The Journal of Experimental Biology 210, 2091-2103
Published by The Company of Biologists 2007
doi:10.1242/jeb.003186

# Specific endocytosis and degradation of naked DNA in the endocardial cells of cod (*Gadus morhua* L.)

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*Accepted 2 April 2007*

## Summary

**DNA vaccines are administered in the form of plasmid DNA (pDNA) carrying a strong promoter and the gene of interest. In this study we investigated the tissue distribution, cellular uptake and the fate of intravenously (i.v.) and intramuscularly (i.m.) injected pDNA in Atlantic cod (*Gadus morhua* L.). The anatomical distribution of pDNA was determined using both morphological and radiotracing methods. Cellular uptake and receptor specificity were studied in cultures of cod atrial endocardial endothelial cells (aEEC) and head kidney leukocytes. The short-term fate of the endocytosed pDNA *in vivo* and *in vitro* was investigated by Southern blot. Expression of the pDNA (R70pRomiLuc)-derived gene was investigated in cod tissues and cultures of cod aEEC by means of real-time RT-PCR and luciferase activity assay.**

**$^{125}$I-labelled pDNA was rapidly eliminated from the blood by the aEEC of the cod heart atrium and ventricle. Co-injection of trace amounts of $^{125}$I-labelled pDNA with excess amounts of non-labelled pDNA or formaldehyde-treated albumin (FSA), a ligand for the cod EEC scavenger receptor, significantly inhibited the accumulation of the radiotracer in the heart. The organ to blood ratio of radioactivity after inhibition of the cod EEC scavenger receptor demonstrated that the radioactivity not taken up by the EEC remained in the blood. Fluorescence microscopy of tissue sections from cod injected with fluorescein-labelled pDNA confirmed intracellular uptake of pDNA by the endocardial cells of the atrium and ventricle. In purified cultures of cod aEEC the fluorescein-labelled pDNA was taken up in structures reminiscent of endosomal/lysosomal vesicles. Uptake of $^{125}$I-labelled pDNA in cultures of cod aEEC was specific. Incubation of cultures with $^{125}$I-labelled pDNA together with excess amounts of FSA and fucoidan, which are molecules also known to bind to the scavenger receptors, reduced the uptake of the pDNA by at least 70%. Mannan, a ligand for the mannose receptor, did not inhibit the uptake of $^{125}$I-labelled pDNA. Despite, low uptake of $^{125}$I-fluorescein-pDNA in the kidney of the cod, the uptake of pDNA in cultured cod head kidney leukocytes was significant.**

**Southern blot analysis of cod tissues after injection of pDNA and culture of aEEC given 10 μg pDNA per $10^6$ cells demonstrated the presence of degradation products in tissues and in the cell cultures. Real-time RT–PCR studies showed expression of luciferase mRNA only at the injection site 168 h after injection. Neither expression of luciferase mRNA nor luciferase activity was present in cod aEEC incubated for 48 h with 10 μg pDNA.**

**These results suggest that the EEC are very important for removal of blood borne pDNA in cod and that the uptake by these cells was mediated in a scavenger–receptor-like manner. Uptake of pDNA by head kidney leukocytes was only observed *in vitro*. The endocytosed DNA was subjected to intracellular degradation and was not expressed by the cod EEC. Despite the low amount of radioactivity found in the head kidney after i.v. injection of $^{125}$I-labelled pDNA, the head kidney leukocytes seem to have a high capacity for uptake of $^{125}$I-labelled pDNA *in vitro*.**

Key words: DNA, scavenger receptor, endocytosis, endothelium, endocardium, Atlantic cod, *Gadus morhua*.

## Introduction

DNA vaccines hold prospects for rapid and long-lasting protection against a variety of diseases caused by intracellular pathogens (e.g. viruses, some bacteria) that are difficult to eradicate with traditional vaccines and antibiotics. There are at present no efficient vaccines based on either live, attenuated virus or vaccines containing recombinant viral antigens towards a range of viral diseases in fish. DNA vaccines represent a means to protect against diseases, and hence improve animal welfare, reduce antibiotic usage and spread of disease. The development of DNA vaccines has several attractive benefits: low cost, ease of production and improved

quality control, heat stability, identical production processes for different vaccines, and the possibility of producing multivalent vaccines (Kwang, 2000). A DNA vaccine consists of a bacterial plasmid with a strong viral promoter, the gene(s) of interest and a polyadenylation and transcriptional termination sequence. The plasmid is grown in bacteria, purified, dissolved in a saline solution, and then administered by intramuscular (i.m.) injection of naked DNA (ng and μg amounts) to activate protein expression *in vivo* and to ultimately induce an immune response and disease protection. Studies with reporter genes have demonstrated that fish cells efficiently express foreign proteins encoded by non-viral eukaryotic expression vectors (Anderson et al., 1996b; Kanellos et al., 1999).

Intramuscular injection of a plasmid encoding the glycoprotein of the infectious haematopoietic necrosis virus (IHNV) and the G-protein of viral hemorrhagic septicaemia virus (VHS) have been reported to induce protection against experimental challenge in rainbow trout and in sockeye and Chinook salmon fry (Corbeil et al., 1999; Garver et al., 2005; Traxler et al., 1999), and to induce antibody production (Anderson et al., 1996a). In Canada, an IHNV DNA vaccine (Apex-IHN®) developed by Aqua Health, Ltd, an affiliate of Novartis, was cleared for marketing by the Canadian Food Inspection Agency on July 15 2005 (Novartis media release July 19, 2005). It is highly likely that this is the first of several DNA-based vaccines for aquacultured fish. A prerequisite for production of a transgene is that the DNA vaccine (naked pDNA) is taken up by the cells, transferred to the cytosol and eventually transported to the nucleus before any expression occurs. Several passive and active mechanisms have been described for receptor binding and/or uptake of DNA. The uptake processes are different forms of endocytosis: phagocytosis (cell eating) and pinocytosis (cell drinking). Pinocytosis is utilised by essentially all cell types and occurs by multiple pathways, i.e. clathrin-mediated endocytosis, caveolin-mediated endocytosis, clathrin- and caveolin-independent endocytosis and macropinocytosis (Belting et al., 2005). Macrophages, granulocytes and dendritic cells carry out phagocytosis (e.g. dead cells, bacteria, large molecular complexes), and they are found in many parts of an animal. In particular, those macrophages residing in close connection to the bloodstream, are highly phagocytic thus functioning as an important element in the reticuloendothelial system – together with scavenger endothelial cells in liver of mammals and in the kidney or heart of pisciformes (fish) (Seternes et al., 2002). The endocytic pathway normally confers total degradation of DNA – especially if the DNA is transported to the end-point terminal (lysosomes). However, tiny amounts of (non-protected) DNA may escape the endocytic compartments and degradation. This DNA may be trafficked through the nuclear membrane into the nucleus where transcription occurs.

The endocardial endothelial cells (EEC) lining the muscular trabecula of the Atlantic cod (*Gadus morhua* L.) heart represent a general vertebrate non-phagocytic scavenger endothelial cell (SEC) system with an extensive capacity to endocytose and degrade soluble physiological and foreign macromolecular waste substances/molecules from the circulation by receptor-mediated endocytosis (Seternes et al., 2002). Functional studies indicate that the cod aEECs express a set of at least four types of functional endocytic receptors for this purpose: (i) the collagen α chain receptor (Koren et al., 1997; Smedsrød et al., 1995), (ii) the hyaluronan receptor (Seternes et al., 2001b; Sørensen et al., 1997a), (iii) the mannose receptor (Sørensen et al., 2001) and (iv) the scavenger receptor (Seternes et al., 2001a; Sørensen et al., 1998).

Several studies in mammals have shown that the liver is the main organ responsible for rapid clearance of DNA from the circulation (Emlen and Mannik, 1978; Emlen and Mannik, 1984; Gauthier et al., 1996). In the rat liver the scavenger endothelial cells rather than Kupffer cells have been shown to be responsible for the highest uptake and degradation of pDNA (Hisazumi et al., 2004). Whether fish scavenger endothelial cells are active in uptake and degradation of pDNA is at present not known. The injected pDNA vaccine is subjected to many hurdles before any expression of the transgene occurs. The first being mucosal-derived and blood plasma nucleases (Kawabata et al., 1995), the second hurdle is cellular degradation (Odaka and Mizuochi, 1999), the third is cytosolic degradation of DNA vaccine, and the process of nuclear transfer and localisation of pDNA/DNA fragments represents an obstacle for transgene expression (Lechardeur et al., 1999). The aim of this study was to examine the tissue distribution of pDNA assisted by recepto-specific uptake, transgene expression and persistence of pDNA in Atlantic cod – a fish species emerging as highly interesting for aquaculture and a fish species that have been shown to be susceptible to several viral diseases (Samuelsen et al., 2006).

