

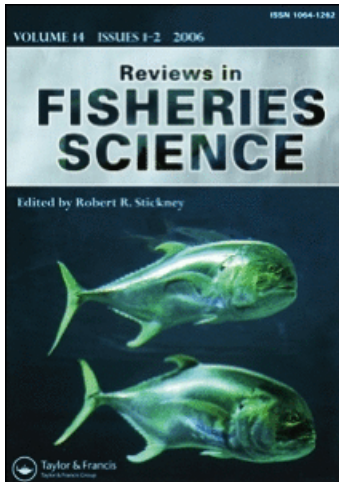
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Genomic Tools for Examining Immune Gene Function in Salmonid Fish

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The health status of farmed fish is central to the efficient production for harvest, as infection, either acute or chronic, will have serious effects on survival, growth, and quality of the final product. A correctly functioning immune system is vital to efficient aquaculture for both defense against invading pathogens and response to vaccines. The increase in sequence data for salmonid fish has allowed the development of tools to examine the immune response at both the transcriptional and the cellular level. This review will focus on identification of novel fish immune-related mRNA species by suppression subtractive hybridization, the expression pattern of mRNAs by transcriptome analysis (microarray), and the use of salmonid cell lines to examine the functional aspects of some key fish immune genes.

Keywords microarray, subtractive cloning, cell lines, cell engineering, immunology, salmon

INTRODUCTION

Fish health within aquaculture has been intensively studied for good reason, in that infection can be devastating. Ways to improve vaccination success require understanding of the immune response elicited. Thus, thorough knowledge of functioning and the control of the immune system is vital for efficient aquaculture and its future development. The immune system is broadly split into innate immune responses and adaptive immune responses. The innate system (Magnadottir, 2006) provides fish with a first line of defense against a pathogen, and the correct innate response needs to be elicited in order to fight off infection. Bacterial, viral, protozoal, and fungal infections all can elicit a different response (Akira et al., 2006), many of which are highly conserved throughout the vertebrates. The invasive pathogen is recognized by cell receptors and a complex series of signaling induces cells to activate response genes. These cell surface molecules, often TOLL receptors, recognize

pathogen-associated molecular patterns (PAMPS) (Janeway and Medzhitov, 2002). The immune response of rainbow trout and, more recently, the Atlantic salmon have been extensively studied in terms of both the innate and the adaptive response. The interest has stemmed from the importance and value of these species to aquaculture and also the rainbow trout as a representative of lower vertebrates on the evolutionary scale. Advances in functional knowledge of the immune system has led to many commercial vaccines, in particular against bacterial diseases (Quentel et al., 2007).

The emphasis on identifying genes related to the immune response has increased significantly over recent years. Initially, this involved cloning of specific immune genes by virtue of the homology with known mammalian genes (Bird et al., 2006). More recently, large EST data sets (Adzhubei et al., 2007; Govoroun et al., 2006; Rise et al., 2004b) have been generated from cDNA libraries that can be screened *in silico* for immune-related genes. Unfortunately, many of these libraries were constructed from healthy fish and contain few transiently activated immune genes. An approach to overcome this has been to generate cDNA libraries that are enriched for differentially expressed genes. This genomic data has enabled the development

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of new tools to functionally examine and characterize these newly discovered genes. This article will examine the use of subtractive cloning, microarray analysis, and the development of engineered salmonid cell lines to investigate the biological function of immune mediators.

SUBTRACTIVE CLONING

The most useful method adopted to date (2008) has been the use of suppressive subtractive hybridization (SSH) (Diatchenko et al., 1996). This method uses a PCR approach, and a commercial kit is now available from Clontech (PCR-select cDNA Subtraction Kit). The PCR products generated, after test and control cDNA, hybridization resulting in selection of differentially expressed or subtracted cDNAs are cloned directly into a plasmid vector, routinely a "TA" cloning vector such as the pGEMTeasy vector from Promega, which can then be sequenced. The clones generated are usually not full length, as cDNA is digested with restriction enzymes prior to the subtraction procedure, and are typically in the region of 200–800 bp in length. This method both normalizes the cDNA in that it removes highly abundant cDNAs but maintains the rare differentially expressed cDNA species. The use of SSH cloning in salmonid fish has been successfully used many times for identifying novel immune genes from both antibacterial responses (Bayne et al., 2001; Martin et al., 2006; Tsoi et al., 2004) and antiviral activity (O'Farrell et al., 2002). These papers have demonstrated this to be a highly efficient method for generating enriched libraries. Once clones have been identified, then full-length cDNA sequences can be routinely obtained by a variety of PCR-based approaches such as RACE. This suite of methodologies have been used to clone several cytokines recently including interleukin-11 (Wang et al., 2005) and IL-6 (Iliev et al., 2007).

