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Hybridization array technology coupled with chemostat culture: Tools to interrogate gene expression in *Saccharomyces cerevisiae*

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Abstract

Hybridization array technology is increasingly being used for the analysis of gene expression in the yeast *Saccharomyces cerevisiae*. It is a powerful technique in which the relative abundance of all the mRNA molecules transcribed under a particular condition may be simultaneously measured. However, most studies performed using this technique are carried out in batch culture where the growth rate and environment are continuously changing. Often, the experimental condition being studied also impacts on the growth rate of the cells. Changes in growth rate affect the pattern of gene expression. Consequently, the analysis and interpretation of experimental results obtained in this way are inherently problematic due to the difficulty in discriminating between effects due to the experimental condition per se and concomitant growth rate-related effects. Here, we present a method that addresses this problem by exploiting chemostat culture, in which the cells can be grown at a fixed growth rate, in combination with hybridization array technology. We use two experimental examples to illustrate the advantages of using this approach and then describe a specific application of this approach to investigate the effect of carbon and nitrogen limitation at the transcriptome level.

1. Introduction

A variety of techniques have been developed recently for the genomewide assessment of gene expression. Whether by the serial analysis of gene expression (SAGE) [1], differential display [2], or hybridization array [3,4], these techniques offer massively parallel approaches to unraveling the "bigger picture" of gene expression on a genomewide scale. Perhaps the most widely used of these techniques is currently that of hybridization arrays in which an ordered arrangement of DNA molecules tethered to a support matrix is used to quantitatively measure the abundance of labeled transcripts from a particular experimental condition. Several different variations on this theme have been developed and these have been comprehensively reviewed [5,6].

Since the inception of this technology, gene expression studies have been performed using baker's yeast, *Saccharomyces cerevisiae*. The reasons why yeast has emerged as a model organism are manyfold, not the least being the fact that it was the first eukaryotic organism for which the complete genome sequence was available [7] and the relative ease with which it may be cultured and genetically manipulated. For these reasons, the scientific literature abounds with research focusing on yeast gene expression studies using hybridization arrays [3,4,8–13]. All these studies, with the notable exception of ter Linde et al. [14], have been performed using batch culture systems. The drawback of this method is that it precludes the study of specific, individual, physiological parameters separated from any secondary effects. In batch culture systems, the growth environment is continuously changing. Secondary effects such as growth rate-dependent factors, therefore, tend to obscure the identification of genes that are really

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pertinent to a particular experimental treatment, mutation, or physiological condition. One way to address these issues is to use continuous chemostat cultures. Here, the growth conditions (and growth rate) are carefully controlled. This enables experiments to be performed in which a single parameter may be varied while all others are maintained constant.

In this article, we include two examples to illustrate this point and to highlight the advantages of using continuous culture over batch culture. First, yeast cells were grown in batch culture and in two chemostat cultures at two different specific growth rates. Labeled cDNAs were then synthesized from these three cultures and hybridized to genomewide macroarrays as described under Description of the Method. Portions of the array, naturally, revealed genes that were expressed at equivalent levels regardless of the growth conditions. However, genes were also evident whose expression was clearly dependent on the growth rate. Often, in transcriptome studies, the experimental condition under investigation (whether it be a particular mutation or addition of a drug) will impact the growth rate of the cells. Many changes in gene expression will be a consequence of that growth rate change and specific, primary effects of the treatment will be obscured. Thus, identification of genes directly involved in the process under investigation is inherently problematic. To further illustrate this point, the second example details experiments comparing a mutant yeast strain with the corresponding wild type. The mutant strain carried a mutation in the region encoding the DNA binding domain of the Mcm1 protein. Both mutant and wild type strains were grown first in batch and then in continuous chemostat culture. A set of genes that were upregulated in the mutant with respect to the wild type were identified from the chemostat experiments. In contrast, the corresponding batch cultures failed to reveal this gene set.

Having illustrated the advantages of chemostat culture in gene expression studies, the second part of the Results section describes an application for this approach in which we examine gene expression under different nutritional limitations.

2. Description of the method

2.1. Yeast strains

Three strains of *S. cerevisiae* were used in this study. Strain FY1679 was the homozygous diploid strain generated by crossing FY23 and FY73 strains [15] in each of which the *HO* locus had been replaced with the *kan*MX4 marker as described [16]. For the *MCM1* work the L68E mutant strain and corresponding wild type were derived from strain YY1901. The wild type has the genotype *MAT*α, *leu2-3*, *112*, *trp1-1*, *his3-11*, *15*, *ura3*, *ade2-1*, *can1-100*, *MCM1::LEU2*/pAS797, whereas the L68E strain has the plasmid pAS1210 instead of pAS797.

