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Highly efficient production of GFP and its derivatives in insect cells for visual in vitro applications

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Abstract We have generated recombinant baculoviruses for expression of the green fluorescent protein (GFP), a bright GFP mutant (S65T), and a GFP-streptavidin fusion protein in Sf9 and High Five insect cell lines. At 3–4 days post infection, about 30% of the total protein contents was represented by the recombinant protein products, giving the infected insect cells a bright green color which was clearly visible by eye in daylight. The isolated GFP-streptavidin fusion protein, which possessed fluorescence properties identical to those of the native GFP, was capable of binding biotin as shown by using biotinylated beads as well as biotinylated antibody complexes decorating surface expressed GluR-6 glutamate receptor in live and fixed insect cells. The exceptionally high expression levels of GFP and GFP (S65T) and the GFP-streptavidin fusion protein in recombinant baculovirus infected insects should facilitate production of GFP derivatives for in vitro applications.

Key words: Green fluorescent protein; Baculovirus; *Autographa californica*; Nuclear polyhedrosis virus; *Aequorea victoria*; Fluorescence; Microscopy; Expression; Streptavidin; Chimeric protein; *Spodoptera frugiperda*; *Trichoplusia ni*

1. Introduction

The green fluorescent protein (GFP) of the jellyfish, *Aequorea victoria*, has become highly attractive as a visual marker for gene expression [1,2]. The protein consists of 238 amino acids [3,4] and migrates with an apparent molecular weight of 25 kDa. The protein undergoes intramolecular cyclization resulting in a brightly fluorescent chromophore that absorbs violet light at a maximum of 395 nm and emits green light with a peak at 509 nm [5]. Functional GFP has been expressed in both procaryotic [4] and eucaryotic systems including yeast cells [6], plant cells [7,8], and animal cells [9]. The introduction of spectral mutants has further increased the use of GFP as a visual marker molecule [10–13].

Native streptavidin molecules of the soil bacterium, *Streptomyces avidinii*, are assembled to tetrameric protein complexes, where each subunit has the capacity of binding one biotin residue. The gene encoding streptavidin has been cloned and sequenced [14]. A chimeric protein, streptavidin-protein A of *Staphylococcus aureus*, has been constructed and expressed in *Escherichia coli* [15]. In addition, a fusion protein containing luciferase of the click beetle, *Pyrophorus plagiophthalamus*, and streptavidin has been recently successfully expressed in *Spodoptera frugiperda* insect cells using recombinant baculovirus technology [16].

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Most baculovirus expression vectors utilize the strong polyhedrin gene promoter of the *Autographa californica* nuclear polyhedrosis virus, AcNPV, [17–20]. The aim of the present study was to use the system in order to evaluate recombinant baculovirus infected lepidopteran insect cells for production of GFP and GFP fusion proteins to be used essentially in vitro. This was conducted by preparing three different recombinant baculoviruses containing genes encoding a) native GFP, b) a spectral GFP mutant (S65T), and c) GFP fused to streptavidin (SA).

2. Materials and methods

2.1. Cells and media

The lepidopteran insect cell lines derived from, *Spodoptera frugiperda* (Sf9: American Type Culture Collection, no. CRL1711; Rockville, MD) and *Trichoplusia ni* (High Five: Invitrogen, CA) were maintained as monolayer cultures in plastic flasks (Nunc, Roskilde, Denmark) or in suspension by using Erlenmayer flasks (250 ml) on orbital shakers (125–135 rpm) at 27°C or room temperature. Both cell lines were grown in serum free SF900 SFM II medium (Gibco BRL, Grand Island, NY). The Sf9 cells were also cultivated in TNM-FH medium (Sigma Chemicals, St. Louis, MO) supplemented with 2 mM glutamine, 10% fetal calf serum. Antibiotics (100 U/ml penicillin, 40 μg/ml streptomycin, and 2.5 μg/ml Fungizone; Gibco BRL) were added to both culture media.

