RESEARCH ARTICLE

# Proteome analysis of the Atlantic salmon (*Salmo salar*) cell line SHK-1 following recombinant IFN-γ stimulation

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Type II IFN exists as a single molecule (IFN-γ) in contrast to type I IFN, of which there are a number of different forms. IFN-γ is involved both directly and indirectly in antiviral activity, stimulation of bactericidal activity, antigen presentation and activation of macrophages. Recently IFN-γ was cloned from a salmonid fish, the rainbow trout and a functional recombinant protein produced exhibited IFN-γ activity. This recombinant IFN-γ was used to stimulate an Atlantic salmon cell line, SHK-1, to monitor the changes in protein expression by proteomic analysis 24 h after stimulation compared to unstimulated control cells. An SHK-1 cell proteome map was developed and proteins altered in abundance by the IFN-γ stimulation were identified. Under the analytical conditions used, 22 proteins were found to be altered in abundance, 15 increased and 7 decreased. Several proteins were excised from the gel and identified, following trypsin digestion and MALDI-MS/MS/LC-MS and database interrogation. Transcriptional analysis of five mRNAs encoding proteins increased in abundance by IFN-γ in the proteome analysis was determined by real-time PCR. We assessed the correlation between gene expression and change in abundance of proteins for these genes.

Received: January 8, 2007
Revised: March 28, 2007
Accepted: March 28, 2007

**Keywords:**
Atlantic salmon / Gene expression / Interferon-γ / Mx / *Salmo salar*

## 1 Introduction

Interferons (IFN) are widely expressed cytokines that exhibit antiviral, antitumour activity and immunoregulatory roles. There are two key types of IFN, type I and type II [1]. Type I IFN include IFN-α and IFN-β, and are expressed in many cell types and are mainly associated with the antiviral response, initiating the expression of a panel of proteins that inhibit the replication of viruses. There is only one type II IFN molecule, IFN-γ, which is expressed mainly by activated T cells, natural killer cells and some antigen presenting cells [2]. IFN-γ signalling also plays a key role in macrophage activation to help mount an effective innate immune response to invading intracellular microorganisms, and to promote the production and presentation of antigenic peptides *via* the MHC during the adaptive immune response [3].

IFN-γ exerts its effect on a cell *via* a signal transduction pathway which is believed to be highly conserved in verte-

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**Abbreviations: GRP78**, glucose regulated protein 78; **IFN,** interferon

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brates [4]. The IFN molecules bind to the IFN receptor on the cell surface. The receptor complex consists of a heterodimer of IFN-γ receptor 1 (IFNGR1) and IFNGR2. The receptor is associated with two Janus activated kinases (JAK) which, when activated, cause the phosphorylation of signal transducer and activator of transcription (STAT). Two of these phosphorylated STAT molecules form a homodimer which can then act as a transcription factor stimulating genes containing IFN-γ activating sites (GAS) in their promoters. Type I IFN has a similar signal transduction pathway, but in this case one of the JAK molecules is replaced with a tyrosine kinase (Tyk2) and upon activation two different STAT molecules, STAT1 and STAT2, in addition to IFN regulatory factor 9 form a trimer which binds to specific IFN stimulated response elements (ISRE) in the promoter of IFN type I responsive genes [5]. There is believed to be coregulation and crosstalk between these signalling pathways in mammals [6] and additional signalling mechanisms are being discovered that may be required to induce the correct IFN response [6]. It is estimated in mammals that up to 200 or more genes may be affected by IFN-γ stimulation [7].

The IFN-γ response in lower vertebrates is much less understood. Recent advances in genome analysis, including whole genome sequences for puffer fish [8], stickleback and zebra fish as well as large EST data sets for salmonid fish [9], have allowed the identification and sequencing of many molecules from fish including many cytokines [10, 11]. This has led to the identification and cloning of both type I and type II IFN molecules in a variety of fish species including salmonids [12, 13], catfish [14, 15] and fugu [16]. Recently, we have produced a functional rainbow trout IFN-γ protein and have shown this to be a potent activator of macrophages. It also stimulates transcription of characteristic IFN-γ inducible genes such as γ-IP, and this expression can be inhibited by blocking the JAK STAT pathway, suggesting a signal transduction pathway similar to that known in mammals exists in fish [13].

To further our studies regarding IFN-γ responses in fish we used the recombinant IFN-γ protein to stimulate a salmonid cell line, SHK-1. An established cell line was chosen, since it will give better results (*i.e.* less variation) than mixed primary cultures which is important when doing this analysis for the first time. The cell line response was analysed in terms of protein expression by proteomics. This cell line was derived from macrophage-enriched cultures from the head kidney of Atlantic salmon and the cells are adherent with a typical macrophage-like morphology [17, 18]. Therefore, they are expected to be highly responsive to IFN-γ (a macrophage-activating factor). Rainbow trout and Atlantic salmon IFN-γ genes show 94% nucleotide identity and the translated proteins share 89% amino acid identity, suggesting cross-reactivity of the trout protein to salmon cells will occur, and this was confirmed in the present study. A 2-DE protein map was generated for the SHK-1 cell line and proteins found to be increased or decreased in abundance by the recombinant cytokine stimulation were identified by gel image analysis. Candidate protein spots that were increased or decreased in abundance were excised from the gel and subjected to trypsin digestion and subsequent separation of the peptides by MS and identification by database interrogation. In parallel to genome information enabling identification of cDNA sequences, the genomic information greatly enhances protein spot identification following trypsin digest fingerprinting and MS analysis. This approach has enabled successful proteomic analysis to be performed in fish [19–21]. Real-time PCR was then used to assay the mRNA expression encoding three proteins found increased in abundance by IFN-γ during the proteome analysis. These included Mx, an antiviral protein [22], a cell surface receptor C-type lectin and a 78 kDa glucose regulated protein.

## 2 Materials and methods

### 2.1 Cell culture and stimulation with recombinant IFN-γ

SHK-1 cells, derived from macrophage-enriched head kidney cells of Atlantic salmon [17], were maintained in L15 medium containing 10% FCS (Invitrogen Life Technologies) and antibiotics (100 μg/mL penicillin and 100 U/mL streptomycin) at 20°C [17] in 80 $cm^2$ flasks. Cells were passaged to fresh flasks at 80% confluence and cultured for 2 days before stimulation. The SHK-1 cells were stimulated with recombinant IFN-γ at 20 ng/mL for 24 h. This concentration was chosen since previous studies have shown no significant difference in effects on induced gene expression when using doses of 10–100 ng/mL [13]. The time (24 h poststimulation) was chosen as this is expected to give us maximal protein expression differences following the IFN-γ stimulation. Early responding genes such as γ-IP have transcription levels increased by 6 h but significant increases of other genes such as MHC II β occur between 6 and 24 h [13], and in both cases protein expression is expected to occur somewhat later. Stimulated and nonstimulated control cells were (four flasks each) processed for protein extraction and proteome analysis. A further three stimulated and control flasks were used for RNA extraction and transcriptome analysis by real-time PCR. Prior to performing the proteomic analysis, RT-PCR was carried out to assess the induction of γ-IP, an IFN-γ inducible gene to confirm good stimulation of the cells had occurred.