2.2. Culture conditions

Yeast strains were grown in fermenters essentially as described [17]. The vessels used were Applikon fermenters equipped with ADI 1030 controllers (FT Applikon Ltd., UK) with a 1-liter working volume. The cultures were stirred at 750 rpm, the temperature was controlled to 30 °C, and the pH was automatically controlled to 4.5 using 2.5 M NaOH. Aeration was maintained at 1 v.v. m^{-1}. A defined minimal medium was used that contained per liter: KH_2PO_4 (2.0 g), $MgSO_4 \cdot 7H_2O$ (0.55 g), NaCl (0.1 g), $CaCl_2 \cdot 2H_2O$ (90 mg), $ZnSO_4 \cdot 7H_2O$ (70 μg), $FeCl_3 \cdot 6H_2O$ (50 μg), $CuSO_4 \cdot 5H_2O$ (10 μg), H_3BO_3 (10 μg), KI (10 μg), inositol (62 mg), thiaminhydrochloride (14 mg), pyridoxine (4 mg), calcium pantothenate (4 mg), biotin (0.3 mg) and uracil (20 mg). The carbon and nitrogen sources were glucose and ammonium sulfate respectively. For carbon-limited medium, 2.5 g/liter glucose and 3.13 g/liter ammonium sulfate were used. For nitrogen-limited medium, 21 g/liter glucose and 0.46 g/liter ammonium sulfate were used.

The carbon source was sterilized separately, then added to the medium. The vitamin component was also prepared separately and sterilized by filtration before addition to the medium. A stationary-phase culture (10 ml) was used to inoculate the fermenter vessels. Batch culture samples were harvested at an OD_{600} of 1.0. Following batch growth, as the cultures approached the end of the exponential phase, the fermenters were switched to continuous culture at a dilution rate of 0.1 h^{-1}. Steady-state conditions were deemed established once biomass levels remained constant over three retention times. Following biomass sampling, the dilution rate was increased to 0.2 h^{-1} and the cultures were allowed to achieve a steady state.

2.3. RNA extraction

Many methods are available, based on chemical and/or physical disruption techniques, for the extraction of total RNA from yeast cultures. The method we have adopted, based on that of Hauser et al. [18], uses mechanical cellular disruption at very low temperature. A monophasic, chaotropic agent is then used to extract the RNA. This method has proven to be rapid and very efficient and, most importantly, yielded RNA that was reverse transcribed with consistently high efficiency. The labeled cDNA targets synthesized in this manner produced consistently reproducible results in subsequent hybridizations.

1. Biomass samples (20 ml) were taken via the sample port of the Applikon fermenters. The cells were pelleted by centrifugation for 5 min at 5000 rpm. The supernatant was removed and the RNA pellet resuspended in the residual medium to form a slurry. This was added in a dropwise manner directly into a 5-ml Teflon flask (B. Braun Biotech, Germany) containing liquid nitrogen and a 7-mm-diameter tungsten carbide ball. After allowing evaporation of the liquid nitrogen the flask was reassembled and the cells disrupted by agitation at 1500 rpm for 2 min in a Microdismembranator U (B. Braun Biotech, Germany).
2. The frozen powder was then dissolved in 1 ml of TriZol reagent (Sigma–Aldrich, UK), vortexed for 1 min, and then kept at room temperature for a further 5 min.
3. Chloroform extraction was performed by addition of 0.2 ml chloroform, shaking vigorously for 15 s, then 5 min incubation at room temperature.
4. Following centrifugation at 12,000 rpm for 5 min, the RNA (contained in the aqueous phase) was precipitated with 0.5 vol of 2-propanol at room temperature for 15 min.
5. After further centrifugation (12,000 rpm for 10 min at 4 °C) the RNA pellet was washed twice with 70% (v/v) ethanol, briefly air-dried, and redissolved in 0.5 ml diethyl pyrocarbonate (DEPC)-treated water.
6. The single-stranded RNA was precipitated once more by addition of 0.5 ml of LiCl buffer (4 M LiCl, 20 mM Tris–HCl, pH 7.5, 10 mM EDTA), thus removing tRNA and DNA from the sample.
7. After precipitation (20 °C for 1 h) and centrifugation (12,000 rpm, 30 min, 4 °C), the RNA was washed twice in 70% (v/v) ethanol prior to being dissolved in a minimal volume of DEPC-treated water. Total RNA extracted using this procedure was used to synthesize labeled targets for hybridization to macro- and microarrays.

2.4. Labeled target synthesis

For generating labeled targets, two essentially similar protocols were employed depending on the type of label being used. For radioactively labeled first-strand cDNA targets, the method of Nguyen et al. [19] was used. In the case of fluorescently labeled targets, the method of Hegde et al. [20] was used. [The authors of the latter paper also provide Internet access to their protocols [21].] Two modifications to the published methods were used. First, the amount of starting material (total RNA) was increased to 50 μg, and second, an anchored oligonucleotide (oligo $dT_{15}VN$, where V is any nucleotide except thymidine, and N is any nucleotide) was used as a primer for the first-strand syntheses. These modifications resulted in consistently higher specific incorporation of labeled nucleotides.

2.5. Hybridization arrays

Both membrane-based macroarrays, and glass slide-based microarrays were used. Each contained spotted DNA in the form of PCR products representative of each open reading frame (ORF) of the yeast genome. More specifically, the macroarrays (which were used in conjunction with radiolabeled targets) contained PCR products spanning the full length of each ORF spotted in duplicate onto polypropylene membranes. Details are provided in Ref. [18]. The microarrays contained single copies of PCR products immobilized onto coated glass microscope slides and were used with fluorescently labeled targets.

