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Functional Expression of the *Aequorea victoria* Green Fluorescent Protein in Insect Cells Using the Baculovirus Expression System

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A DNA fragment encoding the green fluorescent protein (GFP) was isolated via PCR from a jellyfish *Aequorea victoria* cDNA, cloned and sequenced. Subsequently, a recombinant baculovirus bearing the coding region of the GFP under the transcriptional control of the *Autographa californica* nuclear polyhedrosis virus (*Ac*MNPV) polyhedrin gene promoter was constructed and isolated. High-level expression of GFP could be easily monitored in *Spodoptera frugiperda* (Sf9) insect cells after infection with recombinant baculovirus, due to the intrinsic fluorescence (λ_{max} = 508 nm) of the recombinant protein after excitation with blue light (λ_{max} = 400 nm). The functional recombinant GFP displayed an apparent molecular mass of ≈43 kDa and the fluorescence emission spectrum of the recombinant protein was virtually identical to that of the native green fluorescent protein.

Green *in vivo* light emission from the margin of the umbrella in the jellyfish *Aequorea victoria* is accomplished by the interaction of two proteins: aequorin, a Ca^{2+}-binding protein (21.4 kDa) and a green fluorescent protein (GFP; 27 kDa) [1]. GFP emits green light (λ_{max} = 509 nm) when it absorbs violet/blue light maximally at 395 nm. In jelly fish the excitation of GFP has been proposed to occur by radiative energy transfer from the Ca^{2+}-activated photoprotein aequorin [2]. The GFP-gene has been cloned and sequenced [3] and has already been successfully tested as a reporter for monitoring gene expression *in vivo,* in situ and in real time [4–8]. The advantage of GFP as a reporter protein is that its function is not dependent on other proteins, substrates or cofactors. The unique chromophore structure of GFP is composed of a cyclic tripeptide (serine-dehydrotyrosine-glycine) which is covalently linked through the protein's peptide backbone [9, 10]. When GFP is expressed in either eukaryotic and prokaryotic cells, strong intrinsic fluorescence can non-invasively be monitored after illumination by blue- or UV light.

Autographa california multiple nuclear polyhedrosis virus (AcMNPV) is the prototype virus of the *Baculoviridae* family [11] and has become famous as a system for the heterologous production of foreign proteins [12]. The late gene coding for polyhedrin is not essential for the survival of the virus in cell culture and therefore its strong promoter has been successfully used for the heterologous expression of a variety of foreign genes [12–17].

In this study, the ability of GFP as a reporter was tested in the *Spodoptera frugiperda* (Sf9) insect cell line infected with a respective recombinant baculovirus. The results presented reveal that the cells are excellent high level producers of the GFP, and because of the sensitive monitoring of the activity of GFP, this protein should be useful as a reporter protein for either analyzing baculovirus gene expression as well as for the screening and selection of recombinant baculoviruses.

MATERIALS AND METHODS

Strains and transformations. E. coli strain XL-1 Blue (recA1, endA1, gyr96, thi-1, hsdR17, supE44, relA1, lac-, (F'proAB, lacI^{q}ZδM15, Tn10(tet^r)) (Stratagene) was used for propagation of the recombinant plasmids. For *E. coli* transformation, the $CaCl_2$ procedure was used as described [18].

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procedures were performed using established techniques [18, 19]. All plasmid constructs were ensured by DNA sequencing using the dideoxy-termination method [20]. Two oligonucleotide primers (GFPfor: 5′-GC*TCTAGA*AATAAAGGAGATATACATATGAG-TAAAGGAGAAGAACTTTTC-3′; GFPrev: 5′-GGAATTC*AAGCTT*ATTTGTATAGTTCATCC-ATGCC-3′; restriction sites used for cloning are underlined) were designed according to the published sequence of GFP [3] and synthesized using an Applied Biosystems DNA Synthesizer. These primers were used in a polymerase chain reaction to amplify the GFP gene from first strand cDNA of *Aequorea victoria* which was kindly provided by Dr. S. Thulin (Novo Nordisk, Copenhagen, Denmark).

