Oncorhyncin III: a potent antimicrobial peptide derived from the non-histone chromosomal protein H6 of rainbow trout, *Oncorhynchus mykiss*

Jorge M. O. FERNANDES*, Nathalie SAINT†, Graham D. KEMP‡ and Valerie J. SMITH*[1]

*Gatty Marine Laboratory, School of Biology, University of St Andrews, Fife KY16 8LB, Scotland, U.K., †Centre de Biochimie Structurale, UMR 5048 CNRS, 554 INSERM, 34090 Montpellier, France, and ‡Centre for Biomolecular Sciences, School of Biology, University of St Andrews, Fife KY16 9QT, Scotland, U.K.

A 6.7 kDa antimicrobial peptide was isolated from trout skin secretions using acid extraction followed by cation-exchange chromatography, $^{t}C_{18}$ solid-phase extraction, and C_{18} reversed-phase HPLC. The molecular mass of this peptide, which is tentatively named oncorhyncin III, is 6671 Da, as determined by matrix-assisted laser-desorption ionization MS. N-terminal amino acid sequencing revealed that the first 13 residues of oncorhyncin III are identical with those of the non-histone chromosomal protein H6 from rainbow trout. Hence these data combined with the MS results indicate that oncorhyncin III is likely to be a cleavage product of the non-histone chromosomal protein H6 (residues 1–66) and that it probably contains two methylated residues or one double methylation. The purified peptide exhibits potent antibacterial activity against both Gram-positive and Gram-negative bacteria, with minimal inhibitory concentrations in the submicromolar range. The peptide is sensitive to NaCl, and displays no haemolytic activity towards trout erythrocytes at concentrations below 1 μM. Scanning electron microscopy revealed that oncorhyncin III does not cause direct disruption of bacterial cells. Reconstitution of the peptide in planar lipid bilayers strongly disturbs the membranes, but does not induce the formation of stable ion channels. Taken together, these results support the hypothesis that oncorhyncin III plays a role in mucosal innate host defence.

Key words: antimicrobial peptide, histone, mucosal immunity, oncorhyncin III, teleost, trout.

INTRODUCTION

Antimicrobial peptides are increasingly recognized as playing crucial roles in innate immunity, serving not only to kill micro-organisms directly but also, in mammals, to modulate other immune processes [1]. Over 750 such peptides from a variety of eukaryotic species are now listed on databases (see, for example, http://www.bbcm.univ.trieste.it/~tossi/), although relatively few have been described for teleost fish. For several animal taxa, antimicrobial peptides are key components of the secretions of the mucosal surfaces, where they undoubtedly help to protect the underlying tissues from opportunistic or pathogenic bacterial attack [2,3]. Indeed, for fish, the skin and its mucus secretions seem to be the main sites where peptides or small proteins with antibacterial activity are found [4–11]. This is not surprising, as in teleosts the skin is constantly exposed to a microbe-rich environment, but has no keratin, and so is vulnerable to abrasion or damage. The skin epithelium is thus a high-risk locale for foci of infection. There is growing interest in identifying the immune factors of teleosts, especially salmonids, because of the growth of finfish aquaculture in the last 20 years and the need to prevent mucosal and systemic infection in the stock animals.

Surprisingly, several of the antibacterial peptides already described for fish have been found to be molecules derived from proteins that are not traditionally associated with antimicrobial defence. These include, for example, histone H2A [5] or a fragment of this molecule [8] and a 6676.6 Da 40 S ribosomal protein, S30 [6]. As yet the extent to which other histones or other cytosolic proteins also contribute antibacterial protection is unclear.

During our isolation of H2A from rainbow trout, *Oncorhynchus mykiss* [5], it was noted that the 20 % acetonitrile eluate from the $^{t}C_{18}$ solid-phase extraction contained at least one additional antibacterial factor. Therefore, the present study was performed to ascertain the character of this protein and establish its potency, spectrum of activity and likely mechanism of action.

EXPERIMENTAL

Animals

Adult female rainbow trout (College Mill Trout Farm, Almondbank, Perthshire, U.K.), weighing 400–500 g, were maintained in flow-through freshwater tanks at ambient temperature and fed daily with an amount of Dynamic Red M trout pellets (Ewos, Bathgate, West Lothian, U.K.) equivalent to 1 % (w/w) of their body weight.

Sample collection and preparation of epidermal extracts

Some 4 h after gentle surface stimulation with ultrafine sand paper to enhance mucus secretion, 10 fish were killed humanely by immersion in a solution containing 0.6 $g \cdot l^{-1}$ aminobenzoic acid ethyl ester (Sigma, Poole, Dorset, U.K.). After the opercula movements had ceased and no reflexes could be observed, 150 ml of skin secretions was scraped from the dorso-lateral surfaces of

Abbreviations used: HMG, high-mobility group; MALDI-TOF MS, matrix-assisted laser-desorption ionization–time-of-flight MS; MIC, minimal inhibitory concentration; SEM, scanning electron microscopy; TFA, trifluoroacetic acid.

[1] To whom correspondence should be addressed (e-mail vjs1@st-and.ac.uk).

The partial N-terminal amino acid sequence of the antimicrobial peptide reported in the present paper has been submitted to the TrEMBL database under the accession number P83338.

Table 1 List of the micro-organisms used for antibacterial assays

Identification codes and culture conditions are also indicated. NCIMB, National Collections of Industrial, Food and Marine Bacteria.

Species	Identification code	Culture conditions (medium/temperature)
A. viridans	NCIMB 1120	Nutrient (Oxoid)/30 °C
A. hydrophila	NCIMB 1134	Nutrient/30 °C
A. salmonicida	MT 004*	Yeastrel†/20 °C
B. subtilis	ATCC 6051	Nutrient/30 °C
E. coli	NCIMB 12210	Nutrient/37 °C
L. anguillarum	NCIMB 2129	Blood base (Difco)/20 °C
M. luteus	NCIMB 376	Blood base/20 °C
P. citreus	NCIMB 1493	Nutrient containing 1.5 % NaCl/20 °C

* Strain provided kindly by Dr Tony Ellis (Marine Laboratory, Aberdeen, U.K.) and isolated by Dr Mary Tatner (hence the designation MT; University of Glasgow, Glasgow, U.K.).