## Materials and methods

### *Chemicals and medium*

Carrier-free Na[$^{125}$I] was purchased from the Institute of Energy Technology (Kjeller, Norway). Sephadex G-25 (PD-10, disposable column) and Percoll® density medium was obtained from Pharmacia (Uppsala, Sweden). Leibovitz 15 (L-15) medium were obtained from Gibco (Life Technologies, Paisley, Scotland), adjusted to 380 mOsmol $l^{-1}$ tonicity using 0.5 mol $l^{-1}$ NaCl and supplemented with 0.33 g $l^{-1}$ glucose and 0.05 g $l^{-1}$ gentamycin. Foetal calf serum and heparin were purchased from Hyclone (Logan, UT, USA) and Novo Nordisk (Copenhagen, Denmark), respectively. RPMI 1640, supplemented with L-glutamine (2 mmol $l^{-1}$), gentamicin (200 μg $ml^{-1}$), and fungizone (50 μg $ml^{-1}$) was from Flow Laboratories, Irvine, Scotland. Human serum albumin and trypsin (1:250) were purchased from Octapharma (Wien, Austria) and Difco (Detroit, MI, USA), respectively. Luciferase Assay System (cat. No. 1501) was obtained from Promega (Madison, WI, USA). All other chemicals, unless stated, were purchased from Sigma Chemical Co. (St Louis, MO, USA).

### *Animals*

Fish for *in vivo* studies, Atlantic cod (*Gadus morhua* L.; 50–100 g) were obtained from Tromsø Aquaculture station (Norwegian Institute of Fisheries and Aquaculture Research, Tromsø, Norway). The fish were kept in plastic tanks (2000 litre) supplied with running seawater at 4°C. All fish were adapted to the test conditions for at least 1 week before the experiments started and fed a commercial diet daily before and during the adaptation and experimental period. For cell isolation, hearts from wild captured Atlantic cod (1–4 kg) were used. The fish were kept in plastic tanks supplied with running seawater (4°C) and fed a commercial diet. The experiments reported in this manuscript were conducted in accordance with The European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes of 18 March 1986.

### *Plasmids and ligands for endocytosis studies*

The R70pRomiLuc plasmid contained a firefly (*Phontinus pyralis*) luciferase gene under the control of a murine cytomegalovirus immediate early promoter (CMV-IEP), a generous gift from Dr Üwe Fischer (Federal Research Centre for Virus Disease of Animals, Germany). Plasmid DNA was propagated by transformations of *Escherichia coli* DH5α grown in Luria Bertani medium supplied with 100 μg $ml^{-1}$ ampicillin and isolated using Qiagen Plasmid Mega Kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's recommendation. Purity and concentration of pDNA were measured using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and $A_{260}/A_{280}$ ratios between 1.8 and 2.0 were considered pure. Gel electrophoresis analysis confirmed that all pDNA preparations were free of detectable genomic DNA and RNA contamination. Expected endotoxin content was given as 9.3 EU $\mu g^{-1}$ $mg^{-1}$ purified pDNA by the manufacturer. Mannan and fucoidan were purchased from Sigma Chemical Co.

### *Preparation of fluorescein-labelled pDNA*

The nucleic acid labelling kit *Label*IT-fluorescein (MIR 3200) and *Label*IT-TM-Rhodamine (MIR 4100) was obtained from Mirus Corp. (Madison, WI, USA). Synthesis of the *Label*IT reagents is described in US Patent US06262252. Plasmid DNA was modified using *Label*IT reagents according to the manufacturer's recommendations. Briefly, *Label*IT reagent and pDNA were combined in 10× labelling buffer A at 0.5:1 (v:w) ratio of *Label*IT reagent to pDNA and incubated at 37°C for 1 h. The labelled pDNA was separated from unattached label using G50 Microspin purification columns (Amersham Bioscience, Buckinghamshire, UK). Fluorescein–pDNA and rhodamine–pDNA were digested with DNase (TURBO DNA-free; Ambion, Austin, TX, USA) to verify the attachment of fluorescein to the pDNA. Plasmid DNA and fluorescein–pDNA and rhodamine–pDNA were then run on 0.7% agarose gels.

### *Preparation of $^{125}$I-labelled pDNA*

The fluorescein–pDNA was radiolabelled with carrier-free Na[$^{125}$I] in a direct reaction using 1,3,4,6-tetrachloro-3α, 6α-diphenylglycoluril (Iodogen; Pierce, Rockford, IL, USA) as oxidising agent, according to the manufacturer's instructions. Free iodine was removed from the solution by gel filtration on a PD-10 column eluted with phosphate-buffered saline (PBS). The specific activity of $^{125}$I-fluorescein–pDNA was determined to be $1.1\times10^6$ c.p.m. $\mu g^{-1}$. Radioactivity was measured using a Packard gamma counter (Packard Instrument Company, Meriden, IL, USA). Radiolabelled fluorescein–pDNA was digested with DNase (Ambion) or proteinase K (Qiagen) to verify the attachment of radiolabel to the fluorescein–pDNA. To investigate if any free active fluorescein label was still present, human serum albumin (HSA) was added to the radiolabelled fluorescein–pDNA before running the samples on a 0.7% agarose gel and visualisation on a Hyperfilm-HP (Amersham Bioscience).

### *Transfection efficiency of native and modified pDNA*

To ensure that the luciferase gene could still be correctly expressed after labelling pDNA with fluorescein, 0.4 μg of labelled or unlabelled plasmid was transfected into semiconfluent Caco-2 cells in the presence of Lipofectamine Plus according to the manufacturer's instructions (Invitrogen, Oslo, Norway). In short, approximately $2.7\times10^4$ cells were seeded per well in 24-well plates and cultured at 37°C in 5% $CO_2$ for 24 h to obtain 80% confluence in Eagle's minimum essential medium (EMEM) supplemented with 20% foetal bovine serum (FBS). Plasmid DNA (0.4 μg), 4 μl Plus reagent and 1.5 μl Lipofectamine reagent were added to each well and incubated at 37°C for 3 h. The transfection medium was replaced with growth medium. After 48 h of incubation the monolayers of cells were harvested for luciferase assay. Luciferase activity was assayed according to manufacturer's protocol using a Luminoskan Ascent® microplate illuminometer (Thermo Electron Oy, Vantaa, Finland). The relative light units were normalised to the protein concentrations in the samples determined by the Bio-Rad RC DC protein assay (Bio-Rad Laboratories).

### *Anatomical distribution*

Atlantic cod (50–100 g) were anaesthetised by immersion in 0.004% (w/v) metacaine solution and injected intravenously (35 fish) or intramuscularly (25 fish) with trace amounts of $^{125}$I-fluorescein–pDNA (0.5–1 μg $kg^{-1}$ body mass) in a total injection volume of 100 μl PBS per fish for the i.v. injection and 100 μl PBS per fish for the i.m. injection. At specified time intervals, blood samples were collected from the caudal vein approximately 2 cm caudal to the injection site of anaesthetised fish. The fish were killed by a blow to the head immediately after blood sampling, and the heart, anterior kidney, spleen, liver, intestine and blood were removed and analysed for radioactivity. The carcass of the fish, including head, gills, muscle tissue and skin were put in one tube and analysed for radioactivity. Parallel series of five fish were collected 5 min, 15 min 1, 4, 24, 48 and 168 h after intravenous (i.v.) injection, and 1, 4, 24, 48 and 168 h after

i.m. injection. Total blood mass was set to 5% of body mass (Skov and Steffensen, 2003).

Inhibition studies were performed by co-injecting i.v. trace amounts of $^{125}$I-fluorescein–pDNA (0.5–1 μg kg$^{-1}$ body mass) with unlabelled pDNA (3.2 mg kg$^{-1}$ body mass) or formaldehyde-treated albumin (FSA; 5–10 mg kg$^{-1}$ body mass) in a total injection volume of 100 μl PBS per fish. A total of 15 cod were used in this experiment, five were injected with $^{125}$I-fluorescein–pDNA/pDNA, five with $^{125}$I-fluorescein–pDNA/FSA and five with $^{125}$I-fluorescein–pDNA only. After 1 h, blood samples were collected from the caudal vein approximately 2 cm caudal to the injection site. The fish were killed with a blow to the head immediately after blood sampling. The heart, anterior kidney, liver and blood were analysed for radioactivity.