The use of targeted approaches for immune gene discovery by the combination of EST and SSH analysis are well justified and provide important tools for the identification of gene cohorts, which are expressed dynamically and differentially in response to different immune insults. These gene sets have been central to the development of microarrays that enable the study of large-scale gene expression changes as described below.

MICROARRAY

Recent years have seen a rapid development of microarrays for many fish species, including salmonids (Douglas, 2006). Microarrays rely on having a comprehensive EST data set generated by either random sequencing of cDNA libraries from which cluster analysis can determine how many unique transcripts are present (although many of these sequences may not be annotated) or from clone sets enriched for known annotated genes. Both Atlantic salmon and rainbow trout have now vast numbers of ESTs, the majority being generated by a small number of

laboratories. For Atlantic salmon, the genomics research in all salmonids project (GRASP, Canada) (Rise et al., 2004b) consortium and Norwegian salmon genome project (SGP) (Hagen-Larsen et al., 2005) have contributed the majority of clones, whereas the AGENAE project at INRA (Govoroun et al., 2006) and USDA (Rexroad et al., 2003) have generated the bulk of the rainbow trout sequences. A considerable amount of these data has been deposited in public access databases, at NCBI, and the TIGR database. At the time of writing this review, there are 437,510 Atlantic salmon and 265,551 rainbow trout sequences, the majority of which (over 90%) are ESTs. TIGR has performed cluster analysis on all sequences and generated transcript contigs (TC) for these clones, which have been annotated for identity and gene ontology where possible.

Transcriptome wide arrays aim at maximum coverage, whereas system targeted arrays may have fewer selected features representing specific biological processes. The features on an array can either be PCR-generated products, referred to as cDNA arrays or specific oligonucleotides of 25 to 70 bases (oligo arrays) unique to the gene to be hybridized. For oligo arrays, a number of different platforms are being developed, including conventional printed slides and *in situ* synthesized oligos (Agilent and Affimetrix). At the present time, the majority of salmonid microarray platforms are cDNA arrays. Several cDNA microarray platforms have been established with varying numbers of features: (Ewart et al., 2005) 4104; (Rise et al., 2004b) 3700; (von Schalburg et al., 2005) 16008; (Koskinen et al., 2004) 1380; (Martin et al., 2007b) 16951. Cross species hybridization of cDNAs between salmonids has been shown to be extremely high (Rise et al., 2004b; von Schalburg et al., 2005), suggesting the arrays could be used with equal confidence for both Atlantic salmon and rainbow trout as a result of their high level of homology and the cDNA features being several hundred bases in length.

All of the above microarrays have been used in a wide variety of immune-challenged salmonid tissues. These have included tissues from fish given bacterial challenges with *Piscirickettsia salmonis* (Rise et al., 2004a), *Aeromonas salmonicida* (Ewart et al., 2005; Martin et al., 2006), and *Listonella (Vibrio) anguillarum* bacteria (Gerwick et al., 2007). Viral challenge has also been used in vaccination experiments for infectious hematopoietic necrosis virus (Purcell et al., 2006). It is interesting to note that many similar genes are found up-regulated in response to quite different pathogens. Often these are related to the innate immune response, resulting from conserved response pathways, as is indicated by the stimulation of several key transcription factors including CAAT/EBP and Jun-b (Martin et al., 2006). Iron metabolism is altered, with transferrin and ferritin mRNAs being regulated under a number of different immune challenges demonstrating common innate responses. Parasitic and fungal infections have been analyzed by microarray for both amoebic gill disease (Morrison et al., 2006) and for the fungal infection *Saprolegnia* (Roberge et al., 2007). Again some key inflammatory response genes are also observed to change in expression level in common with bacterial and