† Yeastrel medium: 0.5 % (w/v) Lab-Lemco (Oxoid), 0.7 % (w/v) Yeastrel (Natex, Gloucs., U.K.), 0.95 % (w/v) peptone (Difco, West Molesey, Surrey, U.K.) and 0.5 % (w/v) NaCl.

the fish with a glass slide, taking particular care to avoid sample contamination with blood. Acid-soluble protein extracts were prepared by homogenizing the mucus in 450 ml of a solution containing 50 % (v/v) ethanol (Merck, Poole, Dorset, U.K.), 3.3 % (v/v) trifluoroacetic acid (TFA; Sigma) and 2 % (v/v) general-use protease-inhibitor cocktail (Sigma) that comprised 4-(2-aminoethyl) benzenesulphonyl fluoride, NaEDTA, leupeptin, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane, bestatin and aprotinin. Following extraction by stirring for 60 min at 4 °C, the preparation was centrifuged at 29 000 ***g*** for 60 min at 4 °C and the supernatant lyophilized. The resulting extract was resuspended in 100 ml of 20 mM Hepes (Acros, Loughborough, Leics., U.K.) and the pH adjusted to 7.0 with 5 M NaOH (BDH, Poole, Dorset, U.K.) before centrifuging at 29 000 ***g*** for 30 min at 4 °C.

Test bacteria

Antibacterial activity was tested against the following species: *Aerococcus viridans*, *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Bacillus subtilis*, *Escherichia coli*, *Listonella* (*Vibrio*) *anguillarum*, *Micrococcus luteus* and *Planococcus citreus*. Their identification codes and culture conditions are listed in Table 1. Each micro-organism was grown to logarithmic growth phase at the appropriate temperature (Table 1) before washing in sterile saline [1.5 % (w/v) NaCl (Sigma) for *P. citreus*; 0.8 % (w/v) NaCl for the remaining non-marine strains] and resuspension in Mueller–Hinton broth (Oxoid, Basingstoke, Hants., U.K.) to a concentration of 10^5 colony-forming units/ml.

Antibacterial assays

Antibacterial activity was assessed by a modified version of the two-layer radial diffusion assay of Lehrer et al. [12], as reported previously [11]. The following modifications were introduced: Tween 20 was omitted, as it was found to affect bacterial growth negatively, and 0.6 % (w/v) agarose (Sigma) was used to lower the melting point below 37 °C and thus improve the survival rate of thermosensitive bacteria. The Gram-positive bacterium *P. citreus* was used as the test organism throughout the protein-purification procedure. Qualitative evaluation of antibacterial activity of the purified peptide against each of the strains listed above was also achieved by radial diffusion assay.

The minimal inhibitory concentration (MIC) of the isolated peptide against *E. coli* or *P. citreus* was determined using a microtitre broth dilution assay based on that described by Friedrich et al. [13], according to the protocol described by Fernandes et al. [5].

To ascertain the effect of NaCl concentration on the antibacterial activity of the purified peptide, MIC assays were performed using Mueller–Hinton broth supplemented with NaCl to a final concentration of 0.8, 1.5 or 3.2 % (w/v).

Muramidase assay

Muramidase activity was tested by radial diffusion assay as detailed by Smith et al. [11], using hen egg white lysozyme (Sigma) at a concentration of 50 μg $\cdot$ ml^{-1} as standard.

Peptide purification

The reconstituted extract was subjected to cation-exchange chromatography at pH 7.0 as described previously by Fernandes et al. [5]. The fractions eluted between 85 and 90 min were acidified to a final concentration of 0.15 % (v/v) TFA and subjected to solid-phase extraction on Sep-Pak Vac 5 g $^tC_{18}$ cartridges (Waters, Watford, Herts., U.K.), equilibrated previously with 0.15 % (v/v) TFA. The proteins of interest were eluted with 0.15 % (v/v) TFA in 20 % (v/v) acetonitrile (BDH) and lyophilized. The lyophilized eluate was resuspended in deionized water containing 0.1 % (v/v) TFA and fractionated by C_{18} reversed-phase HPLC on an ODS2-Inertpak C_{18} reversed-phase HPLC column (particle size 5 μm, 4.6 mm $\times$ 250 mm; Capital HPLC, Broxburn, West Lothian, U.K.). The HPLC system comprised a series 410 LC pump (Perkin Elmer, Beaconsfield, Bucks., U.K.) coupled with a 996 photodiode array detector (Waters) and a 2110 fraction collector (Bio-Rad, Hemel Hempstead, Herts., U.K.). Elution was executed at 25 °C with a biphasic gradient of 0.1 % (v/v) TFA in water and 0.09 % (v/v) TFA in acetonitrile (see Figure 1, left-hand panel) at a flow rate of 1 ml $\cdot$ min^{-1}. The active fractions eluting between 49 and 51 min were lyophilized, reconstituted in 200 μl of 0.1 % (v/v) TFA and purified further by C_{18} reversed-phase HPLC on the same column but under the shallower gradient depicted in Figure 1 (right-hand panel); 1-min fractions were collected, lyophilized and reconstituted in 200 μl of deionized water.

At each step of the purification procedure, the purity and molecular mass of the proteins were estimated by high-resolution PAGE in the presence of SDS [14].

Protein quantification

Total protein concentration was estimated by the method of Bradford [15] using BSA (Pierce, Rockford, IL, U.S.A.) as standard. Amino acid analysis of the purified peptide was performed at the Protein and Proteomics Facility (University of Aberdeen, Aberdeen, U.K.) employing the phenylthiocarbamyl derivatization method on an Applied Biosystems 420A amino acid analyser. Norleucine was used as internal standard for derivatization and myoglobin was employed as the hydrolysis standard.

Matrix-assisted laser-desorption ionization–time-of-flight MS (MALDI-TOF MS)

The molecular mass of the purified antimicrobial peptide was determined by MALDI-TOF MS at the Centre for Biomolecular Sciences (University of St Andrews, St Andrews, U.K.) according to the protocol described in Fernandes et al. [5].

