A DNA microarray for fission yeast: minimal changes in global gene expression after temperature shift

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ABSTRACT

Completion of the fission yeast genome sequence has opened up possibilities for post-genomic approaches. We have constructed a DNA microarray for genome-wide gene expression analysis in fission yeast. The microarray contains DNA fragments, PCR-amplified from a genomic DNA template, that represent >99% of the 5000 or so of annotated fission yeast genes, as well as a number of control sequences. The GenomePRIDE software used, attempts to design similarly sized DNA fragments corresponding to gene regions within single exons, near the 3’-end of genes that lack homology to other fission yeast genes. To validate the design and utility of the array we studied expression changes after a two-hour temperature shift from 25 to 36 degrees, conditions widely used when studying temperature-sensitive mutants. Obligingly, the vast majority of genes do not change more than two-fold, supporting the widely held view that temperature-shift experiments specifically reveal phenotypes associated with temperature-sensitive mutants. However, we did identify a small group of genes that showed a reproducible change in expression. Importantly, most of these corresponded to previously characterised heat-shock genes whose expression has been reported to change after more extreme temperature shifts that those used here. We conclude that the DNA microarray represents a useful resource for fission yeast researchers as well as the broader yeast community, since it will facilitate comparison with the distantly related budding yeast, *Saccharomyces cerevisiae*. To maximise the utility of this resource, the array and its component parts are fully described in on-line supplementary information and are also available commercially.
INTRODUCTION

Comparison of the recently completed genome sequence of the fission yeast, *Schizosaccharomyces pombe*, with that of the budding yeast, *Saccharomyces cerevisiae* [Goffeau, et al., 1997], reveals the power of comparing genomic information between species [Wood, et al., 2002]. The latter study, combined with a previous study based on partial sequences of several yeast species [Souciet, et al., 2000], showed that proteins can be classified, according to homology, as eukaryote-specific, yeast-specific and organism-specific. 67% of fission yeast proteins have homologues in both budding yeast and the nematode, *Caenorhabditis elegans* and are thought to be common to all eukaryotes, a further 16% are also found in budding yeast, while 14% are not found in either of the other two species and are therefore specific to fission yeast [Wood, et al., 2002]. Thus at the level of structural genomics we can distinguish groups of proteins that will help us to understand the properties of organisms that are conserved in all eukaryotes as well as determinants that define species-specific properties.

The power of having complete genome sequences of two yeasts is that the organisms grow in similar conditions and have comparable life styles. Thus it is possible to extend structural genomic comparisons to the functional level under comparable physiological conditions. DNA microarrays for measuring changes in genome-wide transcription patterns have proved to be of central importance for functional genomic approaches [Horak and Snyder, 2002]. Fission yeast is a natural choice for comparative studies with the budding yeast because it is only distantly related to the family of hemiascomycetes that contains most other yeast species, including *Saccharomyces cerevisiae* [Sipiczki, 2000]. The utility of this approach has already been demonstrated by the group of Jurg Bähler and his collaborators, using a
fission yeast microarray developed at the Sanger Institute. In two recent studies they have shown that the sporulation process [Mata, et al., 2002] and stress response [Chen, et al., 2003] in fission yeast show similarities to the analogous responses in budding yeast but there are also critical differences in each case. Furthermore, we have used the microarray described here to identify genes regulated by the inhibitory RNA (RNAi)/dicer system in fission yeast [Provost, et al., 2002] for which there is no budding yeast equivalent.

The aim of this paper is to describe the construction and validation of a fission yeast microarray for genome-wide analysis of gene expression. The contents of the paper provide the information required to construct the array from scratch and to use the complete array, which is also available commercially. We believe that this will provide a useful resource that is readily available to the entire yeast community.
MATERIALS AND METHODS

Yeast strains, growth and RNA preparation

Strains used were 972h- and 367(h+, ade1-210, leu1-32, ura4-D18). YE medium [Alfa, et al., 1993] was used throughout. A pre-culture was grown at 25°C to a cell density 5x10^6–1x10^7 cells/ml. 2-5-5ml of this culture was used to inoculate a fresh 200ml culture (500ml Ehrlemeyer flask) that was incubated in a 25°C water bath (Grant OLS200, short stroke length) with agitation at 100 strokes/min. When the culture reached a cell density of 5x10^6–1x10^7 cells/ml, half the culture was transferred to an identical flask in a second water bath at 36°C. Both cultures were incubated for a further 2 hours at 25°C and 36°C, respectively, prior to harvesting the cells. The duplicate cultures of strain 367 were prepared by inoculating two 200 ml cultures from the same pre-culture.

Cells were harvested and total RNA extracted using hot phenol [Ausubel, 2002] with minor alternations as follows. Yeast cells were harvested in 50ml tubes by centrifugation at 3700rpm for 5min at room temperature. The medium was carefully removed and the cell pellet was resuspended in 2ml TES solution (10mM Tris.Cl, pH7.5, 10mM EDTA, 0.5% SDS). 2ml of acid phenol (Sigma) was added and the cells were vortexed vigorously prior to incubation at 65°C for 30min with occasional, brief vortexing. After incubation on ice for 5 min the samples were transferred to 2ml tubes and centrifuged in a microfuge at top speed for 5 min. The upper aqueous phase was saved and extracted twice with 2ml chloroform. Total RNA was then precipitated by ethanol and washed with 70% (v/v) ethanol. The RNA pellet was resuspended in 200μl of DEPC water and purified using a Qiagen RNeasy column (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The quality and quantity of RNA was
determined by measuring absorbance at 260nm and 280nm and by gel electrophoresis. The yield of purified RNA was typically 100-250\(\mu\)g from 50ml of culture.