viral infections. Care needs to be taken to eliminate the possibility that responses observed are not a result of secondary bacterial or fungal infections, and this is especially true when examining parasitic infections. Current knowledge gained from the analysis of the available platforms has identified key antibacterial and inflammatory proteins, which are expressed as a result of bacterial infection. To further analyze the regulation of the early immune response, recombinant trout cytokines have been used to stimulate both rainbow trout and Atlantic salmon cell lines and to study the transcriptome wide responses by microarray (Martin et al., 2007a, 2007b). Interleukin- 1β (IL- 1β) and interferon- γ (IFN- γ) were used to stimulate a rainbow trout macrophage cell line RTS 11. The expression profile indicated that the IL- 1β causes genes associated with the acute phase response to be increased, whereas the IFN- γ induced genes, particularly those involved in MHC I antigen presentation, are up-regulated (Martin et al., 2007b). An Atlantic salmon cell line (SHK-1) derived from salmon head kidney (Dannevig et al., 1997) was used to examine the differences in the transcriptional response to type I and type II interferons. Here it was found that a number of genes were stimulated by both molecules at both 6 and 24 hr following exposure, with other genes being expressed preferentially by either type I or type II molecules. Of interest was the fact that in the SHK-1 cells exposed to IFN- γ , no obvious increase in genes related to antigen presentation were observed in contrast to the situation when using RTS11 cells. This demonstrates the variability of response in the cell lines being used and can confound interpretation of results.

Depending on the questions being addressed, the cDNA microarray results will enable mRNAs to be grouped according to function and give indications as to the immunological pathway being stimulated. However, there are several drawbacks to the use of cDNA microarrays, which are particularly relevant for salmonid fish. The tetraploid nature of the salmonid genome results in many duplicated genes, and also the high level of polymorphisms present in salmonid genomes means a hybridized feature could be one of a number of gene transcripts. Additionally, transposons or repeated elements in the cDNA may occur in the 3' UTR and lead to false positives. The cDNA may be unable to distinguish between transcription of alternate alleles or between differential expression of highly homologous genes.

Oligonucleotide microarrays have the ability to overcome some of the inherent limitations of cDNA microarrays, but the design and use of oligoarrays in salmonid research is still at an early stage. A number of groups have designed and used oligo arrays, although to date there is only one published salmonid oligoarray that has been reported. A rainbow trout 70-mer oligoarray has been generated at Oregon State University (Tilton et al., 2005) based on preselected genes that may be related to processes, including carcinogenesis, immunology, environmental toxicology, stress, physiology, and endocrinology. This array has been used for studies on the effect of the dietary contaminant aflatoxin B1 (AFB1) on liver function (Tilton et al., 2005) and the acute phase response in liver following stimulation with

Listonella (Vibrio) anguillarum bacteria (Gerwick et al., 2007). For the majority of the genes, oligos were designed close to the 3' end of the transcript with mostly one oligo per gene, although a number of genes had two oligos to confirm accuracy in hybridization of different oligos to the same gene. To reduce the technical variation and increase statistical precision, features can be printed on the slides in replicates; for example Koskinen et al. (2004) printed a targeted array of 1,380 cDNAs in sextuplicate, while Ewart et al. (2005) printed their features in quadruplicate. However, in the most widely used salmonid array developed by GRASP (von Schalburg et al., 2005), the majority of cDNAs are printed only once per array.

