THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 10, Issue of March 15, pp. 9125–9138, 2004

Printed in U.S.A.

Role of Transcriptional Regulation in Controlling Fluxes in Central Carbon Metabolism of *Saccharomyces cerevisiae*

A CHEMOSTAT CULTURE STUDY*

Received for publication, August 28, 2003, and in revised form, November 20, 2003
Published, JBC Papers in Press, November 20, 2003, DOI 10.1074/jbc.M309578200

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In contrast to batch cultivation, chemostat cultivation allows the identification of carbon source responses without interference by carbon-catabolite repression, accumulation of toxic products, and differences in specific growth rate. This study focuses on the yeast *Saccharomyces cerevisiae*, grown in aerobic, carbon-limited chemostat cultures. Genome-wide transcript levels and *in vivo* fluxes were compared for growth on two sugars, glucose and maltose, and for two C2-compounds, ethanol and acetate. In contrast to previous reports on batch cultures, few genes (180 genes) responded to changes of the carbon source by a changed transcript level. Very few transcript levels were changed when glucose as the growth-limiting nutrient was compared with maltose (33 transcripts), or when acetate was compared with ethanol (16 transcripts). Although metabolic flux analysis using a stoichiometric model revealed major changes in the central carbon metabolism, only 117 genes exhibited a significantly different transcript level when sugars and C2-compounds were provided as the growth-limiting nutrient. Despite the extensive knowledge on carbon source regulation in yeast, many of the carbon source-responsive genes encoded proteins with unknown or incompletely characterized biological functions. *In silico* promoter analysis of carbon source-responsive genes confirmed the involvement of several known transcriptional regulators and suggested the involvement of additional regulators. Transcripts involved in the glyoxylate cycle and gluconeogenesis showed a good correlation with *in vivo* fluxes. This correlation was, however, not observed for other important pathways, including the pentose-phosphate pathway, tricarboxylic acid cycle, and, in particular, glycolysis. These results indicate that *in vivo* fluxes in the central carbon metabolism of *S. cerevisiae* grown in steady-state, carbon-limited chemostat cultures are controlled to a large extent via post-transcriptional mechanisms.

The yeast *Saccharomyces cerevisiae* is widely used as a model organism to study carbon source-dependent metabolic regulation in eukaryotes. Wild-type *S. cerevisiae* strains have a narrow set of carbon sources that can support fast growth in synthetic media (1). The most widely studied of these are the hexoses glucose, fructose, galactose, and mannose, the disaccharides maltose and sucrose, and the C2-compounds ethanol and acetate. The metabolic networks employed for the metabolism of the hexoses and disaccharides are very similar and differ only in the initial steps of metabolism (Fig. 1). For example, glucose and maltose metabolism differ only with respect to two reactions. The first reaction is the sugar transport through the plasma membrane; maltose uptake is catalyzed by an energy-dependent maltose-proton symport mechanism (Fig. 1, step 30) (2), whereas glucose uptake is catalyzed exclusively by a facilitated diffusion mechanism (step 33) (3). The second reaction is the intracellular breakdown of maltose into glucose, which involves a specific α-glucosidase ("maltase," step 29) (4). Similarly, the metabolism of the C2-compounds ethanol and acetate only differ by the initial substrate-uptake step (steps 31 and 32) (5–7) and by two sequential $NAD(P)^+$-dependent oxidation reactions that convert ethanol into acetate (steps 22 and 23).

Drastic changes are observed in central metabolism when the metabolism of sugars is compared with that of the C2-compounds. During growth on sugars, all metabolic building blocks can be derived from glycolysis, tricarboxylic acid cycle, and pentose phosphate pathway. During growth on C2-compounds, gluconeogenesis and glyoxylate cycle are essential for the provision of some of these precursors. Furthermore, the higher ATP requirement for biosynthesis during growth on the C2-compounds (and in particular acetate (Ref. 8)) implies that, at a fixed specific growth rate, dissimilatory fluxes have to be higher with the C2-compounds than with a sugar as the sole carbon source.

So far, most studies on regulation of central carbon metabolism in *S. cerevisiae* have been performed in batch mode in shake-flask or reactors. This cultivation method, however, has several drawbacks for quantitative analysis. First of all, the concentrations of substrates and products change throughout cultivation, which makes it difficult to accurately measure fluxes through specific pathways or to assess the influence of carbon sources on cellular regulation. Furthermore, batch cultivation by definition requires the use of excess concentrations of the carbon source. When different carbon sources are compared, this will lead to different specific growth rates, which, in itself, may already lead to changes in the make-up and activity of the metabolic network. Finally, the relatively high substrate concentrations in batch cultures lead to catabolite repression and inactivation phenomena (9, 10).

Cultivation of microorganisms in chemostats offers numerous advantages for studying the structure and regulation of metabolic networks (11). In chemostat cultures, individual culture parameters can be changed, while keeping other relevant phys-

* This work was supported by a Technologiestichting STW grant and by a Marie-Curie fellowship (to P. D.-L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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ical and chemical culture parameters (composition of synthetic medium, pH, temperature, aeration, etc.) constant. An especially important parameter in this respect is the specific growth rate, which, in a chemostat, is equal to the dilution rate, which can be accurately controlled. This allows the experimenter to investigate the effects of environmental changes or genetic interventions at a fixed specific growth rate, even if these changes result in different specific growth rates in batch cultures. In a chemostat, growth can be limited by a single, selected nutrient. The very low residual concentrations of this growth-limiting nutrient in chemostat cultures alleviate effects of catabolite repression and inactivation. Furthermore, these low residual substrate concentrations prevent substrate toxicity, which, for example, occurs when *S. cerevisiae* is grown on ethanol or acetate as the carbon source in batch cultures (12, 13).

The central goal of the present study is to assess to what extent carbon source-dependent regulation of fluxes through central carbon metabolism in *S. cerevisiae* is regulated at the level of transcription. To this end, we compare the transcriptome of carbon-limited, aerobic chemostat cultures grown on four different carbon sources: glucose, maltose, ethanol, and acetate. Data from the transcriptome analysis are compared with flux distribution profiles calculated with a stoichiometric metabolic network model. Questions that will be addressed are as follows: (i) does glucose-limited aerobic cultivation lead to a complete alleviation of glucose-catabolite repression; (ii) how (in)complete is our understanding of the genes involved in the transcriptional response of *S. cerevisiae* to four of the most common carbon sources for this yeast; and (iii) to what extent do transcriptome analyses with microarrays provide a reliable indication of flux distribution in metabolic networks?

The complete data set used in this study is available for download at www.bt.tudelft.nl/carbon-source.

EXPERIMENTAL PROCEDURES

Strain and Growth Conditions—Wild-type *S. cerevisiae* strain CEN.PK113–7D (*MAT**a***) (14) was grown at 30 °C in 2-liter chemostats (Applikon), with a working volume of 1.0 liter as described in Ref. 15. Cultures were fed with a defined mineral medium that limited growth by glucose, ethanol, acetate, or maltose with all other growth requirements in excess. The dilution rate was set at 0.10 h^{-1}. The pH was measured on-line and kept constant at 5.0 by the automatic addition of 2 M KOH with the use of an Applikon ADI 1030 biocontroller. Stirrer speed was 800 rpm, and the airflow was 0.5 liters·min^{-1}. Dissolved oxygen tension was measured online with an Ingold model 34-100-3002 probe, and was between 60 and 75% of air saturation. The off-gas was cooled by a condenser connected to a cryostat set at 2 °C and analyzed as previously described (16). Steady-state samples were taken after ~10–14 volume changes to avoid strain adaptation caused by long term cultivation (17). Dry weight, metabolite, dissolved oxygen, and off-gas profiles had to be constant over at least five volume changes prior to sampling for RNA extraction.

Media—The defined mineral medium composition was based on that described by Verduyn *et al.* (18). The carbon source was 256 ± 19 mmol of carbon/liter.

Analytical Methods—Culture supernatants were obtained after centrifugation of samples from the chemostats. For the purpose of glucose, ethanol, acetate, and maltose determination and carbon recovery, culture supernatants and media were analyzed by HPLC,[1] fitted with an Aminex HPX-87H ion exchange column using 5 mM H_2SO_4 as the mobile phase. Culture dry weights were determined via filtration as described by Postma *et al.* (19).

Metabolic Flux Distribution—Intracellular metabolic fluxes were calculated through metabolic flux balancing using a compartmented stoichiometric model derived from the model developed by Lange (20).

Because the intracellular localization of certain enzymes as well as the trafficking of certain metabolites in *S. cerevisiae* are still a matter of debate, assumptions had to be made on these aspects. The main assumptions concern acetyl-coenzyme A metabolism. It has been shown that transport of acetyl-CoA through the mitochondrial membrane cannot be performed in *S. cerevisiae* by the carnitine shuttle in the absence of exogenous carnitine (21, 22). It is, however, possible to conceive growth on sugar and gluconeogenic carbon sources without acetyl-CoA transport. During growth on sugar, mitochondrial acetyl-CoA is synthesized directly in this compartment via the pyruvate dehydrogenase complex, whereas the small amounts of necessary cytosolic acetyl-CoA are synthesized in the cytosol by the acetyl-CoA synthase (encoded by *ACS1* and *ACS2* (Refs. 15 and 23)). The gluconeogenic carbon sources acetate and ethanol can be converted by acetyl-CoA synthase into acetyl-CoA in the cytosol, acetyl-CoA being further converted in the cytosol to citrate by citrate synthase (*CIT2* (Ref. 23)). Citrate can then be transported trough the mitochondrial membrane by the well described citrate transporter (*CTP1* (Ref. 24)).

The set-up of the stoichiometric models for growth of *S. cerevisiae* on glucose, maltose, ethanol, and acetate, as well as the flux balancing, was performed using dedicated software (SPAD it, Nijmegen, The Netherlands). The theory and practice of metabolic flux balancing has been described well in literature and will not be repeated here (25–29). For each carbon source the specific rates of growth, substrate consumption, carbon dioxide production, and oxygen consumption during steady-state chemostat cultivation were calculated from the measured concentrations and flow rates from three independent experiments.