*Localisation of radiolabelled pDNA in blood*

Uptake of pDNA by blood cells was investigated by density gradient sedimentation of whole blood after i.v. and i.m. injection of $^{125}$I-labelled pDNA. A total of 10 cod were anaesthetised and injected i.v. (5 fish) and i.m. (5 fish) with $^{125}$I-fluorescein–pDNA (0.5-1 μg kg$^{-1}$ body mass) in a total injection volume of 100 μl PBS per fish. Blood samples were collected in heparin-coated tubes 1 h and 24 h after injection. A discontinuous gradient was made up of 10 ml 25% and 30 ml 50% Percoll® density medium. Whole blood (2 ml) diluted in 8 ml of L15 cell culture medium supplemented with heparin (50 i.u. ml$^{-1}$) were loaded onto each density gradient for centrifugation at 400 ***g*** for 40 min at 4°C in a centrifuge equipped with a swing-out rotor (Kubota 8800, Kubota Corporation, Tokyo, Japan). After centrifugation, 10 fractions of 5 ml were collected from the gradients. Fractions 1–4 contained plasma, fractions 5–7 contained the 25–50% interface where leukocytes are trapped, and fractions 7–10 contained red blood cells. The fractions were analysed for radioactivity.

*In vivo and in vitro stability of pDNA in blood*

The stability of pDNA in blood of Atlantic cod was assayed at 4°C *in vivo* and *in vitro* by gel electrophoresis. A total of five fish were injected i.v. with pDNA (5 mg kg$^{-1}$ body mass). Blood samples were collected 1, 5, 15, 30 and 60 min post-injection in EDTA-coated syringes. For the *in vitro* study, blood was collected from five fish in heparin-coated tubes and mixed with pDNA to a final concentration of 50 μg ml$^{-1}$ blood, corresponding to the calculated initial blood concentration after i.v. injection. The pDNA containing blood was incubated for 1, 5, 15, 30 and 60 min. The blood was then immediately mixed with the deoxyribonuclease inhibitor EDTA to a final concentration of 25 mmol l$^{-1}$ to stop pDNA degradation. Plasmid DNA was extracted from the plasma with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1, w/v; Invitrogen). The aqueous phase containing pDNA was ethanol precipitated and 500 ng of DNA applied to a 0.7% agarose gel for electrophoresis, stained with ethidium bromide and visualised in a transilluminator.

*Histological preparations*

Rhodamine–pDNA was detected by fluorescence microscopy 1 h after i.v. injection of pDNA (0.5 mg kg$^{-1}$ body mass) into Atlantic cod. The fish were killed by a blow to the head, and the heart, anterior kidney, spleen and liver were dissected out and fixed in 4% formaldehyde. Paraffin sections were prepared and then dewaxed in xylene and mounted in Histokitt. Specific fluorescence due to rhodamine–pDNA was observed as a bright red colour. Sections were examined using a Leica photomicroscope equipped with incident-light fluorescence optics. Pictures were taken with a Leica digital camera (Leica Microsystems, Wetslar, Germany).

*Isolation and cultivation of atrial endocardial endothelial cells*

Functionally intact atrial endocardial endothelial cells (aEEC) from cod were purified according to (Koren et al., 1997). In short, the heart was dissected out and perfused with L-15 medium supplied with heparin (10 i.u. ml$^{-1}$). The atria were dissected free and cut open. Ostial tissue rich fibrocytes and macrophages were discarded before transfer to a 50 ml sterile plastic tube with 25 ml of calcium-free buffer (Pertoft and Smedsrod, 1987). After 30 min incubation with horizontal shaking (250 cycles min$^{-1}$) the buffer was changed. The atria were then incubated with the following solutions: (i) trypsin (0.5 mg ml$^{-1}$) and EDTA (0.1 mg ml$^{-1}$) in PBS for 5 min; (ii) collagenase (0.5 mg ml$^{-1}$ in L-15 medium supplemented with 0.7 mg ml$^{-1}$ $CaCl_2$ $2H_2O$) for 30 min. The contents of the tube were transferred to a sterile Petri dish, and the atrium was flushed several times with the jet from a 10 ml plastic syringe, re-using the obtained cell suspension. The remaining tissue was discarded and the cell suspension was centrifuged for 5 min at 400 ***g***, and the pellet was resuspended in L-15. Contaminating macrophages (adherent cells) were removed according to the method of Sørensen et al. (Sørensen et al., 1998). The non-adherent cells were seeded on plastic (Falcon, Becton Dickinson & Company, NJ, USA) or glass tissue culture slides precoated with fibronectin (0.5 mg ml$^{-1}$). The incubation medium was L-15 supplemented with 10% FBS. The isolation procedure and incubations were carried out at 12–14°C. The cells were washed with L-15 medium after 24 h and used in experiments the same or the next day. The number of cells seeded per 2 cm$^2$ was approximately $10^6$. Contamination by cardiomyocytes and a few macrophages were observed. On average, more than 90% of the cells were aEEC, as evaluated by morphological characteristics.

*Isolation and cultivation of cod head kidney macrophages*

Cultures of head kidney macrophages were established as described previously (Braun-Nesje et al., 1981), with some modification by (Sørensen et al., 1997b). Briefly, the bilobular head kidney tissue was aseptically removed by ventral incision and minced through a 70 μm sterile nylon cell strainer (Falcon®; Becton & Dickinson Company, NJ, USA) with 10 ml L-15 medium containing heparin. A discontinuous gradient was made up of 10 ml 25% and 30 ml 51% Percoll solutions

and no more than 10 ml of the cell suspension were loaded onto each density gradient for centrifugation at 400 ***g*** for 40 min at 4°C. The cells banding in the 25–51% interface were collected for cultivation of macrophages. Aliquots of the cell suspension, containing 1–5×$10^6$ cells $ml^{-1}$, were plated in dishes for endocytosis studies.

### *Endocytosis of radiolabelled pDNA by cultured cod aEEC and cod head kidney leukocytes*

AEEC and head kidney leukocytes cultures established in 2 $cm^2$ wells (approximately 3×$10^5$ cells attached and spread per $cm^2$) were washed three times with L-15 medium and supplied with fresh medium containing 1% HSA and trace amounts of $^{125}$I-fluorescein–pDNA (20 000 c.p.m.) in a total incubation volume of 200 μl per well. Incubations of $^{125}$I-fluorescein–pDNA at 12°C were terminated after various times by removing incubation medium along with one washing volume of 500 μl PBS. Cell-associated ligand was quantified by solubilising the cell layer with 1% SDS, followed by counting in a gamma counter. Receptor-specific endocytosis of $^{125}$I-fluorescein–pDNA was examined in cod aEEC by inhibition studies. Monolayer cultures were incubated for 2 h with trace amounts of labelled ligand alone (control) or together with excess amounts of non-labelled macromolecules (100 μg $ml^{-1}$). Endocytosis experiments were terminated after 2 h at 12–14°C by removal of incubation medium and one washing volume of 500 μl PBS to a PD-10 column. Intact ligand was eluted in the void volume and degraded ligand in the total volume. Cell-associated ligand was quantified by solubilising the cell layer with 1% SDS, followed by counting in a gamma counter.

### *Fluorescence microscopy of cod aEEC after incubation with rhodamine–pDNA*

Cultures of aEEC were established on glass coverslips. Rhodamine–pDNA (10–50 μg $ml^{-1}$) was incubated together with the cultures for 2 h at 12–14°C. The cultures were fixed in 4% formaldehyde and embedded in an antifading medium (Dako fluorescent mounting medium, Glostrup, Denmark). The specific fluorescence due to Rhodamine–pDNA was observed as a bright red colour. Sections were examined using a Leica photomicroscope equipped with incident-light fluorescence optics. Pictures were taken using a Leica digital camera.

### *Gene transfer*

The pDNA (1.0 mg $kg^{-1}$ body mass) was injected i.m. into Atlantic cod in an injection volume of 100 μl PBS. Parallel series of three fish were collected 24, 48 and 168 h after injection. The fish were killed with a blow to the head, and heart, kidney, liver, spleen and injection site were aseptically removed. Blood samples were taken from the caudal vein, with EDTA as an anticoagulant. Tissues harvested for Southern blot analysis, real-time reverse transcription polymerase chain reaction (RT–PCR) and luciferase assay were put directly into liquid nitrogen, then kept at –86°C.

Monolayer cultures of aEECs (pooled cells from three fish, approximately 3×$10^5$ cells $cm^2$) were established in 9.6 $cm^2$ dishes. The cells were incubated with 10 μg pDNA in a total volume of 600 μl L-15 medium supplemented with 1% HSA. Incubation of pDNA at 12°C were terminated after 1, 4, 24 and 48 h of incubation. For Southern blotting, the cells were washed in PBS, trypsinised, collected and kept at –86°C. For measuring of luciferase activity the cells were solubilised according to manufacturer's instructions (Promega).

