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Inducible expression of green fluorescent protein within channel catfish cells by a cecropin gene promoter

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Abstract

The activity of an insect promoter of the cecropin B gene (*Cec B*) was investigated using green fluorescent protein (*gfp*) as a reporter in cells of channel catfish (*Ictalurus punctatus*). The expression vector pQZ-1 containing the *Cec B* promoter and a modified *gfp* cDNA sequence was delivered by lipofection to three catfish cell types: fibroblast and leukocyte cell lines, and primary cultures of leukocytes. No resistance genes were included in the vector for selection of GFP-expressing cells. The GFP mRNA was detected in all three cell types with 5 to 10 times higher concentrations observed in leukocytes than in fibroblasts. Expression was enhanced with the addition of irradiated *Flavobacterium columnare* (7.0×10^6 cells/ml) or *Escherichia coli* LPS (125 μg/ml). Quantitative RT-PCR showed GFP mRNA reached maximum levels 24 h after bacterial challenge in fibroblast cells, and at 10–12 h after LPS challenge in fibroblasts and leukocytes. The number of fibroblasts expressing GFP increased by 0.8%, and the average of green fluorescence intensity increased by 52.8%, whereas the increase in leukocytes was 0.13% in cell number and 3.4% in fluorescence intensity. These results suggest that the transcription of the *Cec B* promoter in channel catfish cells exhibited an inducible pattern and could be placed under the control of the immune system (in vivo). The mechanisms for endogenous activation of the *Cec B* promoter and for production of *gfp* RNA in unchallenged cells remain to be studied.

Keywords: *Cec B*; *gfp*; Lipofection; *Ictalurus punctatus*

1. Introduction

Although many regulatory DNA sequences have been tested to produce transgenic fish (Iyengar et al., 1996), none can be activated by pathogenic substances or environmental stressors. The cecropins are one of four major families of lytic peptides in insects to produce a non-specific acute phase response to invading pathogens (Boman and Hultmark, 1987). Cecropin B, native in the giant silkmoth *Hyalophora cecropia*, was found to be one of the lytic peptides with antibacterial activity produced in response to injection of live bacteria (Xanthopoulos et al., 1988). The inducible features of insect cecropin genes were demonstrated in vitro with the addition of bacterial substances such as LPS to a cultured blood cell line (mbn-2) of *Drosophila melanogaster* (Samakovlis et al., 1992). Selective expression and acute phase response of the cecropin gene promoter presents an important application in transgenic studies. In addition, a combination of this promoter with a reporter gene can be used for studying processes such as development of the animal immune system.

The GFP, originally isolated from the jellyfish *Aequorea victoria*, is a useful reporter molecule for monitoring regulation of promoter activity in a host cell line (Chalfie et al., 1994). The fluorescence emitted by

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Abbreviations: APR, acute phase response; Bac, irradiated bacteria; bp, base pair(s); cDNA, DNA complementary to mRNA; *Cec B*, cecropin B gene; CMF-PBS, Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline; con ctl, contamination control; (−) ctl, negative control; FITC, fluorescein isothiocyanate; GFP, green fluorescence protein; *gfp*, gene encoding green fluorescence protein; IS, insertion sequence; Lac Z, gene encoding β-galactosidase; LPS, lipopolysacchride; M, DNA marker, PI, propidium iodide; RT-PCR, reverse transcription-polymerase chain reaction.

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GFP can be visualized without addition of exogenous substrates and, therefore, is a real-time indicator of protein synthesis. Furthermore, results from a variety of studies show that this gene with minor changes can be expressed in cells from diverse organisms, ranging from bacteria (Chalfie et al., 1994), to insects (Plautz et al., 1996), to fish (Long et al., 1997), to human (De Giorgi et al., 1996).

Channel catfish is the most important food fish cultured in the United States, with an economic value of about 297 million dollars in 1997 (USDA, 1997). Although this industry has experienced rapid growth in the past 15 years, the annual loss of channel catfish due to bacterial diseases poses a tremendous problem for catfish farmers (Thune et al., 1993). Production of disease-resistant catfish by vaccination, hybridization between species, selective breeding, and gene transfer has therefore become an important research topic for the industry. Previous studies have demonstrated that native and synthetic lytic peptides are capable of killing pathogenic bacteria of channel catfish (Kelly et al., 1990). The objectives of the present study were to investigate the function and inducibility of an insect acute phase promoter in different channel catfish cell types and to evaluate the feasibility of using GFP as a reporter gene in channel catfish cells.