Quality of starting RNA material is imperative to successful microarray analysis to ensure efficient reverse transcription allowing equal labeling of cDNA samples. If there is poor quality cDNA, which contains impurities, high background can be observed during hybridization. Depending on the tissues or cells to be used for analysis, 5–20 μ g total RNA is commonly used per sample. If starting RNA quantity is limited, linear amplification methods (mRNA MessageAmp II aRNA Amplification Kit, Ambion, Austin, Texas, USA) can be used to generate cRNA from as little as 20 ng of mRNA. It is best not to amplify cDNA by PCR-based methods for probe labeling as this can lead to unevenly labeled probes. The majority of microarray studies performed on currently available platforms are hybridized with cDNAs labeled with fluorochromes. Typically, one sample is labeled with Cy3 and the other with Cy5. These are then mixed before hybridization, and the ratio of hybridization signal relates to gene expression. To avoid bias for dye labeling of particular genes, dye swap design is often used, where each sample is also labeled with the alternate dye and hybridized to a separate slide (Parmigiani et al., 2003). Careful consideration should be given to experimental design, and of great importance is biological replication (Allison et al., 2006; Yang and Speed, 2002), which often limits the numbers of samples that can be analyzed (e.g., time points, concentrations, etc.). The most simple experiments can be control vs. experimental, including dye swaps, but when time course or dilution series are used, the design becomes more complex and can involve a common reference design or loop designs that minimize the number of slides required to obtain maximum data output (Vinciotti et al., 2005).

The analysis of the data generated by the microarray is possibly the most challenging part of the experiment, with each step having multiple variations. The initial steps required after scanning are background subtraction followed by normalization. This is often Lowess normalization (Cleveland, 1981), which allows for variation in signal intensity variation across the slide. Once normalized data is generated, statistical analysis (*t*-test or ANOVA) can indicate genes that are differentially expressed. Of fundamental importance is the calculation of the false discovery rate (FDR). Due to the nature of microarray analyses, with multiple hypotheses testing, data must be corrected for falsely significant results. Typically, the Benjamini-Hochberg test is used for correction, and a cut-off value set, often <0.05 , where false positives are controlled in relation to the number of genes

in the dataset. There are a number of commercial software programs to aid in the data analysis, such as GeneSpring, Acuity, and GeneSight, among others. As methods for microarray analysis improve, labs are moving toward more adaptable methods of analysis, with focus on the statistical programs contained within Bioconductor (<http://www.bioconductor.org/>), which contains packages specifically for microarray analysis. However, this still requires a reasonable competence in the use of the “r” programming language. Microarray platforms and data sets are now routinely submitted to either the gene expression omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) or the Array Express data base (<http://www.ebi.ac.uk/arrayexpress/>) using the minimum information about a microarray experiment (MIAME) guidelines (Brazma et al., 2001), allowing other users to retrieve published data.

In conclusion, microarray technology linked with bioinformatic analysis is fueling the development of transcriptomics in fish. As more platforms become available, in conjunction with precise annotation of genomic resources, the response to pathogens and vaccines will be established. It has already become apparent that many physiological processes interact, especially stress/immune function/growth/maturation, and the application of functional genomic technologies to these processes will provide a clearer view of these relationships at the cellular and tissue level. However, the identification of genes and their expression alone does not result in a holistic view of cellular immune function, and the next section of this review addresses how this can be achieved in salmonid fish.

IMMUNE-RELATED ENGINEERED CELL LINES

In the case of genes regulated post-translationally, the measurement of gene expression gives limited information. In addition, the interaction of a particular gene with other genes in a response pathway is not easily addressed through direct analysis of mRNA expression. For genes encoding secreted immune molecules, post-translational activation can be investigated by producing the recombinant molecule in a prokaryotic or eukaryotic expression system and utilizing the protein to characterize its biological function, as described for a number of trout cytokines (Harun et al., 2008; Zou et al., 2005). In other cases, the genomic information can be used in fish cells to artificially increase or decrease the level of expression of a given gene by manipulating the promoters or inhibiting expression, generating valuable information on its biological function. The possibility of artificially over-expressing or suppressing the expression of a given gene in cells has received almost no consideration in salmonid research. In the following section, we will review the technology available for salmonid species and evaluate the potential for future developments.

The development of stable recombinant cell lines over-expressing key immune genes has many applications, although to date this has not been achieved for salmonid immune genes.