**Primer design**

The design of PCR primers was performed using the GenomePRIDE software package details of which can be found elsewhere [Haas, et al., 2003]. Briefly, all predicted genes were compared using BLAST (blastn, default parameters, e-value cutoff: 0.001) in order to tag regions of similarity, with a minimal length of 40 bp. GenomePRIDE selected an optimal, 'gene-specific' target region within each by minimizing the fraction of similarity regions, as well as the fraction of intron-containing sequence within a PCR fragment. Additionally, target regions located close to the 3' terminus of the genes were preferentially selected and were optimized for an exon content of 420 bp.

**Microarray construction**

Primer pairs for each of the microarray probes were synthesized at a 40 nmol scale using standard cyanoethyl phosphoramidite chemistry. The forward and reverse primers were approximately 35 nucleotides in length and were composed of approximately 20 nucleotides specific for each fragment and a common 5'-tag sequence (forward primer-tag: 5'-CGACGCCCGCTGATA; reverse primer-tag: 5'-GTCCGGGAGCCATG). All primers were synthetized by Eurogentec SA. (http://www.eurogentec.com) and are commercially available.

In addition to *S. pombe* coding sequences, the microarray contains probes for protein Tags (GFP, TAP, GST), marker genes (KanMX, LacZ), a positive control for
spiking experiments (luciferase gene from Renilla, RNA commercially available from Promega, Inc), dynamic range controls (serial dilution of a fragment from the ef1-b+ translation initiation factor gene), negative controls (printing buffer, 6 predicted introns and 10 predicted intergenic regions), 3 PCR products covering the large (c5kb) 3’ exon of the rpb1+ gene (5’-end, middle region and 3’-end) for checking the efficiency of the reverse transcription during probe synthesis and a cross-hybridization control from the S. cerevisiae ACT1 gene that has 80% homology to the S. pombe act1+ gene.

PCR amplification reactions were done in two rounds. In the first round, one hundred-microliter reactions were performed using each primer pair with the following reagents: 5 ng of S. pombe DNA template, 50 pmoles of each primer, 0.25 units of GoldStar™ Taq DNA polymerase (Eurogentec), 125 μM each dNTP, 75 mM Tris-HCl, pH 8.8 (25°C), 20 mM (NH₄)₂SO₄, 0.01% tween 20, 1.8 mM MgCl₂. Thermal cycling was carried out in a Gene Amp PCR system (Applied Biosystems) with a 5 min denaturation step at 95°C, followed by 40 cycles of 1 min at 95°C, 30 sec at 55°C, 1 min at 72°C, and a final cycle at 72°C for 10 min. All PCR reactions were analyzed by agarose gel electrophoresis. Reactions that failed to amplify or showed multiple bands were repeated using different conditions (e.g. higher annealing temperatures) to favour amplification of the desired product. The second round of PCR amplification follows the same protocol as the first round except for the use of ~5 ng of first round PCR products as templates, 50 pmoles each of forward (with an amino-group NH₂-[CH₂]₁₂- at the 5’-end of the oligonucleotide) and reverse tag-primers, and the annealing temperature of 45°C. PCR reactions were purified using 96-well Millipore MultiScreen-FB filters, then eluted in a final volume of 30 μl water, and finally air-dried. Prior to drying, DNA concentrations were estimated by agarose gel electrophoresis in the
presence of ethidium bromide. The DNA was finally brought to a concentration of 200 ng/µl in printing (3x SSC, 0.05% SDS).

PCR products were spotted with the robotic system ChipWriterPro (Biorad) using 24 stealth Micro Spotting Pins SMP3 (TeleChem International), in duplicates onto microarray aldehyde functionalized glass slides (TeleChem International) with 230-µm spacing between neighbouring spot centers. The complete array is composed of 32 subgrids of 18 rows by 18 columns. Subsequent microarray treatments were performed as recommended by the manufacturer (TeleChem International).

RNA labelling, array hybridisation and data quantification

For each experiment, about 25 micrograms of total RNA were converted to either Cy3- or Cy5-labelled cDNA using a custom-made direct labelling kit using a mix of all reverse primers (S. pombe specific Reverse Transcription primer Set supplemented with oligoT18-21 primers, 0.2 pmoles per reaction), CyDye-labelled dCTP (Amersham) and Supercript II enzyme (Invitrogen). The labelled cDNA products were then purified using Qia-Quick clean up columns (Qiagen). The amount of cDNA as well as the incorporation of Cy3 and Cy5 dyes into cDNA targets were quantified by measuring the absorbance of each sample at 260 nm, 550 nm and 650 nm, respectively. For each experiment, labelled-Cy3 and -Cy5 cDNA targets were then combined in equal amount, dried by Speed-vac, and resuspended in 60 µl DIGeasy hybridization buffer (Roche Diagnostics) containing 1 mg/ml of heat-denatured salmon sperm DNA (Sigma).

After covering the array with a 24 x 60 mm coverslip, the slide was placed in a humidified Corning hybridization chamber and hybridization was performed with
resuspended CyDye-labelled cDNA at 37°C for approximately 16 h. Following hybridization, slides were washed once in 0.2x SSC, 0.1% SDS for 5 min and finally in 0.2x SSC for 5 min. Then, slides were immediately dried by centrifugation (5 min at 500 x g).

Hybridized microarrays were scanned for Cy3- and Cy5-labelled targets with the Axon Instruments Genepix 4000A scanner with a resolution of 10 μm. According to the manufacturers specifications, the photomultiplier tube settings were adjusted in order to balance the signal intensities of the two channels, and to get the greatest dynamic range for each scan. Signal quantification for each probe on the microarray was performed with Genepix image acquisition software version 3.0 (Axon Instruments). Primary data files used in this study are available via http://www.biosci.ki.se/users/towr/microarray.