2.2. Construction of plasmids

The GFP coding sequence in the plasmid TU#58 [4] was amplified by PCR [21] with primers 5′-GGTGTGCTAGCTATAAATATGAGTAAAGGAGAAG AAC-3′ (primer #1805, sense, *Nhe*I site underlined) and 5′-GGTGGTGAATTCTTATTTGTAGAGCTCATCCATGCCATGTGTAATC-3′ (primer #1804, antisense, *Eco*RI site underlined), treated with *Nhe*I and *Eco*RI and cloned into a similarly treated pFASTBAC1-derivative (Gibco-BRL; K.K., unpublished). The plasmid was designated as pK410-2. The S65T mutant of GFP was generated by PCR with primers 5′-GGTGTTCCATGGCCAACACTTGTCACTACTTTCACTTATGGTGTTCAATGCTTTTC-3′ (primer #1950, antisense, *Nco*I site underlined) and #1804 (antisense). The amplified product was treated with *Nco*I and *Eco*RI and cloned into similarly treated pK410-2. The presence of the mutation was verified by DNA sequencing and the plasmid was designated pK503-10. For generation of the GFP-streptavidin fusion protein, an *Xba*I site was introduced into the 3′-end of the GFP coding region by PCR, with primers #1805 (sense) and 5′-GTTGGTGAA TTCAAGCTTAATCCCGAAGTTCCATTTTCATGGCTTCGAAGATACGCCGAGTTCTAGACCTTTGTAGAGCTCATCCATGCC-3′ (#1855, antisense, *Xba*I site underlined). The amplified product was digested with *Nco*I and *Eco*RI and cloned into a similarly treated pK410-2 to yield plasmid pK412-1. Then, a 492-bp *Xba*I/*Hin*dIII fragment containing the sequence encoding amino acid residues 25–183 of the streptavidin of *Streptomyces avidinii* [14] was isolated from the plasmid pVL1393-LucGR-StreptAv [16], and cloned into similarly treated pK412-1. The final plasmid encoding the GFP-SA fusion protein was designated pK501-1. For details, see Fig. 1A.

2.3. Recombinant baculoviruses

The recombinant baculoviruses containing native GFP (v410-1), the spectral GFP mutant (v503-10), and the GFP-SA fusion construct

