matchmaker

A Bioinformatics Tool for an Integrated Analysis of Proteomic and Genomic Expression

Master of Science Thesis
2001

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Preface

This report is a Master's Thesis in Bioinformatics, a 1½ year International Master's Programme involving Chalmers and the University of Göteborg (GU). The thesis will conclude the degree of Master of Science in Chemical Engineering with Engineering Physics at Chalmers.

The research project has been carried out at AstraZeneca R&D Mölndal (AZM), within the department of Cell Biology & Biochemistry. The departments of Molecular Biology, Biostatistics, and Discovery IS have also been involved.

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The system developed within the project is available on AZM's intranet at the address (URL):

http://bioinfo.seml.astrazeneca.net/farmmc/matchmaker.html

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Göteborg, November 2001

Abstract

The aim of this Master's Thesis has been to explore ways in which bioinformatics can be applied to proteomics data and research to create additional value. The idea is that bioinformatics can make current research methods more effective and create new valuable information and visualizations that can spark novel hypotheses. Our efforts have resulted in a program called Matchmaker, a useful tool for comparison of genomics and proteomics data.

We have based our work on a study where obese diabetic mice have been treated with the substance rosiglitazone in the hopes of normalizing their condition. Rosiglitazone is a ligand that binds to peroxisome proliferator-activated receptor γ (PPARγ), which in turn activates the transcription of a large number of genes involved in lipid metabolism. The rosiglitazone study was conducted both at the proteomic and the genomic levels, making expression data available both for proteins and mRNA.

The initial task we undertook involved automating a search method for finding PPAR Response Elements (PPRE) in the promoter region of certain mouse genes. After further analysis this proved not to be feasible, primarily due to incompleteness of the mouse genome.

The central task of our thesis has been to create a tool for the automation of a genomic and proteomic comparative analysis. Using the rosiglitazone study as a testing ground, we created Matchmaker, a program that given genomic and proteomic expression data respectively, matches the identified proteins with their corresponding genes and provides visualization options for the results. To get an idea of the statistical significance of the results, we chose to calculate confidence intervals for the matches.

Creating a user-friendly interface for Matchmaker was of primary importance. Therefore we have created a clear and easy-to-use web interface with drop-down menus for genomic data selection and a text area for proteomic data submission. The program subsequently matches the data sets and moves on to a page where the results are shown in table format. From the results page, buttons automatically export the data to Excel and Spotfire, where the data can be analysed in various ways.

Although the design of the program has been our primary effort, we also wanted to perform an analysis of the results in the case of the rosiglitazone study to evaluate the usefulness of the program. We found that protein and gene expression levels were moderately correlated. A number of expected trends were also confirmed.

Integrated analysis of expression levels is very important for the understanding of systems biology, and will play an increasing role when more experiments become coordinated, expression technologies are refined and sequence databases grow. We are confident that our program Matchmaker will make broader perspectives possible and that analysis of the results will lead to new and useful hypotheses.

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1. Introduction

This Master's Thesis has been performed at AstraZeneca in Mölndal. The proteomics researchers at the department of Cell Biology and Biochemistry conduct experiments with 2D-gels and mass spectrometry to, among other things, be able to tell whether proteins have been up- or down-regulated after treatment with a substance. The ultimate goal of the research is to find a substance that can be used as a drug to normalize an unhealthy condition.

The idea for our thesis has been to create a bioinformatics tool to extract more valuable information around these experiments. Bioinformatics can be defined as information technology applied to the management and analysis of biological data. Computing power can be a useful assistant in automation, organisation, and analysis. Databases store great amounts of information effectively, and bioinformatics tools make use of these to analyse a problem in a specific way. Our central task in this thesis has been to combine proteomic and genomic data in a comparative analysis.

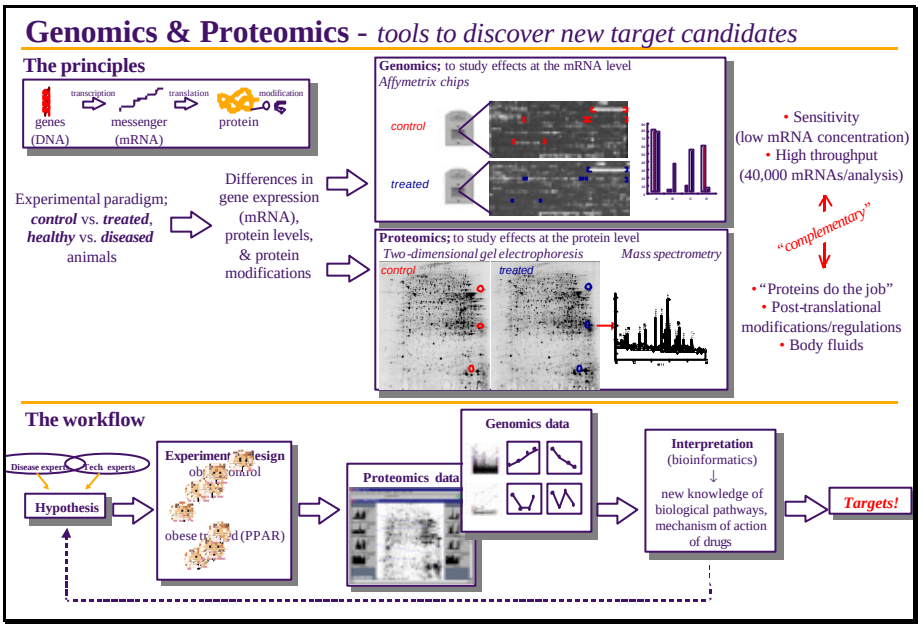

Figure 1.1 – *The principles and workflow of a proteomic and genomic comparative study (kindly provided by Björn Dahllöf)*

1.1 Background

To describe why we have written this thesis, it is important to relate some background information on the technologies that we base our work on. We also want to describe the disease area connected with the insulin resistance syndrome (IRS). This will give an idea of the importance of and meaning behind IRS research, which the proteomics group at AstraZeneca Mölndal is primarily involved with. It is data from experiments within IRS which we have used in our thesis.

1.1.1 Proteomics

Proteomics is the large-scale analysis of the protein complement of the genome, the so-called "proteome". One of the main uses for proteomics is in 'differential display'. By studying differences in protein abundance in cell samples before and after certain perturbations, (such as a comparison of sick tissue with healthy, or sick with treated) conclusions can be drawn as to cell functionality and potential drug candidates. The most common technique today for

this analysis is the use of 2D-PAGE (PolyAcrylamide Gel Electrophoresis) for separation followed by Mass Spectrometry (MS) for identification.[1]

In 2D-PAGE, proteins first migrate towards their iso-electric point along the pH scale (the first dimension). In the second step the proteins are solubilized and evenly negatively charged by the detergent SDS. When an electric field is applied, proteins will move through the porous polyacrylamide gel with a speed inversely correlated to their size, and the separation will instead reflect their molecular weight (the second dimension).

After separation the gels are stained in order to visualize the protein spots, which are then analysed using image analysis software tools. Usually the focus is on spots that differ between different groups of samples, and their intensities can be compared and tested for significance. The proteins in the interesting spots must be identified using MS before any conclusions can be drawn.

There are commonly two MS identification methods in use today. The proteins are initially digested in-gel. In Matrix Assisted Laser Desorption/Ionization – Time Of Flight (MALDI-TOF) the resulting peptides are then fired at by a laser and ionized so that they fly to a detector, resulting in time of flight distributions according to their masses. These flight times work as fingerprints which are searched against databases to finally determine the protein identity. The second method uses two mass spectrometers in tandem (MS/MS) that ionize the peptides by "electro spray" and break the peptides down into even shorter fragments that allow for sequencing. This method is far more specific for identification than the MALDI "fingerprint" method, but also more complex and time consuming. A recent development has been a system that combines the two above mentioned techniques, thus benefiting from both specificity and speed.

It should be mentioned that this relatively straightforward approach to protein expression analysis can not identify and determine all proteins expressed in a cell at a given time point. Only the most abundant spots (~20% of all proteins) are visible enough to be quantified, and the interesting group of membrane proteins does not come out well at all on the gels. In addition, proteins that have yet not been identified and annotated in databases can not be determined using MALDI MS.

1.1.2 Microarrays

Microarray technology allows us to monitor the interactions among thousands of genes simultaneously on a single chip. Hybridisation (i.e. base-pairing: A-T and G-C for DNA; A-U and G-C for RNA) is the underlying principle of microarray technology. Arrays are orderly arrangements of samples, and microarrays get their name from the very small sample size, typically measured in 10s of microns. They provide a medium for matching known and unknown DNA or RNA samples based on base-pairing rules. They require specialized robotics and imaging equipment. The so-called "probe" is the tethered nucleic acid on the microarray plate with known sequence, whereas the "target" is the free nucleic acid sample whose identity/abundance is being detected (although this nomenclature is sometimes reversed in literature). There are two major areas of application for the microarray technology, identification of sequence (gene/gene mutation) and determination of expression level (abundance) of genes.

The Affymetrix GeneChip is a microarray method invented by the company Affymetrix. The GeneChip involves probes of oligonucleotides (25mer) synthesized in situ (on-chip).[2] Instead of using amplification techniques such as PCR, the oligonucleotides are synthetically produced by the techniques of photolithography and solid-phase DNA synthesis directly on the chip. This allows for the production of all possible combinations of sequences. The chemical steps involved are:

1. Synthetic linkers with photochemically removable protecting groups are attached to a glass substrate.
2. A filtering mask directs light to specific areas on the glass surface and thereby removes the protecting groups.
3. Single deoxynucleosides with a protecting group, brought to the surface, bind to the unprotected sites.
4. A new mask is applied and the procedure is repeated until a highly dense collection of any desired oligonucleotides is obtained.

The array is then taken to a hybridisation chamber where fluorescent-labelled nucleotide samples are injected and hybridised to the complementary oligonucleotides. Laser excitation makes the samples fluorescent and a 2D fluorescence image of hybridisation intensity is obtained by a scanner.

The short chains in the Affymetrix technique with only single points of constraint at either end are highly accessible for hybridisation. This potentially allows for more accurate mRNA quantification and the number of dynamic possibilities for detection increases. However, disadvantages of the short-chain Affymetrix technique include the variations in melting temperature due to AT-GC composition, and the reduction in specificity due to the small number of nucleotides (~25).

The Affymetrix GeneChip is a very high-density microarray, where a single 1.28x1.28 cm array today can contain probe sets for approximately 40,000 human genes and ESTs (Expressed Sequence Tags). This compactness is advantageous because it allows more genes to be analysed simultaneously. The use of perfect match probes as well as mismatch probes (where a single nucleotide is substituted) greatly reduces the contribution of background noise due to cross-hybridisation and increases the quantitative accuracy and reproducibility of the measurements. These probe sets will be described in more detail in *Section 2.3.2.*

1.1.3 The Insulin Resistance Syndrome

One of the most rapidly increasing diseases among nations with a high standard of living is Type II Diabetes Mellitus (T2DM). According to WHO (World Health Organisation), the number of people affected will double up to 300 million within the next 25 years.[3] Not only today's welfare states, but also developing countries with food and exercise habits resembling the industrialized world's, will see a dramatic increase of this disorder.

T2DM is preceded by *insulin resistance*, which means that the signalling properties of the insulin molecules have less effect in the cell. Insulin is a peptide hormone whose main function is to control glucose levels in the blood, and lack of these leads to elevated glucose levels with glucose intolerance and diabetes as a result. When the cells first become less sensitive towards insulin, pancreas increases its insulin production to overcome the "resistance" and thereby keeping blood glucose on a normal level. Eventually the insulin producing ß-cells will

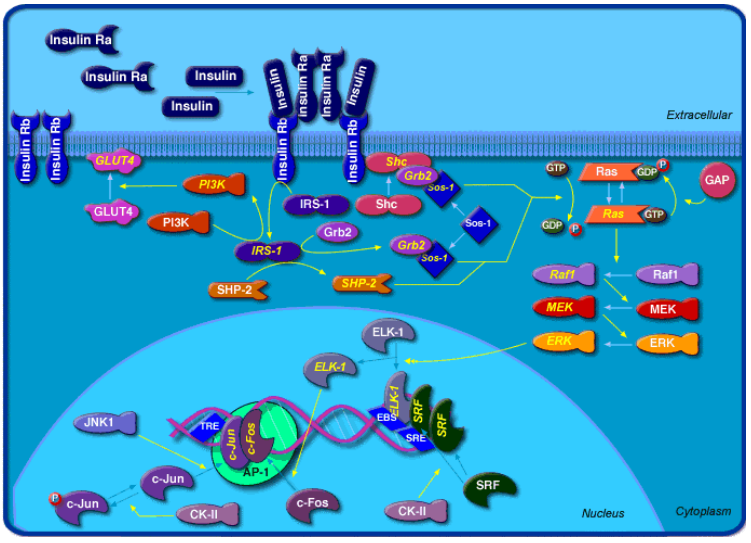

Figure 1.2 - *The appropriate signalling through the insulin pathway is critical for the regulation of glucose levels and the avoidance of diabetes*[4]

become exhausted, the production will halt and diabetes evolves.
In addition to being a precursor to diabetes, the Insulin Resistance Syndrome (IRS) has other serious implications, such as hypertension, atherosclerosis and dyslipidemia (high triglyceride levels and low high-density lipoprotein levels) with probable cardiovascular disease as a consequence.[5] It is therefore obvious that a successful treatment of IRS would directly improve the health of millions of people across the world and lower national medical costs.

