Transduction of cultured fish cells with recombinant baculoviruses

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Five fish cell lines were tested for their ability to be transduced by Ac-CAlacZ, a recombinant baculovirus that is capable of expressing a β-galactosidase reporter gene from the CAG promoter (consisting of a cytomegalovirus enhancer element, a chicken actin promoter and rabbit β-globin termination sequences). TO (Tilapia ovary), EPC (carp), CHH-1 (Chum salmon heart fibroblast) and CHSE-214 (chinook salmon embryo) cells were transducible, as demonstrated by an in situ β-galactosidase assay, whereas RTG-2 (rainbow trout gonad) cells were not. The EPC cell line was used for more detailed studies on baculovirus transduction. The transduction frequency was found to be higher at 28°C than at 21°C. Addition of the histone deacetylase inhibitor sodium butyrate increased the number of blue cells detected 5- to 7-fold. The m.o.i. was positively correlated with transduction frequency, although the relationship did not appear to be strictly linear, as has been observed with mammalian cells. The temperature at which baculoviruses were adsorbed to EPC cells did not affect levels of β-galactosidase expression. We also examined expression levels of β-galactosidase in EPC cells after infection with a baculovirus construct that overexpresses the vesicular stomatitis virus G protein and displays it on the virion surface. Expression levels with this virus were approximately 15-fold higher than were observed with Ac-CAlacZ.

INTRODUCTION

The Baculoviridae is a diverse family of arthropod-specific viruses of which most members infect Lepidoptera (butterflies and moths). Members have also been found that are infectious for other insect orders including Diptera, Hymenoptera and Trichoptera (Martignoni & Iwai, 1986). Baculovirus virions consist of large, enveloped, rod-shaped nucleocapsids that contain circular double-stranded DNA genomes ranging in size from 100 to 180 kbp (Hayakawa et al., 2000). Baculoviruses produce two distinct virion phenotypes. One form, referred to as budded virus, matures by budding through the surface of infected cells and is responsible for the spread of virions to tissues within infected larvae and in cell culture. In the second form, called the occlusion-derived virus, virions are present in large proteinaceous crystals known as occlusion bodies or polyhedra. This form of the virus is normally only infectious after the polyhedra dissolve in the highly alkaline environment of the insect midgut and hence are responsible for the spread of virus from one insect to another.

Baculoviruses, in particular Autographa californica multinucleocapsid nucleopolyhedrovirus (AcMNPV), are widely used for overexpression of cloned genes (O’Reilly et al., 1992). In this system, the gene encoding polyhedrin (the protein comprising the polyhedron matrix in which the occluded viruses are imbedded) is usually replaced with a gene of interest and the very high transcriptional activity conferred by the polyhedrin promoter is exploited for overexpression of the selected gene. Because of the high levels of expression and the fidelity of protein processing, this system is frequently favoured over other eukaryotic expression systems.

In another recent application of baculoviruses, several investigators have reported that AcMNPV is capable of transducing genes into a variety of mammalian cell types (reviewed by Kost & Condreay, 2002). For these investigations, recombinant AcMNPV constructs have been produced that carry expression cassettes consisting of a reporter gene linked either to the human cytomegalovirus (CMV) immediate-early (IE) promoter/enhancer or to the CAG promoter (consisting of the CMV IE enhancer, the chicken β-actin promoter and the rabbit β-globin polyadenylation signal) (Niwa et al., 1991). With these constructs, it was found that a wide variety of mammalian cell types were capable of being transduced, including a number of human, monkey, porcine and rat cell lines (Condreay et al., 1999; Shoji et al., 1997).

Although the ability of baculoviruses to transduce genes into mammalian cells is well established, investigations on their...
application to other vertebrates have been limited. Because fish are subjected to a variety of virus diseases, the development of efficient methods to prevent these infections is of great importance. Therefore we have initiated detailed experiments to determine whether baculovirus vectors are capable of delivering genetic vaccines to fish. In this report, we demonstrate that baculovirus vectors are effective at transferring a lacZ reporter gene under the control of the CAG regulatory elements into different fish cell lines. One cell line, epithelioma papulosum cyprini (EPC) (Fijan et al., 1983), derived from carp, was selected for further studies and the effects of m.o.i., temperature and treatment with a histone deacetylase inhibitor (sodium butyrate) were investigated. Expression from a recombinant baculovirus that displays a heterologous viral fusion protein on the virion surface was also examined.