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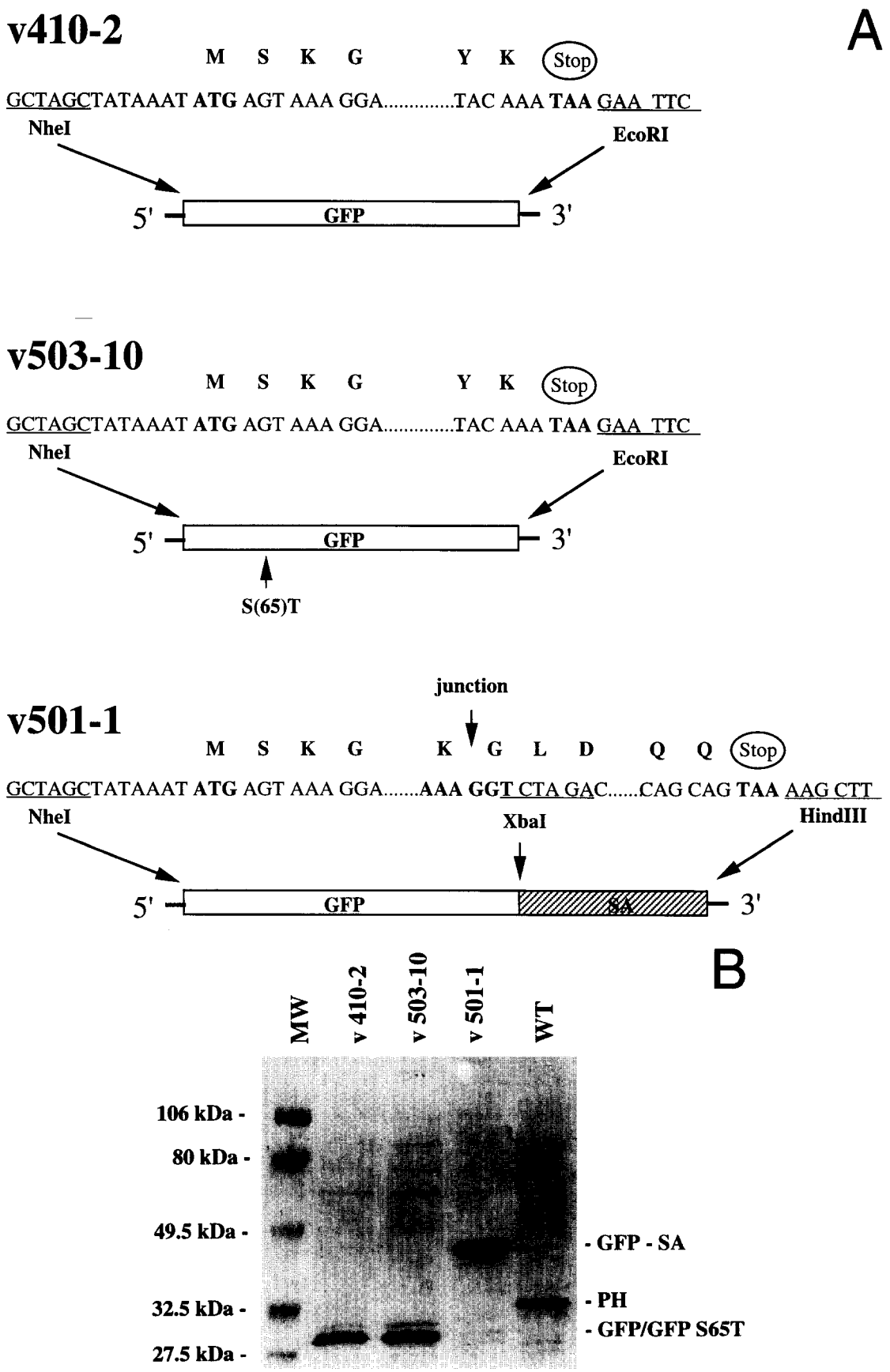

Fig. 1. (A) Genetic constructs used for generation of the recombinant baculoviruses v410-2, v503-10, and v501-1. (B) Synthesis of GFP, GFP (s65T), and GFP-SA in High Five cells during viral infection. Cellular extracts were prepared at 96 h post infection (p.i) and separated on 10% vertical SDS-PAGE slab gels followed by Coomassie blue staining. Cells infected with wild-type AcNPV, producing the polyhedrin protein of 32 kDa, was used as a control. The molecular weight markers in thousands are shown on the left.

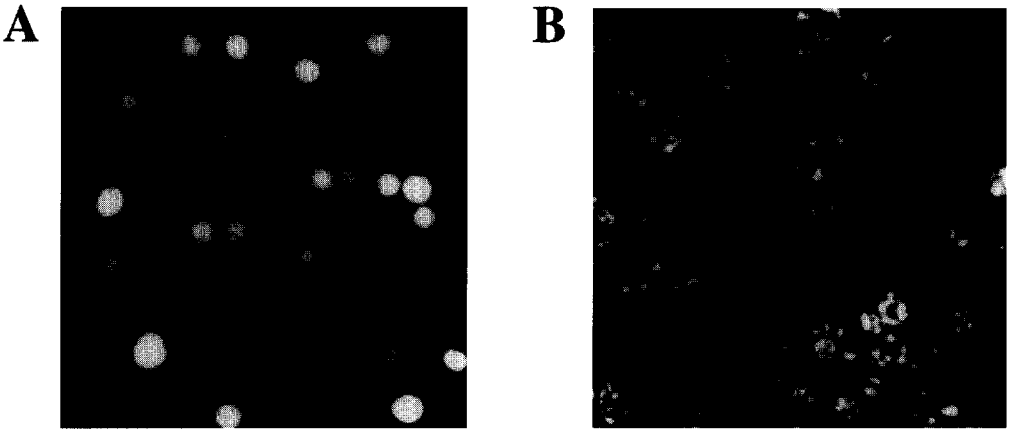

Fig. 2. Fluorescence micrographs of recombinant baculovirus infected Sf9 cells producing (A) native GFP (infected with v410-2) and (B) GFP-SA (infected with v501-1), respectively (magnification ×200).