Advances within this research have led to the discovery of a suitable target: The Peroxisome Proliferator-Activated Receptors (PPARs). These are ligand-activated nuclear hormone receptors that work as transcription factors bound to the DNA, ready to activate and regulate genes responsible for glucose and lipid metabolism. There are three main types of PPARs: PPARa, PPARd and PPAR? commonly present in different cell types.

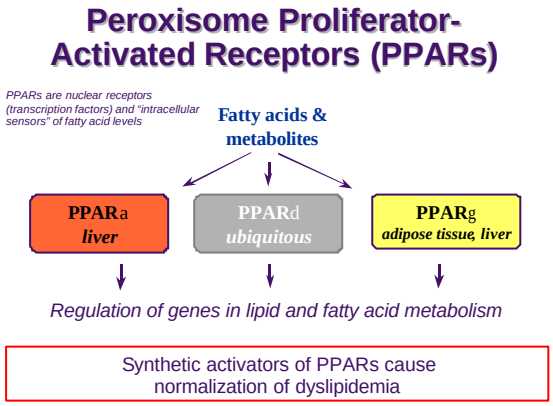

Figure 1.3 – *A picture diagram of PPARs (kindly provided by Björn Dahllöf)*

PPARs exist as heterodimers together with another nuclear receptor RXR and bind to PPAR Response Elements (PPREs) in the genes' promoter regions. When the genes in question are inactivated a co-repressing protein complex keeps the histones deacetylated, thereby inhibiting transcription. If a ligand is added, a co-activating protein complex instead binds to the PPAR-RXR heterodimer and the histones are acetylated. This allows for gene transcription.

A certain group of small molecules have proven to have activating ligand effects for the PPARs, the so-called thiazolidinediones (TZDs). When insulin resistant obese and diabetic animals are treated with these agents, insulin sensitivity and many of its other associated pathological effects are normalized.

1.2 Purpose

The purpose of our thesis is to explore ways in which bioinformatics can be applied to proteomics data and research to create additional value. The idea is that bioinformatics can make current methods more effective and bring in new valuable information and visualizations that can spark novel ideas for the researcher.

1.2.1 PPAR Response Elements

In a search for proteins that are up-regulated by PPARs, it is natural to ask the question: "Which proteins have PPAR Response Elements (PPRE) in the promoter region of their complementary genes?" PPREs are known to be a seat for the PPARs which induce transcription. Response elements have a high degree of conservation and most of them have a certain sequence motif. The PPRE is a so-called DR-1 motif, meaning a direct repeat of a nucleotide sequence with one intervening nucleotide. The spacing nucleotide is usually an A or T. The repeating sequence can also vary somewhat, although the consensus motif is AGGTCA[AT]AGGTCA.[6] Any promoter region with this DR-1 sequence has a high likelihood of binding PPARs.

Localisations of PPREs in the promoter regions of a few known genes are described in the literature. These are, however, not all the PPREs in the mouse genome and a method for finding additional such response elements would be very desirable. If a method could be developed into an automated application, it would be useful within biological research.

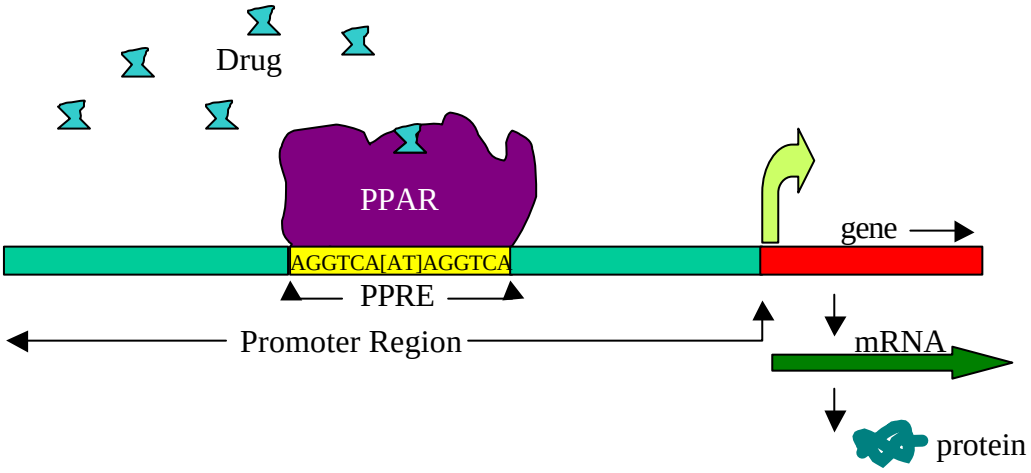

Figure 1.4 – *A schematic drawing of a drug ligand binding to PPAR, in turn activating the PPRE sequence in the promoter region of a gene*

Thus, we began by deriving a method for searching PPREs in the promoter region of genes corresponding to mouse proteins of interest, and tested this on a small number of proteins. We then proceeded with an evaluation of whether our method was suitable for full-scale automation.

1.2.2 Gene/protein correlations

A second and larger task we have undertaken is to create a user friendly program to match proteomics and genomics data and visualize the extent of correlation graphically. Prior to starting our thesis, we read an article in Science with the title *Integrated Genomic and Proteomic Analyses of a Systematically Perturbed Metabolic Network*.[7] This article emphasized the importance of an integrated analysis to more fully understand the interacting networks in living cells.

Our idea has been to investigate correlation between gene and protein expression data to be able to verify old conclusions as well as gain new understanding of metabolic pathways and mechanisms of action of drugs. A question of interest for many is: "To what degree can expression at the mRNA level be correlated to expression at the protein level, and what are the reasons for non-correlation?"

The process of protein production from the original DNA sequence is not entirely straightforward. An understanding of the process will yield hints as to why mRNA levels and protein levels are not strictly correlated.

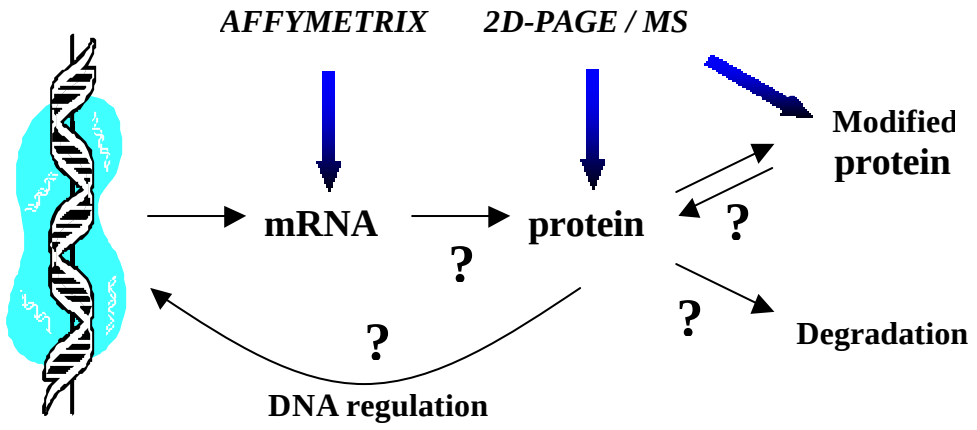

Figure 1.5 - *Model of subsequent processes in a cell. Integrated expression analysis on both the genomic and proteomic level can help in answering questions about the intermediary mechanisms.*

The initial RNA molecule produced by transcription contains both intron and exon sequences.[8] Its two ends are modified, and the introns are removed by an enzymatically catalysed RNA splicing reaction. The resulting mRNA is then transported from the nucleus to the cytoplasm, where it is translated into protein. The final level of a protein depends on the efficiency of each step and on the rates of degradation of the RNA and protein molecules.

Matchmaker

Comparing mRNA and protein data can give clues to answering the question marks in *Figure 1.5* and ultimately lead to the localization of new and more effective drug targets. So what is an effective way of producing and visualizing a comparison? Our answer to that question has been to develop a program we have called Matchmaker.

Microarray analysis generates huge amounts of data. One chip detects the expression of thousands of genes and ESTs. Proteomics does not operate on quite the same level, but there are still potentially hundreds of protein spots. Matching these two manually is an extremely time consuming process that would never be economically justifiable. Therefore, automation in Matchmaker opens up the possibility of a comparison at minimal time-cost.

Creating an easy-to-use interface for the comparison has been a very important part of our project. Without this, the program would not be used. We analysed what visualization methods would be the most effective and what information these would entail. It was important to receive feedback from the users. Through Matchmaker we have provided researchers with a helpful tool in sparking new ideas and insight into the original data.

2. Analysis and Strategy

For a comparative analysis we needed an experiment conducted similarly on both the protein and gene levels. There has been an interest for such a combination at AstraZeneca Mölndal, but as of yet only a few of these studies exist. One such study, however, served as the testing ground for our program, as well as the basis for the PPRE search. The chapter is divided into a description of the study and an analysis of PPRE existence and gene/protein linkage respectively.

2.1 The rosiglitazone study

The proteome study we have primarily looked at involves lean mice, obese control mice, and obese mice treated with rosiglitazone (a TZD, see *Section 1.1.3*) for seven days.[9] Tissue samples from liver and white adipose tissue have been extracted. The treated group consisted of four animals and the control group consisted of five animals. After image processing of the fluorescently stained 2D gels, thousands of protein spots were readily quantified. From these, hundreds of spots differed significantly from the control group spots according to a Student's t-test ($P<0.05$). 111 spots representing 58 unique proteins were identified by mass spectrometry. Failures in spot identification were due either to very low chemical quantity of the proteins, or to the lack of a hit in the databases queried. Although only proteins whose expression showed significant changes were chosen, we were also able to include a number of "unchanged" proteins in our analysis. The reason these had been identified was that they had showed significant changes in the other comparison (*lean vs. obese control*).

The treatment effects of rosiglitazone were explored in a similar study at the mRNA level with Affymetrix Mu6k chips (about 6000 genes/ESTs).[10] Tissue samples were extracted from liver, mesenterial fat, epididimys fat, brown fat and quadriceps. Groups of three mice were treated one, three and seven days. The conditions were similar to the proteome study, except for the fact that the mice were treated with ten times as high a dose.

We have used the *obese treated vs. obese control* comparison in liver tissue as our primary means of testing our program.

2.2 PPRE

Before dwelling deeper into our methods, a few concepts used in bioinformatics need to be explained:

- EMBL (European Molecular Biology Laboratory) is a laboratory that maintains Europe's primary nucleotide sequence data resource.[11,12] The EMBL Nucleotide Sequence Database is a comprehensive database of DNA and RNA sequences collected from the scientific literature, patent applications and directly submitted from researchers and sequencing groups. It collaborates with GenBank in the USA and the DNA Database of Japan (DDBJ).

- BLAST (Basic Local Alignment Search Tool) is a set of similarity search programs designed to explore all of the available sequence databases regardless of whether the query is protein or DNA.[13] It uses a heuristic algorithm, which seeks local as opposed to global alignments and is therefore able to detect relationships among sequences that share only isolated regions of similarity.

- ZSearch is a multiple sequence similarity search tool that performs similarity searches to compare query sequences against a database of sequences.[14] It makes use of the BLAST algorithm. Similarity searching is a key bioinformatics tool, enabling the identification of regions of similarity between sequences that may indicate a shared structure or function.

- There are three types of quality classes assigned to sequenced genomic DNA.[15] The first two classes are found in the EMBL High Throughput Genome (HTG) division, while the last class is moved to the Primary division (*Figure 2.1*). All first-time sequenced contigs (pieces of cloned genomic DNA) greater than 2 kb get an accession number and are deposited as Phase 1 sequence in HTG. This accession number never changes during the following progress. The contigs are at that point unordered, unoriented and contain gaps. As sequencing progresses the quality increases. Phase 2 contains ordered and oriented sequences that may contain gaps. Finished sequences with no gaps belong to Phase 3 and are found in the Primary divisions (Rodent division in the mouse genome case). Sequences in Phase 1 and 2 are also called "working draft sequences".