### 2.2 Protein extraction

The SHK-1 cells were washed three times with 1 × HBSS, before protein extractions. The cells were lysed directly in lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 0.3% w/v DTT, 1% v/v 4–7 IPG buffer (GE Healthcare)), 1% protease inhibitor cocktail (10 μL/mL lysis buffer) (Sigma P 8340) incubated at room temperature for 10 min and clarified by centrifugation at 14 000 × *g* for 20 min at 15°C. Aliquots of the proteins prepared as described above were analysed by 1-D SDS-PAGE and stained using colloidal CBB G250 to ensure

 

that protein concentrations were similar for all samples and equal loading for 2-DE was achieved. Subsequent gel loading was determined based on the intensity of the stained 1-D profiles. Soluble salmon cell proteins (50 μL) were mixed with 210 μL reswelling buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 0.3% w/v DTT, 1% v/v 4–7 IPG buffer (GE Healthcare)), samples are centrifuged on a microfuge (11 000 × *g*) for 5 min and the supernatant taken for gel loading, the supernatant (250 μL) was used to rehydrate pH 4–7, 13 cm IPG gel strips (Immobiline DryStrip; GE Healthcare). The IPG gels were rehydrated overnight at room temperature and the proteins focused on a Multiphor II electrophoresis apparatus (GE Healthcare) using the following steps: 200 V for 1 min and then increasing to 3500 V over 1 h 30 min and finally held for 5 h at 3500 V (at 2 mA and 5 W). For second dimension electrophoresis, the IPG strip was laid onto a 12% polyacrylamide slab gel (16 × 15 $cm^2$) and protein electrophoresed at 100 V for 1 h and 200 V for a total of 5 h [23]. The resolved proteins were detected using Colloidal CBB G250 staining. p*I*s were determined based on the linearity of the IPG strip. Molecular weights were determined by co-electrophoresis with prestained molecular weight standards (SeeBlue Plus2, Invitrogen).

### 2.3 Analysis of 2-D gels

Protein profiles were digitised using a Molecular Dynamics Personal Densitometer (GE Healthcare) as 12-bit grey images at a resolution of 50 μm and stored as *.gel files. Subsequent image analysis was carried out using Progenesis PG200 v2006 (Nonlinear Dynamics, Newcastle upon Tyne, UK). Protein spots were detected using automated routines from the software combined with manual editing to remove artefacts. Matching was performed using a combination of seed matching and automatic matching by the Progenesis program followed by further manual editing. Individual protein spot abundance was determined by the integrated intensity of the spot and referred to as the volume. Background was removed using the 'Mode of Nonspot' method (Progenesis) and the spot volumes were normalised to the total volume of all proteins detected on each gel. Four replicate gels were analysed for the IFN-γ stimulated and non-stimulated conditions. Averaged gels were constructed using the Progenesis software and proteins upregulated between treatments were assigned if they were significant using a Student's *t*-test ($P<0.05$) and were only considered for further spot identification if they were at least a mean two-fold difference increased or decreased. The 2-D gel image can be viewed at the University of Aberdeen fish proteomics web page (http://www.abdn.ac.uk/fishprom/).

### 2.4 PMF

Proteins of interest were excised from preparative gels and subjected to in-gel trypsin digestion [24]. Protein digestion, peptide extraction and the preparation of MALDI targets were carried out using ProPic, ProGEST and ProMS robots (Genomic Solutions, Huntingdon, UK) and the excised spots were washed, reduced, *S*-alkylated and digested within the gel using trypsin (sequencing grade modified trypsin; Promega), as described elsewhere [25]. Briefly, proteins were reduced with DTT (60°C, 20 min), *S*-alkylated with iodoacetamide (25°C, 10 min) then digested with trypsin (37°C, 8 h). The resulting tryptic peptide extract was dried by rotary evaporation (SC110 SpeedVac; Savant Instruments, Holbrook, NY, USA) and dissolved in 0.1% formic acid for LC-MS/MS analysis. An aliquot of the peptide extract produced by this process was passed through a GELoader tip containing a small volume of POROS R2 sorbent (PerSeptive Bio-Systems, USA). The adsorbed peptides were eluted in 0.5 μL saturated solution of CHCA in 50% v/v ACN, 5% v/v formic acid. The mass spectra of the peptide fragments were obtained on a PerSeptive Biosystems Voyager-DE STR MALDI-TOF mass spectrometer. The instrument was operated in the reflection delayed extraction mode. Spectra were internally calibrated using trypsin autodigestion products.

Selected protein spots were also analysed by LC-MS/MS on an HCT Ultra PTM Discovery System (Bruker Daltonics, Coventry, UK) coupled to an UltiMate 3000 LC System (Dionex, Camberley, Surrey, UK). Peptides were separated on a Monolithic Capillary Column (200 μm id × 5 cm; Dionex part no. 161409). For the LC fractionation of the peptides eluent A was 3% ACN in water containing 0.05% formic acid and eluent B 80% ACN in water containing 0.04% formic acid with a gradient of 3–45% B in 12 min at a flow rate of 2.5 μL/min. Peptide fragment mass spectra were acquired in data-dependent AutoMS(2) mode with a scan range of 300–1500 *m/z*, three averages and up to three precursor ions were selected from the MS scan (100–2200 *m/z*). Precursors were actively excluded within a 1 min window, and all singly charged ions were excluded.