The calculated specific conversion rates and their variances were used as input for the metabolic flux balancing procedure. In all cases the ATP balance was omitted as constraint in the flux balancing. This is a prerequisite for proper balancing in case the ATP stoichiometry of some reactions is insufficiently known (*e.g.* maintenance energy requirements, P/O ratio, etc.). However, without the ATP balance, the number of measurements was sufficient to result in an overdetermined system, thus making data reconciliation possible. In all cases the degree of redundancy was equal to 2.

The complete list of reactions and components used to build the model can be found at www.bt.tudelft.nl/carbon-source.

Microarray Analysis—Sampling of cells from chemostats, probe preparation, and hybridization to Affymetrix GeneChip® microarrays were performed as described previously (30). The results for each growth condition were derived from three independently cultured replicates.

Data Acquisition and Analysis—Acquisition and quantification of array images and data filtering were performed using the Affymetrix software packages Microarray Suite version 5.0, MicroDB version 3.0, and Data Mining Tool version 3.0.

Before comparison, all arrays were globally scaled to a target value of 150 using the average signal from all gene features using Microarray Suite version 5.0. From the 9,335 transcript features on the YG-S98 arrays, a filter was applied to extract 6,383 yeast open reading frames, of which there were 6,084 different genes. This discrepancy was the result of several genes being represented more than once when suboptimal probe sets were used in the array design.

To represent the variation in triplicate measurements, the coefficient of variation (standard deviation divided by the mean) was calculated as previously described by Boer *et al.* (31).

For further statistical analyses, Microsoft Excel running the Significance Analysis of Microarrays (SAM, version 1.12) add-in was used (32) for all possible pairwise comparisons of the four data sets. Genes were considered as being changed in expression if they were called significantly changed using SAM (expected median false discovery rate of 1%) by at least 2-fold from each other's conditions. Hierarchical clustering of the obtained set of significantly changed expression levels was subsequently performed by GeneSpring (Silicon Genetics).

Promoter analysis was performed using web-based softwares Regulatory Sequence Analysis Tools[2] (RSA Tools (Ref. 33)) and AlignAce[3] (34). The promoters (from −800 to −50) of each set of co-regulated genes were analyzed for over-represented motifs. When motifs shared largely overlapping sequences, they were aligned to form longer conserved elements. All the individual promoter sequences contributing to these elements were then aligned, and redundant elements were determined by counting the base representation at each position. The relative abundance of these redundant elements was then determined from a new enquiry of the co-regulated gene promoters and the entire set of yeast promoters in the genome using RSA Tools. The cluster coverage (Table V) is then expressed as the number of genes in the cluster containing the motif at least once, divided by the total number of genes

[1] The abbreviations used are: HPLC, high performance liquid chromatography; STRE, stress response element; CSRE, carbon source-responsive element.

[2] URL is rsat.ulb.ac.be/rsat/.

[3] URL is atlas.med.harvard.edu//.

TABLE I

Carbon source concentrations and physiological parameters of cultures used in this study

Data represent the average and S.D. of three separate chemostat cultivations grown to steady states at $D = 0.1\ h^{-1}$. Residual substrates in the culture medium were below detection limit.

Carbon source	Carbon source in feed	Y_{sx}[a]	$q_{carbon\ source}$	q_{O_2}	q_{CO_2}	RQ[b]	Carbon recovery
	mmol·liter^{-1}	*g·Cmol*$^{-1}$	*mmol·(g of dry biomass)*$^{-1}$*·h*$^{-1}$				%
Glucose	41.4 ± 0.2	14.8 ± 0.0	1.15 ± 0.02	2.74 ± 0.03	2.85 ± 0.04	1.04 ± 0.02	97 ± 1
Maltose	19.6 ± 0.8	13.6 ± 0.3	0.61 ± 0.02	3.05 ± 0.18	3.05 ± 0.17	1.02 ± 0.01	97 ± 3
Ethanol	131.6 ± 3	13.4 ± 0.3	3.78 ± 0.06	6.87 ± 0.15	3.26 ± 0.04	0.47 ± 0.01	95 ± 2
Acetate	139.5 ± 4	8.4 ± 0.0	5.89 ± 0.09	7.4 ± 0.23	7.45 ± 0.18	1.01 ± 0.00	96 ± 2

[a] Yield of biomass (g of dry biomass formed/mol of carbon source consumed).
[b] Respiratory coefficient (q_{CO_2}/q_{O_2}).

in the cluster. Similarly, the genome coverage is expressed as the genome-wide number of genes containing the motif at least once, divided by the total number of genes used by RSA Tools (6,451 open reading frames).

RESULTS

Biomass Yields and Respiration Rates in Carbon-limited Chemostat Cultures—Carbon-limited, aerobic chemostat cultures were grown on glucose, maltose, ethanol, and acetate as single growth-limiting nutrients. At a dilution rate of 0.1 h^{-1}, the concentration of all four carbon sources in the reservoir medium was ~250 mmol of carbon/liter (Table I), whereas their residual concentrations in steady-state cultures were below their respective detection limits (*i.e.* less than 0.5 mM). For the glucose- and maltose-grown cultures, the respiratory quotient (ratio of the specific rate of carbon dioxide production and oxygen consumption) was close to 1.0, indicating a fully respiratory metabolism of these sugars. In all cultures over 95% of the substrate carbon was recovered as either biomass or carbon dioxide (Table I), and HPLC analysis of culture supernatants did not reveal the production of any low molecular weight metabolites.

Biomass yields, as well as the specific rates of oxygen consumption and carbon dioxide production, were very similar for cultures grown on maltose and glucose. This was expected, as maltose metabolism is initiated by the uptake and hydrolysis of the disaccharide to two glucose molecules (Fig. 1). In contrast to glucose uptake, however, the uptake of maltose occurs via an energy-dependent proton-symport mechanism (2), which is likely to be responsible for the slightly lower (8%) biomass yield on maltose as compared with glucose (Table I). Consistent with earlier studies, biomass yields on ethanol and, in particular, acetate were lower than that on glucose (8, 35). These lower biomass yields and correspondingly higher respiration rates can be explained from the lower ATP yield from respiratory dissimilation of these substrates (which is largely the result of the investment of 2 ATP equivalents in the acetyl-coenzyme A synthetase reaction) and from the necessity to synthesize biosynthetic precursors via the glyoxylate cycle and gluconeogenesis. The difference in biomass yield between ethanol and acetate can be attributed to two factors: (i) energy-dependent uptake of acetate via a proton symport mechanism (6, 7) and (ii) the higher degree of reduction of ethanol, for which the ethanol and acetaldehyde dehydrogenases (Fig. 1, *reactions 22* and *23*, respectively) can yield NAD(P)H that can either be used for biosynthesis or yield ATP via oxidative phosphorylation.

Flux Distribution in Central Carbon Metabolism—In vivo fluxes through central pathways in carbon metabolism were estimated by metabolic flux analysis, using a stoichiometric model of the *S. cerevisiae* metabolic network. As *S. cerevisiae* is a eukaryote, this model took into account metabolic compartmentation by discriminating between reactions that occur in the yeast cytosol and in the mitochondrial matrix (36, 37). To calculate intracellular fluxes, the model was fed with quantitative data on the biomass composition of *S. cerevisiae* (38) and with the substrate consumption and product formation rates observed in the carbon-limited chemostat cultures. The estimated fluxes of central carbon metabolism relevant for this study are summarized in Tables II and III.

With the exception of the sugar transport and maltose hydrolysis steps, there were only very few predicted changes in central carbon metabolism when either glucose or maltose was used as the carbon source (Tables II and III). The slight increase of catabolic fluxes in maltose-grown cultures was caused by the ATP requirement for maltose uptake (2). Larger changes were predicted between the C2 substrates ethanol and acetate. When ethanol is the carbon source, NADP-dependent acetaldehyde dehydrogenase can make an important contribution to fulfill the cellular demand for NADPH (Table II). In contrast, in acetate-grown cultures, this important reduced cofactor must be regenerated by NADP-dependent isocitrate dehydrogenase and possibly by the pentose-phosphate pathway (39). Furthermore, the lower degree of reduction of acetate and the associated lower yield of reducing equivalents during its dissimilation result in a lower ATP yield and necessitate higher fluxes through dissimilatory pathways. This was reflected by a substantially higher predicted *in vivo* activity of the tricarboxylic acid cycle in acetate-grown cells (Table III).

Major metabolic rearrangements were predicted when ethanol or acetate replaced glucose or maltose as the sole carbon source for growth of *S. cerevisiae*. Predicted fluxes through acetyl-coenzyme A synthetase (Fig. 1, *reaction 24*) were low in glucose-grown cultures, where this enzyme is only required for the provision of relatively small amounts of cytosolic acetyl-coenzyme A that are needed for lipid and lysine biosynthesis (40, 41). In contrast, predicted fluxes through this enzyme were high in ethanol- and acetate-grown cultures (Table II). Another important difference between growth on sugars and growth on C2-compounds is the involvement, for the latter substrates, of the glyoxylate cycle (Fig. 1, *reactions 14*, *15*, *21*, *25*, and *26*) and gluconeogenesis (*steps 27* and *28*). Furthermore, growth on C2-compounds led to a dramatic increase of predicted fluxes through the tricarboxylic acid cycle relative to sugar-grown cultures (Table III). Conversely, the flux through glycolysis was reversed (gluconeogenesis) and much lower in ethanol- and acetate-grown cells. Finally, the predicted flux through the oxidative pentose-phosphate pathway was reduced, as NADP-dependent acetaldehyde and/or isocitrate dehydrogenases provide alternative sources of NADPH during growth on C2-compounds.