Figure 2.1 – *The orientation and relative size of contigs in the different classes of the sequenced genome*

In order to evaluate the possibility of an automated search for PPRE DR-1 sequences from an initial list of interesting proteins, it was important to first derive a method for finding such motifs. We used AstraZeneca's Electronic Laboratory (E-Lab)[16] and the following work flow to search for the DR-1 sequences:

1. The SWISS-PROT accession number is taken from an Excel sheet with a list of proteins that are to be examined.

2. The SWISS-PROT entry is found using E-Lab. (In most cases, the protein was from mouse, but could also be a rat or human homologue.)

3. On the entry's DR-line (Database Reference) there are one or many links to the corresponding genes' EMBL entries. We always choose the first one.

4. This EMBL entry is BLAST searched via ZSearch, against the EMBL divisions Rodents and High Throughput Genomes, to find genomic DNA that could contain a promoter region for the gene in question.

5. A list of hits is produced and sorted according to Percent Identity (percentage of matching nucleotides). Successful hits will be significantly longer than the query sequence since genomic DNA that contains a promoter region as well as the coding region is needed. The Query Percent (percentage of the total query sequence that was used in the match) is another important value to look at. Since BLAST is a local similarity search tool, a hit could match only one or a few of the exons within the gene, making the Query Percent less than 100. Even if the hit only matches the first exon, we can still go further upstream and look for the promoter region.

   If the hit sequence is from Phase 1 or 2 in HTG it is very important to be aware of the size, order and orientation of the contigs. Below is an example of an acceptable hit, although the query sequence is quite short.

Score = 77.8 bits (39), Expect = 5e-14
Identities = 39/39 (100%)
Strand = Plus / Minus

Query: 1 gaaagatggcaccagttgctggcaagaaggccaagaagg 39
||||||||||||||||||||||||||||||||||||||
Sbjct: 45710 gaaagatggcaccagttgctggcaagaaggccaagaagg 45672

Sbjct contig: 24863 - 66286: contig of 41424 bp in length

As can be seen in "Sbjct contig", the contig has an uninterrupted section between the start of the query gene and about 20 kb upstream. Thus, here is a proper location to search for the DR-1. However, with the unfinished genome we can not be sure how large the promoter region is, and where the previous gene ends. Therefore we scan 10 kb (10 000 nucleotides) ahead of the query match and assume that this covers most of the promoting region. The problem is in some cases more complex than this; for example, certain parts of a promoter region can exist hundreds of kb away from the gene. This is not something we can take into account.

If a hit meets the criteria Percent Id > 95% (errors in sequencing taken into account), Query Percent > 15%, and it contains genomic DNA about 10 kb (10.000 nucleotides) upstream of the gene in a single contig (without gaps) it is fine to proceed. A contig must thus contain both the beginning and the full promoter region of the gene to be valid for further searching. It is not possible to jump to the next contig because there is a gap of unknown size between the contigs.

6. When proper genomic DNA is found, ZSearch is used to search for the DR-1 motif. If a hit coincides well with the consensus DR-1 it is likely to be a PPRE.

2.3 Matchmaker

To design a program that would automate the linkage between proteomic and genomic expression, we needed to analyse the technologies behind the data to discover possibilities and pitfalls.

2.3.1 2D-PAGE analysis

The Proteomics group uses a program called PDQuest to analyse spot intensities from 2D-gels. As an example, *Figure 2.2* below shows 6 gels being matched in another study. The left two gels are from obese mice, the middle two from lean mice, and the right two from treated obese mice. After manual "landmarking" of a number of spots, the program attempts to match the spots on the different gels automatically. However, there is a lot of manual labour involved in checking that matches are correct and removing noise (spots that are artefacts rather than proteins). The histogram in the figure represents one spot. Each bar shows the intensity of that spot in one of the gels. In the example, the last six bars are the treated obese gels and it can be seen that the protein is strongly up-regulated.

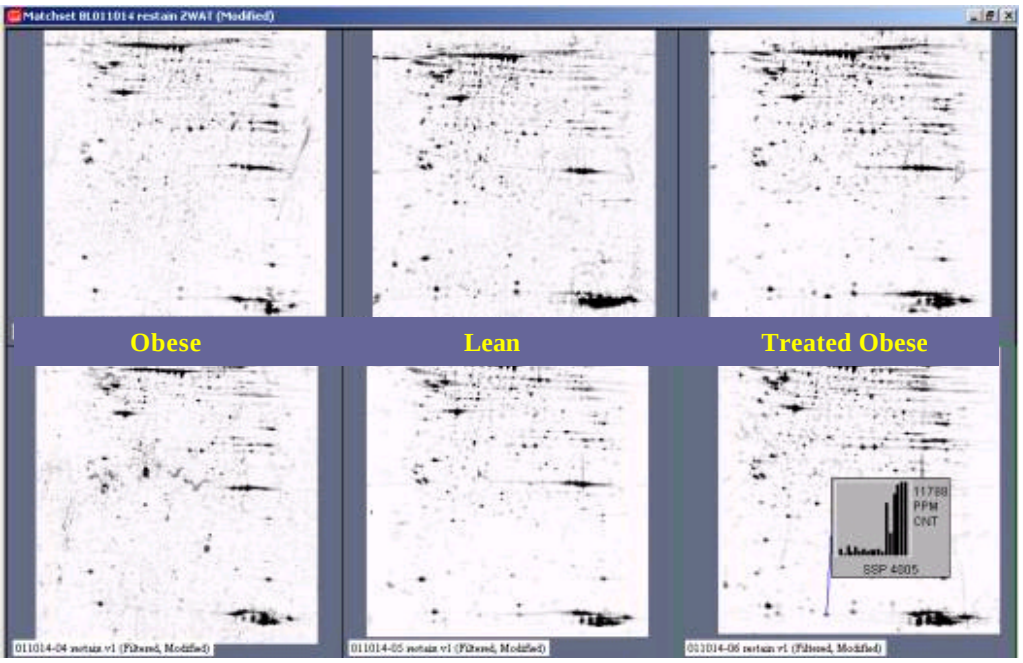

Figure 2.2 – *A PDQuest window where gels are being matched (Kindly provided by Boel Lanne)*

PDQuest has built-in statistical features, but the proteomics group instead uses an Excel macro. The macro is based on the assumption that the *logarithmic* intensity values are normally distributed, and can thus make use of Student's t-tests to calculate a P-value (see *Section 2.3.4*).

2.3.2 Affymetrix analysis

The Affymetrix system is built so that one DNA probe set is designed to detect one cRNA transcript.[17] A probe set usually consists of 16-20 probe pairs. A probe pair in turn consists of two probe cells, a perfect match (PM) and a mismatch (MM). The PM probes are designed to be complementary to a reference sequence. The MM probes are the same, except for a homomeric base mismatch at the central position (e.g. 13th of 25 base length probe array). These serve as a control for cross-hybridization.

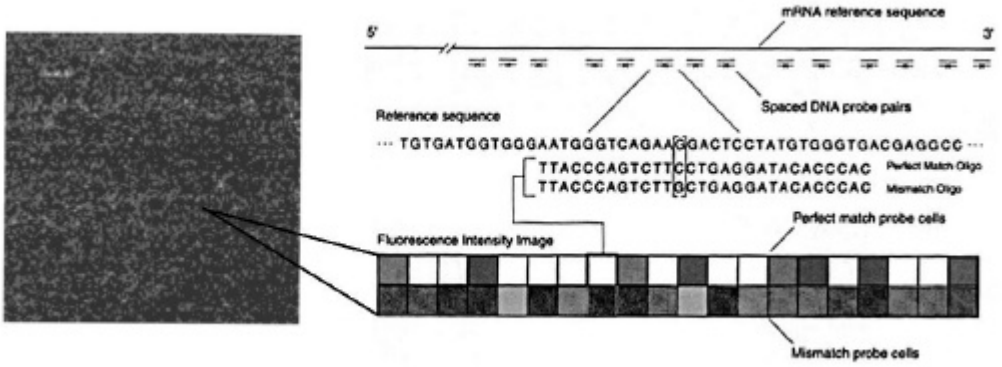

Figure 2.3 – *Affymetrix gene expression monitoring with oligonucleotide arrays. A single 1.28 x 1.28 cm array containing features smaller than 22 x 22 µm. Oligonucleotide probes are chosen based on uniqueness criteria and composition design rules. For eukaryotic organisms, probes are chosen typically from the 3´ end of the gene or transcript (nearer to the poly(A) tail) to reduce problems that may arise from the use of partially degraded mRNA. The use of the PM minus MM differences averaged across a set of probes greatly reduces the contribution of background and cross–hybridisation and increases the quantitative accuracy and reproducibility of the measurements.*[2]

Affymetrix uses a number of absolute analysis algorithms to compare the intensities of the PM and MM probe cells to determine if a transcript is present (P), marginal (M), or absent (A; undetected). This is called the Absolute Call. If, for example, the MM intensity is close to the PM intensity, cross-hybridization is frequent, producing a lot of noise that makes the PM intensity unreliable. When this is the case, the Affymetrix algorithms will tend to yield an A.

A metric that makes use of the probe cell intensities directly is the Average Difference. It is an average of the differences between every PM probe cell and its control MM probe cell. The Avg Diff is thus directly related to the level of expression of the transcript.

Affymetrix has designed a great number of additional metrics, but we have found Avg Diff and Abs Call to be the most important for our purposes, and have chosen to rely on them for further analysis. Together they allow for creation of an expression ratio, filtering of poor data, and calculation of confidence intervals.

The probe sets on a GeneChip will naturally be of varying quality after an experiment has been performed. Before matching the mRNA data with the protein data, it is preferable to sort out the poor quality values from the mRNA data so that we get reasonably reliable plots. We have done this by setting criteria on the Absolute Call: at least two thirds of the experiments in one of the two cases compared (e.g. treated or untreated) must be P or M for the probe set to be included. In other words, comparing treated and untreated with 3 experiments in each, we would accept values PPA/AAA and PPP/PPA, but reject PAA/PAA and AAA/AAA. We have decided to keep cases such as PPP/AAA and vice versa even though their Avg Diff ratios are unreliable, because they clearly imply an up- and down-regulation respectively.

The Avg Diff values in Affymetrix experiments can sometimes be negative. This implies that the average MM intensity is stronger than the average PM intensity for the probe set. An explanation for this could be an extreme form of cross-hybridization where other transcripts have lodged themselves on the MM probe. Another explanation could be that it is actually the MM probe that is correct, and the PM instead acts as the mismatch. In either case, the Avg Diff values can not be trusted. Essentially all negative values are labelled as A, so due to our criteria we get rid of most of the negative values. The negative values left are included in our calculations for statistical reasons. If they happen to make the entire average ratio negative, the probe set will be excluded from the comparison.

Affymetrix has included a certain number of probe sets that do not follow their standard selection rules. One example is an incomplete probe set, meaning that there are not as many probe cells as usual. Another example is when a probe set is not specific enough to detect a single gene, but rather a family of similar genes. We have decided to filter out these cases from the comparison, since they would not give reliable values.

2.3.3 Matching genes and proteins

An Affymetrix probe set is designed to represent a gene or EST, and every gene codes for at least one protein. Thus, we should in many cases be able to find a corresponding probe set on an Affymetrix chip for every protein identified in a proteomics experiment. If the experiments at the mRNA and protein levels respectively have been carried out identically or at least in a similar fashion, it should be possible to directly compare the expression ratios for the two levels. The advantage of using ratios in both cases is that it gives a relative measure of the change in expression rather than an absolute measure, and thus is better suited for a comparison.

Affymetrix supplies information on the reference gene/EST that each probe set represents. The next step involves the decision on how to match the gene or EST with a corresponding protein. The peptide masses from the mass spectrometry analysis are matched to a protein

database, and since this database primarily contains SWISS-PROT and TrEMBL entries, we have decided to build our program to cope with these.

To check whether a gene matches a protein, a BLAST search must be done. After studying the EMBL and SWISS-PROT/TrEMBL databases, we came across the fact that these databases already are cross-referenced with each other. In the EMBL gene entry, the db_xref row in an entry's Feature Table section has a link to a SWISS-PROT /TrEMBL protein accession number if the criteria are good enough. In the case of an EMBL EST entry, there often exists a link in the Description (DE) row directly to protein, or to a gene which we can further link to a protein. The above described method (without BLAST) is time saving and easy to follow.