2. Materials and methods

2.1. Vector construction

An expression vector designated pQZ-1 was constructed for use in this study (Fig. 1). The plasmid contained a *Cec B* promoter sequence from the giant silkmoth cecropin (Xanthopoulos et al., 1988) and a modified GFP cDNA sequence removed from plasmid vector pS65T-C1 (Clontech Laboratories, Palo Alto, CA). The promoter sequence (from −644 to 63) was amplified by PCR with an upstream primer 1 and downstream primer 2 (Fig. 2a). A *Bam*HI restriction site was added at the 5′-end of the upstream primer and the *Age*I and *Hin*dIII sites added at the 3′-end of the downstream primer to allow cloning in the correct orientation. The PCR products were subcloned into pBluescript SK (±), and the nucleotide sequence analyzed with a Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City, CA) and an ABI Prism™M 310 Genetic Analyzer (Perkin Elmer). The *gfp* cDNA sequence consisting of the gene encoding red-shifted green fluorescent protein and a SV40 mRNA polyadenylation signal was removed from pS65T-C1 with restriction enzymes *Age*I and *Afl*II. The *gfp* gene and *Cec B* promoter were ligated in frame using the *Age*I restriction site. The ligated product was inserted into the frame of a mini Tn*10* transposon derived from pNK2859 (Kleckner et al., 1991) with the kanamycin-resistance gene removed by enzyme *Bam*H I. The resulting vector was electroporated to *Escherichia coli* DH5α cells by BioRad Gene Pulser (model 1652076) and Pulser Controller (model 1652098, BioRad Laboratories, Hercules, CA) with the following settings: voltage, 2.0 kV; resistance, 200 Ω; and capacity, 25 μF.

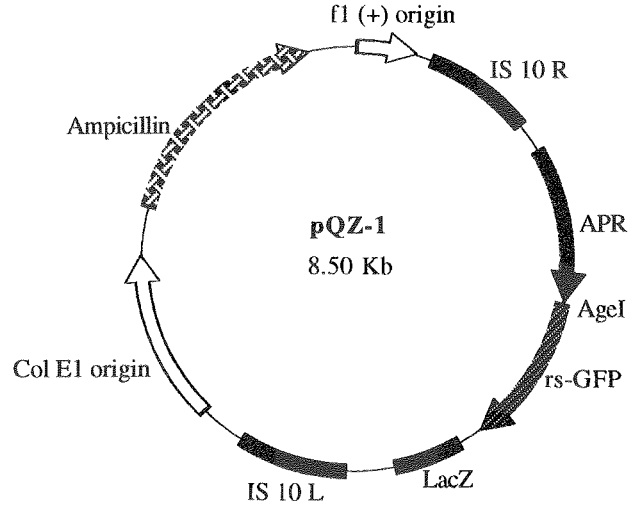

Fig. 1. A plasmid map of the expression vector pQZ-1 constructed for use in this study.

2.2. Cell culture

A channel catfish (CCF) fibroblast cell line derived from fin tissue (Zhang et al., 1998), a CCF leukocyte cell line (Miller et al., 1994), and freshly isolated CCF leukocytes from peripheral blood were used in this study. The fibroblast cells were cultured at 27°C with Leibovitz L15 medium supplemented with 5% fetal bovine serum and 5% catfish serum. The leukocytes were cultured in a 1:1 mixture of AIM V® and Leibovitz L15 media with addition of 5% catfish serum. Cultures were set up in a humidified environment supplemented with 5% CO_2 (Miller et al., 1994).

2.3. Gene delivery by lipofection

E. coli DH5α/pQZ-1 was grown in LB broth overnight at 37°C in a controlled environment incubator shaker (Series 25, New Brunswick Scientific, Edison, NJ). Plasmid DNA was extracted and purified from DH5α using QIAfilter Plasmid Maxi Kits (QiaGen, Chatsworth, CA). The CCF cells were transfected with pQZ-1 complexed by SuperFect Transfection Reagent (QiaGen) according to the manufacturer's instructions.