(v501-1) under the transcriptional regulation of the polyhedrin gene promoter (PHP) were produced using the Bac-to-Bac system [22] according to the procedures described by the manufacturer (Gibco BRL). The system is based on transposon mediated insertion of the foreign genes into the baculovirus genome. Briefly, the pFastBac1 plasmid derivatives, pK410-2, pK501-1, and pK503-10 (Fig. 1) containing the genetic inserts, were used to transform *E. coli* DH10Bac by electroporation. The cells were plated on LB agar containing, ampicillin, kanamycin, and gentamycin as well as IPTG/Bluogal as described [22]. Bacmid miniprep DNAs were prepared from large white colonies and used to transfect Sf9 cells using Cellfectin (Gibco BRL). At 3–4 days post transfection, the supernatant media containing the recombinant baculoviruses were collected. Green fluorescence of the transfected cells was determined by fluorometry as described below. Propagation of the recombinant viruses as well as wild-type AcNPV (strain E2), was performed according to procedures described by Summers and Smith [17].

2.4. Fluorescence measurements

Aliquots (10–50 μl) of intact non-infected or infected cells were resuspended in water and transferred into a quartz cuvette. The fluorescence was then measured by using a Shimadzu RF 500 Fluorometer. The excitation spectra were recorded from 300 to 500 nm with a fixed emission wavelength of 510 nm. The emission spectra were measured from 420 to 600 nm with the excitation fixed at 395 nm or for the GFP mutant at 488 nm.

2.5. Fluorescence microscopy

Immunofluorescence was performed on live and fixed Sf9 or High Five cells infected with v503-16, a recombinant baculovirus containing the cDNA sequence of GluR-6 fused to the FLAG epitope [23] under the polyhedrin gene promoter of AcNPV (K.K., unpublished). Non-infected cells and cells infected with wild-type baculovirus (AcNPV: strain E2) were used as negative controls. Monolayer cultures of non-infected or infected cells in 50 ml tissue culture flasks were collected by low-speed centrifugation (2 min at 800 rpm) at 48 h p.i. Cells were either kept on ice or fixed for 30 min with 3% of paraformaldehyde in 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 250 mM HEPES, pH 7.4. Free aldehyde groups were quenched with 50 mM NH_4Cl in HBS (150 mM NaCl, 1 mM $CaCl_2$, 20 mM HEPES, pH 7.4). Samples of 10 μl, containing 80 000 cells were added to a cover slip previously coated with 10 μl of polylysine (1 mg/ml) for 30 min. Nonspecific binding of antibodies was blocked with 0.1% BSA in HBS for 30 min. Samples were incubated with primary, anti-FLAG M1 antibody (Eastman Kodak Co., New Haven, CT) at a concentration of 29 μg/ml for 90 min followed by another 90 min incubation with sheep antimouse-Ig conjugated with biotin (Bio-Rad, Richmond, CA,) at a concentration of 20 μg/ml. 10 μl of partially purified GFP-SA (0.18 mg/ml), produced in Sf9 cells infected with the recombinant baculovirus v501-1, were further added to the cell samples for 30 min. After each step, at least 3 washes with HBS were carried out. Cells were finally mounted in Mowiol (Calbiochem, San Diego, CA) for fluorescence microscopy. The samples were viewed with an Olympus research microscope, Model AHB5 equipped with a fluorescence attachment AH2-RFL and a FITC filter set and the cells photographed using Kodak 400 ASA film.

