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Signaling pathways regulating the transcriptional response of the sodium ATPase *ENA1* to saline and alkaline stress in the yeast *Saccharomyces cerevisiae*

Doctoral Thesis

Dept. Bioquímica i Biologia Molecular
Universitat Autònoma de Barcelona

May 2008

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Departament de Bioquímica i Biologia Molecular
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Thesis dissertation for the degree of Doctor in Biochemistry, Molecular Biology and Biomedicine, presented by **Maria Platara**, graduated in Pharmacy. This work was performed at the Veterinary Unit of the Department of Biochemistry and Molecular Biology of the 'Universitat Autònoma de Barcelona' under the direction of **Dr. Joaquín Ariño Carmona.**

signature

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May 2008
Bellaterra, Cerdanyola del Vallés
BARCELONA

If we knew what it was we were doing,
it would not be called research, would it?

Albert Einstein

Στους γονείς μου

To my parents

Desde aquí quiero agradecer su apoyo a todas estas personas que han hecho posible el desarrollo de este trabajo.

En primer lugar quiero agradecer a mi director Joaquín Ariño, por haberme dado la oportunidad de entrar en el mundo de la ciencia al aceptarme en su grupo, por confiarme un proyecto y luchar conmigo por su realización, enseñándome incansablemente y corrigiendo con paciencia mis errores y sobre todo por enseñarnos que hay que poner mucha ilusión y trabajar duro para sobrevivir en el mundillo científico.

Al Dr. Fernando Moreno por la colaboración excelente en el desarrollo de este trabajo. También a la Dra. María Carmen Martínez y la Dra. Carme Espunya, por la fructífera colaboración que hemos tenido y que fue la causa de que me interesara por la quinasa CK2.

En especial a todos los compañeros de laboratorio, porque aparte de ayudarme en todos los aspectos, más que nada han sido una familia grande para mí, estando allí tanto en los momentos difíciles pero también en los momentos mas bonitos de todo ese periodo duro que ha sido la realización de la tesis. A Amparo por cogerme de la mano desde el primer día enseñándome a pipetear y a la vez intentando entenderme cuando no se me entendía, por explicarme todo con paciencia todas la veces que hizo falta, pero sobre todo por estar allí con un abrazo en los momentos mas difíciles y desanimados. A Raquel por compartir conmigo todos sus conocimientos sobre pH, sus truquillos en los experimentos, por defenderme en los momentos más críticos y por estar allí, toda una doctora, como asistente personal en uno de los momentos mas importantes de mi vida. Al Antonio, por aparte de saberlo todo (o casi) y contestar a mi preguntas, por las comidas y las cenas compartidas, los cafés de la tarde, su humor que te anima en momentos desanimados y por ser el mejor científico-fotógrafo que he conocido. A Maribel por todos los momentos que hemos compartido, los congresos, y las tardes interminables del laboratorio y las risas en momentos difíciles.

A los que ya no están aquí. A Ernesto por aceptarme como un miembro más en la gran familia del laboratorio, por enseñarme el castellano poco 'correcto' y diseñar oligos en tiempo record. A Ivan, por no negarme nunca su ayuda en cualquier aspecto, por transmitirme sus conocimientos sobre proteínas y westerns pero sobre todo por ser mi psicoanalista personal. A Laia Viladevall por los cafés compartidos y su manera

positiva de afrontar las cosas que me ha transmitido y a la Loli por los momentos compartidos. Y a los que llegaron después, a Martí por aguantar mi lado insoportable estoicamente, por sus análisis sobre cualquier tema, aunque nunca me ha convencido, y sobre todo por ser amigo. A Asier por estar al pie de cañón siempre (porque en un laboratorio pasa de todo), por corregir mis errores lingüísticos, por hacerme reír tantas veces y por ser compañero en lo bueno y en lo malo. A Xavi por sus clases de català, per las cerveses belges, y por demostrar que alguien puede hacer las cosas a su manera.

A los más novatos del grupo. A Carlos, por ser casi mi 'saco de boxeo' durante todo este periodo duro de la escritura de este trabajo y aguantarlo sin quejarse demasiado y por ser colega desde el primer momento que entró en el laboratorio, y a Jofre por hacerme sentir 'jefa' por un minuto al día. A Lina, por haber traído al grupo aires nuevos y un sentido de alegría que se contagia (y eso siempre se agradece). A Dani Valverde por enseñarme que hay vida científica más allá de las levaduras y por aceptar adoptar mis manías en la ejecución de los protocolos.

A los de la otra punta del pasillo, pero aún así parte de la gran familia de nuestro grupo. A la Dra. Anna Barceló por estar siempre dispuesta a explicarnos hasta lo inexplicable y apoyarnos en la lucha contra la tecnología mas avanzada (¿como salen las burbujas en los chips?). Y a Roger, por las fiestas que hemos compartido, las comidas y por secuenciar mejor que nadie nuestras 'creaciones'.

A nuestros técnicos de laboratorio porque sin ellos nada funcionaria igual. A Anna Vilalta por su ayuda incondicional en cualquier momento que la he necesitado, por ser la persona más inventiva que he conocido, teniendo siempre una solución para todo, y por ser el alma del departamento y hacer que todo funcione como 'ha de ser'. A María Jesús por ser la 'madre' de nuestro grupo apoyándonos siempre, por estar dispuesta a hacer lo imposible hasta al momento mas inesperado, por pedir con maestría por nosotros las cosas que queríamos y por apoyar nos en cualquier sentido. Y a Montse por hacerlo todo por nosotros incansablemente desde el primer momento que entró en el grupo, que no siempre es fácil.

A los vecinos que son nuestra familia al fin y al cabo. A la Dra. Anna Bassols y sus becarias (y el único becario pero no por eso menos importante) por compartir fiestas, comidas y 'batallas' (inundacioooon) y por estar siempre al lado ayudando cuando hacía

falta. A Laia, perque ets la meva profe de practiques y em portes al teatre, a María José, por su firmeza en los momentos mas críticos (nadie mas quiso coger el ratón), al Dani por ser el mejor papa pitufo y a Anna Marco, la ‘nena’ del grupo. A Clelia por todos los abrazos y las lecturas de cultura mexicana (te echamos de menos) y a Montse, la veterinaria mas elegante que he conocido.

A Néstor, porque nunca nos ha negado nada, por ser el espíritu no políticamente correcto del departamento y por sus ‘xerradas’ políticas. A sus becarias, Lourdes y Fátima por pasar tantas horas juntas, a Luis y a Carla, los fichajes portugueses, y como no a Josep, por ser la única persona que me puede mover de mi nirvana personal.

A todos los ‘Fátimos’, que con ellos hemos convivido durante mucho tiempo en el mismo pasillo, y especialmente a Vicky, a Judith, Sergio, Alba, Edu, Carles, Albert, Antonio, Ariana, Ivet, Xavi, María, Miguel, Marta, Mireia, Tura, Sylvie y Pedro por todos los momentos que hemos compartido, las charlas y las fiestas.

A nuestras administrativas, que sin ellas nuestra vida seria mucho mas complicada, a Ester, a Montse, a Silvia, a Gloria y a Mercedes, por siempre facilitarme las cosas, y especialmente a Rosa, por ayudarme con cualquiera cosa que surgiera en aspecto burocrático.

A Orfeas por aguantar todo este tiempo a mi y a mis levaduras sin quejarse y por seguir mis pasos.

A mis padres por apoyarme en todas las decisiones que he tomado y por creer en mí, incluso cuando yo no lo consigo.

ABBREVIATIONS

A	absorbance
A. nidulans	*Aspergillus nidulans*
AMP	adenosine 5′ monophosphate
AMPK	AMP-dependent kinase
ARR1	alkaline responsive region 1
ARR2	alkaline responsive region 2
ATP	adenosine triphosphate
BSA	bovine serum albumin
C. albicans	*Candida albicans*
c-AMP	cyclic AMP
CDK	cycline dependent kinase
CDRE	calcineurin dependent response element
ChIp assay	chromatin immunoprecipitation assay
clonNAT	nourseothricin
cpm	counts per minute
CRE element	c-AMP response element
dCTP	deoxycytidine triphosphate
DNA	deoxyribonucleic acid
DTT	dithiotreitol
E. coli	*Escherichia coli*
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
EMSA	electrophoretic mobility shift assay
ESCRT	endosomal sorting complex required for transport
GST	glutathione-S-transferase
GTP	guanosine 5′ triphosphate
H	histone
HDAC	histone deacetylase
HEPES	N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid
HOG	high osmolarity glycerol
IPTG	isopropyl-β–thiogalactopyranoside
kbp	kilo base pair
kDa	kilo Dalton
M	molar
MAPK	mitogen activated protein kinase

MCIR	minimum calcineurin independent response
MEK	MAP kinase kinase
MEKK	MAP kinase kinase kinase
min	minute
mol	mole
mRNA	messenger ribonucleic acid
MVB	multivesicular body
nm	nanometer
nt	nucleotide
N-terminal	amino terminal
OD	optical density
ONPG	o-nitrophenyl-β-D-galactopyranoside
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
pBS	plasmid Bluescript
PCR	polymerase chain reaction
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PP1	protein phosphatase 1
RNA	ribonucleic acid
rpm	revolutions per minute
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulphate
TAF	transcription initiation factor
TAPS	N-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid
TBE	tris-borate–EDTA
TBP	TATA binding protein
TEMED	N,N,N′-N′-tetraethylethylenediamine
TOR	target of rapamycin
Tris	tris-(hydroxylmethyl)-aminoethane
Triton X-100	t-octylphenoxylpolyethoxy ethanol
U	unit
URS	upstream repressing sequence
VPS	vacuolar protein sorting
VTC	vacuolar transporter chaperone

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Publication

'The transcriptional response of the yeast Na^+-ATPase *ENA1* gene to alkaline stress involves three main signaling pathways.'

Maria Platara, Amparo Ruiz, Raquel Serrano, Aarón Palomino, Fernando Moreno, and Joaquín Ariño.

Journal of Biological Chemistry, Vol. 281, Issue 48, 36632-36642, December 1, 2006

I. SUMMARY

The adaptive response of the yeast *Saccharomyces cerevisiae* to environmental alkalinization results in remodeling of gene expression. A key target is the gene *ENA1*, encoding a Na^+-ATPase, whose induction by alkaline pH was shown to integrate at least two different signals, mediated by the calcineurin and the Rim101 pathways, respectively.

Early work in our laboratory identified two regions in the *ENA1* promoter required for full response to sudden alkalinization of the environment: ARR1 and ARR2 (from *A*lkaline *R*esponsive *R*egion 1 and 2). ARR1, the upstream region, integrates the calcium/calcineurin signal, by means of a CDRE element to which the calcineurin-activated transcription factor Crz1 binds upon alkaline stress. ARR1 possibly integrates Rim101-mediated signals also. The Rim101 pathway activates *ENA1* transcription indirectly, by controlling expression of Nrg1, a transcriptional repressor that binds consensus NRG sequences. In contrast, expression from ARR2, which accounts for 60% of the response to high pH, was found to be calcineurin-independent. This region comprises 83 nucleotides and its alkaline response is also reduced in strains lacking Rim101. However, ARR2 contains no previously described NRG-binding sites, and the signaling mechanisms responsible for its response were unknown.

In this work we have restricted the alkaline responsive DNA region present in ARR2 to a smaller fragment of 42 nucleotides that we named MCIR (from *M*inimum *C*alcineurin *I*ndependent *R*esponse). MCIR consists of the 5´-end of ARR2 and contains a MIG element, able to bind Mig1 and Mig2 repressors, which are transcriptional factors involved in the repression of genes regulated by availability of carbon sources. We observe that high pH-induced response driven from MCIR is largely abolished in cells lacking Snf1, the protein kinase that regulates repressor activity of Mig1 with respect to glucose availability, and results moderately reduced in a *rim101* strain. Cells lacking Mig1 or Mig2 have a nearly wild type response, but the double *mig1 mig2* mutant presents high levels of expression upon alkaline stress. In addition, deletion of *NRG1* (but not that of its close homologue *NRG2*) results in increased expression and, finally, induction from the MCIR region is marginal in a quadruple mutant lacking Nrg1,2 and Mig1,2 repressors. Despite that no previously described consensus motifs for Nrg1-binding were identified within the MCIR region, we demonstrate that Nrg1 binds to the 5´-end of the ARR2 region *in vitro* and *in vivo*. Therefore, the calcineurin-independent response of the *ENA1* gene is under the regulation of Rim101 (through Nrg1) and Snf1 (through Nrg1 and Mig2). Accordingly, induction by alkaline stress of the entire *ENA1* promoter in a *snf1 rim101* mutant in the presence of the calcineurin inhibitor FK506 is completely abolished. Thus, the

transcriptional response to alkaline stress of the *ENA1* gene integrates three different signaling pathways, whose relative potency is Snf1 > calcineurin > Rim101.

The protein kinase CK2 is a ubiquitous and pleiotropic kinase well conserved among eukaryotes that participates in many different cellular processes, including survival, cell cycle regulation, polarity, transcription and translation. In the yeast *Saccharomyces cerevisiae,* strains lacking the regulatory subunits Ckb1 and/or Ckb2 of this kinase are hypersensitive to sodium and lithium cations. However, the mechanism by which CK2 affects yeast salt tolerance is not known.

In this study we confirm previous observations that the alkaline and saline response of *ENA1* is decreased in strains lacking Ckb1 and/or Ckb2. Furthermore, we show that *ckb* mutants present a growth defect at alkaline pH environments. The three pathways (Rim101, calcineurin and Snf1) responsible for *ENA1* regulation under alkaline stress conditions were examined for any possible interaction with the protein kinase CK2. Our results suggest that CK2 and calcineurin regulate *ENA1* expression under alkaline and lithium stress in an independent fashion. Moreover, we show that deletion of *RIM101* results in decreased *ENA1* induction under lithium stress conditions and that simultaneous mutation of *CKB* only slightly aggravates the defect that presents the *rim101* strain in *ENA1* alkaline and saline expression. In addition, mutation of the Nrg1 transcription factor in a *ckb* background leads to relatively high levels of *ENA1* expression under alkaline and lithium stress conditions. These results, together with previous data showing that Nrg1 is phosphorylated by CK2 under these stress conditions, support a possible interaction between the CK2 and the Rim101 pathways. Remarkably, deletion of *CKB* partially counteracts the defect that *snf1* mutants present in *ENA1* saline and alkaline expression, and *ckb1,2 snf1* mutants are as tolerant as wild type cells to lithium ions, revealing a complex interaction between CK2 and Snf1. Nonetheless, this effect is specific for lithium sensitivity, as the *ckb1,2 snf1* mutant remains hypersensitive to sodium ions and high pH. Finally, CK2 seems to participate in glucose signaling since lack of Ckb subunits also results in a substantial decrease of *SUC2* expression, the gene that encodes the sucrose hydrolyzing enzyme invertase and whose transcription is mainly regulated by the Snf1 pathway.

II. INTRODUCTION

1. Adaptation mechanisms of filamentous fungi and the yeast *Saccharomyces cerevisiae* to ambient alkalinization.

The ability of microorganisms to adapt to a changing environment is essential for their survival and growth. Adaptation is a complex matter that requires initial sensing of the changes and then development of the appropriate response. Such response often implies the integration of multiple signals that are transmitted through one or various signal transduction pathways and lead to remodeling of gene expression. Nonetheless, post-translational mechanisms are also involved in adaptive responses mainly by controlling activity and stability of proteins.

Extracellular pH is a key environmental parameter and seriously influences growth, physiology and differentiation of many microorganisms. In filamentous fungi (Peñalva and Arst, Jr., 2002; Peñalva and Arst, Jr., 2004) and the yeast *S. cerevisiae* (Lamb *et al.*, 2001; Causton *et al.*, 2001; Serrano *et al.*, 2002; Viladevall *et al.*, 2004) ambient alkalinization triggers specific transcriptional responses that have been extensively characterized in the last years.

The ascomycete *Aspergillus nidulans* is the first eukaryotic organism whose adaptation to ambient pH was characterized. This fungus can grow over a wide pH range from 2.5 to 9.0 and apparently disposes of a regulatory mechanism for controlling gene expression according to the circumstances (Caddick *et al.*, 1986; Rossi and Arst, Jr., 1990). Such a mechanism ensures that extracellular secreted enzymes, permeases and exported metabolites are produced only under pH conditions where they can be functional: i.e. acid phosphatases under acidic pH conditions and alkaline phosphatases under alkaline pH conditions. The transcriptional pH response of *A. nidulans* is regulated by the PacC transcription factor, which is active only at alkaline pH conditions and directly represses or activates expression of pH-responsive genes (Tilburn *et al.*, 1995). Orthologs of PacC, designated as Rim101, have been identified in other fungi, such as in *Saccharomyces cerevisiae*, *Candida albicans* and *Yarrowia lipolytica*, revealing a conserved alkaline responsive mechanism known as the PacC/Rim101 Alkaline Responsive Pathway (Li and Mitchell, 1997; Lambert *et al.*, 1997; Ramon *et al.*, 1999; Lamb *et al.*, 2001).

The budding yeast *S. cerevisiae* also has the capacity to survive over a relatively wide pH range, but optimally proliferates at acidic environmental conditions. The plasma membrane H^+-ATPase Pma1 ensures maintenance of such conditions by actively extruding protons out of the cell. This activity generates an electrochemical proton gradient that regulates cytoplasmic pH strictly between 6.2 and 6.8 and favors nutrient and cation uptake across the plasma membrane (van der Rest *et al.*, 1995; Serrano, 1996). Therefore, ambient alkalinization represents a severe stress condition for this fungus, to which must adapt in order to survive. Adaptation is achieved by means of a complex mechanism that constitutes the alkaline response in *S. cerevisiae*.

In neutral and alkaline environments two other ion pumps are also vital for growth: the plasma membrane sodium ATPase Ena1/Pmr2A (Haro *et al.*, 1991; Garciadeblas *et al.*, 1993) that is described in detail in the section 2 of the introduction, and the multi-subunit vacuolar membrane H^+-ATPase (Stevens and Forgac, 1997). This ATPase is required for vacuolar acidification, which cannot occur through endocytosis at alkaline media (Nelson and Nelson, 1990; Munn and Riezman, 1994). Null mutants lacking these pumps are hypersensitive to alkali and elevated cation concentrations, but grow normally at acidic media. Studies prior to the systematic analysis of the alkaline transcriptional response in yeast indicated that expression of *ENA1* is substantially induced under alkaline pH conditions (Garciadeblas *et al.*, 1993; Mendoza *et al.*, 1994). The systematic approaches performed later confirmed the alkaline induction of *ENA1* (Lamb *et al.*, 2001; Serrano *et al.*, 2002; Lamb and Mitchell, 2003).

In the last few years the alkaline response of this eukaryotic model organism has been studied quite thoroughly, mainly through analysis of the transcriptional remodeling that occurs or the identification of mutants that grow poorly under such conditions (Hong *et al.*, 1999; Lamb *et al.*, 2001; Causton *et al.*, 2001; Serrano *et al.*, 2002; Viladevall *et al.*, 2004). As reflected by the diverse groups of genes that respond transcriptionally to alkali or confer sensitivity when deleted, alkaline pH appears to have a wide-range physiological impact on *S. cerevisiae*. More precise studies focused on specific groups of these genes have revealed that alkaline adaptation in *S. cerevisiae,* indeed, involves the interplay of several regulatory pathways.

The Rim101 Pathway (analyzed in the section 1.1.2), homolog to the PacC pathway of *Aspergillus nidulans*, seems to govern only part of the alkaline response in budding yeast. Adaptation to alkaline environments also requires the activation of the Calcineurin Pathway (Alepuz *et al.*, 1997; Serrano *et al.*, 2002; Viladevall *et al.*, 2004). The main component of this pathway (see section 1.2) is calcineurin, a calcium-activated phosphatase that regulates gene expression indirectly, through the

transcriptional activator Crz1 (Stathopoulos and Cyert, 1997; Matheos *et al.*, 1997). Moreover, a more recent work (Serrano *et al.*, 2006) has demonstrated that the Slt2 MAPK pathway, which is responsible for the maintenance of cell wall integrity, also participates in alkaline adaptation of yeast cells (see section 1.3). Finally, ambient alkalinization seems to affect the availability of phosphate, as well as that of copper and iron and also to evoke oxidative stress in yeast cells (see section 1.4) (Lamb *et al.*, 2001; Causton *et al.*, 2001; Serrano *et al.*, 2002; Serrano *et al.*, 2004).

It has become evident that the alkaline response of *S. cerevisiae* is quite more complex than that characterized in *A. nidulans,* where the PacC/Rim101 pathway accounts for the most important part of the response (Peñalva *et al.*, 2002; Peñalva *et al.*, 2004). However, it seems that *S. cerevisiae* is not the only fungus whose adaptation to alkaline environments requires the integration of signals transmitted through different transduction pathways. Studies performed in *Candida albicans* have pointed out the existence of Rim101-independent pH responsive pathways in this pathogen (Davis *et al.*, 2000). Recently, it has been reported that the Calcineurin Pathways is also involved in alkaline adaptation of *C. albicans* (Kullas *et al.*, 2007), although its modulation presents some differences compared to that described in *S. cerevisiae.*

II. INTRODUCTION

1.1 The PacC/Rim101 signaling pathway in filamentous fungi and yeast.

1.1.1 Regulation of the alkaline response in *Aspergillus nidulans* by the PacC signaling pathway.

The first step to elucidate the mechanism of transcriptional regulation by ambient pH in *Aspergillus nidulans* was made when a large number of genes affecting production of alkaline *(Pal* genes) and acid (*Pac* genes) phosphatases were identified. (Dorn, 1965a; Dorn, 1965b). Subsequent studies demonstrated that many of the *Pal* and *Pac* genes are members of the PacC signal transduction pathway, which governs the alkaline response of this fungus.

PacC, the major component of this pathway, is a transcription factor with three zinc finger domains that recognizes specific DNA motifs (5´-GCCARG-3´) on gene promoters (Espeso *et al.*, 1997; Mingot *et al.*, 2001). In the current model (see figure 1), under acidic conditions the full-length form of the protein of 72 KDa, $PacC^{72}$, is inactive and located in the cytosol, having no apparent role in transcriptional regulation. At neutral to alkaline pH conditions, $PacC^{72}$ undergoes a two step proteolysis to form $PacC^{27}$, which is the functional form of the transcription factor (Orejas *et al.*, 1995; Mingot *et al.*, 1999; Diez *et al.*, 2002). The activated form $PacC^{27}$ then moves to the nucleus, where it binds to gene promoters that contain the PacC recognition sequence, thus activating transcription of alkaline-expressed genes and repressing transcription of acid-expressed genes (Tilburn *et al.*, 1995; Espeso and Arst, Jr., 2000).

Transduction of the alkaline pH signal to PacC and the following proteolytic processing requires six *Pal* proteins: PalA, PalB, PalC, PalF, PalH and PalI (Peñalva *et al.*, 2002; Peñalva *et al.*, 2004). PalH and PalI are located on the plasma membrane and they have seven and four putative transmembrane domains respectively. Presumably, they are the sensors that receive the signal from the environment when pH becomes alkaline (Negrete-Urtasun *et al.*, 1999). PalF is an arrestin-like protein, that strongly binds the cytosolic tail of PalH, and is phosphorylated and ubiquitinated in a PalH- and alkaline pH-dependent manner. It has been proposed that PalF

phosphorylation and ubiquitination lead to the endocytosis of the PalF-PalH complex, thus transmitting the pH signal to the downstream components (Herranz *et al.*, 2005).

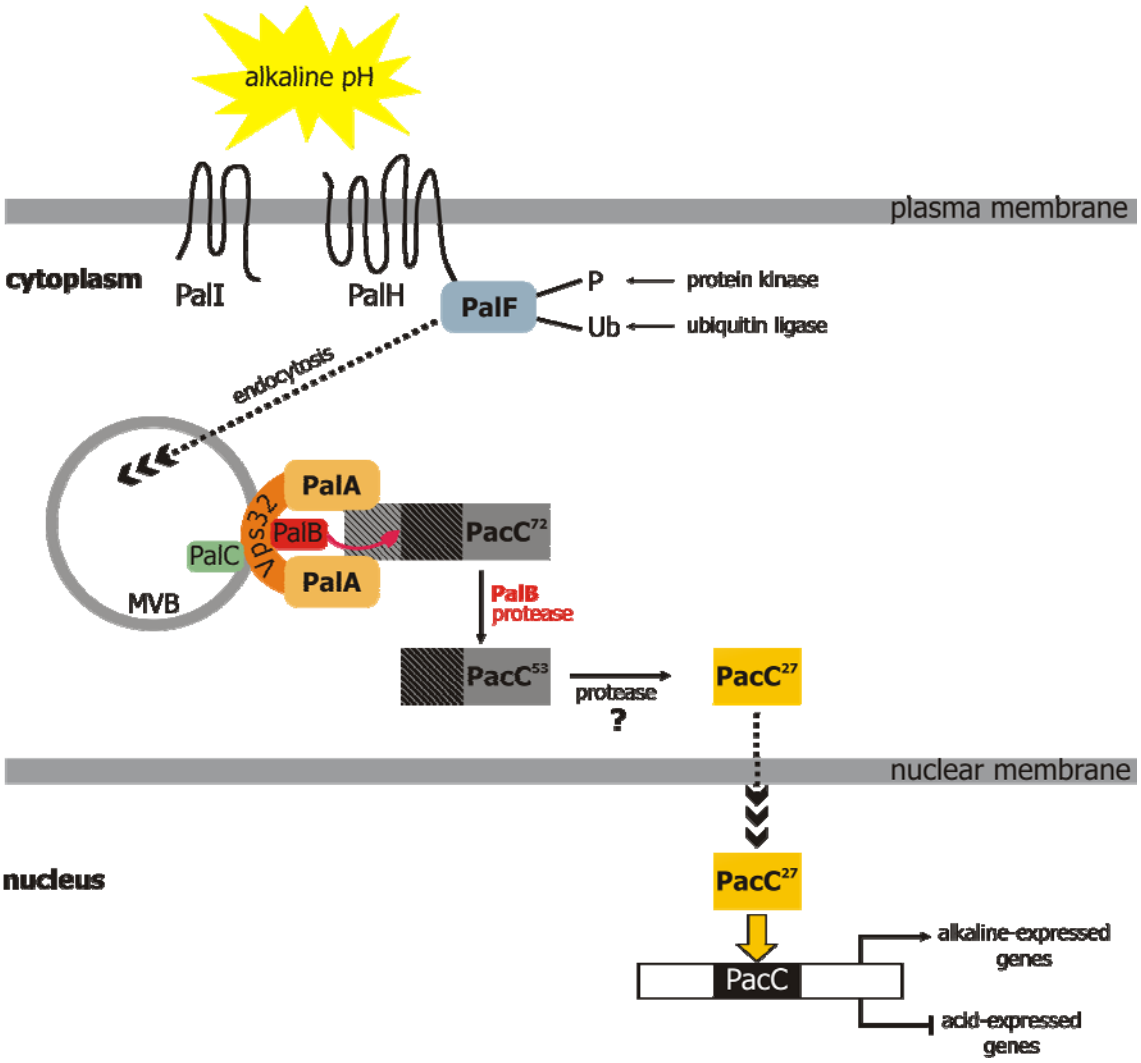

Figure 1. The PacC pathway in *A. nidulans*. Exposure to alkaline pH activates the PalH and PalI membrane sensors and PalF (the arrestin-related protein that is bound to the cytoplasmatic tail of PalH) gets phosphorylated and ubiquitinated. These post-translational modifications lead to the endocytosis of the PalF-PalH complex, transducing the pH signal to the downstream components. Then PalA and PalC which contain a BRO1-domain can interact with Vps32, connecting it with $PacC^{72}$. This interaction is required for the first proteolytic step performed by the PalB protease that leads to $PacC^{53}$. A second proteolytic step leads to the final active form of the transcription factor, $PacC^{27}$, which enters the cell nucleus and binds to the PacC sites of specific gene promoters, regulating their expression.

Transduction of the received signal requires binding of the PalA protein to two YPXL/I motifs located on either side of the signaling protease box in $PacC^{72}$ (Vincent *et al.*, 2003). PalA contains a BRO1-domain which interacts with Vps32, a subunit of the Endosomal Sorting Complex Required for Transport III (ESCRT-III) (Katzmann *et al.*, 2002). The main function of this complex (Babst, 2005) that belongs to a larger group of gene products known as the Vacuolar Protein Sorting (VPS) gene products, is to sort endocytic 'cargo' into the Multivesicular Bodies (MVBs). PalA is located on the membrane of the endosomal system and is thought to connect $PacC^{72}$ with Vps32. PalC, like PalA, also contains a BRO1-domain (Tilburn *et al.*, 2005) and localizes to structures at or near the plasma membrane in a pH-dependent manner. It is believed that PalC binds to Vps32 through its BRO1-domain thus constituting the link between the upstream and the downstream components of the PacC signaling pathway (Galindo *et al.*, 2007).

PalB is the signaling protease that performs the first processing of $PacC^{72}$ (Denison *et al.*, 1995; Penas *et al.*, 2007). PalB resembles the large subunits of m- or μ-calpain, which are modulatory proteases that recognize bonds between domains, rather than specific sequences (Suzuki *et al.*, 2004). At neutral to alkaline pH, PalB cleaves $PacC^{72}$ within a conserved sequence of 24 residues designated as the 'signaling protease box', removing 180 C-terminal residues (Diez *et al.*, 2002). The product is an intermediate form of PacC that consists of ~495 N-terminal residues ($PacC^{53}$). Then an additional fragment of ~245 C-terminal residues is removed, to yield finally the functional form $PacC^{27}$. This step is not dependent on the environmental pH.

1.1.2 Regulation of the alkaline response in *Saccharomyces cerevisiae* by the Rim101 pathway.

Rim101 is the yeast ortholog to the PacC of *A. nidulans* and was first identified in a mutant analysis, as a positive regulator of meiotic gene expression and sporulation (Su and Mitchell, 1993a; Su and Mitchell, 1993b). Further investigation showed that Rim101 defines a pathway that similarly to the PacC of *A. nidulans* plays a certain role in adaptive response to alkaline pH in yeast, thus establishing the PacC/Rim101 pathway as a conserved signaling mechanism in fungi. Nevertheless differences do exist in many aspects of its activity and regulation comparing to that of PacC in *A. nidulans.*

Rim101 is a 628-residue protein, which contains three zinc finger-like domains and an acidic region essential for function (Su *et al.*, 1993b). Despite the similar sizes of PacC and Rim101, sequence homology is almost completely restricted to the zinc finger region. In common with PacC, the full-length inactive form of Rim101 predominates in acidic environments. At alkaline pH conditions, proteolytic removal of a 100-residue fragment is stimulated, thus the processed active form of this transcription factor is more abundant (see figure 2). Nevertheless, unlike PacC that undergoes a two-step proteolytic processing which occurs exclusively at alkaline environments, Rim101 activation requires a unique proteolytic step and occurs under both acidic and alkaline growth conditions (Li *et al.*, 1997; Xu and Mitchell, 2001).