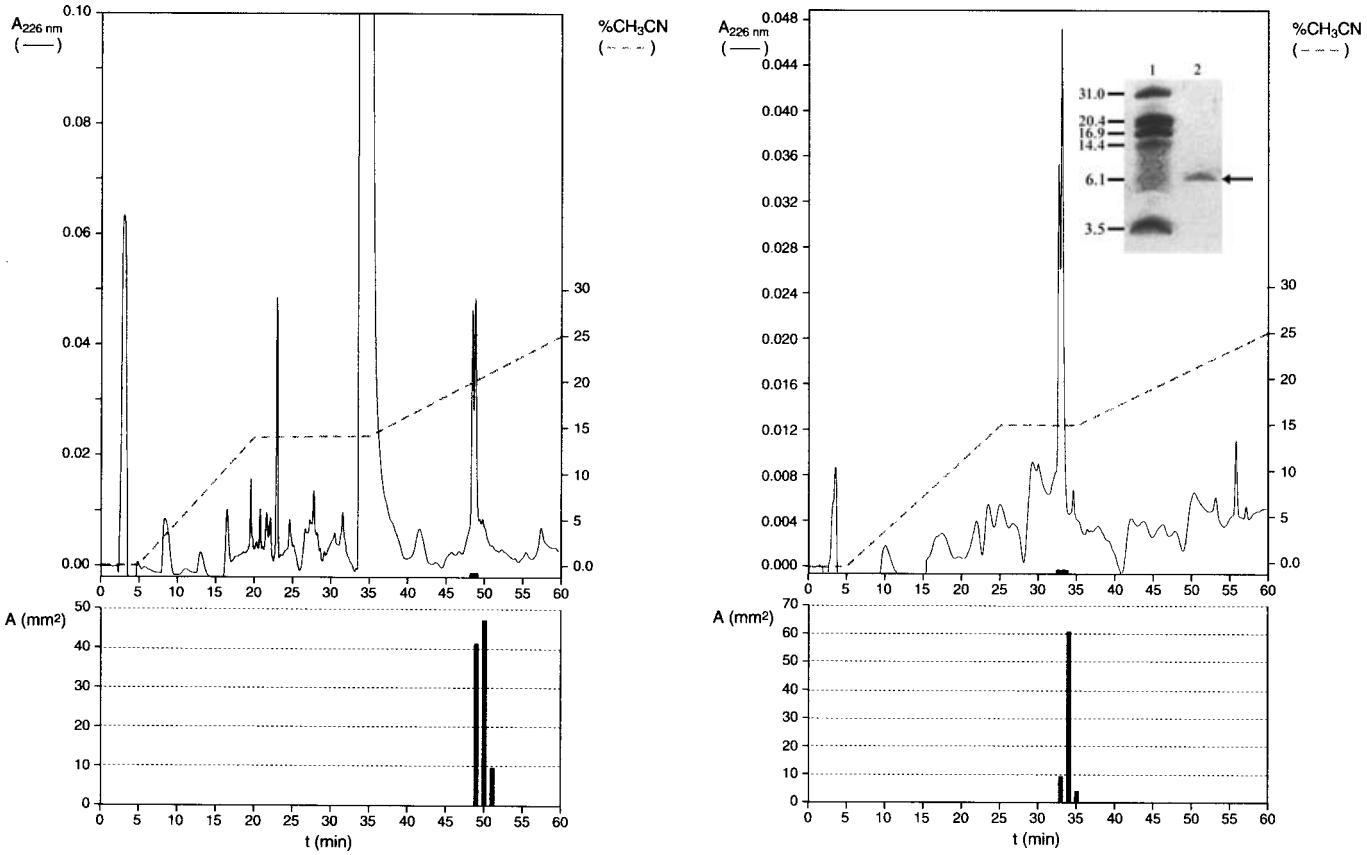

Figure 1 Purification of a 6.7 kDa antimicrobial peptide from skin secretions of rainbow trout

The chromatograms were obtained at 226 nm (solid line). Antibacterial activity profiles against *P. citreus* are represented by the histogram. The dashed line shows the acetonitrile concentration gradients. The peaks of interest are emphasized by the bars below. Left-hand panel: the active fractions eluted between 85 and 90 min during cation-exchange chromatography of acid-soluble mucus extracts from rainbow trout skin were pooled and concentrated by $^{t}C_{18}$ solid-phase extraction, prior to fractionation by C_{18} reversed-phase HPLC. Right-hand panel: the active C_{18} reversed-phase HPLC fractions eluted between 20 and 21 % acetonitrile were pooled and further subjected to C_{18} reversed-phase HPLC under a shallower water/acetonitrile gradient. The fractions corresponding to the peaks eluted at 33.2 and 33.5 min were found to be antibacterial to *P. citreus*. The inset shows the Tris/Tricine SDS/PAGE profile (silver stained) of peak fraction 34 (7.5 μl). The numbers on the left-hand side correspond to the molecular masses of the markers in kDa. The peptide of interest is indicated by the arrow.

Equine cytochrome *c* (12360.1 Da), bovine insulin (5733.5 Da) and corticotropin ('ACTH') 'clip' peptide (residues 18–39, 2465.7 Da) were used as internal standards.

Partial primary structure determination

N-terminal amino acid sequencing of the purified peptide was performed by standard automated Edman degradation on a Procise Sequencer (Applied Biosystems) at the Centre for Biomolecular Sciences (University of St Andrews).

Sequence analyses

Homology searches were performed against the SwissProt, NR and Month databases with the basic local alignment search tool (BLAST) [16] provided by the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). Peptide mass, amino acid composition, isoelectric point and hydropathicity were predicted by the Expert Protein Analysis System (ExPASy) proteomics server of the Swiss Institute of Bioinformatics (http://www.expasy.ch/). Sequence alignments were executed with the Omiga 2.0 sequence analysis software (Oxford Molecular/Accelrys, Cambridge, U.K.), using the Clustal W 1.6 algorithm [17].

Proteolytic digestion

The proteinaceous nature of the purified antimicrobial factor was assessed by radial diffusion assay as above, before or after digestion with 60 μg $\cdot$ ml^{-1} (final concentration) proteinase K (Sigma) for 60 min at 37 °C.

Haemolysis assay

The purified peptide was tested for haemolytic activity against trout erythrocytes according to the protocol described by Fernandes et al. [5].