### 2.5 Protein identification

For protein identification, peptide masses from trypsin digests derived using the MALDI-TOF MS were used to search against the National Centre for Biotechnology Information nonredundant (NCBInr) sequence database using the MASCOT program [26]. The MASCOT search parameters were as follows: peptide mass accuracy was 100 ppm, protein modifications: cysteine as *S*-carbamidomethyl-derivative and oxidation of methionine allowed. For data generated using LC-MS/MS, the peptide peaks were detected and deconvoluted automatically using Data Analysis software (Bruker Daltonics). The mass lists in the form of MASCOT generic files were created automatically and used as the input for MASCOT MS/MS ions searches of the NCBInr database using the Matrix Science web server (www.matrixscience.com). The default search parameters used were: enzyme, trypsin; max. missed cleavages, 1; fixed modifications, carbamidomethyl (C); variable modifications, oxidation (M); peptide tolerance, ± 1.5 Da; MS/MS tolerance

 

± 0.5 Da; peptide charges, 2+ and 3+; instrument = ESI-TRAP. Additional searches were performed using an in-house generated database containing all salmonid cDNA (EST) sequences available downloaded from the Genomics research in all salmonids project (GRASP) consortium where contigs have been generated from 246704 rainbow trout ESTs and 436629 Atlantic salmon ESTs (October 2006). This database was searched using Protein Prospector MS-fit [27]. Parameters for searching MS-fit were: all six frames to be searched: cysteine as *S*-carbamidomethyl-derivative and oxidation of methionine allowed, only peptides generated by MALDI-TOF MS was used in these searches.

### 2.6 RNA isolation and gene expression analysis by real-time PCR

The SHK-1 cells were washed with HBSS prior to being lysed directly in RNA STAT60 (AMS Biotechnology), according to the manufacturer's instructions for the purification of total RNA. Following precipitation, RNA was resuspended in DEPC treated water. The integrity of the RNA was determined using an Agilent Bioanalyser 2100 (Agilent Technologies) and the concentration determined by spectrophotometry using a nanodrop analyser (Labtech International). RNA was stored at −80°C until required.

For real-time PCR, RNA was denatured (65°C, 10 min) in the presence of 1 μL of oligo $dT_{17}$ primer (500 ng/mL), cooled on ice, and cDNA was synthesised using 15U Bioscript reverse transcriptase (Bioline, UK) in the presence of dNTPs (final concentration 200 μM each) at 42°C for 1 h in a final volume of 20 μL. The cDNA was diluted five-fold to 100 and 3 μL used as the template for PCR using primers designed against the Atlantic salmon genes of interest (Table 1). An Opticon qPCR machine was used for monitoring the cDNA amplification using ready prepared 2 × master mix SYBR green PCR mix (BioRad). PCR reactions were performed in 25 μL volumes on a white (BioRad) 96-well PCR plate covered with transparent film. A negative control (no template) reaction was also performed for each primer pair. A sample from the serial dilution was run on an agarose gel and stained with ethidium bromide and viewed under UV light to confirm a band of correct size was amplified.

Efficiency of amplification was determined for each primer pair using ten-fold dilutions (1-, 10-, 100- and 1000-fold dilutions). In addition to those candidate genes β-actin was used as a control for normalisation of expression. PCR conditions were 95°C for 5 min followed by 94°C for 15 s, 57°C for 15 s, 72°C for 20 s, for 35 cycles. The fluorescence signal output was measured and recorded at 78°C during each cycle for all the wells. A melting curve for each analysis was obtained by reading fluorescence every degree between 55 and 95°C to ensure only a single product had been amplified.

To determine the relative expression levels of candidate genes the method of Pfaffl [28] was used to obtain relative expression of candidate genes to both β-actin and elongation factor 1α. The efficiency of the PCR reaction for each primer set was performed on the same plate as the experimental samples. The efficiency was calculated as $E = 10^{(-1/s)}$, where $s$ is the slope generated from the serial dilutions, when log dilution is plotted against ΔCT (threshold cycle number). For all real-time PCRs, triplicate reactions were performed.

## 3 Results

### 3.1 Stimulation of cells with IFN-γ

The SHK-1 cells remained adherent to the flask and showed no visual difference following the 24 h of stimulation with IFN-γ compared to the control cells suggesting that the cells were still healthy. To confirm that the cells had responded to the recombinant IFN-γ, preliminary gene expression analysis was performed with a well-characterised IFN-γ responsive gene γ-IP [29]. This gene encodes a chemokine known to be highly induced in both mammals [30] and fish [13] by IFN-γ. The reverse transcription real-time PCR confirmed the cells responded to the stimulation as predicted, with a significant increase in γ-IP mRNA expression (37.3-fold ± 8.5 SEM, $p<0.05$) in RNA isolated from stimulated cells compared to nonstimulated control cells.

### 3.2 2-D gel analysis

Cellular proteins were extracted from Atlantic salmon SHK-1 cells and analysed by 2-DE to generate 2-D reference maps for stimulated and nonstimulated cells. A representative 2-D gel for stimulated SHK-1 cells is shown (Fig. 1). As part of the analysis average gels were generated for the control and stimulated cells; the average gels contained 503 protein features for the control group and 582 features for the stimulated group. The majority of the additional proteins from the stimulated group were minor spots that were difficult to match to the control gel. The abundance of the individual protein spots, detected on the gels, ranged between 0.006 and 11.8% of the total detected protein on the gel. The feature that contained 11.8% of the protein is likely to represent >1 individual highly abundant protein spots which did not resolve well on our gels in the area of approximately 49 kDa, p*I* 5.5.

Twenty-two protein spots were found altered in abundance due to IFN-γ stimulation compared to nonstimulated cells; 15 of these were increased in abundance whereas 7 were found to be reduced (Table 2). The positions of the altered proteins are shown on the stimulated cell reference gel (Fig. 1) indicating that a range of proteins of different molecular weights and p*I*s were altered following IFN-γ stimulation. The protein spot that was increased by the greatest magnitude was reference spot 12 477 that had an 11-fold increase in abundance following the IFN-γ stimulation (Fig. 2a). The protein that showed the

 

