studies whether or not ODV particles in OB from the AcRFP recombinants were less infectious than those occurring in AcMNPV-HPP occlusion bodies but the budded virus particles from recombinants appear to be normal since the purified clones gave relatively high infectious titers as observed in Table 1.

The irregular shaped greyish material in the electron microphotographs (Figs. 4-6), showing virus assembly in the nucleus, is incompletely formed polyhedrin protein matrix and supports the fluorescent light microscopy observation of irregularly shaped fluorescent bodies. Many viral particles do not have a membrane or envelope and this may be the reason why more viral particles are not occluded. In a normal assembly process, once polyhedrin matrix is detected in the nucleus, most of the nucleocapsids would have their envelopes through the intranuclear nucleocapsid envelopment process to become ODV. The membrane on ODV is a requirement for virion occlusion and thus the observations in this electron microscopy study suggest that there is an impairment in the intranuclear nucleocapsid envelopment process. The absence of nucleocapsids in the virogenic stroma (Fig. 4) may be a reflection of a late stage in the infection cycle.

One of the major disadvantages in the use of microbial biological control agents is their inactivation following exposure to UV light in the environment (Jacques 1968, Bullock et al. 1970, Jones and McKinley 1986). UV-B (280-320 nm) is believed to be the major cause of inactivation of microbials (Jacques 1977, Jones and McKinley 1986) with pathogens losing 50% of their activity within several days (Ignoffo et al. 1977) and in some cases within 24 hours (Broome et al. 1974). One of the approaches that has been

Figure 3. (A) *Trichoplusi ni* larvae infected with AcRFP14 at 4 d following inoculation and (B) un-inoculated control larva. Magnification 7X.