### *Nucleic acid purification from blood and tissue*

Total DNA was extracted from blood, tissue and cell samples with a DNeasy® Tissue Kit (Qiagen) according to the manufacturer's recommendation. DNA was treated according to the protocol with RNase A (Qiagen) to remove any RNA. Samples with an $A_{260}/A_{280}$ ratio between 1.8 and 2.0 were considered pure. DNA concentrations were measured using NanoDrop® ND-1000. DNA was eluted in nuclease-free water (Ambion, Austin, TX, USA).

Total RNA was extracted using the Trizol method (Chomczynski and Sacchi, 1987) with some modifications. Briefly, tissue samples were homogenised in 1 ml TRIzol reagent using a rotor-stator homogeniser (UltraThurax; IKA Werke, Staufen, Germany). For additional removal of DNA and proteins, the water phase of the initial TRIzol/chloroform separation was added to a second volume of TRIzol reagent. To remove any contaminating DNA, samples were treated with DNase (TURBO DNA-free, Ambion) according to the manufacturer's recommendation and eluted in nuclease-free water. Purified RNA was confirmed to be intact by gel electrophoresis. Samples with an $A_{260}/A_{280}$ ratio between 1.8 and 2.2 were considered pure. RNA concentrations were measured using the RiboGreen® RNA Quantitation Kit (Molecular Probes, Eugene, OR, USA) (Jones et al., 1998).

### *Southern blot analysis*

Ten micrograms of total DNA from fish and 2 μg of total DNA from cells were digested with *Mlu*I (Promega) and run on a 0.7% agarose gel. DNA was blotted to a Nytran N membrane (Schleicher and Schuell, Dassel, Germany) with a Turboblotter as recommended by the manufacturer and visualised by enzyme-linked chemiluminescence using digoxigenin (DIG)-labelled PCR probe. The DIG-labelled PCR probe was constructed using the PCR DIG Probe Synthesis Kit from Roche (Roche Diagnostics, Mannheim, Germany) and Luc 1 primers (Table 1), amplifying a 397 bp fragment in the firefly luciferase gene. Primers were designed using the Primer Express software (version 2.0; Applied Biosystems) and synthesized by Operon Biotechnologies Inc. (VWR, West Chester, PA, USA).

### *Complementary DNA synthesis*

RNA was reversely transcribed according to the manufacturer's instructions using random hexamers (TaqMan RT-reagents; Applied Biosystems, CA, USA) under the following conditions; 25°C for 10 min, 37°C for 60 min, 95°C

Table 1. *Primers and probes used in digoxigenin (DIG) PCR probe labelling and real-time RT–PCR (TaqMan assays)*

| Method | Primer | | Oligonucleotides | Concentration (nmol $l^{-1}$) | Amplicon (bp) |
|---|---|---|---|---|---|
| DIG PCR probe labelling | Luc 1 | Forward | 5′-caaatcattccggatactgcg-3′ | 400 | 397 |
| | | Reverse | 5′-cccggtttatcatcccct-3′ | 400 | |
| Real-time PCR assays | Luc 2 | Forward | 5′-tgggctcactgagactacatca-3′ | 900 | 64 |
| | | Reverse | 5′-cgcgcccggtttatcatc-3′ | 900 | |
| | | Probe | FAM-tcgggtgtaatcagaatag | 250 | |
| Real-time PCR assays | 18S | Forward | 5′-gccctgtaattggaatgagtgtact-3′ | 900 | 101 |
| | | Reverse | 5′-acgctattggagctggaattacc-3′ | 900 | |
| | | Probe | FAM-cttgccctccaattgg | 250 | |

for 5 min. Reaction volumes were 10 μl and contained 50 ng of total RNA.

### *Real-time RT–PCR*

Real-time RT–PCRs were performed in duplicate with an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems) at the following cycling conditions: 50°C for 2 min, 95°C for 15 min, 40 cycles of 95°C for 15 s and 58°C for 60 s. Every PCR contained 2× TaqMan Universal PCR Master Mix; Applied Biosystems [AmpliTaq Gold DNA Polymerase, dNTPs, passive reference (ROX) and optimised buffer components], 20× TaqMan Assay Mix, 50 ng of cDNA template and nuclease-free water to a final volume of 25 μl. Primers and probes were designed and synthesised by Applied Biosystems (Custom TaqMan® Gene Expression Assays) based on the firefly luciferase gene and 18S rRNA sequence (Acc. No. AF518205; Table 1). The relative expression ratio (R) of the luciferase gene was calculated based on primer efficiencies (E) and the $C_t$ deviations ($\Delta C_t$) of the unknown samples *versus* a calibrator after normalisation to 18S rRNA (Pfaffl, 2001). The amplification efficiencies of the primers were assessed from twofold liver cDNA dilutions. DNA contamination of the RNA was evaluated by subjecting parallel samples to real-time PCR without preceding reverse transcription. All data were captured using Sequence Detection Software (SDS version 1.1; Applied Biosystems).

### *Extraction and measurement of luciferase protein in cod tissues and in cultures of cod aEEC*

Tissue samples from Atlantic cod injected i.m. with pDNA (1.0 mg $kg^{-1}$ body mass) were collected 24, 48 and 168 h postinjection and processed as described previously (Manthorpe et al., 1993). Monolayer cultures of aEECs (pooled cells from three fish, approximately $3\times10^5$ cells $cm^2$) were established in 9.6 $cm^2$ dishes. The cells were incubated for 1, 4, 24 and 48 h with 10 μg pDNA in 600 μl L-15 medium supplemented with 1% HSA. The experiment was terminated at different time points by adding 300 μl lysis buffer (luciferase assay system; Promega, Madison, Wisconsin, USA). Enzyme activity in the solubilised tissues or cell suspension was measured according to the manufacturers recommendation (Promega). The results were recorded using a Luminoscan Ascent® microplate luminometer (Thermo Electron Oy, Vantaa, Finland). The background, defined as the average luciferase levels plus 2 s.e.m. from tissues or cells that were not injected or incubated with pDNA, were deducted from all values. The relative light units (RLU) were normalised to the concentration as determined by the Bio-Rad RC DC protein assay. The Bio-Rad RC DC protein assay was performed according to the manufacturer's description (Bio-Rad, Hercules, California, USA). To compensate for the colour masking/difference of the solubilised tissues known amounts of tissues were incubated with a fixed amount recombinant luciferase protein (Promega).

## Results

### *Characterisation of native and modified plasmids*

Gel electrophoresis demonstrated no topoformic differences in pDNA after labelling with fluorescein (not shown). Following labelling with *Label*IT-fluorescein, fluorescent pDNA was visible in the absence of ethidium bromide staining due to covalently attached fluorescein (Slattum et al., 2003). Fluorescent bands were detected only as open circular and supercoiled pDNA topoforms (data not shown). Fluorescent bands and pDNA disappeared after DNase treatment. Neves et al. (Neves et al., 2000) demonstrated a 60% reduction of gene transfer efficiency when conjugating fluorescent molecules to pDNA by photoactivation. To ensure that the luciferase gene was capable of expressing the transgene after labelling pDNA with fluorescein, labelled and unlabelled pDNA was transfected into semiconfluent Caco-2 cells. The gene transfer efficiency of the labelled plasmid in Caco-2 cells was reduced by 75% compared to transfection with non-labelled pDNA.

### *Anatomical distribution of $^{125}$I-fluorescein–pDNA*

The anatomical distribution of i.v. and i.m. injected pDNA was investigated by injecting trap-labelled pDNA. The trap molecule (fluorescein) is unable to pass through biological membranes and is not actively taken up by cells unless it is conjugated to a macromolecule. When inside the cell the radioactive trap label will remain within the cell independent of the fate of the attached macromolecule. One hour after i.m. injection of trace amounts of $^{125}$I-fluorescein–pDNA, approximately 90% of the radioactivity was recovered from the injection site, 6.6% was found in the blood, 2% in the heart, 2.3% in the liver and 3.2% in the intestine. Transport of labelled pDNA (not taken up) from the injection site to other organs

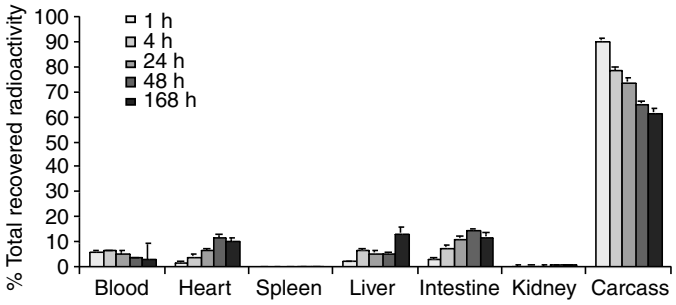


Fig. 1. Time course of the appearance of radioactivity in Atlantic cod tissues after intramuscular administration of trace amounts of $^{125}$I-fluorescein–pDNA (0.5–1 μg kg$^{-1}$ body mass). The results are expressed as means + s.e.m. of five fish per time-point.

studied at 1, 4, 24, 48 and 168 h showed that the labelled pDNA disappeared from the injection site and accumulated in the intestine, heart and liver almost at the same rate (Fig. 1). After 48 h approximately 15% of the radioactivity was found in the intestine, 12% in the heart and 5.5% in the liver. The specific activity of the heart tissue at 48 h was much higher than the specific uptake (c.p.m. g$^{-1}$ tissue) of the intestine and the liver ($1.09\times10^5$ c.p.m. g$^{-1}$ tissue for the heart tissue compared to $5\times10^3$ c.p.m. g$^{-1}$ tissue for the intestine and $1.0\times10^3$ c.p.m. g$^{-1}$ tissue for the liver). The kidney and spleen contained less than 1% of the recovered label at all time-points. Total recovered radioactivity was never less than 66% of the injected dose of labelled ligand.