The live cells, kept on ice, were incubated for 45 min with the primary (29 mg/ml) and secondary (2 mg/ml) antibodies, respectively. Unspecific binding was blocked with 0.2% BSA in HBS for 30 min. GFP-SA (0.18 mg/ml) was finally added and incubated for 30 min. After each step, the cells were carefully pelleted and washed with Tris-buffered saline (TBS).

2.6. Binding to biotinylated agarose beads

Agarose beads labelled with iminobiotin (Sigma Chemicals, St. Louis, MO) were incubated in cytoplasmic extracts prepared from High Five cells infected with the recombinant viruses as well as with wild-type AcNPV.

Samples were agitated for 30 min at room temperature. The beads were collected by brief centrifugation using 1.5 ml Eppendorf tubes and washed several times with PBS containing 1% Triton X-100.

2.7. SDS-PAGE and immunoblotting

Immunoblot procedures were performed essentially as described by Towbin and co-workers [24]. High Five or Sf9 cells infected with the recombinant baculoviruses 410-2, 501-1, 503-10, and 503-16, as well as with wild-type AcNPV at a multiplicity of infection (MOI) of 5–10 plaque forming units (PFU) per cell were collected at 2–4 days post infection by low speed centrifugation at 800 rpm for 10 min. Cytoplasmic extracts were prepared by solubilizing the cells (5×10^4 cells/10 μl) in sample buffer in the absence (non-reducing conditions) or presence (reducing conditions) of β-mercaptoethanol [24]. The reduced samples were boiled for 3 min whereas the non-reduced ones were analyzed unboiled. Proteins were separated by electrophoresis on 10% SDS-polyacrylamide slab gels along with molecular weight markers (Bio-Rad). The proteins were either visualized by Coomassie Brilliant Blue or transferred to nitrocellulose for western blot analysis. The Coomassie blue stained gels were analyzed by scanning using an ImageMaster™ (Pharmacia Biotech, Uppsala, Sweden). In blots, non-specific binding was blocked with either 3% non-fat milk or 1% BSA in TBS (10 mM Tris-HCl, 150 mM NaCL, pH 7.4). Alkaline phosphatase conjugated with biotin (0.38 g/ml, New England Biolabs) was used for detection of the GFP-SA fusion protein whereas GluR-6 was detected using the M1 monoclonal anti-FLAG antibody (Scientific Imaging Systems) and sheep anti-mouse-Ig conjugated with alkaline phosphatase (Bio-Rad).

3. Results

3.1. Expression of GFP, GFP S65T, and GFP-SA

High Five cells infected with v410-2, v503-10, and 501-1 (Fig. 1A) as well as with wild-type AcNPV (strain E2) were collected at 96 h post infection The protein contents were analyzed by SDS-PAGE and Coomassie blue staining (Fig.