Cell culture of insect cells. Spodoptera frugiperda (Sf9) cells were routinely propagated in cell culture flasks at 27°C in TNM-FH medium (= Graces insect medium supplemented with 3.3 g/l of Tc-yeastolate and 3.3 g/l lactalbumin hydrolysate (DIFCO, Nordwald, Hamburg)) supplemented with 5% fetal calf serum and 50 μg/ml gentamycin [21, 22]. Suspension cultures of Sf9 cells were maintained in 100 ml or 250 ml spinner flasks (Techne, England) which were stirred at 70 rpm in culture medium supplemented with 0.2% Pluronic F-68 (kindly provided by BASF, Ludwigshafen, Germany). For small scale production of GFP, insect cells at a density of 2×10^6 per ml or 2×10^6 per tissue culture flask were infected with recombinant baculovirus at a multiplicity of infection (m.o.i.) of 5–10 and harvested 72 h after infection.

Generation of recombinant baculoviruses. The recombinant plasmid pVL92GFP was purified by a CsCl density gradient and used for the co-transfection of Sf9 insect cells for the generation of the recombinant baculovirus. Co-transfections of Sf9 cells with 5–10 μg of the recombinant plasmid and 1 μg of linearized GigaGold baculovirus genomic DNA (*Autographa californica* GigaGold; Pharmingen) were mediated by cationic liposomes (Lipofectin; GIBCO) according to the manufacturer's specifications. The transfection mixture was added to 5×10^6 cells in a cell culture flask in Grace insect cell medium which after 4 hours was replaced by TMN-FH medium containing 5% foetal calf serum. After 4–5 days of incubation at 27°C the virus was collected. Cell culture dishes (60 mm diameter) were seeded with 3×10^6 Sf9 cells and after the medium had been removed these cells were incubated for 1 hour at 27°C with various dilutions of the baculovirus recovered from the supernatant of the co-transfections. After removing the virus, cells were overlaid with medium containing 1.5% low melting point agarose (GIBCO) and then incubated at 27°C for 3 days. After occurrence of the first visible plaques a second overlay with 1.5% low melting point agarose containing XGal was put onto the first overlay. Colourless plaques which could be visually discriminated after another day of incubation were picked and virus was eluted in 200 μl of medium. This baculovirus containing medium was used to infect 1×10^6 Sf9 cells in a 6 well multidish. The cells were daily examined for signs of infection. After 5 days of incubation at 27°C the virus in the supernatant was saved and genomic DNA was prepared from the infected cells and used for Southern hybridization using a digoxigenine labeled DNA-fragment enclosing the GFP-coding region. Additionally, the correctness of the recombinant baculoviruses was ensured by PCR using either genomic DNA or culture supernatant from infected insect cells [23]. A stock of the recombinant virus, which was named AcpVLGFP according to the plasmid used for transfection, was prepared by infecting Sf9 cells in 30 ml of medium in a large cell culture flask at a multiplicity of infection (m.o.i.) of 0.1 and subsequently incubated for 7 days at 27°C. Supernatant was collected, titered and stored in the dark at 4°C. A small aliquot of the virus was stored at −80°C.

Detection of recombinant GFP and measurement of fluorescent spectrum. Heterologous expression of GFP in Sf9 insect cells was analyzed with a fluorescence microscope (Axiophot, Zeiss, Oberkochen, Germany) equipped with an epifluorescence device and using the filter set BP395-440, FT460, LP470 (Zeiss). The fluorescence emission spectrum of soluble proteins prepared from insect cells producing GFP was measured with a luminescence spectrophotometer (Perkin Elmer LS50). For this measurement infected insect cells were pelleted and the pellet was resolved in 1/10 vol. of TE-buffer. Cells were lysed by several bursts with a sonicator and soluble proteins were separated from the membranes by a high speed centrifugation step.