The fundamental differences in the two hybridization systems necessitated the use of two different hybridization and washing regimens. For macroarrays, radiolabeled targets were synthesized from each RNA preparation and hybridized to membranes in a serial manner. This experimental design is particularly appropriate when a large number of samples are being compared. Careful consideration, however, must be given to choosing appropriate normalization routines to allow quantitative data comparisons. Membranes were hybridized with target from the first RNA, the image was acquired, the array was stripped and then rehybridized with the target generated from a second RNA, and so on according to the following procedure:

2.6. Macroarray hybridizations

1. The membranes were incubated in glass tubes rotated in a hybridization oven (Techne, UK) at 68 °C for 1 h in 50 ml of prewarmed prehybridization solution (0.25 M Na_2HPO_4 pH 7.2, 1 mM EDTA, 20% w/v sodium dodecyl sulfate (SDS), and 0.5% w/v freshly added Boehringer blocking reagent). The solution was then decanted and replaced with 15 ml of fresh prewarmed solution to which the denatured probe was added. Alternatively, formamide-based systems could be used that allow a lower hybridization temperature (42 °C). In our hands, however, the nonformamide, aqueous system resulted in the lowest nonspecific background signal.
2. Following 16 h incubation at 68 °C, the membrane was washed three times for 20 min each at 65 °C with 50 ml of prewarmed washing buffer (20 mM Na_2HPO_4 pH 7.2, 1 mM EDTA, 1% w/v SDS).
3. The membrane was then wrapped in Saran wrap and exposed to a storage phosphor screen prior to image acquisition using a Storm 860 phosphorimager (Molecular Dynamics Inc., USA). To reuse the membranes the signal was removed by placing the membrane in 500 ml of boiling stripping buffer (5

mM sodium phosphate buffer, pH 7–7.5, 0.1% w/v SDS). The membranes were then stored in stripping buffer prior to reuse.

For the microarrays, a different regimen was used. Here, labeled targets were generated from each RNA preparation (labeled with either Cy3- or Cy5-dUTP) and cohybridized against the arrays in a pairwise manner on a single-use basis according to the protocol outlined below. This technique is particularly useful for generating ratio-based measurements when two experimental conditions such as a mutant *versus* wild type are being compared.

2.6.1. Microarray hybridizations

1. Two labeled targets, each in 9.25 μl of distilled water, were combined and added to 6.25 μl of 20× SSC. Following denaturation, 1.25 μl of SDS solution (10% w/v) was added, the solution pipetted onto the surface of the microarray, and a glass microscope slide coverslip placed over the array. Automated machines such as the GeneTac HybStation (Genomic Solutions Inc., Michigan) are available with which to perform the hybridization of such arrays. In our hands, a somewhat "lower-tech" (but far cheaper) option provided consistently reproducible results. Plastic sandwich boxes were used to provide a humid environment to prevent drying out of the slides during hybridization. The base of the container was lined with tissue paper that had been wetted with 5 ml of distilled water. The array was placed onto glass pipets resting on top of the prewetted tissue papers. The plastic container was then sealed and incubated at 62 °C overnight.
2. Following hybridization, the array was placed in a Coplin jar containing washing solution I (2× SSC/0.1% w/v SDS) for 10 min at room temperature. With gentle agitation of the vessel, the coverslip was allowed to slide off the array.
3. The array was then transferred to another Coplin jar, containing wash solution II (0.2× SSC/0.1% w/v SDS), and incubated at 60 °C for 10 min. A further wash was carried out in another Coplin jar, containing wash solution II, at room temperature for 10 min.
4. The slides were then rinsed briefly, first in 50 ml of 0.2× SSC and then in 50 ml of 0.05× SSC, before being dried with the aid of a stream of nitrogen.

Important considerations when using this two-color approach are the potential for preferential incorporation and differences in stability and photolability of the different fluorescent labels. Consequently, spurious results maybe obtained depending on which label is used for the "experimental" sample and which for the "control." To compensate for these phenomena, it is important to ensure that the labels used for the "experimental" and the "control" samples are reversed in replicate experiments.

2.7. Image analysis

Images of macroarrays were acquired by scanning at 50-μm resolution using a Storm 860 phosphorimager (Molecular Dynamics Inc., USA). The spots on these images were then detected and quantified using the ArrayVision Version 4.0 Rev. 1.1 software (Imaging Research Inc., Canada). For the microarrays, the images were acquired using a GenePix 4000A microarray scanner (Axon Instruments Inc., USA), and the spots quantified using GenePix Pro 3.0 software.

2.8. Results

2.8.1. Advantages of using chemostat culture

Strain FY1679 yeast cells were grown in carbon-limited medium under three different culture conditions and harvested as described earlier. The conditions were batch culture, chemostat culture at a dilution rate (and hence specific growth rate) of 0.1 h^{-1}, and chemostat culture at a higher specific growth rate of 0.2 h^{-1}. Radiolabeled cDNAs were synthesized from RNA from these three cultures and hybridized to genomewide macroarrays. Portions of these arrays are shown in Fig. 1. Naturally, these revealed genes that were expressed at equivalent levels regardless of the growth conditions. However, genes were also evident whose expression was clearly dependent on the growth rate. These observations have wide-ranging implications for transcriptome studies. In many cases, the experimental conditions under investigation will affect the growth rate of cells grown in batch culture. This will also elicit concomitant effects such as expression of growth rate-related genes, as shown in Fig. 1. In chemostat cultures, however, this would not be the case since the specific growth rate can be maintained at the same level throughout the course of the experiment.

