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Release of Two *Saccharomyces cerevisiae* Cytochrome Genes, *COX6* and *CYC1*, from Glucose Repression Requires the *SNF1* and *SSN6* Gene Products

RICHARD M. WRIGHT AND ROBERT O. POYTON*

Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80309-0347

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We show here that *SNF1* and *SSN6* are required for derepression of the glucose-repressible yeast genes *COX6* and *CYC1*, which encode the mitochondrial proteins cytochrome *c* oxidase subunit VI and iso-1-cytochrome *c*, respectively. In an *snf1* mutant genetic background, the transcription of both *COX6* and *CYC1* continued to be repressed after cells were shifted into derepressing media. In an *ssn6* mutant genetic background, both *COX6* and *CYC1* were expressed constitutively at high levels in repressing media. *SSN6* acted epistatically to *SNF1* in the regulation of both cytochrome genes. These findings are similar to previous findings on the effects of *SNF1* and *SSN6* on *SUC2* expression in *Saccharomyces cerevisiae* and are consistent with a model proposing that *SNF1* exerts its effect through *SSN6* on *COX6* and *CYC1*.

Yeast cells respond to growth in glucose by up-regulating fermentative metabolism, with the consequent production of ethanol. At the same time, enzymes in numerous metabolic pathways, including those involved in gluconeogenesis, the tricarboxylic acid cycle, and mitochondrial electron transport and oxidative phosphorylation (7, 9), are repressed. This process, referred to as glucose repression, is complex. It affects the activity of some enzymes and the synthesis of others (6–8, 13). For many genes, the effect of glucose repression on synthesis operates at the level of transcription (18, 22) and is mediated by upstream *cis*-acting sites, i.e., upstream activation sequence elements (10, 18, 21, 23).

Through the isolation of nonrepressible and nonderepressible mutants and their suppressors, it has been possible to gain initial insight into the genetic regulation of glucose repression. Of the dozen or so genes so far implicated in glucose repression (1, 6–9, 13, 19), some of the best understood are *SNF1* (*CAT1*, *CCR1*) and *SSN6* (*CYC8*). The *SNF1* gene (together with other *SNF* genes) (1–4, 15) is essential for sucrose fermentation because it is required for derepression of the synthesis of the secreted form of invertase, a product of *SUC2*. *SNF1* also affects the derepression of a number of other glucose-repressible enzymes of intermediary metabolism; thus, it appears to be a global regulator of glucose repression (7, 9). Through the isolation of suppressor mutants of *SNF1*, Carlson et al. (1, 2) were able to identify *SSN6* as an additional gene involved in the regulation of *SUC2* by glucose. Based on the observations that *ssn6* mutants produce high levels of the *SUC2* gene product constitutively and suppress mutations in *SNF1* and that *SSN6* acts epistatically to *SNF1*, Neigeborn and Carlson (15) have proposed that the *SSN6* gene product acts, directly or indirectly, to repress *SUC2* transcription and is probably affected by the *SNF1* gene product. Insofar as the *SNF1* gene product is a protein kinase (5), it is possible that *SNF1* exerts its effect on *SUC2* and other glucose-repressible genes through phosphorylation of the *SSN6* gene product or other regulatory factors.

Are the *SNF1* and *SSN6* genes involved in transcriptional regulation of glucose-repressible respiratory proteins? To address the question, we studied *COX6*, (25, 26) and *CYC1* (11), the genes that encode cytochrome *c* oxidase subunit VI and iso-1-cytochrome *c*, respectively. Previous studies demonstrated that the transcription of both genes is glucose repressible in conjunction with the product of the *HAP2* gene (11, 23). We report here that *SNF1* and *SSN6* are also required for the release from glucose repression of *COX6* and *CYC1* and that they affect the transcription of these genes in a manner analogous to the way that they regulate *SUC2*.