The one-step processing of Rim101 requires the activity of several upstream gene products that comprise the Rim101 pathway in *S. cerevisiae*. This pathway is reminiscent to the PacC/Pal pH responsive pathway of *A. nidulans* and includes Rim20, an ortholog to the PalA, Rim13, Rim8, Rim21 and Rim9 orthologs to PalB, PalF, PalH and PalI respectively (Li *et al.*, 1997; Davis *et al.*, 2000; Xu *et al.*, 2001). The mechanism of Rim101 cleavage in yeast is not completely understood but there is evidence that it follows the same pattern as that of PacC. According to this scenario, Rim20 (ortholog to PalA) that contains a BRO1-domain, promotes interaction between Rim101 and Rim13 (ortholog to PalB), which is the protease that performs the processing of Rim101 (Futai *et al.*, 1999; Xu *et al.*, 2001). This model is further supported by the finding that both Rim13 and Rim20 interact with Snf7 (Ito *et al.*, 2001). Snf7, like the Vps32 of *A. nidulans,* is a component of the ESCRT-III in *S. cerevisiae* (Kranz *et al.*, 2001; Babst *et al.*, 2002). Further investigation revealed that Snf7, as well as Vps20, another VPS protein, are required for Rim101 processing. Interestingly, proteolytic cleavage of Rim101 also depends on many other subunits of the ESCRT, suggesting a relationship between MVB and the Rim101 pathway in yeast (Xu *et al.*, 2004; Rothfels *et al.*, 2005; Boysen and Mitchell, 2006). These findings are consistent with a model where the BRO1-domain of Rim20 interacts with Snf7 to form a scaffold that brings the protease Rim13 in proximity with the substrate Rim101 in order to perform cleavage (Xu *et al.*, 2004).

There is evidence that the Multivesicular Bodies (MVBs) formation machinery of *S. cerevisiae*, in collaboration with the Rim101 pathway, is employed in the sensing of ambient pH or even responds to it. First, apart from Snf7, Rim20 also interacts with Vps4, a VPS ATPase required for ESCRT-III dissociation (Ito *et al.*, 2001; Bowers *et al.*, 2004). Second, the MVB trafficking protein Bro1 (Nikko *et al.*, 2003; Odorizzi *et al.*, 2003) contains regions that present homology to PalA *of A. nidulans* and to the Rim20 orthologs of *S. cerevisiae* and *C. albicans* (Negrete-Urtasun *et al.*, 1997). Third, large

scale functional profiling studies indicate that many mutants of the MVB pathway grow poorly at alkaline pH (Giaever *et al.*, 2002; Serrano *et al.*, 2004).

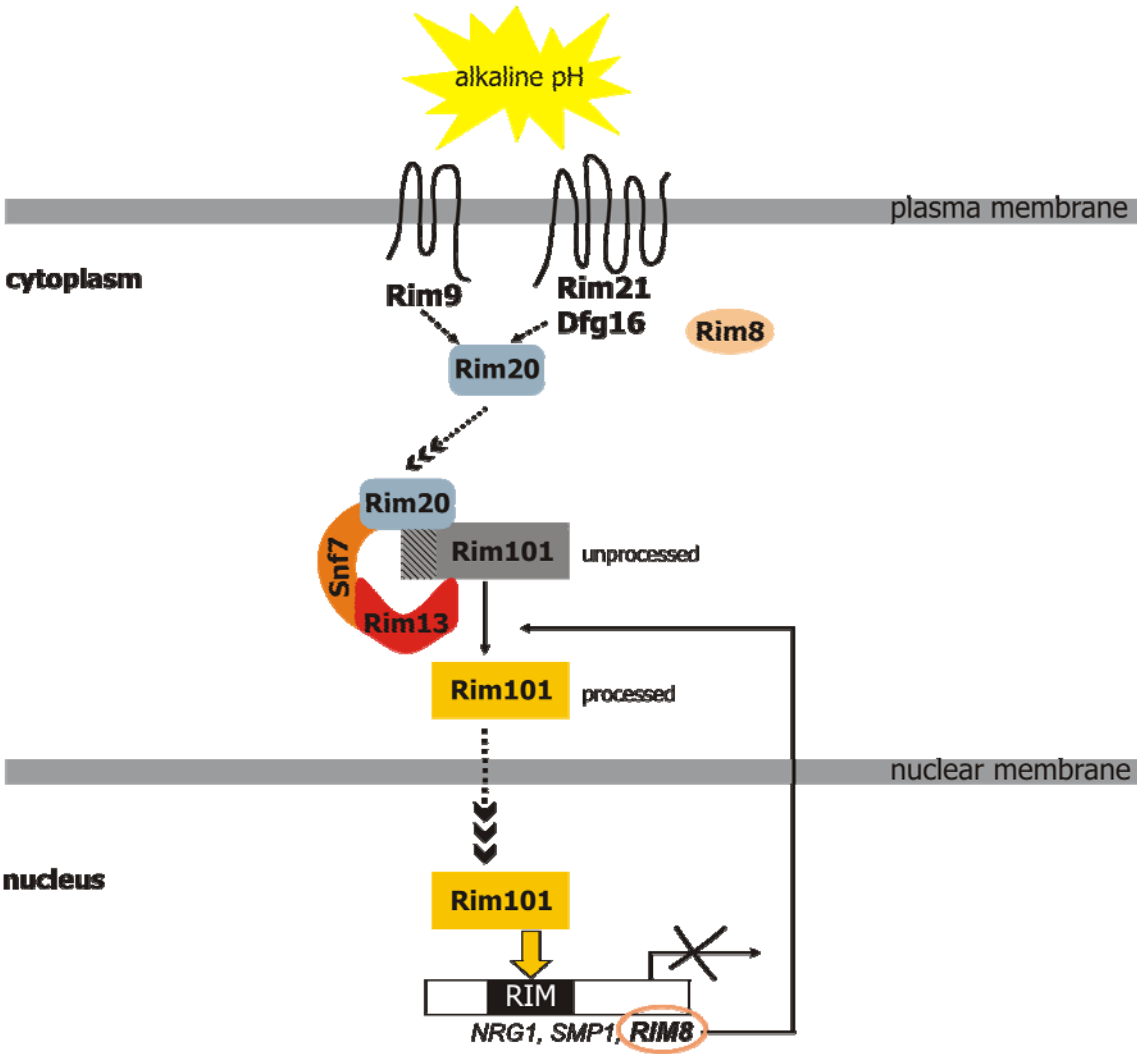

Figure 2. Schematic model of the Rim101 pathway in *S. cerevisiae*. Rim9 and Rim21 are located on the plasma membrane and are the putative alkaline-pH sensors. Rim13 is the calpain-like protease and is thought to be the proteolytic enzyme performing the single proteolytic step that leads to the active form of Rim101. Rim20 interacts with the C-terminal domain of Rim101 and with Snf7 to form a scaffold that brings Rim13 into position to cleave full-length Rim101. Rim8 (ortholog to PalF) is necessary for Rim101 processing. The gene encoding Rim8 is directly repressed by the active form of Rim101, revealing a negative-feedback loop between the two components.

Rim8 (ortholog to PalF) is also a member of the Rim101 pathway and is encoded by *RIM8*, a gene mainly expressed under acidic pH conditions. Interestingly, this gene is negatively regulated directly by the processed form of Rim101, revealing a relationship between these two proteins that have properties of a negative feedback loop (see figure 2). This mechanism might serve to prevent either hyperaccumulation of

processed Rim101 or hyperactivity of the Rim13 protease (Lamb *et al.*, 2003). Rim9 (ortholog to PalI), Rim21 (ortholog to PalH) and Dfg16 (also ortholog to PalH) are the putative membrane proteins and they possibly serve as sensors of extracellular pH (Davis, 2003; Barwell *et al.*, 2005).

The Rim101 pathway seems to play a significant role in the alkaline adaptive response of *S. cerevisiae.* In agreement with this role, yeast cells lacking *RIM101* are defective in haploid invasive growth and sporulation, processes that are stimulated by alkaline pH (Li *et al.*, 1997; Hayashi *et al.*, 1998). In addition, *rim101* mutants grow poorly under alkaline pH conditions (Futai *et al.*, 1999; Lamb *et al.*, 2001).

Apart from the revealing phenotypes of the *rim101* mutants, gene expression analyses in this genetic background have also contributed to the consolidation of Rim101 as an alkaline-responsive pathway. It has been demonstrated that the expression of a considerable number of genes that are induced under alkaline pH conditions depends on this pathway (Lamb *et al.*, 2001; Lamb *et al.*, 2003). There are two classes of *RIM101*-dependent alkaline-expressed genes. The first class includes those that are totally *RIM101*-dependent, such as *ARN4*, a siderophore-iron transporter and *VMA4* that encodes a subunit of the vacuolar H^+-ATPase (Foury, 1990; Ho *et al.*, 1993). Alkaline induction of these genes is abolished when *RIM101* is mutated. The second class includes alkaline-expressed genes whose induction is only partially dependent on Rim101. *ENA1*, encoding the plasma membrane Na^+-ATPase belongs to this class (Haro *et al.*, 1991; Lamb *et al.*, 2001; Serrano *et al.*, 2002). In mutants lacking Rim101, the alkaline induction of *ENA1* is decreased but not fully abolished, suggesting that the Rim101 pathway is not the only one involved in the alkaline regulation of this gene (details in the section 2 of the introduction). *NRG2,* encoding a transcription factor that mediates glucose repression and negatively regulates filamentous growth (Kuchin *et al.*, 2002) also belongs to the class of genes whose alkaline expression partially depends on the Rim101 pathway (Lamb *et al.*, 2001).

The function of Rim101 in *S. cerevisiae* is different from that observed in *A. nidulans*. Whereas in *A. nidulans* PacC acts as both an activator and a repressor by binding directly to the promoter of alkaline responsive genes, Rim101 in *S. cerevisiae* promotes expression of alkaline-responsive genes indirectly, through repression of Nrg1 (Lamb *et al.*, 2003). *NRG1* contains a putative PacC site (TGCCAAG) in its promoter, to which Rim101 binds directly, inhibiting expression. Nrg1 is a repressor itself and seems to have a key role in pH-responsive gene regulation and ion tolerance in yeast by negatively regulating the sodium ATPase *ENA1* (see section 2.1 of the introduction). Correspondingly to that role, mutation of *NRG1* confers ion tolerance and

fully suppresses the growth defect of a *rim101* mutant at alkaline pH (Lamb *et al.*, 2003).

Nrg1, together with its homologue Nrg2, were first identified as transcriptional repressors of several glucose-repressed genes such as *STA*, *FLO11*, *SUC2*, *DOG2* and *GAL* (Park *et al.*, 1999; Zhou and Winston, 2001; Kuchin *et al.*, 2002). Nrg1 and Nrg2 also regulate negatively haploid invasive growth, which is a cellular response to glucose limitation (Cullen and Sprague, 2000; Kuchin *et al.*, 2002; Vyas *et al.*, 2003). These two repressors interact with the Snf1 kinase, a key component of the glucose signaling pathway (see section 2.4 of the introduction) and there is genetic evidence that they function downstream of this protein kinase (Kuchin *et al.*, 1993; Vyas *et al.*, 2001; Kuchin *et al.*, 2002). However, direct phosphorylation of Nrg1 or Nrg2 by Snf1 has not been demonstrated (Vyas *et al.*, 2001; Berkey *et al.*, 2004). Apart from the glucose repressed genes, Nrg1 and Nrg2 were further shown to regulate a set of stress responsive genes and mutations of these transcription factors enhanced resistance to salt, oxidative and alkaline pH stress (Vyas *et al.*, 2005).

1.1.3 Regulation of alkaline response in *Candida albicans* by the Rim101 pathway.

Candida albicans is the most common fungal pathogen in humans and can cause life threatening infections in susceptible individuals (Odds, 1994). It is a dimorphic fungus that undergoes reversible morphological transition between unicellular yeast-like and hyphal and pseudohyphal forms. The ability to grow in an hyphal or else called filamentous form is an adaptive response to ambient stress stimuli such as temperature and alkaline pH (de Bernardis *et al.*, 1998) and interestingly is required for pathogenicity (Lo *et al.*, 1997; Davis, 2003). Therefore, mechanisms governing adaptation to alkaline pH conditions in *Candida albicans* are of great importance and have received considerable attention.

The ability of *Candida albicans* to adapt to alkaline environments is mediated by the conserved Rim101/PacC signal transduction pathway as in case of *Aspergillus nidulans* and *Saccharomyces cerevisiae* (Davis *et al.*, 2000). Rim101, as PacC in *A. nidulans*, acts as a positive regulator of alkaline expressed genes and as a negative regulator of acid expressed genes (Davis *et al.*, 2000) and is critical for the morphological development of this fungus to a filamentous form (Ramon *et al.*, 1999).

RIM101 in this fungus specifies a protein of 785 aminoacids that presents only a 20 % sequence homology with Rim101 from *S. cerevisiae*. However they share three structural features: the zinc finger domain, a D/E-rich C-terminal domain and a similar size. Moreover, genetic evidence suggests that Rim101 in *C. albicans* is also activated by proteolytic removal of its C-terminus (Davis *et al.*, 2000; El Barkani *et al.*, 2000; Porta *et al.*, 2001). Two other members of the PacC/Rim101 pathway, Rim20 and Rim8 that are orthologs to PalF and PalB of *A. nidulans* respectively, have been identified. These two proteins are required for alkaline-induced filamentation, as well as for alkaline induction of specific genes (Davis *et al.*, 2000). However, the processing mechanism of Rim101 has not been defined yet biochemically (Davis, 2003). *C. albicans* has a homolog to the *DFG16* gene of *S. cerevisiae* that corresponds to the ORF *IPF9013* that is predicted to be a transmembrane protein functioning as a sensor of the extracellular stimuli (Barwell *et al.*, 2005).

Despite the conservation of the Rim101 pathway in this fungus, there is evidence that the function of Rim101 is different comparing to that of *S. cerevisiae* and *A. nidulans*. First, the consensus DNA sequence recognized by Rim101 is 5´-CCAAG-3´ followed preferentially by A or C in the adjacent 3´ position and is not the same as that recognized by PacC (Ramon and Fonzi, 2003). Second, there is evidence that in *Candida*, unlike with what happens in *S. cerevisiae,* Rim101 can act both as a transcriptional activator and a repressor (Braun *et al.*, 2001). Third, although an Nrg1 protein has been identified in this fungus, it does not seem to act downstream of Rim101, as growth defects at alkaline pH or high lithium concentrations associated with loss of Rim101 function are not rescued when *NRG1* is mutated (Bensen *et al.*, 2004).

Nevertheless, the contribution of the Rim101 pathway to alkaline adaptation of *Candida* is very important, as reflected by the transcriptional profiling of strains lacking this protein. More specifically, the Rim101 pathway seems to regulate positively certain aspects of the alkaline response, including hyphal formation, ion transport and adaptation to iron starvation (Bensen *et al.*, 2004). However, only 23 % of the genes implicated in these pH-dependent functions are clearly governed by the Rim101 pathway, suggesting that this pathway is dispensable for other aspects of the alkaline response. This is consistent with the idea that Rim101-independent pathways also contribute to the adaptation of this fungus to environmental pH (Davis *et al.*, 2000; Davis *et al.*, 2002; Bensen *et al.*, 2004).

1.2 Regulation of the alkaline response by the calcineurin pathway.

Calcineurin is a serine/threonine protein phosphatase that is well conserved among eukaryotes and is a critical component of calcium-regulated signaling in a wide range of unicellular and multicellular organisms. In response to a variety of external stimuli, transitory increases in cytosolic calcium occur, thus activating the Ca^{2+}-binding protein calmodulin, which in turn activates calcineurin (Aramburu *et al.*, 2004; Clapham, 2007). The immunosuppressants FK506 and cyclosporine A specifically inhibit calcineurin, making them valuable either as drugs or as experimental tools for exploring functions of calcineurin *in vivo* (Liu, 1993).

In *Saccharomyces cerevisiae*, calcineurin is an heterodimer that consists of one catalytic subunit encoded by two genes, *CNA1* and *CNA2,* that are functionally redundant and one regulatory subunit encoded by the gene *CNB1* (Cyert *et al.*, 1991; Cyert and Thorner, 1992). As in other eukaryotes, calcineurin in yeast is activated by increments of the intracellular calcium concentration, caused by high temperature (Zhao *et al.*, 1998), addition of glucose to starving cells (Nakajima-Shimada *et al.*, 1991), sphingosine (Birchwood *et al.*, 2001), prolonged exposure to mating pheromone (α-factor) (Iida *et al.*, 1990; Moser *et al.*, 1996; Withee *et al.*, 1997; Muller *et al.*, 2001), or elevated extracellular concentrations of ions such as OH^-, Mn^{2+} and Na^+/Li^+ (Farcasanu *et al.*, 1995; Batiza *et al.*, 1996; Denis and Cyert, 2002; Matsumoto *et al.*, 2002; Viladevall *et al.*, 2004). As a result, whereas this phosphatase is dispensable for normal growth, under certain stressing conditions, as those mentioned above, becomes essential for survival (Cyert *et al.*, 1992; Nakamura *et al.*, 1993; Mendoza *et al.*, 1994). In addition, calcineurin seems to be indispensable for growth of mutants with cell wall defects such as *mpk1/slt2*, *pkc1* and *fks1* (Eng *et al.*, 1994; Garrett-Engele *et al.*, 1995).

The mechanisms that regulate raises of intracellular calcium concentration in yeast are not yet fully understood. As a general rule, increments are possible due to either influx of calcium cations (Ca^{2+}) from the extracellular environment or release from intracellular stores, such as the vacuole, the major calcium store in yeast (Halachmi and Eilam, 1989; Strayle *et al.*, 1999). The mechanism that is triggered mostly depends on the stimulus. Exposure to pheromones and endoplasmic reticulum stress leads to entry of extracellular Ca^{2+} through the plasma membrane Mid1-Cch1 calcium channel (Iida *et al.*, 1994; Paidhungat and Garrett, 1997; Bonilla *et al.*, 2002). On the other hand, in case of hypotonic stress, Ca^{2+} comes out of the vacuole through

the Yvc1, a Ca^{2+}-channel related to the Transient Receptor Potential (TRP) family of ion channels (Batiza *et al.*, 1996; Palmer *et al.*, 2001). There are some contradictory results about the origin of the cytosolic Ca^{2+} increments observed under hyperosmolarity conditions. Initially it was reported that upon saline stress Ca^{2+} is released from the vacuole through the Yvc1 channel (Denis *et al.*, 2002), but another study published some months later sustained that the origin is extracellular and Ca^{2+} enters cells through the Mid1-Cch1 channel (Matsumoto *et al.*, 2002)

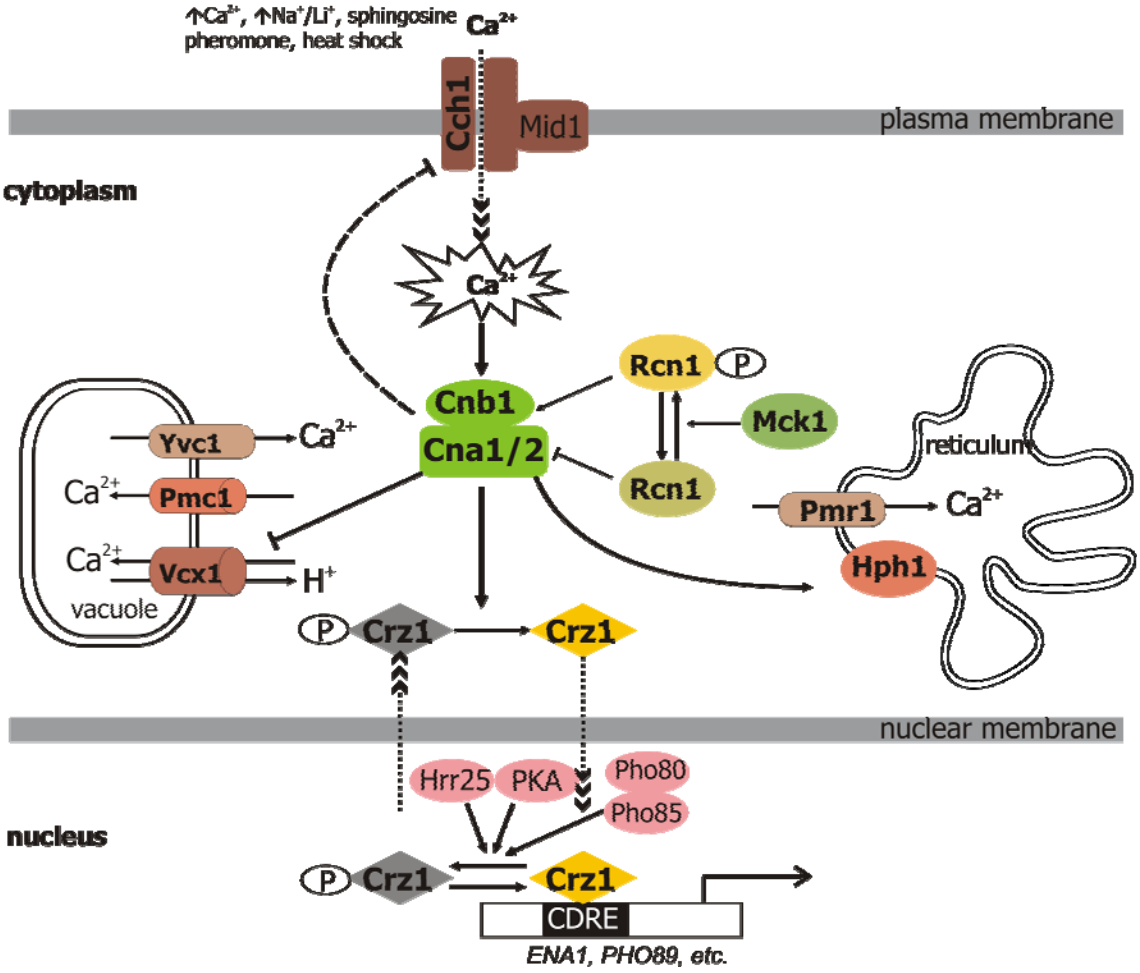

Figure 3. Outline of the calcineurin pathway. The main transporters involved in calcium homeostasis are depicted: Mid1-Cch1 and Yvc1 which contribute to intracellular [Ca^{2+}] raises and Pmc1, Vcx1 and Pmr1 which eliminate calcium from the cytoplasm. Specific environmental conditions provoke increases of intracellular [Ca^{2+}] and the phosphatase calcineurin is activated, dephosphorylates Crz1 which then enters the nucleus to activate CDRE-containing genes. Deactivation of Crz1 occurs upon phosphorylation by PKA, Hrr25 and the Pho85-Pho80 CDK complex. Apart from Crz1, calcineurin also targets directly Cch1, Vcx1 and Hph1 (a reticulum protein). Calcineurin activation depends on the phosphorylation state of the calcipressin Rcn1.

Calcineurin controls gene expression indirectly, through activation of the transcription factor Crz1/Tcn1/Hal8 (Stathopoulos *et al.*, 1997; Matheos *et al.*, 1997). Under non-stressing conditions, intracellular levels of calcium are low, calcineurin is inactive and Crz1 is mainly distributed in the cytoplasm. Exposure of cells to stress signals like those mentioned above, leads to cytosolic Ca^{2+} increments, thus calcineurin becomes active and dephosphorylates Crz1 (see figure 3). The dephosphorylated form of Crz1 can then enter the nucleus by binding to the Nmd5 importin that recognizes a specific Nuclear Localization Sequence (NLS) of Crz1 (Polizotto and Cyert, 2001). Once in the nucleus, this transcription factor binds to the promoter of calcineurin-dependent genes, at a specific consensus DNA motif (5´-GNGGC(G/T)CA)-3´) denominated Calcineurin Dependent Response Element (CDRE) (Stathopoulos *et al.*, 1997). Large scale transcriptional studies performed in yeast using DNA microarrays revealed that calcineurin activation induces expression of more than 160 genes, most of which contain one or more CDREs on their promoters (Yoshimoto *et al.*, 2002).

Exit of Crz1 from the nucleus is regulated by its phosphorylation state. When calcineurin is inactive, Crz1 is phosphorylated and can bind to the Msn5 exportin and translocate from the nucleus to the cytoplasm (Boustany and Cyert, 2002). Three protein kinases have been described to phosphorylate Crz1: PKA (the cyclic AMP-dependent protein kinase A), Hrr25 (a casein kinase I isoform) and the Pho85-Pho80 CDK complex (Kafadar *et al.*, 2003; Kafadar and Cyert, 2004; Sopko *et al.*, 2006).

Studies performed in yeast cells lacking either the unique regulatory (Cnb1) or both catalytic subunits (Cna1 and Cna2) of calcineurin have demonstrated that this phosphatase is critical for adaptation to high salt stress conditions. Calcineurin enhances active Na^+ export out of the cell through activation of the Na^+-ATPase *ENA1* (described in detail in the section 2.2 of the introduction). Moreover, calcineurin regulates posttranslationally Trk1 and Trk2, the principal potassium uptake system in this fungus (Nakamura *et al.*, 1993; Mendoza *et al.*, 1994). The same studies revealed that calcineurin is also important for adaptation to alkaline pH conditions. Growth of *cnb1* single or *cna1 cna2* double mutants is severely deteriorated at pH >8 and this deterioration might be, at least partially, due to *ENA1* decreased expression.

In a genome-wide analysis focused on the transcriptional response of *S. cerevisiae* to mild alkaline conditions performed in our laboratory (Serrano *et al.*, 2002), the calcineurin-dependent response of *ENA1* to alkaline pH was verified and characterized in some detail (see section 2.2). In addition, in this study it was demonstrated that the expression of another alkaline pH induced gene, *PHO89,* is blocked by the calcineurin inhibitor FK506, as well as by the absence of either Cnb1 or

Crz1/Tcn1. The above results prompted our laboratory to further analyze the role of the calcineurin pathway in the transcriptional response of yeast under alkaline stress conditions.

Monitoring of *in vivo* changes in intracellular calcium concentration, showed that alkaline stress provokes a rapid and transient burst of Ca^{+2} cations in yeast cells. This effect is similar to what has been observed for hypotonic and saline stress and faster than that described for sphingosine treatment. The calcium cations that enter the cytoplasm in this case have an extracellular origin and are conducted through the plasma membrane Mid1-Cch1 channel (Viladevall *et al.*, 2004).

Moreover, a systematic screen for mutations conferring sensitivity to yeast cells in the presence of the calcineurin inhibitor FK506 led to the identification of 48 genes. Interestingly, 42 % of these mutations were also found sensitive to alkaline pH in another screen of the same library performed earlier (Serrano *et al.*, 2002), thus revealing a significant overlap between the alkaline-response genes and those affecting growth when the calcineurin pathway is inhibited.

The above results were indicative of a possible role for the calcineurin pathway in the transcriptional response of yeast to alkaline pH. To corroborate such a possibility, DNA microarray experiments were performed in cells lacking either the regulatory subunit of the calcineurin (*cnb1* mutants) or the transcription factor Crz1 (*crz1* mutants) at pH 8. It was shown that the transcriptional response of 10 % of the alkaline-responsive genes is partially or totally dependent on the calcineurin pathway. Most of these genes contain in their promoter one or more putative Crz1-binding sites, supporting the notion that this transcription factor is the main mediator of the calcineurin-dependent transcriptional response (Viladevall *et al.*, 2004).

However, Crz1 is not the unique target of this phosphatase. Other direct targets of calcineurin have been described, such as the calcium channel Mid1-Cch1 (Bonilla and Cunningham, 2003) and Hph1, an endoplasmic reticulum membrane protein. Calcineurin directly dephosphorylates Hph1, thus modifying its distribution within the endoplasmic reticulum, and is required for full Hph1 activity *in vivo*. Hph1, together with its homolog Hph2, mediate responses to high salinity, alkaline pH and cell wall stress, both in a calcineurin-dependent manner, although Hph2 does not interact directly with this phosphatase (Heath *et al.*, 2004). Moreover, calcineurin dephosphorylates Rcn1, a calcipressin homolog of *Saccharomyces cerevisiae*, that was first identified as an endogenous inhibitor of calcineurin. Subsequent studies revealed that Rcn1 can also act as a stimulator of calcineurin, when phosphorylated by the kinase Mck1, and return to the inhibitory form when dephosphorylated by calcineurin, in a mechanism with the characteristics of a negative feedback (Hilioti *et al.*, 2004; Hilioti and Cunningham,

2004). Hsl1 and Yap1 are also direct substrates of the phosphatase calcineurin and it has been suggested that their dephosphorylation leads to cell cycle blockage (Mizunuma *et al.*, 1998; Mizunuma *et al.*, 2001; Yokoyama *et al.*, 2006).

More targets of calcineurin have been described such as the potassium transport system Trk1-Trk2 mentioned before (Mendoza *et al.*, 1994), the membrane proton pump Pma1 (Withee *et al.*, 1998) and the vacuolar H^+/Ca^{2+} exchanger Vcx1 (Cunningham and Fink, 1996). Nonetheless, it has not been demonstrated whether the effect of calcineurin in these cases is direct or implies intermediate components.

The calcineurin pathway is conserved in *Candida albicans* and, similarly to what occurs in *S. cerevisiae,* it seems to be important for adaptation to ambient pH. Recently it has been reported that *C. albicans* strains lacking the transcription factor Crz1 or the calcineurin regulatory subunit Cnb1 grow poorly at alkaline and acidic pH environments. *CRZ2*, a homolog of *CRZ1*, is also required for normal growth at acidic and alkaline pH and for the pH-dependent filamentation of this fungus, but in a calcineurin-independent manner. Surprisingly, activity of Crz2 seems to depend on Rim101 (Kullas *et al.*, 2007).

1.3 Role of the cell wall integrity pathway in the yeast alkaline response.

The principal function of the cell wall in *S. cerevisiae* is to maintain cell shape and intracellular osmotic homeostasis and, at the same time, acts as a shield against physical or chemical external stresses (Klis *et al.*, 2006). Thereby integrity of this structure is substantial for survival and, for this reason, it is under the tight control of a regulatory mechanism known as the Cell Wall Integrity (CWI) Pathway. This pathway is comprised of a group of cell wall sensors (Wsc1-3, Mid2 and Mtl1) coupled to a small G-protein called Rho1. Rho1 is a GTPase that has two main functions: it is the regulatory subunit of the cell wall synthesizing enzyme complex whose catalytic subunits are Fks1 and Gsc2 and the signal transducer that binds and activates the protein kinase C (Pkc1). The protein kinase Pkc1 is member of the Mitogen Activated Protein (MAP) kinase pathway and in response to certain stimuli phosphorylates Bck1, a MAP kinase kinase kinase (MEKK) that in turn phosphorylates and activates Mkk1/2, a pair of redundant MAP kinases kinases (MEKs) and Slt2, a MAP kinase (MAPK). Activation of Slt2 results in phosphorylation of several nuclear and cytosolic targets

(see figure 4). Among its nuclear targets are the transcription factor Rlm1, which seems to be responsible for most of the transcriptional effects derived from Slt2 activation, and the SCB-binding factor, comprised of Swi4 and Swi6.

The CWI pathway is active during vegetative growth and pheromone-induced morphogenesis of yeast cells, but also mediates the response triggered in case of cell wall damage provoked by chemical agents (e.g. Congo red, calcofluor white, zymolyase, caffeine), mutations (e.g. *fks1*) or environmental conditions (e.g. heat shock, hypoosmotic shock) which compromise cell wall integrity (Levin, 2005).

There are evidences suggesting that alkaline pH could provoke cell wall stress in yeast. First, the alkaline transcriptional response presents a considerable overlap with that observed after mutation of genes related to cell wall components or after exposure to cell wall damaging agents (Lagorce *et al.*, 2003; Viladevall *et al.*, 2004; Garcia *et al.*, 2004). Second, cells lacking certain genes related to cell wall biogenesis or maintenance, such as *RHO4*, *SLT2* and *BCK1* exhibit a severe alkaline sensitive phenotype (Giaever *et al.*, 2002; Serrano *et al.*, 2004).