Global Transcriptome Changes in Chemostat Cultures Limited for Different Carbon Sources—Independent triplicate chemostat cultures were run for each carbon limitation, followed by genome-wide transcriptional analysis with oligonucleotide DNA microarrays. Consistent with the excellent reproducibility reported in earlier studies in which DNA microarray analysis was applied to chemostat cultures (30, 32), the average coefficient of variation for the independent triplicate analyses did not exceed 0.18 (Table IV). Furthermore, the levels of *ACT1*

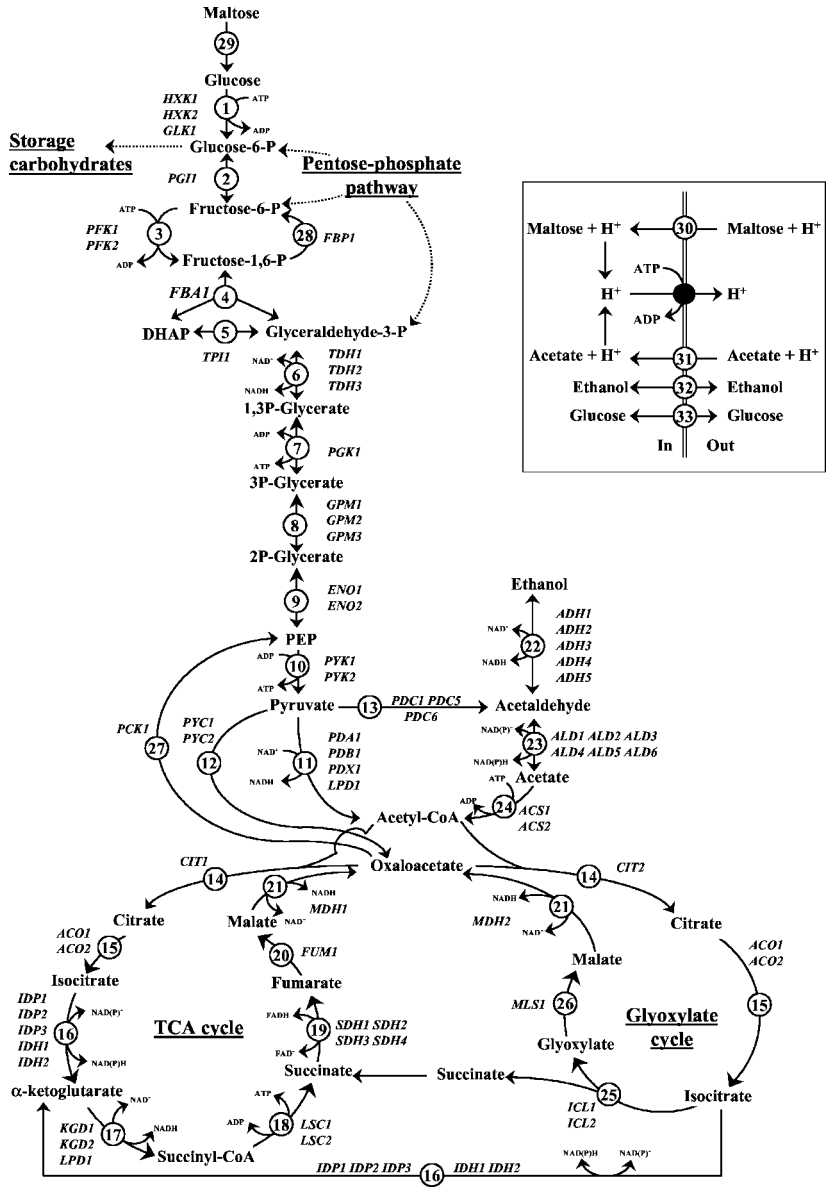

FIG. 1. **Central carbon metabolism in *S. cerevisiae*.** *1*, hexokinase; *2*, phosphoglucose isomerase; *3*, phosphofructokinase; *4*, fructose-bisphosphate aldolase; *5*, triose-phosphate isomerase; *6*, glyceraldehyde-3-phosphate dehydrogenase; *7*, phosphoglycerate kinase; *8*, phosphoglycerate mutase; *9*, enolase; *10*, pyruvate kinase; *11*, pyruvate dehydrogenase; *12*, pyruvate carboxylase; *13*, pyruvate decarboxylase; *14*, citrate synthase; *15*, aconitase; *16*, isocitrate dehydrogenase; *17*, α-ketoglutarate dehydrogenase; *18*, succinyl-CoA ligase; *19*, succinate dehydrogenase; *20*, fumarase; *21*, malate dehydrogenase; *22*, alcohol dehydrogenase; *23*, acetaldehyde dehydrogenase; *24*, acetyl-CoA synthase; *25*, isocitrate lyase; *26*, malate synthase; *27*, phosphoenolpyruvate carboxykinase; *28*, fructose bisphosphatase; *29*, maltase; *30*, maltose permease; *31*, acetate transporter; *32*, ethanol diffusion; *33*, glucose permease. *DHAP*, dihydroxyacetone phosphate; *PEP*, phosphoenolpyruvate.

and *PDA1* transcripts, which are commonly applied as loading standards for conventional Northern analysis, were not significantly different for cultures grown on different growth-limiting carbon sources (Table IV). The lowest measurable signal was 12 (arbitrary units) in all conditions (Table IV). 833 transcripts (13.7% of the genome) remained below this detection limit for all four growth-limiting carbon sources tested.

As few as 180 genes (only 2.8% of the genome) were carbon source-responsive, as defined by a significant change in the transcription profile (-fold change greater than 2 and false discovery rate of 1%; see "Experimental Procedures"). This number seems low, especially when considering the major rearrangements in metabolism that must occur when, for example, ethanol replaces glucose as the sole carbon source for growth (Table I). As shown on Fig. 2, many of the carbon source-responsive genes (72 genes, 40%) have not yet been assigned a biological function, as defined by MIPS[4] (42). A significant number (50 genes, 28%) were found to be related to carbon metabolism-encoding enzymes (35 genes), transporters (6 genes), or proteins involved in regulation (9 genes). Finally, 11 genes (6%) were involved in nitrogen metabolism.

Specific Transcriptional Responses to Growth-limiting Carbon Sources—To analyze the specific transcriptional response of *S. cerevisiae* to the four growth-limiting carbon sources, the transcriptome data were subjected to hierarchical cluster analysis. This resulted in six distinct clusters (Fig. 3). Most of the C2-responsive genes found in the present study (117 of 180) were present in clusters 2 and 5 (Fig. 3), which yielded different transcript levels for growth limited by sugar (glucose or maltose) and for growth limited by C2-compounds (ethanol or acetate), respectively. The other clusters consisted of genes that specifically responded to a single growth-limiting carbon source: low expression under acetate limitation (cluster 1, 16 genes), high expression under glucose limitation (cluster 3, 13 genes), high expression under maltose limitation (cluster 4, 16 genes), and, finally, low expression under maltose limitation (cluster 6, 18 genes). No genes were found with a specific response to growth under ethanol limitation, or with a specifically increased transcript level in acetate- or glucose-limited cultures. The six clusters that were identified will be briefly discussed below.

Different Transcript Levels in Sugar and C2-compound-limited Cultures (Clusters 2 and 5)—Of the 117 genes that

[4] Munich Information Center for Protein Sequences URL is mips.gsf.de/.

TABLE II

Flux distribution and transcript levels in S. cerevisiae grown in chemostat cultivation under maltose, glucose, ethanol, and acetate limitation in glycolysis and metabolic steps around pyruvate

In gray background are indicated significantly up-regulated transcripts and increased fluxes. Significantly down-regulated transcripts and decreased fluxes are underlined. G3P, glyceraldehyde-3-P; Alc, alcohol.