Anatomical distribution of blood-borne pDNA was investigated after i.v. injection of trace amounts of $^{125}$I-fluorescein–pDNA, the amount of radioactivity in various tissues was recorded after 5 min, 15 min, 1 h, 4 h, 24 h, 48 h and 168 h. Radiolabelled pDNA was rapidly eliminated from the circulation during the first 15 min of the experiment. The total recovered radioactivity in the blood decreased from 73.2% after 5 min to 14.7% after 60 min and to 2.7% after 168 h (Fig. 2). Approximately 30% of the total radioactivity was found in the heart 5 min after injection of $^{125}$I-fluorescein–pDNA; 168 h later the amount increased slightly to 44%. The specific activity in the heart during the same period did not increase. After 15 min the specific activity was $2.66\times10^5$ c.p.m. g$^{-1}$ tissue and $2.71\times10^5$ c.p.m. g$^{-1}$ tissue after 168 h. The amount of radioactivity found in the kidney and liver was never higher than 3.8% of the total recovered radioactivity for the kidney and 8.6% for the liver. In the spleen and the intestine the amount of radioactivity recovered was low. The high heart to blood ratio (30.3:1) compared with the much lower ratios in liver, anterior kidney and spleen (0.18:1, 1.14:1 and 0.99:1, respectively) indicates a very effective uptake mechanism for pDNA in the cod heart. The total amount of radioactivity found in the carcass after injection of $^{125}$I-fluorescein–pDNA decreased from 38% after 5 min to 33% after 168 h. The specific activity in the carcass was never higher than $1.0\times10^2$ c.p.m. g$^{-1}$ tissue.

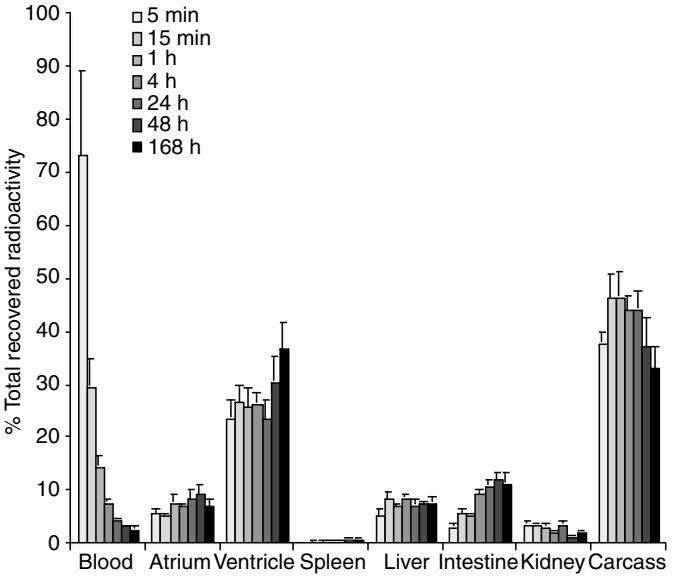


Fig. 2. Time course of the appearance of radioactivity in Atlantic cod tissues after intravenous administration with trace amounts of $^{125}$I-fluorescein–pDNA (0.5–1 μg kg$^{-1}$ body mass). The results are expressed as means + s.e.m. of five fish per time-point.

Injection of $^{125}$I-fluorescein–pDNA together with excess amounts of unlabelled pDNA or FSA, a ligand for the cod aEEC scavenger receptor, effectively blocked uptake of radiotracer by the heart (Table 2). This indicated that the uptake of pDNA by the cod heart was specific and was mediated by the cod aEEC scavenger-like receptor (Sørensen et al., 1998). The uptake of radiotracer was inhibited in the kidney as well, indicating that there was a specific uptake of pDNA in this organ. The uptake in spleen, liver, intestine and carcass was not

Table 2. *Ratio of tissue to blood specific activity* following intravenous injections of $^{125}$I-fluorescein-DNA (0.5–1 μg kg$^{-1}$ body mass)*

| | $^{125}$I-fluorescein-pDNA | | |
|---|---|---|---|
| | No inhibitor | + pDNA | + FSA |
| Heart | 90.8±20.6 | 13.5±2.90 | 2.89±1.01 |
| Kidney | 5.79±2.14 | 2.54±0.63 | 1.17±0.27 |
| Spleen | 2.26±1.26 | 3.80±2.28 | 1.61±0.38 |
| Liver | 0.26±0.08 | 0.18±0.09 | 0.13±0.04 |
| Intestine | 0.24±0.81 | 0.20±0.05 | 0.16±0.02 |
| Carcass | 0.97±0.07 | 0.15±0.05 | 0.23±0.19 |
| Blood | 1 | 1 | 1 |

Labeled ligands were injected alone, or along with excess amounts of non-labelled DNA or formaldehyde-treated serum albumin (FSA). The fish were killed 1 h after intravenous injection of $^{125}$I-fluorescein-DNA. Values are means ± s.e.m. (*N*=5).

*Ratio of tissue to blood specific activity was calculated as (c.p.m. g$^{-1}$ tissue)/(c.p.m. g$^{-1}$ blood). The specific activity in blood 1 h after injection of $^{125}$I-fluorescein-DNA alone was 14.08±$1.91\times10^3$ c.p.m. g$^{-1}$ tissue, with non labeled DNA 24.24±$6.75\times10^3$ c.p.m. g$^{-1}$ tissue and with FSA 14.92±$6.34\times10^3$ c.p.m. g$^{-1}$ tissue.

influenced by co-injection of pDNA and FSA. The tissue to blood ratio, after inhibition of the cod aEEC scavenger-like receptor indicates that the radiotracer, not taken up by the heart, was not taken up by other organs and most likely continued to circulate in the blood.

Furthermore, we investigated whether $^{125}$I-fluorescein–pDNA was bound to or taken up by blood cells or if it remained in the plasma fraction 1 h after i.v. injection. Density gradient centrifugation demonstrated that more than 93% of the radioactivity was present in the serum/plasma fraction and only minor amounts were found in the cellular fractions containing white blood cells (4%) and red blood cells (0.5%).

*In vivo and in vitro stability of native pDNA in cod blood*

The degradation of pDNA in blood *in vivo* and *in vitro* was investigated. The supercoiled topoform of pDNA was completely changed to open circular and linear topoforms within 5 min after injection and then degraded to low molecular mass products (Fig. 3A). The *in vitro* degradation of pDNA by blood was similar (Fig. 3B).

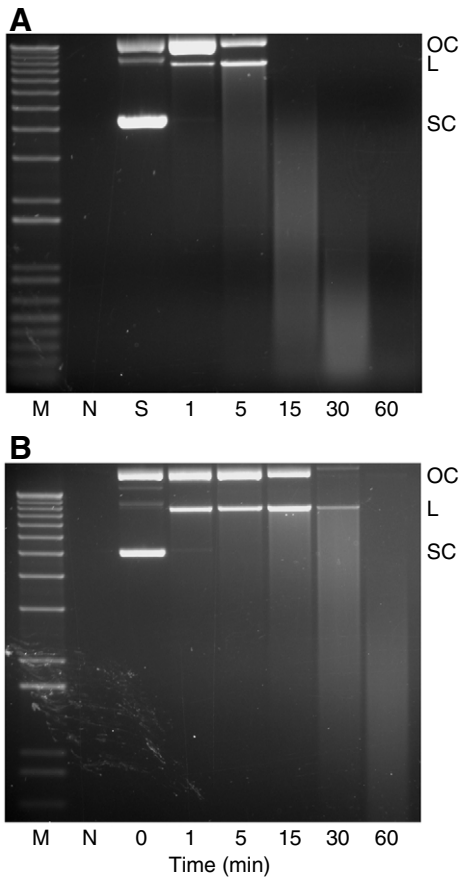


Fig. 3. Stability of R70pRomiLuc in Atlantic cod blood after intravenous administration (A) and *in vitro* in whole blood (B). Supercoiled (SC) and open circular (OC) topoform were reduced to linear (L) topoform and degradation products, both *in vitro* and *in vivo*. M, molecular marker 1 kb plus (Invitrogen); N, negative control (blood without R70pRomiLuc); S, standard (R70pRomiLuc). The negative control demonstrates that there is no detectable endogenous pDNA in the blood.