A more detailed study centered in the repercussions of alkaline stress to cell wall integrity of *S. cerevisiae* performed in our laboratory while this thesis was in progress, demonstrated that indeed exposure of yeast cells to alkaline environmental conditions results in specific activation of the Slt2 MAPK pathway (Serrano *et al.*, 2006). Upon alkalinization, Slt2 is rapidly phosphorylated and this phosphorylation is transient and fully depends on the integrity of the MAPK cascade, as demonstrated by the absence of phosphorylation in *bck1* mutants. The alkaline pH signal transmitted through Slt2 is perceived mainly by the Wsc1 sensor, whereas Mid2 plays a less significant role.

The nature of the changes that occur to yeast cell wall at alkaline pH conditions remains unknown. A transcriptional profiling performed in *slt2* cells exposed to alkaline stress shows that expression levels of many alkaline-induced genes are lower than in wild type strains. Most of these genes are induced upon exposure to cell wall damaging agents and interestingly, several such as *GSC2/FKS2*, *SKT5, CRH1*, or *DFG5* are related to cell wall biogenesis or maintenance, supporting a possible effect of alkaline pH on cell wall structure.

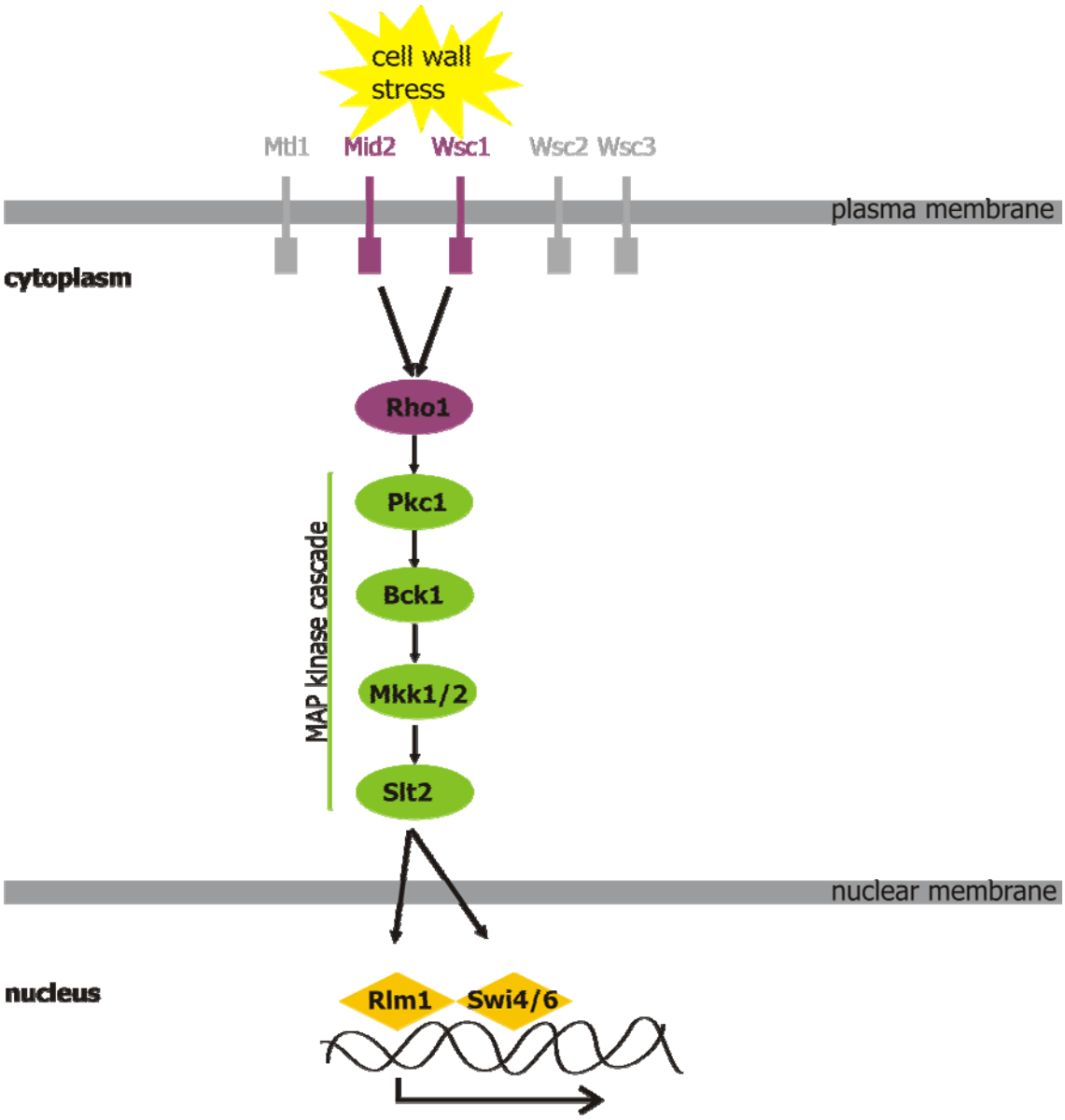

Figure 4. Cell Wall Integrity pathway. Cell wall stress is perceived from the cell surface sensors Wsc1-3, Mid2 and Mtl1 and the signal is transmitted through the G-protein Rho1 to the Pkc1 protein kinase. When activated, Pkc1 in turn activates the other members of the MAP kinase cascade, Bck1, Mkk1/2 and Slt2. Two transcription factors, Rlm1 and the SBF complex (Swi4/Swi6), are targets of the Slt2 MAP kinase. The sensors Wsc1 and Mid2 that are important for Slt2 activation by alkaline pH are depicted.

Interestingly, it has been demonstrated that the Rim101 alkaline responsive pathway also contributes to the assembly of the yeast cell wall and that this function becomes essential in absence of the MAPK Slt2. These findings are in accordance with a scenario where both pathways cooperate in parallel in order to maintain cell wall integrity (Castrejon *et al.*, 2006).

Additional evidences supporting the above theory come from independent studies performed in *Candida albicans.* In this fungus, the transcription of two cell wall genes *PHR1* and *PRA1* is induced at neutral and alkaline pH conditions in a Rim101-dependent manner (Saporito-Irwin *et al.*, 1995; Sentandreu *et al.*, 1998; Ramon *et al.*, 1999; Davis *et al.*, 2000). In addition, *PHR2*, encoding a Rim101-dependent glucosidase with functions equivalent to *PHR1*, participates in cell wall assembly when environmental pH becomes acid (Muhlschlegel and Fonzi, 1997; de Bernardis *et al.*, 1998). In a later study, transcriptional profiling experiments led to the identification of additional cell wall genes that are either repressed or induced by Rim101 in response to environmental pH changes, suggesting that the adaptive response to alkaline pH mediated by the Cell Wall Integrity pathway could be conserved among fungi (Bensen *et al.*, 2004; Lotz *et al.*, 2004).

1.4 Other physiological alterations caused by alkaline pH stress in *S. cerevisiae.*

Inorganic phosphate (Pi) is an essential nutrient and phosphate homeostasis in *S. cerevisiae* is subjected to the strict control of the Phosphate Regulatory Pathway also known as the PHO regulon (Oshima *et al.*, 1996; Lenburg and O'Shea, 1996). Analysis of the transcriptional response of yeast cells when shifted to alkaline pH conditions showed that mild alkaline stress results in transcriptional induction of several genes related to the PHO regulon, such as *PHO84* and *PHO89*, encoding two high-affinity phosphate transporters, *PHO11/PHO12* encoding an acid phosphatase and also *VTC1/PHM4*, *VTC3/PHM2* and *VTC4/PHM3* (Lamb *et al.*, 2001; Causton *et al.*, 2001; Serrano *et al.*, 2002). These *VTC* (from Vacuolar Transporter Chaperone) genes are members of a yeast chaperone family and presumably they are involved in the distribution of the vacuolar H^+-ATPase and possibly in the polyphosphate synthesis in the vacuole (Cohen *et al.*, 1999; Ogawa *et al.*, 2000). Moreover, lack of some components of the PHO regulon, such as the CDK inhibitor Pho81, or the transcriptional factors Pho2 and Pho4, severely deteriorates growth of yeast cells in alkaline environments and completely abolishes alkaline response of *PHO84* and *PHO12*. The above data suggest that alkaline stress possibly generates a situation of phosphate starvation, hypothesis that is reinforced by the fact that the kinetics of the transcriptional responses of both *PHO84* and *PHO12* to alkaline and low phosphate conditions are quite similar (Serrano *et al.*, 2002). Nevertheless, this is not the case for

PHO89, which is induced to a much higher level by mild alkalinization treatment than by phosphate starvation. In addition, the alkaline response of this gene is faster than that of *PHO84* and *PHO12* and depends on the calcineurin pathway, suggesting that it could be triggered by the alterations of intracellular calcium concentration occurring upon alkalinization and not by a possible low phosphate availability (Serrano *et al.*, 2002; Viladevall *et al.*, 2004).

Under alkaline pH conditions, a set of genes related to the copper and iron homeostasis machinery also show induced expression levels (Lamb *et al.*, 2001; Causton *et al.*, 2001; Serrano *et al.*, 2002). Most of these genes are involved in the response of yeast cells to iron deprivation and two of them (*CTR1* and *CTR3*) encode high-affinity copper transporters. A more detailed study, focused on the role of these two metals in alkaline adaptation of yeast, showed that mutation of a considerable number of genes related to the transport of these metals confers sensitivity to yeast cells at alkaline pH, suggesting that copper and iron are possibly limiting factors for alkaline growth (Serrano *et al.*, 2004). This theory is further supported by the fact that supplementation of the growing media with copper and iron improves alkaline growth of many of these alkaline sensitive mutants. Thereby, ambient alkalinization seems to result in decreased availability of copper and iron for yeast cells and this could be in part the result of the decreased solubility of these metals at this pH range (Serrano *et al.*, 2004).

The study of the alkaline transcriptional response of *S. cerevisiae* also revealed that a significant number of the alkaline-responsive genes are related to the yeast response to oxidative stress (Viladevall *et al.*, 2004). Additionally, genome-scale phenotypic assays performed at alkaline pH conditions have shown that strains lacking *SOD1* or *SOD2,* encoding the cytosolic and mitochondrial superoxide dismutase, respectively, or *CCS1/LYS7*, encoding the copper chaperone that specifically delivers copper to Sod1, are extremely sensible to alkali (Serrano *et al.*, 2004). The above data suggested that exposure of yeast cells to alkaline environments triggers to some degree oxidative stress.

2. Function and regulation of the *ENA1* Sodium ATPase in *Saccharomyces cerevisiae.*

Sodium is an abundant cation in the environment and enters easily yeast cells through several low-affinity cation transport systems, mainly those involved in potassium uptake. However, whereas potassium is accumulated in cells at a fairly high concentration, threshold for Na^+ (and its analog Li^+) toxicity is low and active efflux is required when their extracellular concentrations are elevated.

The major sodium and lithium efflux system in *S. cerevisiae* is the P-type ATPase *ENA/PMR2.* This pump is able to actively extrude sodium, lithium but also calcium and potassium cations in reactions coupled to ATP hydrolysis. In *S. cerevisiae* there is an unusual tandem of one to five (depending on the strain), almost identical open reading frames that code for *ENA* proteins. *ENA1/PMR2A* was the first member of this tandem to be identified and as demonstrated later the most important (Haro *et al.*, 1991; Martinez *et al.*, 1991; Wieland *et al.*, 1995; Benito *et al.*, 2002). Yeast cells tolerate deletions of the entire *ENA* cluster, but such mutants show a dramatic phenotype of sensitivity when exposed to high salinity environments or under alkaline pH conditions (Haro *et al.*, 1991; Garciadeblas *et al.*, 1993; Rodriguez-Navarro *et al.*, 1994).

Under standard growth conditions expression of *ENA* genes is marginal, but when cells are exposed to elevated sodium and lithium levels or alkaline pH, the expression of *ENA1/PMR2A*, but not that of other members of the cluster, is potently induced (Garciadeblas *et al.*, 1993; Mendoza *et al.*, 1994). In addition, transcriptional response of *ENA1* is observed under glucose starvation conditions (Alepuz *et al.*, 1997) or upon activation of the TOR pathway, triggered when nitrogen is limited (Withee *et al.*, 1998; Crespo *et al.*, 2001). However, this ATPase does not seem to be involved in the general environmental stress response of yeast, as the environmental challenges that affect its expression are rather specific.

The transcriptional response of *ENA1* to the diverse stimuli mentioned above requires the intervention of multiple pathways that transmit the signals to its promoter. The promoter of this gene contains diverse regulatory elements recognized by certain transcription factors that activate or inhibit transcription according to the signal (see table 1 and figure 5).

The alkaline response of *ENA1* is remarkable, considering that can be similar or even higher than that evoked by high salinity. The alkaline responsive region of the

promoter of this gene was initially identified between nucleotides -742 and -358 (Alepuz *et al.*, 1997). A more detailed functional mapping performed later (Serrano *et al.*, 2002) showed that two smaller separated fragments account for the alkaline response of *ENA1*: an upstream region denoted as Alkaline Responsive Region 1 (ARR1) that is located between nucleotides -742 and -577 and a downstream region denoted as Alkaline Responsive Region 2 (ARR2) that is located between nucleotides -573 and -490 (see table 1 and figure 5). The ARR1 contains a CDRE element and its alkaline response is calcineurin-dependent, whereas that driven from ARR2 is essentially calcineurin-independent.

The complex regulatory mechanisms governing *ENA1* expression are analyzed in the following paragraphs, focusing on those that are relevant for its alkaline response.

motif	sequence	position	transcription factor	signaling pathway
GATA	5´-GATAA-3´	-1370/-1366	Gln3	**TOR**
GATA	5´-GATAAG-3´	-1107/-1102	Gln3, Gat1	**TOR**
GATA	5´-GATAAG-3´	-1075/-1070	Gln3, Gat1	**TOR**
GATA	5´-GATAA-3´	-972/-969	Gln3	**TOR**
CDRE	5´-GAATGGCTG-3´	-813/-821	Crz1	**calcineurin**
NRG	5´-CCCTC-3´	-725/-729	Nrg1	**Rim101,Snf1**
CDRE	5´-GGGTGGCTG-3´	-727/-719	Crz1	**calcineurin**
STRE	5´-AGGGG-3´	-651/-647	NF	
NRG	5´-CCCCT-3´	-651/-647	Nrg1	**Rim101,Snf1**
GATA	5´-GATAA-3´	-636/-632	Gln3	**TOR**
MIG	5´-ATTTTGCGGGG-3´	-544/-534	Mig1, Mig2	**Snf1**
CRE	5´-TGACGTTT-3´	-509/-502	Sko1	**HOG**
GATA	5´-GATAA-3´	-243/-239	Gln3	**TOR**

Table 1. The main regulatory DNA motifs identified on *ENA1* promoter. For each motif is denoted the name (first column), the sequence (second column), the distance from the initiating ATG of the ORF (third column), the transcription factor that recognizes the motif (fourth column) and the cognate signaling pathway (fifth column). The STRE element of *ENA1* promoter is non-functional (NF). The two boxes show the regulatory elements included in ARR1 (-751 to -667) and ARR2 (-573 to -490) as defined in (Serrano *et al.*, 2002). Modified figure from the thesis of Amparo Ruiz, March 2006, UAB.

2.1 Regulation of *ENA1* by the HOG osmoresponsive pathway.

The transcription of *ENA1* gene is induced in response to mild saline stress through the High Osmolarity Glycerol (HOG) pathway (Marquez and Serrano, 1996). This pathway responds to moderate concentrations of osmotic agents by rapidly activating the MAP kinase Hog1 via a multistep phosphorylation mechanism (Brewster *et al.*, 1993; Maeda *et al.*, 1994; Posas *et al.*, 1996)

Hence, under osmotic stress Hog1 is phosphorylated and accumulates in the cell nucleus where it phosphorylates Sko1 at multiple sites. Sko1 under non-stressing conditions acts as a repressor (Proft and Serrano, 1999; Proft *et al.*, 2001), by recruiting the general co-repressor complex Tup1-Ssn6 to a CRE site (from c-AMP Response Element) situated on the *ENA1* promoter at the position -509 to -502 (see figure 5). Upon phosphorylation by Hog1, the Sko1 repressor is converted to an activator and this conversion requires the additional recruitment of the chromatin-modifying complexes SAGA and Swi/Snf (Proft and Struhl, 2002). Moreover, the nuclear localization and the repressor function of Sko1 are also regulated by the protein kinase A (PKA). However, phosphorylation of Sko1 by PKA has the opposite effect as it enhances its repressor activity (Proft *et al.*, 2001; Pascual-Ahuir *et al.*, 2001).

Hog1 also seems to regulate transcription of *ENA1* by an additional mechanism that involves recruitment of the Rpd3-Sin3 histone deacetylase complex on its promoter that in turn leads to histone deacetylation, entry of the RNA polymerase II complex and thus induction of expression (Alepuz *et al.*, 2001; Nadal E. *et al.*, 2004). There is data suggesting that apart from recruitment of RNA Pol II, Hog1 kinase activity may also play a role in the activation of the holoenzyme. Nevertheless, no direct phosphorylation target of Hog1 has been identified within the components of the general transcription machinery (Alepuz *et al.*, 2001; Alepuz *et al.*, 2003).

In contrast to saline stress, the transcriptional response of *ENA1* under alkaline stress conditions is not affected by mutation of the *HOG1* gene, although the CRE element is located within the ARR2 region (Serrano *et al.*, 2002).

2.2 Regulation of *ENA1* by the calcineurin pathway.

In addition to the HOG-dependent mechanism, under severe saline stress conditions the transcription of *ENA1* is also subjected to a positive control mediated by the calcineurin pathway. Yeast strains lacking Cnb1, the single regulatory subunit of

calcineurin or treated with the calcineurin inhibitor FK506 accumulate sodium or lithium cations and this accumulation is attributed partially to impaired induction of *ENA1* expression (Mendoza *et al.*, 1994; Hirata *et al.*, 1995; Marquez *et al.*, 1996). On the contrary, hyperactivation of calcineurin increases *ENA1* expression, thus improving salt tolerance (Mendoza *et al.*, 1996; Park *et al.*, 2001).

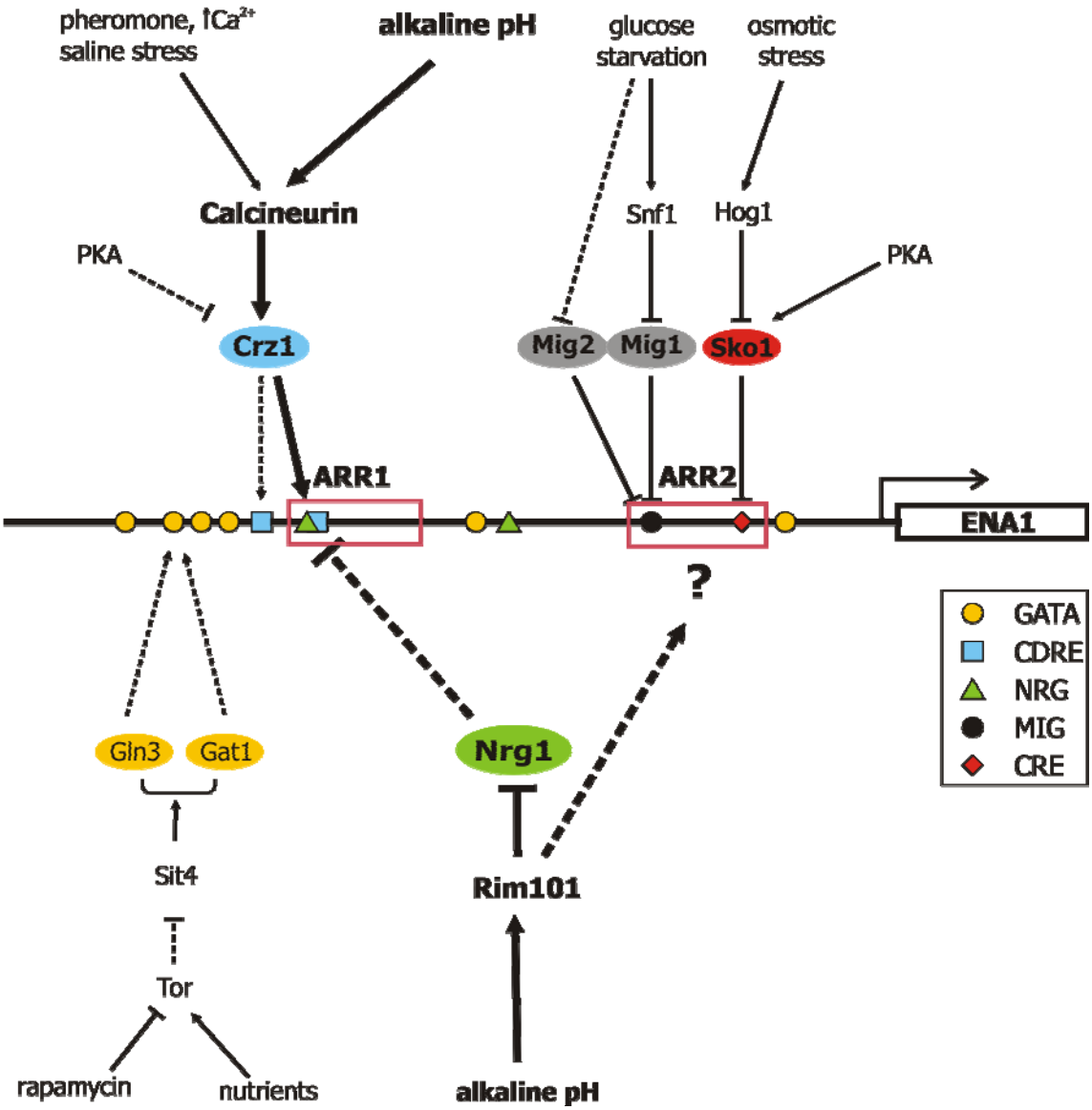

Figure 5. Schematic outline of the regulation of the *ENA1* promoter in *S. cerevisiae*. Only the major components of the signal transduction pathways and the corresponding stimuli that activate them are denoted. Discontinuous lines represent inputs to the promoter that were not documented when this work started or that still remain obscure. The regulatory DNA-motifs are represented to their relative position on the promoter (which is not drawn to scale) with basic shapes and indicated in the inset. The signaling pathways known to participate in the alkaline response of *ENA1* when this work started are depicted with thick black arrows (figure based on Ruiz and Ariño, 2007).

Interestingly, in the same study reporting that the calcineurin pathway is required for transcriptional induction of *ENA1* under severe saline stress conditions, it was found that calcineurin is also necessary for full alkaline induction of this gene (Mendoza *et al.*, 1994). Posterior studies showed that the calcineurin-directed transcriptional response of *ENA1* is mediated by Crz1 (see section 1.2). Mutants lacking Crz1 exhibit decreased *ENA1* expression and are hypersensitive to calcium and salt stress (Mendizabal *et al.*, 1998; Serrano *et al.*, 2002).

Two CDREs have been identified on the *ENA1* promoter, at positions -813 to -821 and -727 to -719 (see figure 5 and table 1), the downstream element being the most relevant for the transcriptional induction of the gene (Mendizabal *et al.*, 2001). This functional CDRE is located within ARR1, whose alkaline response was proved to be calcineurin-dependent (Serrano *et al.*, 2002). A subsequent study suggested that alkaline response from the ARR1 fragment is triggered by the calcium burst that takes place upon ambient alkalinization, as it is severely decreased when the calcium-calcineurin signaling is interrupted (Viladevall *et al.*, 2004). On the contrary, alkaline response of ARR2, which does not contain any consensus Crz1-binding site, is essentially calcineurin-independent (Serrano *et al.*, 2002; Viladevall *et al.*, 2004).

The calcineurin pathway also mediates expression of *ENA1* in response to Mg^{2+} depletion. Apparently, low concentration of Mg^{2+} in yeast cells environment results in a rise of intracellular Ca^{2+} concentration that in turns activates calcineurin and the transcription factor Crz1 thus inducing expression of *ENA1* (Wiesenberger *et al.*, 2007).

Finally, transcriptional induction of *ENA1* through the calcineurin pathway is observed in mutants lacking Pmc1 and Pmr1, two ATPases located on the vacuole and the Golgi apparatus, respectively. Pmc1 and Pmr1 contribute to intracellular calcium homeostasis by sequestering cations into these organelles (see figure 3), hence their mutation induces *ENA1* expression presumably because of an increase in intracellular Ca^{2+} concentration (Cunningham *et al.*, 1996; Park *et al.*, 2001).

2.3 Regulation of *ENA1* by the Rim101 pathway.

Together with calcineurin, the Rim101 pathway was the first alkaline responsive pathway described in *S. cerevisiae* (see section 1.1). Cells lacking Rim101, the main component of this pathway, are sensitive to both saline and alkaline stress and display decreased expression of *ENA1* at alkaline environments (Lamb *et al.*, 2001; Serrano *et al.*, 2002).

Rim101 regulates expression of *ENA1* indirectly, through repression of the transcription factor Nrg1 (Lamb *et al.*, 2003) that presumably binds directly to consensus NRG sequences on its promoter (see Figure 5). Nrg1 is a repressor protein that also negatively regulates invasive growth and the expression of several glucose repressed genes, under the tight control of the Snf1 protein kinase (Vyas *et al.*, 2001). Cells lacking Nrg1 show increased expression levels of *ENA1*, even in the absence of stress (Lamb *et al.*, 2003), and this effect is accentuated by further deletion of *NRG2* (Vyas *et al.*, 2005), encoding a close homolog of Nrg1 with partially redundant functions (Vyas *et al.*, 2001; Kuchin *et al.*, 2002; Vyas *et al.*, 2005).

Sequence analysis suggested that the *ENA1* promoter contains two putative Nrg1 binding sites: CCCCT and CCCTC, located at positions -725 to -729 and -651 to -647, respectively (Lamb *et al.*, 2003). Interestingly, the first upstream site partially overlaps with the functional CDRE binding site of this promoter (see Figure 7) spanning from nucleotides -727 to -719 (Mendizabal *et al.*, 2001). However, this might be a biologically important binding site, as it is located within the ARR1 region of *ENA1* promoter, whose alkaline expression is considerably decreased in *rim101* mutants. The downstream element appears to be less important or ineffective, as functional mapping of the promoter showed that it is situated between the two ARR of *ENA1*. The ARR2 region does not contain any previously suggested consensus Nrg1-binding sequence. Nevertheless, alkaline induction driven from this region is noticeably decreased in *rim101* mutants (Serrano *et al.*, 2002), suggesting the presence of a cryptic Nrg1 site in this promoter segment.

2.4 Regulation of *ENA1* by glucose: the Snf1 pathway.

In *S. cerevisiae* glucose, which is the preferred carbon source, negatively regulates the expression of many genes involved in the metabolism of alternative carbon sources. This phenomenon, known as glucose repression, requires complex interactions between DNA-binding transcription factors, their cognate elements and several components of the transcriptional machinery.

ENA1 belongs to the set of genes that are subjected to glucose repression (Alepuz *et al.*, 1997). Under non-limiting glucose conditions, transcription of *ENA1* is inhibited because of the repression activity of the transcription factors Mig1 and Mig2. Mig1 is a zinc finger protein that in the absence of any stimuli is located in the cell nucleus and directly represses transcription by binding to a specific DNA-sequence denominated as MIG regulatory element (see Table 1 and Figure 6). The MIG element of *ENA1* spans from nucleotides -533 to -544 and has been characterized as an Upstream Repressing Sequence ($URS_{MIG\text{-}ENA1}$) regulated by carbon source. Similarly to Sko1, recruitment of the Ssn6-Tup1 co-repressor complex is required for repression activity of Mig1 (Keleher *et al.*, 1992; Treitel and Carlson, 1995; Proft *et al.*, 1999).

The repressor activity of Mig1 is subjected to the control of the Snf1 protein kinase, which plays a central role in the transcriptional regulation by glucose in yeast (Gancedo, 1998; Hedbacker and Carlson, 2008). Snf1 is the catalytic subunit of the yeast homolog to the mammalian AMP-activated protein kinase (AMPK), a highly conserved kinase among eukaryotes that is involved in responses to metabolic stress (Hardie *et al.*, 1998; Hardie and Hawley, 2001). Like AMPK, the yeast homolog is a heterotrimeric enzyme comprised of the catalytic α-subunit Snf1, the γ-subunit Snf4 and one of the three alternative β-subunits, Sip1, Sip2 or Gal83, which regulate the subcellular localization of the kinase complex (Erickson and Johnston, 1993; Yang *et al.*, 1994). Upon glucose depletion (see figure 6) Gal83 moves to the nucleus thus directing Snf1 translocation, whereas Sip1 relocalizes to the vacuolar membrane and Sip2 remains cytoplasmatic (Vincent *et al.*, 2001). The γ-subunit Snf4 is constitutively localized at both the nucleus and the cytoplasm, independently of glucose availability, but under limiting conditions binds to Snf1 causing a conformational change that releases Snf1 from its autoinhibitory state (Celenza and Carlson, 1989; Celenza *et al.*, 1989; Jiang and Carlson, 1996). The activated nuclear Snf1 in turn phosphorylates Mig1 that exits from the nucleus and moves to the cytoplasm thus relieving repression (De Vit *et al.*, 1997; Treitel *et al.*, 1998; Ostling and Ronne, 1998). However, posterior studies suggest that phosphorylation of Mig1 by Snf1 alters its interaction with Ssn6-

Tup1 to alleviate repression, but does not release DNA-bound Mig1 from the promoter (Papamichos-Chronakis *et al.*, 2004).

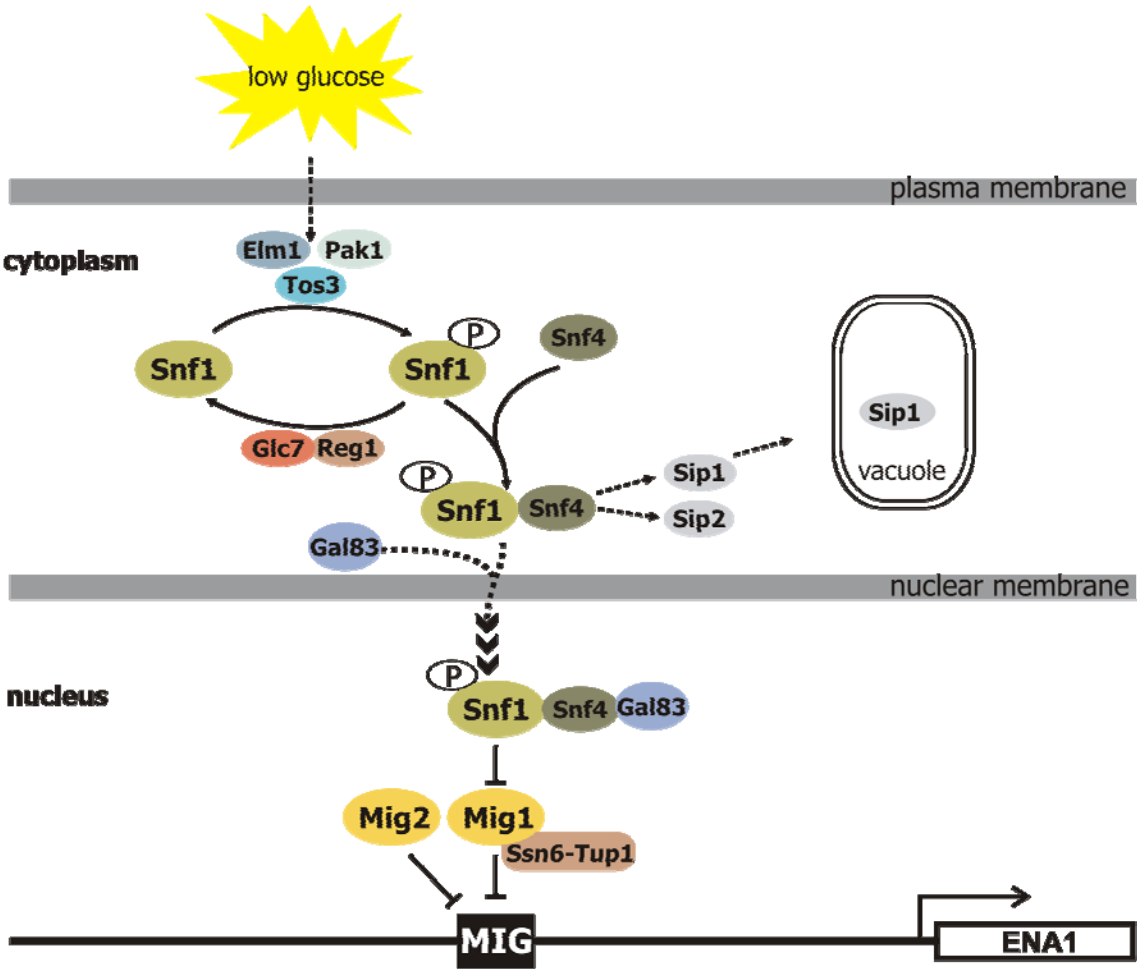

Figure 6. Transcriptional regulation of the *ENA1* gene by the Snf1 pathway. In the presence of glucose, the repressor Mig1 is nuclear and represses *ENA1* in conjunction with Mig2 and the Ssn6-Tup1 co-repressor complex, whereas Snf1 is cytoplasmatic and remains inactive in its autoinhibitory form. The PP1 phosphatase (Reg1-Glc7) keeps Snf1 dephosphorylated. Under glucose-limiting conditions, the Snf4 γ–subunit relieves Snf1 from its autoinhibitory state and Snf1 gets phosphorylated by three upstream kinases (Pak1, Elm1, Tos3). Gal83, the regulatory β–subunit moves to the nucleus directing Snf1 translocation. Nuclear Snf1 phosphorylates Mig1, thus relieving repression of *ENA1* promoter. Mig2 also relieves *ENA1* repression in response to glucose starvation, but the mechanism remains unknown.