Protein analysis. Insect cells were counted in a hemocytometer and 1×10^5 cells were pelleted and resolved in SDS-sample buffer. SDS-PAGE was carried out under non-reducing conditions using a 10% gel according to Laemmli [24]. After electrophoresis the gels directly were examined under UV-light (λ_{max} = 395 nm) for intrinsic fluorescence of the recombinant GFP. Afterwards, gels were stained with Coomassie brillant blue R250 (BioRad).

RESULTS AND DISCUSSION

GFP has already been successfully used as a reporter in several divergent organisms, including bacteria, fungi, invertebrates (Drosophila, *C. elegans*), vertebrates, and plants [4–8]. My intention was to test, if the GFP also can be used as a reporter in the baculovirus expression system.

To obtain a DNA fragment encoding for GFP a PCR on first strand cDNA from *Aequorea victoria* was performed. The two PCR-primer GFPfor and GFPrev containing a *Xba*I and a *Hin*dIII restriction site, respectively, were used to amplify the gene from first strand cDNA and after 30 cycles a ≈700 bp DNA-fragment could be detected in an agarose gel. The amplified fragment was restricted with *Xba*I and *Hin*dIII, isolated from a gel, ligated into appropriate cleaved vector pBluescriptSK+ and transformed into *E. coli* XL-1Blue. The primers which were used for amplification were designed in the way that after cloning of the PCR fragment into pBluescript, a

bicistronic mRNA (lacZ, GFP) could be transcribed from the inducible lac-promoter located on vector pBluescriptSK+. Therefore, one hundred recombinant colonies were picked from the transformation plates and striked onto IPTG containing plates for induction of the lac-promoter. Plates were cultured at 37°C overnight and after two days at 4°C, they were tested for expression of the GFP by illumination with a UV handlamp (λ = 366 nm). Six clones bearing an amplified fragment encoded a functional protein which could be easily detected by the intrinsic green fluorescence of the colonies on the plates. From one clone named pKS + GFP4 the complete DNA insert was sequenced. The amino acid sequence which could be deduced from the complete nucleotide sequence was identical to the amino acid sequence of GFP reported previously [3]. Therefore, the ≈700 bp *Xba*I/*Hin*dIII DNA fragment from pKS + GFP4 encoding the GFP was cloned into appropriate restriction sites of baculovirus vector pVL1392 where the gene was now under transcriptional control of the strong viral polyhedrin promoter. The resulting plasmid pVL92GFP (Fig. 1) was co-transfected with chromosomal GigaGold-baculovirus DNA into Sf9 insect cells and the supernatant of the transfection was saved after seven days of incubation at 27°C.

The main step in the isolation procedure of a recombinant baculovirus is the so-called 'plaque assay' where a cell lawn of Sf9 cells is overlayed with soft agarose after infection with a certain dilution of the virus containing supernatant from the initial co-transfection. After two to three days plaques develop due to the stop in cell growth of infected insect cells which then can be picked after visual inspection for further propagation. Even when non-recombinant plaques can be stained blue