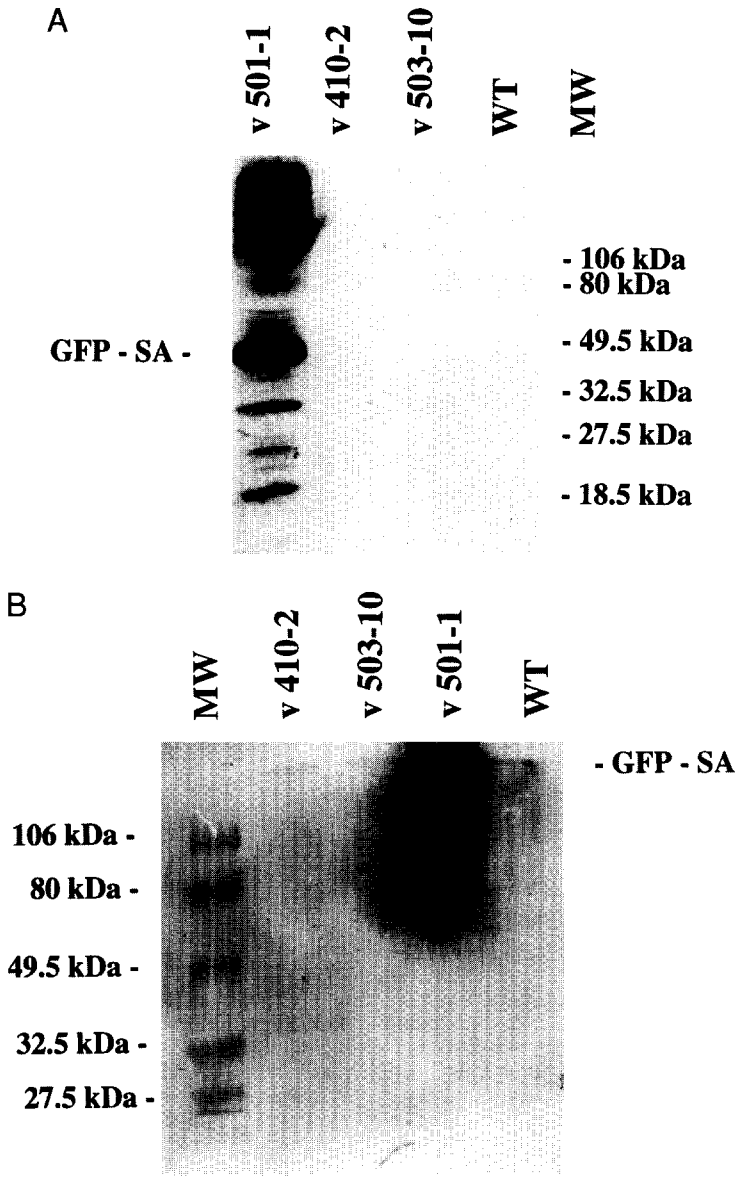

Fig. 3. Western blot analysis of v410-2, 503-16, and 501-1 infected High Five cells producing GFP, GFP S65T, and GFP-SA, respectively. Cells were collected 48 h after infection and proteins were either reduced with β-mercaptoethanol (A) or analyzed non-reduced (B). Cells infected with wild-type AcNPV were used as a control. The molecular weight markers in thousands are shown or indicated (for details, see Section 2).

1B). In all cases, a polypeptide of the expected size was the major species in whole cell extracts. Recombinant GFP and GFP S65T migrated with apparent molecular weights of about 27 kDa, respectively, whereas the chimeric protein product, GFP-SA, had an apparent molecular weight of 45 kDa. The intensity of all three recombinant protein products was comparable to that of polyhedrin (32 kDa) expressed by the wild-type AcNPV infected cells (Fig. 1B). Scanning of the Coomassie blue stained gels revealed that the recombinant protein products amounted to 25–30% of the total cellular protein. None of the indicated proteins were seen in the mock-infected cells (data not shown).

Cells infected with the recombinant viruses were further studied by fluorescence microscopy at 48 h post infection (Fig. 2A) Recombinant GFP produced by cells infected with v410-2 was found to be distributed throughout the cytoplasm, whereas the fusion protein produced by v501-1 appeared to form intensely fluorescing granules within the infected cells. The fluorescence spectra of the recombinant GFP and the GFP-SA were identical as compared to each other and indistinguishable from that described for native GFP [4], whereas the S65T mutant of GFP [13] was different, but identical to that described for the corresponding protein (data not shown).

3.2. *Binding of GFP-SA to biotin*

Total homogenates of High Five cells infected with v410-2, v503-10, and v501-1 as well as with wild-type AcNPV (strain E2) were separated by SDS-PAGE, and transferred to nitrocellulose. Detection of the proteins was carried out by using biotinylated alkaline phosphatase. In samples reduced with β-mercaptoethanol, a protein migrating with an apparent molecular weight of 45 kDa was clearly identified from cells expressing the GFP-SA fusion protein, whereas no bands were visualized in samples from cells expressing GFP, GFP S65T or polyhedrin (Fig. 3A). This is in agreement with the theoret-