To further illustrate this point, a second experiment was performed in which the transcriptome of a yeast strain, carrying a mutation in the region encoding the DNA binding domain of Mcm1p, was compared with that of the corresponding wild-type strain. Mcm1 is a transcription factor that is involved in regulating multiple processes including the transcription of key cell cycle genes. We have identified a mutation (L68E) that leads to alterations in DNA binding characteristics, transcriptional defects, and slow growth of the yeast (F.L.L. and A.D.S., unpublished data). Both mutant and wild-type strains were grown using the same growth medium in both batch culture and continuous chemostat culture. Fluorescently labeled cDNA targets were generated from both mutant and wild-type strains and hybridized to microarrays as described. In the case of the

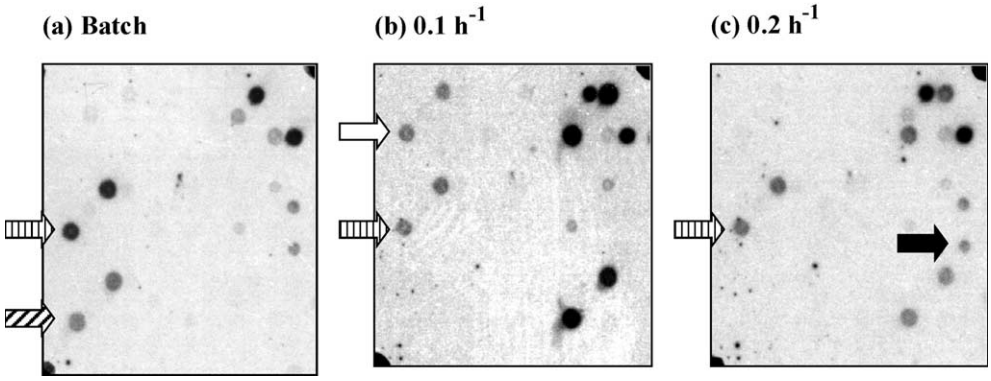

Fig. 1. Subsections of *S. cerevisiae* macroarrays. Radiolabeled cDNAs were synthesized from RNA extracted from yeast cells growing at: (a) mid-exponential phase of batch growth, (b) steady-state chemostat culture $D = 0.1\ h^{-1}$, and (c) steady-state chemostat culture $D = 0.2\ h^{-1}$. The expression of certain genes is dependent on the growth conditions: ▨, batch only; ▬, 0.2 h^{-1} chemostat and batch; ▭, 0.1 h^{-1} chemostat. The expression of other genes (▥) is independent of the culture conditions.

batch culture (Fig. 2a) the vast majority of genes for which transcripts were detectable were downregulated in the mutant with respect to the wild-type strain. This observation is not unexpected since the growth rate is reduced in this mutant; all the growth rate-associated genes would likely be downregulated also. It is therefore inherently difficult to distinguish genes whose regulation is dependent on the *mcm1* mutation per se from those whose regulation occurs simply as a consequence of the growth rate changes. In the case of the chemostat-grown cultures, however, the situation was completely different. Here, the specific growth rates for both strains were maintained at the same level. Any differences between the expression profiles of the two strains would therefore be directly attributable to the mutation in question and not be a secondary consequence of the change in growth rate. As Fig. 2b illustrates, the chemostat experiments identified a set of genes that were upregulated in the mutant with respect to the wild type. In contrast, the corresponding batch cultures failed to reveal these genes.

2.8.2. Nutrient limitation, growth rate, and gene expression

Having illustrated the advantages of using chemostat culture, we undertook a series of experiments to examine the effects of different nutritional limitations and specific growth rates on gene expression in *S. cerevisiae*. To achieve this a series of chemostat cultures were performed using different nutritional limitations as described earlier. In the first phase of this experiment, total RNA samples were taken from steady-state cultures at a dilution rate of 0.2 h^{-1}. Macroarray hybridizations were then performed as described and a series of informatic