**Table 1.** PCR primers for real-time PCR analysis to assay gene expression of cDNAs found to be stimulated by recombinant IFN-γ. Amplified cDNA was monitored by SYBR green incorporation on an Opticon real-time PCR machine

| Gene | Primer | Primer sequence | Accession number | Length | PCR annealing temp (°C) |
|---|---|---|---|---|---|
| γ-IP | γ-IPF | AAGGCCAAGTGGGGTCATTCTAA | DR696064 | 360 | 58 |
| | γ-IPR | AACGTATTCAGGCAGTCTTCAGG | | | |
| Mx | sMxF1 | TGAGGACTCGGCAGAAAGGATGTA | SSU66475 | 415 | 58 |
| | sMxR1 | CTTCGCGGATTTCAGGAGGAGGTTAGG | | | |
| C-type lectin | sCLF1 | AATCAGTTTGGCAAGCAGCAGA | CB516930 | 374 | 58 |
| | sCLR1 | AAGCGATTTGAGATGTTTTAGTG | | | |
| GRP78 | sGRP78F1 | TTCCGTAAAGCTTGCTGATGATG | AM042306 | 266 | 58 |
| | sGRP78R1 | TGCCGATGAGGACAAGAAGT | | | |
| EEF-2 | ES24F | CCCCCGTCACCGCCCAGAAGTA | DY699581 | 187 | 58 |
| | ES24R | GACACGCAGCCGGAGAAGACACG | | | |
| UBMO[a)] | ES30F | TGAGGCAGGCAACAAGAAAGTG | DW572586 | 187 | 58 |
| | ES30R | TGTAATCAGGGCATCCGAGCAG | | | |
| ATPaseB | ES26F | GGAGAGCCCATTGACGAGAGG | DW537729 | 261 | 58 |
| | ES26R | TCCGGCAAACACAGAGTAACCAC | | | |
| IFN type I | ES29F | AGCTACGCCCTTACCAGTTTA | AY216594.1 | 170 | 58 |
| | ES29R | TAGACCGGCAATACAGTTCAGT | | | |
| β-Actin | β-actinF | CCAGGCATCAGGGAGTGA | AF012125 | 287 | 58 |
| | β-actinR | GTACATGGCAGGGGTGTTGA | | | |

a) Ubiquinone biosynthesis monooxygenase.

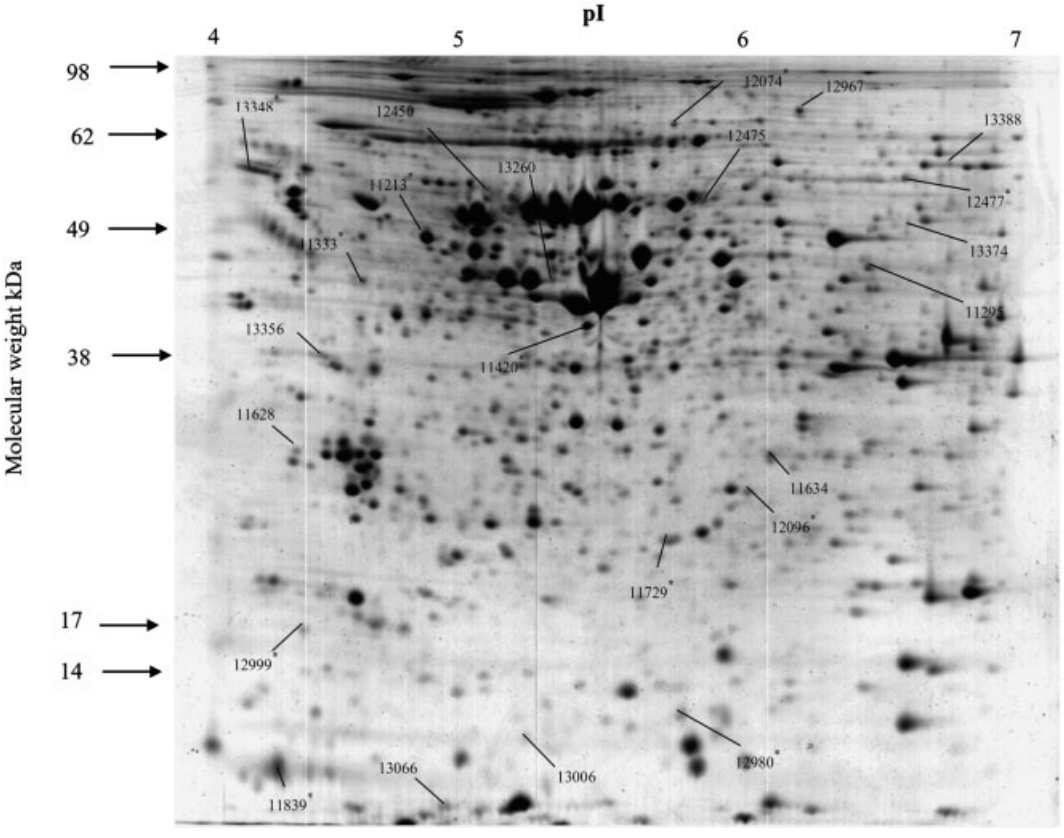


**Figure 1.** Representative 2-D gel of Atlantic salmon cell line SHK-1 proteins analysed. The gel shows the 2-D protein profile of cell extracts from SHK-1 cells stimulated by recombinant IFN-γ. Protein spots that were altered by the stimulation are shown by the arrows. Protein spots indicated by * were excised for identification by PMF and MS. The molecular weight and p*I* scales are indicated.

 

**Table 2.** Proteins altered in abundance in SHK-1 cells following recombinant IFN-γ stimulation for 24 h (>1.5-fold difference)

| Normalised spot volume | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Control | | Stimulated | | | |
| Spot | kDa | p*I* | Mean | SE | Mean | SE | Fold change | *p* value |
| 12 477[a)] | 60.6 | 6.5 | 49.6 | 12.4 | 559.1 | 117.5 | 11.3 | 0.050 |
| 13 348[a)] | 62.0 | 4.2 | 1224.3 | 305.0 | 3664.6 | 163.2 | 3.0 | 0.010 |
| 11 420[a)] | 39.5 | 5.4 | 643.1 | 52.1 | 1751.7 | 182.2 | 2.7 | 0.001 |
| 11 333[a)] | 45.0 | 4.5 | 165.2 | 26.0 | 429.5 | 3.9 | 2.6 | 0.004 |
| 11 729[a)] | 21.1 | 5.7 | 318.7 | 39.2 | 828.0 | 141.3 | 2.6 | 0.010 |
| 11 839[a)] | 10.8 | 4.2 | 1233.2 | 440.8 | 3105.3 | 474.7 | 2.5 | 0.036 |
| 13 066 | 9.7 | 4.9 | 183.7 | 46.5 | 458.4 | 104.4 | 2.5 | 0.044 |
| 12 999[a)] | 16.2 | 4.4 | 94.0 | 43.2 | 220.6 | 39.1 | 2.3 | 0.10 |
| 11 628 | 27.4 | 4.3 | 103.2 | 22.4 | 226.9 | 5.3 | 2.2 | 0.006 |
| 12 074[a)] | 71.1 | 5.7 | 150.3 | 12.7 | 308.9 | 41.8 | 2.1 | 0.009 |
| 13 260 | 45.2 | 5.2 | 260.9 | 51.1 | 491.7 | 28.2 | 1.9 | 0.042 |
| 12 450 | 59.1 | 5.0 | 322.3 | 29.3 | 554.4 | 78.9 | 1.7 | 0.026 |
| 12 475 | 56.9 | 5.8 | 297.4 | 43.2 | 506.6 | 39.9 | 1.7 | 0.039 |
| 13 006 | 11.8 | 5.1 | 80.4 | 6.8 | 135.7 | 7.6 | 1.7 | 0.008 |
| 13 356 | 36.2 | 4.4 | 325.5 | 20.1 | 521.8 | 51.9 | 1.6 | 0.011 |
| 12 967 | 74.0 | 6.1 | 870.3 | 54.0 | 595.4 | 37.0 | −1.5 | 0.012 |
| 11 634 | 26.9 | 6.0 | 1016.7 | 57.2 | 630.9 | 70.3 | −1.6 | 0.008 |
| 11 295 | 46.8 | 6.4 | 1051.8 | 78.1 | 647.8 | 61.3 | −1.6 | 0.012 |
| 12 980[a)] | 12.6 | 5.7 | 314.1 | 48.0 | 147.2 | 62.9 | −2.1 | 0.084 |
| 13 388 | 61.0 | 6.7 | 165.2 | 24.7 | 63.4 | 9.3 | −2.6 | 0.020 |
| 13 374 | 53.3 | 6.5 | 320.9 | 21.2 | 108.7 | 26.8 | −3.0 | 0.001 |
| 12 096[a)] | 24.3 | 6.0 | 302.1 | 42.3 | 100.6 | 76.2 | −3.0 | 0.055 |