The only stable recombinant salmon cell line in existence is that of CHSE cells constitutively expressing an exogenous gene, the infectious pancreatic necrosis virus non-structural VP5 protein (Helmrich et al., 1988). The establishment of this cell line allowed clear demonstration of the anti-apoptotic properties of the VP5 protein. More generally, in other fish species, a European flounder cell line over-expressing the flounder antimicrobial peptide pleurocidin has recently been established and used to produce sufficient amounts of the recombinant antimicrobial peptide so that its bactericidal abilities may be further characterized (Brocal et al., 2006). In addition, a zebrafish stable cell line over-expressing a gene belonging to the anti-apoptotic Bcl2 family was isolated (Chen et al., 2006), as well as a grouper GB3 brain cell line expressing constitutively the gene encoding for the interferon-induced antiviral protein Mx (Lin et al., 2006).

With the recent development of transfection methods for non-mammalian cell lines and inducible expression systems (Chambard and Pognonec, 1998), the potential for establishing inducible stable fish cell lines has greatly increased, which in turn should open the door to better functional characterization of newly isolated genes.

Stable recombinant cell lines expressing a reporter gene have now been established in salmonid cell lines (Collet et al., 2004) and can be used to characterize the regulation of a specific gene. Reporter genes are used in the development of sensitive and reliable cell-based functional assays (Fent, 2001), where a reporter gene is linked to a genomic regulatory sequence, promoter, and transfected into fish cells. Commonly used reporter genes, such as luciferase or the green fluorescent protein (GFP), have a low toxicity to most fish cell lines even when they are highly expressed and accumulate at high levels. In a few cases, a permanent recombinant fish cell line has been generated in this way for the characterization of genes (Collet et al., 2004), promoters (Ikeuchi et al., 2003), or to localize a gene product generated by different expression plasmids (Castro et al., 2008; Collet and Secombes, 2005).

The expression of a gene can be artificially decreased (“knocked-down”, KO) by RNA interference, and the effect of this decrease gives valuable information on the identification of its function. However, although RNA interference has been efficiently used with fish muscle tissue (Xie et al., 2005), it has never been successful in cultivated fish cells (Molina et al., 2002). An alternative approach is to permanently modify the genome at the whole organism level through modification at the embryonic stage or on a somatic cell line by homologous recombination leading to the disruption of a given gene and the production of a KO model (Van der Weyden et al., 2002). Homologous recombination involves the specific replacement of the gene of interest in the genome by an artificially created disrupted version of the gene, using the cell’s own recombination enzymes.

The generation of transgenic salmonids is difficult and slow because, unlike the situation in cyprinids or other fish groups (Hong et al., 2004), there are no embryonic stem (ES) cells available, the generation time is long, and the ploidy is more

complex as all salmonids are tetraploid. A number of well-characterized somatic cell lines are available in Atlantic salmon and rainbow trout, including the macrophage-like cell line RTS 11 (Ganassin and Bols, 1998) and SHK-1 (Dannevig et al., 1997). However, to date no attempt has been made to develop KO somatic fish cell lines.

Besides the obvious application in the characterization of gene function, a KO cell model could greatly improve diagnostic methods for intracellular pathogens based on cell culture of the pathogens. Permissive fish cell lines represent an essential component of diagnostic procedures for viruses or some bacterial pathogens (Villena, 2003), where samples potentially containing live viruses or intracellular bacteria are applied to a monolayer of sensitive cells. However, most of the fish cell lines used to date (CHSE, RTG-2, and EPC) exhibit a potent interferon activity that leads to an antiviral state conferring partial resistance to the viruses, thereby delaying the onset of the cytopathic effect. This results in diagnostic methods being time-consuming. A number of the genes involved in the viral resistance of these cell lines are known (Lin et al., 2006), and the ability to permanently disrupt their expression would allow the development of cell lines for improved diagnostics.

CONCLUSIONS

The ongoing development of genomic and cellular tools related to the functional genomic analysis of the fish immune system is allowing a much deeper insight into how salmonid fish respond to either vaccination or infection. Key goals for the future include increasing annotation of sequences, developing new technologies for ultra-high throughput sequencing (454 technology) (Margulies et al., 2005), coupled with the development of cell lines and KO protocols to examine cell signaling. Combined, these tools are very powerful, and the data generated will have an increasing relevance to the aquaculture industry, helping to ensure more sustainable farming of salmonid fish into the future.

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