The phosphorylation state of Snf1 is regulated by a set of three kinases and a phosphatase complex and is the determinant factor for its activity. The three upstream kinases that activate Snf1 by phosphorylation are Sak1 (formerly Pak1), Elm1 and Tos3. They are highly similar and exhibit overlapping functions. Nevertheless, Sak1 seems to be the most important of the three kinases as it also regulates the nuclear localization of Snf1 (Hong *et al.*, 2003; Nath *et al.*, 2003; Sutherland *et al.*, 2003;

Hedbacker *et al.*, 2004). On the other hand, the catalytic activity of Snf1 is negatively regulated by the Reg1-Glc7 protein phosphatase 1 (PP1): Reg1 corresponds to the regulatory subunit and interacts with Snf1 under non-limiting glucose conditions thereby targeting the Glc7 phosphatase to the kinase complex. Reg1 is cytoplasmatic independently of glucose availability, but it is phosphorylated in response to glucose limitation in a Snf1-dependent manner and dephosphorylated by Glc7 when glucose is present. The hexokinase Hxk2 that principally phosphorylates glucose also plays a role in regulating the phosphorylation state of Reg1, either by stimulating this phosphorylation or by inhibiting the dephosphorylation of Reg1 by Glc7 when glucose is scarce (Tu and Carlson, 1994; Tu and Carlson, 1995; Ludin *et al.*, 1998; Sanz *et al.*, 2000). However, the hexokinase Hxk2 apparently is also essential for long term repression maintenance under non-limiting glucose conditions, by interacting directly with Mig1 and Snf1 in the nucleus thus inhibiting phosphorylation of the transcription factor by the kinase (Ahuatzi *et al.*, 2004; Moreno *et al.*, 2005; Ahuatzi *et al.*, 2007).

Besides Mig1, another zinc finger transcription factor, Mig2, contributes almost equally to glucose repression of *ENA1* via the same DNA binding site (MIG regulatory element). *mig1 mig2* strains show a salt tolerant phenotype and increased expression levels of *ENA1* under basal and derepressing (high salt or glucose limiting) conditions (Proft *et al.*, 1999). Nonetheless, Mig2 seems to be regulated differently, since it is not phosphorylated by the Snf1 protein kinase and is permanently located in the nucleus (Lutfiyya *et al.*, 1998).

2.5 Regulation of *ENA1* by the Hal3/Ppz system.

The yeast Ppz1 and Ppz2 protein phosphatases and their inhibitory subunit Hal3 are important determinants of salt tolerance, cell wall integrity and cell cycle progression in *S. cerevisiae*. Mutants lacking Ppz1 display a marked tolerance to sodium and lithium cations that is enhanced upon further deletion of Ppz2. This tolerance is at least partially justified by an increase of *ENA1* expression observed in these strains, fact that supports the initial notion that Ppz1 and Ppz2 regulate negatively *ENA1* (Arino, 2002).

The two phosphatases can also modulate activity of the Trk1/2 potassium transport system thus influencing potassium uptake, saline tolerance and intracellular pH (Yenush *et al.*, 2002). The mechanism suggested for this modulation is that upon internal alkalinization the interaction between Ppz1 and its inhibitory subunit Hal3 is destabilized, thus Ppz1 is activated and binds Trk1. There is evidence suggesting that the Ppz1 phosphatase regulates negatively the activity of the Trk1 potassium transporter by modulating its phosphorylation state. Nonetheless, direct dephosphorylation of Trk1 by Ppz1 has not been demonstrated (Yenush *et al.*, 2005). Hal3 also participates in the regulatory activity that Ppz1 and Ppz2 exert over expression of *ENA1* (Arino, 2002).

The mechanisms by which the Ppz phosphatase influence *ENA1* expression are rather complex. The effect of single *PPZ1* mutation to the expression of *ENA1* depends exclusively on the CDRE element located within the ARR1 of its promoter and requires participation of the calcineurin pathway. The requirement for calcineurin signaling is justified by the negative effect that Ppz1 exerts to the calcium entrance through the Cch1-Mid1 calcium channel (Cunningham, K. W., *et al.,* personal communication). In case that both phosphatases are absent (mutants *ppz1 ppz2*) apart from the CDRE and calcineurin signaling, ARR2 is also required for full induction of *ENA1* expression. It is likely that in this mutant, deregulation of the Trk transporters results in intracellular alkalinization that would activate additional alkaline responsive pathways that act through the ARR2 (Yenush *et al.*, 2002; Ruiz *et al.*, 2003; Yenush *et al.*, 2005).

2.6 Regulation of *ENA1* by the CK2 protein kinase.

CK2 (formerly known as casein kinase 2) is a highly conserved serine/threonine protein kinase which is ubiquitous among eukaryotic organisms. CK2 is pleiotropic and participates in many different cellular processes, including survival, cell polarity, cell cycle regulation, stress responses, transcription and translation (Glover, 1998; Ahmed *et al.*, 2002).

In *S. cerevisiae* CK2 is a tetramer that consists of two distinct catalytic subunits α and α´ encoded by the genes *CKA1* and *CKA2* and two regulatory subunits, β and β´ encoded by the genes *CKB1* and *CKB2*, respectively. The catalytic subunits are functionally redundant but collectively essential as simultaneous disruption of both *CKA1* and *CKA2* is lethal for yeast cells. The regulatory subunits are dispensable for growth under normal, non-stressing conditions (Glover, 1998). However, deletion of *CKB1* and/or *CKB2* results in specific sensitivity to sodium and lithium cations, implying a role for CK2 in cation homeostasis (Bidwai *et al.*, 1995). A slight sensitivity of *cka1* and *cka2* mutants to high sodium has also been reported (Berkey and Carlson, 2006).

Yeast strains *ckb1, ckb2* and *ckb1 ckb2* lacking the regulatory subunits of CK2, were shown to be defective in expression of *ENA1*. Expression levels were tested by measuring β-galactosidase activity in cells transformed with plasmids of *lacZ* fusions to *ENA1* promoter. Furthermore, overexpression of *ENA1* from a heterologous promoter completely suppressed salt sensitivity of these mutants. Interestingly, induction of *ENA1* expression after cell treatment with alkali was also considerably decreased, but growth on alkaline media was normal. The above results were in accordance with a possible role of CK2 in modulation of *ENA1* expression (Tenney and Glover, 1999). However, another study performed almost simultaneously (Nadal *et al.*, 1999) yielded quite contradictory results, despite the fact that the experimental approach followed was the same (β-galactosidase assays by using *lacZ* fusions to *ENA1* promoter). Nevertheless, it should be noted that the stress conditions tested (NaCl concentration, time of exposure to stress) were different. In any case, the mechanism by which CK2 may influence *ENA1* expression remains unknown although, on the basis of saline tolerance studies, calcineurin and the Hal3/Ppz system were excluded as possible targets for the protein kinase (Nadal *et al.*, 1999).

CK2 seems to play a quite fundamental role in signaling and gene expression in eukaryotic cells. Apart from phosphorylating directly numerous transcription factors (Meggio *et al.*, 1994; Allende and Allende, 1995), this kinase also has substrates among the subunits of the RNA polymerase complexes, which regulate initiation of

transcription (Pinna, 2002). The mechanism of the CK2-dependent regulation of Pol III transcribed genes was studied in some detail (Hockman and Schultz, 1996; Ghavidel and Schultz, 2001; Saez-Vasquez *et al.*, 2001). The target of CK2 in this case is the TATA Binding Protein (TBP) of the Pol III transcription initiation factor IIIB (TFIIIB). CK2 presumably phosphorylates TBP and this step is indispensable for activation of TFIIIB and therefore transcription. Although the mechanism that activates CK2 to phosphorylate TBP is not known, several evidences suggest that the regulatory subunit CK2β plays an important and direct role in the regulation of Pol III transcription by CK2 (Maldonado and Allende, 1999; Ghavidel *et al.*, 2001).

ENA1 represents the first gene transcribed by RNA Pol II found to be regulated by CK2, but the mechanism of this regulation remains unknown (Tenney *et al.*, 1999). In a subsequent study it was suggested that CK2 is the kinase that phosphorylates Bdf1 (Sawa *et al.*, 2004), a component of the RNA Pol II preinitiation complex that corresponds to the C-terminal half of higher eukaryotic TAF1 (TBP-associated factor 1). Bdf1 is associated with the basal transcription factor TFIID and contains two bromodomains that bind to hyperacetylated histone H4, thus promoting association with chromatin and modulating transcription (Matangkasombut *et al.*, 2000; Matangkasombut and Buratowski, 2003; Ladurner *et al.*, 2003). *BDF1* is nonessential, but cells lacking both *BDF1* and *BDF2* (encoding a homolog of Bdf1 that is also phosphorylated by CK2) are not viable. Interestingly, *bdf1* mutants exhibit phenotypes such as temperature sensitivity, slow growth, defects in utilization of alternative carbon sources and salt sensitivity (Lygerou *et al.*, 1994; de Jesus Ferreira *et al.*, 2001). Recently, a global analysis of the transcriptional response of yeast cells to saline stress showed that expressions levels of *ENA1* are not altered in *bdf1* mutants, suggesting that the effect of Bdf1 on salt tolerance is not mediated by the sodium ATPase (Liu *et al.*, 2007).

Nrg1, the transcriptional repressor that negatively regulates *ENA1* expression (see paragraph 2.3) was also found to be phosphorylated by CK2 in response to a variety of stresses such as high salinity, alkaline pH and glucose starvation. Interestingly, phosphorylation of Nrg1 by CK2 depends on the Cka1 catalytic isoform and both Ckb1 and Ckb2 regulatory subunits, whereas the Cka2 isoform is dispensable (Berkey *et al.*, 2006).

CK2 also phosphorylates the mammalian histone deacetylase enzymes HDAC1-3 that are transcriptional co-repressors. These enzymes are homologues to the Rpd3 histone deacetylase of yeast (class I HDACs) and remove acetyl groups from the histone tails thus contributing to closed nucleosome conformations and repression

of transcription. Phosphorylation by CK2 seems to promote the enzymatic activity of HDACs (Pflum *et al.*, 2001; Cai *et al.*, 2001; Tsai and Seto, 2002; Sun *et al.*, 2002).

Regulation of CK2 remains an unsolved matter. Although this pleiotropic enzyme is constitutively active, in many cases it was observed that its activity is enhanced. The β subunits of the tetramer seem to have a key role in such regulation, not by modulating the kinase activity, but by targeting substrates or forming docking surfaces to facilitate their interaction with the kinase. Moreover, interaction between the subunits of the tetramer plays a decisive role in regulation of the CK2. In most cases the holoenzyme is more active than the isolated catalytic subunits. However there are exceptions (*e.g.* calmodulin), where the holoenzyme is inactive, and the substrate is only phosphorylated by the isolated catalytic subunits (for a review see Pinna and Meggio, 1997; Pinna, 2002, and references therein).

2.7 Regulation of *ENA1* by the Ptc1 protein phosphatase.

Ptc1 is a type 2C protein phosphatase (PP2C) that negatively regulates the HOG pathway by dephosphorylating the MAP kinase Hog1 (Saito and Tatebayashi, 2004). Deletion of *PTC1* confers to yeast cells a specific sensibility to lithium cations that is not additive to that observed in *ena1-4* cells. Furthermore, *ptc1* mutants show decreased levels of *ENA1* expression under both basal and high lithium conditions and this effect is not attributed to the regulatory activity that Ptc1 exerts to the HOG pathway (Ruiz *et al.*, 2006). There are several evidences pointing out the Hal3/Ppz system as target of this phosphatase, but the regulatory mechanism by which Ptc1 regulates expression of *ENA1* remains elusive (Ruiz *et al.*, 2006). Apart from intolerance to lithium ions, deletion of *PTC1* also results in impaired alkaline growth of yeast (Gonzalez *et al.*, 2006) and more recent data show that alkaline expression of *ENA1* is also impaired in these mutants (Gonzalez and Ariño, unpublished data).

2.8 Regulation of *ENA1* by nitrogen sources.

TOR (*T*arget *O*f *R*apamycin) is a highly conserved cascade that in *S. cerevisiae* is represented by two kinases encoded by the *TOR1* and *TOR2* genes. Inactivation of this kinases, either by shifting cells to poor nitrogen medium or by treating them with rapamycin, triggers a transcriptional response which leads to repression of translation and cell cycle arrest (Crespo and Hall, 2002).

The TOR cascade is one of the pathways that can modulate expression of the sodium ATPase *ENA1*. It seems that under saline stress conditions the TOR cascade is inhibited and as a result the phosphatase Sit4 is activated and releases the GATA-binding transcription factors Gln3 and Gat1 allowing their translocation from the cytoplasm to the nucleus. *ENA1* promoter contains six putative GATAA motifs (see table 1 and figure 5), an indicator that Gln3-dependent transcription might be possible. In addition, two of them are followed by a G (GATAAG), the consensus sequence recognized by Gat1. Hence it has been suggested that regulation of *ENA1* by the TOR pathway at saline environment is possibly mediated by Gat1 and Gln3, through binding on the cognate GATA elements of its promoter (Beck and Hall, 1999; Crespo *et al.*, 2001).

III. MATERIALS & METHODS

1. Yeast strains and growth media

The *S. cerevisiae* strains used throughout this work derive from DBY746 (*MAT*a*, ura*3-52 *leu2*-3,112 *his*3-*Δ*1 *trp1-Δ*239) and BY4741 (*MAT*a, *his3*Δ1 *leu2*Δ *met15*Δ *ura3*Δ) haploid wild-type strains and are listed in table 2 and 3, respectively. The only exception are the strains used for *Chromatin Immunoprecipitation Assays* which derive from the W303-1A background (*MAT*a *ade2-1 his3-11,15 can1-100 ura3-1 leu2-3,112 trp1-1*).

Yeast cultures were grown at 28 ºC in YPD medium (10 g/l yeast extract-20 g/l peptone-20 g/l dextrose) or complete minimal (CM) medium. When indicated, synthetic complete (SC) medium lacking appropriate supplements was used to maintain selections for plasmids (Adams *et al.*, 1997).

2. Recombinant DNA techniques

Escherichia coli DH5α was used as a host for DNA cloning experiments. Bacterial cells were grown at 37 °C in LB (Luria Bertani) medium containing, when needed, 50 μg/ml ampicillin for plasmid selection. *Escherichia coli* cells were transformed by standard treatment with calcium chloride (Sambrook *et al.*, 1989). *S. cerevisiae* cells were transformed by a modification of the lithium acetate method (Ito *et al.*, 1983).

Restriction mapping, DNA ligations, and other recombinant DNA techniques were carried out by standard methods (Sambrook *et al.*, 1989).

Purification of DNA fragments from complex mixtures, including PCRs and restriction endonuclease digests was performed by agarose gel electrophoresis. The appropriate bands were recovered from agarose gel slices using the *Agarose Gel DNA Extraction Kit* (Roche).

Table 2. Saccharomyces *cerevisiae* strains used in this study. All strains have the DBY746 (*MATa ura*3-52 *leu2*-3,112 *his*3-*Δ*1 *trp1*–*Δ*239) genetic background.

Name	Relevant genotype	Source /reference
DBY746	*MATa ura*3-52 *leu2*-3,112 *his*3-*Δ*1 *trp1-Δ*239	D. Botstein
RSC10	DBY746 *snf1*::*LEU2*	Serrano *et al.* 2002
RSC13	DBY746 *mig1*::*LEU2*	This work
RSC21	DBY746 *rim101*::*kanMX4*	Serrano *et al.* 2002
RH16.6	DBY746 *ena1-4::LEU2*	Haro *et al.* 1991
MAR15	DBY746 *cnb1*:: *kanMX4*	Serrano *et al.* 2002
MP005	DBY746 *rim101*::*kanMX4 snf1*:*LEU2*	This work
MP008	DBY746 *nrg1*::*kanMX4*	This work
MP009	DBY746 *mig1*::*LEU2 nrg1*::*kanMX4*	This work
MP010	DBY746 *mig2*::*TRP1*	This work
MP011	DBY746 *mig2*::*TRP1 nrg1*::*kanMX4*	This work
MP012	DBY746 *mig1*::*LEU2 mig2*::*TRP1*	This work
MP013	DBY746 *mig1*::*LEU2 mig2*::*TRP1 nrg1*::*kanMX4*	This work
MP014	DBY746 *snf1*::*LEU2 mig1*::*kanMX4*	This work
MP015	DBY746 *snf1*::*LEU2 mig*2::*TRP1*	This work
MP016	DBY746 *snf1*::*LEU2 mig2*::*TRP1 mig1*::*kanMX4*	This work
MP017	DBY746 *nrg2*::*kanMX4*	This work
MP018	DBY746 *nrg2*::*TRP1*	This work
MP019	DBY746 *nrg1*::*kanMX4 nrg2*::*TRP1*	This work
MP020	DBY746 *snf1*::*LEU2 nrg1*::*kanMX4*	This work
MP021	DBY746 *snf1*::*LEU2 nrg2*::*kanMX4*	This work
MP022	DBY746 *nrg1*::*kanMX4 nrg2*::*TRP1 snf1*::*LEU2*	This work
MAR194	DBY746 *mig1::LEU2 mig2::TRP1 nrg2::kanMX4*	This work
MAR195	DBY746 *nrg2::TRP1 rim101::kanMX4*	This work
MAR198	DBY746 *mig1::LEU2 mig2::TRP1 nrg1::nat1*	This work
MAR199	DBY746 *mig1::LEU2 mig2::TRP1 nrg2::kanMX4 nrg1::nat1*	This work
MAR200	DBY746 *nrg2::TRP1 rim101::kanMX4 nrg1::nat1*	This work
MAR206	DBY746 *rim101::kanMX4 nrg1::nat1*	This work

Table 3. Saccharomyces *cerevisiae* strains used in this study. All strains have the BY4741 (*MATa his3*Δ1 *leu2*Δ *met15*Δ *ura3*Δ) genetic background.

Name	Relevant genotype	Source /reference
BY4741	*MATa his3*Δ1 *leu2*Δ *met15*Δ *ura3*Δ	Winzeler et al., 1999
	BY4741 *snf1::kanMX4*	Winzeler et al., 1999
	BY4741 *rim101::kanMX4*	Winzeler et al., 1999
	BY4741 *ckb1::kanMX4*	Winzeler et al., 1999
	BY4741 *ckb2::kanMX4*	Winzeler et al., 1999
	BY4741 *nrg1::kanMX4*	Winzeler et al., 1999
	BY4741 *nrg2::kanMX4*	Winzeler et al., 1999
	BY4741 *ena1-5::loxP*	Sychrova
MP050	BY4741 *ckb1::kanMX4 ckb2::nat1*	This work
MP044	BY4741 *ckb1::kanMX4 snf1::LEU2*	This work
MP045	BY4741 *ckb2::kanMX4 snf1::LEU2*	This work
MP051	BY4741 *ckb1::kanMX4 ckb2::nat1 snf1::LEU2*	This work
MP046	BY4741 *ckb1::kanMX4 rim101::nat1*	This work
MP047	BY4741 *ckb2::kanMX4 rim101::nat1*	This work
MP053	BY4741 *ckb1::kanMX4 nrg1::nat1*	This work
MP054	BY4741 *ckb2::kanMX4 nrg1::nat1*	This work

3. Deletion cassettes and gene disruptions

The *mig2::TRP1* cassette was constructed as follows. A 1.76-kbp region of the *MIG2* genomic locus, spanning from nucleotides -809 to +1149 from the start codon, was amplified by PCR using primers mig2_5´_SalI and mig2_3´_PstI, which contain artificial SalI and PstI restriction sites (the sequence of all oligonucleotides used for generation and verification of gene disruptions in this work can be found in table 4). The amplification fragment was cloned into the SalI/PstI sites of plasmid pBluescript-SK (Stratagene), to give pBS-*MIG2*. A 387-bp SnaBI/NheI fragment of the open reading frame was replaced by a 1.0-kbp *TRP1* marker recovered from plasmid YDp-W

by digestion with SmaI/NheI, yielding plasmid pBS-*MIG2::TRP1*. This plasmid was digested with StuI/AfeI and the 2.0-kbp fragment obtained was used to transform strain DBY746. Homologous integration of the cassettes was verified by colony PCR (Adams *et al.*, 1997) using primers mig2_5´_SalI and TRP1-3, which amplify a fragment of 1.15-kbp.

The *nrg1*::*kanMX4* disruption cassette was amplified using oligonucleotides 5´-nrg1_disr and 3´-nrg1_disr from genomic DNA obtained from a *nrg1* mutant in the BY4741 background, in which the entire *NRG1* open reading frame was replaced by the heterologous marker *kanMX4*, which confers resistance to geneticin (Wach, 1996; Winzeler *et al.*, 1999). The same strategy was followed to generate the cassettes *nrg2*::*kanMX4* (using primers 5´-nrg2_disr and 3´-nrg2_disr) and *mig2*::*kanMX4* (using primers 5´-mig1 _disr and 3´-mig1_disr).

Disruption of *NRG1* by the *nat1* marker was accomplished as follows. A 2.1-kbp DNA fragment encompassing the *NRG1* gene was amplified from genomic DNA by PCR using oligonucleotides 5´-nrg1_comp and 3´-nrg1_disr. The fragment was digested with XbaI and PmlI, yielding a 1.7-kbp fragment that was cloned into the XbaI and HincII sites of pBluescript-SK (Stratagene) to generate pBS-*NRG1*. This construct was digested with EcoRI and HincII and the 0.8-kbp fragment was replaced by the 1.27-kbp *nat1* gene fragment recovered from plasmid pAG25 (Goldstein and McCusker, 1999) by digestion with EcoRI and PvuII. The resulting construct, pBS-*NRG1*::*nat1*, was then digested with XbaI and XhoI and the 2.15-kbp fragment released was used to transform yeast cells. Clones that contain the *nrg1*::*nat1* cassette are resistant to nourseothricin, thus can be selected on media that contain this antibiotic (clonNAT, Werner Bioagents). Homologous integration of the cassette into the genome was verified by colony PCR, using primers 5´-nrg1_comp and 3´-nrg1 that amplify a 2.0-kbp DNA fragment.

Disruption of the *NRG2* gene by the marker *TRP1* was generated as follows. A 2.0-kbp region of the *NRG2* locus was amplified by PCR from genomic DNA, prepared from the DBY746 wild type strain, using oligonucleotides 5'-nrg2_disr, which contains an artificial restriction EcoRI site, and 3'-nrg2_disr, which contains an artificial PstI site. This fragment was cloned into the same sites of plasmid pBluescript-SK to give plasmid pBS-*NRG2*, which was digested with NheI/HincII to remove a 652-bp internal region. This region was replaced by a 1.0-kbp *TRP1* marker recovered from plasmid YDp-W (Berben *et al.*, 1991) by digestion with SmaI/NheI, to yield plasmid pBS-*NRG2::TRP1*.

This plasmid was digested with EcoRI/SmaI and the 2.2-kbp fragment obtained was used to transform the appropriate yeast strain.

Gene disruptions performed in a DBY746 genetic background.

The strain MP005 (*rim101 snf1*) was generated by transformation of strain RSC21 (*rim101*) with the *snf1::LEU2* cassette recovered from plasmid pCC107::*LEU2* (Rodriguez *et al.*, 2003) after digesting with restriction enzymes BamHI and HindIII. Homologous integration was verified by colony PCR using oligonucleotides 5´-snf1_disr and leu2_3´_bis that amplify a 1.0-kbp fragment.

The strain RSC13 (*mig1*) was generated by transformation of the wild type strain DBY746, with the cassette *mig1::LEU2*, recovered from plasmid pMIG3 (Nehlin and Ronne, 1990) after digesting with the restriction enzyme SacI. Homologous integration was verified by colony PCR using oligonucleotides 5´-mig1_disr and leu23´_bis that amplify a 1.0-kbp fragment.

Strains MP012 (*mig1 mig2*) and MP015 (*snf1 mig2*) were made by introducing the *mig2::TRP1* cassette into strains RSC13 (*mig1*) and RSC10 (*snf1*), respectively and homologous integration was confirmed by colony PCR using primers mig2_5´_SalI y TRP1-3 that amplify a fragment of 1.15-kbp.

Strains MP009 (*mig1 nrg1*), MP011 (*mig2 nrg1*), MP013 (*mig1 mig2 nrg1*) and MP020 (*snf1 nrg1*) were constructed by transformation of strains RSC13 (*mig1*), MP010 (*mig2*), MP012 (*mig1 mig2*) and RSC10 (*snf1*), respectively, with the *nrg1*::*kanMX4* cassette. Clones resistant to geneticin (G418) were recovered and correct integration of the deletion cassette was confirmed by colony PCR with primers 5´-nrg1_comp and K2 that amplify a DNA fragment of 1.0-kbp approximately.

Similarly, strains MP014 (*snf1 mig1*) and MP016 (*snf1 mig2 mig1*) were generated by transformation of strains RSC10 and MP015, respectively, with the cassette *mig1*::*kanMX4*. The primers used for verification of proper integration by colony PCR, were 5´-mig1_comp and K2, yielding a DNA band of 1.1-kbp approximately.

Strain MP019 (*nrg1 nrg2*) was constructed by transforming strain MP008 (*nrg1*) with the *nrg2*::*TRP1* disruption cassette and homologous integration was confirmed by

PCR with primers 5´-nrg2_comp y TRP1-3´ that amplify a DNA fragment of 1.4-kbp. Strains MP021 (*snf1 nrg2*) and MAR194 (*mig1 mig2 nrg2*) were obtained similarly using strains RSC10 and MP012 as a genetic background, respectively.

Strain MP022 (*nrg1 nrg2 snf1*) was generated by introducing the *snf1*::*LEU2* cassette into MP019 strain. Strain MAR195 (*nrg2 rim101*) was obtained by transformation of strain MP018 (*nrg2*::*TRP1*) with the *rim101*::*kanMX4* disruption cassette (Serrano *et al.*, 2002). Homologous integration of the cassette was verified by colony PCR using the primers 5´-rim101_bis and K2 that amplify a 0.9-kbp fragment.

Strains MAR198 (*mig1 mig2 nrg1*), MAR199 (*mig1 mig2 nrg2 nrg1*), MAR200 (*nrg2 rim101 nrg1*), and MAR206 (*rim101 nrg1*) were constructed by introducing the cassette *nrg1*::*nat1* in strains MP012, MAR194, MAR195, and RSC21, respectively. Homologous integration of the cassette to the genome was verified by colony PCR using primers 5´-nrg1_comp y 3´-nrg1 that amplify a 2.0-kbp DNA fragment.

Gene disruptions performed in a BY4741 genetic background.

Strain MP027 (BY4741 *mig2 mig1*) was generated by transformation of the BY4741 *mig2::kanMX4* strain with the cassette *mig1::LEU2*, recovered from plasmid pMIG3 (Nehlin *et al.*, 1990) after digesting with the restriction enzyme SacI. Homologous integration was verified by PCR using oligonucleotides 5'-mig1_disr and leu2_3'_bis that amplify a 1.0-kbp fragment.

Strain MP050 (*ckb1 ckb2*) was generated by replacing sequence from +40 to +740 of the *CKB2* ORF in the isogenic strain BY4741 *ckb1::KAN*, by the heterologous marker *nat1* according to the short flanking homology replacement strategy (Wach *et al.*, 1994). The primers that were used to amplificate the *nat1* gene fragment from plasmid pAG25 (Goldstein *et al.*, 1999) are 5´-disr_ckb2_nat and 3´-disr_ckb2_nat. Clones resistant to clonNAT were selected and homologous integration of the cassette to the genome was verified by colony PCR using the primers 5´-ckb2 and 3´-nat1 that amplify a 1.5-kbp DNA fragment.

Strains MP044 (*ckb1 snf1*), MP045 (*ckb2 snf1*) and MP051 (*ckb1 ckb2 snf1*) were constructed by introducing the *snf1::LEU2* cassette, recovered from the plasmid pCC107::*LEU2* (Rodriguez *et al.*, 2003) after digesting with restriction enzymes BamHI and HindIII, into strains *ckb1*, *ckb2* and *ckb1 ckb2* (MP050), respectively.

Table 4. Oligonucleotides used in this work for generation and verification of gene disruptions.