The proteins are ordered by fold change. Only proteins that had greater than two-fold change were considered for MS analysis.
a) Protein selected for MS.

greatest reduction in abundance was reference spot 13 374, which showed a three-fold reduction following IFN-γ stimulation.

### 3.3 Peptide mass mapping

Eleven protein spots were chosen to be excised from preparative 2-D gels for PMF (Table 3). These included eight upregulated, two downregulated protein spots as well as one additional protein spot that was not differentially regulated. All of the 11 protein spots generated peptide profiles that were used in database searches, the number of peptide fragments generated by the samples ranged from 37 for reference spots 11 333 and 12 999 to 52 for reference spot 12 074. The peptide fragments produced were used to search against the NCBI nonredundant database using the MASCOT search program, and were also searched against an in-house salmonid EST sequence database generated from GRASP (http://web.uvic.ca/cbr/grasp/) where contigs of all salmon or trout EST sequences have been generated. The MASCOT searches resulted in five significant identities (Table 3), two of these were to salmonid protein sequences, reference spot 12 074 was found to be Mx (protein montage, Fig. 2b) and reference spot 11 333 was identified as glucose regulated protein 78 (GRP78), also known as an Ig-binding protein. An MHC antigen (reference spot 13 348) and a C type lectin (reference spot 11 729) were also identified homologous to the human and rat molecules, respectively. The final positive identification obtained by MASCOT was for the constitutively expressed protein spot (11 213) that was identified as an ATPase β-subunit.

A greater number of identities were obtained for the protein spots when peptide profiles were used to search against the salmonid sequences using MS-fit, where identities were found for all trypsin digest fingerprints (Table 4). The identification of Mx, GRP 78 and the ATPase β chain confirmed the results obtained from the MASCOT searches. However, protein spot 13 348 was identified as a nucleosome assembly protein as opposed to an MHC antigen by MASCOT. The different identities for some of the proteins are most likely due to the lack of coverage in the sequence databases rather than being due to the different programs used.

 

**Table 3.** Results using MASCOT program from trypsin digest fingerprinting of protein spots excised from the Atlantic salmon cell line SHK-1 following stimulation with recombinant IFN-γ

| | MASCOT | | | | | |
|---|---|---|---|---|---|---|
| Protein spot | MASCOT score | Masses matched#/(%)[a)] | % Sequence coverage | Protein | Species | Accession no. |
| Increased | | | | | | |
| 11 333 | 83 | 11/(31) | 19 | Glucose-regulated protein 78 kDa | *O. mykiss* | BAD90025.1 |
| 11 420 | – | – | | – | – | – |
| 11 729 | 72 | 6/(26) | 38 | C-type lectin | *R. rattus* | ABA47206 |
| 11 839 | – | – | | – | – | – |
| 12 074 | 76 | 6/(12) | 10 | Mx | *Salmo salar* | AAB40996 |
| 12 477 | – | – | | – | – | – |
| 12 999 | – | – | | – | – | – |
| 13 348 | 90 | 6/(60) | 28 | MHC I Antigen | *H. sapiens* | BAD82817 |
| Decreased | | | | | | |
| 12 096 | – | – | | – | – | – |
| 12 980 | – | – | | – | – | – |
| Constitutive | | | | | | |
| 11 213 | 113 | 15/(48) | 37 | ATPase β | *C. elegans* | NP_498111 |

a) Peptide masses matched number of peptides (#); percent of peptides (%) matched in relation to total peptides searched.