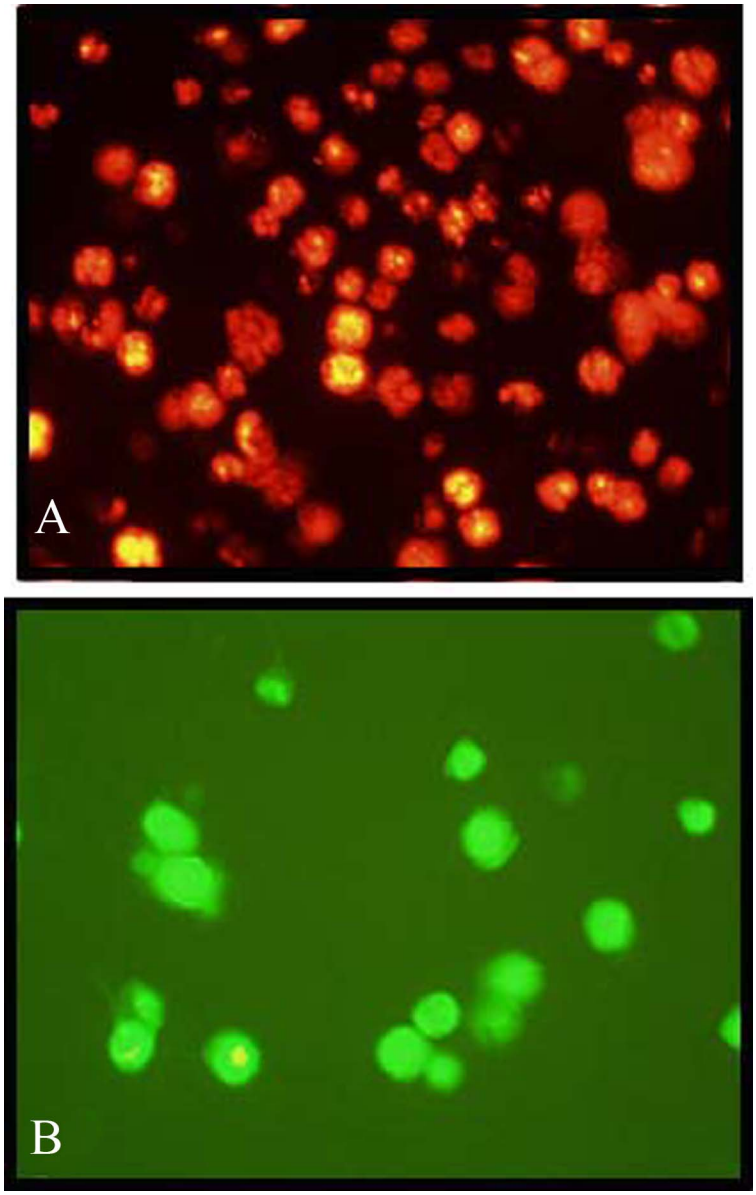

Figure 2. (A). TN-CL1 cells infected with AcRFP CL14 (72 h, p.i) and (B) AcuW21-23GFP (5 days after infection). 200X magnification.

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Table 2. Average percentage mortality of *Trichoplusia ni* larvae exposed to AcRFP recombinants and AcMNPV-HPP.[a]

Virus	Dilution	No. insects	Dead	% Mortality
AcRFPCL1	10os	75	24	32
	1-Oct	75	9	12
	2-Oct	75	3	4
AcRFPCL2	10os	75	75	100
	1-Oct	75	38	50.7
	2-Oct	75	9	16
AcRFPCL14	1-Oct	75	75	100
	2-Oct	75	35	46.6
	3-Oct	75	0	0
AcMNPV-HPP	3-Oct	75	75	100
	4-Oct	75	68	92
	5-Oct	75	21	28

[a]Mortality studies were conducted employing 24 h old *Trichoplusia ni* larvae.
OS = original suspension from one T-75 cm^2 of TN-CL1 cells inoculated at a MOI = 5; All dilutions were made from the OS of each virus.

Table 3. Effect of UV-B treatment on the infectivity of AcMNPV recombinants and a clone (AcMNPV-HPP) of the wild type virus.[a]

UV-B Exposure Time					
LC_{50} (OB/mm^2)					
	2 h		4 h		
Virus	S	NS	S	NS	Ratio (NS/S)
AcRFPCL14	8.6 (L_1 = 5.6; L_2 = 15.6)	20.03 (L_1 = 8.9; L_2 = 47.1)	-	-	2.33
pAcUW21-23GFP	1.11 (L_1 = 0.82; L_2 = 1.46)	0.85 (L_1 = 0.54; L_2 = 1.21)	0.68 (L_1 = 0.55; L_2 = 0.84)	4.1 (L_1 = 0.55; L_2 = 0.84) 8.04 (L_1 = 4.1; L_2 = 16.0)	0.77 (2h) 6.03 (4h) 11.8 (4h)
Ac-HPP	0.34 (L_1 = 0.23; L_2 = 0.48)	26.72 (L_1 = 19.4; L_2 = 37.2)	-	-	78.6

[a] Bioassays performed on 24 h old *Trichoplusia ni* larvae employing 75 larvae per dilution for infectivity studies.
S = shielded ; NS = not shielded; (-) not determined; (L_1 = lower 95% C.I.; L_2 = upper 95% C.I.). Values in the 4 h, NS column of pAc-23GFP are from two replications.

employed with the expectation of decreasing inactivation by UV-B has been the addition of UV protectants such as dyes and optical brighteners to formulations (Shapiro and Robertson 1990, Shapiro and Vaughn 1995). Also, Grasela *et al.* (2002) demonstrated that insect cells can protect virus from inactivation by UV-B. In that study, it was not known whether such protection was due to some DNA repair mechanism or to physical protection by the cells. It is unlikely such an approach would find applicability of baculoviruses in the environment since the presence of insect cells would be required. A more recent report (Petrik et al. 2003) employed a DNA repair enzyme whose gene was engineered into AcMNPV for repairing any damage that might have occurred to DNA following exposure to UV light. This approach, however, involves repair after damage has occurred and would require the presence of insect cells for the replication and repair of the damaged viral DNA. Furthermore if ultraviolet inactivation of the repair enzyme gene