**METHODS**

**Virus strains.** Two recombinant baculoviruses (Ac-CAlacZ and AcVSVG-CAlacZ (Tani et al., 2001) (a gift from Y. Matsuura) were tested for their ability to transduce cultured fish cells. Both baculoviruses contain the lacZ gene under the control of the CAG regulatory cassette, consisting of a CMV enhancer element, a chicken actin promoter and rabbit β-globin termination sequences (Niwa et al., 1991). AcVSVG-CAlacZ expresses the vesicular stomatitis virus (VSV) G gene from the polyhedrin promoter, resulting in exhibition of VSV G-protein on the virion surface.

**Cell lines tested.** The fish cell lines tested for transduction with recombinant baculoviruses were: Tilapia ovary (TO-2) (Chen et al., 1983); epithelioma papulosum cyprini (EPC) cells (Fijan et al., 1983); rainbow trout gonad (RTG-2) cells (Wolf & Quimby, 1962); Chum salmon ( Oncorhynchus keta) heart fibroblast (CHH-1) cells (ATCC CRL-1680; Lannan et al., 1984); and chinook salmon embryo (CHSE-214) cells (Lannan et al., 1984). All cells were maintained in complete MEM-5, which consisted of autoclaved minimal essential medium (GibcoBRL) supplemented with 5% foetal bovine serum (FBS; Intergen) and 2 mM l-glutamine (GibcoBRL).

**Infections and β-galactosidase assays.** The high-sensitivity β-galactosidase assays were performed by incubating 1 × 10^6 cells per well in 24-well tissue culture plates with 100 μl of complete MEM-5 culture medium containing Ac-CAlacZ or AcVSVG-CAlacZ baculovirus at the indicated m.o.i.s. At 1 h post-treatment, 1 ml MEM-5 was added to each well, with or without the addition of sodium butyrate (Sigma), as indicated. At the indicated times, cells were treated with lysis buffer [0.25 M Tris, pH 7-4, 0.25 % (v/v) NP-40, 2.5 mM EDTA] and assayed for β-galactosidase activity using a high-sensitivity β-galactosidase assay kit (Stratagene). Each data-point represents a single β-galactosidase activity determination divided by the protein concentration of the extract. The protein concentration was determined from the average of three assays for each extract, using the DC protein assay (Bio-Rad).

For in situ β-galactosidase staining assays, 2 × 10^5 cells per well in 96-well plates were incubated with 30 μl of complete MEM-5 culture medium containing Ac-CAlacZ or AcVSVG-CAlacZ baculovirus at the indicated m.o.i.s, with or without the addition of sodium butyrate (Sigma), as indicated. At 1 h post-treatment, 100 μl MEM-5 was added to each well. At the indicated times, cells were stained using an in situ β-galactosidase assay kit (Stratagene).

**RESULTS**

**Susceptibility of fish cells to baculovirus-mediated transduction**

In order to determine whether AcMNPV budded virus is capable of entering and expressing genes in fish cells, five different fish cell lines were tested using Ac-CAlacZ (Tani et al., 2001), a recombinant baculoviruses that contains the lacZ gene under the control of the CAG transcriptional regulatory elements (Niwa et al., 1991). The cell lines tested were TO, EPC, RTG-2, CHH-1 and CHSE-214. Using an in situ β-galactosidase assay, individually transduced cells expressing β-galactosidase (blue cells) could be detected among baculovirus-treated TO, EPC, CHH-1 and CHSE-214 cells, but were not observed with RTG-2 cells. Fig. 1 shows the typical appearance of blue cells observed in this assay. Blue CHH-1 and CHSE-214 cells were rarely observed unless sodium butyrate was added to the culture media following infection (see below). The transduction frequency observed was highest for TO cells (approximately 20% without and approximately 30% with sodium butyrate). For EPC cells without sodium butyrate, the number of blue cells detected ranged from 0-04 to 2% in different experiments. Addition of sodium butyrate generally results in a 5- to 7-fold increase in apparent transduction frequency with this cell line. The transduction frequency of CHSE-214 and CHH-1 cells was approximately 0-05% in the presence of sodium butyrate. EPC and TO cells were maintained at a higher temperature (28–29°C) than the other cells and this may account for the elevated transduction efficiency observed in these cell lines (see below). CHSE-214 and CHH-1 cells could not be tested at 28°C because they do not survive prolonged incubation at this temperature.