The use of the accession number in the linking is due to it being the "most unique" identifier. Both the ID and AC rows are supposed to act as "unique" identifiers. However, the ID can change due to its inherent construction. It is built up using an alphanumeric code (X_Y) that is supposed to reflect the protein name and the species it comes from. "X" is a mnemonic code of at most 4 alphanumeric characters representing the protein name (e.g. INS for Insulin). "Y" is a mnemonic species identification code of at most 5 alphanumeric characters representing the biological source of the protein. This code is generally made of the first three letters of the genus and the first two letters of the species. If a protein is suddenly found to belong to a different class or needs a new name, the ID can change.

"Accession numbers are the primary means of identifying sequences and provide a stable way of identifying entries from release to release. For reasons of consistency it sometimes required to change the entry name (ID) between releases (e.g. to ensure that related entries have similar names). An accession number, however, always remains in the accession number list of the latest version of the entry in which it first appeared. Accession numbers allow unambiguous citation of database entries. Researchers who wish to cite entries in their publications should always cite the first accession number in the list (the 'primary' accession number) to ensure that readers can find the relevant data in a subsequent release. Readers wishing to find the data thus cited must look at all the accession numbers in each entry's list. Secondary accession numbers allow tracking of data when entries are merged or split. For example, when two entries are merged into one, a new 'primary' accession number goes at the start of the list, and those from the merged entries are added after this one as 'secondary' numbers."[18]

With this in mind, linking genes and proteins by accession number is a safe method as long as we make sure to check the entire row of accession numbers, not just the primary one. In *Appendix C*, an example of an EMBL gene entry and its related SWISS-PROT protein entry is shown.

2.3.4 Statistical considerations

To understand the underlying complications and limitations of the proteomics and genomics technologies, some statistics is necessary. Of course to do a proper statistical comparative study, experiments at both protein and mRNA level would have to be carried out in exactly the same way. It would be ideal to use the same tissue, the same number of animals, the same substance concentration, and so on. The primary studies we have to work with do not entirely meet up to these criteria. However, our goal has been to get a statistical feel for the technologies and also to come with suggestions on how to make future comparisons more statistically significant.

The P-values used by Proteomics give a picture of whether a protein's expression can be said to be significantly changed. However, the P-value does not take into account the chance occurrences of certain values that are likely to be present the more values we have. To then get a proper idea of significance, an adjusted P-value should be used (see *Appendix D*).

The confidence interval is a good measure to get an idea of the variance in a number of samples. The 95% confidence interval, commonly used, gives us a region in which we could find the point with 95% certainty given our experimental data. We have calculated confidence intervals for both the proteomic and genomic variances and used somewhat different formulas for the two levels (see *Appendix D*). Considering that the intensities are skewed, we have assumed a log-normal distribution in the protein case. This method is not possible for the Affymetrix intensity values (Avg Diff), because these can in certain cases be negative. Instead we have used an approximation called Fieller's Theorem.[19]
When the confidence intervals have been worked out, we have chosen to plot them along with the average intensity values in log scale (those points with negative average genomic intensities are filtered out prior to this). Log scale is more appropriate considering the span in intensity values and also places points at the origin when expression is unchanged on both the proteomic and genomic levels.

Affymetrix has, as described in a *Section 2.3.2*, several of their own statistical metrics for their data. They do not, however, give a detailed explanation of the underlying statistics, and can thus be hard to rely upon.

Worth mentioning is that we have not taken into account the inter-chip variation. This is the variation within a single probe set, between the PM and MM values (see *Section 2.3.2*). Knowledge of the intra-chip variation can affect the confidence interval in both directions, but we did not consider it necessary to take into consideration for our purposes.

3. Program Design

This chapter discusses the design of our program Matchmaker. A section on usability is followed by a description of the program's functional and technical structure.

3.1 Usability

The usability aspect of our program was of primary importance. We felt the need to create a clear, concise, attractive, and informative web interface to make the usage of the program simple and pleasant.

3.1.1 User analysis

Matchmaker is intended for use primarily by the proteomics team (cell biologists). Molecular biologists can also make use of the program. The users are not expected to have any programming or UNIX experience, and thus a web interface is used and kept as simple and clear as possible. The users have a good knowledge of the underlying biology and at least a basic knowledge of both expression techniques, so these do not need to be explained in the program.

3.1.2 System design

WEB INTERFACE

In the first stage of the program, the user must select the two studies to compare. The genomics data is stored in databases. Thus, it was felt that building an invisible database interface which would allow the user to select a study from the database list was the best option.

Certain parts of a proteomics study are stored in a database, but the intensity values we needed are not. Instead, this information is handled in Excel sheets. Since it was beyond the scope of the project to expand the proteomics database to contain this data, we decided to make use of a text area that the user can paste the data into. Copying from the Excel sheet to the web page text area is easy and intuitive for the user. The user has to order the columns of the sheet in a specific way so that the program understands the input. See *Section 3.4* and *Appendix A* for more detail.

VISUALIZATION

For the visualization, we wanted to be able to make 1D bar plots with error bars (the confidence intervals) and 2D scatter plots with large flexibility in viewing the data. We found Spotfire and Excel had these capabilities and were commonly used by our user group. We decided to incorporate these two applications into the design of our program, with the benefits of the power of the applications and their familiarity and accessibility within the user group. Excel has good functionality with bar plots, allowing the user to easily create these plots with the error bars using the values from the program results. Spotfire is a powerful visualization tool. With the use of its Application Programming Interface (API), we were able to program certain settings so that a scatter plot opens in the correct way with our result data at the click of a button. All the result data is imported into Spotfire (not only the x- and y- values) so that the user has the ability to view extensive information about points in the graph. The user also has the ability to modify the plot in a number of ways throughout the analysis.

3.2 Functional structure

The following figure shows our design in functional terms.

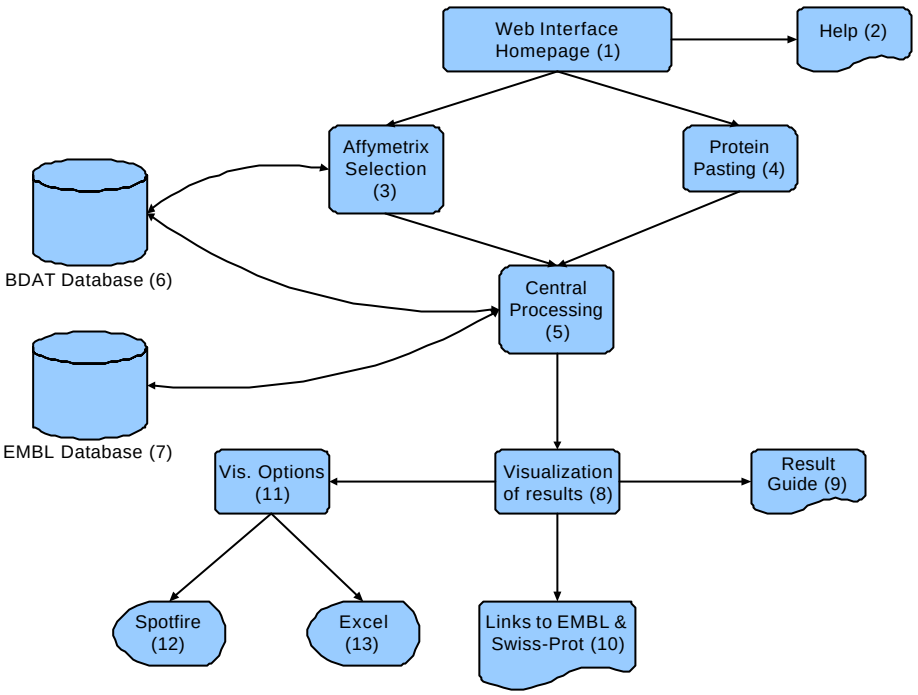

Figure 3.1 – *Matchmaker's functional structure from a user's perspective*

1. Homepage
The homepage is the user's first view of the program Matchmaker. From here the user makes his/her selections and can view the help section.

2. Help
This is a help section that describes what Matchmaker is capable of and a step-by-step guide in using the program. The help section is embedded in the homepage, avoiding unnecessary extra windows.

3. Affymetrix selection
The user must select which Affymetrix study to use in the comparison from the drop-down menus.

4. Protein pasting
The user must also decide which protein study to use in the comparison. The data from this study is pasted into a text area.

5. Central Processing
This is the core of the program where the proteomics and genomics studies are matched and the results organized for subsequent presentation.

6. BDAT Database
BDAT stands for the Biological Data Analysis Team. After conducting an experiment, the researchers working with genomics data extract the most useful information from the Affymetrix databases and store it in the BDAT database.

7. EMBL Database
The protein-gene/EST links are found in EMBL entries.

8. Visualization of results
The results are shown in a table on the web page. There is a choice of further visualization options in Spotfire and Excel. There are also hypertext links from each accession number to AstraZeneca's Electronic Laboratory (E-Lab), where AstraZeneca locally stores their version of a number of public databases.

9. Result Guide
The result guide helps the user to understand the results and continue with further analysis.

10. Links to E-Lab
Each EMBL, SWISS-PROT or TrEMBL accession number has a hypertext link to the database entry in E-lab.

11. Visualization Options
The visualization options in Excel or Spotfire are activated by pressing on the appropriate button.

12. Spotfire
Spotfire.net Desktop 5.1 plots protein log-ratio against gene log-ratio. It is a powerful tool for further graphical analysis.

13. Excel
Microsoft Excel 2000 is useful for viewing the data and adding/making adjustments. It is also useful for creating bar graphs with error bars.

3.3 Technical structure

Matchmaker is built on a Perl platform.[20,21] The web interface is in HTML and CGI scripts enable selection and forms.[22,23] Perl DBI allows connection to an Oracle database and SQL commands extract data from the Oracle database.[24,25] SRS commands allow for connection to the EMBL and SWISS-PROT/TrEMBL databases. The API scripts for accessing Spotfire and Excel are written in VBScript.

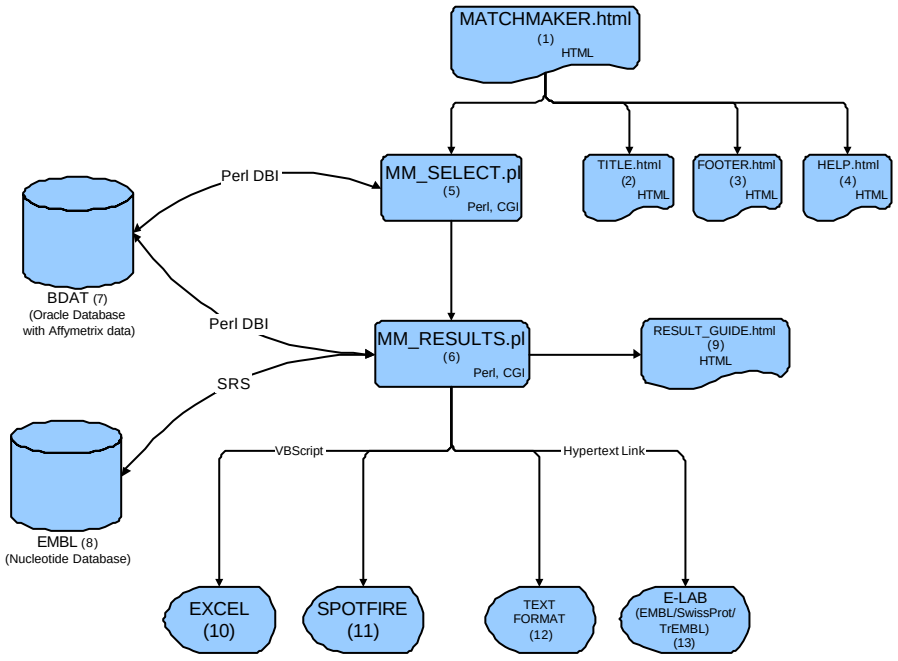

Figure 3.2 – *Matchmaker's technical structure*

1. MM_FRAMES.html
An HTML file that defines the four frames of Matchmaker's homepage.

2. TITLE.html
An HTML file that creates the title frame.

3. FOOTER.html
An HTML file that creates the footer frame.

4. HELP.html
An HTML file that creates the help frame.

5. MM_SELECT.pl
A Perl CGI and HTML file that controls the selection of genomics data. The connection with the Oracle BDAT database is controlled using the Perl database interface (DBI). The choices selected, as well as the protein data pasted into the text area, are saved as parameters that are sent on to MM_RESULTS.pl.

6. MM_RESULTS.pl
A Perl and HTML file that matches the two data sets.

7. BDAT database
A denormalized Oracle database with Affymetrix data. The bioinformatics group has extracted some of the more useful Affymetrix data into this database. BDAT table columns include probe set name, time point, tissue, Avg Diff, Abs Call, and individual. The probe set name has to be linked with another table that has the matching EMBL accession number for each probe set.