*Southern blot analysis of cod tissues and cells*

The topoform of the re-isolated pDNA in blood, liver, kidney, spleen, heart, and injection site was examined after injection of pDNA (1 mg kg$^{-1}$ body mass). Southern blot revealed extra-chromosomally super-coiled, open circular and linear pDNA in all the investigated tissues 48 h after i.v. injection. Degradation products were only detected in the muscle tissue at the site of injection (Fig. 4). No pDNA was detected in tissues from PBS injected fish.

*Expression of luciferase mRNA and protein after intramuscular injection of pDNA into Atlantic cod*

Expression of luciferase mRNA and protein was investigated after i.m. injection of pDNA (1 mg kg$^{-1}$ body mass). Real-time RT–PCR demonstrated luciferase mRNA in the muscle tissue at the site of injection 168 h after injection. Luciferase mRNA was not found in any other tissue or in controls.

Enzymatically active luciferase was observed in muscle tissue at the injection site 168 h after injection. In the muscle tissue we measured 115.71±65 relative light units (RLU) per mg protein, compared to 0.25 RLU mg$^{-1}$ protein in the control fish. In heart, kidney, spleen, liver and muscle tissue no expression of luciferase protein was found.

*Cellular distribution of rhodamine–pDNA*

Tissue sections prepared from fish 1 h after i.v. injection with fluorescein–pDNA revealed the presence of fluorescence in endocardial cells in both the atrium (Fig. 5A) and the ventricle (Fig. 5B).

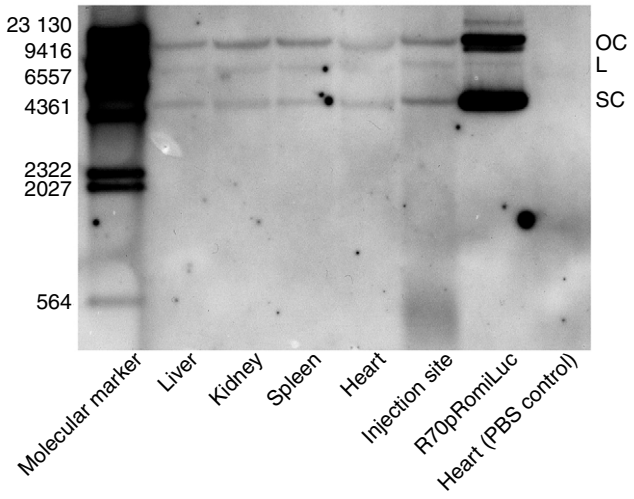


Fig. 4. Southern blot analysis of tissues 48 h after intramuscular administration of native pDNA (1 mg kg$^{-1}$ body mass). SC, supercoiled topoform; OC, open-circular topoform,; L, linear topoform. All topoforms of pDNA (R70pRomiLuc) were detectable in all the investigated tissues, but degradation products were only visible in samples from the injection site

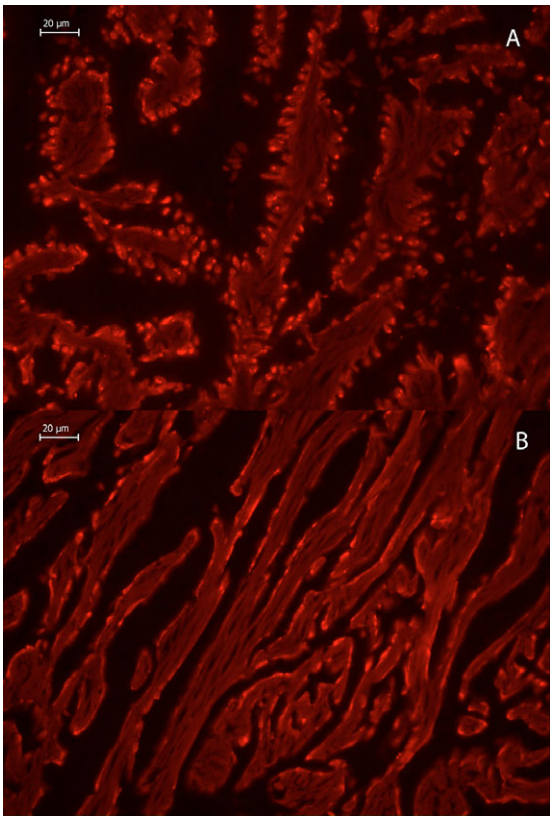


Fig. 5. Fluorescence micrograph of atrium (A) and endocardium (B) of cod heart 24 h after intravenous injection of rhodamine–pDNA. The fluorescence can be seen in discrete vesicles evenly distributed throughout the cytoplasm of the endocardial cells.

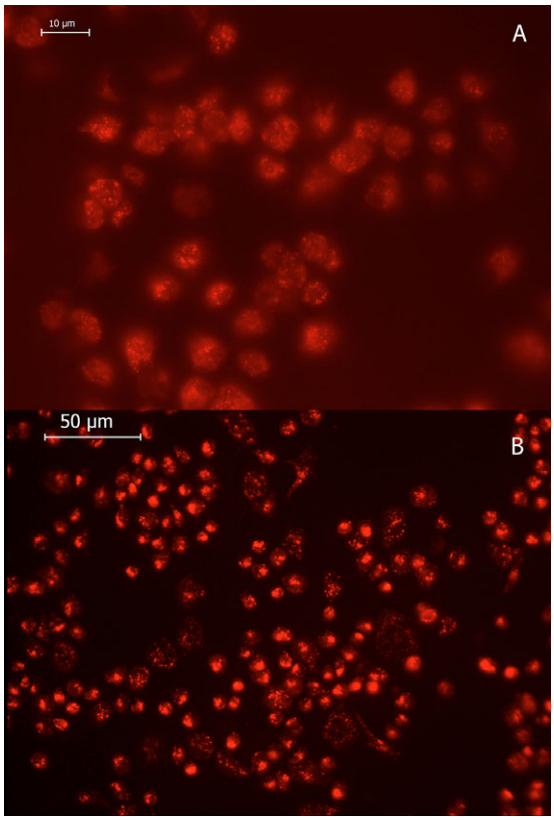


Fig. 6. Fluorescence micrograph of atrial endocardial endothelial cells (A) and cod head kidney leukocytes (B) cultured on glass coverslip and incubated with rhodamine–pDNA for 2 h. All cells in the monolayer cultures accumulated the pDNA. Note that fluorescence is confined to discrete vesicles which are probably endocytic vesicles.

### *Endocytosis of rhodamine–pDNA in cod aEEC and head kidney leukocytes in vitro*

Cultures of aEECs (Fig. 6A) and head kidney leukocytes were incubated with rhodamine–pDNA for 2 h. Subsequent fixation and examination in the fluorescence microscope revealed that all cells accumulated large amounts of fluorescent Rhodamine in distinct vesicles.

### *Kinetics of endocytosis of pDNA in cultured cod aEEC and leukocytes*

To study the kinetics of endocytosis, cultured cod aEEC or leukocytes were incubated with trace amounts of $^{125}$I-fluorescein–pDNA for 1, 2, 4, 24 and 48 h. Of the $^{125}$I-fluorescein–pDNA added to the cod aEEC, 2.1% was endocytosed after 1 h and approximately 32% after 48 h (Fig. 7A). For the head kidney macrophages, the corresponding numbers were 2.7% after 1 h and approximately 28.8% after 48 h (Fig. 7B). The specificity of endocytosis of $^{125}$I-fluorescein–pDNA was studied by attempting to inhibit the uptake of radiolabelled ligands in cultured cod aEEC and head kidney leukocytes using excess amounts of unlabelled macromolecules (100 μg ml$^{-1}$). Incubation of aEEC with $^{125}$I-fluorescein–pDNA in the presence of pDNA or ligands for the cod aEEC scavenger receptor (FSA, fucoidan and dextran sulphate) inhibited the uptake by at least 70% (Fig. 8). Mannan, a ligand for the cod aEEC mannose receptor did not inhibit the uptake of $^{125}$I-fluorescein–pDNA. The uptake of $^{125}$I-fluorescein–pDNA by the head kidney leukocytes was inhibited by 50% by non-labelled pDNA and less than 30% by ligands for the cod aEEC scavenger receptor (results not shown).