Oligonucleotide	Sequence
5´-snf1_disr	CTCTTACTGCGCATTCGTGTC
leu2_3´_bis	CAGTACCACCGAAGTCGGTG
5´-nrg1_disr	CGAAACGCGTTGAGCGCGCAG
3´-nrg1_disr	CTGACGTACATCACGTGAACTG
5´-nrg1_comp	CAGTAATCTACAAGGACCGTTGC
K2	CACGTCAAGACTGTCAAGGA
5´-nrg2_disr	CGGAATTCTCTGAGTGGCGCACC
3´-nrg2_disr	GATCAATGGCACTCGAGATC
5´-nrg2_comp	ACGTGGCTACACCGGCTG
5´-mig1 _disr	CTAGCAGTGTAACTCGATAGG
3´-mig1_disr	GATGTCTACTCGTCCACATC
5´-mig1_comp	GTTAGCGAGCCAGCACGTTTT
mig2_5´_SalI	TACGCGTCGACAAACTCGCTCGATACAGTA
mig2_3´_PstI	AAAACTGCAGTCTTGGTGGTGTATCTATTG
TRP1-3´	CATCTCCATGCAGTTGGACG
3´-nat1	GTGAAGGACCCATCCAGTGC
5´-ckb2	GGCCAGTAGGATCTATTATGCCTG
5´-rim101_bis	GCGAATTCGCCCATTCAAAAGCAACAC
5´-rim101_XbaI	GCTCTAGACCGCGTTCGTGCGAGACA
5´-disr_rim101_nat1	AGATGGTGCCATTGGAAGATCTGCTTAATAAAGAAAATGGCACTGCG TACGCTGCAGGTCGAC
3´-disr_rim101_nat1	TTGGGATACTTGGTCAAGATGCGAACTGAGTTAGTTCCCATTTGGAT CGATGAATTCGAGCTCG
5´-disr_ckb2_nat1	TCATGGGCAGTAGATCGGAGAATGTGGGAACAGTGACTAGAGCGTAC GCTGCAGGTCGAC
3´-disr_ckb2_nat1	TTAATAGTTAGGTTTTAAAACCACCACTTTTCGTCAAATCAACATCG ATGAATTCGAGCTCG

Strains MP046 (*ckb1 rim101*) and MP047 (*ckb2 rim101*) were generated by replacing sequence from +44 to +1865 of the *RIM101* ORF in the isogenic strains *ckb1::KAN* and *ckb2::KAN*, respectively, by the heterologous marker *nat1* according to the short flanking homology replacement strategy (Wach *et al.*, 1994). The primers that were used to amplificate the *nat1* gene fragment from plasmid pAG25 (Goldstein *et al.*, 1999) are 5´-disr_rim101_nat1 and 3´-disr_rim101_nat1. Positive clones were tested for homologous integration of the cassette to the genome by colony PCR, using primers 5´-rim101_XbaI and 3´-nat1 that amplify a 0.9-kbp DNA fragment.

Strains MP053 (*ckb1 nrg1*) and MP054 (*ckb2 nrg1*) were constructed by introducing the cassette *nrg1*::*nat1* in strains *ckb1::kanMX* and *ckb2::kanMX* respectively. Clones resistant to nourseothricin (clonNAT) were selected and homologous integration of the cassette to the genome was verified by PCR using primers 5´-nrg1_comp y 3'-nrg1 that amplify a 2.0-kbp DNA fragment.

4. Constructions of β-galactosidase reporters

In order to evaluate the response of different fragments of the *ENA1* promoter to alkaline pH or saline stress a number of β-galactosidase reporter plasmids were used (see figure 8).

The pKC201 plasmid (Alepuz *et al.*, 1997) contains the *ENA1* gene upstream region from nt -1385 to +35 (distance from the starting Met codon) fused to the *lacZ* gene.

Table 5. Oligonucleotides used for construction of β-galactosidase reporter plasmids. Underlined sequences represent added restriction sites for cloning purposes.

Plasmid	Fragment position	Sequences
pMRK213	-490 to -573	TACGCGTCGACAGGGTCTTAAACATCGCCGT CACGCTCGAGAATCTACATCCCTGCCCTAA
pMRK525	-511 to -573	TACGCGTCGACAGGGTCTTAAACATCGCCGT CACGCTCGAGAGAAGTAGGAAATAAATGAT
pMRK505	-531 to -573	TACGCGTCGACAGGGTCTTAAACATCGCCGT CACGCTCGAGGAGCCCCGCAAAATGACGCC
pMRK564	-490 to -531	TACGCGTCGACATCATTTATTTCCTACTTCT CACGCTCGAGAATCTACATCCCTGCCCTAA
p*SUC2*	-745 to +125	GGAATTCGGTGAGCTGTCGAAGGTATC GGCTGCAGTCATCGGAAGTAGCATGGCCC

Plasmid pMRK213 (Serrano *et al.*, 2002) that contains the ARR2 DNA fragment from nt -573 to -490 (see figure 8) was constructed by excising the appropriate promoter fragment from the pMP vector (Proft *et al.*, 1999) by PstI-XhoI digestion, and then cloning into these sites of plasmid pBluescript-SK. The fragment was recovered by digestion with SmaI-XhoI and cloned into the same restriction sites of plasmid

pSLFΔ-178K. This plasmid is a *CYC1* promoter-lacZ fusion from which the *CYC1* UAS elements have been deleted (Idrissi *et al.*, 1998).

Plasmids pMRK525, pMRK505 and pMRK564 were constructed as follows: the corresponding *ENA1* promoter region (see figure 8) was amplified by PCR using the appropriate primers (see table 5) and plasmid pKC201, which contains the entire *ENA1* promoter, as a template. The PCR products were digested by the restriction enzymes XhoI and SalI and then cloned into the XhoI restriction site of pSLFΔ-178K. All inserts were verified by sequencing using the Big Dye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems).

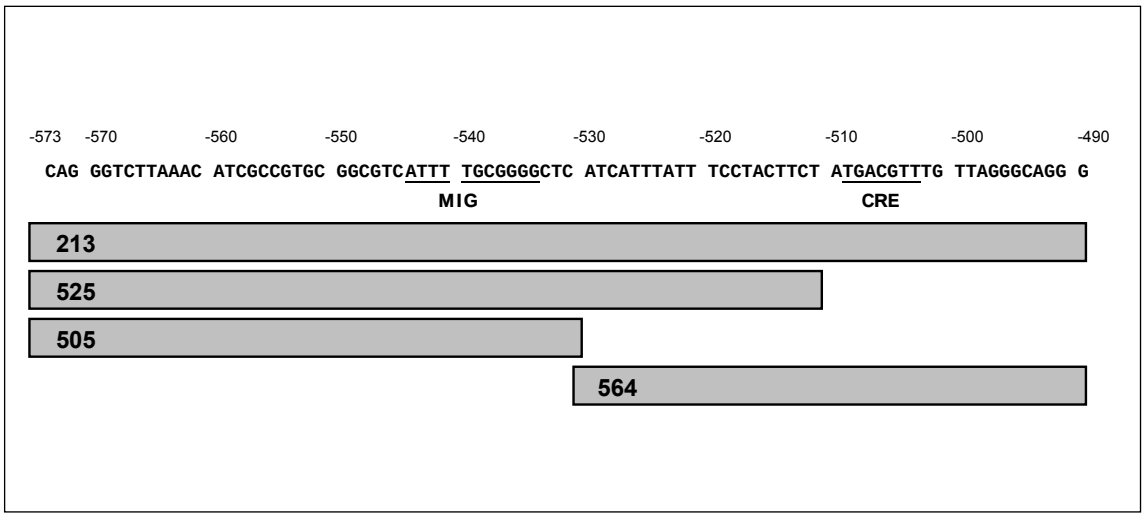

Figure 8. Segments of the ARR2 (corresponding to 213 fragment) region of *ENA1* promoter used for reporter plasmid construction. Numbers correspond to the distance from the initiating ATG of the *ENA1* ORF. The regulatory elements MIG and CRE are underlined.

In order to evaluate expression of the *SUC2* gene that encodes the sucrose hydrolyzing enzyme invertase in different yeast strains, a reporter plasmid that contains the *SUC2* promoter fused to the *lacZ* gene was used. Construction of this plasmid was performed as follows: the *SUC2* upstream region from nt -745 to +125 (positions relative to the starting ATG of the ORF) was amplified by PCR using primers 5'_prom*SUC2*_EcoRI and 3'_prom*SUC2*_PstI (see table 5) that contain an artificial EcoRI and PstI restriction site, respectively. The PCR product was digested by the restriction enzymes EcoRI/PstI and cloned into the same sites of the plasmid YEp357 (Myers *et al.*, 1986).

5. β-galactosidase activity assays

Yeast cells were grown to saturation in the appropriate drop-out media overnight and then inoculated in YPD at an A_{660} of 0.2. Growth was resumed until an A_{660} of 0.5 to 0.7 and cultures were then distributed into 1.0 ml aliquots and centrifuged for 5 minutes at 1620 *x g*. The supernatant was discarded and cultures were resuspended in the appropriate media. Unless otherwise stated, alkaline stress was provoked by resuspending cells in YPD containing 50 mM TAPS adjusted to pH 8.5 by addition of potassium hydroxide (autoclaving decreases the pH of the medium up to pH 8.0 approximately). Saline stress was provoked by addition of 0.4 M NaCl or 0.2 M LiCl to the medium. In order to cause glucose starvation, cells were washed with sterile MilliQ water and then resuspended to a medium that contains only 0.05 % of glucose. Non-induced cells in all cases were resuspended in YPD medium adjusted to pH 6.3 after autoclaving. Growth was resumed and cells were collected after 60 minutes of exposure to alkaline or saline stress and after three hours of exposure to glucose limiting conditions.

In order to perform β-galactosidase assays cells were centrifuged for 5 minutes at 1620 *x g* and resuspended in 300 μl of buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 35 mM 2-mercaptoethanol). 100 μl of the cell suspension were diluted in 900 μl of buffer Z in assay tubes and cells were permeabilized by addition of 40 μl of chloroform and 20 μl of 10 % SDS (sodium dodecyl sulfate) and then vortexing for fifteen seconds. The rest of cell suspensions were stored at 4 ºC, whereas the assay mixture was equilibrated by placing the assay tubes at 30 ºC in a water bath. After 15 minutes, 0.2 ml of ONPG (o-nitrophenyl-β-D-galactopyranoside) were added to the assay mixture to start the enzymatic reaction (Reynolds *et al.*, 1997). After sufficient yellow color was developed, the enzymatic reaction was stopped by adding 500 μl of 1 M Na_2CO_3, which shifts pH of the reaction solution to 11. The product formation was evaluated spectrophotometrically at 405 nm in 96-well plates using an iEMS Reader MF apparatus (Labsystems). Concentration of the cell suspensions was monitored similarly, with the difference that the wavelength was adjusted to 620 nm. β-galactosidase activity was calculated in Miller Units using the formula [(1000 x A_{405})/ (time of reaction x A_{620})].

6. Invertase activity assay

Yeast cells were grown to saturation in rich YPD medium overnight and then inoculated in YPD at an A_{660} of 0.2. Growth was resumed until A_{660} of 0.5 to 0.7 and cultures were then distributed into 10 ml aliquots and centrifuged for 5 minutes at 1620 *x g*. Cell pellets were washed twice with 4 ml of MilliQ water and then resuspended in YP medium containing 2 % (non-inducing conditions) or 0.05 % (inducing conditions) of glucose. Growth was resumed and cells were collected after 3 hours of incubation at 28 ºC.

In order to perform invertase activity assays cells were centrifuged for 5 minutes at 1620 *x g*, washed twice with MilliQ water and resuspended in 0.1 M sodium acetate buffer adjusted at pH 5 (20 mg cell wet weight /ml). Samples of 75 and 150 μl of non-induced or 10 and 20 μl of induced yeast cell suspension were diluted with the same sodium acetate buffer to a final volume of 180 μl and equilibrated for 5 minutes at 30 ºC. Then 20 μl of 0.5 M sucrose solution was added in each tube and after 15 min of incubation the enzymatic reaction was stopped by adding to the reaction mixture 0.2 ml of 0.4 M potassium phosphate solution (pH 7) and incubating for 3 min at 100 ºC. Then cell suspensions were centrifuged for 5 min at 16,000 x *g* and glucose concentration in the supernatant was measured using the Cromatest Kit (Linear Chemicals, S. L.) based on the glucose oxidase method. Invertase activity is expressed as nanomoles of glucose released per minute per mg of yeast cells (wet weight).

7. Real-time Reverse Transcriptase-PCR (RT-PCR)

Saturated cultures of the appropriate strains were diluted to A_{660} of 0.2 in YPD and grown up to A_{660} of 0.5–0.7. Then, two aliquots of 10 ml of each culture were centrifuged during 5 min at 1620 x *g* at room temperature and pellets were resuspended in 10 ml of fresh YPD containing 50 mM TAPS buffered at pH 8.0 (alkaline induction) or YPD (no induction), respectively. After 10 min, cells were harvested by centrifugation for 2 min at 1620 x *g* at 4 °C, washed once with cold water, and centrifuged again to obtain cell pellets that were immediately frozen and stored at –80 °C until RNA purification. When inhibition of calcineurin was desired, FK506 (Astellas Pharma) was added at a final concentration of 1.5 µg/ml in the medium, 1 h prior initiation of the alkaline treatment. The drug was also present in final resuspension media during stress.

Total RNA was purified using the RiboPure-Yeast kit (Ambion) following the manufacturer's instructions. RNA quality was assessed by denaturing 0.8 % agarose gel electrophoresis and RNA quantification was performed by measuring A_{260} in a BioPhotometer (Eppendorf). Real time PCR was performed in a SmartCycler (Cepheid) apparatus, using the QuantiTect SYBR Green reverse transcriptase-PCR kit (Qiagen) and 10 ng of total RNA. For *ENA1* amplification, oligonucleotides *ENA1* fw and *ENA1* rev were used (see table 6). Control experiments were carried out by amplifying a fragment of the *PHO84* gene, using oligonucleotides *PHO84* fw and *PHO84* rev. In this case, due to the slower induction of this gene after alkaline stress, cell samples were taken after 30 min of induction. Reverse transcription was performed for 30 min at 50 °C, followed by incubation at 95 °C for 15 min. Finally, 45 PCR cycles (15 s at 94 °C, 30 s at 50 °C, and 30 s at 72 °C) were carried out.

Table 6. Oligonucleotides used in this work for Real Time PCR experiments.

Gene	Sequences
***ENA1* fw**	AAAGGCTGCTCCAGATTTGA
***ENA1* rev**	GTGATCCAGTAAATGAAGCCA
***PHO84* fw**	TGCTAGAGACGGTAAGCCAA
***PHO84* rev**	ATGGGCTGGAAGATTCAATG

8. Expression in *E. coli* of *S. cerevisiae* Nrg1

For expression in *E. coli*, the *NRG1* open reading frame (approximately 0.7 kbp) was amplified by PCR using oligonucleotides 5'-Nrg1 and 3'-Nrg1. The amplification fragment was cleaved with BamHI and XhoI restriction enzymes and cloned into the BamHI-XhoI site of pGEX-4T-1 (Amersham Biosciences) to yield plasmid pGEX-4T1-Nrg1. *E. coli* BL21-Codon Plus (DE3)-RIL cells (Stratagene) were transformed with plasmid pGEX-4T1-Nrg1 and grown overnight at 37 °C in LB medium containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. The culture was then diluted 1/10 in the same medium and cultures were grown to an A_{660} of 0.6–0.8. At this point isopropyl β–D-thiogalactopyranoside (IPTG) was added to the medium to a final concentration of 1 mM and cells were induced for 3 h at 37 °C. Cells were centrifuged at 1620 x *g* at 4 °C, washed twice, and resuspended in ice-cold lysis buffer (20 ml/liter of culture) that contained 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM dithiothreitol, 10 % glycerol, and 0.1 % Triton X-100, plus a protease inhibitor mixture (0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine, 1 µg/ml pepstatin A, 1 µg/ml leupeptin). Cells were then sonicated (3 x 15 s) and centrifuged at 800 x *g* at 4 °C for 5 min, and the supernatant was collected. To purify the GST-Nrg1 fusion protein the bacterial crude lysate was incubated with Glutathione-Sepharose 4B beads (Amersham Biosciences), allowing gentle shaking for 1 h at 4 °C. Then the mixture beads-bound protein was spun down by centrifugation (13,000 *x* g, 10 minutes) at 4 ºC and washed twice with 10 volumes of buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 % glycerol and 0.1 % Triton X-100. A third wash was made with the same buffer but without Triton X-100 and finally the GST-Nrg1 fusion protein was eluted with a 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM dithiothreitol buffer supplemented with 10 mM glutathione.

Table 7. Oligonucleotides used in this work for EMSAs and Chromatin Immunoprecipitation Assays

Oligonucleotide	Sequence
5´-Nrg1	CGGGATCCCCATGTTTTACCCATATAAC
3´-Nrg1	AGCTCGAGGATACCGTCAATTATTGTC
ena1_pr_5´_640	TGTGATAATTCATGTACGGA
ena1_pr_3´_541	CGCCGCTCGAGAAATGACGCCGCACGGCCCCGGGGGA
ena1_pr_3´_551	CGCCGCTCGAGGCACGGCGATGTTTAAGCCCGGGGGA
ena1_pr_3´_561	CGCCGCTCGAGGTTTAAGACCCTGTACTCCCGGGGGA
ena1_pr_3´_578	CACAATGTAACCACTTCGTG
A	ACTACGAGTTTTCTGAACCTCC
B	TAATTTCGTGGATCTCGCAATC
O1	AATGAGGGTGGCTGTGCAA
O2	GGGTGTGATAATTCATGTACGGA
O3	TGCAACGAAGTGGTTACATTGTG
O4	TTCGGGAATACTGCTCTAAGACC
O5	GATAATTGCTTGCAACACTATAGAACACATTTGAAAAAGGGACA ATCCGGTTCTGCTGCTAGT
O6	ACAGGAAGTCAGGAGGTAGTCACAGTCTCGTATTAAGCAAAAGT GCCTCGAGGCCAGAAGAC

9. Electrophoresis Mobility Shift Assays (EMSAs)

DNA probes were labeled with ^{32}P by PCR using [α-^{32}P]dCTP and the appropriate primers (listed in table 7) and purified using S-200 HR microspin columns (Amersham Biosciences). Various amounts of GST-Nrg1 protein were incubated with 10 ng of α-^{32}P-labeled probe (~10,000 cpm) and 1 µg of poly(dI-dC) for 30 min at 30 °C in a reaction volume of 20 µl containing 20 mM HEPES (pH 7.6), 1 mM $MgCl_2$, 60 mM KCl, 12 % glycerol, 6 µg of bovine serine albumin, 10 µM $ZnCl_2$, and 1 mM dithiothreitol. Probe 578, which was added as a nonspecific competitor where indicated, was obtained by amplification from genomic DNA of the –578/–640 region of the *ENA1* promoter, using primers ena1_pr_5´_640 and ena1_pr_3´_578 (see table 7). The reaction samples were loaded onto 5 % non-denaturing polyacrylamide gels, prerun at 100 V for 30 minutes in a TBE electrophoresis buffer (22.5 mM Tris base, 22.5 mM boric acid, and 0.63 mM disodium EDTA adjusted to pH 8.0) and then electrophoresed at 150 V for 3 h in the same buffer. The non-denaturing gel mix was prepared using the TBE buffer by adding 150 µl of 30 % ammonium persulfate and 70 µl of TEMED (final

volumen of the mixture was 60 ml). After electrophoresis the gel was vacuum dried for 1 h at 80 ºC using a slab gel dryer (DrygelSr-Hoefer Scientific Instruments, San Francisco) and subjected to autoradiography.

10. Chromatin Immunoprecipitation Assay

The *NRG1-HA* strain used for the assay was obtained by transformation of the W303-1A wild type strain with a modification module that contains as selectable marker the *E. coli kan*^{r} gene. The C-terminal modification module was obtained by PCR using as a template the pFA6a-3HA-kanMX6 plasmid (Longtine *et al.*, 1998) and a pair of primers that contain *NRG1*-specific sequences. The forward primer (O5) was chosen to end just upstream of the stop codon, preserving the reading frame of the tag and the reverse primer (O6) to end just downstream of the stop codon (primers are listed in table 7). The resulting strain has the 3´ end of *NRG1* tagged with 3 hemagglutinin epitope (HA) sequences. To test the Nrg1-HA construct immunoblot analysis was used. Twenty to 40 µg of proteins from a *NRG1-HA* yeast extract were separated by SDS-12% polyacrylamide gel electrophoresis and transferred to enhanced chemiluminescence nitrocellulose membranes (Amersham Biosciences) by electroblotting, which were then incubated with an anti-HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase-conjugated protein A was used as secondary reactant. The complex was detected by the ECL detection system (Amersham Biosciences).

Chromatin immunoprecipitation assays were performed essentially as described previously (Alepuz *et al.*, 2001; Tomas-Cobos *et al.*, 2004) with the following modifications. Yeasts cells were grown in 100 ml of low pH (pH 6.2) YPD medium at 28°C to an A_{600} of 1.0. The cells from one-half (50 ml) of the culture were shifted from low to high pH (pH 8.0) YPD medium for 30 min. Each sample was treated with formaldehyde (final concentration 1 %) for 60 min at 20 °C, and 2.5 ml of 2.5 M glycine was added to stop the cross-linking reaction. Cells were harvested and disrupted by vortexing in the presence of glass beads, and the lysate was sonicated to generate DNA fragments that ranged in size from 300 to 500 bp. To immunoprecipitate HA-tagged proteins, the extract was incubated overnight at 4 °C with anti-HA antibodies, and the extract/antibody mixture was incubated for 3–4 h with protein A-Sepharose beads (Amersham Biosciences). Immunoprecipitates were washed 4-fold with 1 ml

each of lysis buffer (50 mM HEPES, pH 7.5, 250 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.1 % sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, and 1 µg/ml pepstatin A). The DNA was eluted with elution buffer (100 mM sodium bicarbonate and 1 % SDS). After reversal of the formaldehyde-induced cross-links, 1/5000 of input DNA and 1/45 of each immunoprecipitated DNA were used as templates for amplification by PCR (35 cycles), using primers O1 (sense), O2 (sense), O3 (sense), and O4 (antisense). Band intensities were quantified using the 1D Image Analysis Software (Kodak Digital Science).

11. Growth tests

The sensitivity of different yeast strains to alkaline pH and saline stress was assayed by drop test on YPD plates. In order to test alkaline stress sensitivity, plates containing 50 mM TAPS adjusted with KOH at different pH values were used. Yeast saturated cultures were diluted to an A_{660} of 0.05 and 3 µl were spotted. In some cases dilutions of the cultures to an A_{660} of 0.01 and 0.002 were also dotted consecutively. Plates were then incubated at 28 °C for various periods of time. Saline sensitivity tests were performed similarly, but in this case NaCl or LiCl was added to the YPD plates at different concentrations. Details about pH values, salt concentrations and time of incubation are given on the corresponding figure legends.

Sensitivity of yeast cells to high pH was also evaluated by growth test in liquid media that was performed in 96-well plates. Yeast cultures (250 µl) at an initial A_{660} of 0.01 were grown without shaking for 14–20 h at 28 °C in YPD containing 50 mM TAPS buffered at the indicated pH values. Growth was monitored in an iEMS Reader MF apparatus (Labsystems) at 620 nm.

IV. RESULTS & DISCUSSION

1 The transcriptional response of the yeast Na^+-ATPase *ENA1* gene to alkaline stress involves three main signaling pathways.

Whereas the response of *ENA1* gene to saline stress has been fairly well defined, its transcriptional induction by alkaline pH was not fully understood when this work started. In a previous functional mapping of *ENA1* promoter performed in our laboratory (Serrano *et al.*, 2002), two separated alkaline responsive regions were identified: ARR1 (nt -751 to -667) that contains the functional CDRE of *ENA1* promoter (see table 1 and figure 8) and exhibits a calcineurin-dependent response and ARR2 (nt -490 to -573) that contains two known regulatory elements, MIG and CRE, and whose response is calcineurin-independent.

In addition, it was shown that the alkaline response of both regions is decreased in cells lacking Rim101, giving evidence for involvement of the Rim101 pathway (Serrano *et al.*, 2002). The Rim101 transcription factor promotes induction of alkaline responsive genes indirectly, through the Nrg1 repressor, which binds directly consensus NRG sequences of specific gene promoters (Lamb *et al.*, 2003). However, whereas ARR1 contains a putative NRG-binding site at position -759 to -729, the ARR2 region does not contain any previously described NRG motif (see table 1 and figure 8). Additionally, a substantial part of the alkaline response of ARR2 is conserved in *rim101* mutants, suggesting the participation of additional components in the regulation of *ENA1* alkaline expression targeting this region.

In this work, our purpose was to identify the components of the alkaline response of *ENA1* that are calcium-independent. To this end we performed functional mapping of the calcineurin-independent ARR2 region, to identify putative regulatory elements located on this part of the promoter. The identification of these elements would be crucial for the characterization of additional pathway(s) and their respective components involved in the alkaline response of this gene.

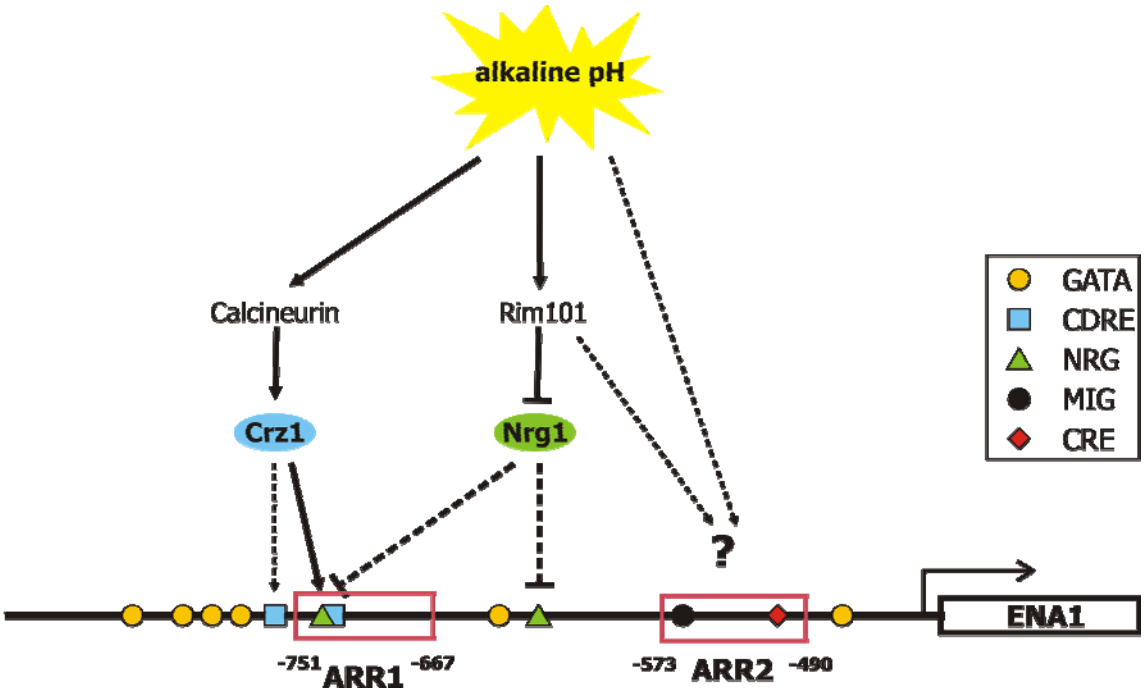

Figure 8. Schematic depiction of the known pathways (at the starting point of this work) involved in regulation of *ENA1* expression under alkaline stress conditions in *S. cerevisiae*. Only the major components of the signal transduction pathways are denoted. Discontinuous lines represent possible inputs to the promoter that were not documented at this moment. The regulatory DNA-motifs are represented to their relative position on the promoter (which is not drawn to scale) with basic shapes and are indicated in the inset. The two boxes represent the alkaline responsive regions ARR1 and ARR2 and the numbers refer to their relative position on *ENA1* promoter (nt from the starting ATG codon). See text for more details.

1.1 Functional mapping of the calcineurin independent response of the *ENA1* promoter.

To further characterize the ARR2 region of *ENA1* promoter, we constructed several reporter plasmids (pMRK213, pMRK525, pMRK505 and pMRK564) containing overlapping portions of the ARR2 region fused to the *lacZ* gene (see figure 9). In this manner expression levels of *ENA1* would be reflected in β-galactosidase activity, which can be easily detected and quantified.

As can be observed in Figure 9, full response to alkaline stress was retained in pMRK525 and pMRK505, containing the -573/-511 and the -573/-531 regions of the promoter, respectively. This part of ARR2 contains the MIG binding motif (nt -544 to -533), which is an upstream-repressing sequence relevant for the derepression of *ENA1* observed under glucose-limiting conditions (Alepuz *et al.*, 1997; Proft *et al.*, 1999). However, there was no previous evidence about a role of this element or its cognate

repressors Mig1 and Mig2 (see Figure 5) in the alkaline response of *ENA1*. In fact, it was evidenced that a *mig1* strain retains the ability to induce an *ENA1-lacZ* reporter when cells are shifted to alkaline pH media (Alepuz *et al.*, 1997). Remarkably, smaller overlapping fragments of the region represented by pMRK505 (-573/-531) encompassing sequences from nt -567 to -551, -557 to -531 and -547 to -531 (which corresponds to the MIG element) failed to give full response of ARR2, suggesting that more than one regulatory elements may exist within this part of the promoter (data not shown).

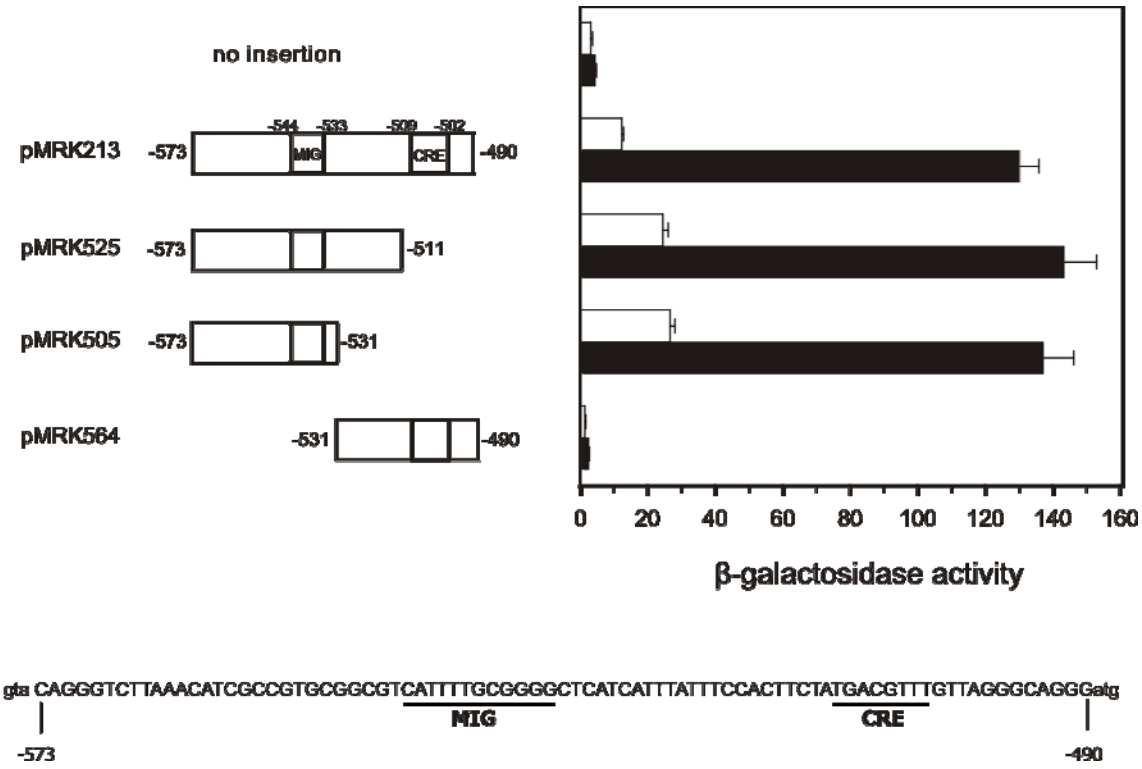

Figure 9. Functional mapping of the ARR2 region of the *ENA1* promoter in response to alkaline stress. Wild type DBY746 cells were transformed with the indicated constructs and cultures were grown for 60 min at pH 6.3 (*empty bars*) or 8.0 (*filled bars*). Cells were collected and β-galactosidase activity measured as described in *Materials & Methods*, section 5. Data are mean ± S.E. from at least six independent clones. The segments of the *ENA1* promoter included in each plasmid are denoted by *boxes*, and their relative position is indicated by *numbers* (nucleotide positions from the initial Met codon) flanking each box. Relevant known regulatory elements are depicted schematically and placed on the sequence of the ARR2 fragment at the *bottom* of the graphic. Details on the constructions of plasmids can be found in *Material & Methods*, section 4.