Enzyme[a,b]		Fluxes (mol/Cmol/h[c])				Gene	Transcripts (hybridisation intensities)				Loc[a]
		Glucose	Maltose	Ethanol	Acetate		Glucose	Maltose	Ethanol	Acetate	
MALTOSE UTILIZATION											
29	Maltase	0	**30.9 ± 0.4**	0	0	*MAL32*	363 ± 97	**2834 ± 389**	45 ± 2	44 ± 8	C
						FSP2	74 ± 13	**641 ± 87**	66 ± 11	56 ± 19	C
						YJL216C	19 ± 7	**165 ± 61**	15 ± 6	25 ± 5	C
						YGR287C	30 ± 1	**66 ± 4**	12 ± 1	12 ± 3	C
GLYCOLYSIS											
1	Hexokinase	29.6 ± 0.2	30.9 ± 0.2	**0**	**0**	HXK1	1563 ± 159	1225 ± 550	**271 ± 37**	**212 ± 51**	C
						HXK2	885 ± 113	685 ± 193	617 ± 267	513 ± 105	C
						GLK1	1512 ± 315	1427 ± 259	897 ± 58	645 ± 129	C
2	Glucose-6P isomerase	12.7 ± 0.1	14.1 ± 0.3	**-5.4 ± 0.1**	**-4.9 ± 0.1**	*PGI1*	1852 ± 129	2393 ± 1308	1308 ± 197	1273 ± 98	C
3	Phosphofructokinase	18.8 ± 0.1	20.2 ± 0.3	**0**	**0**	*PFK1*	752 ± 93	662 ± 260	556 ± 137	509 ± 69	C
						PFK2	899 ± 38	1055 ± 71	715 ± 81	596 ± 48	C
4	Fructose-1,6P aldolase	18.8 ± 0.1	20.2 ± 0.3	**-6.2 ± 0.1**	**-5.7 ± 0.1**	*FBA1*	3585 ± 98	6042 ± 300	3989 ± 632	4366 ± 410	C
5	Triose-P isomerase	18.5 ± 0.1	19.9 ± 0.3	**-6.5 ± 0.1**	**-6 ± 0.1**	*TPI1*	3690 ± 249	6504 ± 576	4113 ± 476	3799 ± 351	C
6	G3P dehydrogenase	40.1 ± 0.3	42.9 ± 0.7	**-13.3 ± 0.1**	**-12.4 ± 0.2**	*TDH1*	1557 ± 173	1890 ± 82	**606 ± 205**	**470 ± 75**	C
						TDH2	3623 ± 322	5936 ± 737	3956 ± 737	3406 ± 244	C
						TDH3	4300 ± 340	7633 ± 2052	6118 ± 1165	6259 ± 940	C
7	3P-glycerate kinase	40.1 ± 0.3	42.9 ± 0.7	**-13.3 ± 0.1**	**-12.4 ± 0.2**	*PGK1*	3474 ± 339	5565 ± 1269	3222 ± 342	3016 ± 442	C
8	P-glycerate mutase	38.4 ± 0.3	41.2 ± 0.7	**-15.2 ± 0.1**	**-14.3 ± 0.2**	*GPM1*	3107 ± 128	4356 ± 315	2967 ± 321	2572 ± 301	C
						GPM2	71 ± 2	46 ± 25	53 ± 27	50 ± 10	C
						GPM3	37 ± 5	34 ± 19	29 ± 3	30 ± 6	C
9	Enolase	38.4 ± 0.3	41.2 ± 0.7	**-15.2 ± 0.1**	**-14.3 ± 0.2**	*ENO1*	3219 ± 379	4883 ± 1304	3106 ± 345	2662 ± 133	C
						ENO2	2509 ± 299	2206 ± 1088	1575 ± 333	1546 ± 346	C
10	Pyruvate kinase	37.2 ± 0.3	40± 0.7	**4.6 ± 0**	**4.9 ± 0.1**	*PYK1*	2104 ± 400	2468 ± 210	1696 ± 208	1257 ± 91	C
						PYK2	51.4 ± 14	47 ± 10	55 ± 12	35 ± 3	C
PYRUVATE BRANCHPOINT											
11	Pyruvate dehydrogenase	21.6 ± 0.2	24.5 ± 0.7	**0**	**0**	*PDA1*	488 ± 67	385 ± 17	499 ± 12	406 ± 31	M
						PDB1	401 ± 19	272 ± 69	451 ± 34	436 ± 46	M
						PDX1	125 ± 19	81 ± 18	150 ± 30	141 ± 20	M
						LPD1	1220 ± 186	1357 ± 78	1932 ± 406	1860 ± 364	M
12	Pyruvate carboxylase	5.3 ± 0.1	5.3 ± 0.1	**0**	**0**	*PYC1*	654 ± 171	305 ± 32	834 ± 185	730 ± 111	C
						PYC2	543 ± 110	417 ± 94	551 ± 68	398 ± 46	C
13	Pyruvate decarboxylase	5.9 ± 0.1	5.8 ± 0.1	**0**	**0**	*PDC1*	1462 ± 139	2408 ± 228	1415 ± 49	1226 ± 155	C
						PDC5	95 ± 25	84 ± 64	89 ± 10	75 ± 11	C
						PDC6	80 ± 31	48 ± 38	39 ± 8	40 ± 9	C
22	Alc. dehydrogenase, C	0	0	**-94.4 ± 0.7**	0	*ADH1*	2935 ± 106	3705 ± 59	2057 ± 196	1496 ± 170	C
						ADH2	4616 ± 129	7371 ± 245	6873 ± 1285	6681 ± 647	C
						ADH4	117 ± 40	83 ± 10	125 ± 24	128 ± 12	C
						ADH5	198 ± 30	146 ± 44	234 ± 67	108 ± 11	C
	Alc. dehydrogenase, M	0	0	0	0	*ADH3*	625 ± 127	590 ± 2	996 ± 132	1177 ± 381	M
23	Acetald. dehydrogenase, NAD	0	0	**62.7 ± 0.7**	0	*ALD6*	1046 ± 88	1322 ± 329	1518 ± 366	1303 ± 36	C
						ALD4	3040 ± 173	4377 ± 340	3452 ± 518	2978 ± 256	M
						ALD5	309 ± 28	359 ± 158	226 ± 9	**134 ± 10**	M
	Acetald. dehydrogenase, NADP	6 ± 0.1	6 ± 0.1	**31.7 ± 0.3**	**0.1 ± 0**	*ALD2*	34 ± 5	35 ± 24	31 ± 9	28 ± 9	C
						ALD3	45 ± 5	59 ± 38	26 ± 4	31 ± 10	C
24	Acetyl-CoA synthase	6.1 ± 0.1	6.1 ± 0.1	**94.5 ± 0.7**	**149 ± 1.5**	*ACS1*	2221 ± 126	2416 ± 384	4120 ± 565	3866 ± 173	NM
						ACS2	675 ± 38	950 ± 45	1070 ± 266	1018 ± 146	NM

[a] Subcellular localization of the protein is indicated by: C, cytosolic; M, mitochondrial; P, peroxisomal; NC, non-cytosolic; NM, non-mitochondrial.
[b] The number preceding the enzyme name corresponds to its metabolic step number on Fig. 1.
[c] Fluxes are expressed as biomass specific conversion rates, *i.e.* as mol of reactant converted per Cmol of biomass (*i.e.* the amount of biomass containing 1 mol of carbon) per hour.

yielded different transcript levels in sugar-limited cultures and cultures limited by either ethanol or acetate, 79 were up-regulated (cluster 5) and 38 down-regulated (cluster 2) in cultures limited by the C2-compounds. Among the up-regulated transcripts, 21 encoded enzymes or regulatory proteins related to carbon metabolism and included the four structural genes for gluconeogenesis and glyoxylate cycle enzymes: *PCK1* and *FBP1* (encoding the gluconeogenic enzymes phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase) and *ICL1* and *MLS1* (encoding the glyoxylate cycle enzymes isocitrate lyase and malate synthase, respectively). An additional seven of these genes encoded enzymes or subunits active in the tricarboxylic acid cycle. This included five cytosolic enzymes (*IDH1*, *IDH2*, *FUM1*, *SDH1*, *SDH3*) and two mitochondrial enzymes

TABLE III

Flux distribution and transcript levels in S. cerevisiae grown in chemostat cultivation under maltose, glucose, ethanol, and acetate limitation in the TCA cycle, the glyoxylate cycle, gluconeogenesis, and the pentose-phosphate pathway

In gray background are indicated significantly up-regulated transcripts and increased fluxes. Significantly down-regulated transcripts and decreased fluxes are underlined. TCA, tricarboxylic acid. PEP, phosphoenolpyruvate.

Enzyme[a,b]		Fluxes (mol/Cmol/h[c])				Gene	Transcripts (hybridisation intensities)				Loc
		Glucose	Maltose	Ethanol	Acetate		Glucose	Maltose	Ethanol	Acetate	
TCA CYCLE											
14	Citrate synthase, M	21.6 ± 0.2	24.5 ± 0.7	0	0	*CIT1*	1725 ± 55	2472 ± 81	2794 ± 284	3144 ± 400	M
						CIT3	367 ± 100	298 ± 78	606 ± 83	719 ± 90	C
	Citrate synthase, C	0	0	59.3 ± 0.7	114.8 ± 1.4	*CIT2*	515 ± 67	329 ± 216	2421 ± 386	2481 ± 299	M
15	Aconitase	21.6 ± 0.2	24.5 ± 0.7	59.3 ± 0.7	114.8 ± 1.4	*ACO1*	1065 ± 123	1255 ± 79	1594 ± 142	2114 ± 145	M
						ACO2	266 ± 46	290 ± 65	226 ± 33	247 ± 21	M
16	Isocitrate dehydrogenase NAD	19.2 ± 0.2	22.1 ± 0.7	30 ± 0.7	54.6 ± 1.7	*IDH1*	523 ± 57	536 ± 63	921 ± 79	1227 ± 87	M
						IDH2	1060 ± 94	1308 ± 309	1750 ± 238	2752 ± 246	M
	Isocitrate dehydrogenase NADP, C	0	0	0	31.1 ± 0.5	*IDP2*	610 ± 20	740 ± 130	1075 ± 122	1121 ± 12	C
	Isocitrate dehydrogenase NADP, NC	2.4 ± 0	2.4 ± 0	2.6 ± 0	2.7 ± 0.5	*IDP1*	230 ± 32	221 ± 33	383 ± 62	315 ± 24	M
						IDP3	182 ± 7	127 ± 64	141 ± 29	120 ± 34	P
17	α-ketoglutarate dehydrogenase	18.7 ± 0.2	21.7 ± 0.7	29.5 ± 0.7	85.1 ± 1.5	*KGD1*	971 ± 84	1343 ± 231	1759 ± 284	1663 ± 205	M
						KGD2	526 ± 66	531 ± 17	550 ± 93	609 ± 40	M
						LPD1	1220 ± 186	1357 ± 78	1932 ± 406	1860 ± 364	M
18	Succinyl-Co A ligase	18.7 ± 0.2	21.7 ± 0.7	29.5 ± 0.7	85.1 ± 1.5	*LSC1*	1323 ± 206	1655 ± 116	1345 ± 48	1466 ± 81	M
						LSC2	868 ± 149	1005 ± 152	1074 ± 133	921 ± 126	M
19	Succinate dehydrogenase	18.7 ± 0.2	21.7 ± 0.7	56.2 ± 0.7	111.6 ± 1.4	*SDH1*	593 ± 93	604 ± 115	1386 ± 181	1348 ± 160	M
						SDH2	2073 ± 68	2147 ± 114	3185 ± 221	2964 ± 116	M
						SDH3	1289 ± 230	1792 ± 29	2473 ± 427	2870 ± 282	M
						SDH4	1530 ± 53	2066 ± 374	2701 ± 245	2962 ± 292	M
20	Fumarase[d]	1 ± 0	1 ± 0	27.7 ± 0.3	27.6 ± 0.4	*FUM1*	1352 ± 146	1638 ± 110	2571 ± 155	2980 ± 136	C
		18.7 ± 0.2	21.7 ± 0.7	29.5 ± 0.7	85.1 ± 1.5						M
21	Malate dehydrogenase, M	21.9 ± 0.2	24.8 ± 0.7	0.3 ± 0	0.3 ± 0	*MDH1*	1792 ± 613	2729.5 ± 412	2040 ± 122	2450 ± 134	M
	Malate dehydrogenase, NM	-2.1 ± 0	-2.1 ± 0	83.7 ± 0.7	138.9 ± 1.4	*MDH2*	844 ± 90	762 ± 61	3918 ± 727	3420 ± 206	C
						MDH3	1133 ± 120	1106 ± 46	1126 ± 192	1108 ± 74	P
GLYOXYLATE CYCLE and GLUCONEOGENESIS											
25	Isocitrate lyase	0	0	26.7 ± 0.2	26.5 ± 0.4	*ICL1*	865 ± 183	954 ± 86	4076 ± 932	3865 ± 448	C
						ICL2	514 ± 44	381 ± 20	914 ± 166	1220 ± 55	M
26	Malate synthase	0	0	26.7 ± 0.2	26.5 ± 0.4	*MLS1*	503 ± 199	311 ± 55	3332 ± 1009	3041 ± 76	C
						MLS2	27 ± 6	10 ± 8	245 ± 7	25 ± 5	C
27	PEP carboxykinase	0	0	21.1 ± 0.2	20.5 ± 0.3	*PCK1*	204 ± 31	156 ± 53	3599 ± 587	3474 ± 563	C
28	Fructose-bisphosphatase	0	0	6.2 ± 0.1	5.7 ± 0.1	*FBP1*	240 ± 7	166 ± 67	2150 ± 501	1766 ± 207	C
PENTOSE-PHOSPHATE PATHWAY											
NS	Glucose-6P dehydrogenase	10.2 ± 0.1	10.1 ± 0.1	0	0	*ZWF1*	343 ± 50	350 ± 68	250 ± 101	141±40	C
NS	6P-gluconolactonase	10.2 ± 0.1	10.1 ± 0.1	0	0	*SOL1*	758 ± 54	1708 ± 270	492 ± 177	367±45	C
						SOL2	383 ± 54	334 ± 132	512 ± 60	402±18	C
						SOL3	631 ± 91	607 ± 84	307 ± 60	291±46	C
						SOL4	658 ± 49	730 ± 200	435 ± 99	211±86	C
NS	6P-gluconate dehydrogenase	10.2 ± 0.1	10.1 ± 0.1	0	0	*GND1*	1546 ± 94	1433 ± 161	736 ± 99	734±32	C
						GND2	365 ± 54	357 ± 139	150 ± 22	94±36	C
NS	Ribose-P isomerase	3.3 ± 0	3.3 ± 0	-0.1 ± 0	-0.01 ± 0	*RKI1*	107 ± 14	65 ± 13	278 ± 57	290±27	C
NS	Ribulose-P epimerase	6.1 ± 0.1	6 ± 0.1	-0.7 ± 0	-0.8 ± 0	*RPE1*	483 ± 122	315 ± 112	541 ± 138	437±37	C
NS	Transketolase 1	3.3 ± 0	3.3 ± 0	-0.1 ± 0	-0.1 ± 0	*TKL1*	652 ± 30	713 ± 118	686 ± 91	726±80	C
NS	Transaldolase	3.3 ± 0	3.3 ± 0	-0.1 ± 0	-0.1 ± 0	*TAL1*	626 ± 17	664 ± 83	450 ± 27	437±26	C
NS	Transketolase 2	2.7 ± 0	2.7 ± 0	-0.7 ± 0	-0.7 ± 0	*TKL2*	139 ± 8	134 ± 50	41 ± 7	41±22	C