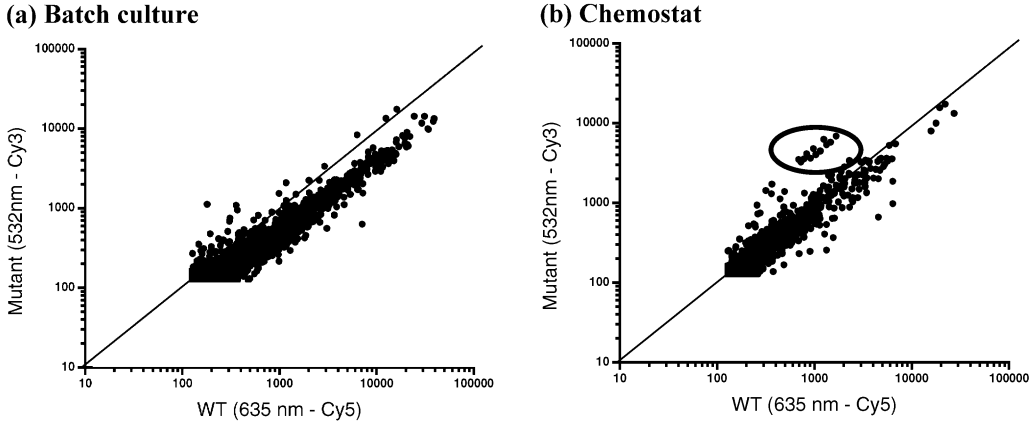

Fig. 2. Relative gene expression profiles of WT and *mcm1* (L68E) mutant strains. RNA was extracted from both mutant and wild-type yeast cells grown in both batch and chemostat cultures. The mutant strain harbored the L68E mutation in the DNA binding domain of Mcm1p. Microarray hybridizations were performed, and the abundance of transcripts in the mutant is plotted against the corresponding level in the wild type. In the chemostat-grown cultures (b) a group of genes were revealed (circled) that were upregulated in the mutant strain. In the case of the batch cultures (a) this cluster of genes was not revealed. Moreover, in batch growth, significantly more genes appeared to be upregulated in the wild type with respect to the mutant than was the case for the chemostat cultures.

analyses undertaken. The aims of each these analyses are described in the following section.

2.9. League tables of expression levels

As a primary analysis, a pairwise comparison of the steady-state nitrogen- and carbon-limited transcriptomes was undertaken. Those ORFs that were expressed at higher levels in one condition with respect to the other were arranged into "league tables" on the basis of their fold regulation (Table 1). To illustrate this approach we can examine, as an example, the well-characterized, low-affinity, hexose transporter *HXT1*, which is known to be induced by high levels of glucose [22]. In the case of the nitrogen-limited condition, the glucose concentration is relatively high. Consequently, under these conditions, *HXT1* is preferentially expressed at a level greater than eight fold higher (Table 1b) than is found in the carbon-limited condition. There is, however, a caveat to the interpretations of these results, namely, increased expression of a gene under nitrogen-limited conditions (with respect to the carbon-limitation condition) may be due to nitrogen limitation or carbon sufficiency. To address this issue we have carried out a series of transcript profile experiments using chemostat cultures grown under a variety of nutritional limitations in which neither carbon nor nitrogen was limiting. The results from these experiments are beyond the scope of this article and will be published elsewhere. However, these data did confirm that overexpression of all but five of the genes listed in Table 1a (*ISF1*, *CBP4*, *YMR31*, *YGL015c*, and *CYT1*) was due to carbon limitation per se. The situation was somewhat different in the case of the nitrogen-limited league table (Table 1b). For these genes nitrogen limitation alone was not sufficient to induce overexpression. By examining the preferential expression of an individual gene under a particular condition one can then infer that the function of that gene is, in some way, related to that condition. This gives vital clues in assigning categories to genes that hitherto had no known function.

Table 1
"League table" of ORFs[a]

Gene	Function	Fold Regulation
(a) Carbon limitation		
HXK1	Hexokinase I (PI) (also called hexokinase A)	59.86
YLR327c		**55.56**
YGR243w		**30.16**
HXT6	Hexose transporter	26.97
HXT7	Hexose transporter	21.25
YER067w		**13.77**
ALD4	Mitochondrial aldehyde dehydrogenase	11.73
YGL037c		**11.70**
GLK1	Glucokinase	7.56
CIT1	Citrate synthase; nuclear encoded mitochondrial protein	6.87
COR1	44-kDa core protein of yeast coenzyme QH2 cytochrome *c* reductase	6.33
ISF1	Involved in mitochondrial RNA splicing	6.20
DLD1	Mitochondrial enzyme D-lactate ferricytochrome c oxidoreductase	5.99
CBP4	Ubiquinol–cytochrome c reductase assembly factor	5.51
YMR31	Mitochondrial ribosomal protein (precursor)	5.51
YGL015c		**5.38**
CYT1	Cytochrome c_1	5.23
(b) Nitrogen limitation		
ADH4	Alcohol dehydrogenase isoenzyme IV	10.21
YGL258w		**10.10**
YDR033w		**9.32**
HXT1	High-affinity hexose (glucose) transporter	8.92
YHL047c		**6.03**
RAD54	DNA-dependent ATPase	5.68
YOL153c		**5.23**
RHR2	DL-Glycerol-3-phosphatase	4.48
YPR157w		**3.78**
YOR107w		**3.77**
YGR138c		**3.68**
YRO2	Homolog to HSP30 heat shock protein YRO1 (*S. cerevisiae*) 7	3.60
ARC40	Component of Arp2∨Arp3protein complex	3.58
TID3		3.44
ZRT1	High-affinity zinc transport protein	3.28
YGR245c		**3.18**
NIT1	Nit1 nitrilase	3.09

[a] Preferentially expressed under conditions of (a) carbon- and (b) nitrogen-limited chemostat culture at the same dilution rate (0.2 h^{-1}). This type of analysis gives new information for the possible function of genes (highlighted in bold face type) whose function was previously unknown.