### *Southern blot analysis of endocytosed pDNA in cod aEEC in vitro*

Southern blot analyses were performed on solubilised cells 1, 4, 24 and 48 h after incubation of 10 μg pDNA. Southern blot analysis of cultured aEEC showed a complete degradation of supercoiled, open-circular and linear pDNA to low molecular mass products after 24 h (Fig. 9). No pDNA was detected in control aEEC or in the last PBS washing solution, prior to DNA isolation.

### *Expression of luciferase mRNA and enzyme activity in aEEC*

Expression of luciferase mRNA and enzyme activity was investigated after adding 10 μg of pDNA to cultured aEEC. Real-time RT–PCR demonstrated no luciferase mRNA

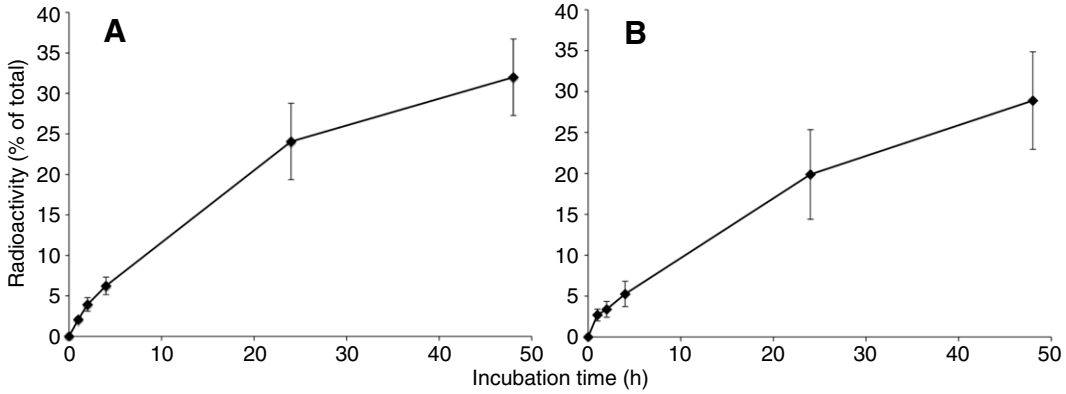


Fig. 7. Kinetics of endocytosis of $^{125}$I-fluorescein–pDNA in cultured cod atrial endocardial endothelial cells at 12°C (A) and head kidney leukocytes at 6°C (B). Monolayer cultures of aEEC or head kidney leukocytes in 2 cm$^2$ wells were incubated with trace amounts of $^{125}$I-fluorescein–pDNA (approximately $2\times10^4$ c.p.m.; 20 ng). The results are presented as the cell-associated percentage of the total added radioactivity and are the mean of three independent experiments (aEEC). The result from the head kidney leucocyte study are the mean of cells from eight different fish.

transcripts in the aEEC. Luciferase enzyme activity was not measurable in aEEC.

## Discussion

The aim of the present study was to determine the tissue distribution, cellular uptake and fate of naked DNA in Atlantic cod (*G. morhua* L.). Plasmid DNA or pDNA tagged with fluorescence or radioactivity, was administered i.m. or i.v. to cod or incubated with cod aEECs or cod head kidney leukocytes *in vitro*. The specificity was examined *in vivo* and *in vitro* by competitive ligand inhibition. For the *in vivo* and *in vitro* receptor studies, fluorescein–pDNA was labelled with $^{125}$I. The resulting specific activity of $^{125}$I-fluorescein–pDNA was sufficiently high to allow studies of binding of DNA in cultured aEECs and leukocytes. Uptake of conjugated DNA was inhibited by unlabelled pDNA demonstrating that the conjugation did not alter structures in the DNA molecule necessary for uptake and receptor recognition. Expression of the luciferase gene mediated by the CMV-IEP promoter in Atlantic cod was demonstrated in muscle tissue 168 h after injection of unlabelled pDNA. The reporter gene was expressed after transfection of the conjugated plasmid in Caco-2 cells, although gene transfer efficiency was decreased by 75% as compared with unlabelled DNA.

The present results revealed that trace amounts of $^{125}$I-fluorescein–pDNA after i.m. injection disappeared from the injection site and accumulated almost at the same rate in the heart, liver and intestine. The use of a radiolabelled fluorescein adduct that is trapped after endocytosis (Li et al., 2003), with almost no escape of the label from the intracellular compartments, allows a reliable measurement of the anatomical and cellular site of uptake of pDNA. Local degradation of pDNA in the muscle tissue (at the site of injection) of mice has previously been reported by (Barry et al., 1999; Manthorpe et al., 1993). In those studies, 95–99% of the injected pDNA was degraded and was no longer present in tissue 90 min after injection, as detected by Southern blot and PCR. In our experiment, Southern blot analysis of muscle tissue from the injection site showed presence of degradation products in addition to intact pDNA 48 h after injection. A total of 30% the radiolabel escaped from the site of injection during a period of 48 h. Furthermore, the finding of 10% of the recovered radioactivity in the cod heart after 48 h indicated that a part of the injected pDNA escaped the local degradation and entered the blood where it was rapidly cleared by the cod heart cells.

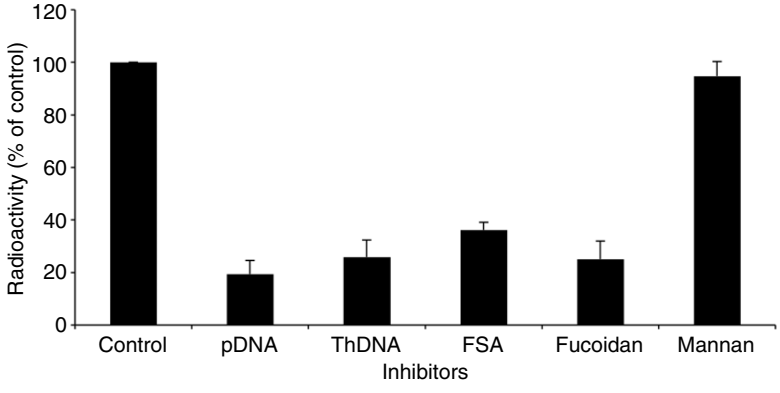


Fig. 8. Specificity of endocytosis of $^{125}$I-fluorescein–pDNA in cultured cod atrial endocardial endothelial cells. Monolayer cultures were incubated for 2 h at 12°C with trace amounts of labelled ligand (approximately $2\times10^4$ c.p.m.; 20 ng) alone (control) or together with excess amounts of unlabelled macromolecules (100 μg ml$^{-1}$). The following macromolecules were used: plasmid DNA (pDNA), thymus DNA (ThDNA), formaldehyde-treated serum albumin (FSA), fucoidan and mannan. The results are presented as a percentage of the control value and are mean + s.e.m. of three independent experiments.

This result was confirmed by Southern blot analysis that demonstrated intact pDNA in heart tissue 48 h after injection. The $^{125}$I-fluorescein has been shown not to be recognised or taken up by the heart (Sørensen et al., 1997a). The radioactivity in the intestine and liver after i.m. injection may be a result of drainage of low molecular mass $^{125}$I-fluorescein or $^{125}$I-fluorescein-labelled oligonucleotides from the injection site to the general circulation where it was secreted by the kidney or bile to the intestine. However, Southern blot analysis of liver and intestine tissues showed the presence of intact pDNA. Accumulation of macromolecules in the intestine after intravenous injection has been reported previously by Dalmo et al. (Dalmo et al., 1996).

Gradient centrifugation of the blood after injection of $^{125}$I-fluorescein-pDNA showed that 90% of the radioactivity was present in the plasma fraction. An *in vitro* incubation of pDNA with whole blood or plasma showed a complete degradation of the pDNA within minutes. These findings, together with the observation that the amount of radiolabel in the blood was low at all time-points investigated, suggested that the transit time of labelled pDNA or its degradation products in the circulation was very short. Sørensen et al. (Sørensen et al., 1997a) reported that subcutaneously injected hyaluronan (a connective tissue macromolecule), was transported to the blood circulation before being finally eliminated by cells in the cod heart. In their experiment 65% of the injected radiolabelled hyaluronan was found in the heart after 48 h.