[a] Subcellular localization of the protein is indicated by: C, cytosolic; M, mitochondrial; P, peroxisomal; NC, non-cytosolic; NM, non-mitochondrial.
[b] The number preceding the enzyme name corresponds to its metabolic step number on Fig. 1 (NS, not shown on Fig. 1).
[c] Fluxes are expressed as biomass specific conversion rates, *i.e.* as mol of reactant converted per Cmol of biomass (*i.e.* the amount of biomass containing 1 mol of carbon) per hour.
[d] Although found both in the cytosol and in the mitochondria, fumarase is encoded by one gene and one transcript only (94). According to recent findings, its localization depends on the folding of the protein (95).

(*CIT2*, *MDH2*) that were already known to be up-regulated during growth on non-fermentable carbon sources in batch cultures (43). Five additional genes encoded enzymes involved in acetyl-coenzyme A metabolism and its trafficking across intracellular membranes (*ACS1*, *ACH1*, *CAT1*, *YAT1*, *YAT2*), consistent with the key role of this intermediate in the metabolism of C2-compounds. Three transcripts that showed an increased level in cultures limited by C2-compounds are involved in transcriptional regulation of carbon metabolism: *SIP4*, a transcriptional activator of gluconeogenic genes *SIP2* and *REG2*. Finally, *RKI1* (encoding ribose-phosphate isomerase, involved in the pentose-phosphate pathway), *INO1* (encoding inositol-1-phosphate synthase), and *ICL2* (encoding a 2-methylisocitrate lyase involved in propionate metabolism) transcripts were also up-regulated in the presence of ethanol or acetate.

Eight of the genes that showed increased transcript levels in cultures limited by C2-compounds encoded proteins involved in

TABLE IV
Summary of microarray experiment quality parameters for each carbon limitation

Culture-limiting nutrient	Average coefficient of variation[a]	*ACT1*[b]	*PDA1*[c]	Lowest "measurable" signal[d]
Glucose	0.13	2556 ± 157	488 ± 67	12 ± 2
Maltose	0.18	3119 ± 446	385 ± 8	13 ± 5
Ethanol	0.17	3028 ± 449	499 ± 12	12 ± 2
Acetate	0.14	2615 ± 103	406 ± 30	12 ± 6

[a] Represents the average of the coefficient of variation (standard deviation divided by the mean) for all genes except the genes with mean expression below 12.
[b] Encoding actin.
[c] Encoding pyruvate dehydrogenase complex E1-α subunit.
[d] Corresponds to the signal from the open reading frame with the lowest reliably detectable abundance.

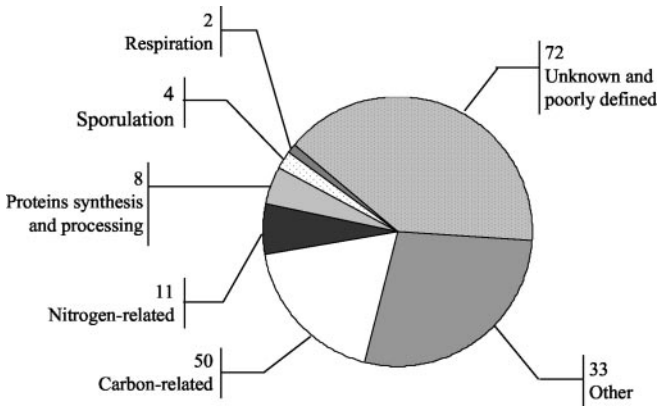

FIG. 2. **The 180 genes transcriptionally regulated by carbon source were sorted by their functional category as defined by MIPS.** The two major categories were found to be genes with poorly described functions (40%) and genes related to carbon metabolism (28%).

transport across the cytosolic and the mitochondrial membranes. In addition to the *CAT1*, *YAT1*, and *YAT2* genes mentioned above, two further up-regulated genes were involved in acetyl-CoA trafficking via the L-carnitine shuttle. *AGP2* encodes a plasma membrane carnitine transporter, whereas the gene product of *CRC1* transports acetylcarnitine across the mitochondrial inner membrane. Consistent with published results on batch cultures, *SFC1*, which encodes the mitochondrial succinate-fumarate exchanger, was expressed at increased levels in ethanol- and acetate-limited chemostat cultures (44). Surprisingly, *STL1* also exhibited elevated transcript levels in ethanol- and acetate-limited cultures (~4-fold higher compared with sugars). Although Stl1p has been described as a member of the hexose transporter family (45) because of its homology to these transport proteins (26–28% identity), its physiological role in sugar transport has not been clearly established. Its transcriptional induction by gluconeogenic carbon sources may indicate its involvement in the transport of other compounds than hexoses. Three further transporter-encoding genes (*DUR3*, *MEP2*, and *SAM3*) involved in nitrogen metabolism, as well as four genes (*DAL2*, *DAL5*, *GDH3*, and *GCV2*) encoding enzymes involved in nitrogen metabolism showed higher transcript levels during carbon-limited growth on C2-compounds. Despite the high specific rates of respiration in the ethanol- and acetate-limited cultures as compared with the glucose- and maltose-limited cultures (Table I), only a single gene (*NDE2*) involved in respiration showed a significantly higher transcript level in cultures limited for the C2-compounds. The 31 other transcripts responding to these carbon sources had unknown or poorly described functions.

Of the 38 genes down-regulated in the presence of ethanol and acetate (cluster 2), more than half (20 genes) have not yet been assigned a clear biological role. Remarkably, of the remaining 18, 10 were linked to carbon metabolism: 2 glycolytic genes (*HXK1*, *TDH1*), 4 genes from the pentose-phosphate pathway (*TKL2*, *GND1*, *GND2*, and *SOL3*), *VID24* encoding a protein involved in fructose-6-bisphosphate vacuolar transport and degradation, 2 members of the hexose transport family (*HXT2* and *HXT7*), and finally *MTH1*, involved in glucose signaling and repression (46). Surprisingly, one gene (*CYC7*) encoding iso-2-cytochrome *c* and involved in respiration was repressed, whereas respiration rate was increased in cells grown with C2-compounds compared with sugars (Table I). Among the remaining seven genes were *BAP2*, a branched-chain amino acid permease; *SPS100* and *SWM1*, both involved in sporulation; and *FDH1* and *FDH2*, encoding formate dehydrogenases for which a precise role in yeast metabolism has not been clearly defined yet (47). Two other down-regulated genes respond to stress conditions, *DOG2* responds to oxidative and osmotic stress and *PDR12* is involved in weak organic acid resistance.