The region from nt -490 to nt -531 (pMRK564), which roughly represents the 3´-half of ARR2, was completely unable to sustain a transcriptional response to alkaline stress. This fragment of ARR2 contains the Sko1-binding motif denoted as CRE (-502 to -513), which is responsible for transcriptional regulation of *ENA1* by osmotic stress (Proft *et al.*, 1999). This result is in accordance with previous data, showing that the transcriptional response driven from the whole *ENA1* promoter (*ENA1-lacZ* reporter) was not altered in strains lacking Hog1 (Serrano *et al.*, 2002), the kinase that phosphorylates the Sko1 transcriptional repressor under osmotic stress conditions, thus inducing *ENA1* expression.

The above evidences suggest that the CRE element and, consequently, the High Osmolarity Glycerol (HOG) pathway do not participate in the regulation of *ENA1* by ambient pH. In contrast, a possible involvement of the MIG element cannot be excluded, as this is the unique previously described regulatory motif located in the promoter area represented by the pMRK505 construct (see Figure 9). The fact that mutation of Mig1 did not affect *ENA1* alkaline expression (Alepuz *et al.*, 1997), could be attributed to the function of the Mig2 repressor which exerts its repressor activity through the same MIG element (Lutfiyya and Johnston, 1996). In fact, it has been shown previously that both Mig1 and Mig2 repressors participate in the regulation of *ENA1* expression through its URS-MIG element under standard and glucose-limiting conditions, since the double *mig1 mig2* mutant showed a higher degree of derepression than either of the single mutants (Proft *et al.*, 1999).

Therefore, the calcineurin independent response of the *ENA1* promoter could be mapped to the fragment contained in pMRK505, spanning from nt -573 to -531. This region was named MCIR (for Minimum Calcineurin Independent Response) and, consequently, the pMRK505 reporter plasmid was used for further characterization of the response.

1.2 Effect of lack of the Snf1 protein kinase and the Mig1,2 transcriptional repressors.

Because of the presence of the MIG element in the MCIR region, we considered necessary to evaluate the possible role of the Mig1 and Mig2 repressors in the alkaline response of this region. Furthermore, we decided to test whether the protein kinase Snf1 participates in the alkaline response of the MCIR region, since this is the kinase that under glucose limiting conditions phosphorylates Mig1 leading to *ENA1* derepression (Alepuz *et al.*, 1997; Treitel *et al.*, 1998). To this end, we performed β-galactosidase assays in mutants lacking either or both repressors Mig1 and Mig2, the protein kinase Snf1, as well as in strains *mig1 snf1*, *mig2 snf1*, and *mig1 mig2 snf1*.

As shown in Figure 10, mutation of Mig1 or Mig2 barely affects the response to high pH, although slightly higher basal levels (that is, in the absence of stress) are observed. In contrast, mutation of both genes results in markedly increased basal levels and enhanced response to alkaline stress. Interestingly, mutation of the Snf1 protein kinase, which is known to inhibit Mig1, results in a dramatic decrease of this response, suggesting that this kinase plays an important role in controlling activation of the *ENA1* promoter by acting on the ARR2 region.

We also observed that the expression from the MCIR region in a strain lacking both Snf1 and Mig1 is identical to that found in the *snf1* mutant. In contrast, the response in the *snf1 mig2* mutant was almost as strong as that observed in *mig2*. Interestingly, mutation of *SNF1* markedly decreased the very high expression characteristic of the *mig1 mig2* strain.

Alkaline and saline sensitivity of cells lacking Mig repressors and/or the protein kinase Snf1 was also tested. As shown in Figure 11, strains *mig2* and *mig1 mig2* are tolerant to high salt and alkaline pH, whereas strain *mig1* does not differ from the wild type isogenic strain. Remarkably, a *mig2 snf1* mutant displays identical tolerance to NaCl and alkaline pH as observed in *mig2* cells.

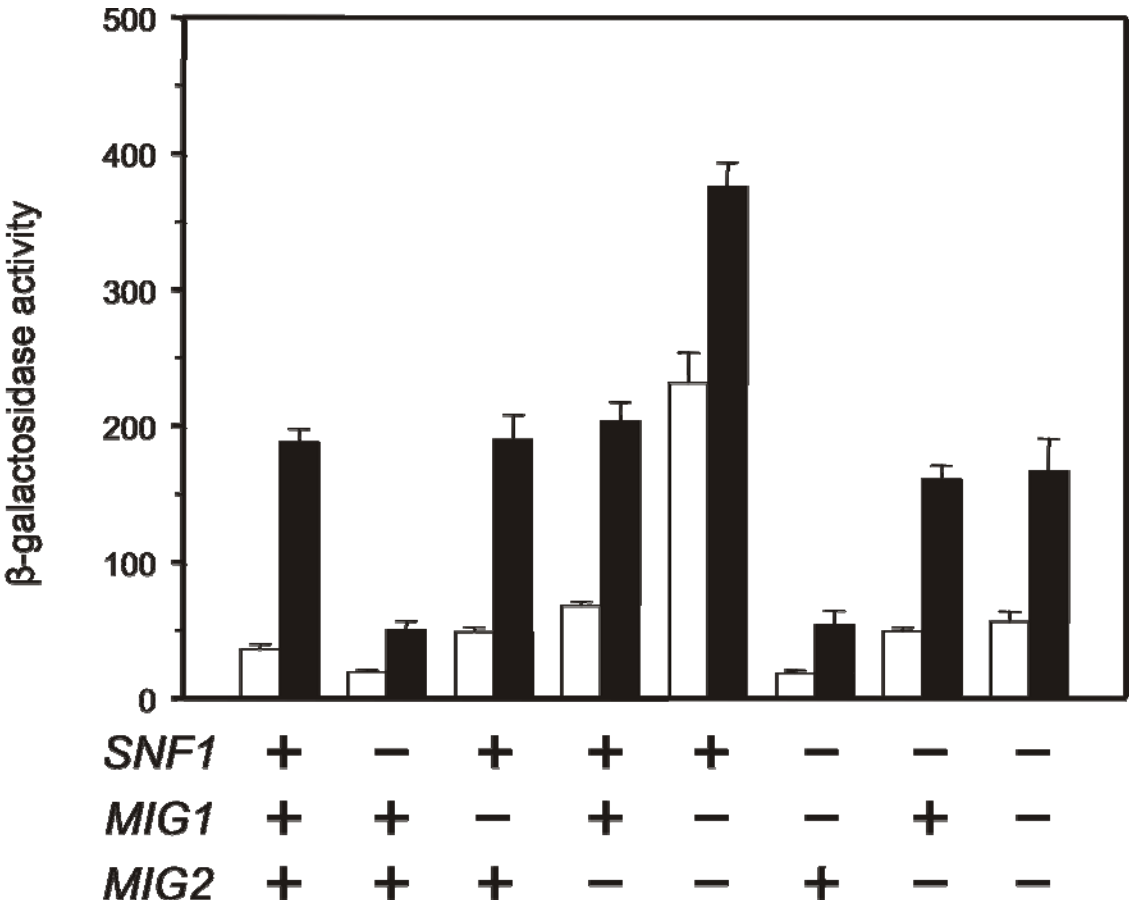

Figure 10. Evidence for Snf1- and Mig1/2-dependent activation of the *ENA1* promoter MCIR region under alkaline stress. The indicated strains were transformed with the reporter-plasmid pMRK505 and β-galactosidase activity was tested in cells grown at pH 6.3 (*empty bars*) or 8.0 (*filled bars*). Data are mean ± S.E. from at least six independent clones.

Our data suggest that both Mig1 and Mig2 act on the ARR2 region, as expression from the MCIR is stronger in the *mig1 mig2* strain than in each single mutant. This result is in accordance with previous data reported by Alepuz and collaborators (Alepuz *et al.*, 1997), evidencing that in a *mig1* strain, full alkaline response of *ENA1* is conserved. Apparently, whenever one of the two repressors is absent, its homologue is sufficient to regulate negatively transcription exerted through the MIG regulatory element and no alteration is observed in the expression levels of the ATPase. The redundancy of the repressor activity of Mig1 and Mig2 has been described previously, since a large number of glucose-repressed genes are regulated by both transcription factors (Lutfiyya *et al.*, 1998). Remarkably, the fact that a strain lacking both Mig1 and Mig2 proteins was still able to promote induction from the MCIR after alkaline shock suggests that additional regulatory elements must be present in this region.

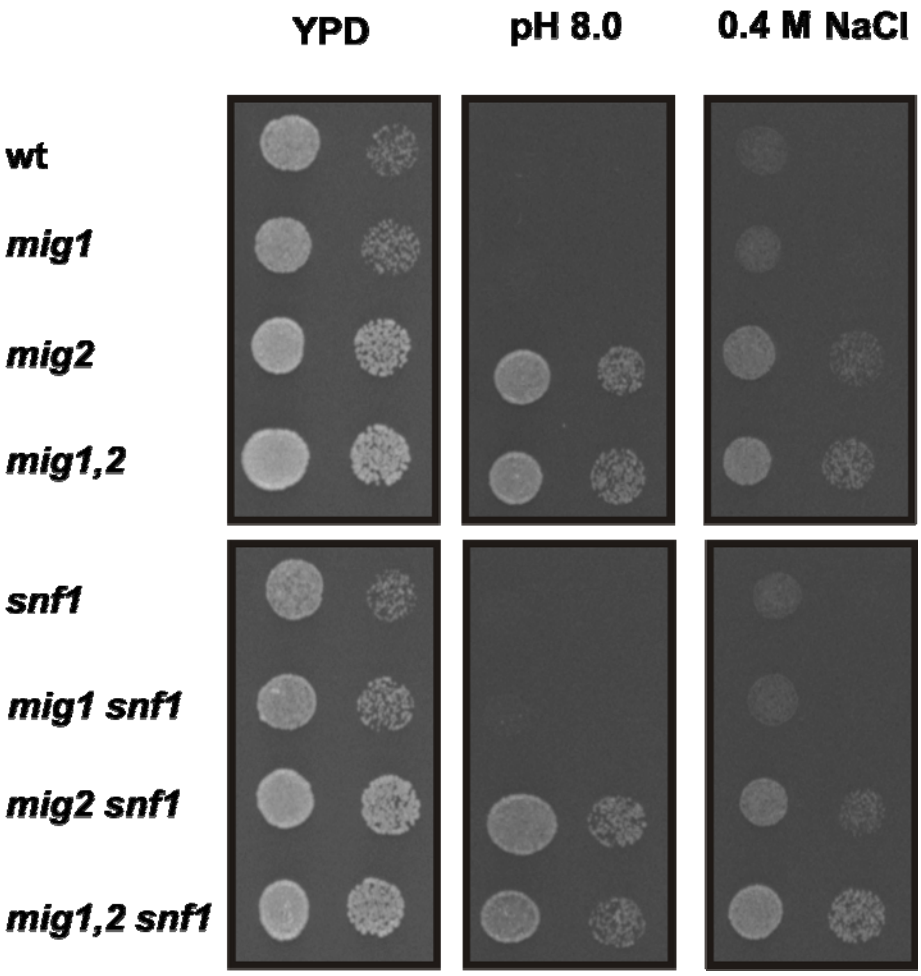

Figure 11. Effect of Mig1,2 and/or Snf1 mutations on alkaline and saline tolerance of yeast cells. The indicated strains were grown to saturation and two consecutive dilutions of each culture were made to an A_{660} of 0.05 and 0.005, respectively. Then 3 μl of each dilution were dotted onto YPD plates adjusted to pH 8.0 or containing 0.4 M NaCl. Growth was monitored after two days. For more details see *Materials & Methods*, section 11.

In addition, the above data indicate that Snf1 protein kinase mediates alkaline response of MCIR in a positive manner, which is in accordance with the early observation made by Alepuz *et al.* 1997 that a *snf1* mutant displays decreased *ENA1* expression at pH 8.5 when compared with a wild type strain. The Snf1 kinase is a key regulator of the yeast response to decreased glucose availability (see section 2.4 of the introduction and reference Sanz, 2003) and the activation of this kinase in response to alkaline stress suggests that exposure to high pH could mimic a situation of glucose starvation. This notion is supported by the observation that severe alkaline stress induces the expression of many genes that are also induced by low glucose availability (Viladevall *et al.*, 2004). Nevertheless, in contrast to what happens under glucose-

limiting conditions, where Snf1 exerts its activity through Mig1 (Treitel *et al.*, 1998; Ostling *et al.*, 1998), under alkaline pH conditions Snf1 seems to act through Mig2 (strain *mig1 snf1* shows expression of MCIR similar to strain *snf1*, whereas strain *mig2 snf1* shows expression levels similar to *mig2*). Furthermore, deletion of *MIG2* and not that of *MIG1* conferred yeast cells tolerance to alkaline pH and sodium ions (Figure 11), suggesting that Mig2 has a key role in alkaline and saline adaptation. However, the fact that *mig2* strains showed expression levels from the MCIR region similar to the wild type strain (Figure 10), would suggest that Mig2, apart from *ENA1,* probably would have additional targets that are important for alkaline and saline adaptation of yeast. Interestingly, mutation of *SNF1* markedly decreased the very high expression characteristic of the *mig1 mig2* strain under both basal and alkaline stress conditions (see expression levels of strain *mig1 mig2 snf1*).

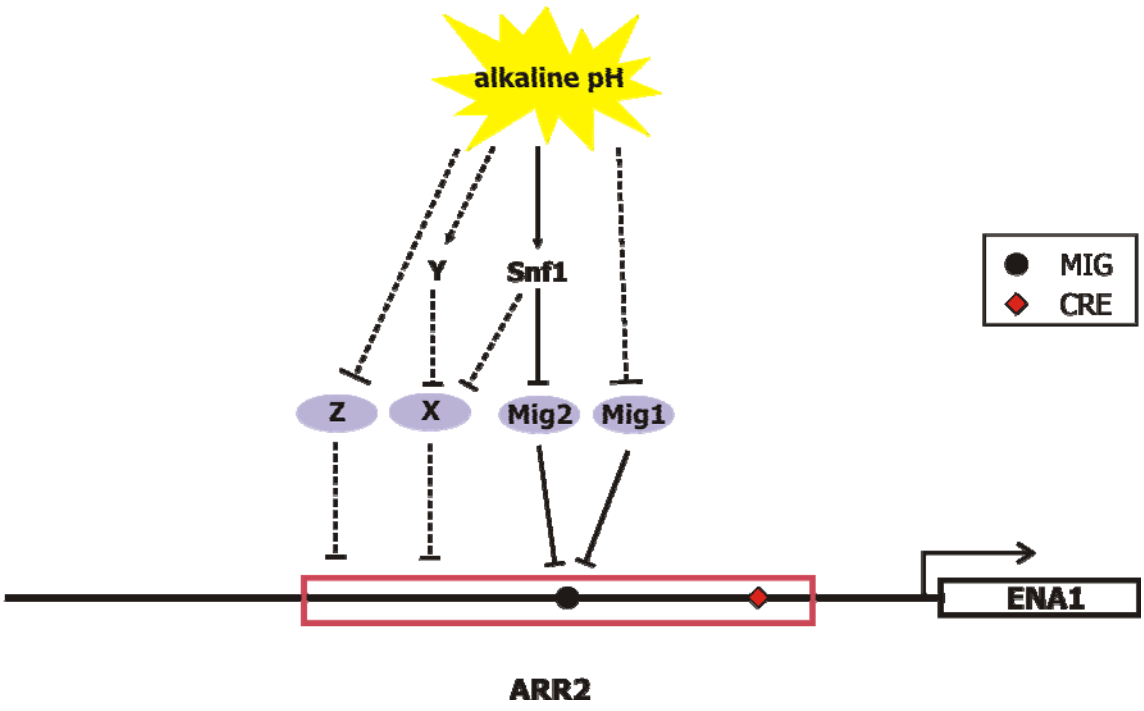

Figure 12. Schematic model for the calcineurin-independent pH signaling that mediates alkaline response of ARR2. Mig1 and Mig2 are deactivated in response to alkaline pH and relieve repression exerted through the MIG regulatory element. Deactivation of Mig2 is Snf1-dependent, whereas Mig1 is deactivated in a Snf1-independent manner. The presence of presumable additional repressor(s) is denoted with discontinuous lines, as well as the hypothesis that they may be under control of Snf1. The CRE regulatory element is irrelevant for the alkaline response of ARR2. Relative positions of the regulatory elements are not drawn to scale.

This could be explained if one assumes that Snf1 is controlling an additional negative regulator of *ENA1* expression located within the ARR2 region that in absence of the protein kinase represses constitutively transcription driven through this region. Figure 12 presents a working model that integrates the above observations.

1.3 The Rim101 pathway and the Nrg1 repressor regulate the alkaline induction from MCIR.

We had previously observed that mutation of *RIM101* decreased the response from the ARR2 region of the *ENA1* promoter (Serrano *et al.*, 2002). Therefore, it was reasonable to assume that the Rim101 pathway could provide the missing regulatory mechanisms mentioned above (see Figure 12).

To test this hypothesis, we constructed strains lacking the *RIM101* gene and/or *NRG1* and *NRG2* encoding its downstream targets (Lamb *et al.*, 2003; Rothfels *et al.*, 2005) and used them to evaluate expression of the reporter plasmid pMRK505. As observed in Figure 12, cells lacking *RIM101* presented a decreased alkaline response. However, this decrease was not as strong as in the case of *snf1* cells. The absence of Nrg1 resulted in increased basal expression and response to high pH, whereas mutation of *NRG2* had little effect and the expression level in the double mutant was only slightly higher than in the *nrg1* strain. These results were indicative of a repressor role of the Nrg1 transcription factor in the alkaline response from the MCIR region. We observed that mutation of *NRG1* strongly increases the basal levels and alkaline response from plasmid pMRK505 in the *rim101* mutant, whereas mutation of *NRG2* has almost no effect. Therefore, lack of Nrg1, but not Nrg2, abolishes the decrease of high pH response from the MCIR region observed in *rim101* cells.

Nrg1 and Nrg2 are also known to interact physically with Snf1 (Vyas *et al.*, 2001) and to regulate negatively transcription of various Snf1-dependent genes such as *GAL, SUC2*, *STA1* and *FLO11,* which like *ENA1* are repressed under non-limiting glucose conditions (Park *et al.*, 1999; Zhou *et al.*, 2001; Kuchin *et al.*, 2002). To investigate any possible epistatic relationship between the *nrg1,2* and *snf1* mutations, we assayed expression from pMRK505 in cells deficient in all possible combinations of

these genes. As shown in Figure 13, mutation of *NRG1* drastically counteracts the effect of the *snf1* mutation, whereas lack of Nrg2 has no effect.

Taken together, these results suggested that both Snf1 and Rim101 may participate in regulation of alkaline response of ARR2 by controlling the repressor effect of Nrg1, being the role of Nrg2 virtually negligible.

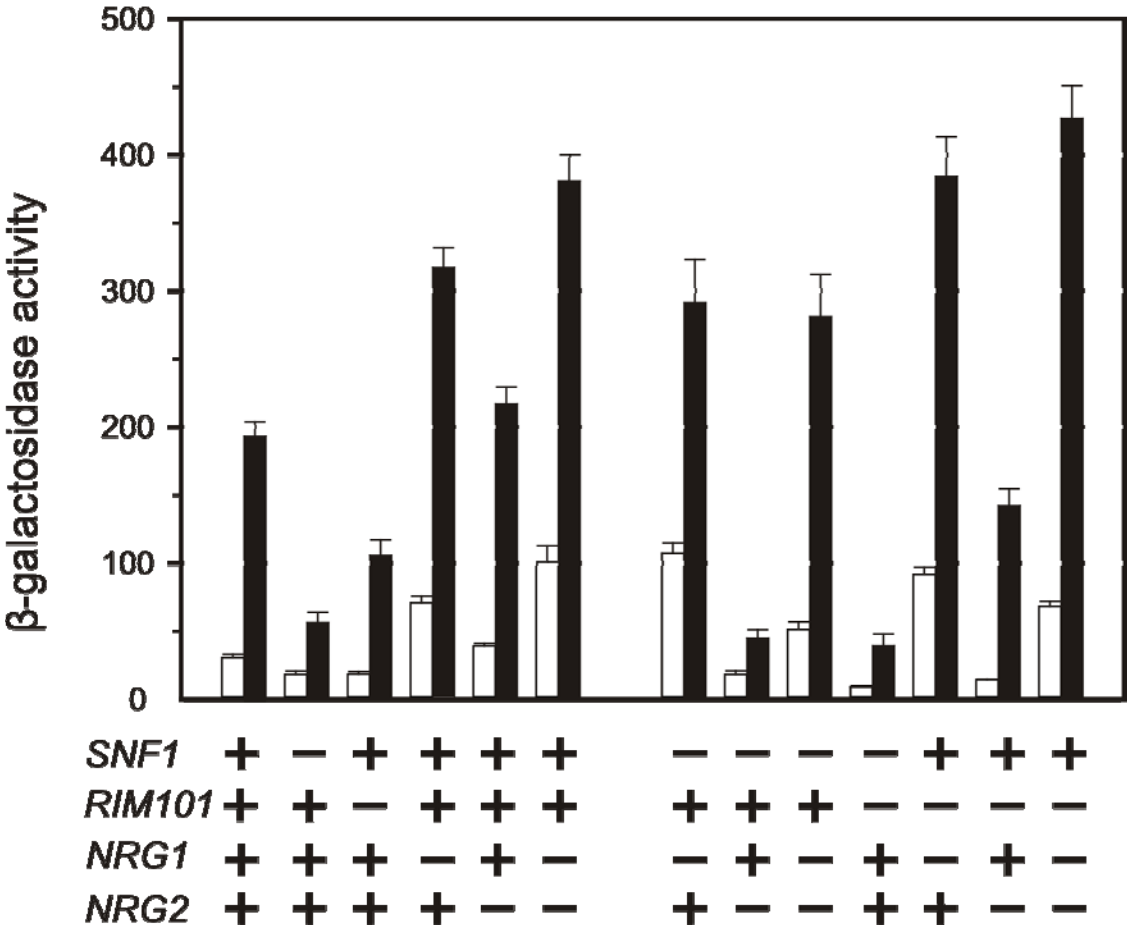

Figure 13. Both Snf1 and Rim101 pathways participate in the alkaline response of the MCIR region by regulating the activity of Nrg repressors. The indicated strains were transformed with the plasmid pMRK505 and cells processed as described in the legend of the figure 10. Data are mean ± S.E. from at least six independent clones.

The data collected so far suggested that expression from the MCIR region in response to alkaline pH is mostly controlled through the release from the negative effect of the Mig1,2 and Nrg1,2 repressors. If so, one would expect that cells lacking all four repressors, Nrg1,2 and Mig1,2, would show a very high basal activity from the pMRK505 reporter and, in addition, further increase induced by alkaline shock would be either very small or null.

In agreement with this hypothesis, in a quadruple *mig1,2 nrg1,2* mutant (see Figure 14), expression from the MCIR gives rises to higher basal levels than in *mig1,2* or *nrg1,2* cells and the activity of the reporter after alkaline stress increases only marginally (1.3-fold, in comparison with 8.5-fold in the wild type strain). It is worth noting that lack of Mig1,2 results in basal expression levels from pMRK505 that largely exceeds those observed in *nrg1,2* cells, suggesting that the Mig1,2 repressors exert a stronger repressor control on this promoter region.

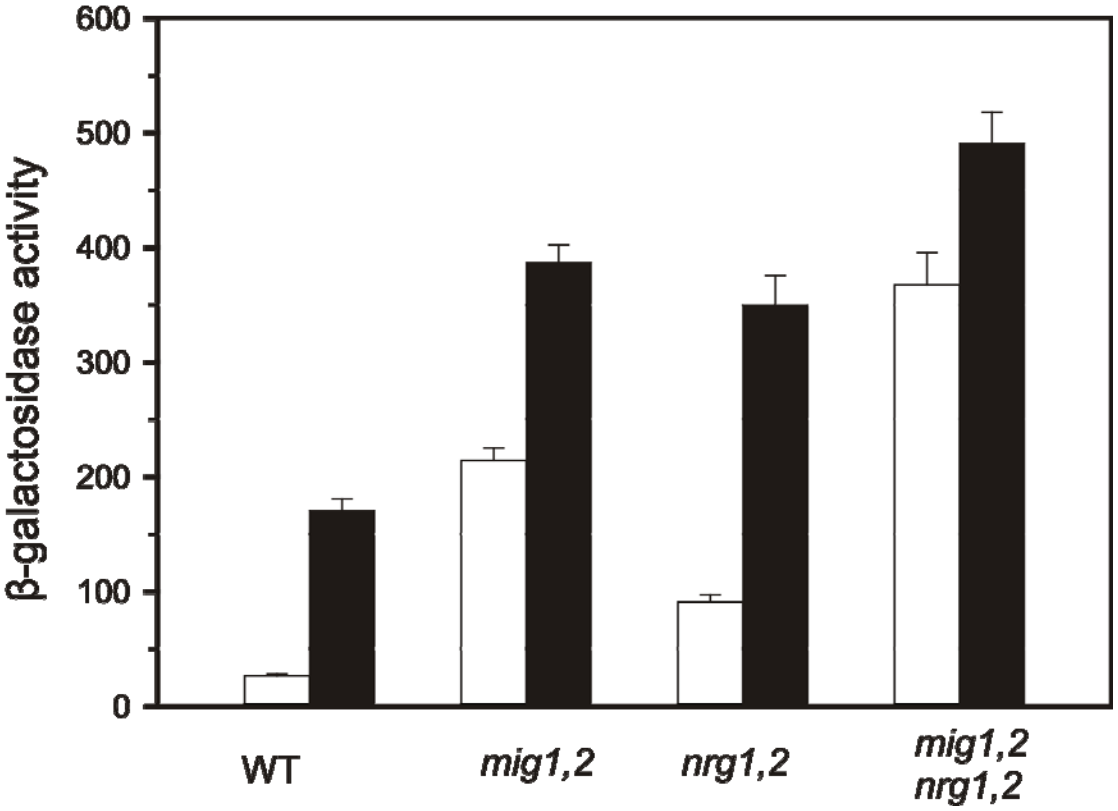

Figure 14. Response of the MCIR region to alkaline stress in cells lacking repressors Mig and Nrg. The indicated strains were transformed with plasmid pMRK505 and cells were processed as described in the legend of figure 10. Data are mean ± S.E. from at least six independent clones.

The above results suggested that the Rim101 pathway indeed participates in the alkaline response of ARR2, fact that would explain the previous observation that in *rim101* mutants expression levels from this region are lower that those observed in a wild type background (Serrano *et al.*, 2002). In addition, there is evidence that this activity is exerted mainly through the Nrg1 repressor, since mutation of *NRG2* in a *rim101* background has almost no effect in expression levels of MCIR.

Our initial hypothesis for additional components that regulate alkaline expression from ARR2 under the control of Snf1 protein kinase is reinforced by our observation that the triple *nrg1,2 snf1* mutant shows stronger expression from MCIR than that of a *snf1* strain. Furthermore, comparison of the expression levels observed in mutants *nrg1 snf1* and *nrg2 snf1* supports the notion that Nrg1 has a much more relevant role than its homologue in the regulation of alkaline response from this region.

The Nrg1 and Nrg2 repressors have very similar DNA-binding domains and exhibit a considerable degree of functional overlap with respect to their roles in the regulation of glucose-repressed genes (Vyas *et al.*, 2001; Kuchin *et al.*, 2002; Vyas *et al.*, 2005). However, there are previous studies evidencing that Nrg1 and Nrg2 are distinct, as far as their regulation and function is concerned, in response to carbon source availability. Similarly to our observations about a more important role of Nrg1 in the calcineurin-independent alkaline response of *ENA1*, the *nrg1* mutation relieves glucose repression of the *STA2* gene (encoding the glucoamylase enzyme) more effectively than *nrg2* and also enhances derepression under glucose limiting conditions to a greater extent (Vyas *et al.*, 2003; Berkey *et al.*, 2004). Such differences in the regulatory activity of the two repressors could be attributed to the differences that Nrg1 and Nrg2 present in the sequence outside their DNA-binding domains, which may allow Nrg1 to interact more efficiently with regulatory proteins, such as Snf1 or Rim101.

1.4 Identification of the Nrg1-binding site within the ARR2 region.

In contrast to the evidence described above, canonical sequences previously defined for Nrg1 (Park *et al.*, 1999; Lamb *et al.*, 2003) were not identifiable within the ARR2 region (see Figure 8). Therefore, we considered necessary to directly test whether or not Nrg1 was able to interact with this region and the possible functional relevance of such an interaction.

In order to isolate sufficient amount of the Nrg1 protein needed for our experiments, we decided to express recombinant GST-Nrg1 in bacterial cells. To this end the sequence corresponding to the *NRG1* coding region was subcloned *in frame* with glutathione S-transferase (GST) in the pGEX-4T1 vector. The construct was then introduced into *E. coli* BL21 (DE3) cells and expression of the recombinant protein was induced with IPTG for 3 hours at 37 ºC, as described in *Materials & Methods*. Crude lysates of both induced and non-induced samples were analyzed by SDS-PAGE. As observed in Figure 15 A the *E. coli* cells induced with IPTG exhibited high levels of expression of the fusion protein GST-Nrg1 of the predicted molecular weight (53 kDa).

Purification of the recombinant protein was performed by binding the cell lysate to Glutathione Sepharose 4B beads, then washing to remove unbound or non-specifically bound proteins and finally eluting with the appropriate glutathione buffer (see *Materials & Methods* for further details). Three consecutive elution fractions were obtained and then were analyzed by SDS-PAGE to detect purified recombinant protein. The amount of the purified GST-Nrg1 in each fraction was evaluated by comparing with equal volumes of BSA standard solutions of different concentration loaded on the same gel (see Figure 15 B).