Scanning electron microscopy (SEM)

An *E. coli* culture (90 μl) in exponential growth phase at 5×10^6 colony-forming units $\cdot$ ml^{-1} was incubated at 37 °C for 1 h with 10 μl of purified trout antimicrobial peptide (10 μM) or cecropin P1 (20 μM; Sigma). After washing with 0.8 % (v/v) NaCl, the specimens were fixed in 2.5 % (v/v) glutaraldehyde (Sigma) and then dehydrated in ascending concentrations of ethanol (Merck), from 70 to 100 % (v/v). They were then transferred to a Samdri 780 (Tousimis, Rockville, MD, U.S.A.) for critical-point drying

with liquid CO_2 (BOC Gases, Guildford, Surrey, U.K.). Dry specimens were mounted on 3-cm-diameter aluminium SEM stubs (Agar Aids, Stansted, Essex, U.K.) using double-sided sticky tape, placed in an SC500 sputter coater with a gold target (Emscope) and coated for 3 min at 15 μA under an argon (Boc Gases) atmosphere. The samples were then observed under a 35 CF scanning electron microscope (JEOL, Tokyo, Japan), using an accelerating voltage of 10 kV. Photographs were taken using 125 ASA FP4+ Ilford film (H. A. West, Edinburgh, U.K.).

Planar lipid bilayer assay

The ion-channel properties of the purified antimicrobial peptide were tested by macroscopic and single-channel conductance measurements in planar lipid bilayers. Virtually solvent-free planar lipid bilayers were formed using the Montal and Mueller technique [18] over a 100–150 μm hole in a Teflon film (10 μm thick), pre-treated with a mixture of 1:40 (v/v) hexadecane/hexane, separating two half glass cells. Lipid monolayers were spread on top of electrolyte solution (1 M KCl/10 mM Tris, pH 7.4) in both compartments. Bilayer formation was achieved by lowering and raising the electrolyte level in one or both sides and monitoring capacity responses. Planar lipid bilayers were formed using either asolectin IV-S from soya bean (Sigma) or a 7:3 (molar) mixture of phosphatidylcholine/phosphatidylethanolamine grade 1 from egg yolk (Lipid Products, Redhill, Surrey, U.K.). Voltage was applied through an Ag/AgCl electrode in the *cis*-side.

With regard to the macroscopic conductance experiments, the purified protein was added in the concentration range 5×10^{-8}–5×10^{-7} M from a 10^{-5} M stock solution in 0.3 % (v/v) Triton X-100. The doped membranes were subjected to slow voltage ramps (6.6 mV $\cdot$ s^{-1}) and trans-membrane currents were fed to a 427 Keitley amplifier (Cleveland, OH, U.S.A.). Current-voltage curves were recorded on an X-Y plotter.

In single-channel recordings, the protein concentrations ranged from 2×10^{-9} to 10^{-8} M. Currents were amplified and potentials were applied simultaneously with an RK 300 patch clamp amplifier (Biologic Science Instruments SA, Claix, France). Single-channel currents were monitored on an R5103N oscilloscope (Tektronix, Beaverton, OR, U.S.A.) and stored on a DTR 1202 DAT recorder (Biologic Science Instruments SA) for off-line analysis. Satori (v3.1; Intracel Software, Royston, Herts., U.K.) was used for downstream analysis. All experiments were performed at room temperature.

RESULTS AND DISCUSSION

Purification and characterization of oncorhyncin III

The results of this study show that the skin epithelium of rainbow trout expresses a 6.7 kDa antimicrobial peptide, which was isolated from acid-soluble skin mucus extracts. The 20 %-acetonitrile eluate from solid-phase extraction of the active fractions eluted between 85 and 90 min during cation-exchange chromatography was subjected to C_{18} reversed-phase HPLC (Figure 1, left-hand panel). Active fractions were fractionated further by C_{18} reversed-phase HPLC, yielding one main fraction with antibacterial activity against *P. citreus* that corresponded to two peaks with retention times of 33.2 and 33.5 min (Figure 1, right-hand panel). The antibacterial activity of the purified peptide was thermostable, remaining present even after incubation at 80 °C for 5 min. Activity was abolished after digestion with proteinase K, confirming that this factor has a proteinaceous nature. One protein band with an electrophoretic mobility of approx. 6.5 kDa could be observed by SDS/PAGE after silver staining (Figure 1, right-hand panel, inset). The yield of purified peptide, determined by amino acid analysis, was approx. 30 ng $\cdot$ g of mucus^{-1}. Automated Edman degradation of the purified peptide yielded the following partial N-terminal sequence: PKRKSATKGDEPA. BLAST homology searches established that it is a perfect match with the N-terminus of the non-histone chromosomal protein H6 (histone T) from rainbow trout (Figure 2). The molecular mass of the purified peptide is 6671.4 Da, as determined by MALDI-TOF MS (Figure 3). The amino acid analysis data were in good agreement with the expected composition of the non-histone chromosomal protein H6 from trout gonads (Swiss-Prot P02315; results not shown). MS analysis combined with N-terminal sequence data showed that it is a 66-residue N-terminal fragment of the non-histone chromosomal protein H6 from *O. mykiss*. This 6.7 kDa antimicrobial peptide is provisionally named oncorhyncin III, after the genus of rainbow trout, *Oncorhynchus*. The discrepancy between the predicted (6643.7 Da) and experimental average molecular masses of this peptide suggests that it may contain two methylated residues or one double methylation. This hypothesis is supported by the fact that the non-histone chromosomal protein H6 contains the same consensus sequence Arg-Lys-Ser (residues 3–5) present in histone H3, which is known to be methylated at lysines 9 and 27 [19]. Oncorhyncin III has a high positive net charge at physiological pH, with a theoretical isoelectric point of 10.72, and a grand average of hydropathicity equal to − 1.106.

Table 2 Bacteriostatic activity of oncorhyncin III, a 6.7 kDa antimicrobial peptide purified from trout skin secretions

Shown are the MIC values (μM) of oncorhyncin III and cecropin P1 against the test bacteria.

Species	Gram staining	MIC (μM)	
		Oncorhyncin III	Cecropin P1
A. viridans	+	0.06–0.12	0.9–1.8
B. subtilis	+	0.25–0.5	>3.6
M. luteus	+	0.12–0.25	>3.6
P. citreus	+	0.06–0.12	1.8–3.6
A. hydrophila	−	>0.5	>3.6
A. salmonicida 004	−	>0.5	0.45–0.9
E. coli	−	0.25–0.5	0.45–0.9
L. anguillarum	−	0.25–0.5	0.06–0.12

Quantification of antibacterial activity using the microtitre broth dilution method revealed that oncorhyncin III is active at submicromolar concentrations against several Gram-positive and Gram-negative bacteria (Table 2) and has no detectable muramidase activity. At a concentration of 1 μM, the trout peptide caused only 5 % haemolysis of trout erythrocytes, whereas melittin, a known haemolytic peptide [20], induced 38 % haemolysis under the same conditions (Figure 4).