Up- and Down-regulation in Response to Maltose—A set of 34 genes specifically responded to growth with maltose. Of the 16 up-regulated transcripts, 6 had poorly described biological functions, 4 encoded ribosomal proteins (*RPS10A*, *RPS26B*, *RPL15B*, and *RPL31B*), and 6 coded for proteins involved in maltose utilization. Genes necessary for maltose degradation (*i.e.* maltose permeases *MALx1*, maltases *MALx2* and maltose transcription activators *MALx3*) are clustered on *MAL* loci carried by subtelomeric regions (48). *S. cerevisiae* strains contain up to five highly homologous *MAL* loci, and one locus is enough to sustain growth on maltose (4). Two *MAL* loci have been sequenced in *S. cerevisiae* S288C, the strain used for the genome sequencing program, although this yeast is not able to grow on maltose as the sole carbon source (49, 50). Our laboratory strain, like all members from the CEN.PK family, can grow on maltose and contains four *MAL* loci.[5] The Affymetrix microarrays contain four probe sets that are specific to known *MAL* genes (*MAL11*, *MAL13*, *MAL33*, and *MAL23*) and two that can hybridize to several homologues (*MALx2* and *MALx1*). As expected, genes encoding for maltose permeases and maltases were strongly induced in the presence of maltose (6–10-fold), as did *FSP2*, YJL216C, and YGR287C, genes sharing high homology with maltases. Despite their recent identification as α-glucoside transporters encoding genes and their reported induction in the presence of maltose (51), two maltose permease homologues, YDL247W (*MPH2*) and YJL160C (*MPH3*), were not up-regulated in maltose-limited chemostats. Although their presence in CEN.PK background has been detected by genome-wide genotyping (52), their expression at a very low level in CEN.PK113–7D (average signal intensity below 50) may indicate a strain-specific regulation. Among the three genes coding for the maltose regulator, only *MAL13* and *MAL33* transcripts were detected, *MAL13* displaying a very low transcript level. Surprisingly, none of these transcripts was induced in the presence of maltose, pointing toward a probable control of their activation properties at the post-transcriptional level.

Of the 18 genes down-regulated in the presence of maltose, most have not been assigned a function yet. The remaining genes cover a broad range of functional categories (budding, protein processing, DNA repair, cell wall maintenance, etc.) and could not be directly linked to maltose utilization.

Down-regulation in Response to Acetate—As few as 16 transcriptional changes were observed between cells grown on eth-

[5] P. Daran-Lapujade, J.-M. Daran, T. Petit, and J. T. Pronk, unpublished results.

FIG. 3. **Transcript profiles of carbon source-responsive genes.** Each *column* represents the average expression intensity of three replicate genome-wide transcript profiles for carbon limitation. Each *row* represents a gene. Low expression levels are represented by *green*, whereas *red* indicates high expression levels.

anol or acetate, and all of them were down-regulations in the presence of acetate. Among these changes five were related to carbon metabolism: *ALD5*, *PFK26*, *GPH1*, YNL134C, and *SOL4*. *ALD5*, encoding a mitochondrial minor isoform of acetaldehyde dehydrogenase, has previously been shown to be induced in the presence of ethanol (53). In apparent contrast, genome-wide transcription analysis indicated a mild repression of its expression after the diauxic shift (43). The specific physiological role of this acetaldehyde dehydrogenase isoenzyme has not been elucidated (54), and a proposed role in maintenance of the electron transport chain (53) does not shed light on its down-regulation in the presence of acetate. *PFK26* codes for a 6-phosphofructokinase catalyzing fructose-2,6-bisphosphate production. This metabolite has been shown to activate phosphofructokinase (Fig. 1, *step 3*) and to inactivate fructose bisphosphatase (Fig. 1, *step 28*), although its role in the switch between glycolysis and gluconeogenesis remains unclear (55, 56). This significant down-regulation of *PFK26* in

TABLE V
Over-represented sequences retrieved from the promoters of co-regulated genes

Regulatory cluster	Promoter element[a]	Putative binding protein	Cluster coverage	Genome coverage	Ref.
			%	%	
Cluster 1: low expression on acetate	NS[b]				
Cluster 2: low expression on ethanol and acetate	GsGkrrGGGG	Msn2p/Msn4p?	16	1	61
	AnhArnAGTwCT	?	26	6	
	wwGkCnnmGmAA	?	31	7	
Cluster 3: high expression with glucose	NS				
Cluster 4: high expression with maltose	bbTTTCGCns	Mal63p	60	8	62, 85
	SCCnCdATCC	?	53	1	
	Cky TmCsGym	?	53	4	
	CmnCGTkTbb	?	60	14	
Cluster 5: high expression with ethanol and acetate	CCnnynrnCCG	Cat8p/Sip4p	35	8	96
	CCCsGms	Mig1p	30	15	87, 97
	TCnGCrGCnAww	?	10	2	
	kCsGsGCsrr	?	14	3	
Cluster 6: low expression with maltose	NS				

[a] Redundant nucleotides are indicated as follows: r = A or G; y = C or T; s = G or C; w = A or T; k = G or T; m = A or C; b = C, G, or T; d = A, G, or T; H = A, C, or T; n = A,C,G, or T.
[b] NS, no significant patterns retrieved.

the presence of acetate (2.3-fold) and minor down-regulation (1.6-fold) in the presence of ethanol are, however, indicating a transcriptional control of *PFK26* by carbon source in carbon-limited cultures. This may be relevant for controlling the *in vivo* fluxes through the antagonistic enzymes phosphofructokinase and fructose-1,6-biphosphatase. YNL134C is a member of the zinc-binding dehydrogenase family, which catalyzes the reversible oxidation of ethanol to acetaldehyde. This glucose-repressed gene (31, 57) is the only alcohol dehydrogenase-encoding ORF displaying a significant change in expression in response to carbon source identified in this study; however, its physiological role has not been clearly identified so far. Finally, *GPH1* encodes a glycogen phosphorylase involved in glycogen degradation (58), and *SOL4* expression product is a 6-phosphogluconolactonase involved in the pentose-phosphate pathway.

No transcripts related to acidic stress response were up-regulated, confirming that acetate limitation efficiently resolved acid stress problems that are typically observed in batch cultures. Furthermore, three genes responding to acid and/or oxidative stress were down-regulated (*MSC1*, *AHP1*, *GAD1*).

Up-regulation in Response to Glucose—A puzzling cluster is the small subset of 13 transcripts that were specifically up-regulated when *S. cerevisiae* was grown with glucose as the sole carbon source. Six genes have unknown or poorly described functions. Four genes (*SUC2*, *SUC4*, *HXT4*, and *GIP2*) are involved in carbon metabolism. Invertase, encoded by *SUC* homologues, is a well described target of catabolite repression by glucose, both at the level of transcription and mRNA stability (59). However, it has been shown that low concentrations of glucose (0.1%) are necessary for a maximum expression of *SUC* genes (60). These findings are in good agreement with the significantly higher expression level of *SUC2* and *SUC4* (3–4-fold) measured when cells were grown in glucose compared with maltose, ethanol, or acetate. *HXT4*, encoding a moderate to low affinity hexose transporter, was also responding to low extracellular glucose concentration in our chemostat cultivations. The up-regulation of *GIP2* (protein phosphatase PP1-interacting protein), as well as *GNP1* (glutamine permease), *SKN1* (glucan synthase subunit), and *MCH5* (member of the major facilitator superfamily), in the presence of glucose does not have a clear physiological relevance.

In Silico Promoter Analysis of Carbon Source-regulated Genes—Co-regulation of global transcription is generally controlled by the specific binding of common activating or repressing proteins (transcription factors) to short sequences contained in promoter regions of the regulated genes. Searching the promoter regions of co-regulated genes for over-represented short sequences can identify these binding sites. We analyzed the upstream sequences of the genes from the six clusters defined above using web-based tools (for further information see "Experimental Procedures"). No significantly over-represented sequences were recovered from three clusters, namely low expression on acetate, high expression with glucose, and low expression with maltose (Table V). 11 putative promoter elements were over-represented in the three remaining clusters, of which only 4 could be associated to known transcription factors.

Among the promoter regions of the genes down-regulated in the presence of ethanol and acetate, three sequences were found to be over-represented compared with their genome coverage. One of them resembles the binding site targeted by the transcription factors Msn2p and Msn4p known as stress response element (STRE, Table V). However, the down-regulation of Msn2p/Msn4p-regulated genes seems unlikely as, in our experiments, cells were not exposed to any stress known to trigger the activity of these factors (reviewed by Estruch (Ref. 61)). Furthermore, a comparison of the genome-wide and cluster 2-wide coverage of the core sequence of the STRE (AGGGG) did not show any significant over-representation of this element among the genes down-regulated with C2-carbon sources. This renders it unlikely that the recovered sequence is a "true" STRE element.

The induction of genes necessary for maltose utilization (*MALx1* and *MALx2*) requires the activation by the transcription factor Malx3p (48). It was therefore expected that one of the binding sites targeted by Malx3p would be over-represented in the promoter region of genes up-regulated when *S. cerevisiae* is grown in the presence of maltose. Indeed, a 10-nucleotide sequence with significant homology to the Mal63p binding site (62) was retrieved from cluster 4 (Table V). Surprisingly, three other sequences were significantly over-represented (4–53-fold compared with genome coverage) in this relatively small cluster, but could not be associated to any known DNA-binding protein (Table V).

Four rather well conserved sequences were recovered from the promoter regions of genes up-regulated in the presence of C2-carbon sources (cluster 5). Two of them could be identified as targets of known transcription factors: Mig1p and Cat8p/Sip4p (Table V).