8. EMBL database
Entries for all publicly known genes and ESTs are stored in this database.

9. RESULT_GUIDE.html
An HTML file that guides the user through the results with tips on how to analyse them.

10. E-Lab links
The accession numbers have hypertext links to E-lab, where the specific gene, EST or protein entry can be studied in more detail.

11. Excel
The link to Excel is written in VBScript. It imports the data into an Excel sheet.

12. Spotfire
The link to Spotfire is also written in VBScript. It imports the data into a scatter plot. Additional features using Spotfire's API make sure that the axes are correct and that the points are coloured by protein, and adjust the label density.

13. Text format
A link to the data in tabbed text format. This option is mainly available should the other options fail.

3.4 User interface

The user's first impression of the application is of great importance. Matchmaker's homepage is designed to be clear, simple, and informative (*Figure 3.3*). The initial web page is built in four frames. The program logo is in the top frame, the program in the left frame, and the help section in the right frame. At the bottom there is a frame with creator information and links.

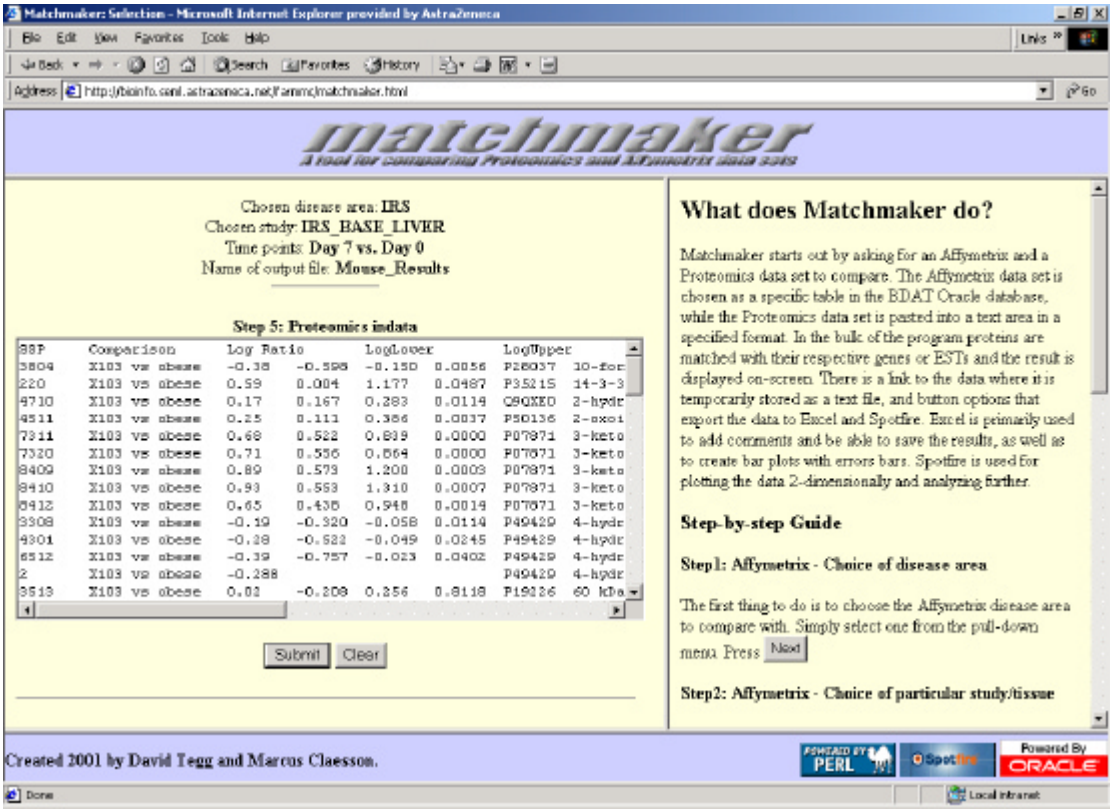

Figure 3.3 – *Matchmaker's selection page. Here the Affymetrix study has been chosen and the proteomics data pasted in.*

We have chosen to build the help section into the initial page for two main reasons: the selection frame does not need the entire width of the page, and having the help section nearby saves the user opening a new screen. The help section with a step-by-step guide through the selection process can be found in *Appendix A*.

The results page (*Figure 3.4*) pops up when the user has submitted the selections and the program has matched the genomic and proteomic data. On this page there is a link to a Results Guide (see *Appendix A*). The guide explains the table columns as well as the visualization possibilities. The guide would not fit on the same page as the results, because of the size of the results table. We have chosen to make the Results Guide link open a new web browser window so that the results page remains intact and can be viewed simultaneously.

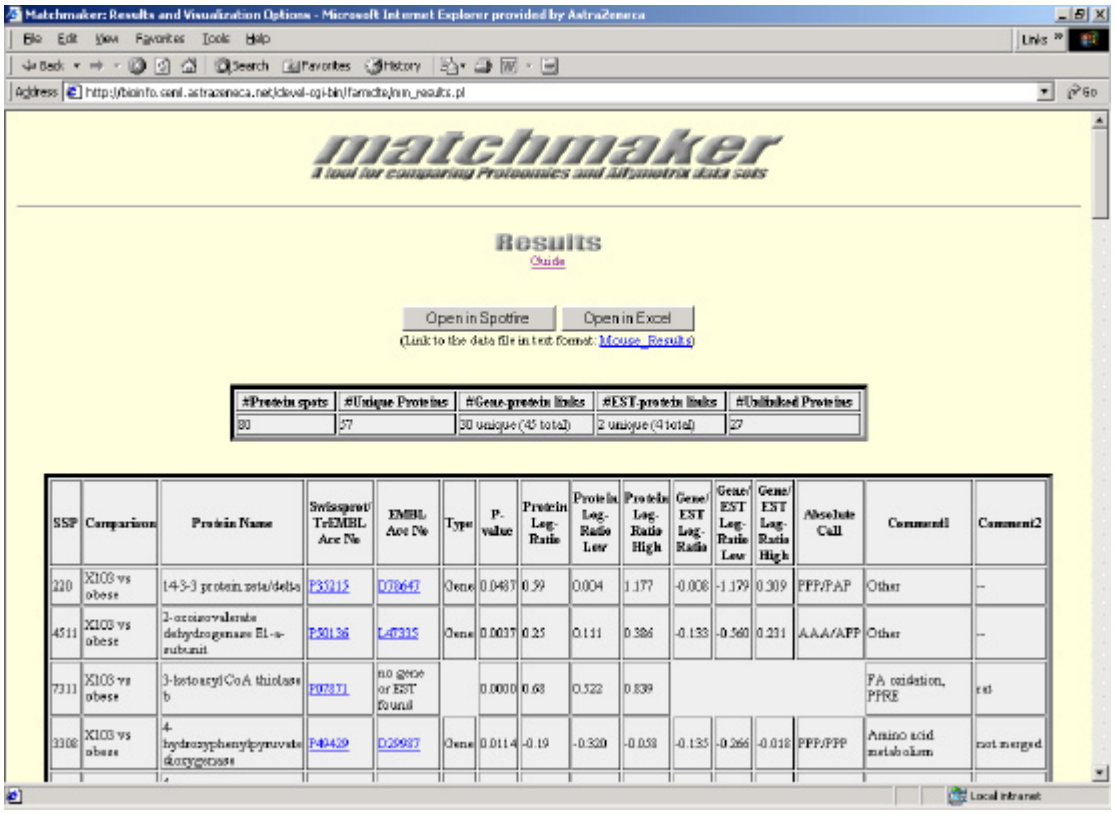

Figure 3.4 – *The results page shows the results in a table and contains the buttons for export to Excel and Spotfire*

From the results page, the data can be exported to Excel and/or Spotfire at the click of a button. The large table contains all the protein spots that have been entered into the program and the accompanying data, as well as genomic data if a match has been found.

4. Results

4.1 PPRE

We examined the same proteins that were identified in the study described in Section *2.1*. In most cases there were no acceptable hits against genomic sequences, since either the Percent Id was too low or a long enough sequence of genomic DNA could not be found. When a hit indeed was found it was usually a Phase 1 HTG sequence with too many gaps in the wrong places, i.e. a continuous sequence long enough to hold a promoter region did not exist. Consequently, no DR-1 motifs could be found in these proteins using the search method mentioned above.

4.2 Matchmaker

After applying Matchmaker on the rosiglitazone study data sets, several proteins could be linked to genes or ESTs. Of the 59 unique proteins from 86 different 2D gel spots, links to 30 genes and 2 ESTs were found. Thus, about half of the proteins could not be assigned to a gene or an EST using Matchmaker's algorithm. We have found three distinct reasons for this:

1. A corresponding gene or EST *was* found on the GeneChip, but the criteria stated in *Section 2.3.2* had not been fulfilled since the proportion of Absent transcripts (A:s rather than P:s) was unacceptable or the probe sets were not reliable in some other way (7% of the non-linked proteins).

2. The protein is a mouse protein, whose corresponding gene did not yet have a transcript on this GeneChip Mu6k - version (55%). However, it is probable that these genes will exist on later chip versions. For example, we found three of these transcripts in the newer Mu11k (11 000 genes/ESTs) chip.

3. The protein is not a mouse protein, but from another organism such as rat or human. Mass spectrometry could not assign the protein to an entry in the mouse database and therefore a homologue from a different organism with a good hit was chosen instead (28% rats and 10% human).

To visualize the gene/EST – protein links that were found, we used Matchmaker's built-in function buttons to transfer the result data to Spotfire and Microsoft Excel. Spotfire provides various ways to plot the "Protein Log-Ratios" against the "Gene/EST Log-Ratios", a couple of which can be seen in *Appendix B, Graphs 1-2 (B.1-B.2)*. However, visualizing the confidence intervals was very complicated since an adequate tool does not yet exist in Spotfire. It also proved to give messy and almost unreadable plots. Instead we plotted confidence intervals in Excel, where they could easily be added using the error bar function in a 1D bar diagram, with expression values from the proteins and their corresponding genes plotted next to each other. *Graph B.3* shows all genes and proteins, where spots from five proteins have been merged.

To reveal expression similarities in different groups of proteins/genes the diagrams were divided according to protein classes. *Graphs B.4-B.5* show similar behaviours in the groups "Amino acid metabolism" and "Proven or presumed PPREs".

Confidence intervals for eight genes were not calculated, since the statistical criteria in *Section 2.3.4* were not fulfilled. The only proteins without confidence intervals were the merged protein spots, "merged" implying an average over all spots matched to the same protein. Since there are dependencies between spots belonging to the same proteins, our method for calculating confidence intervals is not adequate for the merged protein spots.

5. Discussion

5.1 PPRE

The main reason for not finding any DR-1 motifs was that the mouse genome is still incomplete. When a genomic sequence region was found it was usually divided into unordered contigs, which made the search for a promoter region impossible or at least very difficult. In order to produce a fully automated DR-1 search tool, a search method had to be derived on the basis of a test of a small number of proteins with known PPREs. Since the DR-1 motif was not found even for these proteins, automation was not considered.

Currently the EMBL database contains very little genomic DNA. No valid hits were generated starting from the proteins that were used in this study. To find any DR-1 regions in the public genomic material that is present today, much handiwork as well as biological knowledge and experience is needed.

A DR-1 search will most likely become easier in the future. The sequencing of the mouse genome will proceed and the genome databases will be continuously updated. As of October the 9th 2001, only 13.2% of the mouse genome exists as a working draft sequence and only 1.7% has been fully sequenced.[26] The working draft sequence of the mouse genome is planned to be finished 2003, and the fully completed genome 2005. A complete and annotated version of the mouse genome was recently made available from the genomic company Celera. This sequence data is, however, only commercially available.

5.2 Matchmaker

CORRELATION

As can be seen in *Graph B.1* expression levels for both protein and mRNA seem to be moderately correlated, with R ˜ 0.5. This correlation coefficient suggests that mRNA and protein levels are to some degree connected, but that they in certain cases are regulated by more complicated mechanisms. A number of strongly up-regulated proteins with documented PPRE regions in their complementary genes could not be matched in our program because they were rat proteins. Had these been matched, they would most likely have increased the correlation coefficient.

Nevertheless, a clear up- or down-regulation on both levels strengthens experimental results. In addition, a direct correlation would theoretically suggest the possibility of using the gene rather than the protein in pharmaceutical drug targeting. Knowledge of correlation can thus be useful both in proteomics and genomics research.