**Fig. 1.** Susceptibility of EPC cells to baculovirus-mediated gene transfer. EPC cells were transduced with Ac-CAlacZ (m.o.i. = 200) and maintained at 29°C. At 48 h post-transfection, the cells were stained in situ and examined for β-galactosidase-expressing (blue) cells. Blue cells were not observed in baculovirus-negative control wells (not shown).
Effect of virus dose on reporter gene expression

The dose-response characteristics of Ac-CALacZ were determined on EPC cells (Fig. 2). Using the in situ β-galactosidase assay, an average of two blue cells per well (2 x 10⁴ cells per well) was detected at an m.o.i. of 1:6, the lowest m.o.i. tested. The number of blue cells per well increased rapidly as the m.o.i. increased. Assays of cell lysates for β-galactosidase expression yielded similar dose-response kinetics (data not shown).

Effect of sodium butyrate on reporter gene expression

Sodium butyrate is an inhibitor of histone deacetylase and is thought to activate gene expression by maintaining histones in the acetylated form (Kruh, 1982). The addition of 0.1 mM sodium butyrate to EPC cells maintained at 28°C resulted in a 2- to 3-fold increase in β-galactosidase activity (Fig. 3). A maximum increase (5- to 6-fold) occurred when the concentration of sodium butyrate was 1.0 mM. At 21°C, there was no measurable increase in β-galactosidase activity in response to sodium butyrate, although we have noted increases in activity in response to sodium butyrate at 21°C with CHH-1 and CHSE-214 cells (not shown).

Time course of reporter gene expression

The time course of β-galactosidase expression following Ac-CALacZ infection was determined in EPC cells maintained at 21 and 28°C, both in the presence and absence of 1 mM sodium butyrate (Fig. 4). Cells maintained at 28°C with sodium butyrate showed peak levels of expression at approximately 48 h post-infection. Without sodium butyrate, the highest level of expression was achieved at 72 h, the point at which the experiment was terminated. β-Galactosidase levels in cells maintained at 21°C were also highest at 72 h, irrespective of whether sodium butyrate was added to the medium or not.

Expression levels as a function of the temperature of adsorption and incubation

Two possibilities exist for the low level of reporter gene expression observed at 17 and 21°C; either adsorption/entry/uncoating of viruses is inhibited, or the expression of the reporter gene is less robust. To distinguish between these two possibilities, Ac-CALacZ was adsorbed to EPC cells for 1 h at 17, 21 or 29°C and the cells infected at each of these temperatures. Fig. 5 shows that expression levels depended entirely on the temperature at which the cells were incubated following infection. Almost no β-galactosidase activity was measured in extracts from cells maintained...
at 17°C, some activity was present in extracts from cells maintained at 21°C and a further 7- to 8-fold increase over the level expressed at 21°C was produced by cells maintained at 29°C. Varying the temperature at which the virus was adsorbed to the cells had no effect on reporter gene expression, indicating that the virions are efficiently adsorbed at temperatures as low as 17°C.

**Effects of homologous and heterologous membrane fusion proteins on reporter gene expression**

It has been reported that expression and display of the VSV G protein on the surface of recombinant baculovirus virions greatly increases their transducing ability in mammalian cell cultures (Barsoum et al., 1997). At 72 h post-infection and at 29°C, β-galactosidase levels were approximately 15-fold higher when EPC cells were transduced with AcVSVG-CAlacZ, which displays the VSV G protein on its surface (Tani et al., 2001), compared with Ac-CAlacZ, which does not carry an exogenous membrane protein (data not shown).

**DISCUSSION**

Reports on the ability of baculoviruses to transduce cold-blooded vertebrates are limited to frog melanophores (Condrey et al., 1999). In this study, we have investigated the ability of recombinant baculovirus vectors carrying the β-galactosidase gene under the control of the CAG promoter to deliver foreign genes into fish cell lines. We detected gene transfer and expression in four of the five fish cell lines tested. In other studies, we have found that the RTG-2 cell line, which failed to express the baculovirus-delivered reporter gene, also expresses reporter genes poorly from a CMV promoter after liposome-mediated transfection with plasmid DNA. This suggests that RTG-2 cells may be unable to express foreign DNA efficiently, regardless of the manner in which it is introduced into the cell.