**Data analysis**

We used intensity-dependent normalization by fitting a Lowess curve, an approach that has been applied to microarray data analysis previously [Dudoit, et al., 2000]. For most of the chips there was significant but not extreme dye bias. Intensity-dependent effects were visualised by plotting the log₂ signal ratio (R/G) as a function of the log₂ product of the two signals (R*G). The Lowess procedure detects systematic deviations revealed in such a ratio-intensity plot and corrects them by carrying out a local weighted linear regression as a function of the log₂(intensity) and subtracting the calculated best-fit average log₂(ratio) from the experimentally observed ratio for each data point. Fold-change ratios (36/25) calculated from normalised data and used in this study are available via http://www.biosci.ki.se/users/towr/microarray.
GenePix software computes a number of measures of spot quality, such as size, circularity, and standard deviation of pixel intensities within the spot mask and uses them to flag defective spots. We constructed additional quality control measures such as relative difference between mean and median. Each of these measures has a distribution, and we identified by hand, a ‘normal’ range of values for each measure. Then we defined a threshold for each measure as a multiple of the upper limit of the ‘normal’ range. If the measure exceeds this threshold the spot fails to pass the quality control criterion in question. The four quality control criteria that we applied to each spot require that: (1) the median pixel intensity minus the mean pixel intensity is less than 10% of the median pixel intensity for each channel, (2) mean pixel intensity divided by the standard deviation about the mean of the pixel intensities is greater than 2 for each channel, (3) the signal intensity is less than or equal to 20% of the saturation level for each channel and (4) 90% of pixel intensities should be above background+1SD and 80% above background+2SD for both channels. These criteria are similar to those previously described independently [Wang, et al., 2001].

Pairwise correlative analysis was used to calculate the correlation coefficient (r) for comparisons between different array experiments before or after the application of the quality control criteria above. Genes with significantly altered expression in 8 replicate measurements were identified using a Student’s t-test to identify genes with a mean fold change significantly different from 1. In cases where some spots were excluded from the analysis due to poor quality, the appropriate t-test was used for the number of spots analysed. The moving average (window size 201, step size 1) and frequency analyses were performed using the Excel program (Microsoft).
RESULTS

Design and construction of a fission yeast DNA microarray for transcriptome analysis

We have designed and constructed a microarray for transcriptome analysis of fission yeast. The array is based on the use of PCR primers designed to amplify optimal DNA fragments corresponding to all the annotated genes in the recently released genome sequence of fission yeast [Wood, et al., 2002]. The primers were designed using the GenomePride software package that has been described in detail elsewhere [Haas, et al., 2003]. Briefly, the software aims to design primers of equal melting temperature based on thermodynamic criteria and to minimise hybridisation with themselves or with secondary binding sites in the template DNA. The software successfully designed primers for 5006 of the 5039 genes annotated as “CDS” in the fission yeast nuclear and mitochondrial DNA sequence. The design failures were mainly due to the short length of some annotated genes. The primers were synthesised with a universal tag sequence at the 5’ end. A complete list of primer sequences is provided in the Supplementary information, Table 1S. Using these primers 4997 fragments were successfully amplified from a genomic DNA template, representing an amplification success rate of greater than 99.8%. These fragments were further amplified in a second PCR reaction using the universal primers. This step increases the yield of amplified fragments and allows addition of modified groups at the 5’ end of fragments for covalent attachment of the fragments to the microarray slide. Fig 1A shows an example of the gels run to check the amount and quality of the amplified fragments. The amplified fragments represent 4927 nuclear genes encoding known or hypothetical proteins, 48 nuclear pseudogenes, 13 nuclear genes annotated as “mRNA”
genes and 9 mitochondrial genes encoding mitochondrial proteins. Table 2S in the Supplementary Information contains the sequence of the amplified regions and other information about the fragments.

Using the GenomePride software, we aimed to optimise the size, specificity and relative position of the amplified fragments. The relative position of the fragments within each gene is important for two reasons. First, if cDNA probes are made using poly dT primers it is highly desirable to have probe fragments close to the 3’-end of genes on the microarray in order to maximise sensitivity. Second, almost half the genes in fission yeast contain introns. To our knowledge the use of probe fragments that include both intron and exon sequences on microarrays has not previously been evaluated. It was therefore important to maximise the number of probe fragments that could be amplified from individual exons. We chose 420bp as the optimum fragment size in our primer design in order to ensure optimal array performance as well as fragment amplification.

Fig 1B shows the frequency distribution of the selected fragments, the vast majority of which are 420±20bp in size. Fragments less than this size are shorter in order to fulfil the other criteria of specificity and relative position. Longer fragments contain intron sequences. In these cases the software tries to compensate for the presence of intron sequences by aiming to maintain the length of exon sequences at 420bp. Fig 1C shows the exon content of the 153 intron-containing fragments plotted as a function of the proportion of intron in the fragment. The cluster of points at the top left of the plot illustrates the attempt of the software to minimise intron-content and maximise exon length. Fig 1D shows that the frequency distribution of the distances from the 3’-end of the probe fragments to the 3’-end of the respective annotated coding
sequences. This distance is most frequently 100bp or less but there is a significant tail on the distribution to 2000bp. Very few fragments are further away from the 3’-end than this. The GC content of amplified fragments was not used as a variable in the array design process but is nonetheless likely to have an important effect on the rate of hybridisation. Fig 1E shows the frequency distribution for the GC content of the probe fragments. Most fragments have a GC content between 30% and 50% with a mode value close to 40%. This is consistent with the GC content of the protein coding regions of the genome, which is 39.6% [Wood, et al., 2002].