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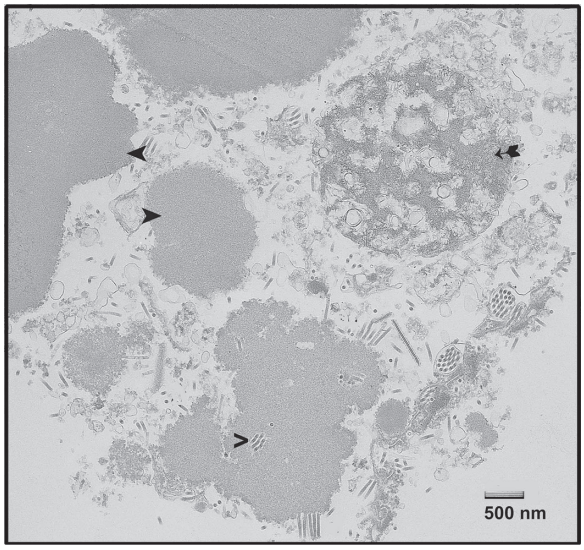

Figure 4. Electron micrograph of AcRFPCL14 in infected TN-CL1 cells showing the presence of irregular shaped polyhedrin matrix protein material (grey structures) with some occluded viral particles. The circular grey structure in upper right is the virogenic stroma.

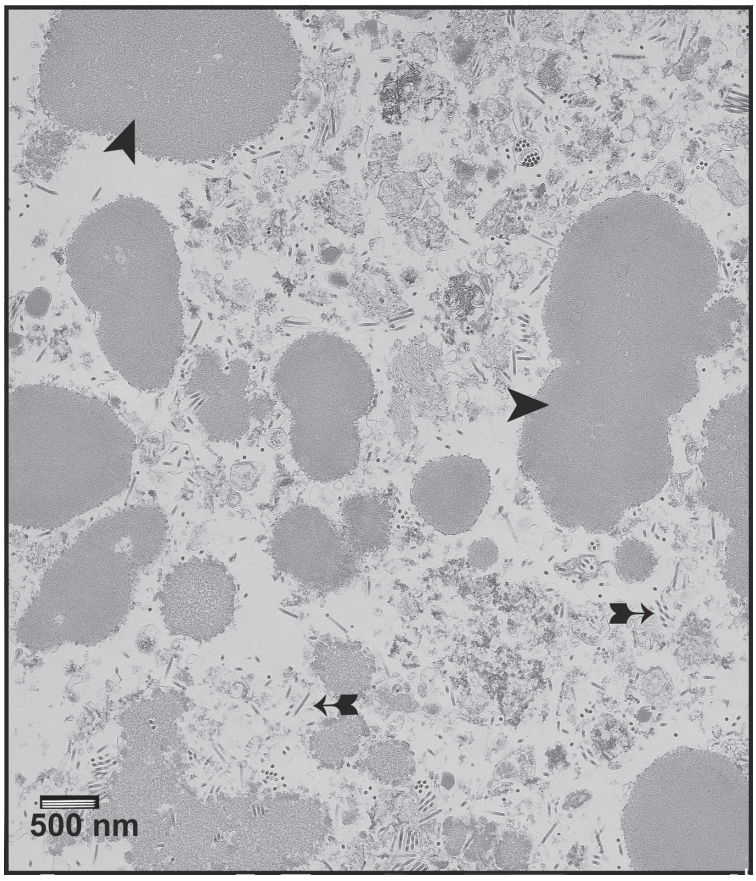

Figure 5. Electron photomicrograph of infected TN-CL1 cells with AcRFP14 showing the presence of numerous viral particles and incompletely formed polyhedra.