Most antimicrobial peptides found throughout the Animal and Plant Kingdoms are small, functionally specialized peptides [1,21]. Nevertheless, an increasing number of antimicrobial peptides are found to be generated by proteolysis of larger proteins with previously known function but with a role not associated with antimicrobial defence [1]. For instance, the murine 40 S ribosomal protein S30 [22] and the trout equivalent [6] were found to have potent antimicrobial properties. Two other examples are provided by histone H2A from rainbow trout skin secretions [5] and parasin I, a 19-residue N-terminal fragment of histone

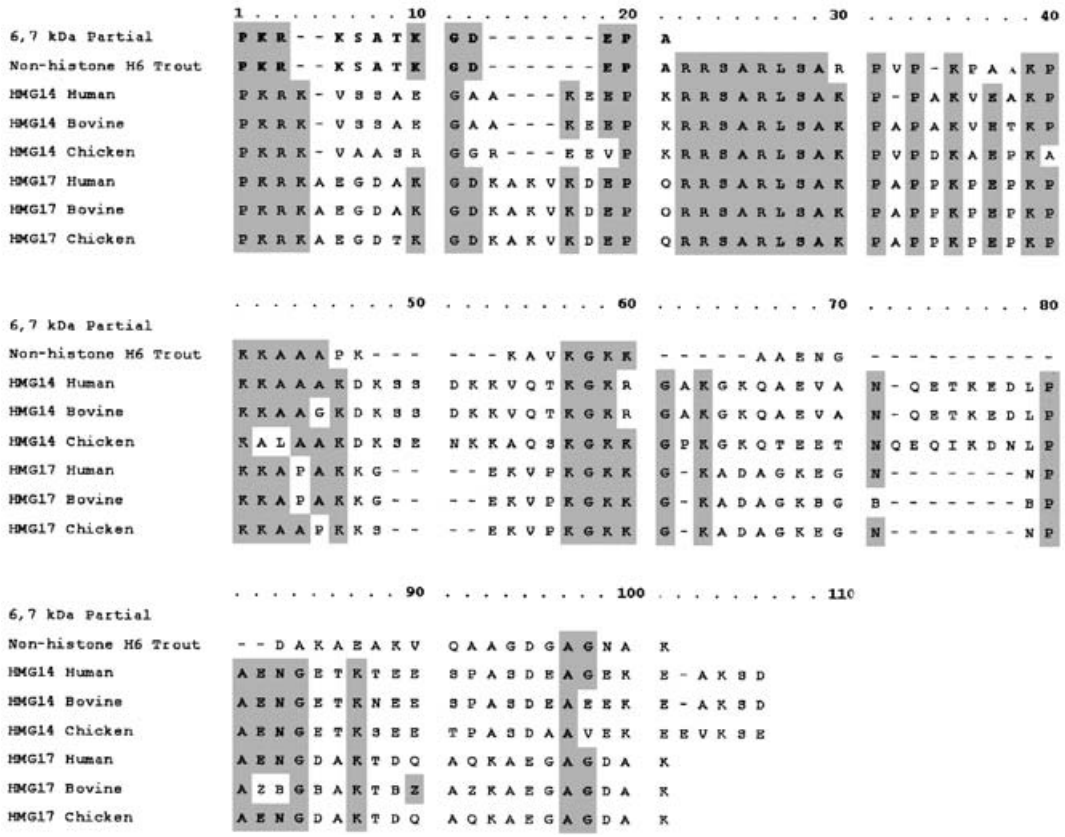

Figure 2 Partial N-terminal sequence of the 6.7 kDa antimicrobial peptide purified from skin mucus of rainbow trout and its homology with high-mobility group (HMG) proteins

Identical residues between the purified antimicrobial peptide, non-histone chromosomal protein H6 from rainbow trout [42], human HMG-14 [43], bovine HMG-14 (Swiss-Prot P02316), chicken HMG-14 [44], human HMG-17 [45], bovine HMG-17 (Swiss-Prot P02313) and chicken HMG-17 [46] are represented by a shaded box.

H2A present in the skin of the catfish, *Parasilurus asotus* [8]. To the best of our knowledge, this is the first report to demonstrate directly that a 66-residue N-terminal fragment of the non-histone chromosomal protein H6 displays potent antimicrobial properties and may therefore assist in mucosal defence. Moreover, the non-histone chromosomal protein H6 belongs to the high-mobility group (HMG) of proteins, which are present throughout the vertebrate subphylum [23]. BLAST homology searches and sequence alignments of oncorhyncin III with H6 from rainbow trout testes, human HMG14, bovine HMG14, avian HMG14, human HMG17, bovine HMG17 and avian HMG17 reveal a high degree of similarity amongst them (Figure 2). These proteins constitute a family of relatively low-molecular-mass non-histone components in chromatin. The proteins bind to the inner side of the nucleosomal DNA, altering the interaction between the DNA and the histone octamer [24]. Indeed, it is currently accepted that the HMG proteins may be involved in the process that confers specific chromatin conformations to transcribable regions in the genome [24]. The purification of oncorhyncin III will permit the development of antibodies that may be employed to ascertain whether this non-histone chromosomal protein can have a cytosolic as well as a nuclear localization, similarly to other histones or histone-derived fragments [25–28]. Furthermore, it would be relevant to investigate the changes in expression of H6 and its processing to oncorhyncin III after antigenic challenge or under immunosuppressive environmental conditions, to investigate the hypothesis that besides their nucleosomal involvement, HMG proteins may be involved in innate host defence.

Mode of action of oncorhyncin III

The mechanism by which antimicrobial peptides kill microbes is still unclear, but it is currently thought that different peptides employ different strategies. These include the fatal depolarization of the cell membrane [29], the formation of pores and subsequent leakage of the cell contents [30] or the damaging of critical intracellular targets after internalization of the peptide [31,32]. Nonetheless, it is well accepted that the primary target of antimicrobial peptides is the bacterial membrane. Regardless of the actual bactericidal mechanism, the peptide has to interact with the membrane, either to disrupt it physically or to access the intracellular space.