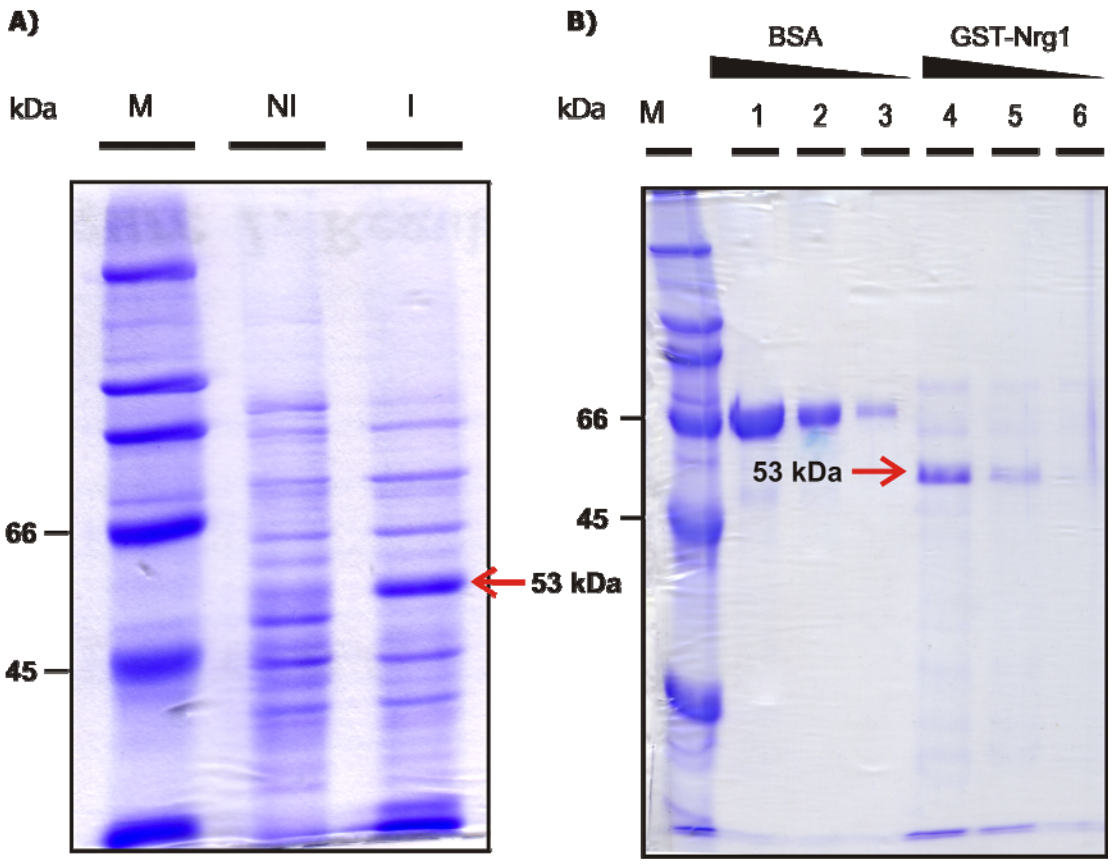

Figure 15. A) SDS-PAGE analysis of crude lysate from *E. coli* cells expressing recombinant protein GST-Nrg1. Aliquots of 15 μl of crude lysate from *non-induced (NI)* and *induced (I) E. coli* BL21 (DE3) cells carrying the plasmid pGEX-4T1-Nrg1 were loaded on a 10 % polyacrylamide gel as indicated and electrophoresed; 5 μl of protein molecular weight standard (Sigma, SDS-6H) was also loaded on lane *M*. **B**) **SDS-PAGE of the purified GST-Nrg1 recombinant protein.** To quantify purified GST-Nrg1 15 μl of BSA standard solutions (0.25, 0.1, and 0.05 μg /μl) were loaded on a 10 % polyacrylamide gel and run in parallel with 15 μl of the three consecutive elution fractions of GST-Nrg1. Lane *M,* molecular mass standard, lanes *1-3,* BSA standard solutions, lanes *4-6*, first, second and third elution fraction of purified GST-Nrg1, respectively. After electrophoresis the gels were stained with Coomassie Brilliant Blue R-250 (Amresco) and dried onto cellophane membrane backing (Sigma-Aldrich). Red arrows indicate the expressed GST-Nrg1 recombinant protein (53 kDa). Fore more details about the expression of GST-Nrg1 see *Materials & Methods*, section 8.

The purified recombinant protein from the first elution was used to test direct binding of Nrg1 to the ARR2 region *in vitro* by Electrophoretic Mobility Shift Assay. As can be observed in Figure 16 A, the presence of GST-Nrg1 in the mixture is able to shift the electrophoretic mobility of a radioactively labeled ARR2 fragment and this fragment can be competitively displaced by unlabeled ARR2. Thus binding of the Nrg1 protein to the ARR2 does occur, despite that no consensus Nrg1-binding motif located within this region of the *ENA1* promoter was previously described.

To further map the interaction region, we generated additional fragments spanning either fully outside (5') of the ARR2 region (fragment 578 that served as a negative control) or overlapping its 5' end (fragments 541, 551, and 561) and tested their ability to bind Nrg1. As expected the 578 fragment (nt -640 to -578) did not bind at all Nrg1 (see Figure 16 B), whereas fragment 561, the smallest of the three ARR2 portions tested, still did. This suggests that the Nrg1 binding region is located at the 5' end of ARR2 and can be mapped between nucleotides –561/–574.

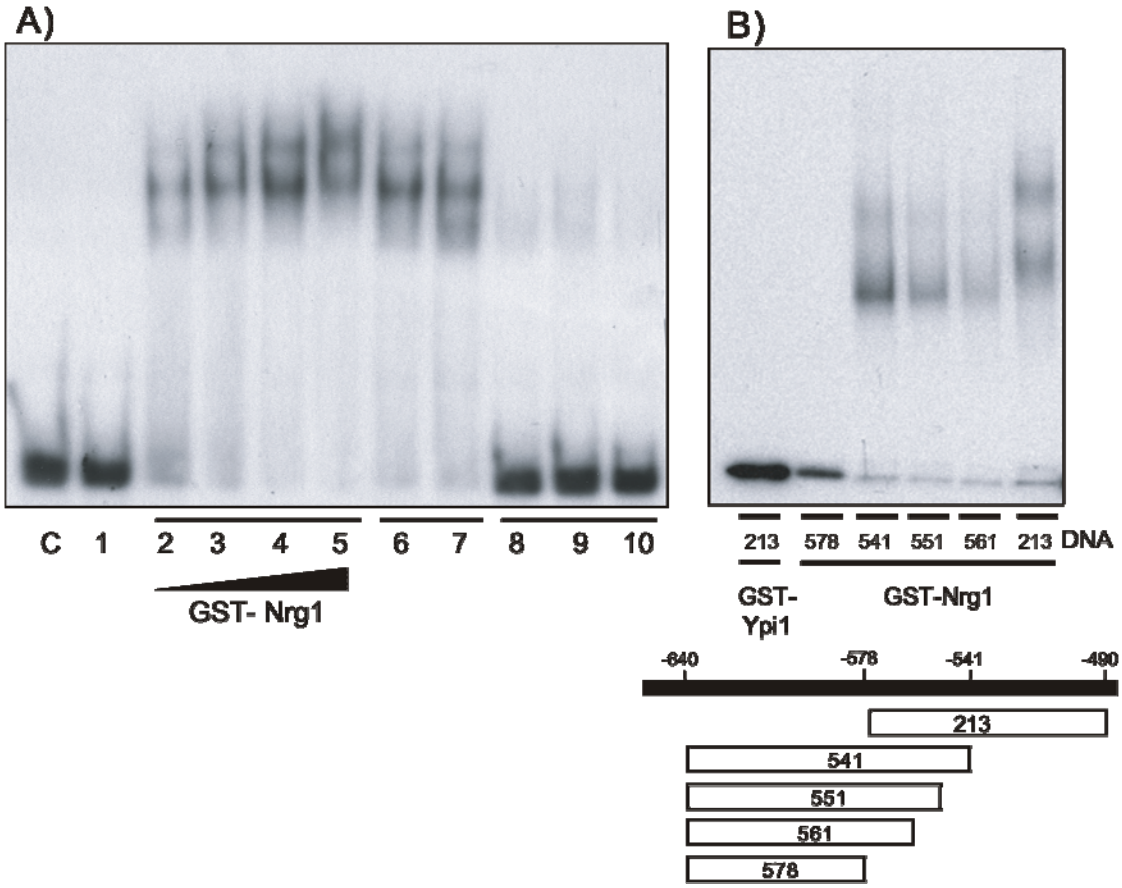

Figure 16. Nrg1 binds *in vitro* to the ARR2 region of the *ENA1* promoter. A) Electrophoretic mobility shift assays were performed with purified protein GST-Nrg1 and ^{32}P-labeled probe 213, which corresponds to the ARR2 region. *C*, no added protein; *1*, GST (0.05 µg); *2–5*, increasing amounts of GST-Nrg1 (0.025, 0.05, 0.1, and 0.2 µg); *6* and *7*, 0.05 µg of GST-Nrg1 plus increasing amount of nonspecific competitor (16- and 32-fold molar excess of probe 578); *8–10*, 0.05 µg of GST-Nrg1 plus unlabeled specific competitor (16-, 32-, and 64-fold molar excess of probe 213) **B)** Electrophoretic mobility shift assays were performed with 0.15 µg of GST-Nrg1 and different ^{32}P-labeled fragments that include segments of the ARR2 region as depicted. GST-Ypi1 (0.15 µg) was included as negative binding control. The labeled 213 probe plus 0.15 µg of GST-Nrg1 is included as a positive control. Fragments of the *ENA1* promoter included in each probe are denoted by *boxes* and named (except for 213) after the corresponding relative position of their 3' extreme (nucleotide positions from the initiating Met codon).

To test the biological relevance of the Nrg1-binding to the ARR2 region chromatin immunoprecipitation (ChIP) experiments were carried out. For this purpose, the native *NRG1* open reading frame of the wild type strain W303-1A was replaced with a HA-tagged version and a ChIP assay was performed under both basal and alkaline pH conditions.

As shown in Figure 17, fragments surrounding the ARR2 region were specifically amplified, indicating that binding was occurring *in vivo* under standard culture conditions (see lanes 4-6). More importantly, when the same experiment was performed in cells subjected to alkaline stress, the amount of amplified material was substantially reduced (compare lanes 4-6 to lanes 7-9). It is worth noting that this does not appear to be a general, non-specific effect, since in a parallel experiment where HA-tagged Med8 (a protein which binds to the *HXK2* gene promoter) was used, binding was not altered in cells exposed to high pH. These results provide evidence about Nrg1 binding *in vivo* to the ARR2 region and more importantly that this binding is abrogated when sudden environmental alkalinization occurs.

The above results provide evidence about the existence of an Nrg1-binding site located close to the 5'-end of the ARR2 region, and more specifically between the nucleotides -573 and -561 (see Figure 16 B). Interestingly, this small region does contain in the non-coding strand a AGACCCT sequence (nt -572/-566) that is somewhat different from the initially proposed binding motifs CCCCT and CCCTC recognized by the Nrg1 repressor (Park *et al.*, 1999), but that closely matches the consensus sequence ggACCCT, identified in more recent studies as a very likely Nrg1-binding element (Harbison *et al.*, 2004; Macisaac *et al.*, 2006). Therefore, we propose that this sequence is responsible for the control exerted by the Rim101 pathway through the Nrg1 repressor on the ARR2 region of *ENA1*. Moreover, it is demonstrated that Nrg1 binds *in vivo* to the ARR2 region and, more importantly, that this interaction is substantially decreased upon exposure to alkaline pH stress, thus providing additional evidence about a relevant physiological role of Nrg1 to the alkaline transcriptional response of this region.

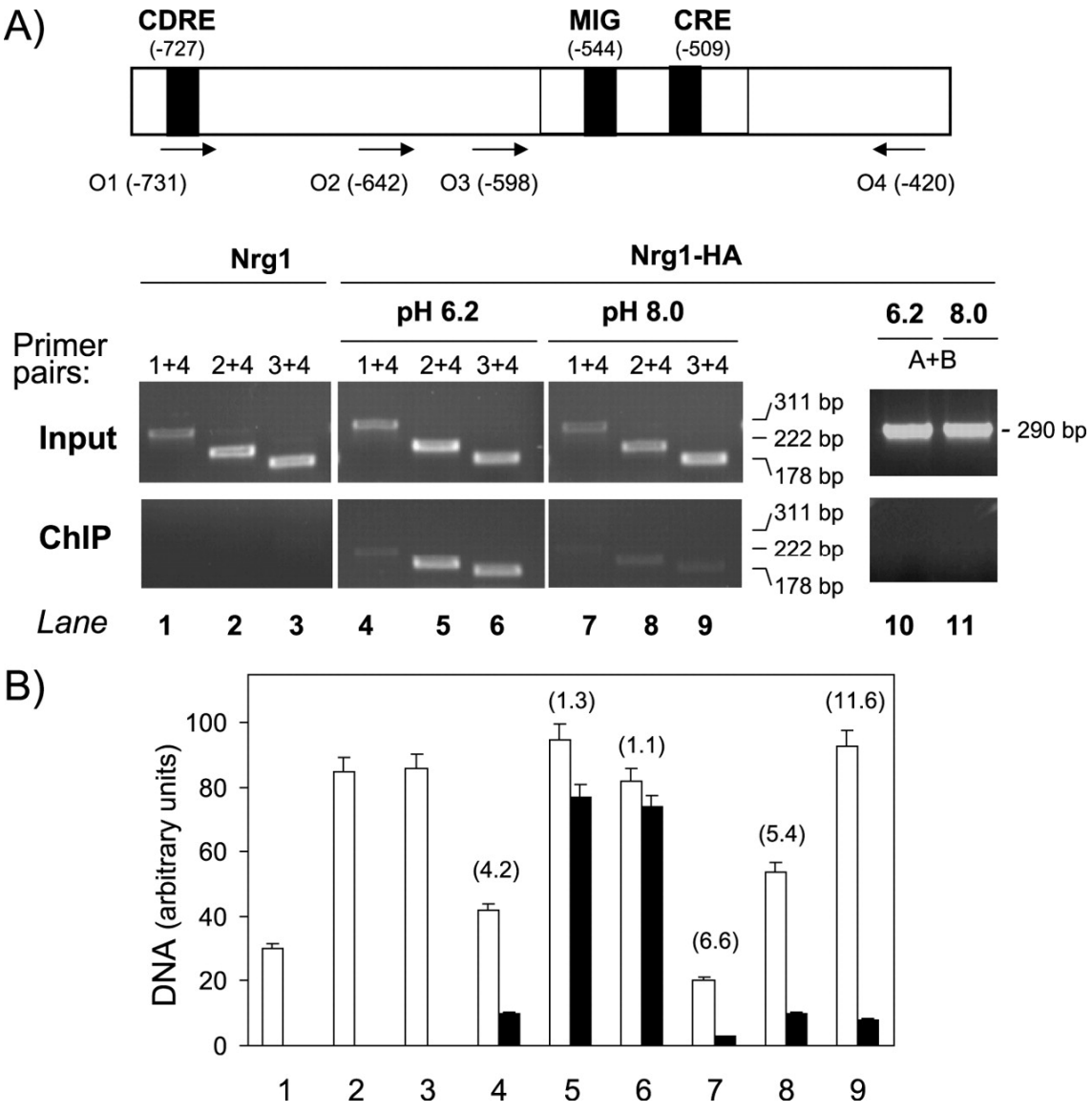

Figure 17. ***In vivo*** **binding of Nrg1 to the ARR2 region of the** ***ENA1*** **promoter. A)** Schematic depiction of the region of the *ENA1* promoter. The *dashed box* denotes the ARR2 region. Wild type strain W303-1A containing the native (Nrg1) or the HA-tagged version of Nrg1 (Nrg1-HA) was grown on YPD medium and switched at the indicated pH values for 30 min before cells were collected. Fragmented DNA was obtained and immunoprecipitated as described under "*Materials & Methods*" and amplified by PCR using the indicated combination of oligonucleotides. *Lanes 10* and *11* show the amplification of a region of the *HXK2* promoter with oligonucleotides A and B that was used as a negative control. This region is devoid of Nrg1 binding sequences. The amplified DNA fragments were resolved by agarose electrophoresis. Migration of standard markers is indicated on the *right.* **B)** The intensity of the amplified DNA band from the input sample (*empty bars*) and the chromatin immunoprecipitate (*filled bars*) obtained for each condition was integrated and the ratio calculated when possible (*numbers in parentheses*). Data represent the mean ± S.D. of three experiments in duplicate.

1.5 Induction of the *ENA1* promoter by ambient alkalinization involves three main signaling pathways.

The results obtained so far indicated that the calcineurin-independent induction of *ENA1* expression in response to high pH is mediated by two pathways: the Snf1 pathway, which exerts its regulatory activity through the repressors Mig2 and Nrg1 and the Rim101 pathway, which exerts control also through the repressor Nrg1. Therefore, calcineurin, Snf1, and Rim101 would define the main elements governing the transcriptional response of the *ENA1* gene to ambient alkalinization. In this case, one would expect that blockage of these three pathways would result in the incapacity of the *ENA1* promoter to respond to alkaline stress. To verify this hypothesis, we combined the *snf1* and *rim101* mutations and tested expression from the reporter plasmid pKC201, which contains the entire *ENA1* promoter, in the presence of the calcineurin inhibitor FK506. As shown in Figure 18 systematic blockage of each component results in additive loss of transcriptional response, whereas loss of all three elements results in a fully insensitive promoter. Therefore, calcineurin, Snf1, and Rim101 define three pathways that account for virtually every positive input to the *ENA1* promoter in response to extracellular alkalinization. However, the relevance of the different pathways in the control of *ENA1* does not seem to be equal, as deduced from the loss of induction levels observed in each strain. Remarkably, under these specific experimental conditions (pH 8), the Snf1 pathway accounts for the most important part of the induction since its inhibition results in loss of more than the fifty per cent of the alkaline response of *ENA1* (see Figure 18). Inhibition of the calcineurin pathway also results in a substantial decrease of *ENA1* expression, although this is somewhat lesser than that observed in cells lacking Snf1. Finally, the Rim101 pathway participates in the alkaline induction of this gene, but its contribution is minor in comparison with Snf1 and calcineurin. Therefore, as far as the importance of the three pathways is concerned, a sequence Snf1 > calcineurin > Rim101 can be established.

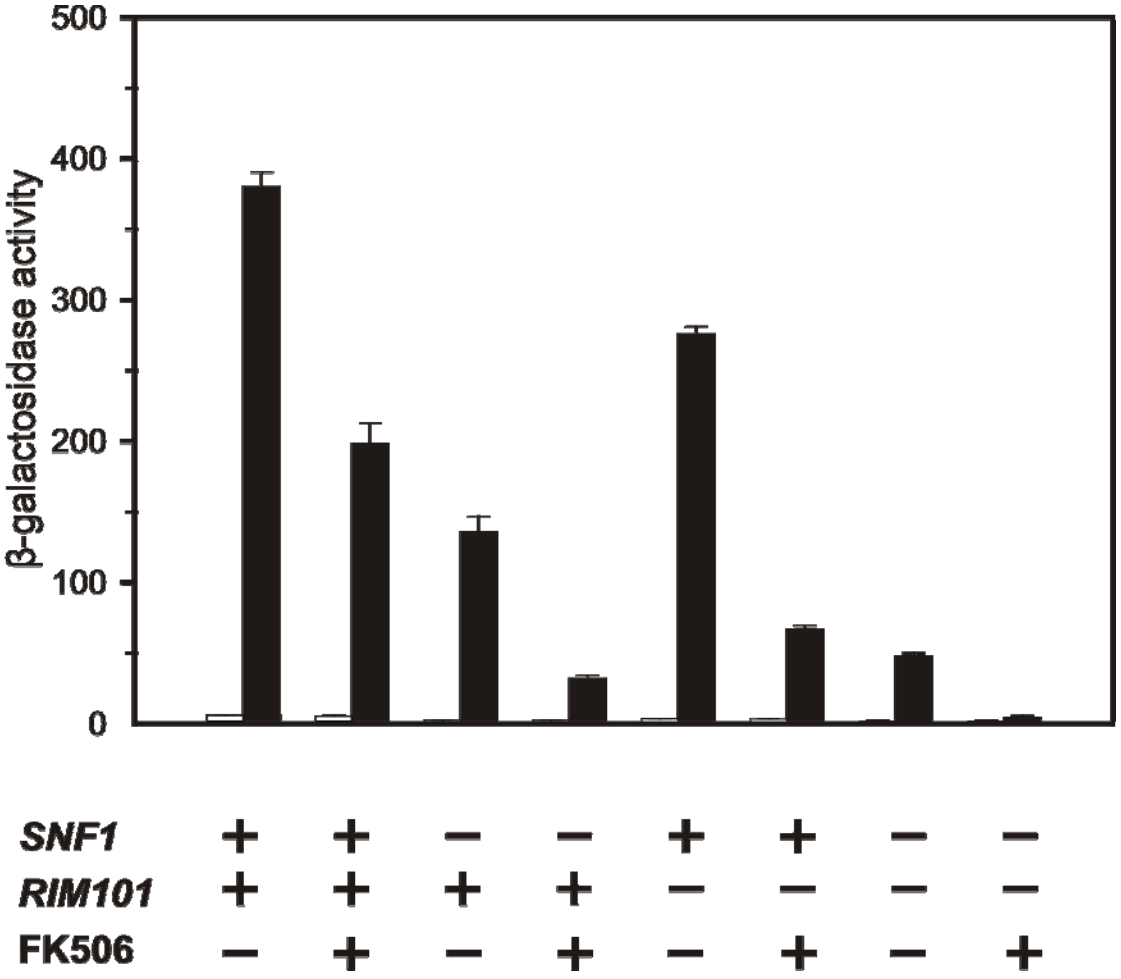

Figure 18. Activation of three signaling pathways, calcineurin, Snf1, and Rim101, accounts for full response of the *ENA1* promoter to alkaline stress. The indicated strains were transformed with the pKC201 plasmid that contains the entire promoter of *ENA1* and cells were subjected to alkaline stress in the presence or absence of FK506 (1.5 μg ml^{-1}). *Empty bars* denote β-galactosidase activity under non-stressing conditions (pH 6.3) and *filled bars* under stressing conditions (pH 8.0). Data are mean ± S.E. from at least six independent clones.

These results were substantiated by direct analysis of the *ENA1* mRNA levels by real-time reverse transcriptase-PCR in different mutants after exposure to alkaline stress. As can be observed in Figure 19, blockage of the calcineurin pathway with the drug FK506 in a strain lacking both Snf1 and Rim101 fully abolishes the high pH-induced expression of the *ENA1* gene (last column). The other strains also exhibited a pattern of expression levels of *ENA1* similar to that obtained by β–galactosidase assays (compare Figure 18 with 19). In contrast, induction of the *PHO84* gene whose expression under alkaline stress is mediated by the Pho2 and Pho4 transcription factors (Serrano *et al.*, 2002) was unaltered in a *rim101 snf1* strain grown in the presence of the calcineurin inhibitor FK506 (not shown).

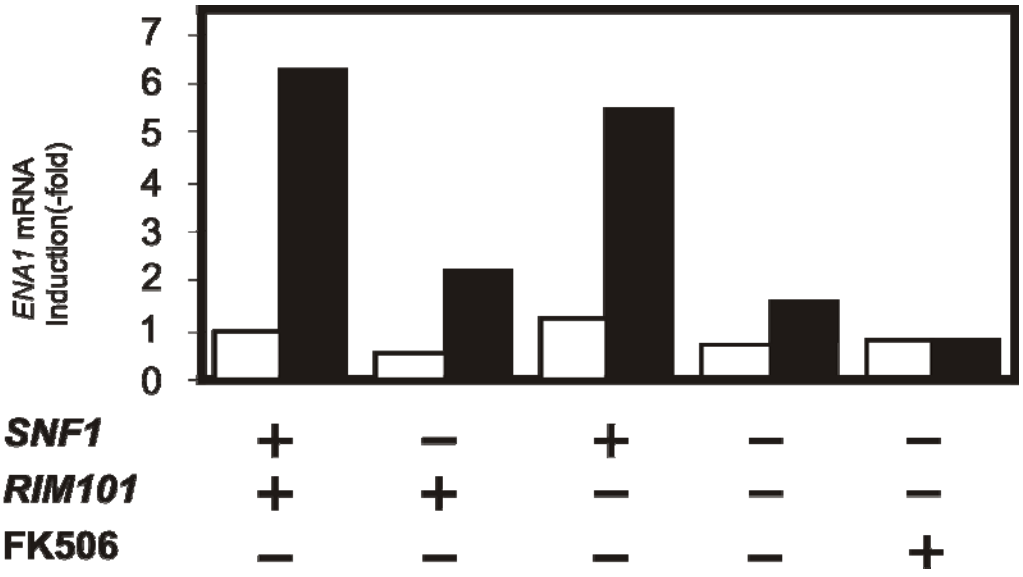

Figure 19. Evaluation by RT-PCR of the *ENA1* mRNA levels after alkaline stress. The induction of the *ENA1* gene was evaluated by quantitative real-time PCR in the indicated genetic backgrounds. The expression level of the non-induced (pH 6.3, *empty bars*) wild type strain was considered as the unit. *Filled bars* denote cells subjected to alkaline stress (pH 8.0) for 10 min. Data represent the mean of two independent determinations.

As mentioned in the introduction, *ENA1* expression can be strongly induced by saline as well as by alkaline stress. Moreover, the calcineurin pathway was proven to be quite important for transcriptional induction of this ATPase under both environmental conditions (Mendoza *et al.*, 1994; Hirata *et al.*, 1995; Marquez *et al.*, 1996; Serrano *et al.*, 2002; Viladevall *et al.*, 2004). In addition, both Rim101 and Snf1 seem to be important for saline tolerance of yeast cells, as mutants lacking either of these protein grow poorly at high salt, whereas Rim101 is also required for growth under alkaline pH conditions (Alepuz *et al.*, 1997; Lamb *et al.*, 2001; Portillo *et al.*, 2005). Thus we decided to compare the relative relevance of the pathways studied in this work in response to both types of stress. To this end, cells were exposed to pH 7.9 or 0.4 M NaCl, which are treatments that provoke an *ENA1* response of similar potency and timing. Under these conditions (see Figure 20) the induction of *ENA1* by saline stress and high pH was similarly decreased in a calcineurin mutant. In contrast, lack of Snf1 had a strong effect on the alkali-induced response but produced only a moderate decrease in expression in cells exposed to NaCl. Mutation of *RIM101* barely affected the response provoked by sodium chloride.

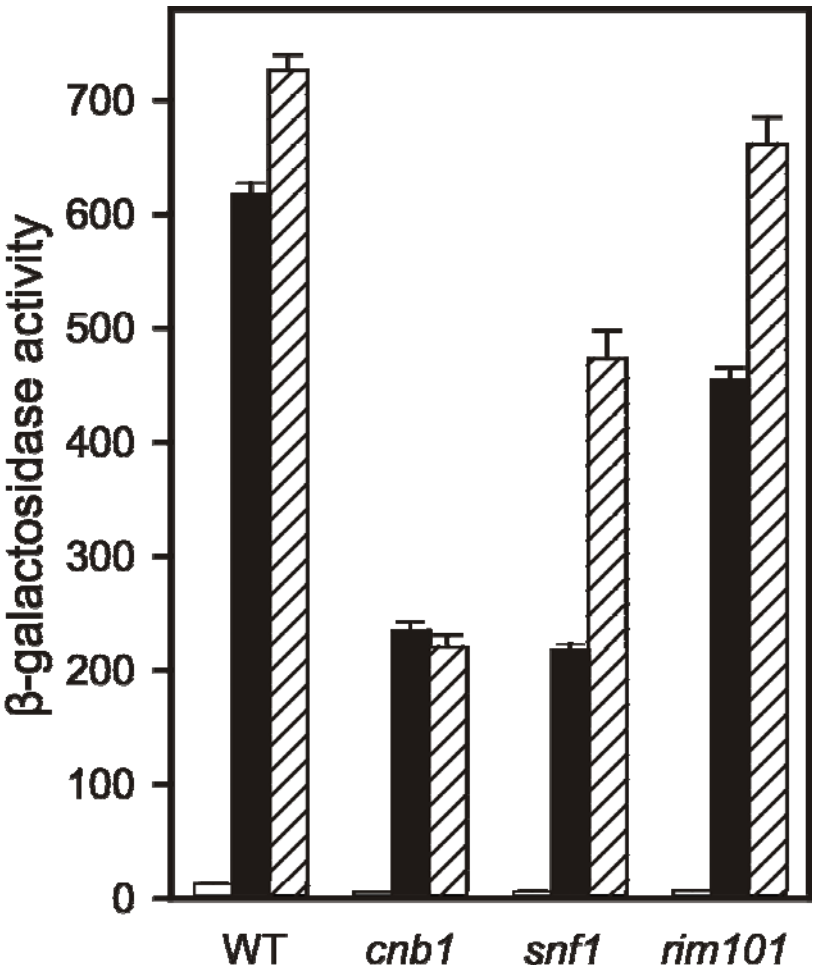

Figure 20. Alkaline and saline expression of *ENA1* in strains lacking the calcineurin subunit Cnb1, Snf1 or Rim101.

The indicated strains were transformed with the pKC201 plasmid and subjected to alkaline (pH 7.9, *filled bars*) or saline stress (0.4 M NaCl, *hatched bars*) for 60 min before cells were collected and β-galactosidase activity was measured. Data represent the mean ± S.E. of six experiments.

We also compared how blockage of the three signaling pathways affects cell tolerance to high pH. As shown in Figure 21, chemical inhibition of the calcineurin pathway with FK506 and mutation of *SNF1* decrease similarly the tolerance to high pH, whereas mutation of *RIM101* has a lesser effect. In all cases, the phenotype observed was less intense than that produced by the absence of the ATPase gene. However, cells lacking both Snf1 and Rim101 grown in the presence of the calcineurin inhibitor FK506 were clearly more sensitive to high pH than *ena1–4* cells.

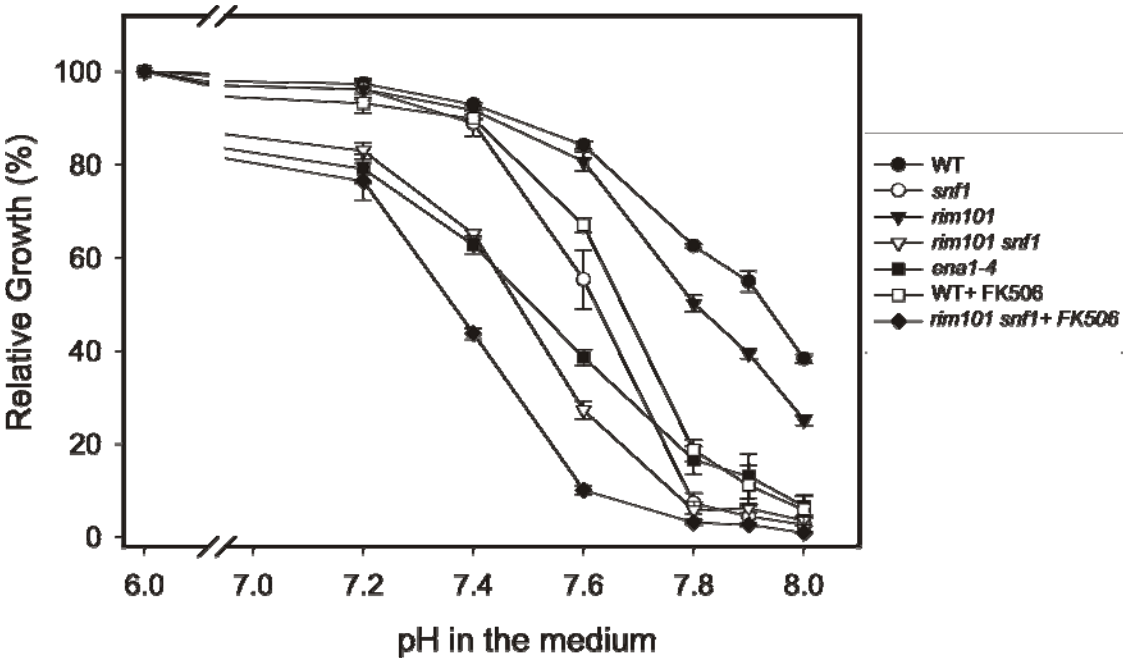

Figure 21. Effect of blockage of calcineurin, Snf1, and Rim101 pathways on high pH tolerance. The indicated strains were inoculated at an initial A_{660} of 0.01 in YPD medium buffered at the indicated pH values. FK506 was used at a concentration of 1.5 μg ml^{-1} where indicated. Growth is represented as the percentage of cell density at a given pH relative to cells growing at pH 6.0. Data correspond to the mean ± S.E. of three experiments.