2.10. Spatial organization on chromosomes

To investigate whether the regulation of gene expression is influenced by the position of a gene on the chromosome we considered those genes that were differentially expressed between the two conditions and examined their spatial arrangements on the chromosomes. As shown in Fig. 3, and in agreement with previous work [23,24], we found no major effects of the position of an ORF on a chromosome and its level of expression. The graphic in Fig. 3 does, however, illustrate the fact that more genes were upregulated (and to higher levels) in the carbon-limited condition. This suggests that the yeast cells call more heavily on their genetic repertoire in this state than in the nitrogen-limited condition.

2.11. Promoter sequence analyses

To identify potential regulatory regions involved in the response to nutritional status we examined the DNA

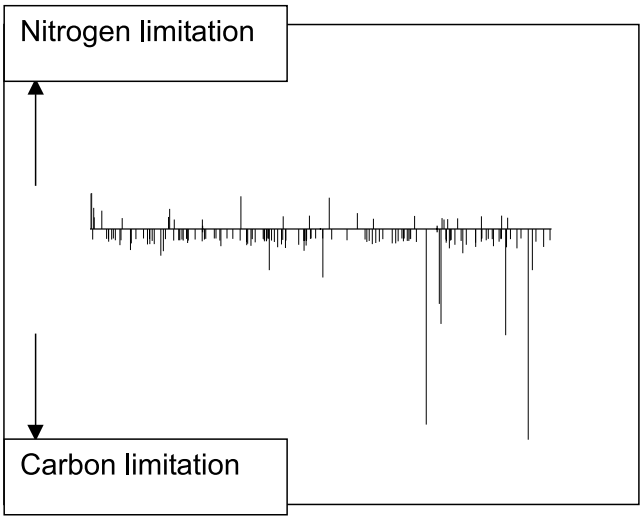

Fig. 3. "Universal" yeast chromosome. All 16 yeast chromosomes were graphically superimposed and normalized to unit length to represent a "universal" chromosome. Those genes that were preferentially expressed under conditions of nitrogen- or carbon-limited chemostat culture were plotted onto this idealized chromosome according to their position relative to the centromere. Each bar represents an individual gene. The height of the bars is proportional to the level of upregulation in the relevant condition.

sequences upstream of genes that were preferentially expressed under conditions of nitrogen- and carbon limitation. Using the regulatory sequence analysis (RSA) tools software [25] sequences upstream of each ORF were retrieved and then analyzed for statistically overrepresented oligonucleotide sequences. Fig. 4 shows a sample of the output from these analyses. The complete output is available on our web site (http://www.yeastresearch.man.ac.uk). The hexanucleotide sequence ARAAAA is overrepresented within the upstream regions of N-limited specific genes and therefore potentially plays a role in the regulation of these genes. In contrast, the carbon-limit specific genes displayed a completely different set of overrepresented sequences. Further experimental work, such as site-directed mutagenesis studies, could then be used to determine whether or not these oligonucleotide sequences actually play an active role in the promoter regions of these genes.

2.12. Functional categories

The Munich Information Centre for Protein Sequences (MIPS) curates a catalog of yeast ORFs which have been classified into categories, according to information about their function [26]. To identify the cellular processes involved in the response to different nutritional states we examined those genes that were preferentially expressed in either nitrogen- or carbon-limited conditions at the same specific growth rate (0.2 h^{-1}). These were then assigned to functional categories according to their MIPS classification. The distribution of these categories was then represented as color-coded pie charts (Fig. 5). On a global scale the two conditions show a broadly similar distribution of functional cate-