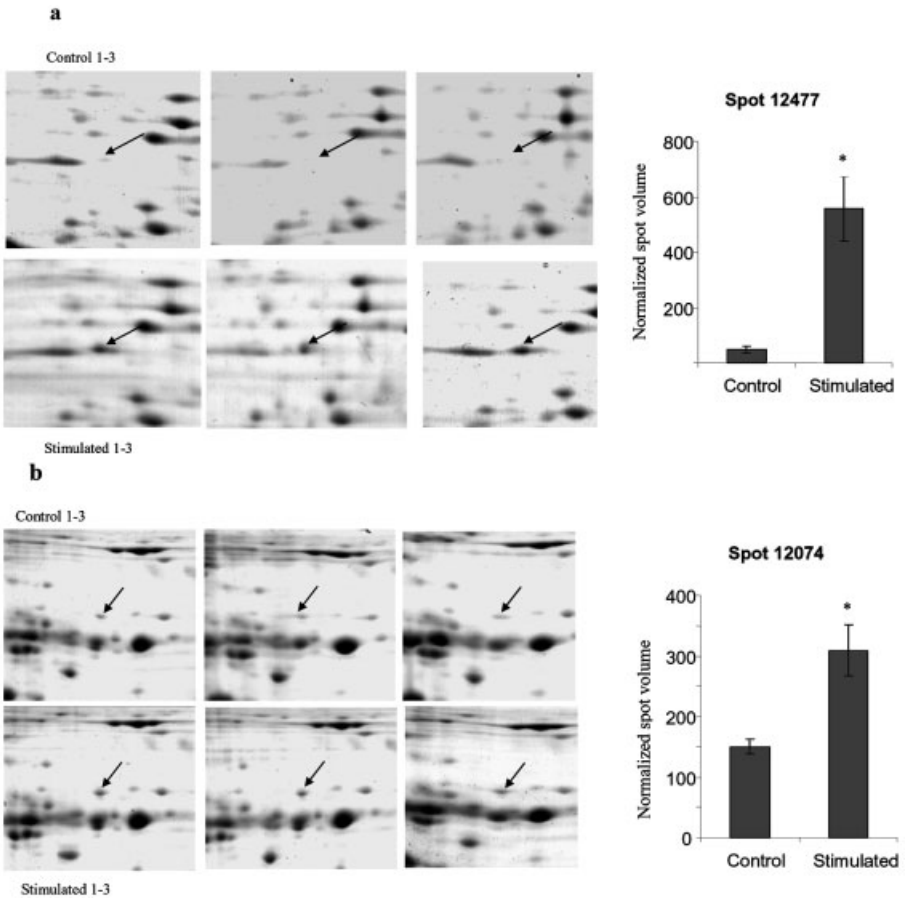


**Figure 2.** Changes in abundance of protein reference spots 12 477 (a) and 12 074 (b) (indicated by arrows in the panels) as observed by the analysis of Atlantic salmon cell line SHK-1 proteins following recombinant IFN-γ stimulation. Each panel shows an enlarged view of the gels spots from Fig. 2 for proteins from 3 control or 3 stimulated flasks of cells and the corresponding graph of the normalised volume of the spot. Error bars represent SEM. * Indicates significant difference relative to the control.

 

**Table 4.** Results using the MS-fit program from trypsin digest fingerprinting of protein spots from the Atlantic salmon cell line SHK-1 following stimulation with recombinant IFN-γ

| | | | | MS Fit | | | |
|---|---|---|---|---|---|---|---|
| Protein spot | Mosie Score | Masses matched#/(%)[a)] | % Coverage | Salmonid seq | Blast score | Protein | |
| Increased | | | | | | | |
| 11 333 | 808 | 4/(33) | 17 | 47174[T] | e-132 | Glucose-regulated protein 78 kDa | BAD90025.1 |
| 11 420 | $1.8 \times 10^6$ | 5/(45) | 51 | 21356[T] | 3e-55 | Ubiquinone biosynthesis monooxgenase | XP_684723.1 |
| 11 729 | 329 | 4/(33) | 18 | 47737[T] | 8e-64 | Complement component C5-2 | BAC23058.1 |
| 11 839 | 5684 | 6/(35) | 57 | 29264[S] | 1e-69 | Myosin light chain | AAY86953.1 |
| 12 074 | $1.5 \times 10^4$ | 7/(38) | 18 | 49905 | 0.0 | Mx3 | AAB40994.1 |
| 12 477 | $3.8 \times 10^4$ | 4/(36) | 55 | 40121[T] | 0.0 | Elongation factor 2 | NP_956752.2 |
| 12 999 | 178 | 5/(29) | 17 | 39315[S] | 0.00 | N-ethylmaleimide-sensitive factor | NP_958898.1 |
| 13 348 | $2.8 \times 10^4$ | 6/(40) | 27 | 37702[S] | 9e-91 | Nucleosome assembly protein 1 | NP_958475.1 |
| Decreased | | | | | | | |
| 12 096 | $3.9 \times 10^3$ | 6/(35) | 32 | 36691[S] | 2e-62 | Proteasome subunit 6α | AAH55520.1 |
| 12 980 | $2.3 \times 10^4$ | 4/(40) | 46 | 21968[S] | 7e-61 | ATP citrate lyase | NP_001025711 |
| Constitutive | | | | | | | |
| 11 213 | $2.5 \times 10^{-4}$ | 7/(14) | 45 | 50192[S] | 4e-79 | ATPase β | |

The salmonid sequence shown is either from GRASP contigs for salmon[S] or GRASP contigs for trout[T]. % Coverage indicates coverage of the predicted ORF from the cDNA contigs.

a) Peptide masses matched number of peptides (#); percent of peptides (%) matched in relation to total peptides searched.

To further investigate these apparent contradictions, LC-MS/MS was performed on four protein spots (Table 5). Under these conditions, reference spot 11 348 was confirmed as a nucleosome assembly protein. The GRP78 was also reconfirmed. Consequently, more confidence was placed on the identification of 13348 as a nucleosome assembly protein. The other two proteins used in LC-MS/MS did not reveal any additional information which may reflect the lack of salmonid protein sequences in the NCBInr database.

### 3.4 Gene expression analysis by real-time PCR

To verify whether the changes in protein expression correlated with transcript level, five upregulated proteins and one constitutively expressed protein were chosen for further analysis. Thus real-time PCR was performed to quantify the transcript level of Mx, GRP78, C-type lectin, eukaryotic elongation factor 2, ubiquitone biosynthesis monooxygenase (UBMO) and ATPase B using a parallel set of stimulated and nonstimulated cells. There was a significant increase in the expression for Mx (Fig. 3a), C-type lectin (Fig. 3c) and elongation factor 2 mRNA following IFN-γ stimulation suggesting that the increase in protein abundance was regulated by the transcription of these genes. However, no significant change was observed for the mRNA of UBMO or GRP78 (Fig. 3b), although the mRNA for UBMO showed an increase but did not reach significance. The constitutively expressed ATPase B showed no difference in mRNA level following the stimulation. To determine if there was stimulation of type I IFN real-time PCR for IFN I mRNA was carried out at both 6 and 24 h following IFN-γ exposure. The mRNA for IFN I was significantly increased at 24 h whereas at 6 h there was a small but nonsignificant increase (Fig. 4).