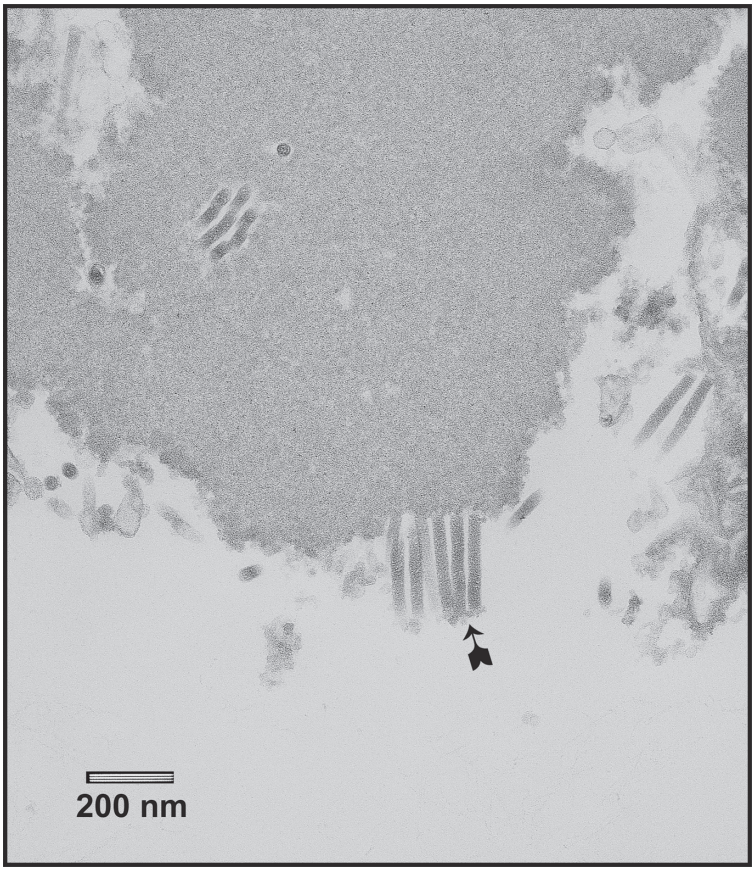

Figure 6. An electron photomicrograph at higher magnification of Figure 4 showing few viral particles incorporated into the polyhedrin matrix protein and several virus particles without a membrane on the periphery of the protein matrix material.

had occurred the potential restorative effect would be negated. In the present investigation, we considered a genetic approach in which we genetically engineered fluorescent protein genes into AcMNPV with the expectation that the expressed protein would absorb some of the damaging ultraviolet radiation. Although the fusion of a red fluorescent protein (RFP) gene to the polyhedrin gene resulted in a decrease in infectivity, nevertheless it did afford some protection to the occluded derived virus. Other fusion attempts of another fluorescent gene (GFP) to the polyhedrin gene did not result in formation of OB but when native polyhedrin was introduced with the fusion gene complex normal infectious occludion bodies were formed (Je et al. 2003). In those studies the recombinant was not tested for resistance to UV-B.

To more accurately assess which of the two, GFP or RFP, gives better protection against UV light, they should be both compared on the same basis (i.e., both recombinants as fusion products of the polyhedrin gene or incorporation of GFP and RFP in the envelopes of budded virus). The excitation maxima of GFP, RFP and an enhanced blue fluorescent protein (EBFP) are 395 nm, 558 nm and 380 nm respectively. Although the mechanism of the protective effect of RFP and GFP in the present study is unknown, and even though these maxima are greater than the wavelength of UV-B (280-320 nm), nevertheless they may have an overlapping protective effect. Also, with regards to sunlight-UV, it has been reported that UV-A (320-400 nm) may also contribute to inactivation of baculoviruses (Morris 1971, Shapiro and Domek 2002) and UV-C (250-280 nm) is even more damaging to DNA.

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