Intravenously administered $^{125}$I-fluorescein–pDNA was rapidly eliminated from the circulation and mainly taken up by the cod heart, where approximately 25% of the total recovered radioactivity was found 5 min after administration. The amount of radioactivity recovered from the heart at later time-points was essentially the same. This result confirmed the high blood clearance capacity and the efficiency of the cod aEEC system. The maximum clearance capacity of these cells was not tested in this study but the total amount recovered from the heart after injecting 10 000 c.p.m. $\mu g^{-1}$ pDNA in the ligand competition study corresponded to a total uptake of 25 μg DNA compared to approximately 0.25 μg in the initial study. This indicated that most of the injected pDNA was cleared from the circulation within 5 min. The amounts of radioactivity found in kidney, spleen, liver and intestine were lower compared with the amounts found in the heart. The total amount of radioactivity found in the carcass was high, but the specific activity was less than $7.1\times10^3$ c.p.m. $g^{-1}$ tissue compared with $3217\times10^3$ c.p.m. $g^{-1}$ tissue in the heart tissue. The efficient uptake of macromolecules by the cod heart has been reported earlier. Intravenously injected radiolabelled gelatinised cod skin collagen was rapidly eliminated from the blood ($t_{\frac{1}{2}}$=15 min), and taken up by the heart, with minor uptake in other organs (Smedsrød et al., 1995). The cod heart is the most important scavenger organ for circulating FSA, aminoterminal propeptides of type 1 procollagen (P1NP) and bacterial lipopolysaccharide (LPS) all of which are ligands for the scavenger receptors. In salmonid fish, the kidney tissue has been reported to be the major organ for DNA clearance, followed by the liver, blood, muscle and gonadal tissue after i.v. administration (Nielsen et al., 2006). In mammals, the liver is the main organ responsible for the rapid clearance of DNA from the circulation. The non parenchymal cells of these mammals are mainly responsible for this clearance, with the liver sinusoidal endothelial cells as the major contributor (Hisazumi et al., 2004). Uptake of DNA in other organs such as kidney and bone marrow has also been reported (Bijsterbosch et al., 1997).

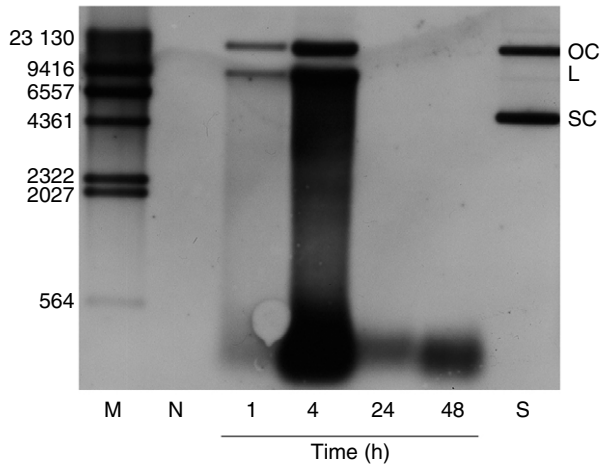


Fig. 9. Southern blot analysis of degradation of pDNA in cultures cod atrial endocardial endothelial cells (aEEC). Monolayer cultures were incubated with 10 μg native pDNA and cells were solubilised after 1, 4, 24, 48 h. SC, super coiled topoform; OC, open-circular topoform; L, linear topoform. Open-circular and linear topoforms of pDNA (R70pRomiLuc) were detectable after 1 h. After 24 h, no intact pDNA was detected in the cells.

Fluorescence microscopy showed that rhodamine pDNA was taken up by endocardial endothelial cells lining the muscular trabecula of both heart chambers. The endocytic (high blood clearance) capacity of the endocardial endothelial cell of cod has been well studied. These special endocardial endothelial cells, together with the sinusoidal endothelial cells of the mammalian liver and salmonid kidney have been characterized as scavenger endothelial cells (SEC). Using specific and extremely effective endocytosis they eliminate soluble waste macromolecules from the circulation at a very high rate (Seternes et al., 2002). *In vitro* studies in cultured atrial endocardial cells have provided functional evidence for the existence of distinct scavenger, mannose and hyaluronan receptors in these endocytically highly active cells (Seternes et al., 2001b; Sørensen et al., 2001; Sørensen et al., 1998). When trace amounts of radiolabelled DNA was co-injected with excess amounts of unlabelled pDNA or FSA, the tissue to blood ratio measured after 1 h in the atrium and ventricle was reduced by more than 90%, indicating that the uptake of pDNA in the cod heart was specific and largely mediated *via* a functional scavenger receptor. The observation that the *in vitro* uptake of $^{125}$I-fluorescein pDNA was effectively inhibited by excess amounts of scavenger receptor ligands supports the idea that

pDNA was taken up *via* the functional scavenger receptor expressed by these cells. Sørensen et al. (Sørensen et al., 1998) and Seternes et al. (Seternes et al., 2001a) have demonstrated the presence of a functional scavenger receptor in cultures of aEEC by ligand competition studies. Similar studies in cultures of rat liver sinusoidal endothelial cells (sLEC) have suggested that the scavenger receptor was responsible for cellular binding and uptake of naked DNA in both rats and mice (Hisazumi et al., 2004).

Ligands taken up by the cod aEEC are subjected to intracellular degradation in endosomes and lysosomes. Southern blot analysis of solubilised cod aEEC cells after incubation with unlabelled pDNA showed total degradation of pDNA. Earlier studies by Sørensen et al. (Sørensen et al., 1998) showed that as much as 45% of the added $^{125}$I-FSA and $^{125}$I-PINP were degraded and recovered as low molecular mass products after 24 h of incubation with cultured aEEC. Moreover, hyaluronan, a connective tissue polysaccharide, was taken up by the aEEC *in vitro* and degraded to acetate (Seternes et al., 2001b). Another ligand for the aEEC scavenger receptor, lipopolysaccharide was not degraded by the aEEC even after 48 h incubation (Seternes et al., 2001a). All together, the aEEC shows an efficient endocytic and degradative capacity towards a whole range of macromolecules. Degradation of pDNA have been demonstrated in primary cultures of rat sinusoidal endothelial cells by release of acid-soluble radioactivity from the cells after incubation with $^{32}$P-pDNA (Hisazumi et al., 2004). The use of the trap-labelled fluorescein in our study prevented us from measuring acid-soluble radioactivity in the culture media. Expression of the luciferase reporter gene was investigated in cod aEEC after incubation with unlabelled pDNA by real-time RT–PCR and luciferase enzyme assay. The construct used in this study was shown to give expression of luciferase protein in cod muscle tissue 168 h after i.m. injection. Our results showed that neither luciferase mRNA nor enzyme were present in the cultured aEEC, even after 48 h of incubation. These results were in accordance with the i.v. and i.m. injection results that demonstrated high amounts of pDNA in the heart but no expression of mRNA and luciferase protein.

The total recovered radioactivity in the kidney was low, but there was still a reduction after co-injection of unlabelled pDNA and FSA. The anterior kidney of Atlantic cod and other teleosts is a haematopoietic organ and a rich source of macrophages (Braun-Nesje et al., 1981; Sørensen et al., 1997b). These results indicated that the cod macrophage was involved in the clearance of circulatory pDNA. A functional scavenger receptor responsible for uptake of particles in fish macrophages was reported in rainbow trout macrophages by Frøystad et al. (Frøystad et al., 1998). However, this macrophage receptor seemed to be of minor importance in the blood clearance of soluble scavenger receptor ligands (Seternes et al., 2001a; Sørensen et al., 1998). *In vitro* studies of head kidney leukocytes from cod demonstrated that these cells have the capability to bind and endocytose pDNA. In spite of these results, that the head kidney cells can take up pDNA from the circulation, the results from the distribution studies with $^{125}$I-fluorescein–pDNA strongly suggest that the head kidney leukocytes contributed less to the total *in vivo* blood clearance of pDNA in cod. The uptake of pDNA in cultures of aEEC is rapid, with approximately 33% of the total added $^{125}$I-fluorescein–pDNA taken up after 48 h. However, compared with the uptake rate of other SR ligands (added in the same concentration, approximately $1\times10^6$ c.p.m. $\mu g^{-1}$ protein) by the aEEC it is markedly slower. Already 1 h after incubation of $^{125}$I-FSA the uptake reached 25%, increasing to 75% after 6 h (Sørensen et al., 1998). The reason for the difference in rate of uptake was not investigated. The uptake of $^{125}$I-fluorescein–pDNA by the head kidney leukocytes was similar with approximately 29% of the total added radioactivity taken up after 48 h.

Based on the results from the present and earlier studies, it seemed that there was drainage of intact pDNA from the site of injection to the general blood circulation. The transit time of pDNA in the circulation was very short, and even if the pDNA was subjected to degradation, most of the blood-borne pDNA was cleared by specific uptake by scavenger endothelial cells lining the muscular trabecula of both heart chambers. The uptake of pDNA was inhibited both *in vivo* and *in vitro* by co-injecting ligands recognised by scavenger receptors. The endocytosed pDNA was degraded by the aEEC *in vitro*. There was no expression of the luciferase mRNA or protein in the cod aEEC. The cod head kidney macrophage was able to take up pDNA by a specific mechanism *in vitro*. However, the head kidney macrophage did not seem to be involved in the *in vivo* clearance of pDNA from the blood.

The present study was financially supported by the Research Council of Norway and by the European Commission Grant IMAQUANIM (contract no. 007103).

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