The probe fragments on a microarray should ideally be specific for their cognate cDNAs in the labelled sample and not cross-hybridise with any other cDNA species. In practice, this is very hard to achieve for all the fragments. There are 266 fragments on the fission yeast microarray that recognise at least one non-cognate gene in a BLAST search of all the annotated fission yeast genes. Fig 1F shows that most of these fragments recognise one or two other genes. These consist mainly of genes encoding ribosomal proteins that are frequently present in two or three copies in the genome. A smaller number of fragments recognise larger numbers of other genes. These correspond to Tf2 transposon sequences and Wtf elements. Table 1 summarises the main families of genes with known functions that cross-react. A complete list of the 266 fragments, including the genes they recognise in a BLAST search, is provided in the Supplementary Information, Table 3S.

**Experimental validation of the design principles**

We have tested the utility of the fission yeast microarray with particular regard to fragment length, relative fragment position and hybridisation specificity, which were
important variables in the design process. We have evaluated the microarrays by studying the level of hybridisation signals as a function of the different design variables. For this, we have accumulated data from 10 different hybridisation experiments that compare the transcriptome of cells shifted from 25°C to 36°C for 2 hours with that of control cells that remained at 25°C. In these experiments over 95% of the spots on the array were detected using the Genepix software. The microarrays contain duplicate spots for each gene, data from which are analysed below as the mean of the values from the ten experiments (the 10,000 or so individual data points are shown in grey, a moving average to show underlying trends is shown in black). Fig 2A shows that the hybridisation signal is not significantly affected by the length of probe fragments ranging from 150-420bp. These data were accumulated from probe fragments within individual exons. Importantly, Fig 2B shows that there is no consistent positive or negative trend in signals from the small proportion of intron-containing fragments as the proportion of intron DNA within the fragments increases. The cDNA probes used in these experiments were generated using both a mix of the fragment-specific primers, used to generate the probe fragments on the array, and a poly-dT primer. Fig 2C shows that under these conditions the distance between the 3’-end of the fragment and the 3’-end of the respective genes has only a small effect. The average signal strength falls by about 2-fold as the distance from the 3’-end of the gene increases from 0 to 4000bp. The microarray contains control spots to evaluate the detection efficiency of probe fragments at different positions in the same coding sequence. In addition to the designed fragment close to the 3’-end of the rpb1 (RNA polymerase II, large subunit) 3’-exon, we have included two further fragments of similar size and CG content in the middle and at the 5’end of this exon. Consistent with the trend in Fig 2C, there is a downward
trend in the sensitivity of the hybridisation as the distance between the fragment and the 3’-end of the gene increases (Fig 2D). As might be expected, the GC content of fragments has a significant influence on the sensitivity of detection by probe fragments. Fig 2E shows that, on average, fragments with the highest GC content are at least an order of magnitude more efficiently detected than those with the lowest GC content. Interestingly, the relationship between average signal strength and GC content appears to be non-linear. Up to about 40% GC there is little change in average signal strength but at higher levels the average signal strength increases sharply.

The last design variable of importance is the specificity of the probe fragments for hybridisation to cognate cDNA fragments in the labelled sample. In order to estimate the contribution of hybridisation to non-cognate cDNAs to the measured signals we included a fragment of the ACT1 gene from *S. cerevisiae* on the array that is 80% identical to the fission yeast act1 gene. The two fragments are of similar length and GC content. Fig 2E shows that the signal strength from the ACT1 probe is only about 5% of the level measured for the cognate fission yeast probe. This experiment suggests that the probe fragments that only recognise themselves in a BLAST search are likely to show good discrimination between cognate and non-cognate cDNA fragments in the labelled samples.

**Reproducibility: slide to slide variability**

To test the reproducibility of data obtained with the microarray, we performed two independent labelling experiments of RNA samples from a culture of the 972h-strain grown at 25°C and 36°C. The fold change in the signal for each gene was calculated for both experiments after normalisation of detectable hybridisation signals
(>95% of spots detected). The results (Fig 3A) correlate very well (correlation coefficient = 0.9) with none of the genes differing from the ideal 1:1 ratio by more than 2-fold. Most genes do not exhibit persistent changes in expression after the transition from 25°C to 36°C. In these two data sets, only 45 genes changed more that 2-fold in both experiments, with a maximum fold change up and down of about 6-fold.

It has been reported that the different chemical properties of the Cy3 and Cy5 dyes lead to a dye bias in array data such that the two dyes are not always equivalent in the way they reflect the expression level of different genes. We therefore labelled the 972h- RNA samples using the opposite dye orientation to label the 25°C and 36°C samples. The fold change values obtained still correlate well with the experiments shown in Fig 3A (Fig 3B) but the correlation coefficients are reproducibly lower (0.6-0.7). This is reflected in a greater spread of the data, with over 10 genes differing from the ideal 1:1 ratio by greater than 2-fold.

Another way to assess the quality of data obtained from each individual slide is to impose criteria that more stringently test the intensity range and homogeneity of the pixel intensities for each spot. We have chosen four such criteria (see Experimental Procedures) to apply to the signals detected by the Genpix software in order to select genes where at least one of the duplicate spots for each gene passes the criteria. Further, we determined the number of genes for which both spots pass the quality control criteria and for which the values from the two passing spots fall within 15% of each other. The results are shown in Table 2 as the mean of 8 hybridisation experiments. On average about three quarters of the genes pass the most stringent selection criteria, although this level varies significantly from slide to slide as indicated by the relatively high standard deviation about the mean. The correlation coefficient for comparisons of fold-change
data after normalisation of dye-swapped data for which both spots pass the criteria generally increases by 0.03-0.04.