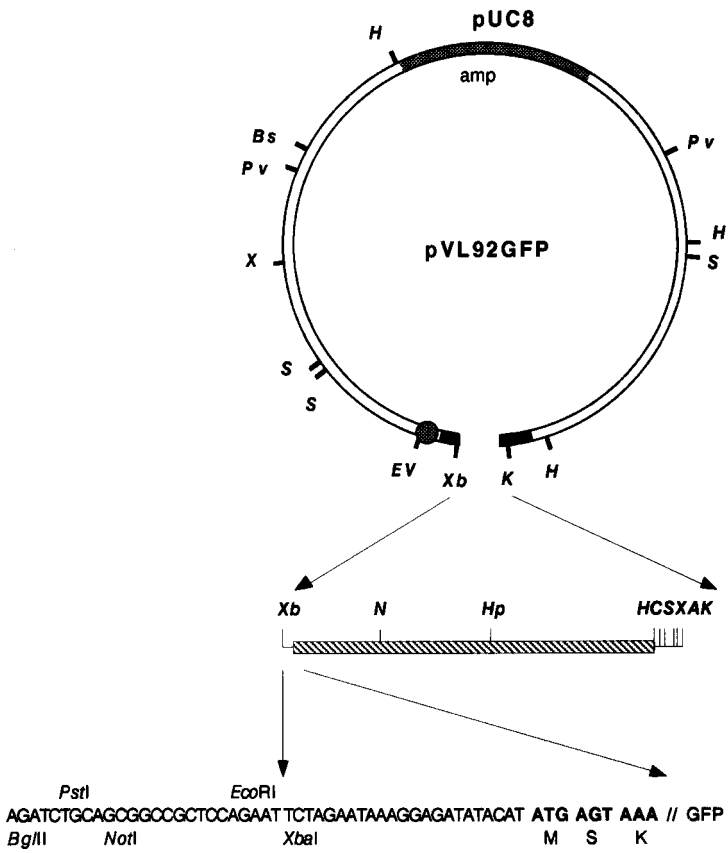

FIG. 1. The transfer plasmid pVL92GFP constructed for the generation of recombinant baculovirus. The GFP coding region was cloned under the transcriptional control of the strong viral polyhedrin promoter. Restriction sites given are: **Bs,** *Bst*EII; **EV,** *Eco*RV; **H,** *Hin*dIII; **Hp,** *Hpa*I; **K,** *Kpn*I; **N,** *Nco*I; **Pv,** *Pvu*II; **S,** *Sal*I; **X,** *Xho*I; **Xb,** *Xba*I.

by addition of XGal (see material and methods), the recognition of recombinant plaques tends to be cumbersome. Still a trained eye is necessary to distinguish between 'real' plaques and artifacts when the assay is not optimal due to too high or too low cell densities seeded into the plates before transfection. Previously, there has been a report where a luciferase gene from Jamaican click beetle, *Pyrophorus plagiophthalamus* has been favoured as a reporter for the detection of recombinant plaques [25]. Luciferase is the only reporter gene whose activity can be quantified using non-invasive methods. Nevertheless, for *in vivo* detection of luciferase bioluminescence the substrate luciferin has to be supplied to the cell culture. The non-invasive method of detecting the expression of GFP here probably offers great potential for using it as a screenable marker in the plaque assay. Therefore, plaques emerging after two days incubation with the virus supernatant of the GFP-transfections were examined in a fluorescence microscope. As shown in figure 2, positive plaques which form when cells have been infected with recombinant baculovirus present in the supernatant of the transfection, can easily be identified in the microscope due to the intensive intrinsic fluorescence of the infected insect cells in the center of 'mature' as well as developing plaques (Fig. 2A). Positive plaques and even separately growing single cells which have been infected by the recombinant baculovirus can clearly be discriminated from non-recombinant plaques and non-infected cells when simultaneously illuminated by transmission (phase contrast modus) and epi-fluorescent light (Fig. 2B). In conclusion, this approach in comparison to the classical plaque screening procedure which has been described in the material and method section allows quick and reliable distinction and isolation of recombinant virus from positive plaques.