Fibroblast cells were placed in six-well plates (Falcon Plastics, Becton Dickinson, Franklin Lakes, NJ), and a coverslip (22 × 22 mm) was placed in each well of the plates for cell attachment. Once cultures were grown to

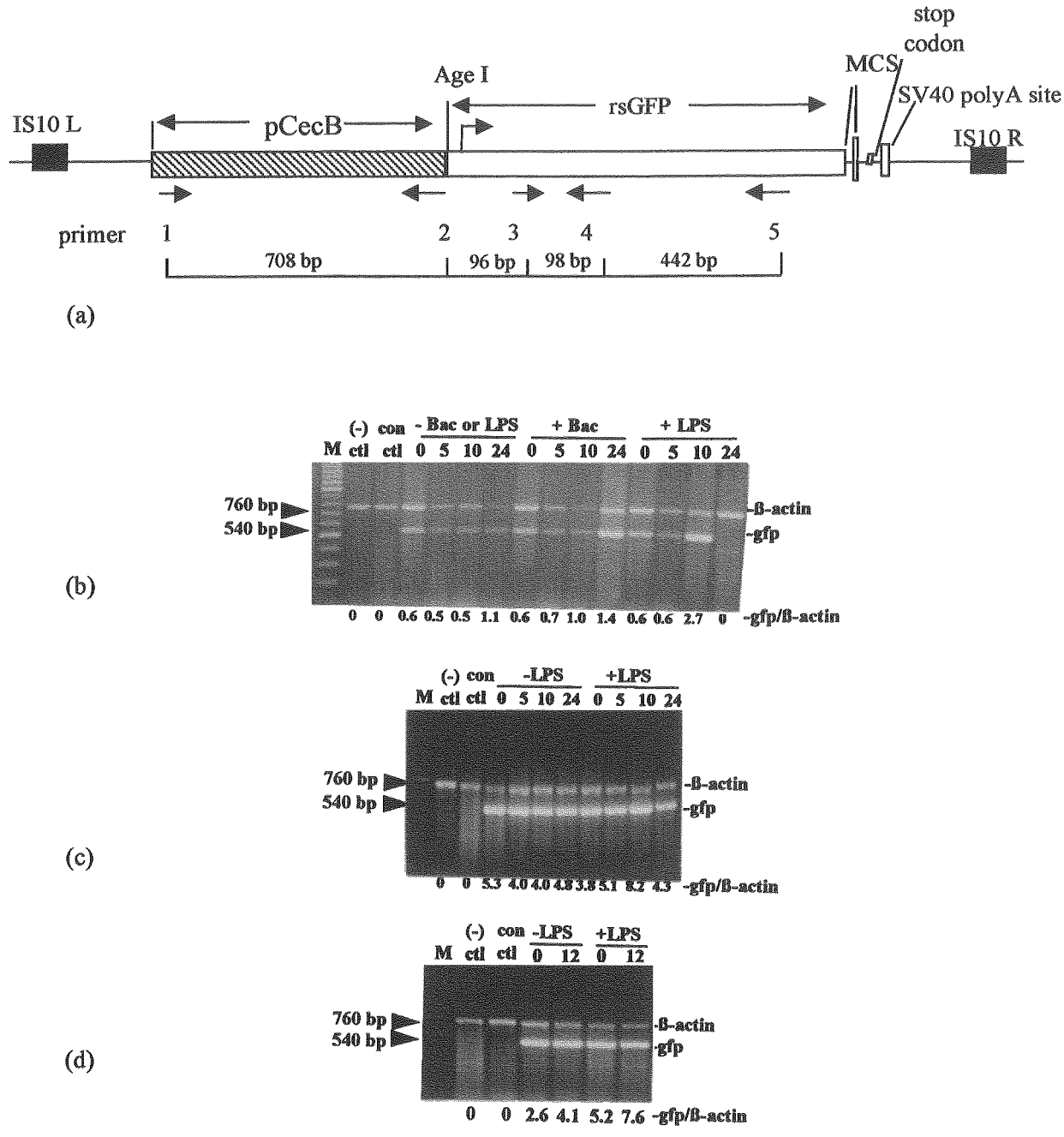

Fig. 2. (**a**) A linearized restriction map of pQZ-1 and (**b**) analysis by quantitative RT-PCR of *gfp* RNA expressed within a fibroblast cell line, (**c**) leukocyte cell line and (**d**) primary leukocytes of channel catfish. The map in (**a**) indicates the locations of the *Cec B* promoter, GFP, poly A site, multiple cloning site, and primers. Primer 1-GCCGGATCCGTGTATTCCTGCACCAAAAA (5′ to 3′) and primer 2-CGGAAGCTTACCGGTCGAAATATTAAATTTAGATA were designed to target the *Cec B* promoter region (sites −644 to −627 and 44 to 63, respectively). Primer 3-GTCAGTGGAGAGGGTGAAGGTGATGCAAC, primer 4-AGTAGTGACAAGTGTTGGCCATGGA, and primer 5-GAAAGGGCAGATTGTGTGGACAGGTAATG targeted different regions of the *gfp* gene (85–114, 165–189, and 594–623, respectively). All PCR reactions (**b**, **c** and **d**) contained primers 3, 5, and a pair of *β*-actin primers (Clontech Laboratories, Inc., Palo Alto, CA) except that primers 1 and 4 were used to replace 3 and 5 to test for plasmid DNA contamination in samples. The nucleotide sequences of actin primers were: actin-1, TTGTAACCAACTGGGACGATATGG (catalog no. 1552–1575) and actin-2, GATCTTGATCTTCATGGTGCTAGG (catalog no. 2991–2844).

about 50% confluency, the spent media was removed and cells incubated for 10 h with medium containing the pQZ-1 plasmid DNA-liposome complex (1:4, w/v) without addition of serum and antibiotics. The media were removed from the cultures, and cells were incubated with fresh media containing serum without antibiotics for another 24 h. For leukocytes, about 1.0×10^6 cells were seeded in each well of six-well plates and incubated with media containing serum without antibiotics. These cells were incubated with the DNA–liposome complex

(1:4, w/v) for 24–36 h before challenge. Controls containing liposomes without DNA were included for each cell type.

2.4. Post-transfection challenge

The transfected fibroblast cells were incubated with irradiated *Flavobacterium columnare* (7×10^6 cells/ml) or with *E. coli* LPS (125 μg/ml) (Sigma Chemical Co., St. Louis, MO) for 5 h, 10 h, or 24 h. The leukocyte cell line was incubated with LPS (125 μg/ml) for 5 h, 10 h, or 24 h. The freshly isolated leukocytes were incubated with LPS (125 μg/ml) for 10 h.