To determine the nature of the signals that are filtered out by the quality control criteria we plotted the ratio of the Cy3 and Cy5 signals for each spot against the product of the two signals (Fig. 4). The raw data spots detected by the Genepix software are shown in Fig. 4A for a representative microarray. By selecting genes for which at least one spot passes the quality control criteria the proportion of genes selected drops from 98% to 84%. Fig. 4B shows spots fulfilling the criteria (black symbols) as well as the spots that are excluded (red symbols). Spots with low intensity are overrepresented in the excluded set, consistent with the application with measures of spot quality that are more rigorous than those applied by the GenPix software. For 80% of genes, both duplicate spots passed the quality control criteria (Fig. 4C, black symbols). The signals selectively excluded by this more stringent criterion (red symbols) are evenly distributed throughout the distribution of values selected by the less stringent criteria in Fig 4B. For these genes the measured values for the duplicate spots differs by more than 15% in less than 1% of the cases. Fig 4D shows the data selected in Fig 4C after Lowess normalisation. We conclude that the quality control criteria provide an efficient test of the overall robustness of each data set but we have also found that data that do not pass these criteria are often still useful for identification of gene expression changes.

**Reproducible changes in gene expression after a temperature shift from 25 to 36 degrees**

The experiment shown in Fig. 3 showed that large changes in gene expression do not occur after a 2 hour shift from 25°C to 36°C. In that experiment, the expression
of around 40 genes was altered more than 2-fold. The low magnitude of the fold-
changes makes the identification of reproducibly altered genes difficult but, at the same
time, it provides a good test of microarray performance. In order to maximise the
identification of reproducibly altered genes, we prepared duplicate cultures at 25°C,
seeded from a common starter culture. These cultures were subsequently divided into
two flasks that were left growing for a further 2 hours at 25°C and 36°C, respectively.
RNA was then extracted from the resulting four cultures, labelled and hybridised to a
total of four arrays in each dye-swap orientation. The Venn diagram in Fig. 5A shows
the number of genes for which the mean-fold change of the normalised dye-swap
experiments was ≥2 for each of the two cultures. Genes detected in both cultures are
shown in the green intersection. It is evident that about 2.5 times as many genes passed
the threshold for culture 1 (red) as for culture 2 (blue). Nonetheless over 60% of the
genes regulated 2-fold or more in culture 2 were also regulated at least 2-fold in culture
1. Reduction of the selection threshold for culture 2 data to account for its lower overall
variance (0.78 of culture 1) increases the number of genes in the intersection with
culture 1 to 97. Thus the number of genes in the intersection is very sensitive to the cut-
off thresholds used to select regulated genes. Fig. 5B compares the fold change values
for the selected genes (>2-fold) in the two experiments using the same colour codes as
in Fig. 5A. This figure shows that almost all the selected genes are regulated
consistently up or down in both cultures but that there is variation in the extent of their
regulation. If the main aim is to identify reproducibly regulated genes, an alternative
approach is to identify genes that are significantly related in both cultures. 493 genes
are significantly regulated in cultures 1 and 2 (p≤0.01). Of these 173 are altered 1.5-fold
in one of the cultures and 113 of these are altered 1.5-fold in both. We conclude that
cultures 1 and 2 are very similar in the genes that are changed after transfer to 36°C but that they show variation in the level of the expression changes for many genes. This may be due to the fact that expression of many genes is altered transiently during temperature transitions.

The high degree of physiological reproducibility in Fig. 5 was obtained using parallel cultures seeded from the same pre-culture. The temperature-shift experiment procedure is however frequently used when studying phenotypes associated with temperature sensitive mutations. It would be advantageous therefore to identify genes that can be expected to show robust changes in their expression irrespective of the strain isolate and the exact growth protocol. In order to address this question we used the prototrophic strain 972h- for the four hybridisation experiments described in Fig. 3 and an auxotrophic isolate of the same strain (367h-) for the 4 hybridisation experiments described in Figs. 4 and 5. The mean fold-change values calculated from the eight data sets identified 424 significantly regulated genes ($p \leq 0.001$). In all but 4 of these cases the eight individual measurements showed consistent regulation, up or down. Furthermore, 122 genes past the reduced p-value threshold ($p \leq 0.00005$) that reduces the probability of selecting a false positive gene to 0.25, according to the very conservative Bonferroni correction for multiple testing [Dudoit, et al., 2000]. As shown in Table 3, 21 significantly changed genes were regulated by more than 2-fold.
DISCUSSION

We have developed and tested a DNA microarray for genome-wide transcription analysis of the fission yeast, *Schizosaccharomyces pombe*. Large-scale users can construct the array by amplification of genomic DNA using the primer pairs and other information described in this paper. However, access to the array described here is also readily available to the many smaller-scale users within the community from commercial sources. After submission of this paper, a paper describing a custom-made fission yeast array used in work referred to previously [Chen, et al., 2003; Mata, et al., 2002] was published [Lyne, et al., 2003]. Generally, that microarray appears to function similarly to the one described here.

While the use of genomic DNA as a template for amplification increases the ready availability of the components needed to construct the array, it has one potential disadvantage. About 3% of the amplified fragments contain predicted intron sequences and in these fragments the exon region available for hybridisation with the cDNA probes is divided into two parts. To our knowledge, the hybridisation performance of small intron-containing fragments on microarrays has not previously been evaluated. Our data show that the intron content of intron-containing fragments does not measurably reduce the average signal from spots and there is no clear adverse trend in signal detection as the proportion of intron sequence in the fragments increases.

We have employed a mix of the primers used to amplify the array fragments, in combination with polydT primers for production of labelled cDNA probes. Under these conditions, we might expect the sensitivity of signal detection to be independent of the position of the array fragment in relation to the sequence of the mRNA transcript. However, to facilitate use of the arrays using only polydT-primed probe synthesis, we
have designed the array fragments so that most are in very close proximity to the 3’ end of the gene. Our results show that, even using our labelling strategy, the sensitivity of signal detection drops slowly as the position of the array fragment gets further away from the 3’ end of the gene. However, the redundant rpbl probes representing sequences up to 5,000 bases from the 3’-end (Fig. 2D) show that there is no significant effect of distance from the 3’-end on the mean fold change value.