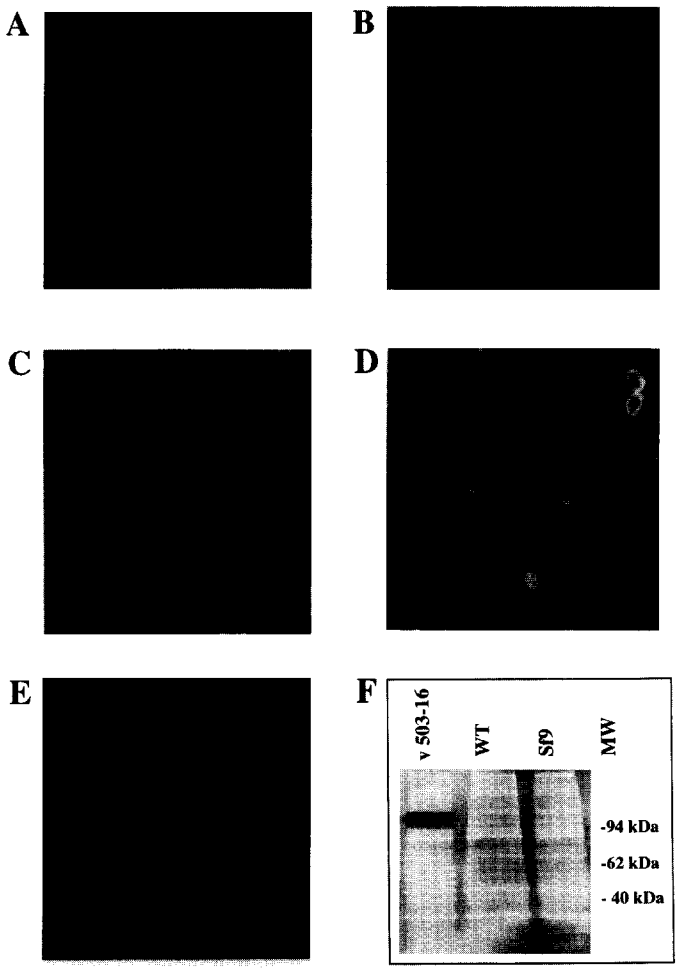

Fig. 4. Fluorescence micrographs of (A) agarose beads coated with iminobiotin after incubation in partially purified GFP-SA produced in High Five cells, (B) agarose beads coated with iminobiotin after incubation in partially purified native GFP produced in High Five cells, (C) fixed Sf9 cells expressing GluR-6 after immunological detection with mouse anti-FLAG antibodies, biotinylated anti-mouse antibodies, and the GFP-SA fusion protein, (D) fixed Sf9 cells expressing GluR-6 after treatment with Triton X-100 and immunological detection with mouse anti-FLAG antibodies, biotinylated anti-mouse antibodies, and the GFP-SA fusion protein, (E) fixed Sf9 cells infected with wild-type AcNPV after identical treatment to that in C (magnification ×200).

ical size of the corresponding chimeric protein where streptavidin has a molecular mass of 15 kDa and GFP of about 26 kDa. Under non-reducing conditions, the fusion protein migrated with a molecular mass of over 180 kDa (Fig. 3B) These results show that the binding of biotin is specific for the fusion protein and that the corresponding protein is capable of forming tetramers (4×45 kDa) which is characteristic for authentic streptavidin.

Binding of the fusion protein, GFP-SA, to immobilized biotin was further analyzed by incubating biotinylated agarose beads with cytoplasmic extracts prepared from High Five cells expressing GFP, GFP (S65T), and GFP-SA. As can be seen from Fig. 4A, beads incubated in lysates containing the fusion protein, GFP-SA, had a bright green color, whereas beads incubated in lysates containing native GFP (Fig. 4B), the spectral mutant of GFP (S65T), or polyhedrin (produced by wild-type AcNPV), were invisible by fluorescence microscopy (data not shown).