2.5. Analysis of RNA by quantitative RT-PCR

Cells were harvested and rinsed three times with CMF-PBS. The RNA was prepared from the cells with a RNeasy™ Total RNA kit (QiaGen). Prior to RT-PCR, total RNA was treated with DNase I (Pharmacia Biotech, Alameda, CA) to eliminate any contamination from plasmid and genomic DNA. Reverse transcription was performed with a First-Strand cDNA Synthesis Kit (Pharmacia Biotech). A poly-T primer *Not*I-d$(T)_{18}$ (Pharmacia Biotech) and 1 μg of total RNA from each sample were included in each reaction. Besides primer 1 (described in Section 2.1), three primers 3, 4, and 5 (Fig. 2a), were designed using PC/Gene computer software (IntellGenetics, Mountainview, CA) for use in polymerase chain reaction. All primers functioned under similar PCR conditions, which was confirmed by amplification with the plasmid DNA of pQZ-1. Primer pair 3 and 5 was used to amplify a 540-bp fragment from *gfp* cDNA, and primers 1 and 4 were used as a control for monitoring contamination from plasmid DNA. A pair of β-actin primers (actin-1 and -2) derived from a rat sequence (Clontech Laboratories) was included in each reaction as an internal standard (Fig. 2a).

The PCR products were analyzed by electrophoresis on a 1.5% agarose gel, and gel images were recorded with the Eagle Eye® II Still Video System (Stratagene Cloning System, La Jolla, CA). The band intensity was determined using ZERO-Dscan™ densitometry and analysis software (Stratagene). The ratio of mRNA of the target and the actin standard were calculated for evaluation of expression of the *gfp* gene for 24 h after challenge.

2.6. Morphological analysis by fluorescence microscopy

Coverslips with adherent fibroblasts were rinsed with CMF-PBS equilibrated to room temperature. Cells were fixed in 2% paraformaldehyde (PFA, pH 7.0) for 30 min at room temperature, rinsed with CMF-PBS, and stained with 0.5 μg/ml of PI (Sigma) dissolved in PBS. Coverslips were mounted on microscope slides with antifading medium (100 mg of *p*-phenylenediamine in 100 ml of glycerol). Alternatively, cells were counterstained with PI dissolved in antifading medium.