The third variable in our design that affects the selection of the amplified fragment is its specificity. To reduce cross-reactivity of the array fragments with non-cognate genes, regions of potential cross hybridisation between coding regions were identified by the BLAST algorithm and masked in the sequence prior to primer design. The GenomePride software then attempts to select fragments that lie outside these regions. However, analysis of the designed fragments using BLAST predicts that 266 of them might cross react with one or more non-cognate sequences. Inspection of these fragments shows that they generally represent members from highly similar gene families (Table 1) for which it is difficult to make discriminating array fragments by this approach. To determine how specifically the fragments that only recognise themselves in the BLAST search hybridise to their respective cognate cDNAs in the labelling mixture, we included a fragment of the budding yeast ACT1 gene on the array. This fragment, which is 80% identical to the homologous region of the fission yeast act1+ gene, was recognised about 20-fold less efficiently than the fission yeast act1+ fragment. Since the BLAST search we performed reliably identified homologous sequences down to the 80% identity level, greater than 95% of the signal for most of the “unique” fragments is likely to result from hybridisation with cognate cDNAs in the probe mixture. Clearly, there may be some cases in which a homologous non-cognate
message is in vast excess compared to the cognate message, thereby leading to a larger degree of cross-reactivity.

We found a very high reproducibility in the detection of regulated genes using different microarrays probed with labelled cDNAs produced from the same RNA templates in two different labelling reactions (Fig. 3). The variability of the results increased significantly when comparison was made between experiments in which the orientation of the labelling dyes was exchanged. Although almost all the results in each experiment still varied from the ideal 1:1 ratio by less than 2-fold, our results suggest that there are some gene-dye specific effects. Similar phenomena have been discussed elsewhere [Kerr and Churchill, 2001]. Most of our data analysis has been done using the >95% of gene signals that are detectable by the Genepix software package. However, we have investigated the effect of imposing stricter quality control requirements on the reproducibility of data. Criteria that select signals more stringently based on (i) the homogeneity of spots, (ii) signal strength in relation to background and (iii) signal strength in relation to the signal saturation level increase reproducibility between experiments (0.03-0.04 increase in correlation coefficients) at the expense of reducing the proportion of genes analysed to around 75%. In our experience, these quality controls provide a useful measure of the overall quality of each data set but they do not enrich the relative size of the subset of reproducibly regulated genes when different experiments are compared. Thus, in replicate experiments we find that many spots excluded by the quality control criteria are still useful for the detection of regulated genes.

The main problem in obtaining reproducible microarray data is often physiological [Grunenfelder and Winzeler, 2002]. This is the case in the data described
here where the relevant fold-changes in gene expression to be detected are similar in magnitude to the noise level associated with the measurement technique (up to 2-fold in this case). Nonetheless, we have been able to identify 21 genes whose level was reproducibly and significantly changed ≥2-fold after a two hour shift to 36°C. Importantly, most of the genes found in individual experiments, but not in all, are regulated consistently up or down but are excluded by the 2-fold threshold level.

We chose to perform the temperature-shift experiment described in this paper because similar conditions are often used in the study of temperature-sensitive mutants. Thus the results have a broad significance in addition to their role in validating the microarray. In studies of temperature sensitive mutants, it cannot be formally excluded that the temperature-sensitive phenotype results from a combination of the mutation together with a physiological change in cells shifted to the restrictive temperature. Our results suggest that any such physiological change does not involve major persistent changes in gene expression. 36°C is the optimal growth temperature for fission yeast, so we would not expect a major induction of gene programs associated with heat stress. However, it is quite likely that the small changes we see might overlap with changes that occur during heat stress when cells are shifted to higher temperatures. In this respect, it is interesting to note that several of the up-regulated genes of known function have been shown previously to be induced during heat-shock conditions (Table 3). Furthermore, during the preparation of this manuscript genome-wide transcription data was published identifying 375 fission yeast genes that are regulated ≥2-fold by heat shock at 39°C for 1 hour [Chen, et al., 2003]. All the up-regulated genes that we identified are also up-regulated after heat shock. As might be expected, the magnitude of the changes induced by heat shock is generally greater than under temperature shift
conditions used in our study. Six of the 9 down-regulated genes that we detected are also down regulated in the heat shock experiment. The three genes that were not down-regulated in the heat shock data (gut2+, SPAC1002.19 and SPAP7G5.03) might differ due to legitimate physiological differences in the experiments. gut2+, for example, is transiently up-regulated in the 39C heat shock experiment (15 min., 5.3-fold up; 60 min., 1.9-fold up). It is therefore, possible that gut2+ mRNA levels might continue to drop as a consequence of the heat treatment, leading to down-regulation of expression at later time points, such as the two hour time point used in our experiments. The correspondence in these two data sets serves to validate our microarray results and confirms the notion that the small gene expression changes we measure are the result of a physiological adaptation to a minor heat shock.
ACKNOWLEDGEMENTS

We thank Val Wood at the Sanger Institute for her generous help in facilitating our access to and use of the fission yeast genome sequence. The work was supported by grants from the Swedish Research Council and the European Commission (QLRT-1999-30174). AW and KE are senior investigators supported by the Swedish Research Council and the Royal Swedish Academy of Sciences, respectively.
REFERENCES


FIGURE LEGENDS

Figure 1. Design and construction of a DNA microarray for fission yeast. (A) Representative ethidium-bromide-stained gel used for quality control of amplified fragments prior to spotting on the microarray. (B) Frequency distribution of amplified fragment size for all the designed fragments. (C) Exon content as a function of the proportion of the fragment that consists of intron sequence for each of the 153 fragments that contain introns. (D) Frequency distribution of distances between the 3’ end of each fragment and the 3’ end of the respective coding sequence. (E) Frequency distribution of the percent GC content for the amplified fragments. (F) Frequency distribution of the number of BLAST hits per fragment for the 266 fragments that recognise one or more non-cognate sequences in a BLAST search.