**Table 5.** Results using MASCOT program on peptides generated by LC-MS/MS from trypsin digestion of protein spots excised from the Atlantic salmon cell line SHK-1 following stimulation with recombinant IFN-γ

| Spot | Protein identity | Species | MASCOT score | Accession no. |
|---|---|---|---|---|
| 11 333 | Glucose-regulated protein 78 kDa | *O. mykiss* | 99 | BAD90025 |
| 11 420 | N/A | | | |
| 12 477 | Unnamed protein product | *T. nigroviridis* | 56 | CAF95669 |
| 13 348 | Nucleosome assembly | *D. rerio* | 155 | AAH44152 |

## 4 Discussion

IFN-γ exerts a large number of functions allowing an infected host to respond correctly to help eliminate a pathogen. The mechanism that IFN-γ uses to exert its effects appears to be highly conserved through evolution [31], particularly in

 

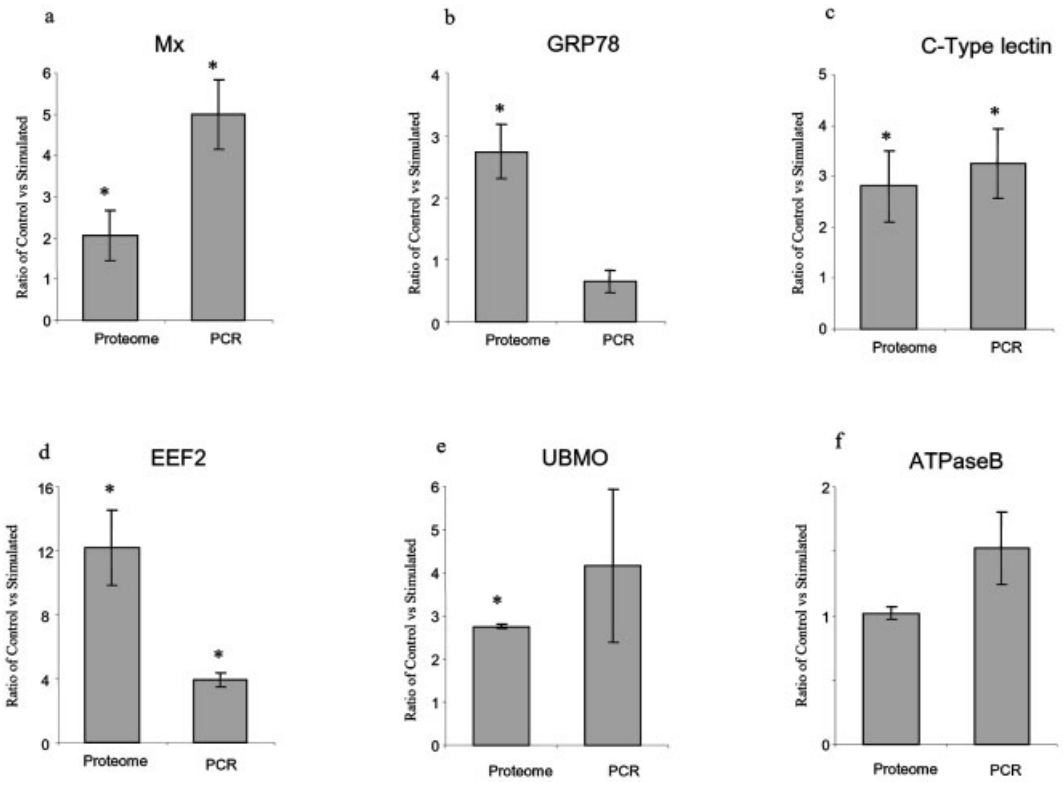


**Figure 3.** Comparison of fold increase for protein abundance observed by 2-DE gel analysis and mRNA expression obtained by real-time PCR for the Mx (a), GRP78 (b), C-type lectin (c), eukaryotic elongation factor 2 (d), ubiquinon biosynthesis monooxygenase (UBMO) (e) and ATPase B (f) genes following stimulation of the Atlantic salmon cell line SHK-1 with recombinant IFN-γ. Error bars represent SEM. * Indicates significant difference relative to the appropriate control.

higher vertebrates with the classic JAK/STAT pathway of signal transduction leading to induction of gene expression in IFN-γ responsive genes. To further the understanding of IFN function in fish, our laboratory has recently produced a recombinant trout IFN-γ protein that has been shown to activate macrophages and stimulate IFN-γ responsive genes such as γ-IP [13]. Here we have used the recombinant IFN-γ protein to stimulate an Atlantic salmon cell line, SHK-1, and studied the resulting alterations in the cellular proteome by 2-DE, and identified altered proteins using PMF. The use of proteomics to investigate gene expression in fish has become much more feasible in recent years with the great increase in genomic information which can be used for the identification of proteins following MS analysis.

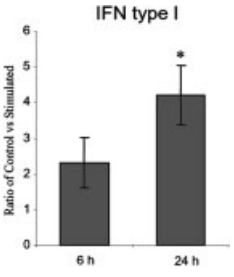


**Figure 4.** Real-time PCR for type I IFN mRNA in the Atlantic salmon cell line SHK-1 following stimulation with IFN-γ for 6 and 24 h. Error bars represent SEM. * Indicates significant difference relative to the nonstimulated control.

There are several characterised cell lines from salmonid fish that have been assessed for use in proteomics [32]. In this study, we have used a cell line derived from Atlantic salmon head kidney cells, which were originally used for the growth of ISA virus, an important viral pathogen of salmon [17]. Subsequent work on this cell line demonstrated that it responded to viral infection by increasing transcription of the MHC class I heavy chain and MHC II β chain [18], which would indicate that it may respond to the virus *via* an IFN-type response. The work presented in this paper is the first time that this cell line has been used as part of a proteomics study. Prior to performing extensive proteomic analysis, we confirmed that the cells responded to the recombinant IFN-γ by studying the transcription of an IFN-γ induced protein γ-IP [29]. γ-IP is a chemokine with most homology to CXCL10, which in mammals is strongly induced by IFN-γ [30]. We found that γ-IP was strongly upregulated in stimulated compared to non-stimulated cells, indicating good stimulation by the recombinant protein.

 