Non-infected Sf9 cells as well as Sf9 cells which had been infected with wild-type baculovirus, GigaGold baculovirus and recombinant baculovirus were collected and total protein was analyzed by SDS-PAGE. After illumination of the SDS-gel with UV light of wavelength 395 nm the recombinant GFP could be detected by its green fluorescence directly in the gel (Fig. 3). No

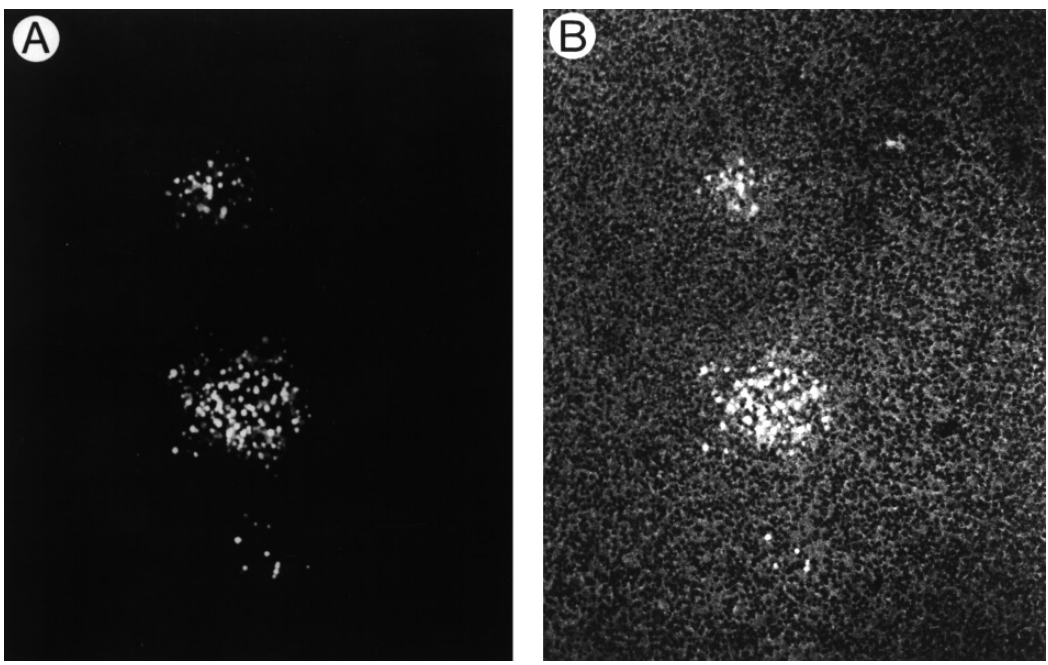

FIG. 2. Expression of the GFP in living Sf9 insect cells after infection with recombinant baculovirus. After infection with baculovirus from transfection supernatant, Sf9 insect cells were overlayed with agarose after two days and developing plaques were analyzed (**A**) by fluorescent light only and (**B**) by simultaneous application of transmission phase contrast and epifluorescent light.

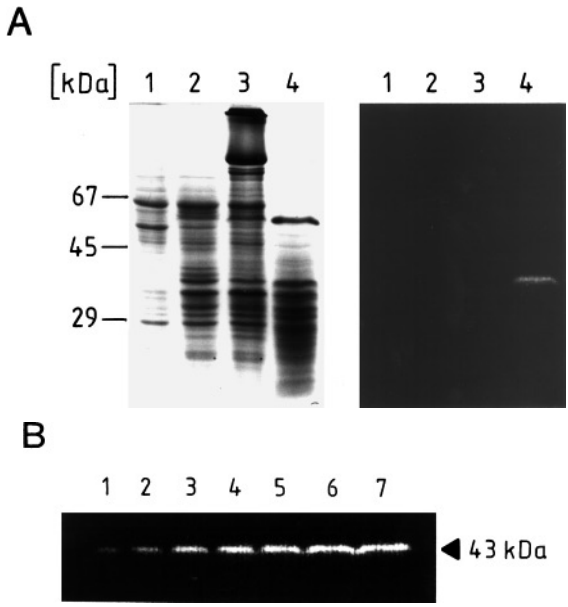

FIG. 3. SDS–PAGE and fluorescence analysis of heterologously produced GFP. Soluble proteins from (lane 1) non-infected Sf9 cells, (lane 2) Sf9 cells infected with wild-type baculovirus, (lane 3) Sf9 cells infected with GigaGold baculovirus, (lane 4) Sf9 cells infected with recombinant baculovirus. (**A, left panel**) SDS-gel stained with Coomassie brillant blue R250, (**A, right panel**) same gel prior to staining. The gel was examined with UV light (λ_{max} = 395 nm). (**B**) Dose dependence of GFP fluorescence. Soluble protein corresponding to 50000, 75000, 100000, 125000, 150000, 175000 and 200000 infected insect cells was applied to a 10% SDS-gel and analyzed as described above.