REASONS FOR POOR CORRELATION

Even though it is natural to expect a correlation between mRNA and protein levels, there are reasons why this is not always the case. There are known alterations that can occur in the DNA>RNA>protein mechanism and that need to be considered. *Post-transcriptional* changes refer to either degradation of mRNA or changes of the translational efficiency, i.e. the efficiency by which mRNA is translated to proteins. *Post-translational* changes refer to degradation or modifications of proteins.

Below are descriptions and possible explanations of drug effects and exceptions from the "mRNA-yields-protein" relation:

1. mRNA level unchanged, protein level up/down: The translational efficiency has changed, which renders more or less protein from the same amount of mRNA (post-transcriptional). The protein is modified or degraded soon after translation (post-translational).

2. mRNA up/down, protein unchanged: Short lived mRNA does not have enough time to produce sufficient amounts of detectable proteins (post-transcriptional). The protein is produced, but is soon degraded or modified (post-translational).

3. mRNA up, protein down or vice versa: More of mRNA is produced but the translational efficiency is reduced even more or vice versa (post-transcriptional *and* post-translational).

INDIRECT PPAR REGULATION

One explanation for up-regulation of genes, without PPREs in their promoter regions, is that they can be indirectly influenced by "PPRE genes". A drug ligand bound to PPAR, activating a PPRE and inducing transcription could result in a gene product that is part of a different gene regulating protein complex. The activation of a new promoter sequence, without PPRE, would then lead to increased levels of other mRNA and ultimately to the production of other proteins. Thus, there is a complicated network of "cause and effect", which is far from wholly understood.

FUNCTIONAL CATEGORIES

When studying expression levels of different functional categories, clear tendencies in especially two categories are evident. Treating obese mice with rosiglitazone shows that genes and proteins involved in amino acid metabolism are down-regulated on both levels (*Graph B.4*). This effect has recently been shown and published.[27] The indication is that PPARα is a key controller of intermediary metabolism during fasting. *Graph B.5* indicates that genes with proven or possible PPREs are up-regulated, although their corresponding proteins are generally not as positively affected. Apparently there have been alterations in the mRNA to protein chain.

Dividing and visualizing proteins according to functional categories can support thoughts about which category non-classified proteins belong to. Points in a certain region of the plot may have similar function. Thus, if a non-classified protein shows a similar expression profile to a classified group of proteins, it may also belong to that group.

STATISTICAL COMMENTS

Regarding the statistical significance of the result data a few things need to be mentioned. About 25% of the genes did not fulfil the statistical criteria for calculating reasonable confidence intervals (see *Appendix D*). In addition, many of the calculated confidence intervals were very large (see *Graph B.3*). These values reflect the limited reliability of the Affymetrix microarray technique. In general, the confidence intervals for the protein expression levels were not as wide as for the gene expression levels. The proteomics team have done certain experiments to test the variance of the 2D-PAGE method. They have come to the conclusion that the method's coefficient of variance (CV, standard deviation/mean) is around 20%. Similar experiments have been done with Affymetrix, but there the results showed that the CV increased with decreasing intensity.[28] For the majority of the intensity values, CV was between 10% and 100%. The variance is clearly larger than in the proteomics case.

In many cases with very wide or unreliable intervals there has been one specific mouse whose mRNA expression value (Avg. Diff.) differs significantly from the others. Since there were only three mice in each group, each individual has a large impact on the intervals. No matter

how accurate measurements will get, they will always reflect individual variance. Therefore, it is important to conduct experiments with several individuals to acquire more statistically significant results.

VISUALIZATION POSSIBILITIES

Through Matchmaker the user can export the result data to both Spotfire and Microsoft Excel, which give excellent graphical representations when combined. By analysing the data from the rosiglitazone study using Matchmaker we had the opportunity to explore suitable ways for visualizing the results. An advantage with Spotfire is that every column in the original data table can be used and displayed in the scatter plot itself, the "Query Devices" window and/or the "Details-on-Demand" window. Size, shape and colour of the markers in the plot can all represent different features (columns), simulating additional dimensions. New columns can also be created by calculations or by binning (organizing data into "bins") old columns. These features allow the user to filter the data visually in ways that can highlight areas of interest.

In Spotfire we coloured the markers according to protein accession numbers and used different shapes for gene and EST transcripts. By binning the P-values in three groups (<0.05;0.05-0.10;>0.10) and making check boxes of the groups it is easy to distinguish proteins that are not changed significantly. The functional classes of the proteins were denoted in "Comment1" and check boxes allowed for the choice of which protein classes to be displayed. "Comment2" contained the reasons for why gene links to certain proteins were not found, or whether a protein was merged or not. We have chosen to leave an unmerged alternative, since there can be multiple reasons behind why the same protein has been identified on many gel spots. Splitting could for example be due to natural degradation or induced by the 2D-PAGE method.

As mentioned earlier, Spotfire is not yet suitable for visualizing confidence intervals, especially when there are lot of markers. Excel, however, has a well developed functionality for error bars, which can be used for confidence intervals in this case. Also here gene/protein bars can be ordered into functional categories or any other suitable way.

A combination of scatter plots in Spotfire and bar diagrams in Excel creates a complete method for visualizing the result data.

TECHNICAL LIMITATIONS

The Affymetrix technology will soon have the capability to fit essentially all of a mouse's genes on a GeneChip. The most recent chip fits 40,000 human genes, but has compromised accuracy by reducing the number of probe cells for each probe set. The biggest problem in the case of the mouse genome is that all genes have not yet been publicly sequenced.

The 2D-gel technique has limitations in the number of proteins that can be detected. A dilemma exists between efficient protein quantification and detectability of proteins with a very low concentration. Also, the proteome has not been fully established.

HOW PROTEOMICS CAN BENEFIT FROM MATCHMAKER

Due to for example the statistical reasons mentioned above, the simultaneous expression levels from proteomics and genomics experiments should not be blindly trusted. They may, however, give useful indications, which can be more thoroughly investigated by examining the raw data from the conducted experiments.

Global and integrated analyses are also important when investigating regulation and interconnections within and between metabolic pathways in cells. In addition, Matchmaker can be powerful when used as a verification of results in literature.

The comparative analysis has its greatest effect when using studies with the exact same set-up. However, it can also be informative to compare two studies that are somewhat different, but where the researcher for example knows that similar functions are affected in the body. These could be used as more of a rough guide to check whether the genomic and proteomic regulation are affected similarly.

5.3 Matchmaker in the future

In the rosiglitazone study neither the same tissue samples nor drug concentrations were used in the two different experiments. It is naturally important for the biological relevance to have the same conditions in both experiments in the future. Therefore, to be able to use Matchmaker more precisely and with maximum benefit, coordinated studies must be strived for.

If MS identification fails to identify a protein, a homologue from another organism is used if the hit is good enough. However, Matchmaker can not match protein and genes from different organisms. If a match still is desirable, the user has the choice of BLAST searching for a hit with a worse score but from the correct organism. The new hit is probably not the correct protein, but could be from the same family or at least have a similar function and therefore be useful in further analysis.

Matchmaker will become even more useful in the future because:

1. More genomics and proteomics experiments will be coordinated.
2. The public gene and protein databases as well as the MS peptide database will grow.
3. The precision of the Affymetrix and 2D-PAGE/MS technologies is likely to improve.
4. More genes will fit on a chip, and ESTs will be replaced by genes.

We have thought of several developmental steps for Matchmaker that do not fit within the scope of this thesis but that can be considered in the future.

- Matchmaker can be more closely intertwined with the local information system NEXIS (Next generation proteomics and EXpression Analysis Information System).
- Links and cross-references to a variety of databases can be added, such as PDB (Protein Data Bank) and Enzyme (Enzyme data bank).
- If all proteomics data were inserted into AstraZeneca Mölndal's Proteome Study database (PS), Matchmaker could offer a selection system for this data in the same way that it does for the genomics data. Pasting into the text area would then be unnecessary.

5.4 Concluding remarks

- Automating the process of finding PPRE motifs in the mouse genome proved not to be feasible, primarily due to incompleteness of genomic mouse DNA.

- Using Matchmaker on the comparison of obese mice with and without rosiglitazone treatment showed that protein and gene expression levels were moderately correlated. In certain cases this implies alterations in the "DNA to protein" process. In addition, a number of expected trends were confirmed.

- Matchmaker's automated matching of gene and protein expression allows for quick and easy comparative analyses of large data sets, making broader perspectives possible. Analysis of the results will lead to new and useful hypotheses.

- Integrated analysis of expression levels is important for the understanding of systems biology, and will play an increasing role when more experiments become coordinated, expression technologies are refined and sequence databases grow.

- Matchmaker is a first step in making use of genomics and proteomics data simultaneously. It has highlighted the potential benefits of such a comparison and will lead the way for more such applications in the future.

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Appendix A - User Documentation

A.1 Introduction

WHAT DOES MATCHMAKER DO?

Matchmaker starts out by asking for an Affymetrix and a proteomics data set to compare. The Affymetrix data set is chosen as a specific table in the BDAT Oracle database, while the Proteomics data set is pasted into a text area in a specified format. In the bulk of the program proteins are matched with their respective genes or ESTs and the result is displayed on-screen. There is a link to the data where it is temporarily stored as a text file, and button options that export the data to Excel and Spotfire. Excel is primarily used to add comments and be able to save the results, as well as to create bar plots with errors bars. Spotfire is used for plotting the data 2-dimensionally and analyzing further.

TECHNICAL DESCRIPTION

Matchmaker consists of a web page (HTML) where selections of genomics and proteomics data are made, and a web page with results and visualization options. Perl CGI scripts run in the background to insure interactivity, and also to match the data sets. The connections to the Oracle databases are handled using SQL commands with the Perl Database Interface (DBI), and SRS commands allow for connection to the EMBL and SWISS-PROT/TrEMBL databases. The Excel and Spotfire visualization buttons are programmed with VBScript.

LOCATION

Matchmaker is available on AstraZeneca Mölndal's intranet at the address (URL):

http://bioinfo.seml.astrazeneca.net/farmmc/matchmaker.html

A.2 System requirements

The program is designed to run faultlessly in the Topaz environment, AstraZeneca's global Windows 2000 platform. Topaz has Internet Explorer 5.0.

The program relies on the bioinformatics group's BDAT server to be available and kept standardised. The program also relies on the availability of the local SRS system for accessing EMBL, SWISS-PROT, and TrEMBL entries.

For the visualization options in Excel and Spotfire respectively, these applications must be installed on the computer.

A.3 Step-by-step guide

This is a step-by-step guide to the selection process.

Step1: Affymetrix - Choice of disease area
The first thing to do is to choose the Affymetrix disease area to compare with. Simply select one from the pull-down menu.
Press "Next"

Step2: Affymetrix - Choice of particular study/tissue

Choose the Affymetrix study to compare with. Simply select one from the pull-down menu.
Press “Next”
Step3: Affymetrix - Choice of the time point ratio
Choose two treatment days to produce a ratio. For example, "treated day 7 vs. untreated" would mean choosing 7 for time point 1 and 0 for time point 2.
Usually you will be wanting to compare studies of exactly the same type in Affymetrix and proteomics. Thus, make sure that also the ratios are inserted into the program in the same way (e.g. treated vs. untreated).
Press “Next”

Step4: Name of output file
Write in a name for your result file. The results will be temporarily stored in this name on a UNIX disk. To safely keep the results for future use, *please save them* on your own disk afterwards.
Press “Next”

Step5: Proteomics - Insertion of data
Now it's time to insert the proteomics data. The data has to be in a specified format to be properly understood by the program.

The file can be created in Excel (or saved as a text file) and then copied and pasted into the text area on the web page.

Important:

- Make sure that all 10 column headings are entered even if they don’t contain any data. Also, make sure that the columns are in the correct order and that the heading of the first column is "SSP".
- Make sure there are no line feeds anywhere within a column (do not press enter or tab when typing in a cell).
- The program requires the *logged* average intensities.

Finally, press “Submit”. The program will now start processing the input. It could take several minutes for the results to appear.

A.4 Result guide

This guide describes the results and the options for visualization of the results.

The results are shown in a large table (see Column Description below). There is also a small table with the following information:

- # of protein spots: Tells us how many different protein spots were pasted into the text area.
- # of unique proteins: Several spots can be the same protein, so the number of unique proteins is less than the number of spots.
- # of gene-protein links: The number of unique gene-protein links. The number in parentheses refers to the spots for each protein being separately counted.
- # of EST-protein links: The number of unique EST-protein links. The number in parentheses refers to the spots for each protein being separately counted.