In glucose-grown batch cultures, many genes involved in the utilization of alternative carbon sources are repressed via a

TABLE VI
CSRE-related sites in the upstream region of the genes up-regulated in the presence of gluconeogenic carbon source

Systematic name	Standard name	Molecular function	CSRE sequence	CSRE localization
Gene with previously characterized CSRE				
YJL089W	*SIP4*	Transcription factor of gluconeogenic genes	CCGTTCGACCG	−275 to −265
YLR377C	*FBP1*	Fructose-1,6-bisphosphatase	CCATCCGTCCG	−504 to −494
YER065C	*ICL1*	Isocitrate lyase	CCATTCATCCG	−398 to −388
YNL117W	*MLS1*	Malate synthase	CCATTGGGCCG	−500 to −490
			CCGGCGAGCCG	−450 to −440
			CCATTGAGCCG	−531 to −521
YKR097W	*PCK1*	Phosphoenolpyruvate carboxykinase	CCTTTCATCCG	−481 to −471
			CCATTCACCCG	−560 to −550
			CCCTTTATCCG	−362 to −352
YOL126C	*MDH2*	Cytosolic malate dehydrogenase	CCTTTAATCCG	−262 to −252
			CCATTCGGCCG	−239 to −229
			CCATTTGGCCG	−295 to −285
YJR095W	*SFC1*	Mitochondrial succinate-fumarate transporter	CCGGTAAACCG	−491 to −481
			CCATTAAACCG	−679 to −669
			CCATTCAACCG	−608 to −608
YML042W	*CAT2*	Carnitine *O*-acetyltransferase	CCTTTCGCCCG	−281 to −271
Proposed new CSRE genes				
YBL015W	*ACH1*	Acetyl-CoA hydrolase	CCGACGGCCCG	−433 to −423
			CCGGCGGGCCG	−430 to −420
YLR308W	*CDA2*	Chitin deacetylase	CCATTTGCCCG	−437 to −427
			CCGACGGCCCG	−288 to −278
YOR316C	*COT1*	Involved in cobalt accumulation	CCGCTCACCCG	−261 to −251
YOR100C	*CRC1*	Mitochondrial carnitine carrier	CCAGTCATCCG	−258 to −248
YKL096W	*CWP1*	Cell wall mannoprotein	CCTTCGGCCCG	−345 to −335
YPL262W	*FUM1*	Mitochondrial and cytosolic fumarase	CCCCTGAGCCG	−334 to −324
YLR205C	*HMX1*	Unknown	CCAATGATCCG	−430 to −420
YDL085W	*NDE2*	Mitochondrial NADH dehydrogenase	CCGGCCATCCG	−386 to −376
YPL156C	*PRM4*	Unknown	CCGCTTGCCCG	−383 to −373
YBR050C	*REG2*	Protein phosphatase type 1	CCATTTGCCCG	−405 to −395
			CCGACGGCCCG	−370 to −360
YOR095C	*RKI1*	Ribose-5-phosphate ketol-isomerase	CCATTAGCCCG	−335 to −325
YGL056C	*SDS23*	Unknown	CCGCTAACCCG	−452 to −442
YGL208W	*SIP2*	Response to glucose starvation	CCCTTGGACCG	−148 to −138
YAR035W	*YAT1*	Outer carnitine acetyltransferase	CCGGCGGTCCG	−167 to −157
			CCGTCCGCCCG	−136 to −126
			CCGCCGGGCCG	−171 to −161
YER024W	*YAT2*	Carnitine *O*-acetyltransferase	CCGTCGGTCCG	−169 to −159
YGR250C		Unknown	CCGCTGATCCG	−476 to −466
YER158C		Unknown	CCCTTCGTCCG	−478 to −468
YDR222W		Unknown	CCGTCTAGCCG	−355 to −345
YOL125W		Unknown	CCATTTGGCCG	−491 to −481
			CCATTCGGCCG	−547 to −537
			CCTTTAATCCG	−524 to −514
YGR067C		Unknown	CCGATCGTCCG	−488 to −478
			CCCTTTGTCCG	−458 to −448
Proposed CSRE consensus[a]			CCnnynrnCCG	

[a] n = A, C, G, or T; y = C or T; r = A or G.

complex of signals known as carbon catabolite repression (10, 63). Glucose repression of transcription is mainly exerted via the Mig1p-binding protein. This is consistent with the finding that 24 genes (30%) up-regulated when *S. cerevisiae* is grown with acetate or ethanol contain a Mig1p binding site in their promoter region. From this set, most of the 18 genes with known function have previously been experimentally shown to contain a Mig1p binding site (*REG2* (Ref. 64), *FBP1* (Ref. 65), and *ICL1* (Ref. 66)) or at least to be repressed by glucose (*ACH1*, *MDH2*, *ICL1*, *NDE2*, *YAT1*, *CAT2*, *YAT2*, *CRC1*, *SFC1*, and *SDH1* (Refs. 31 and 67–69)). *MIG1* transcription itself did not respond to carbon source, which is consistent with previous reports of Mig1p activity control by post-translational modification and nuclear export (70, 71).

An important cis-acting element for the transcriptional response to gluconeogenic carbon sources is Cat8p (for review, see Ref. 72). Cat8p binds to an 11-bp upstream activation sequence element named CSRE (carbon source-responsive element) and most recently defined as CCrTysrnCCG (r = A or G, y = C or T, s = C or G, n = A, T, G, or C) (73). Most of the genes that have been shown to contain functional Cat8p binding sites were indeed up-regulated in response to growth with C2-carbon sources (*SIP4*, *FBP1*, *ICL1*, *MLS1*, *PCK1*, *MDH2*, *SFC1*, and *CAT2*). Furthermore, screening the upstream untranslated regions of all genes up-regulated in the presence of C2-compounds allowed the identification of 20 additional genes containing one or more CSRE-related sequences (Table VI). In contrast to most of its targets, *CAT8* itself was not affected by the carbon source (Figs. 3 and 4). This behavior is consistent with earlier reports of Cat8p activation at the post-translational level by phosphorylation (74).

No other binding sites corresponding to transcription factors known to be involved in carbon source adaptation (Adr1p, Hap complex, Rtg regulators) could be recovered from this set of C2-compound co-regulated genes. *ACS1* and *ADH2*, the main targets of Adr1p (75, 76), were not significantly up-regulated in our culture conditions and were therefore not included in the promoter analysis. Similarly, few of the potential targets of the Hap complex and the Rtg regulators (*SDH1*, *CIT2*, *IDH1*, and *IDH2*) were up-regulated in this study and could therefore not result in a significant over-representation of their binding motifs in the cluster.

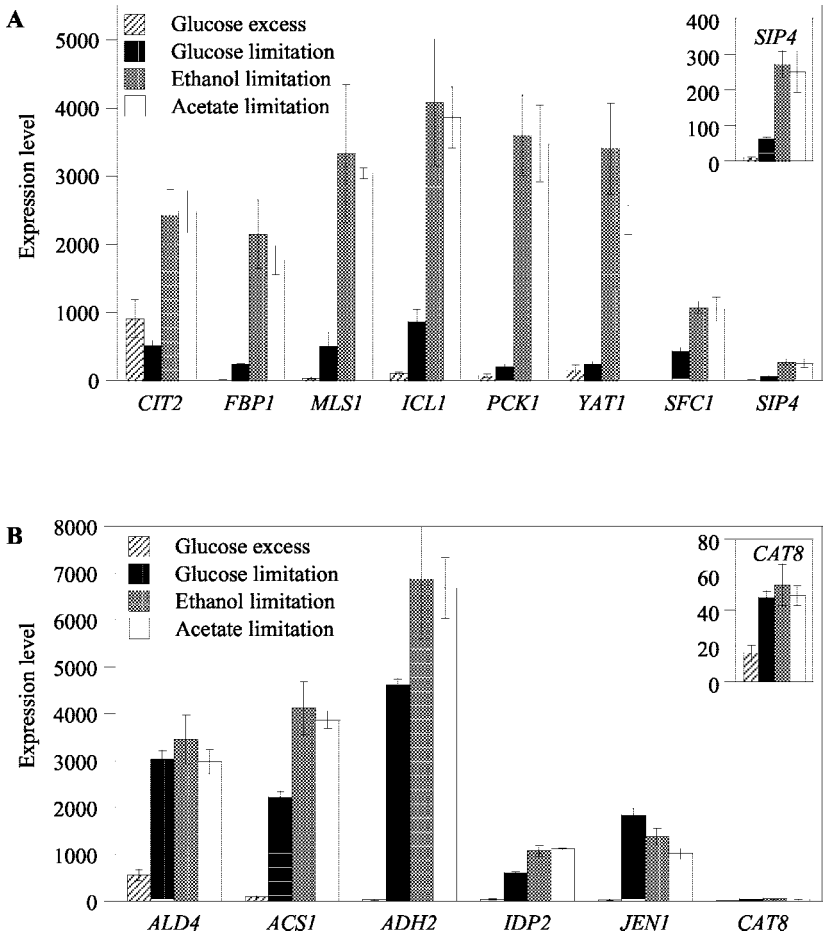

FIG. 4. **Expression profiles in chemostat cultivation of specific genes under glucose excess and glucose, ethanol, or acetate limitation.** Glucose excess expression data represent the average hybridization intensity in aerobic chemostat cultivation under nitrogen, phosphorus, and sulfur limitation for the same *S. cerevisiae* strain and in perfectly identical culture conditions published by Boer *et al.* (31). *A*, C2-compound-responsive genes; *B*, C2-compound-nonresponsive genes.

DISCUSSION

Transcription Analysis of Chemostat Cultures Versus Batch Cultures—In carbon-limited, aerobic chemostat cultures, remarkably few transcripts exhibited significant differences when the growth-limiting carbon source was changed. The change from a sugar (glucose or maltose) to a C2-compound (ethanol or acetate) resulted in only 117 genes with a significantly changed transcript level (Fig. 3). This robustness of the yeast transcriptome in response to changes of the carbon source is in contrast with previously reported data from batch cultures. In a transcriptome analysis of the diauxic shift in *S. cerevisiae*, which essentially represents the transition from growth on glucose to growth on ethanol, over 400 transcripts were found to change by more than 2-fold (43). Similarly, in an independent study on glucose- and ethanol-grown batch cultures, over 600 transcripts were found to change (77). When comparing the carbon source response in chemostat with these previous reports in batch cultivation, approximately half of the 117 genes that responded to C2-compounds in chemostat cultivations were also transcriptionally regulated during these two transcriptome studies.