SEM imaging of *E. coli* cells incubated with oncorhyncin III at a concentration 2-fold greater than its MIC revealed that the general cell morphology remains unchanged, even though the cells appeared smaller (results not shown). In contrast, incubation with cecropin P1 at a concentration 2-fold greater than its MIC induced marked changes in cell shape and even disruption of bacterial integrity (results not shown). The hypothesis that the oncorhyncin III does not cause immediate disintegration of the cell membrane is further corroborated by planar lipid bilayer and ion-channel

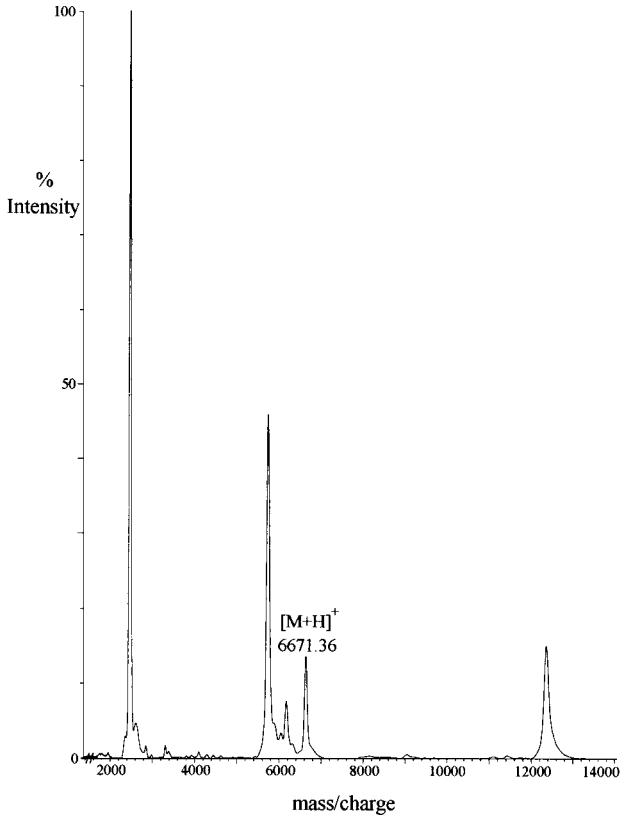

Figure 3 MALDI-TOF mass spectrum of the 6.7 kDa antimicrobial peptide purified from skin exudates of rainbow trout

The single charged molecular ion is labelled. The other peaks that can be observed correspond to the internal standards used.

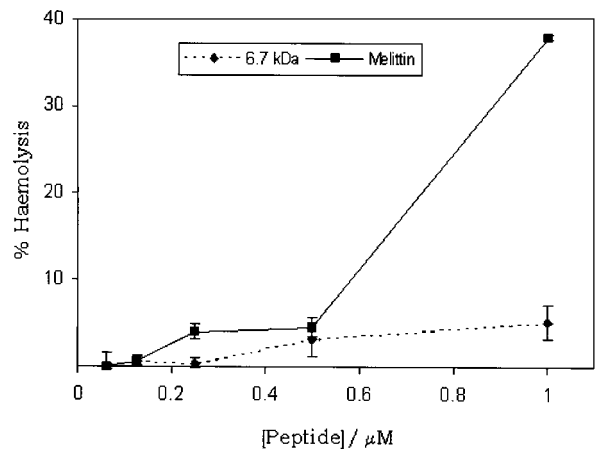

Figure 4 Haemolysis assay of the purified 6.7 kDa antimicrobial peptide against trout erythrocytes

Serially diluted test samples were incubated with a 2% (v/v) suspension of erythrocytes for 30 min at 37 °C. The initial concentrations of 6.7 kDa peptide and melittin were approx. 10 μM. Percentage haemolysis is defined as the ratio of absorbances (read at 545 nm) between each sample and the positive control (Triton X-100). Values are means $\pm$ S.E.M., $n = 3$.

experiments, which showed that the H6-derived trout mucus peptide induced a marked destabilization of planar lipid bilayers, but was unable to form stable channels. These results fit the

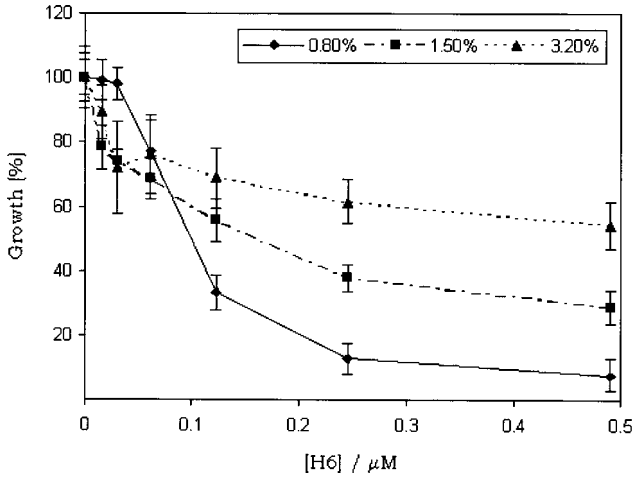

Figure 5 Effect of NaCl concentration on antibacterial activity of the 6.7 kDa trout antimicrobial peptide against *P. citreus*

Bacterial suspensions containing different concentrations of NaCl, as indicated by the key, were incubated with serially diluted purified peptide. The results are expressed as the ratio of absorbances (read at 570 nm) between each sample and the control (no peptide added). Values are means $\pm$ S.E.M., $n = 3$.

'carpet' mechanism of action of antimicrobial peptides described in the Shai–Matsuzaki–Huang model, i.e. the peptide adsorbs to the target membrane and covers it in a carpet-like fashion [33]. The formation of 'wormholes' occurs at high enough local concentration of peptide [34,35], consistent with the observed transient ion-channel activities. Following this phase transition, oncorhyncin III may diffuse on to intracellular targets [1,33], where it may exert its antibacterial function. Buforin II, a linear α-helical peptide derived from histone H2A from *Bufo bufo gargarizans*, also does not cause *E. coli* cell lysis at concentrations up to five times its MIC [36]. Instead, buforin II penetrates the cell membrane and accumulates inside the cell, where it is likely to interact with bacterial nucleic acids [36]. This mode of action is different from the one suggested for another antimicrobial histone, histone H1 from Atlantic salmon (*Salmo salar*). Histone H1 interacts with the cell surface and directly compromises the integrity of the cell membrane, as visualized by SEM [7].

As the initial step of permeabilization of the bacterial membrane involves the electrostatic interaction between the cationic peptides and the negatively charged phospholipid head groups of the outermost leaflet of the lipid bilayer [33], several antimicrobial peptides are inhibited by high NaCl concentrations [37–39], although there are some examples of peptides that seem to be salt-tolerant [10,40].