Leukocytes were processed with procedures similar to those for fibroblast cells. After fixing with PFA, cells were rinsed with CMF-PBS and resuspended in PI. Ten microliters of cell suspension were placed on microscope slides and dried in a laminar flow hood. A coverslip was mounted on each slide with the antifading medium. Slides were examined under a fluorescence microscope (Microphot-SA, Nikon Inc.) equipped with fluorescence filters for FITC and PI.

2.7. Quantitation of expression level by flow cytometry

Transfected cells were harvested after 10 h of incubation with LPS. Cells were rinsed with CMF-PBS, and cell pellets were resuspended in PBS containing 0.5 μg/ml of PI. Fluorescence was detected using a FACSCalibur™ (Becton Dickinson, San Jose, CA) flow cytometer equipped with a 488-nm argon laser. The FL1 photomultiplier tube (PMT) monitored the green fluorescence emitted by GFP, and the red fluorescence emitted by PI was monitored by the FL2 PMT. Ten thousand events were collected from each sample. An analytical gate was set such that less than 0.2% of transfection control cells (as described in Section 2.3) exceeded the gate and fell in the positive region designated as M1 (Figs. 5 and 6).

3. Results and discussion

3.1. Analysis of gfp RNA by quantitative RT-PCR

A preliminary experiment, in which cells were incubated with different DNA–liposome complexes (mixed at 1:1, 1:2, 1:3, 1:4, 1:5, and 1:8 w/v) demonstrated that the highest basal expression was observed in cells transfected with DNA:liposome complexes of 1:3 to 1:5 (data not shown). The presence of *gfp* DNA in host cells was detected by PCR at 3 days and 1 month after transfection (data not shown). The *gfp* RNA was detected in all three cell types before addition of irradiated bacteria or LPS (Fig. 2b–d). The basal level of *gfp* RNA doubled through the 24-h observation period in fibroblasts, whereas there was no change in the cell line leukocytes. In primary leukocytes, the basal *gfp* RNA increased 1.6 times at the end of a 12-h observation period. Overall, the basal *gfp* RNA amount in leukocyes was at least four times that observed in fibroblast cells.

The RNA levels in fibroblast cells exhibited a gradual increase upon challenge with bacteria and reached the highest level (increased by 2.3 times) at 24 h (Fig. 2b). The maximum *gfp* RNA in fibroblasts (increased by 4.5 times) appeared at 10 h after challenge with LPS and