fluorescent band could be detected in the lanes where the controls had been applied to the gel. The recombinant GFP displayed an apparent molecular mass of ≈43 kDa, whereas the calculated molecular mass was ≈27 kDa. However, it is already known, that native GFP tends to self-association which is in agreement with the assumption that the 43 kDa band most likely represents a partially denatured GFP-dimer [6]. As expected, on complete denaturation of the proteins due to subsequent Coomassie staining of the SDS-gel, fluorescence of the band vanished. In the stained gel a band with an apparent molecular mass of ≈29 kDa which corresponds to the monomer of GFP, can be detected only in the lane where the GFP producing cells had been applied. The fluorescent form of GFP in the molecular mass range of 43 kDa can not be distinguished from other viral coded proteins in the same molecular mass range.

To test the dose dependence of GFP fluorescence, varying numbers of infected insect cells were applied to SDS-PAGE and subsequently the gel was analyzed in UV light as described above. As can be seen in figure 3B, the intensity of the GFP-fluorescence was directly proportial to the number of cells applied to the gel.

To more carefully study the recombinant GFP, soluble proteins from infected Sf9 insect cells were prepared and a fluorescence emission spectrum was recorded on a fluorescence spectrophotometer. As presented in figure 4, the fluorescence emission maximum of the crude protein solution was at ≈509 nm, with a shoulder at ≈545 nm. The observed maximum and also the shape of the spectrum were virtually the same as that described for native GFP [26, 27] as well as for recombinant GFP [5].

In conclusion, the GFP from *A. victoria* could be successfully expressed via a recombinant baculovirus in insect cells. This indicates that the GFP might be a useful reporter for the *in vivo* analysis of gene expression as well as for screening and selection of recombinant baculovirus in this expression system. It should also be possible, to sort GFP producing insect cells via fluores-

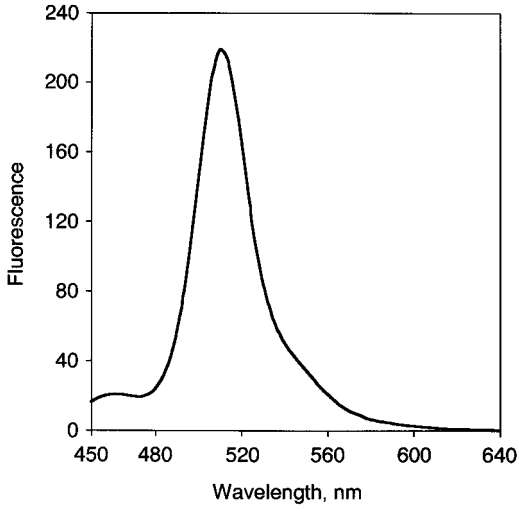

FIG. 4. Fluorescence emission spectrum of soluble protein prepared from GFP producing Sf9 cells. Fluorescence intensity is given in arbitrary units and the spectrum is uncorrected. Excitation wave length was set at 400 nm.

cence-activated cell sorting (FACS) device thereby circumventing the time-consuming and tedious plaque screening. Therefore, two types of baculovirus transfervectors have to be developed: (1) vectors which allow the high-level expression of a desired foreign gene cloned under the control of the polyhedrin promoter and which simultaneously coexpress the GFP, therefore allowing quick isolation of recombinant virus as described above, and (2) vectors which can be used for the construction of N- and C-terminal GFP fusion proteins. Both vector types are under development.

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