To determine whether *SNF1* is involved in *COX6* regulation, we used an *snf1* deletion strain, MCY1595, and its parent carrying a wild-type *SNF1* allele, MCY1093, kindly provided by Marian Carlson (Columbia University, New York, N.Y.). Cells were grown to the early log phase in 2% glucose, harvested, washed in H_2O, and shifted into 2% (wt/vol) glucose (repressing conditions) or 0.05% (wt/vol) glucose (derepressing conditions) as described previously (1). Three hours following the shift, poly(A)$^+$ RNA was prepared and Northern (RNA) blot analysis was conducted. The blots were hybridized with probes specific for the yeast actin gene (a nonresponding control), *ORF-U* (a divergently transcribed gene flanking *COX6* upstream), or *COX6* and *ORF-D* (a gene flanking *COX6* downstream) (26). In the *SNF1*$^+$ strain, both *COX6* and *ORF-U* showed marked derepression (Fig. 1); the levels of the major *COX6* transcripts (26) and the *ORF-U* transcript were higher under derepressing than under repressing conditions. In the *snf1* mutant strain, *COX6* expression was only slightly derepressed and *ORF-U* expression was decreased slightly under derepressing conditions. In contrast to both *COX6* and *ORF-U*, the *ORF-D* gene was expressed at higher levels under repressing than under derepressing conditions. Moreover, the state of the *SNF1* gene had little effect, if any, on the decreased expression of *ORF-D* under derepressing conditions. Data for each of these genes were quantitated (Table 1) by counting the radioactivity associated with the bands shown in Fig. 1. We observed a 4.8-fold level of derepression for *COX6* and a 2-fold level of derepression for *ORF-U* in the *SNF1*$^+$ strain. The *snf1* mutation blocked the derepression of *ORF-U* and diminished the derepression of *COX6* but had little effect on the relative expression of

* Corresponding author.

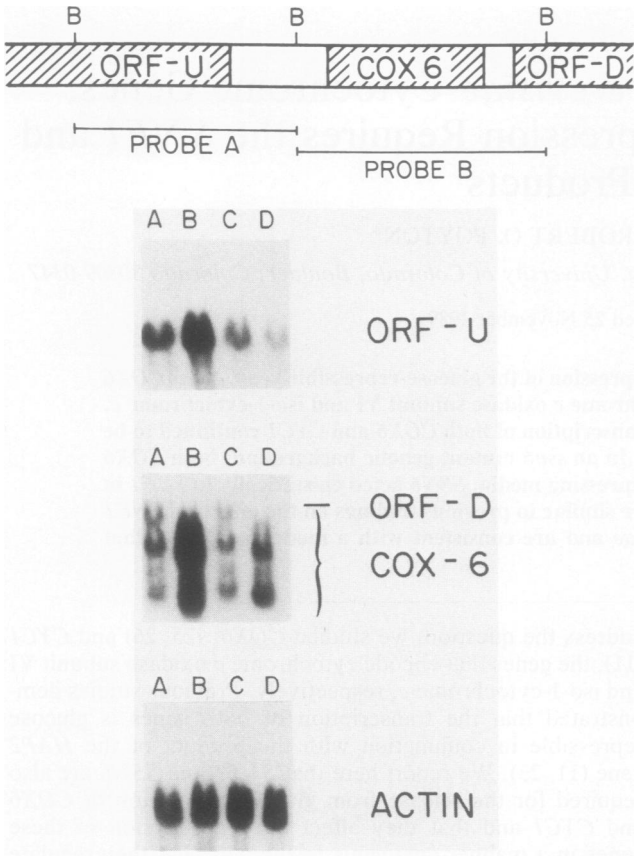

FIG. 1. Northern blot analysis of *COX6* and its two flanking genes, *ORF-U* and *ORF-D*. Shown at the top is the *COX6* genetic region and the probes that were used. Probe A is *ORF-U* specific, and probe B hybridizes to *COX6* RNA and to *ORF-D* RNA. B, *Bgl*II restriction sites that were used to generate probes A and B. Northern blots for *ORF-D*, *COX6* and *ORF-D*, and actin (a nonresponding gene) are shown at the bottom. *SNF1*$^+$ and *snf1* cells were grown in repressing (2% glucose) or derepressing (0.05% glucose) media, as indicated in the text. Lanes: A, *SNF1*$^+$-repressing media; B, *SNF1*$^+$-derepressing media; C, *snf1*-repressing media; D, *snf1*-derepressing media. The region corresponding to the major *COX6* transcripts (26) is identified by a brace.

ORF-D under repressing and derepressing conditions (Table 1 and Fig. 1).

To corroborate these observations, we transformed both strains with a high-copy-number *COX6-lacZ* fusion plasmid, pMC61L (23, 24), that has 1.5 kilobase pairs of the *COX6* upstream DNA and the first 21 codons of *COX6* fused to the coding region of the *Escherichia coli lacZ* gene encoding β-galactosidase. Expression of the *COX6-lacZ* fusion gene carried on this plasmid is glucose repressible and requires heme and *HAP2* in a way that mimics that of the copy of *COX6* carried on the chromosome (23). Transformant cells were grown as described above, and β-galactosidase activity was measured 3 h after the shift into repressing or derepressing media. We observed a fourfold derepression of *COX6* in the *SNF1*$^+$ strain when cells were shifted into derepressing media and a little derepression of *COX6* in the *snf1* mutant strain (Table 2). Thus, the β-galactosidase assay revealed nearly the same level of *SNF1* dependence as the Northern blot measure of steady-state RNA levels. Because the expression of the *COX6-lacZ* fusion gene bearing *COX6* promoter sequences was affected by the *snf1* mutant in the same way as the native *COX6* gene, we concluded that the effect of *SNF1* on *COX6* is exerted at the level of transcription and not at some posttranscriptional step in *COX6* expression.