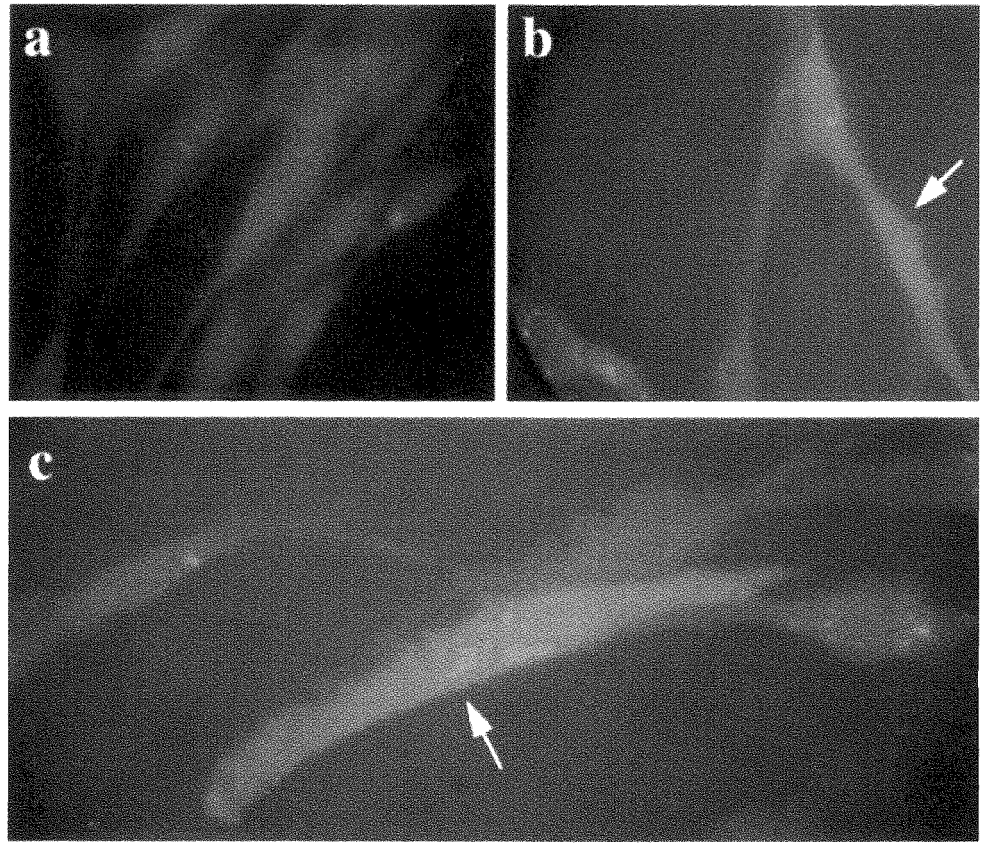

Fig. 3. Examination of transfected fibroblasts of channel catfish by fluorescence microscopy. (**a**) Transfection control cells and (**b** and **c**) the cells transfected with the expression vector pQZ-1 were counterstained with PI (0.5 μg/ml) dissolved in an antifading medium. Arrowheads indicate the GFP-expressing cells.

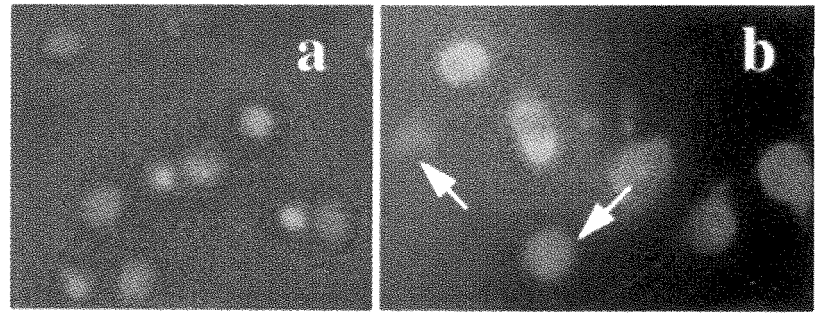

Fig. 4. Examination of transfected leukocytes of channel catfish by fluorescence microscopy. (**a**) Transfection control cells and (**b**) cells transfected with the expression vector pQZ-1 were counterstained with PI (0.5 μg/ml in PBS) and mounted in an antifading medium. Arrowheads indicate the GFP-expressing cells.

dropped to undetectable levels by 24 h (Fig. 2b). In the leukocyte cell line, *gfp* RNA increased 2.2 times at 10 h after challenge with LPS and dropped to the initial level by 24 h after challenge (Fig. 2c). The *gfp* RNA of primary leukocytes increased 1.5 times by 12 h after challenge with LPS (Fig. 2d). However, unchallenged primary leukocytes had a similar increase within 12 h (Fig. 2d).

In native insect cells, the expression of cecropin occurs mainly in fat bodies and hemocytes (Samakovlis et al., 1990; Kato et al., 1993). Our results indicated that the function of the *Cec B* promoter did not show tissue preference in channel catfish, although the basal expression level appeared higher in leukocytes than in fibroblasts. Basal transcription in the absence of inducers was found in all three cell types, suggesting that the transfected cells were activated by potential stressors or mitogenic components present in the serum before challenge. This phenomenon was also observed in studies with insect cells (Trenczek and Faye, 1988). However, it remains unknown as to why the basal expression of transfected fibroblasts and primary leukocytes increased with time.

In this study, the response of the *Cec B* promoter to irradiated bacteria was not as rapid as to LPS. This difference probably resulted from different levels of effective LPS dosage present in the media or was due to LPS of *E. coli* being more potent than LPS of *F. columnaris*, indicating that the activation of this promoter might be dosage- and inducer-dependent in channel catfish cells. Overall, the response of the *Cec B* promoter to LPS was not as rapid as reported for insect cells (Samakovlis et al., 1992) in which maximal expression appeared within 1 h after challenge.

3.2. Fluorescence microscopy

The GFP was evenly distributed within fibroblasts (Fig. 3b and c) and long-term cultures of leukocytes (Fig. 4b). In fibroblasts with a high level of GFP, fluorescence covered the nucleus and cytoplasm. The number of cells found with visible green fluorescence was low for the two cell types (1–2%); however, no selectable marker was used in the vector, and the expression level of *gfp* required for visual detection in channel catfish is unknown. Cells detected with mRNA might not express GFP sufficiently for microscopic identification.