Several factors may have contributed to these different carbon source-dependent transcriptional responses in batch and chemostat cultures. A large number of genes (225 based on work in our laboratory (Ref. 31)) are transcriptionally regulated by glucose, for instance via glucose catabolite repression. This phenomenon does not occur in glucose limited chemostat cultures, where the low residual glucose concentration (~0.1 mM measured using a fast sampling technique (Ref. 78)) prevents glucose catabolite repression. This absence of glucose repression is supported by our data; the transcript levels of several genes that are known to be regulated by glucose repression (*GAL* (Ref. 79) and *MAL* (Ref. 80), for instance) were clearly not further de-repressed when *S. cerevisiae* was grown with non-fermentable carbon sources. Another factor that may have influenced the transcriptional response in batch cultures is the toxicity of substrates and/or products. This is perhaps best illustrated by acetate. This weak acid, which is a normal metabolite of *S. cerevisiae* in glucose-grown batch cultures, uncouples the pH gradient across the yeast plasma membrane and is therefore likely to result in transcriptional stress responses (18). Production of ethanol and acetate does not occur in aerobic, glucose-limited chemostat cultures, and, when these compounds are used for carbon-limited chemostat cultivation, their residual concentration is below 0.5 mM and therefore unlikely to induce any stress responses. An additional factor that complicates interpretation of the data from the classical study of deRisi *et al.* (43) on the diauxic shift is the nature of the culture medium. This deRisi study was performed with a complex medium, in which the yeast not only has to change carbon source at the diauxic shift, but also has to sequentially utilize the many nitrogen sources that are present in the medium. This complication does not influence the data from our chemostat study, in which ammonium ions were the sole nitrogen source. Finally, whereas the specific growth rate drastically decreases after the diauxic transition in batch cultures, our transcriptional analysis was performed at a fixed specific growth rate. All these differences between chemostat and batch cultivation make the former a powerful tool to study the influence of one parameter only, *i.e.* carbon source, without the inherent interferences that occur in batch cultures. Unlike chemostat studies, carbon source response studies performed in batch cultivation can therefore not discriminate between carbon source de-repression and induction mechanisms. In the

present study, three different transcriptional responses to carbon source could be identified: (i) a strong C2 induction in addition to a de-repression, as observed for most of the Cat8-dependent genes (*FBP1*, *MLS1*, *ICL1*, *PCK1*, *SFC1*, and *SIP4*; Fig. 4*A*); (ii) a strong induction by non-fermentable carbon sources without glucose de-repression, as displayed by *CIT2* and *YAT1* (both involved in acetyl-CoA metabolism; Fig. 4*A*). This glucose insensitivity of *CIT2* is consistent with previous reports (81), whereas contradictory reports of *YAT1* repression by glucose may indicate a strain-specific response of *YAT1* to catabolite repression (Refs. 68 and 81). (iii) A more surprising set of genes, which had been so far described as induced by C2-compounds (43, 43, 82–84), were strongly de-repressed under glucose limitation but did not show any further significant induction in the presence of ethanol or acetate (*ALD4*, *JEN1*, *IDP2*, *ACS1*, and *ADH2*; Fig. 4*B*).

Carbon Source-dependent Transcripts and Regulation Mechanisms—Carbon metabolism in *S. cerevisiae* is one of the most intensively studied metabolic systems. Indeed, in many cases, the carbon source-dependent transcription of genes could be teleologically explained from the known catalytic or regulatory functions of their gene products. Some obvious examples include the high transcript levels of glyoxylate cycle and gluconeogenic genes during growth on C2-compounds and the induction of *MAL* genes in maltose-limited chemostat cultures. However, in many other cases, the function of carbon source-responsive genes is either entirely unknown (the frequency of genes with unknown function was 40%, which is significantly higher than that of the entire yeast genome (27% according to YPD™)) or difficult to interpret in terms of biochemical function of the gene product. Although there is no *a priori* proof that carbon source-dependent transcriptional regulation correlates with physiological function, our data provide an interesting lead for future functional analysis research.

As expected, very few transcriptional differences were measured between cultures grown on glucose and maltose (34 changes). The *MAL* structural genes (maltose permeases and maltases), but not the *MAL* activators, were up-regulated in the presence of maltose. Analysis of the upstream region of these co-regulated genes resulted in the identification of the *MAL* regulator binding site (85) (Table V). Cultivation on maltose, however, generated a puzzling set of 18 genes down-regulated compared with glucose cultivations. These changes suggest that maltose utilization, despite its closeness to glucose utilization, results in more metabolic perturbations than is generally recognized. Comparing the transcriptomes of ethanol- or acetate-grown cells resulted in even fewer differences. As few as 16 genes yielded different transcript levels for these two gluconeogenic compounds, all being down-regulated with acetate. Acetate uptake is mediated by a protein for which the encoding gene has not been identified so far (6, 7). Unfortunately no potential candidate for an acetate transporter could be identified from our data set. Finally, comparing C2-compound-limited cultures with sugar-limited cultures resulted in the identification of 117 carbon source-responsive genes. Among these genes, a high proportion have unknown or poorly defined biological functions (48%), but as many as half of the remainder (34 genes, 29%) are involved in carbon metabolism. The promoter analysis of co-regulated genes resulted in the definition of a new set of genes containing one or more sequences closely related to the carbon source-responsive element (Table VI). Using the totally different approach of integrating transcriptome and proteome comparison of a wild-type and a *cat8*Δ *S. cerevisiae* strain, Haurie and co-workers (86) also proposed a list of Cat8p-dependent genes containing a CSRE-related binding site. Their gene list largely overlaps the list proposed in this work. From the set of C2-compound-up-regulated genes could also be identified a large number of genes containing a Mig1p binding site, which is consistent with the role of Mig1p in the repression of the genes involved in the utilization of alternative carbon sources. Two additional over-represented promoter elements were recovered from this set of genes that could not be related to any known transcription factor. A recent study on *FBP1* (87) nicely exemplified the potential complexity of carbon source control of gene expression and strongly suggests, together with earlier studies (9, 74), the involvement of additional, still unidentified transcription factors. The putative promoter elements identified in the present study could be the targets of these additional carbon source regulators.

Transcript Levels Versus Metabolic Fluxes—From a combination of metabolic flux analysis and transcriptome analysis, we were able to compare metabolic fluxes in central carbon metabolism and transcript levels of key structural genes that encode enzymes of the central metabolic pathways (Tables II and III). Three distinct types of correlation between transcript levels and fluxes could be identified.

For the pathways that are specific for maltose metabolism (maltase) or for the metabolism of gluconeogenic carbon sources (gluconeogenesis and glyoxylate cycle), there was a strong qualitative correspondence between transcriptional regulation and estimated *in vivo* metabolic fluxes. The genes involved in these pathways are known to be strongly transcriptionally regulated, but several of the corresponding enzymes are also subject to post-translational regulation (inactivation by phosphorylation and/or ubiquitin-catalyzed degradation (Refs. 88 and 89)). The time scale of these post-translational processes is much shorter than that of transcriptional regulation. Although one would intuitively assume that post-translational processes are predominantly important under dynamic conditions, we cannot presently exclude the possibility that they also contribute to the regulation of *in vivo* enzyme activity during carbon-limited steady-state cultivation in chemostat cultures.

The changes in metabolic fluxes in the tricarboxylic acid cycle and in the pentose-phosphate pathway were only partially mirrored by changes in transcript levels. Little is known about the regulation of these two pathways. From our data it appears clearly that, to meet the new flux requirements when C2 carbon sources are used, *S. cerevisiae* only enhanced or repressed the transcription of a few genes. One can assume that only the rate controlling steps need to have their protein concentration optimized to adjust the flux to the new requirement. In such a case, transcriptome analysis would help to identify the potential rate-limiting steps, *i.e.* succinate dehydrogenase and fumarase for the tricarboxylic acid cycle (*CIT2* and *MDH2* induction mainly reflecting the need of the corresponding proteins in a different compartment rather than a real rate-limiting step) and 6P-gluconolactonase, 6P-gluconate dehydrogenase, ribose-P isomerase, and transketolase 2 for the pentose-phosphate pathway. However, the magnitude of change in transcript levels does not correlate well with the magnitude of change in metabolic fluxes, indicating that transcriptional control alone cannot explain the modifications in the flux distribution in the tricarboxylic acid cycle and in the pentose-phosphate pathway.

The model-predicted fluxes in the glycolytic pathway and in the enzymic reactions surrounding pyruvate were strongly dependent on the studied carbon sources. However, the different *in vivo* activities of the key enzymes of these pathways were not at all mirrored by their transcript levels. In glycolysis, only *HXK1* (encoding hexokinase I) and *TDH1* (encoding a minor

isoform of glyceraldehyde dehydrogenase) displayed a reduced transcription level when the glycolytic flux was decreased at least 3-fold. This is a clear indication that, during carbon-limited cultivation, fluxes through these central metabolic pathways in *S. cerevisiae* are not primarily controlled at the transcriptional level. Further research is required to assess the contribution of translational efficiency, post-translational modification, and regulation by intracellular concentrations of substrates, products, and effectors to the regulation of *in vivo* activity of these pathways. Such discrepancies between fluxes and/or enzyme activities and transcript levels have already been reported by other integrative approaches with *S. cerevisiae* (90) and several bacteria (91–93).

Our study underlines that DNA microarrays, however useful for studying transcriptional regulation, comparative genotyping, and purely correlation-based diagnostics, have limited value as indicators for *in vivo* activity of proteins. This limitation should be considered when applying DNA microarrays as a tool for activities such as metabolic engineering or identification of potential drug targets.

Acknowledgments—The research group of Prof. J. T. Pronk is part of the Kluyver Centre for Genomics of Industrial Fermentation, which is supported by the Netherlands Genomics Initiative. We thank H. C. Lange for providing the compartmented stoichiometric model. We also thank L. M. Serrano, M. de Herdt, and J. M. A. Geertman for technical assistance and Prof. J. P. van Dijken for critically reading the manuscript.

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J. Biol. Chem. 2004, 279:9125-9138.
doi: 10.1074/jbc.M309578200 originally published online November 20, 2003