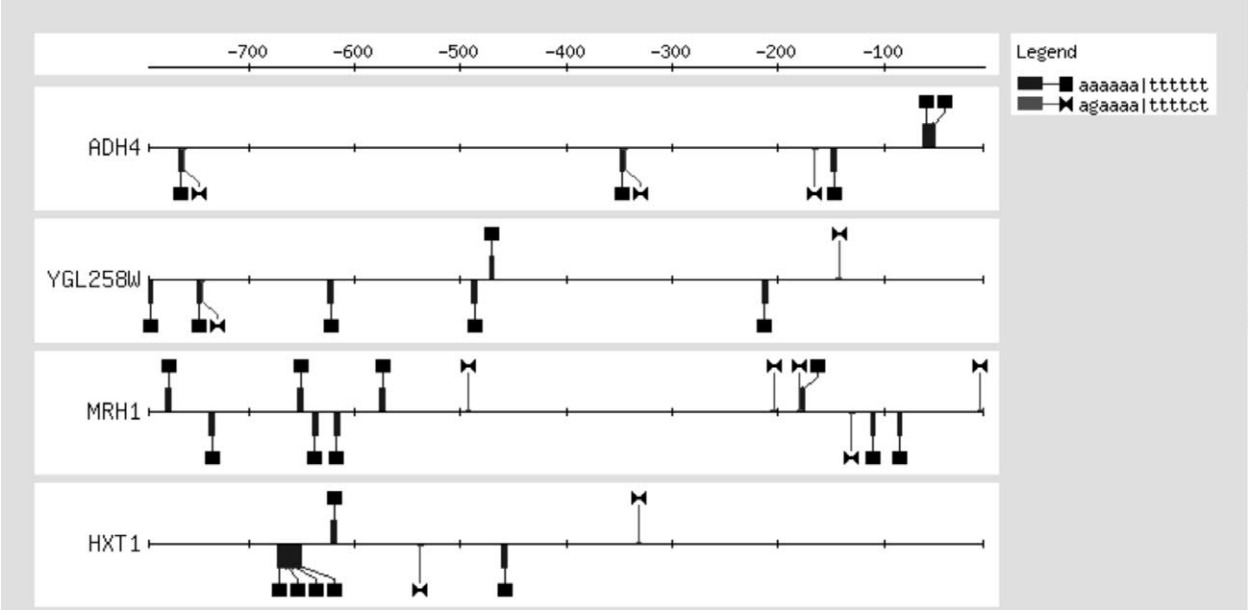

Fig. 4. Distribution of overrepresented hexanucleotide sequences. RSA tools software was used to analyze the upstream regions of N-limit specifically upregulated ORFs. Black horizontal bars represent the regions from the ORF to −800 nucleotides upstream of the start codon. The complete output from this analysis is available on the web site (http://www.yeastresearch.man.ac.uk).

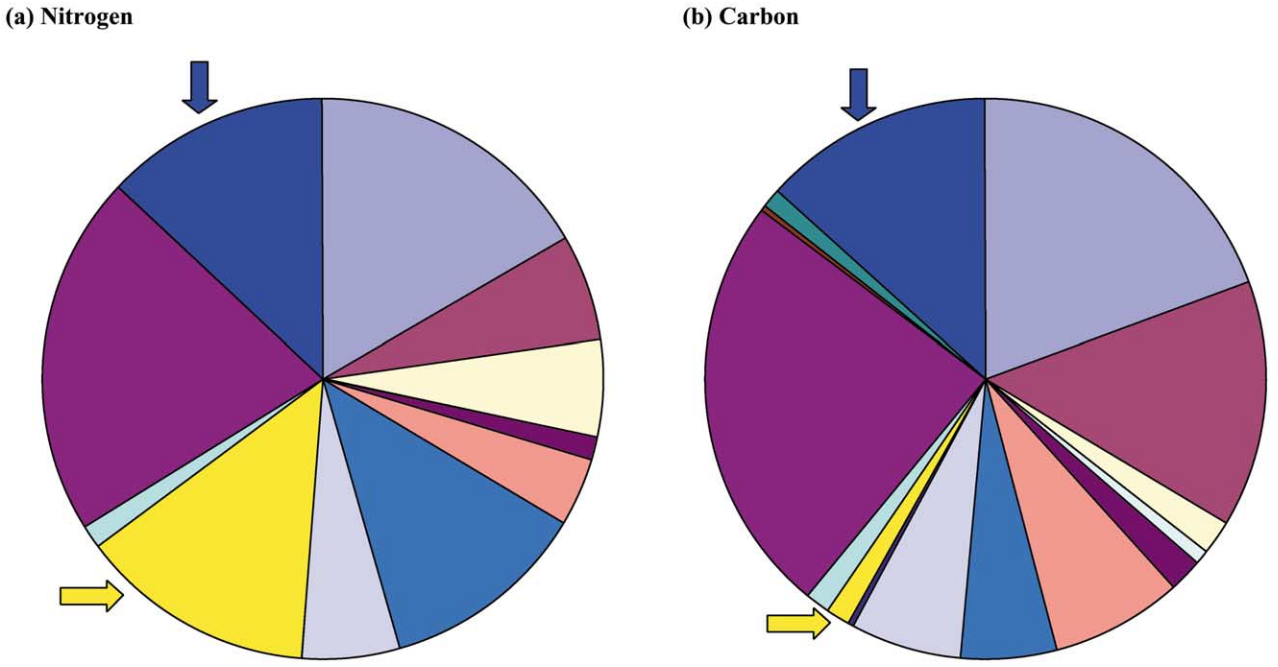

Fig. 5. Distribution of the functional categories of ORFs. ORFs that are preferentially expressed under conditions of (a) nitrogen and (b) carbon limitation at the same dilution rate (0.2 h^{-1}). ⬇, "Unclassified proteins" category. ⇨, "Cell rescue" category. The complete figure together with the identities of the remaining functional categories is available on the web site (http://www.yeastresearch.man.ac.uk).

gories. A notable exception is that of "cell rescue." The fractional contribution of this category, which includes stress responses and DNA damage/repair mechanisms, is much larger under conditions of nitrogen-limitation. The category of "unknown proteins" appears similarly represented under both conditions.

2.13. Genes with growth rate-related expression profiles

In the final phase of our experiment, we expanded the analyses to encompass not only the two different nutritional states but also two different specific growth rates. This enabled us to identify clusters of genes that were associated with changes in growth rate. Transcriptomes were examined from four experimental conditions (carbon and nitrogen limitation at dilution rates of both 0.1 h^{-1} and 0.2 h^{-1}). The "profiles" of expression of each gene were then compared over these four conditions. An ORF was selected that followed a particular profile regardless of nutritional limitation (Fig. 6a). The ORF YKL038w (*RGT1*) was selected since its expression level was relatively low (<40 arbitrary units) at the lower growth rate and high (>600 arbitrary units) at the higher rate. Using software tools MaxDView [27] and GeneSpring 3.0 [28], this profile was saved and used as a pattern to identify other genes whose expression followed a similar, growth rate-specific profile (Fig. 6b). Identifying such a set of genes provided further insight both into the function of these genes and into the molecular basis of growth rate control.