Figure 2. Detection of hybridisation signals using the fission yeast DNA microarray. (A) Strength of measurable hybridisation signals as a function of fragment size for fragments amplified within single exons. The grey spots show the mean values (Cy5 channel only) for each spot on the array, calculated from 10 independent experiments. Eight of the data sets are the same as those discussed in later Figures, two data sets come from a dye-swap experiment that was not analysed further. Since the experiments consisted of five experiments in each of the two dye orientations the mean values for Cy3 channel signals (not shown) were very similar to the Cy5 signals for each spot. The black spots show a moving average (window=201 values, step size=1) of the data to enhance any trends that occur in the signal level as a function of fragment
size. (B) Strength of measurable hybridisation signals as a function of intron content for intron-containing fragments. Annotations are as for (A) except that signals from both the Cy3 and Cy5 channels are plotted. (C) Strength of measurable hybridisation signals as a function of the distance from the 3’ end of the fragments to the 3’ end of the respective coding sequences. Annotations are as for (A). (D) Strength of hybridisation signal as a function of fragment position within the 3’ exon of rpb1+, encoding the large subunit of RNA polymerase II. Each column shows the mean value for spots (Cy5 signals only) from the ten hybridisation experiments (error bars = standard deviation). There are duplicate spots for each fragment. The size ranges, in parentheses, locate the position of the fragments within the exon sequence. The size and (GC content) of the rpb1-3’, rpb1-middle and rpb1-5’ fragments are 368bp (47%), 498bp (39%) and 400bp (45%), respectively. (E) Strength of measurable hybridisation signals as a function of the GC content of the amplified fragments. Annotations are as for (A). (F) Comparison of the levels of hybridisation to a 100% homologous act1+ (act1) fragment from fission yeast with a 80% homologous fragment from budding yeast (SC_ACT1). The bars show the mean of 20 measurements (duplicate spots on 10 arrays) and the standard deviation about the mean is shown by the error bars. The act1 and SC_ACT1 fragments are mainly overlapping and have a length and (GC content) of 413bp (45%) and 418bp (44%), respectively.

**Figure 3. Reproducibility of detecting gene expression changes in different labelling experiments.** (A) Comparison of normalised data from two different labelling and hybridisation experiments. Both sets of probes were produced from the same preparations of 972h- RNA. In each case, the 25°C sample was labelled with Cy3 and
the 36°C sample with Cy5. The dotted lines show the position of a two-fold deviation from the ideal 1:1 relationship between the two experiments. The plotted values are the mean of the values for duplicate spots. (B) Comparison of two different labelling and hybridisation experiments in which one set of samples was labelled in the alternative dye orientation (dye swap). The plot is identical to (A) except that the y-axis 25°C and 36°C samples were labelled with Cy5 and Cy3, respectively.

**Figure 4. Stringent quality control criteria as a test of microarray quality.** (A) The ratio of measurable (GenePix flag = 0), non-normalised 36°C (Cy5) to 25°C (Cy3) signals is plotted as a function of the product of the two signals for a representative data set. Note that there is a dye bias that is related to signal strength. In this experiment, weak signals are favoured in the Cy5 channel, while the Cy3 channel favours strong signals. As described in the Methods section, we used a Lowess normalisation [Cleveland and Devlin, 1988; Dudoit, et al., 2000] to correct for such global dye effects. (B) Red and black spots show the same distribution as (A). Red spots show signals eliminated by the four more stringent quality control criteria that are described in the Methods section. (C) Red and black spots show the spots that were not eliminated by the quality control criteria in (B). Red spots show spots eliminated when we retained only signals for which both spots pass the quality control criteria. (D) Signals for which both spots pass the quality control criteria after Lowess normalisation.

**Figure 5. Reproducibility of detecting gene expression changes in different cultures.** (A) Shows the number of genes that are up- or down-regulated by 2-fold or more at 36°C in two independent cultures of strain 367. In the Venn diagram, the green
intersection shows genes identified in both cultures, while the red and blue areas show genes identified in only one culture. (B) The mean fold changes for each culture are plotted against each other for the genes identified in (A). The colour-coding of the spots is the same as in (A).
<table>
<thead>
<tr>
<th>Number of BLAST Hits</th>
<th>Protein Family</th>
</tr>
</thead>
</table>
| 2                    | Alcohol Dehydrogenase  
|                      | Amino Acid Permease  
|                      | L-Asparaginase Precursor  
|                      | Casein Kinase Homologue  
|                      | Drug Efflux Transporter  
|                      | Glucose Transporter  
|                      | Glutathione S-Transferase  
|                      | Glyceraldehyde-3-Phosphate Dehydrogenase  
|                      | Histone H2A  
|                      | Inorganic Phosphate Transporter  
|                      | Pyridoxine-Pyridoxal-Pyridoxamine Kinase  
|                      | Ribosomal Protein  
|                      | Translation Elongation Factor (eft2)  |
| 3                    | Amino Acid Permease  
|                      | Drug Efflux Transporter  
|                      | Glucose Transporter  
|                      | Histone H3  
|                      | Histone H4  
|                      | Inorganic Phosphate Transporter  
|                      | Malate Oxidoreductase  
|                      | NAD-Dependent Malic Enzyme  
|                      | Ribosomal Protein  
|                      | Translation Elongation Factor (eft1)  
|                      | Wtf Element  |
| 4                    | Ribosomal Protein  |
| 5                    | Ubiquitin-like Protein  
|                      | Wtf Element  |
| ≥7                   | Tf2 Transposon  
|                      | Wtf Element  |
Table 2. Affect of increased spot quality criteria on the proportion of analysable genes.