Counterstaining of cells with PI provided better visibility of the outline of cells that did not express GFP. Cells counterstained with PI dissolved in the antifading medium had a faint red color (Fig. 3), whereas cells stained with PI dissolved in PBS and mounted in the antifading medium exhibited a deep red color (Fig. 4).

3.3. Quantitation of the GFP level by flow cytometry

It was difficult to evaluate differences in expression by fluorescence microscopy because of the low percentage of cells found with visible green fluorescence. Therefore, flow cytometry was utilized for quantitation of fluorescence intensity and counting of GFP-expressing cells in each sample. After challenge with LPS for 10 h, the fluorescence of transfected fibroblasts increased from a mean channel value of 6.53 (unchallenged) to 9.98 (challenged), and cells falling within the positive gated region increased from 0.51% (unchallenged) to 1.30% (challenged) (Fig. 5). The overall fluorescence of leukocytes did not increase with exposure to LPS. The percent of leukocytes in the gated region was around 1.5% (Fig. 6), and this number did not increase with LPS induction. The findings by flow cytometry were consistent with those of RT-PCR in terms of basal expression, indicating that the cellular level of *gfp* RNA was directly related to GFP detection.

3.4. Conclusions

The insect *Cec B* promoter was functionally active in channel catfish cells. The unchallenged expression of

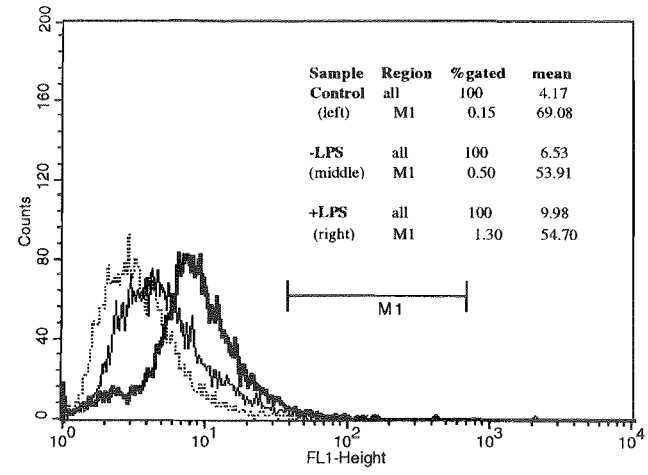

Fig. 5. Detection of GFP in channel catfish fibroblasts by flow cytometry. Left curve, control; middle curve, transfected cells without challenge; right curve, transfected cells with challenge by LPS for 10 h. The gated region M1 was set as described in the Section 2.7.

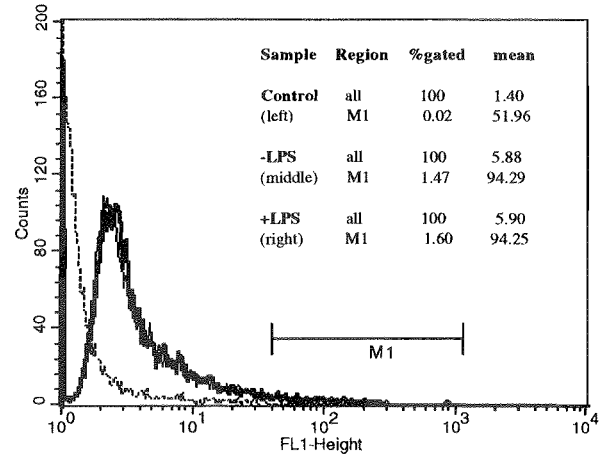

Fig. 6. Detection of GFP in channel catfish leukocytes by flow cytometry. Left curve, control; middle curve, transfected cells without challenge; right curve, transfected cells with challenge by LPS for 10 h. The gated region M1 was set as described in the Section 2.7.

gfp, detected in transfected fibroblasts and leukocytes, could be caused by the existence of mitogenic materials in the culture media or response to stressors. The transcription of this promoter could be enhanced in fibroblasts and leukocytes by challenge with bacterial substances and demonstrates the feasibility of using a promoter under the control of the immune system. The mechanism for expression of the *gfp* RNA in unchallenged cells remains to be studied.

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