During the analysis of the 2-DE gels, 22 proteins were identified as being altered in expression following IFN-γ stimulation, and of these 15 were upregulated proteins and 7 downregulated. Eleven protein spots, eight showing induction, two showing repression and one constitutively expressed protein were excised from the gels for further identification by PMF and MS. Two database search programs, MASCOT [26] and MS-fit [27] were used for protein identification. During the MASCOT search, the trypsin-digested peptides were used to identify identical peptides generated from the NCBI nonredundant protein database. This analysis gave four significant protein identities. However, this search program requires either the salmonid protein sequence to be present in the database, or that the protein is highly conserved and as such may be identified by homology to more distance species. At the time of these searches (October 2006), there were only 1000 Atlantic salmon protein sequences and 2054 rainbow trout protein sequences in the NCBInr database, which was the likely reason for the low number of protein identifications by this approach. To increase the number of protein identifications we used a rainbow trout and Atlantic salmon nucleotide database and searched using the MS-fit program which translates the nucleotide sequence into protein in all six frames and generates trypsin-digest peptides in silico, which are then searched using the experimentally obtained peptides. For these searches, we used two databases generated by GRASP (www.grasp.org), which contain contigs assembled from all of the publicly available ESTs for both Atlantic salmon and rainbow trout. We included the rainbow trout sequences since they have very high sequence conservation with Atlantic salmon due to their close evolutionary relatedness. Using this approach, we were able to give identities for the remaining proteins. In three cases, the searches confirmed the same protein when searched against NCBInr using MASCOT and against the salmonid nucleotide database using MS-fit; these were Mx protein, GRP78 [33] and the constitutively expressed protein taken as a control which had been identified previously during a rainbow trout liver proteomics study as an ATPase β subunit protein [19]. One protein (reference spot 13 348) was identified by MASCOT as homologous to the human MHC I antigen, but was identified as a nucleosome assembly protein [34] by MS-fit. To clarify this ambiguity, we extended our MS analysis to include LC-MS/MS which confirmed the protein spot to be the nucleosome assembly protein. GRP78 was also confirmed by this method. These data clearly show that a variety of approaches are required to identify protein spots from animals that do not have a fully characterised genome. The γ-IP protein was not found to be in the group of upregulated proteins, most likely as the predicted p*I* for this protein is 9.6 and molecular weight of 11.1 kDa, which would put it outside the range of proteins resolved on the gel system used in this experiment as our gels were p*I* 4–7.

The protein that showed the greatest increase in abundance (spot 12 477) was identified as elongation factor 2 [35], a protein involved in protein translation which most likely indicated there was an increase in the translational machinery in the cell as a consequence of the IFN-γ stimulation [36]. In line with this was the identification of a downregulated proteasome subunit (reference spot 12 096), which may indicate decreased levels of protein degradation reflecting a general increased protein production in the cells.

Mx is a protein that is well characterised as an antiviral protein that has a tripartite GTP binding domain and a leucine zipper motif [37]. All Mx proteins identified to date have molecular weights between 70 and 80 kDa, which was confirmed in our study where protein spot 12 074 was found to be 71.1 kDa. The Mx protein has been sequenced in many teleost species including Atlantic salmon [31] and trout [22] amongst others. The rainbow trout promoter sequence of Mx has an ISRE site [38] indicating it is an IFN responsive gene, but did not reveal a GAS site. However, the present results indicated that in fish this gene may also be responsive to IFN-γ. The expression of Mx mRNA is well characterised in salmon and the kinetics of the transcriptional response has been studied in salmon infected with IPNV where Mx peaks 5 days postinfection [39]. The protein has been studied using a polyclonal antibody raised against the trout sequence which demonstrated that there was a significant increase in Mx protein following poly I:C stimulation of rainbow trout gonad, RTG-2 cells [40]. In our study, real-time PCR was used to determine if the increase in protein abundance was a result of increased mRNA transcription. The Mx mRNA was found to be upregulated five-fold, which was in fact greater than the increase observed for the protein. This difference may be due to a multitude of factors including mRNA stability, transcription rate of the Mx protein, or the rate of protein synthesis and half-life of the protein.

In mammals Mx gene is especially responsive to type I IFN, it is possible that secondary stimulation of type I IFN by IFN-γ may be affecting Mx expression. To address this additional real-time PCR was performed to determine type I IFN gene expression. This showed that there was an increase in type I IFN gene expression following the IFN-γ stimulation, and it is possible this may result in secondary responses. Further work will need to be carried out to fully assess the control of Mx expression in fish.

Lectins are cell surface receptors that can bind both viral and bacterial pathogens and initiate an immune response. A protein identified as a C-type lectin, increased in abundance by the IFN-γ stimulation was also studied for mRNA expression. In fish, gene expression of C-type lectins is increased following both bacterial [41] and viral [42] infection. Here we have shown that both mRNA and protein are induced in expression by the IFN-γ stimulation with similar levels of induction (*i.e.* three-fold), indicating protein abundance may be regulated at the transcriptional level.

Another upregulated protein identified here was GRP78. The gene encoding GRP78 has been found to be increased in expression following heat shock in the rainbow trout cell line RTG-2 [43] indicating its role as a heat shock protein (HSP), but in addition to its potential role as a chaperone protein it is

 

also implicated in the immune response. The mRNA is upregulated by bacterial infection in Atlantic salmon [41], is involved in macrophage development in goldfish [44] and in differentiation of murine myeloid leukaemia cells into macrophages by the proinflammatory cytokine interleukin-6 (IL-6) [45]. The same protein was found to be induced during a proteomic analysis of human dendritic cells stimulated with macrophage colony-stimulating factor and IL-4 [46]. Together these data show that GRP78 may have additional regulatory roles in macrophage activity. Surprisingly there was no significant increase in the transcription of the GRP78 mRNA when assayed by real-time PCR, which indicates that there may be additional points of control of this protein expression in the cell.

Indeed the relationship between transcription of mRNA and the abundance of protein is not always a direct one as there are many regulatory mechanisms that can affect these processes. Several recent papers have addressed this question performing parallel proteomic/gene expression studies. Experiments on preosteoblast cells [47], human THP-1 monocyte leukemia cells stimulated by oligonucleotides containing CpG motifs present in bacterial DNA [48] and human umbilical cord endothelial cells undergoing hypoxia [49] have all shown poor correlations. In contrast, a modest correlation between transcriptional and proteome analysis was found by principle component analysis following a bromobenzene treatment of rat hepatocytes [50].

### 4.1 Conclusion

In this paper, we demonstrated that proteomics can be used successfully to study the changes in cellular protein abundance following stimulation of a fish cell line with fish recombinant IFN-γ. The reference gel constructed as part of this work will be of benefit to others performing proteomics on this cell line and will add to fish cell proteomics information now available [32]. In recent years, there has been a dramatic increase in the genomic information for salmonids, mostly from ESTs. Although contigs were used here, many still do not represent full length ORFs which limits our ability to identify all the proteins. However, it is likely in the near future that as more sequencing is performed and protein sequence data are entered into the databases it will become more straightforward to identify proteins from proteomic analysis.

*We thank both Mrs. Evelyn Argo for running the 2-DE gels and Mrs. Liz Stewart for trypsin digest fingerprinting and mass spectrometry analysis. This work was funded by BBSRC grant EGA17675 (Salmon TRAITS). B. P. M. was supported by the Department of Biotechnology, Ministry of Science & Technology, Government of India with a 'Biotechnology Overseas Associateship Award' (BT/IN/BTOA/22/2005). Work in the Aberdeen Proteome Facility is supported in part by grants from The Scottish Funding Council, BBSRC and Aberdeen University.*

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