The assays to investigate the effect of salt on antibacterial activity of oncorhyncin III revealed that, despite being active against *P. citreus* at concentrations up to 3.2% (w/v) NaCl, its MIC value increased 2-fold when the NaCl concentration increased from 0.8 to 1.5% (w/v); furthermore, an 8-fold increase in the MIC value [relative to 0.8% (w/v) NaCl] was observed in the presence of 3.2% (w/v) NaCl (Figure 5). This implies that during the seasonal migrations of rainbow trout to the sea, the protective antimicrobial effect of H6 may be greatly diminished in steelhead (salt-water) rainbow trout. However, the probable intracellular location of oncorhyncin III indicates that, even under high NaCl concentrations, it could still play an important role in host defence against intracellular pathogens.

The likely relevance of oncorhyncin III as an effector of the innate immune system is supported by its very high potency. It is

active against all the Gram-positive and Gram-negative bacteria tested, with MICs in the micromolar range. In particular, its MIC against *P. citreus* is in the range 0.06–0.12 μM, values approx. 30-fold lower than those of cecropin P1 against the same micro-organism (Table 2). Importantly, the peptide is not significantly lytic for trout erythrocytes at the concentrations tested. Hence, this antimicrobial peptide could exert its biological effects *in vivo* against bacteria without damage to the host tissues. Innate host defence factors are unarguably crucial for teleosts, as their adaptive immune system is less sophisticated than that of higher vertebrates and is markedly constrained by environmental variables such as temperature. Oncorhyncin III may be one such factor, probably acting in synergism with other antibacterial factors such as lysozymes or other antimicrobial peptides known to be present in trout skin epithelium. A similar synergistic relationship has been reported for histone H1-derived peptides from coho salmon (*Oncorhynchus kisutch*) and lysozyme [41]. Since the non-histone chromosomal protein H6 is not as tightly bound to chromatin as are the core histones [42], the antimicrobial peptide derived from it may function either in the cytoplasm against intracellular pathogens or extracellularly through release to mucosal surfaces after infection-induced cell lysis or apoptosis. Its ability to destabilize membranes may allow it to enter the cell, where it could exert its antibacterial action by impairing intracellular targets.

We acknowledge Dr Gérard Molle (UMR 5048 CNRS, Montpellier, France) for discussion and invaluable contribution to the lipid bilayer experiments. We thank Mr Irvine Davidson (University of St Andrews) for his expert assistance in scanning electron microscopy. This work was made possible by a research grant (GR3/11099) to V. J. S. by the Natural Environment Research Council, U.K. J. M. O. F. acknowledges the financial support provided by the Fundação para a Ciência e Tecnologia, Portugal (scholarship Praxis XXI, BD/18583/98).