<table>
<thead>
<tr>
<th></th>
<th>Proportion of Genes (%) ±SD (n=8)</th>
</tr>
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<tbody>
<tr>
<td>Detectable Genes</td>
<td>97.6 ± 0.2</td>
</tr>
<tr>
<td>Genes for which at least one spot passes QC</td>
<td>82.7 ± 12.0</td>
</tr>
<tr>
<td>Genes for which both spots pass QC</td>
<td>75.7 ± 16.7</td>
</tr>
<tr>
<td>Genes for which both spots pass QC and differ by &lt;15%</td>
<td>74.1 ± 16.8</td>
</tr>
</tbody>
</table>
Table 3. Genes with significantly (p \leq 0.001) changed expression by \geq 2-fold after a 2 hour shift to 36°C.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Mean Fold Change</th>
<th>Significance (p)</th>
<th>SD (n=8)</th>
<th>Heat Shock (39°C)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsp10</td>
<td>3.11</td>
<td>5.72E-5</td>
<td>38.85</td>
<td>1.95</td>
<td>putative heat shock protein</td>
</tr>
<tr>
<td>SPAC27D7.10c</td>
<td>2.87</td>
<td>7.42E-6</td>
<td>23.46</td>
<td>15.77</td>
<td>hypothetical protein</td>
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<tr>
<td>SPBC1347.11</td>
<td>2.66</td>
<td>1.29E-4</td>
<td>28.81</td>
<td>6.09</td>
<td>hypothetical protein</td>
</tr>
<tr>
<td>SPBC1711.08</td>
<td>2.56</td>
<td>3.16E-4</td>
<td>35.99</td>
<td>3.09</td>
<td>hypothetical protein</td>
</tr>
<tr>
<td>sti1</td>
<td>2.55</td>
<td>2.10E-4</td>
<td>29.37</td>
<td>4.15</td>
<td>activator of Hsp70 and Hsp90 chaperones</td>
</tr>
<tr>
<td>SPAC23G3.13c</td>
<td>2.53</td>
<td>3.38E-5</td>
<td>24.52</td>
<td>7.57</td>
<td>hypothetical protein</td>
</tr>
<tr>
<td>SPAC4H3.08</td>
<td>2.45</td>
<td>3.26E-5</td>
<td>29.21</td>
<td>91.35</td>
<td>putative short chain dehydrogenase</td>
</tr>
<tr>
<td>wis2</td>
<td>2.44</td>
<td>7.35E-4</td>
<td>38.50</td>
<td>3.68</td>
<td>heat shock inducible 40 kDa protein involved in the regulation of mitosis</td>
</tr>
<tr>
<td>SPCC576.03c</td>
<td>2.19</td>
<td>3.53E-4</td>
<td>32.08</td>
<td>3.62</td>
<td>thioredoxin peroxidase</td>
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<tr>
<td>hsp9</td>
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<td>1.75E-5</td>
<td>20.91</td>
<td>10.84</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>SPAC343.09</td>
<td>2.12</td>
<td>3.04E-4</td>
<td>29.59</td>
<td>2.84</td>
<td>possibly involved in glycogen metabolism</td>
</tr>
<tr>
<td>SPAC27D7.11c</td>
<td>2.04</td>
<td>5.64E-4</td>
<td>36.07</td>
<td>3.90</td>
<td>hypothetical protein</td>
</tr>
<tr>
<td>SPAC5H10.03</td>
<td>0.49</td>
<td>3.96E-7</td>
<td>10.97</td>
<td>0.23</td>
<td>putative phosphoglycerate mutase</td>
</tr>
<tr>
<td>gut2</td>
<td>0.47</td>
<td>1.54E-4</td>
<td>28.88</td>
<td>1.91</td>
<td>glycerol-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>SPAC1039.02</td>
<td>0.46</td>
<td>2.26E-6</td>
<td>16.07</td>
<td>0.27</td>
<td>putative calcineurin-like phosphoesterase</td>
</tr>
<tr>
<td>SPAC2E1P3.05c</td>
<td>0.44</td>
<td>1.38E-8</td>
<td>7.79</td>
<td>0.34</td>
<td>hypothetical protein</td>
</tr>
<tr>
<td>SPAC750.08c</td>
<td>0.42</td>
<td>9.38E-6</td>
<td>22.57</td>
<td>0.47</td>
<td>NAD-dependent malic enzyme-homolog 2</td>
</tr>
<tr>
<td>mae2</td>
<td>0.41</td>
<td>7.49E-7</td>
<td>15.56</td>
<td>0.45</td>
<td>malate oxidoreductase [NAD]</td>
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<tr>
<td>SPAC1002.19</td>
<td>0.40</td>
<td>2.37E-5</td>
<td>28.49</td>
<td>1.25</td>
<td>putative GTP cyclohydrolase (bacterial type)</td>
</tr>
<tr>
<td>SPAP7G5.03</td>
<td>0.34</td>
<td>6.79E-7</td>
<td>16.95</td>
<td>1.32</td>
<td>hypothetical protein</td>
</tr>
<tr>
<td>SPAC26H5.09c</td>
<td>0.30</td>
<td>9.05E-8</td>
<td>14.80</td>
<td>0.63</td>
<td>putative oxidoreductase</td>
</tr>
</tbody>
</table>

1 Standard deviation expressed as a percentage of the mean fold change  
Fig. 4

A

B

C

D