3. Concluding remarks

The combination of steady-state chemostat cultures with hybridization arrays provides a powerful tool for the analysis of lower eukaryotic gene expression. By combining highly parallel array technology with carefully controlled and defined physiological conditions, an insight into the impact of changes to a single parameter on global gene expression is gained. This is providing fundamental clues to cellular function at the transcript level. Moreover, from the functional genomics perspective, these techniques yield vast amounts of information regarding the functions of novel genes.

Using the techniques described here, experimental growth conditions can be carefully controlled, thereby greatly reducing the complexity of the system and allowing a much more focused analysis of the factors affecting gene expression. The techniques under pinning hybridization array technology are maturing and generating enormous amounts of data. The biggest challenge now seems to lie with bioinformaticians for the development of robust software tools with which to analyze and interpret these data. Developments in this field are progressing rapidly. Indeed, concerted international efforts, such as the Microarray Gene Expression Database (MGED) group [29], are well underway to standardize microarray experiments and data and to establish databases and software tools. As these tools become available, researchers should be able to exploit the full potential of microarray technology.

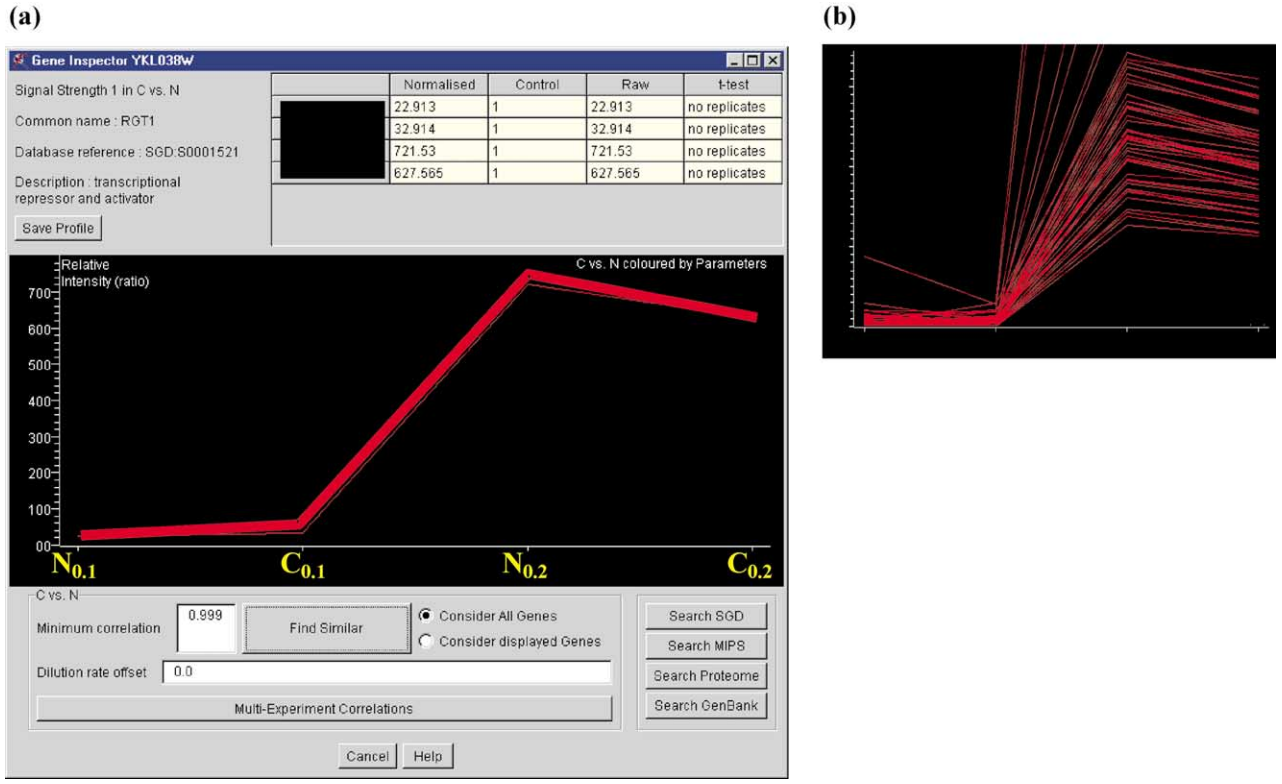

Fig. 6. Gene expression profiles over multiple growth conditions: $N_{0.1}$, nitrogen-limited chemostat $D = 0.1\ h^{-1}$; $C_{0.1}$, carbon-limited chemostat $D = 0.1\ h^{-1}$; $N_{0.2}$, nitrogen-limited chemostat $D = 0.2\ h^{-1}$; $C_{0.2}$, carbon-limited chemostat $D = 0.2\ h^{-1}$. An ORF was selected (in this case YKL038w) that was expressed at low levels at the lower dilution rates and high levels at the higher dilution rates regardless of nutritional limitation. (a) Screen shot from GeneSpring software. Using the software the expression profile of this gene, depicted by the red line, was saved and used to mine the rest of the data set. (b) Expression profiles of a set of genes that followed a trend similar to that of YKL038w.

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