REFERENCES

1 Zasloff, M. (2002) Antimicrobial peptides of multicellular organisms. Nature (London) **415**, 389–396
2 Simmaco, M., Mangoni, M. L., Boman, A., Barra, D. and Boman, H. G. (1998) Experimental infection of *Rana esculenta* with *Aeromonas hydrophila*: a molecular mechanism for the control of the normal flora. Scand. J. Immunol. **48**, 357–363
3 Bevins, C. L. (1994) Antimicrobial peptides as agents of mucosal immunity. In Antimicrobial Peptides, Ciba Foundation Symposium (Marsh, J. and Goode, J. A., eds.), pp. 250–260, John Wiley & Sons, London
4 Alexander, J. B. (1980) Precipitins in the serum of the Atlantic salmon. Dev. Comp. Immunol. **4**, 641–651
5 Fernandes, J. M., Kemp, G. D., Molle, G. and Smith, V. J. (2002) Antimicrobial properties of histone H2A from skin secretions of rainbow trout, *Oncorhynchus mykiss*. Biochem. J. **368**, 611–620
6 Fernandes, J. M. O. and Smith, V. J. (2002) A novel antimicrobial function for a ribosomal protein from skin secretions of rainbow trout. Biochem. Biophys. Res. Commun. **296**, 167–171
7 Richards, R. C., O'Neil, D. B., Thibault, P. and Ewart, K. V. (2001) Histone H1: an antimicrobial protein of Atlantic salmon (*Salmo salar*). Biochem. Biophys. Res. Commun. **284**, 549–555
8 Park, I. Y., Park, C. B., Kim, M. S. and Kim, S. C. (1998) Parasin I, an antimicrobial peptide derived from histone H2A in the catfish, *Parasilurus asotus*. FEBS Lett. **437**, 258–262
9 Robinette, D., Wada, S., Arroll, T., Levy, M. G., Miller, W. L. and Noga, E. J. (1998) Antimicrobial activity in the skin of the channel catfish *Ictalurus punctatus*: characterization of broad-spectrum histone-like antimicrobial proteins. Cell. Mol. Life Sci. **54**, 467–475
10 Cole, A. M., Weis, P. and Diamond, G. (1997) Isolation and characterization of pleurocidin, an antimicrobial peptide in the skin secretions of winter flounder. J. Biol. Chem. **272**, 12008–12013
11 Smith, V. J., Fernandes, J. M. O., Jones, S. J., Kemp, G. D. and Tatner, M. F. (2000) Antibacterial proteins in rainbow trout, *Oncorhynchus mykiss*. Fish Shellfish Immunol. **10**, 243–260
12 Lehrer, R. I., Rosenman, M., Harwig, S. S. L., Jackson, R. and Eisenhauer, P. (1991) Ultrasensitive assays for endogenous peptides. J. Immunol. Methods **137**, 167–173
13 Friedrich, C., Scott, M. G., Karunaratne, N., Yan, H. and Hancock, R. E. W. (1999) Salt-resistant alpha-helical cationic antimicrobial peptides. Antimicrob. Agents Chemother. **43**, 1542–1548
14 Schägger, H. and von Jagow, G. (1987) Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. **166**, 368–379
15 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254
16 Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**, 3389–3402
17 Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**, 4673–4680
18 Montal, M. and Mueller, P. (1972) Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. Proc. Natl. Acad. Sci. U.S.A. **69**, 3561–3566
19 Honda, B. M., Dixon, G. H. and Candido, E. P. (1975) Sites of *in vivo* histone methylation in developing trout testis. J. Biol. Chem. **250**, 8681–8685
20 DeGrado, W. F., Musso, G. F., Lieber, M., Kaiser, E. T. and Kezdy, F. J. (1982) Kinetics and mechanism of hemolysis induced by melittin and by a synthetic melittin analogue. Biophys. J. **37**, 329–338
21 Boman, H. G. (1995) Peptide antibiotics and their role in innate immunity. Annu. Rev. Immunol. **13**, 61–92
22 Hiemstra, P. S., van den Barselaar, M. T., Roest, M., Nibbering, P. H. and van Furth, R. (1999) Ubiquicidin, a novel murine microbicidal protein present in the cytosolic fraction of macrophages. J. Leukoc. Biol. **66**, 423–428
23 Brown, E. and Goodwin, G. H. (1983) Comparison of the high-mobility group chromosomal proteins in rainbow trout (*Salmo gairdnerii*) liver and testis. Biochem. J. **215**, 531–538
24 Bustin, M. and Reeves, R. (1996) High-mobility-group chromosomal proteins: architectural components that facilitate chromatin function. Prog. Nucleic Acid Res. Mol. Biol. **54**, 35–100
25 Kim, H. S., Yoon, H., Minn, I., Park, C. B., Lee, W. T., Zasloff, M. and Kim, S. C. (2000) Pepsin-mediated processing of the cytoplasmic histone H2A to strong antimicrobial peptide buforin I. J. Immunol. **165**, 3268–3274
26 Kashima, M. (1991) H1 histones contribute to candidacidal activities of human epidermal extract. J. Dermatol. **18**, 695–706
27 From, M., Gunne, H., Bergman, A. C., Agerberth, B., Bergman, T., Boman, A., Liden, S., Jornvall, H. and Boman, H. G. (1996) Biochemical and antibacterial analysis of human wound and blister fluid. Eur. J. Biochem. **237**, 86–92
28 Cho, J. H., Park, I. Y., Kim, H. S., Lee, W. T., Kim, M. S. and Kim, S. C. (2002) Cathepsin D produces antimicrobial peptide parasin I from histone H2A in the skin mucosa of fish. FASEB J. **16**, 429–431
29 Westerhoff, H. V., Juretic, D., Hendler, R. W. and Zasloff, M. (1989) Magainins and the disruption of membrane-linked free-energy transduction. Proc. Natl. Acad. Sci. U. S. A. **86**, 6597–6601
30 Yang, L., Weiss, T. M., Lehrer, R. I. and Huang, H. W. (2000) Crystallization of antimicrobial pores in membranes: magainin and protegrin. Biophys. J. **79**, 2002–2009
31 Subbalakshmi, C. and Sitaram, N. (1998) Mechanism of antimicrobial action of indolicidin. FEMS Microbiol. Lett. **160**, 91–96
32 Kragol, G., Lovas, S., Varadi, G., Condie, B. A., Hoffmann, R. and Otvos, Jr, L. (2001) The antibacterial peptide pyrrhocoricin inhibits the ATPase actions of DnaK and prevents chaperone-assisted protein folding. Biochemistry **40**, 3016–3026
33 Shai, Y. (1999) Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-lytic peptides. Biochim. Biophys. Acta **1462**, 55–70
34 Matsuzaki, K., Yoneyama, S., Fujii, N., Miyajima, K., Yamada, K.-i., Kirino, Y. and Anzai, K. (1997) Membrane permeabilization mechanisms of a cyclic antimicrobial peptide, tachyplesin I, and its linear analog. Biochemistry **36**, 9799–9806
35 Ludtke, S. J., He, K., Heller, W. T., Harroun, T. A., Yang, L. and Huang, H. W. (1996) Membrane pores induced by magainin. Biochemistry **35**, 13723–13728
36 Park, C. B., Kim, H. S. and Kim, S. C. (1998) Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. Biochem. Biophys. Res. Commun. **244**, 253–257

37 Turner, J., Cho, Y., Dinh, N. N., Waring, A. J. and Lehrer, R. I. (1998) Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils. Antimicrob. Agents Chemother. **42**, 2206–2214
38 Bals, R., Goldman, M. J. and Wilson, J. M. (1998) Mouse beta-defensin 1 is a salt-sensitive antimicrobial peptide present in epithelia of the lung and urogenital tract. Infect. Immun. **66**, 1225–1232
39 Bals, R., Wang, X., Meegalla, R. L., Wattler, S., Weiner, D. J., Nehls, M. C. and Wilson, J. M. (1999) Mouse beta-defensin 3 is an inducible antimicrobial peptide expressed in the epithelia of multiple organs. Infect. Immun. **67**, 3542–3547
40 Relf, J. M., Chisholm, J. R. S., Kemp, G. D. and Smith, V. J. (1999) Purification and characterization of a cysteine-rich 11.5-kDa antibacterial protein from the granular haemocytes of the shore crab, *Carcinus maenas*. Eur. J. Biochem. **264**, 350–357
41 Patrzykat, A., Zhang, L., Mendoza, V., Iwama, G. and Hancock, R. (2001) Synergy of histone-derived peptides of coho salmon with lysozyme and flounder pleurocidin. Antimicrob. Agents Chemother. **45**, 1337–1342
42 Watson, D. C., Wong, N. C. and Dixon, G. H. (1979) The complete amino-acid sequence of a trout-testis non-histone protein, H6, localized in a subset of nucleosomes and its similarity to calf-thymus non-histone proteins HMG-14 and HMG-17. Eur. J. Biochem. **95**, 193–202
43 Landsman, D., Srikantha, T., Westermann, R. and Bustin, M. (1986) Chromosomal protein HMG-14. Complete human cDNA sequence and evidence for a multigene family. J. Biol. Chem. **261**, 16082–16086
44 Srikantha, T., Landsman, D. and Bustin, M. (1988) Cloning of the chicken chromosomal protein HMG-14 cDNA reveals a unique protein with a conserved DNA binding domain. J. Biol. Chem. **263**, 13500–13503
45 Landsman, D., Soares, N., Gonzalez, F. J. and Bustin, M. (1986) Chromosomal protein HMG-17. Complete human cDNA sequence and evidence for a multigene family. J. Biol. Chem. **261**, 7479–7484
46 Landsman, D. and Bustin, M. (1987) Chicken non-histone chromosomal protein HMG-17 cDNA sequence. Nucleic Acids Res. **15**, 6750

Received 17 February 2003/8 April 2003; accepted 25 April 2003
Published as BJ Immediate Publication 25 April 2003, DOI 10.1042/BJ20030259