Besides the two tables in the browser, you have three more alternatives for viewing the results (all at the top of the page):

- The first button opens an Excel sheet with the data. Excel is primarily used to add comments and be able to save the results, as well as to create bar plots with errors bars. Please save your data in Excel onto your own M-drive!
- The second button opens Spotfire and plots the data immediately (Protein log-ratio vs. Gene/EST log-ratio). This 2-dimensional plot can then be manipulated in many ways for further analysis.

You can save your Spotfire plot in two ways:

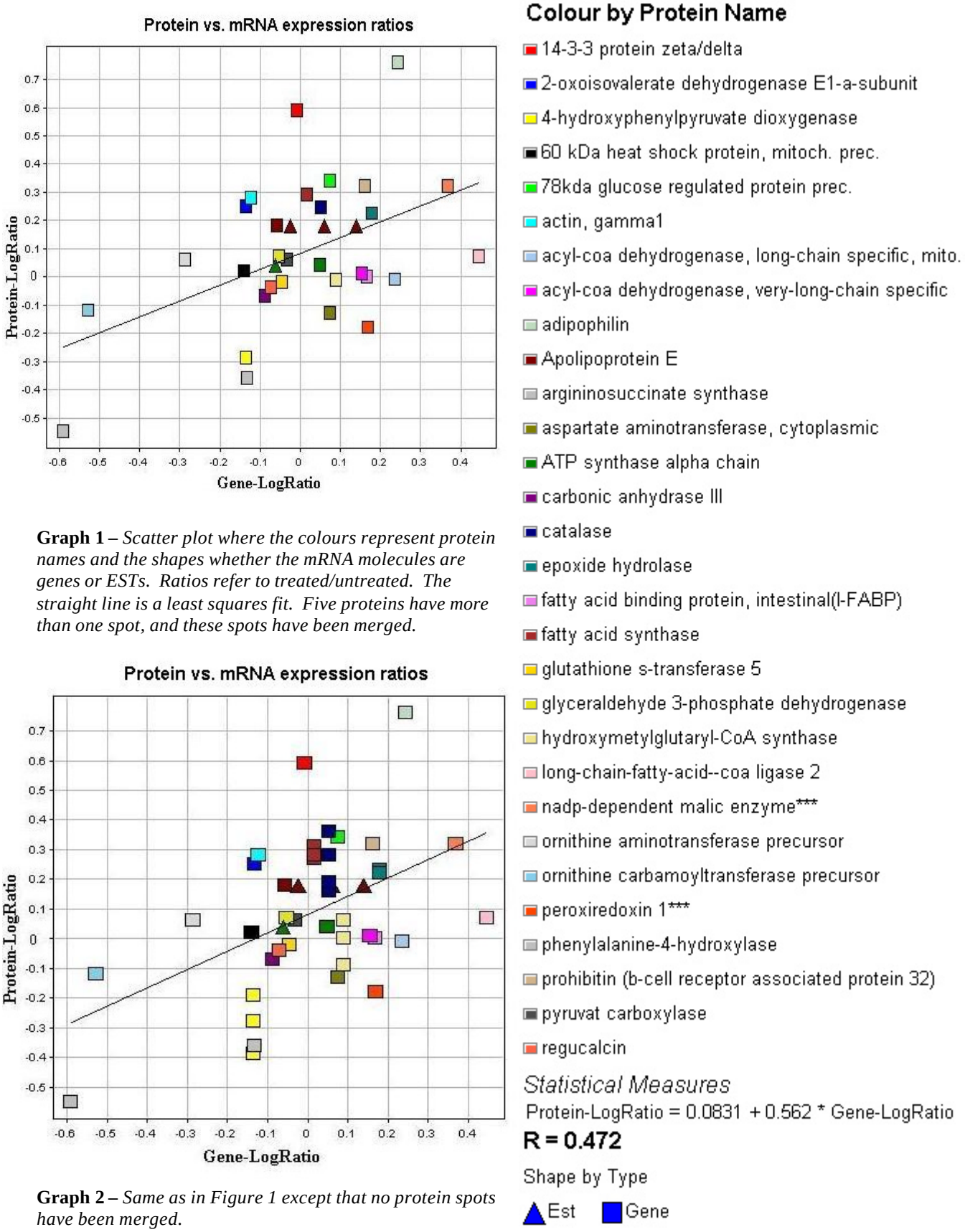
- Saving as a Spotfire Analysis File (*.sfs) will save the plot and the data.
- Saving as a Spotfire Template File (*.sft) will only save the adjustments you have made to the plot. This is a good alternative if, after making adjustments, you would like to add some more information to the data (e.g. in Excel) and then reinsert the data into Spotfire.

The third alternative is primarily for use if the first option for some reason doesn't work. You can click on the link with the name of your output file, and it is then shown as a tabbed text file in the web browser. From here you can save the file by choosing "File/Save As" in the web browser.

Column description
The large result table has several columns:

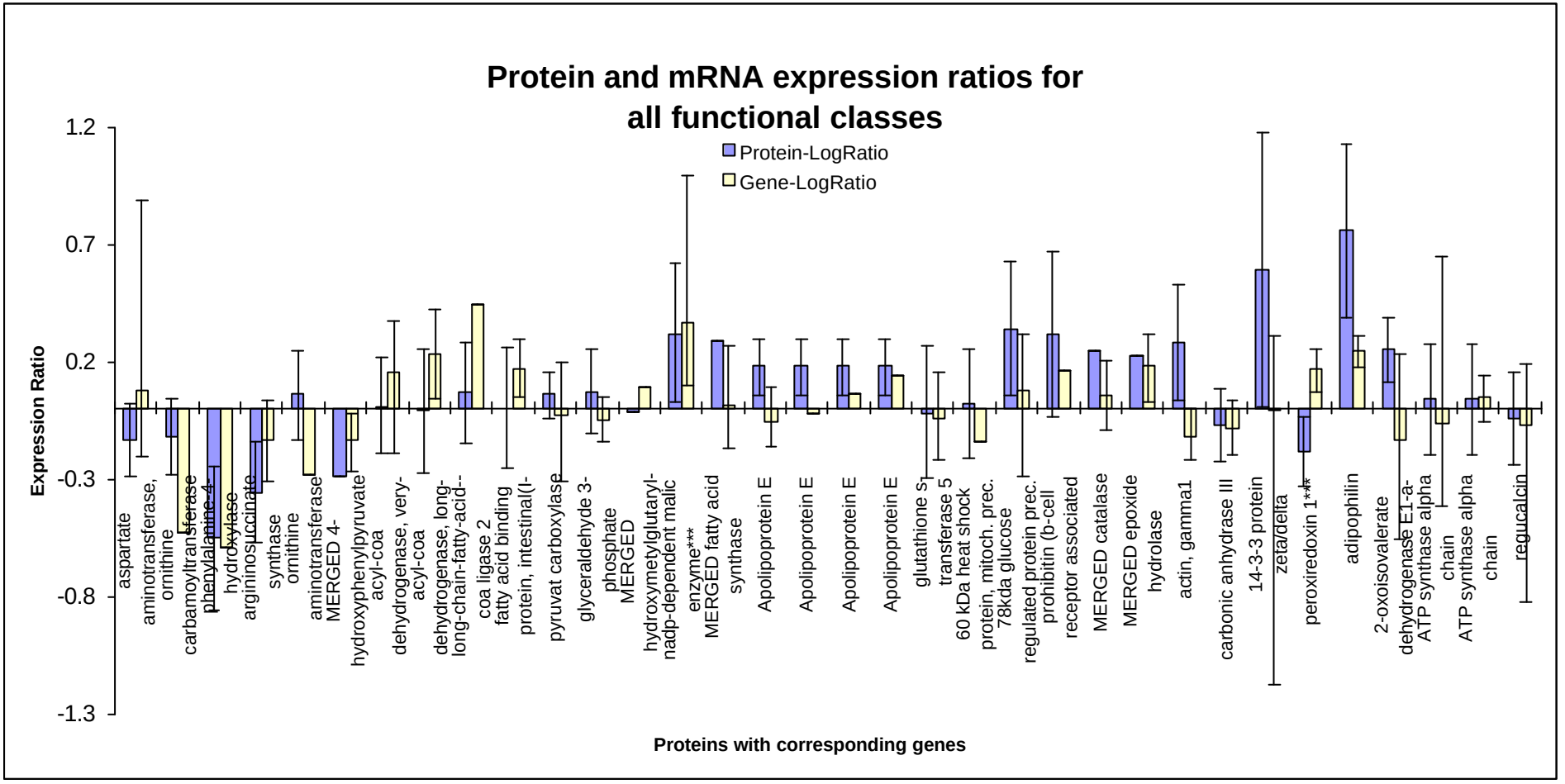
- The Proteomics columns initially pasted into the text area carry through and appear in the table. Each protein accession number is a hypertext link to the SWISS-PROT/TrEMBL entry in E-Lab.
- The "EMBL accession number" is the gene or EST link to the protein. If no link was found, this will be stated. Each EMBL accession number is a hypertext link to the EMBL entry in E-Lab.
- A "Type" column has been added to show whether the link is with a gene or an EST.
- There are three columns for the gene/EST log-ratio and its lower and upper bound according to a 95% confidence interval. In some cases the interval does not satisfy the criteria of the statistical method, and in these cases no interval will be shown (interpret as interval being too big). The interval is based on the inter-chip spread (essentially the variation in individuals) and does not take into account the intra-chip spread (within the probe sets).
- The "Absolute Call" column refers to an Affymetrix statistically based call, that decides on whether it thinks the correct mRNA has indeed attached to the probe set. "A" means absent, "M" means marginal, and "P" means present. The ratio you see shows each mouse (chip) in the two time points respectively. Our criteria for this is that at least 2/3 of the chips in either the first or the second time point are "P" or "M". Otherwise the gene/EST will not be shown, even if it could be linked to a protein.
- Don't forget that you can add useful comments into the "Comment1" and "Comment2" columns before or after running the program!

Appendix B – Result diagrams

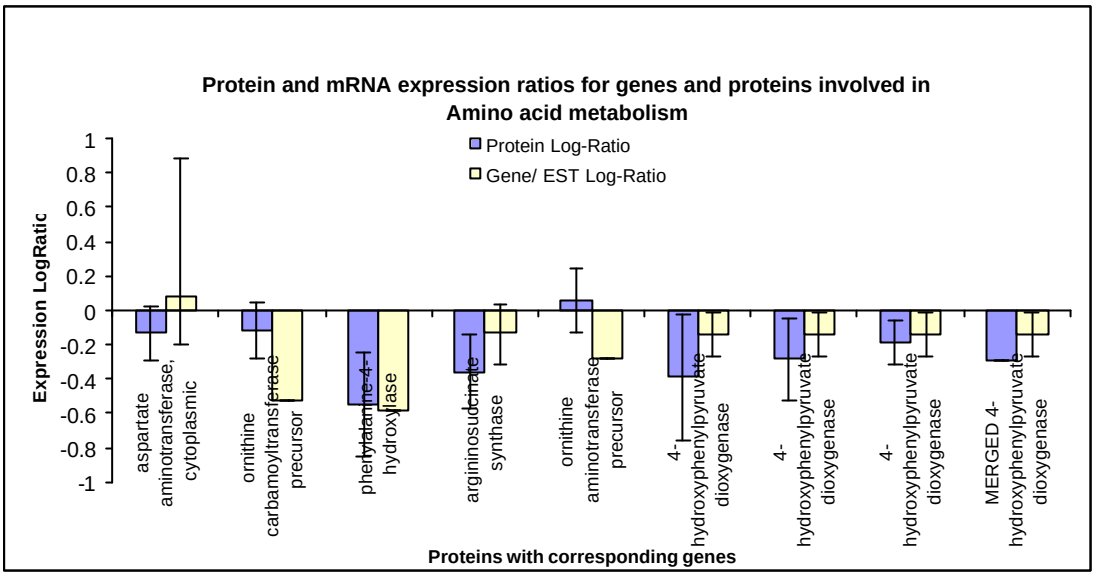


Graph 1 – *Scatter plot where the colours represent protein names and the shapes whether the mRNA molecules are genes or ESTs. Ratios refer to treated/untreated. The straight line is a least squares fit. Five proteins have more than one spot, and these spots have been merged.*