Schultz and Carlson have proposed that one of the possible targets of the *SNF1* protein kinase is the *SSN6* gene product (20). To determine whether the *SSN6* gene product affects *COX6* expression, we transformed *snf1* and *ssn6* single- and double-mutant strains with pMC61L (Table 2). The β-galactosidase activities supported by each strain were determined after the cells were shifted into repressing or derepressing conditions as described above. *COX6-lacZ* was constitutively derepressed in both single- and double-mutant strains carrying an *ssn6* mutation (Table 2). Because an *snf1* mutation is silent in combination with an *ssn6* mutation, these findings suggest that *SSN6* acts epistatically to *SNF1* in regulating *COX6*.

To determine whether the *SNF1* and *SSN6* gene products affect the derepression of another protein of the mitochondrial electron transport chain, we assayed the expression of a *CYC1-lacZ* fusion gene on the high-copy-number plasmid pLG669Z (12) during a glucose shift experiment with the four strains described above (MCY1093, MYC1595, MYC1097, and MYC1640). The derepression of *CYC1* was *SNF1* dependent; the *snf1* mutant strain failed to derepress the *CYC1* gene following a shift into derepressing media (Table 2). In addition, the *CYC1* gene was constitutively derepressed in either *ssn6* background. As indicated above, these observations suggest that *SSN6* acts epistatically to *SNF1* in regulating *CYC1*. Interestingly, it was shown previously that a strain carrying a mutation in *CYC8*, which is allelic to *SSN6* (2), exhibits increased levels of iso-2-cytochrome *c*, the product of *CYC7*, under derepressing conditions (17). Although it is not known if this change is mediated through transcriptional regulation of *CYC7*, this observation, together with those presented here, make it clear that *SSN6* affects the expression of both cytochrome *c* isologs in *S. cerevisiae*.

In summary, the studies described here reveal that both *SNF1* and *SSN6* are involved in the regulation of *COX6* and *CYC1*. They also demonstrate that the derepression of *ORF-U* requires *SNF1* and that the expression of *ORF-D* is glucose dependent and unaffected by *SNF1*. The latter finding for *ORF-D* suggests that the effects of *SNF1* on *COX6* and *CYC1* expression are not exerted via some general change in metabolism but are instead exerted in a gene-specific manner.

Insofar as both *CYC1* and *COX6* are positively regulated

TABLE 1. Quantitation of Northern blot data from Fig. 1[a]

Gene	% of counts hybridized to actin transcript			
	SNF1$^+$		*snf1* mutant	
	Repressing	Derepressing	Repressing	Derepressing
Actin	100	100	100	100
COX6	88.9	422.8	45.2	102.5
ORF-U	77.2	152.8	29.2	22.2
ORF-D	24.6	8.1	8.2	2.2

[a] Spots were cut from the nitrocellulose filters used to obtain the data shown in Fig. 1. Radioactivity bound to each spot was determined by liquid scintillation counting. The data for each lane were normalized to the counts obtained from the actin probe. Raw data for actin were as follows: *SNF1*$^+$ repressing, 14,140 cpm; *SNF1*$^+$ derepressing, 19,291 cpm; *snf1* mutant repressing, 25,440 cpm; *snf1* mutant derepressing, 17,790 cpm. The data presented for *COX6* were obtained by measuring the radioactivity associated with all of its transcripts within the braced region of the blot shown in Fig. 1.

TABLE 2. Mediation of derepression of the expression of *COX6-lacZ* and *CYC1-lacZ* fusion genes by *SNF1* and *SSN6*[a]

Strain	Relevant genotype	β-Galactosidase activity (U/min)			
		COX6-lacZ		*CYC1-lacZ*	
		Repressing	Derepressing	Repressing	Derepressing
MYC1093	*SNF1*$^+$ *SSN6*$^+$	21.9 ± 3.6	83.9 ± 6.3	14.1 ± 0.32	186 ± 9.5
MYC1595	*snf1 SSN6*$^+$	24.8 ± 3.1	29.4 ± 5.3	6.9 ± 1.6	6.1 ± 0.25
MYC1097	*SNF1*$^+$ *ssn6*	116 ± 12.4	137 ± 5.2	188 ± 38	157 ± 10.8
MYC1640	*snf1 ssn6*	110 ± 40.8	145 ± 3.4	471 ± 82.2	343 ± 38.3