The EPC cell line was selected for further study. Dose-response kinetics were examined, both by counting the number of cells expressing β-galactosidase activity and by measuring the amount of β-galactosidase activity in lysates from infected cells. At an m.o.i. of 1-6, we observed, on average, only one β-galactosidase-expressing cell per 2 × 10⁶ cells. Between m.o.i.s of 6 and 10, the number of β-galactosidase-expressing cells was linear with respect to the dose of virus. At m.o.i.s higher than 25, expression levels rose at a rate slightly lower than the increase in dosage level. These results are not in strict agreement with dose-response experiments performed with mammalian cells, which showed a linear relationship with m.o.i., with no saturation occurring even with an m.o.i. as high as 800 (Duisit et al., 1999).

The addition of sodium butyrate to the culture medium of baculovirus-infected EPC cells resulted in a 5- to 6-fold elevation of β-galactosidase activity. Similar results have been reported with mammalian cells (Condrey et al., 1999). Sodium butyrate maintains histones in an acetylated form by inhibiting histone deacetylase. Addition of sodium butyrate to cells transfected with plasmids using liposomes showed only small or negligible increases in reporter gene activity (data not shown). In contrast to purified plasmids that consist of naked DNA, baculovirus virion DNA most likely has a nucleosome-like structure (Wilson & Miller, 1986). These data suggest that sodium butyrate may act on virion DNA in a manner similar to its effects on cellular DNA.

We found that reporter gene expression levels in baculovirus-infected EPC cells were much higher at 29°C than at lower temperatures. Temperature-shift experiments demonstrated that temperatures in the range tested (17–29°C) had no affect on the ability of the cells to adsorb virus, but did affect the level of gene expression after adsorption. We have now tested several different promoters via liposome-mediated transfection of plasmid DNA and found that, in comparison with other promoters tested, the CAG regulatory cassette is particularly temperature-sensitive (data not shown). Since the CAG control elements were used in all of our baculovirus constructs, the observed temperature effects are probably due to reduced transcription of the reporter gene at the lower temperatures.

In AcMNPV, entry into insect cells is mediated by specific interactions between the virion envelope protein gp64 and host receptors (Hefferson et al., 1999). Recent studies suggest, however, that baculovirus binding to mammalian cells may occur through non-specific electrostatic interactions between the viral and cell membranes (Duisit et al., 1999). Although gp64 may not be important for initial
binding and internalization into endosomes, it is probably involved in fusion of the virion envelope to the endosomal membrane, thereby facilitating the release of the nucleocapsid into the cell (Blissard & Wenz, 1992). A recombinant baculovirus that contained about 1.5 times the normal amount of gp64 on the virion surface exhibited 10- to 100-fold more reporter gene expression in a variety of mammalian cells compared with similar viruses with a normal amount of gp64 (Barsoum et al., 1997; Tani et al., 2001). Recombinant baculoviruses that contain heterologous viral envelope glycoproteins, such as the VSV G protein or the mouse hepatitis virus S protein, have also been reported to elevate levels of foreign gene expression in mammalian cells (Tani et al., 2001). These studies suggest that these proteins may mediate binding to specific receptors on the mammalian cells or release of the virions from endosomes. Using a VSV G-containing baculovirus (AcVSVG-CA-
lacZ) and the EPC fish cell line, we observed 15-fold elevated levels of β-galactosidase expression, similar to that observed with mammalian cells (Barsoum et al., 1997).

Recent studies have demonstrated superior efficacy of DNA-based vaccines for immunization of fish compared with more traditional vaccines, such as inactivated or attenuated vaccines. For instance, injection of rainbow trout with a plasmid construct containing the infectious hematopoietic necrosis virus (IHNV) G protein gene under the control of the CMV promoter provided excellent protection against IHNV in viral challenge assays (Anderson et al., 1996). However, the practical application of such DNA-based vaccines is limited because they have only been found to be effective when delivered by injection, gene gun, scarification (Corbell et al., 2000), or by the application of short-pulse ultrasound (Fernandez-Alonso et al., 2001). Simple bath immersion, which is effective for delivery of some traditional inactivated or attenuated vaccines (Corbell et al., 2000), has not been found to be effective for DNA vaccines. We are interested in testing the possibility of using transducing baculoviruses to deliver DNA modules to fish by immersion or ingestion, which would be preferred routes of administration in hatchery situations where large numbers of fry need to be vaccinated.

Many of the features of baculoviruses that make them attractive as gene therapy vehicles could potentially be exploited for the development of fish vaccines that could be delivered by immersion or ingestion. The results presented here demonstrate that baculovirus-vectored foreign genes can be expressed in a variety of fish cell lines, providing incentive for further investigations on the development of baculovirus-based vaccines for use in fish.

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REFERENCES