3.3. Visual detection of biotinylated molecules using GFP-SA

To examine the use of the GFP-SA fusion protein as a fluorescent reagent, Sf9 and High Five cells were engineered for surface expression of FLAG epitope-tagged [23] glutamate receptor protein, GluR-6 (K.K., unpublished). Cells expressing GluR-6 and non-infected or AcNPV infected control cells were incubated with monoclonal anti-FLAG antibody followed by biotinylated anti-mouse antibody and finally with partially purified GFP-SA. The membrane bound GluR-6 protein was clearly visible as a rim of green fluorescence at the surface of intact recombinant virus infected cells (Fig. 4C), whereas the green fluorescence was almost evenly distributed when cells had been permeabilized with Triton X-100 (Fig. 4D). Cells infected with wild-type AcNPV or non-infected cells were not visible in the fluorescence microscope (Fig. 4E). The positive reactivity of the anti-FLAG antibody with GluR-6 was also confirmed by immunoblot analysis with the same samples. The recombinant protein product migrated with an apparent molecular weight of 110 kDa whereas the wild-type and mock-infected cells showed no reactivity (Fig. 4F).

4. Discussion

The aim of the present study was to evaluate the use of

GFP as a reporter and fusion partner for baculovirus infected insect cells. The experimental design was focused on the capability of insect cells to produce abundant amounts of protein and on examining the possibility of using GFP derivatives as fluorescent reagents in in vitro applications, especially in fluorescence microscopy.

Three different recombinant baculoviruses encoding native GFP, a spectral mutant of GFP and a GFP-streptavidin fusion protein were engineered. The recombinant proteins were expressed at exceptionally high levels, equivalent to those of polyhedrin produced by wild-type baculovirus infected cells [17]. According to scanning analysis of Coomassie blue-stained SDS-polyacrylamide gels, the recombinant GFPs and GFP-streptavidin fusion protein represented about 30% of the total protein in the infected cells, amounting several hundreds of micrograms per millilitre of cell culture. Functional expression of GFP in insect cells and the sensitive methods by which the fluorescence can be monitored in real time, should make it very useful for solving problems related to baculovirology, such as gene promoter activity studies and viral distribution within the organism. Recently, GFP has been expressed in Sf9 cells [25] as well as in larvae of a lepidopteran moth, *Plutella xylostella* [26] by using recombinant baculovirus. In addition, bacterial expression of a fusion of GFP and protein A was recently described [30].

The high affinity binding of streptavidin to biotin is widely exploited in biotechnological and biomedical applications [27]. In addition, the corresponding gene has been expressed in both *E. coli* [15,28] and in *Bacillus subtilis* [29]. Streptavidin was therefore considered attractive as a fusion partner for GFP. The functionality of the GFP-streptavidin fusion protein was demonstrated by its binding to biotinylated agarose beads. In the fluorescence microscope, beads were highly fluorescent after exposure to lysates containing the fusion protein, whereas those exposed to native GFP or the spectral mutant thereof, were not fluorescent.

The biotin-binding activity of the isolated GFP-streptavidin fusion protein was exploited as a fluorescent reagent in biotinylated antibody-mediated visualization of a cell surface protein. For this purpose, FLAG-epitope tagged GluR-6 glutamate receptor was expressed in Sf9 cells, and the receptor was detected with an anti-FLAG antibody on both fixed and unfixed cells. The monoclonal mouse antibodies were then allowed to react with biotinylated anti-mouse immunoglobulins. The immunocomplexes were finally visualized with the fusion protein, which gave the GluR-6 expressing cells a bright green fluorescence. The staining appeared to be highly specific and sensitive since uninfected cells or cells infected with wild-type baculovirus gave almost no background fluorescence. The GluR-6 protein was easily visualized on the surface of the intact cells, whereas permeabilization with Triton X-100 resulted in a more even distribution of the green fluorescence as expected. Very little fading was observed and the partially purified protein retained its biological properties even after several months at +4°C or −20°C.

Together, the data presented here show that recombinant baculoviruses can be used to produce exceptionally high levels of functional GFP and fusion protein containing the entire coding sequences of GFP and streptavidin. GFP-streptavidin should be useful as a fluorescent reagent in microscopy and other analytical applications.

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