[a] Each strain was transformed with either the *COX6-lacZ* fusion plasmid pMC61L or the *CYC1-lacZ* fusion plasmid pLG669Z. The strains were grown to the mid-log phase in growth medium containing 2% glucose and shifted into either 2% glucose (repressing media) or 0.05% glucose (derepressing media). Three hours after the shift, the cells were harvested, washed, and assayed for β-galactosidase activity as described previously (23). For each assay 10 independent transformants were pooled. The values given are the mean of six determinations ± the standard deviation for *COX6-lacZ* and the mean of three determinations ± the standard deviation for *CYC1-lacZ*. The units of β-galactosidase activity were determined as follows: $(OD_{420}/OD_{600}) \times 1{,}000$, as defined previously (14). The genotypes of the strains were as follows: MCY1093, *MATa his4-539 lys2-801 ura3-52*; MCY1595, *MATα his4-539 lys2-801 ura3-52 snf1Δ3*; MCY1097, MATα *ade2-101 lys2-801 ura3-52 ssn6-1*; *MCY1640, MATa ade2-101 ura3-52 ssn6-1 snf1-28*.

by the *HAP2* and *HAP3* gene products (11, 24), how is regulation by *HAP2* and *HAP3* related to regulation by *SNF1* and *SSN6*? At present, two general types of models are possible (Fig. 2). In the first model, the *HAP2-HAP3* complex (10) could affect *COX6* or *CYC1* expression independently of *SNF1* and *SSN6*, through two regulatory pathways, an *HAP2-HAP3* activation pathway and an *SNF1-SSN6* repression pathway. In the second model, the products of *HAP2*, *HAP3*, *SNF1*, and *SSN6* would all participate in the same pathway. In the latter model, *SNF1* may regulate the synthesis or activity of the complex formed by the protein products of *HAP2* and *HAP3* (10). As a regulator of synthesis, *SNF1* may act positively on *HAP2* transcription, possibly in conjunction with *SSN6*. Because the transcription of *HAP2* itself is glucose repressible (16), the failure of *COX6* and *CYC1* to derepress could be explained in this model by the failure of the transcription of a positive effector gene (i.e., *HAP2*) to be derepressed. As a regulator of activity, *SNF1*, whose product is a protein kinase, may phosphorylate *HAP2*, *HAP3*, or another protein (e.g., the *SSN6* gene product), with which *HAP2* and *HAP3* may interact to regulate the transcription of *COX6* or *CYC1*. Because the *HAP1* (*CYP1*) gene has no effect on the expression of *COX6* (24), it is unlikely to be a component of either

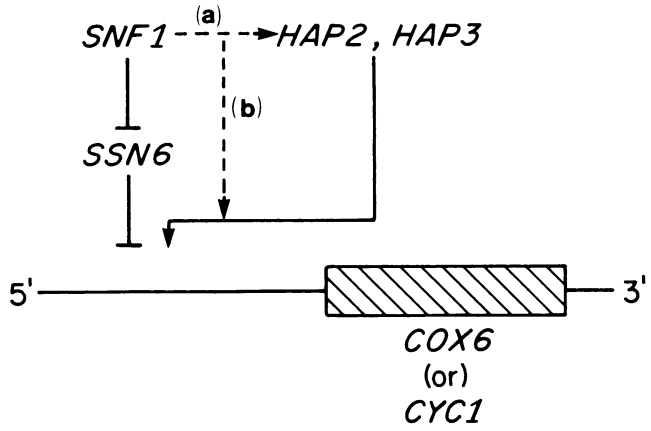

FIG. 2. Possible models for the interrelationships between the regulation of *COX6* and *CYC1* by the *SNF1-SSN6* pathway and the *HAP2-HAP3* pathway. In the first model (solid lines), the *SNF1-SSN6* and *HAP2-HAP3* pathways affect *COX6* and *CYC1* transcription independently of one another. In the second pathway (broken lines), *SNF1* affects either the synthesis of *HAP2* or *HAP3* (a) or the activity of their products (b).

of these models for the regulation of *COX6*. However, we cannot exclude the possibility that it interacts with the *SNF1-SSN6* pathway in the regulation of *CYC1*. Although further study is required before it is possible to decide between these models, the findings presented here, together with results presented previously, indicate that the expression of nuclear genes for mitochondrial respiratory proteins is under the control of multiple common factors.

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