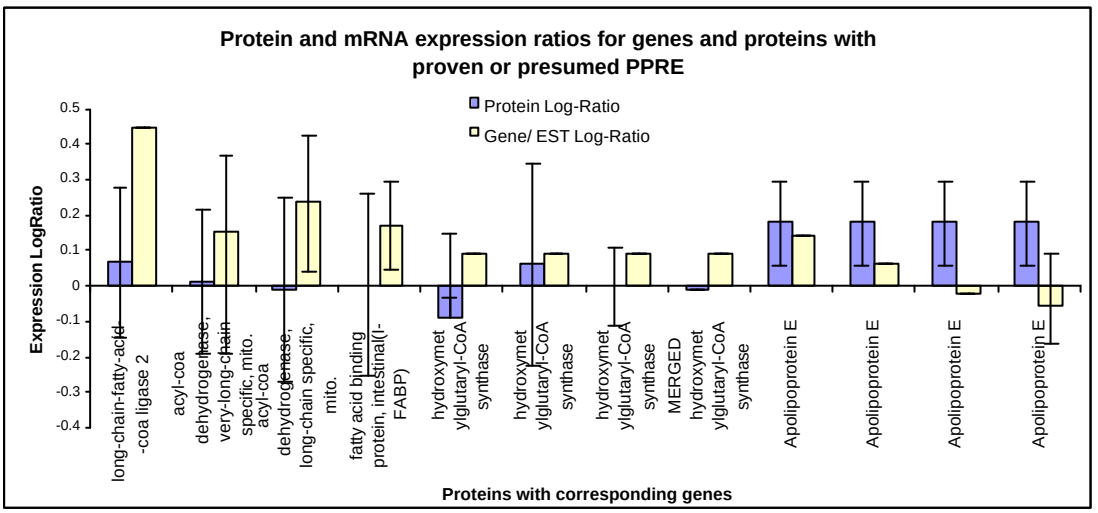
Graph 2 – *Same as in Figure 1 except that no protein spots have been merged.*



Graph 3 – *Bar diagram of protein and mRNA expression ratios (treated/untreated). The error bars represent confidence intervals when applicable Only expression ratios for unique and merged protein spots are shown.*



Graph 4 – *Expression ratios for genes and proteins involved in amino acid metabolism. Proteins with with and without merged spots are shown.*



Graph 5 – *Expression ratios for genes and proteins with proven or presumed PPRE. Proteins with and without merged spots are shown.*

Appendix C – EMBL and SWISS-PROT Entries

EMBL gene entry

In one of the feature table (FT) rows there is a cross-reference (db_xref) to a SWISS-PROT entry with the corresponding protein.

ID MMADFP standard; RNA; ROD; 1680 BP.
XX
AC M93275;
XX
SV M93275.1
XX
DT 15-MAY-1992 (Rel. 31, Created)
DT 04-MAR-2000 (Rel. 63, Last updated, Version 3)
XX
DE Mouse adipose differentiation related protein (ADFP) mRNA, complete cds.
XX
KW adipose differentiation-related protein.
XX
OS Mus musculus (house mouse)
OC Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia;
OC Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.
XX
…
DR MGD; MGI:87920; Adfp.
DR SWISS-PROT; P43883; ADFP_MOUSE.
XX
FH Key Location/Qualifiers
FH
FT source 1..1680
FT /db_xref="taxon:10090"
FT /organism="Mus musculus"
FT /strain="C3H"
FT /cell_line="1246"
FT /tissue_type="adipose"
FT 5'UTR 1..78
FT /note="putative"
FT mRNA 1..1680
FT /evidence=EXPERIMENTAL
FT CDS PEPT 79..1356
FT /codon_start=1
FT /db_xref="SWISS-PROT:P43883"
FT /evidence=EXPERIMENTAL
FT /standard_name="ADRP"
FT /gene="ADFP"
FT /product="adipose differentiation related protein"
FT /protein_id="AAA37176.1"
FT translation="MAAAVVDPQQSVVMRVANLPLVSSTYDLVSSAYVSTKDQYPYLRS
FT AEKGVKTVTSAAMTSALPIIQKLEPQIAVANTYACKGLDRMEERLPILNQPTSEI
FT VTGAKDVVTTTMAGAKDSVASTVSGVVDKTKGAVTGSVERTKSVVNGSINTV
FT VGPFYPQSTEVNKASLKVQQSEVKAQ"
FT 3'UTR 1357..1680
FT /note="putative"
FT polyA_signal 1664..1669
FT /note="putative"
FT polyA_site 1680
XX
SQ Sequence 1680 BP; 422 A; 413 C; 460 G; 385 T; 0 other;
agtggtgatctggaccgtgcggacttgctcgtccc……..

SWISS-PROT e ntry

The following SWISS-PROT protein entry corresponds to the EMBL gene entry above.

ID ADFP_MOUSE STANDARD; PRT; 425 AA.
AC P43883;
DT 01-NOV-1995 (Rel. 32, Created)
DT 01-NOV-1995 (Rel. 32, Last sequence update)
DT 30-MAY-2000 (Rel. 39, Last annotation update)
DE Adipophilin (Adipose differentiation-related protein) (ADRP).
GN ADFP OR ADRP.
OS Mus musculus (Mouse).
OC Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
OC Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.
OX NCBI_TaxID=10090;
RN [1]
RP SEQUENCE FROM N.A.
RC TISSUE=Adipocyte;
RX MEDLINE=92390349; PubMed=1518805;
RA Jiang H.P., Serrero G.;
RT "Isolation and characterization of a full-length cDNA coding for an
RT adipose differentiation-related protein.";
RL Proc. Natl. Acad. Sci. U.S.A. 89:7856-7860(1992).
RN [2]
RP SEQUENCE FROM N.A.
RC STRAIN=C3H; TISSUE=Adipose tissue;
…
CC -!- FUNCTION: MAY BE INVOLVED IN DEVELOPMENT AND MAINTENANCE OF
CC ADIPOSE TISSUE.
CC -!- SUBCELLULAR LOCATION: MEMBRANE-ASSOCIATED.
CC -!- TISSUE SPECIFICITY: ADIPOSE TISSUE SPECIFIC. EXPRESSED ABUNDANTLY
CC AND PREFERENTIALLY IN FAT PADS.
CC -!- INDUCTION: BY DEXAMETHASONE.
CC -!- SIMILARITY: BELONGS TO THE PERIPILIN FAMILY.
DR EMBL; M93275; AAA37176.1; -.
DR EMBL; L09734; -; NOT_ANNOTATED_CDS.
DR MGD; MGI:87920; Adfp.
DR InterPro; IPR004279; perilipin.
DR Pfam; PF03036; perilipin; 1.
KW Membrane.
SQ SEQUENCE 425 AA; 46664 MW; 82624E6CE3429C22 CRC64;
MAAAVVDPQQSVVMRVANLPLVSSTYDLVSSAYVSTKDQYPYLRSVCEMAEKGVKTVTSA
AMTSALPIIQKLEPQIAVANTYACKGLDRMEERLPILNQPTSEIVASARGAVTGAKDVVT
TTMAGAKDSVASTVSGVVDKTKGAVTGSVERTKSVVNGSINTVLGMVQFMNSGVDNAITK
SEMLVDQYFPLTQEELEMEAKKVEGFDMVQKPSNYERLESLSTKLCSRAYHQALSRVKEA
KQKSQETISQLHSTVHLIEFARKNMHSANQKIQGAQDKLYVSWVEWKRSIGYDDTDESHC
VEHIESRTLAIARNLTQQLQTTCQTVLVNAQGLPQNIQDQAKHLGVMAGDIYSVFRNAAS
FKEVSDGVLTSSKGQLQKMKESLDEVMDYFVNNTPLNWLVGPFYPQSTEVNKASLKVQQS
EVKAQ

Appendix D - Statistics

Fieller's Theorem (confidence intervals for the Affymetrix data)

Fieller's theorem is used in finding a confidence set for a ratio of paramaters, $\rho = \theta_1 / \theta_2$. In general there are two statistics, $\hat{q}_1$ and $\hat{q}_2$, which estimate θ_1 and θ_2, respectively. It is assumed that $(\hat{q}_1, \hat{q}_2)$ follows either exactly or approximately a bivariate normal distribution with mean (θ_1, θ_2) with $\sigma_{11} = \text{var}(\hat{q}_1)$, $\sigma_{22} = \text{var}(\hat{q}_2)$, $\sigma_{12} = \text{cov}(\hat{q}_1, \hat{q}_2)$.

…With $t_{1-\alpha/2}(d)$ denoting the 100(1-α/2)th percentile of the *t* distribution with *d* degrees of freedom, $P[H(\rho)^2 \le t_{1-\alpha/2}(d)^2] = 1 - \alpha$. (1)

Equation (1) can be rewritten as $P(Q(\rho) \le 0) = 1 - \alpha$, where $Q(\rho) = f_0 - 2f_1\rho + f_2\rho^2$ is a quadratic function of ρ, with $f_0 = \hat{q}_1^2 - t_{1-\alpha/2}(d)^2 \hat{s}_{11}$, $f_1 = \hat{q}_1 \hat{q}_2 - t_{1-\alpha/2}(d)^2 \hat{s}_{12}$, and $f_2 = \hat{q}_2^2 - t_{1-\alpha/2}(d)^2 \hat{s}_{22}$.

Defining $D = f_1^2 - f_0 f_2$, $r_1 = (f_1 - \sqrt{D}) / f_2$, $r_2 = (f_1 + \sqrt{D}) / f_2$, the confidence set for ρ is:

Case 1: A finite interval $[r_1, r_2]$, if $D \ge 0$ and $f_2 \ge 0$.
Case 2: The complement of a finite interval, $(-\infty, r_2] \cup [r_1, \infty)$, if $D \ge 0$ and $f_2 < 0$.
Case 3: $(-\infty, \infty)$ if $D < 0$ and $f_2 < 0$.

So for our purposes, the equation must fulfil the Case 1 criteria for the confidence interval to be used in the plots.

Confidence intervals for the proteomics data

Assuming X has a log-normal distribution, then $\ln(X) \sim N(m, s^2)$, i.e. normally distributed with expected value m and variance s^2.
Let $X_1, \ldots, X_n$ och $Y_1, \ldots, Y_m$ represent random samples from two log-normal distributions. Assuming also that $\ln(X_i) \sim N(m_X, s^2)$ and $\ln(Y_i) \sim N(m_Y, s^2)$, we have a 95% confidence interval for the estimate of the difference (on the log scale) between the expected values $\hat{m}_X - \hat{m}_Y$ given by

$$\frac{1}{n}\sum_{i=1}^{n} \ln(X_i) - \frac{1}{m}\sum_{j=1}^{m} \ln(Y_j) \pm t_{1-0.05/2}(n+m-2) \cdot s_{pooled} \cdot \sqrt{1/n + 1/m}$$

where

$$s_{pooled} = \sqrt{\frac{(n-1) \cdot s_X + (m-1) \cdot s_Y}{n+m-2}}$$

and

s_X , s_Y are the standard deviations for the random samples, $t_{1-0.05/2}(n+m-2)$ is the 97.5% quantile from the t-distribution with m+n-2 degrees of freedom.

Taking the anti-logarithm of the end points in the interval above we get a confidence interval for the quotient between the medians $\tilde{X} / \tilde{Y} = e^{m_X} / e^{m_Y}$ to our two log-normal distributions.

P-values

A p-value $\hat{a}(x)$ is the smallest α level on which we could reject the null hypothesis, given the data that we have received. The p-value is not a significance level since it is data-dependent (a significance level α is the risk we are willing to take to reject a true null hypothesis).

For a two sample t-test (two-sided) the p-value is given by

$$\hat{a}(x) = P_q\left(H_0 \text{ is rejected} \,|\, T(x) = t, \mathrm{H}_0 \text{ is true}\right)$$

$$= 1 - \int_{-T(x)}^{T(x)} dF(y) = 1 - \int_{-T(x)}^{T(x)} \frac{\Gamma\left(\frac{n+1}{2}\right)}{\sqrt{np}\,\Gamma\left(\frac{n}{2}\right)\left(\frac{1+y^2}{n}\right)^{-\left(\frac{n+1}{2}\right)}} dy$$

where ν is the degrees of freedom, $\Gamma(z)$ is the Gamma function and

$$T(x) = \frac{\left|\bar{x}_1 - \bar{x}_2\right|}{s_{pooled}\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}.$$

The above integral is solved numerically.

Holm's step-down method (adjusted p-values)

Let $p_1, p_2, \ldots, p_k$ denote the original p-values, sorted in ascending order, that have been received from k hypothesis tests, the adjusted p-values are then defined sequentially by

$$\tilde{p}_1 = \min\{kp_1, 1\}$$

$$\tilde{p}_2 = \min\{\max\{\tilde{p}_1, (k-1)p_2\}, 1\}$$

$$\vdots$$

$$\tilde{p}_j = \min\{\max\{\tilde{p}_{j-1}, (k-j+1)p_j\}, 1\}$$

$$\vdots$$

$$\tilde{p}_k = \min\{\max\{\tilde{p}_{k-1}, p_k\}, 1\}$$

This method is used in multiple testing to adjust p-values when the tests are dependent (it works even if they are independent).