The above results taken together reveal the existence of a complex mechanism that is responsible for alkaline induction of the sodium ATPase *ENA1*. Three main signaling pathways define this alkaline response: the calcineurin pathway, through the transcriptional activator Crz1, the Rim101 pathway through the Nrg1 repressor and the Snf1 pathway through the Mig2 and Nrg1 repressors (see Figure 22 for an integral model of the mechanism described). The Mig1 transcriptional repressor is also important for derepression of *ENA1* by alkali, although there is no evidence that its activity is regulated by Snf1. Among the three pathways, the one defined by the protein kinase Snf1 seems to have the greater impact on *ENA1* derepression by alkali, as deduced from the expression levels of the *ENA1* promoter observed in *snf1* strains in comparison with those observed in strains where the Rim101 or the calcineurin pathway are not functional (see Figures 18 and 19). The fact that the protein kinase Snf1 exerts its regulatory activity on *ENA1* through two different transcriptional factors Mig2 and Nrg1 could be, at least in part, the reason for the severe impact that its mutation has on the alkaline expression of *ENA1.* Considering that the sodium ATPase Ena1 plays an important role in the alkaline adaptation of yeast cells, the above results would suggest that the Snf1 pathway is a very important component of the yeast

adaptation mechanisms to alkaline pH. This notion was further supported by the fact that yeast cells lacking Snf1 grew poorly at alkaline environments and this growth defect was more severe than that manifested in strains lacking Rim101 and similar to the one observed when the calcineurin pathway is blocked.

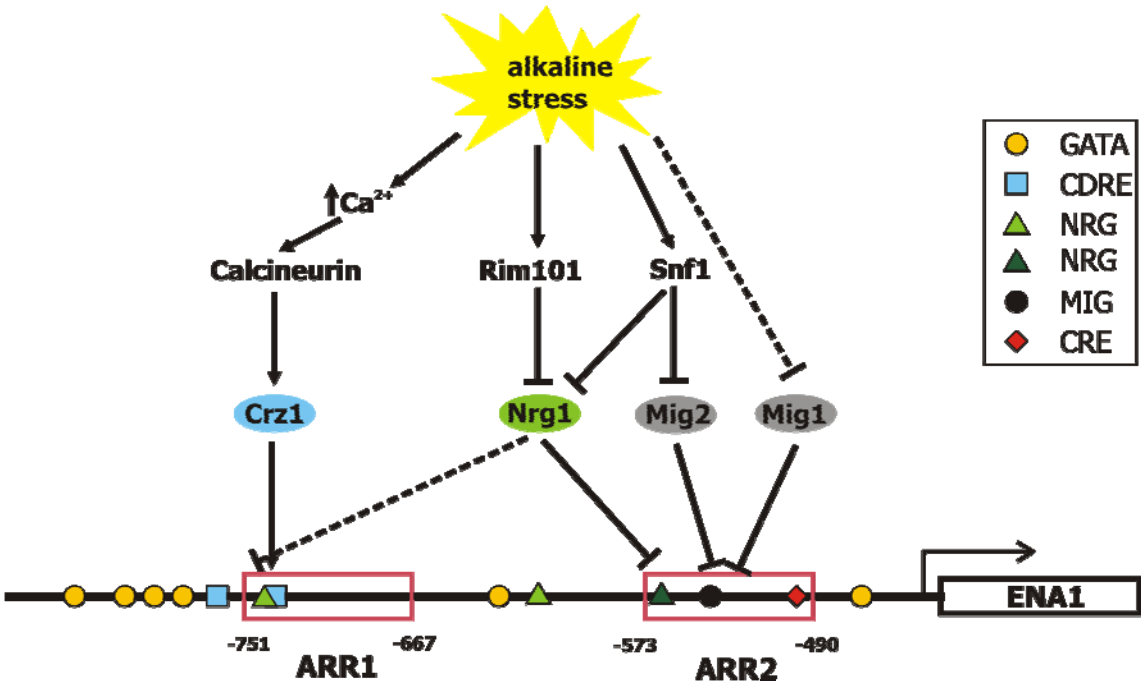

Figure 22. Schematic model for the signaling pathways mediating response of the *ENA1* promoter to alkaline stress. Regulatory elements in the promoter are depicted schematically. *Discontinuous lines* denote still uncharacterized processes. The effect of the calcineurin pathway on *ENA1* expression was reported previously (Serrano *et al.*, 2002; Viladevall *et al.*, 2004). The regulation exerted by Nrg1 on the ARR1 region is presumed from data reported previously using *rim101* mutants and from the identification of a putative Nrg1 binding sequence (Serrano *et al.*, 2002; Lamb *et al.*, 2003).

On the basis of previously reported data and the experiments presented here, it is clear that the mechanisms for controlling *ENA1* expression in response to saline and alkaline stress are somewhat overlapping but not identical. Whereas the calcineurin pathway plays a relevant role in both saline and alkaline stress, mapping of the ARR2 region clearly shows that the CRE response element responsible for derepression of the *ENA1* promoter in response to osmotic stress (Proft *et al.*, 1999; Proft *et al.*, 2001) is irrelevant under alkaline stress. Moreover, lack of the Snf1 protein kinase affects

more severely alkaline expression of *ENA1* than that observed after exposure to NaCl. In addition, our observation that blocking calcineurin, Snf1 and Rim101 pathways fully abolishes the response from the *ENA1* promoter suggests that, in contrast to what has been described for saline stress (Crespo *et al.*, 2001), the TOR pathway and the Gln3/Gat1 GATA transcription factors are not relevant for *ENA1* regulation under alkaline stress. This is remarkable, because Ure2, Gln3, and Gat1 have been found to be required for normal high pH tolerance (Giaever *et al.*, 2002; Serrano *et al.*, 2004; Garcia-Salcedo *et al.*, 2006). Therefore, the pH-related phenotypes of these mutant strains cannot be explained through a role for the corresponding gene products in the control of *ENA1* expression.

The Ena1 ATPase plays an important role in the adaptation to high pH conditions, as evidenced by the intense pH-sensitive phenotype of a strain defective in the ATPase function (see Figure 21). However, we observed that simultaneous blockage of the three main pathways governing *ENA1* expression under alkaline stress resulted in a phenotype substantially stronger than that observed for the ATPase-deficient strain. This would clearly indicate that one or more of these pathways play role(s) relevant for high pH tolerance that are independent of the ATPase function. This notion is supported by several lines of evidence. First, the response of about 10 % of the genes induced under alkaline pH conditions requires, at least in part, the calcineurin pathway (Viladevall *et al.*, 2004), giving evidence that the role of calcineurin in the alkaline response of yeast cells is not restricted to the regulation of the *ENA1*. Second, a large number of genes that are induced after exposure to alkaline pH are genes that are typically induced under low glucose conditions, since they are involved in hexose transport and carbohydrate metabolism in yeast (Serrano *et al.*, 2002; Viladevall *et al.*, 2004). Remarkably, in a recent study performed in our laboratory it was shown that many of these genes integrate inputs from both the calcineurin and the Snf1 pathway (Ruiz *et al.*, 2008). Therefore, blockage of the two pathways (calcineurin and Snf1) would result in impaired expression of the calcineurin- and Snf1-dependent genes, which are up-regulated at high pH, explaining, at least partially, the hypersensitivity to alkali of *rim101 snf1* cells treated with FK506 (Figure 21).

2 Regulation of the alkaline and saline response of *ENA1* by the protein kinase CK2.

CK2 is a tetrameric kinase that in *Saccharomyces cerevisiae* consists of two catalytic subunits α and α´, encoded by the genes *CKA1* and *CKA2* (Chen-Wu *et al.*, 1988; Padmanabha *et al.*, 1990), and two regulatory subunits β and β´ encoded by the genes *CKB1* and *CKB2*, respectively (Reed *et al.*, 1994; Bidwai *et al.*, 1995). As mentioned in the introduction, although the regulatory subunits are dispensable for normal growth, deletion of *CKB1* and/or *CKB2* results in specific sensitivity to sodium and lithium ions, suggesting a role for CK2 in saline homeostasis (Bidwai *et al.*, 1995). However, the molecular basis for the saline sensitivity of *ckb* mutants remains unclear.

In a study performed by Tenney and Glover (Tenney *et al.*, 1999) it was suggested that the function of Ckb1 and Ckb2 in salt tolerance involves the sodium ATPase Ena1. Their conclusions were based on β–galactosidase activity assays showing that saline expression from a reporter-plasmid that contains the entire *ENA1* promoter fused to the gene *lacZ* was decreased in *ckb* mutants. Moreover, in the same study it was shown that overexpression of *ENA1* from a heterologous promoter completely suppresses the salt sensitivity associated with these mutants. Interestingly, *ckb* strains also exhibited a decrease in expression of *ENA1* under alkaline pH conditions, but their alkaline tolerance was similar to that of a wild type strain. However, in another study performed almost simultaneously in our laboratory (Nadal *et al.*, 1999), although the methodology used was the same, the involvement of *ENA1* in the mechanism responsible for saline hypersensitivity of *ckb* mutants was discarded. Other regulatory pathways related to the salt tolerance of *S. cerevisiae* such as the calcineurin pathway, the Trk1/2 transport system, the Nha1 antiporter, or the Hal3/Ppz1 pathway, also seemed to be irrelevant for the sensitivity of *ckb* mutations to saline environments (Nadal *et al.*, 1999). Therefore, the molecular basis of the saline sensitivity of strains lacking the regulatory subunits of CK2 remains unsolved.

A possible reason for the discrepancies observed between the two studies is that the experimental conditions used were different: whereas in the first study (Tenney *et al.*, 1999) salt stress was evoked by incubation of the yeast cells for 30 minutes in YPD medium supplemented with 0.4 M NaCl, in the second study (Nadal *et al.*, 1999) incubation was for one hour and the medium was supplemented with 0.75 M NaCl. Our current knowledge about the kinetics of the saline response of *ENA1* in β-galactosidase assays is that the time of exposure to saline stress required for full induction of the gene depends on the salt concentration in the medium: in a range of 0.7-0.9 M NaCl, 4

hours of incubation are necessary to generate full induction of *ENA1,* whereas for lower concentrations (0.4 M NaCl) 30-60 minutes are sufficient. Thus, it is possible that Nadal and collaborators (Nadal *et al.*, 1999) did not detect changes in *ENA1* saline expression in *ckb* mutants because the time of induction (1 hour) was not sufficient for full response of *ENA1*. The fact that currently the experimental conditions required for full expression of *ENA1* are well established, prompted us to reexamine whether the sodium ATPase expression under saline and alkaline stress is affected by *CKB* mutation.

2.1 Effect of the *ckb* mutations on the alkaline and saline response of *ENA1.*

We first decided to test whether *ckb* mutants were defective in the expression of *ENA1* under conditions similar to those previously reported by Tenney and Glover. To this end, the reporter plasmid pKC201, which contains the entire *ENA1* promoter, was used to transform strains *ckb1, ckb2* and *ckb1,2* and β-galactosidase activity was measured after exposure of yeast cells to 0.4 M NaCl for one hour.

As shown in Figure 23, mutation of either *CKB1* or *CKB2* genes results in a decreased response of *ENA1* to sodium stress, whereas simultaneous deletion of both genes does not have any additive effect. A comparable reduction of *ENA1* expression levels is also observed in the absence of any stimuli.

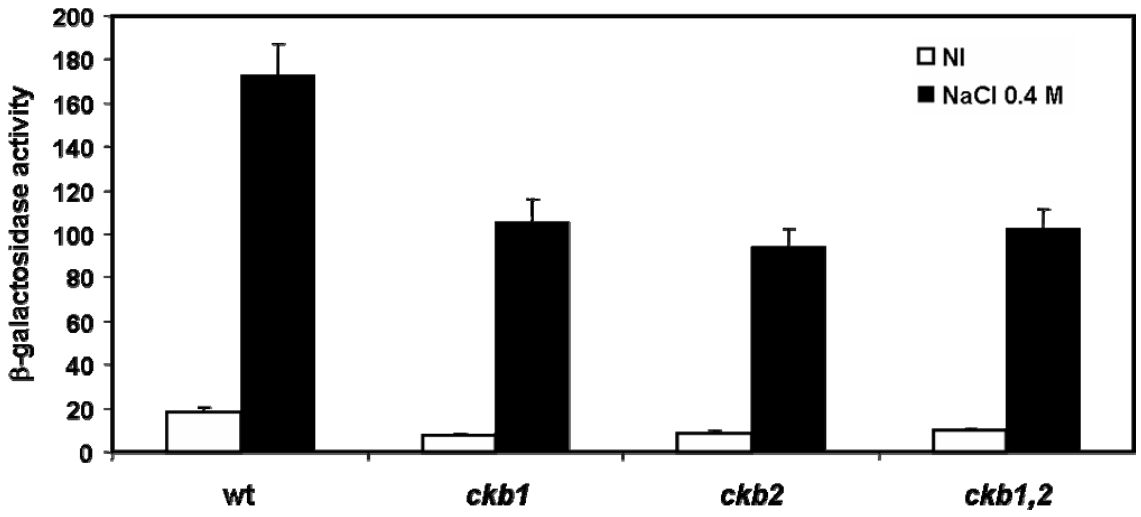

Figure 23. The regulatory subunits Ckb1 and Ckb2 of the protein kinase CK2 participate in the saline response of *ENA1*. The indicated strains were transformed with the reporter plasmid pKC201, which contains the entire *ENA1* promoter fused to *lacZ*, and β–galactosidase activity was measured in cells incubated for 60 minutes at YPD medium (*NI, empty bars*) or at YPD supplemented with 0.4 M NaCl to evoke saline stress (*full bars*). Data are mean ± S.E. from at least six independent clones.

Therefore, our results confirm the findings reported by Tenney and Glover about lower *ENA1* expression levels in *ckb* mutants (Tenney *et al.*, 1999). However, exposure of yeast cells to 0.4 M NaCl also provokes osmotic stress, which is among the stimuli that induce *ENA1* transcription, thus we could not be sure whether the effect observed was due to high osmolarity of the medium or the toxicity of sodium cations. We then decided to monitor *ENA1* expression after exposure to lithium stress. Lithium ions are considered as analogous to sodium ions and it has been demonstrated that low concentrations of this cation (0.15-0.2 M), which invokes a rather insignificant osmotic response, are sufficient for full induction of *ENA1* promoter after 60 minutes of exposure (Ruiz *et al.*, 2006). Because alkaline expression of *ENA1* was found to be affected in *ckb* mutants (Tenney *et al.*, 1999), we also assayed *ckb* cells exposed to high pH stress for 60 minutes.

Similarly with what occurs after cell exposure to sodium stress, mutation of Ckb1 and/or Ckb2 results in a decrease of the transcriptional activation of *ENA1* observed under alkaline and lithium stress conditions (see Figure 24). Therefore, the regulatory subunits of the protein kinase CK2 are indeed involved in the regulation of the expression of the sodium ATPase Ena1 under saline and high pH stress. Furthermore, both subunits seem to have a redundant role in this regulation, as simultaneous deletion of both *CKB1* and *CKB2* genes does not have an additive effect on loss of *ENA1* expression.

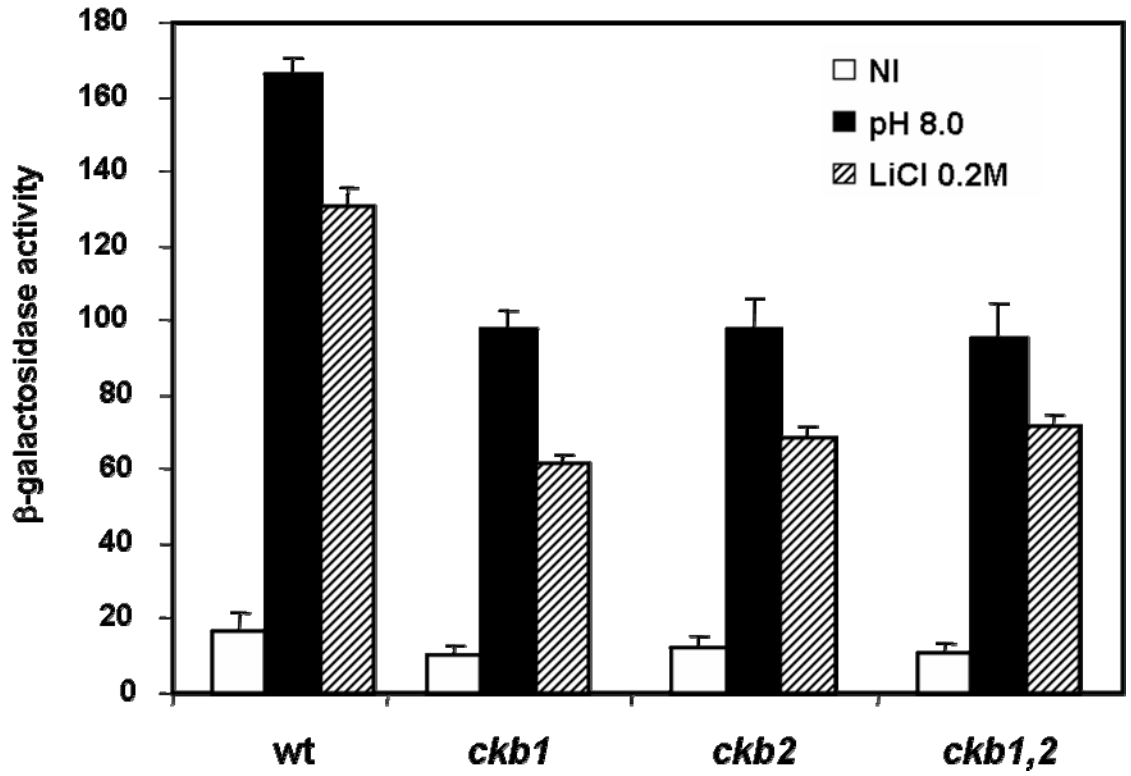

Figure 24. The regulatory subunits of Ckb1 and Ckb2 participate in the alkaline and saline response of *ENA1*. The indicated strains were transformed with the plasmid pKC201 and β–galactosidase activity was tested in cells grown at pH 6.3 (*NI, empty bars*), pH 8.0 (*filled bars*) and LiCl 0.2 M (*hatched bars*) for 60 minutes. Data are mean ± S.E. from at least six independent clones.

Since Ena1 is an important determinant of alkaline and saline tolerance in yeast cells, one would expect that strains lacking *CKB* genes, which are defective in alkaline expression of the ATPase, would grow poorly under alkaline stress conditions. Thus, we tested growth of these strains on solid media whose pH was adjusted to the alkaline range.

As can be observed in Figure 25, cells lacking either of the Ckb subunits are sensitive to alkaline pH. Therefore, our results argue previous data showing that *ckb* mutants are not sensitive to alkali (Tenney *et al.*, 1999). The fact that the genetic background used by these authors (YPH499) was different from ours (BY4741) would be a possible reason for this discrepancy. However, we repeated the same phenotypic assays using strain DBY746 (see *Materials & Methods*) as a genetic background and the result was similar to that shown in Figure 25 (data not shown).

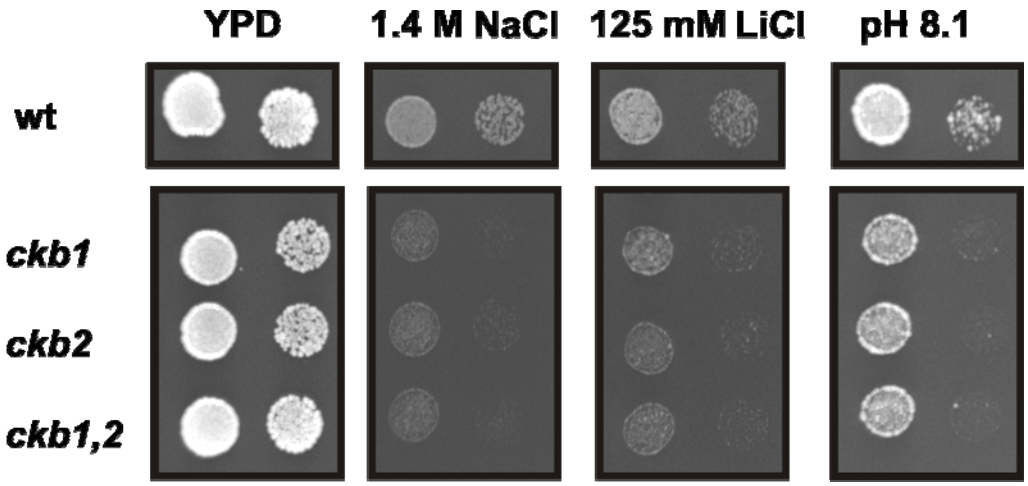

Figure 25. Effect of *ckb* mutations on high salt and alkaline pH tolerance. The indicated strains were grown to saturation, two consecutive dilutions of each culture were made to an A_{660} of 0.05 and 0.005, respectively, and 3 μl were dotted on YPD plates containing the indicated concentration of NaCl or LiCl. Alkaline tolerance was tested on YPD plates containing 50 mM TAPS and adjusted with KOH to pH 8.1. Growth was monitored after three days.

Our results until now support the idea that the regulatory subunits of CK2 are indispensable for full expression of *ENA1* under both alkaline and saline stress conditions. Although the role of the β subunits in regulation of the CK2 catalytic activity is not clear, it has been demonstrated in many cases that they modulate substrate specificity of the kinase and also enhance stability of the catalytic subunits (for a review see Pinna, 2002; Litchfield, 2003). Therefore, a possible explanation would be that lack of Ckb leads to loss of the phosphorylation activity of CK2, which in turn somehow results in a decrease of *ENA1* transcriptional activation. In addition, it seems that the CK2 subunits also contribute to the adaptation of yeast cells to alkaline environments (see Figure 25).

We also compared the sensitivity of *ckb* strains to lithium and alkaline pH with that of *ena1-5* strain, which lacks the whole tandem of *ENA* genes (five in this specific genetic background). As shown in Figure 26, the alkaline sensitivity of *ckb* mutants was similar to that observed in cells lacking the whole tandem of *ENA* genes. On the contrary, lithium sensitivity observed in *ena1-5* cells was much stronger than that of strains lacking Ckb: addition of 5 mM of LiCl to the medium is sufficient to inhibit *ena1-5* cells growth, whereas *ckb* cells grow well. These results suggest that, although *CKB* mutations influences the transcriptional response of *ENA1*, this effect has relatively small impact on tolerance to lithium (or sodium) cations. In contrast, an impaired Ena1 function could possibly explain the effect of Ckb mutation on alkaline tolerance of yeast cells.

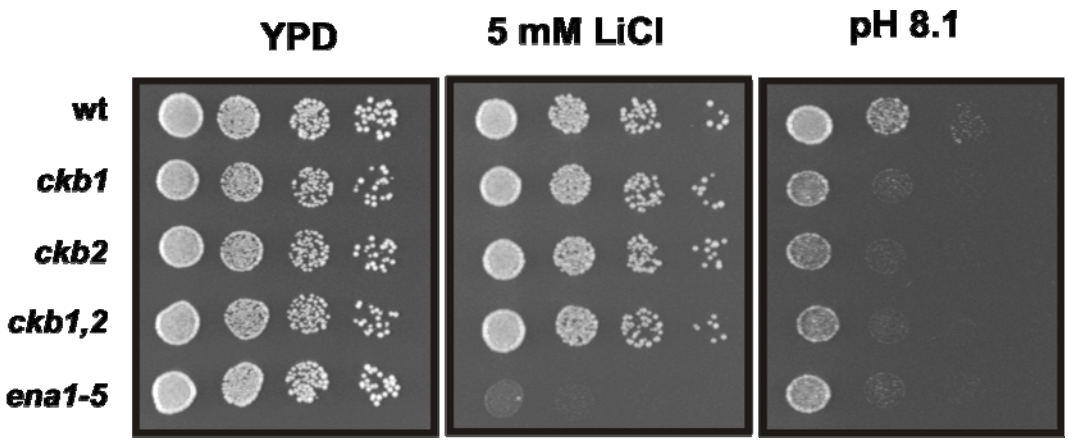

Figure 26. Effect of *ckb* and *ena1-5* mutations on lithium and alkaline pH tolerance. The indicated strains were grown to saturation, four consecutive dilutions of each culture were made to an A_{660} of 0.05, 0.001, 0.002 and 0.0004 respectively, and 3 μl were dotted on YPD plates containing the indicated concentration of LiCl. Alkaline tolerance was tested on YPD plates containing 50 mM TAPS and adjusted with KOH to pH 8.1. Growth was monitored after three days.

Because of the possibility that CK2 function on alkaline tolerance could be mediated by the sodium ATPase Ena1, we decided to explore which of the pathways that we had already establish as responsible for the alkaline response of *ENA1* could be the target of the kinase CK2.

2.2 Regulation of *ENA1* expression by the CK2 kinase is calcineurin independent.

Activation of the calcineurin pathway is required for adaptation of yeast cells to alkaline and saline environments. In addition, this pathway is one of the main regulators of *ENA1* transcriptional activation by salt and high pH. Thus, we decided to test if inhibition of the calcium-calcineurin pathway would affect the already decreased expression of *ENA1* in cells lacking the Ckb subunits. To this end, the wild-type strain and *ckb1* and *ckb2* mutants were transformed with the reporter plasmid pKC201 and β-galactosidase activity was assayed after 60 minutes of exposure to alkaline or saline stress, in the absence or presence of the calcineurin inhibitor FK506.

As shown in Figure 27, inhibition of the calcineurin pathway further decreases expression levels of *ENA1* observed in strains *ckb1* and *ckb2*. Interestingly, the effect of the inhibition of calcineurin is additive to that observed in *ckb* mutants for both stressing conditions (alkaline pH and lithium stress).

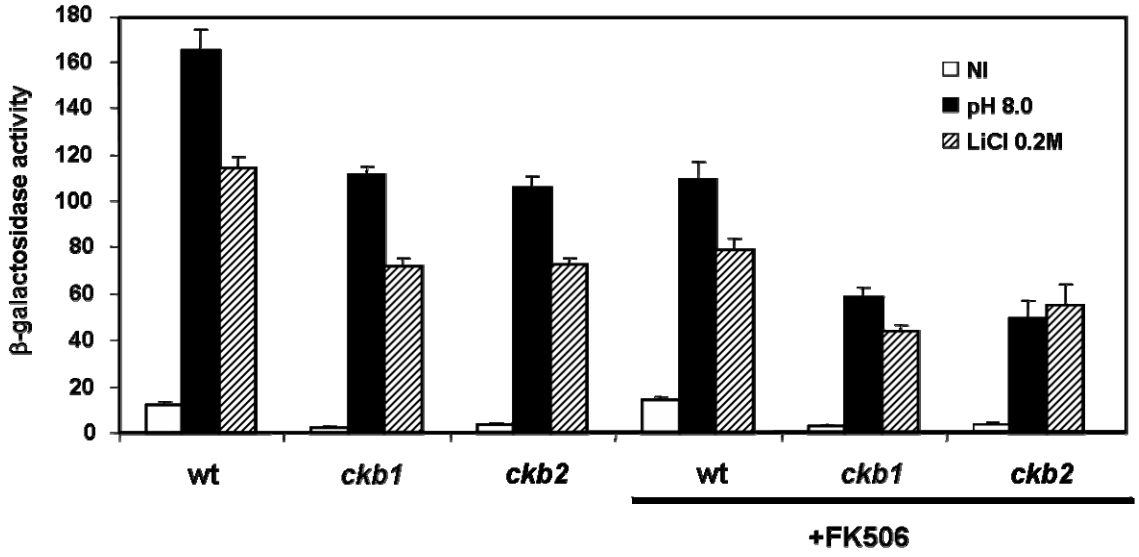

Figure 27. Inhibition of the calcineurin pathway in *ckb1* and *ckb2* strains results in further decrease of *ENA1* expression under alkaline and saline conditions. The indicated strains were transformed with the plasmid pKC201 and cells were processed as described in the legend of figure 24 in the presence or absence of the calcineurin inhibitor FK506 (1.5 μg ml^{-1}). Data are mean ± S.E. from at least six independent clones.

These findings suggest that CK2 plays a regulatory role in *ENA1* expression that is independent of that of calcineurin. Our results are in accordance with earlier observations (Nadal *et al.*, 1999) showing that the decreased lithium tolerance observed in *ckb1* mutants is further reduced by deletion of *CNB1,* the gene that encodes the regulatory subunit of calcineurin.

2.3 The Rim101 pathway is a possible target of the protein kinase CK2.

The Rim101 pathway was the second to explore as a possible target of CK2. This pathway regulates the alkaline response of *ENA1* indirectly, through the repressor activity of Nrg1. In addition, cells lacking the Rim101 protein grow poorly on both alkaline and saline media (Lamb *et al.*, 2001; Lamb *et al.*, 2003).

To test whether regulation of *ENA1* by CK2 is exerted through the Rim101 pathway, we generated double mutants *ckb1 rim101* and *ckb2 rim101,* transformed these strains, as well as the single mutants *ckb1, ckb2* and *rim101* with the reporter plasmid pKC201 and measured β-galactosidase activity after 60 minutes of exposure to alkaline pH and LiCl stress.

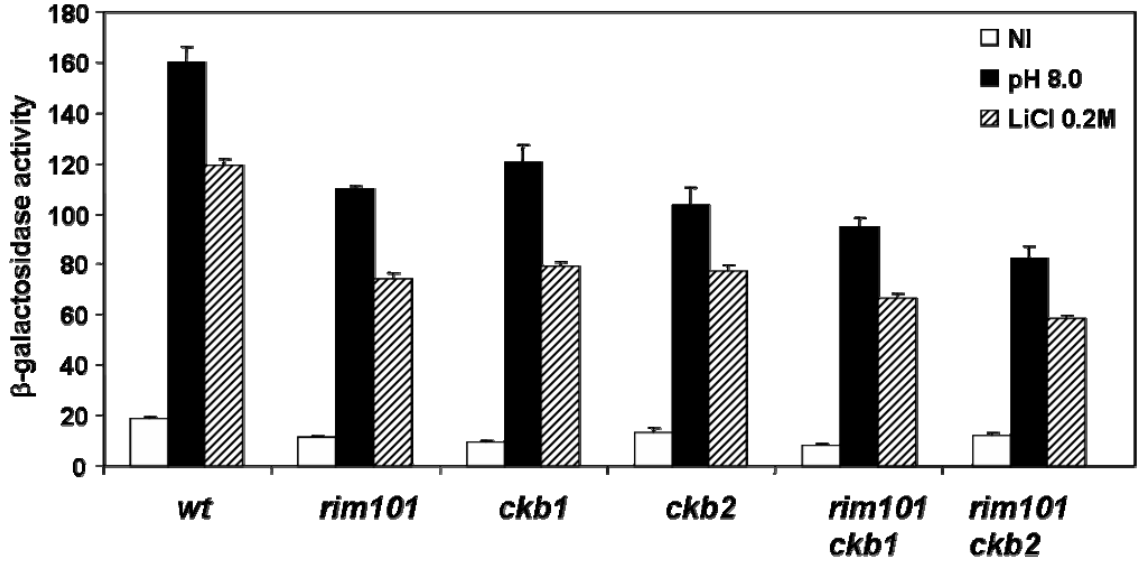

Figure 28. Effect of *RIM101* and *CKB* mutations on *ENA1* expression. The indicated strains were transformed with the plasmid pKC201 and cells were processed as described in the legend of figure 24. Data are mean ± S.E. from at least six independent clones.

As shown in Figure 28, *ENA1* expression levels were similarly reduced in *ckb* and *rim101* mutant strains, under stressing (alkali or salt) and non-stressing conditions. Interestingly, simultaneous deletion of *RIM101* and *CKB1* (or *CKB2)* resulted only in slightly lower expression levels of *ENA1* than those observed in *ckb* cells.

In agreement with the above results, phenotypic analysis performed using the same strains (see Figure 29), showed that strains lacking both Rim101 and Ckb are only slightly more sensitive than the respective single mutants when growing on media supplemented with NaCl or LiCl. In addition these double *ckb rim101* mutants do not show significant differences in alkaline pH tolerance when compared with the single mutants.

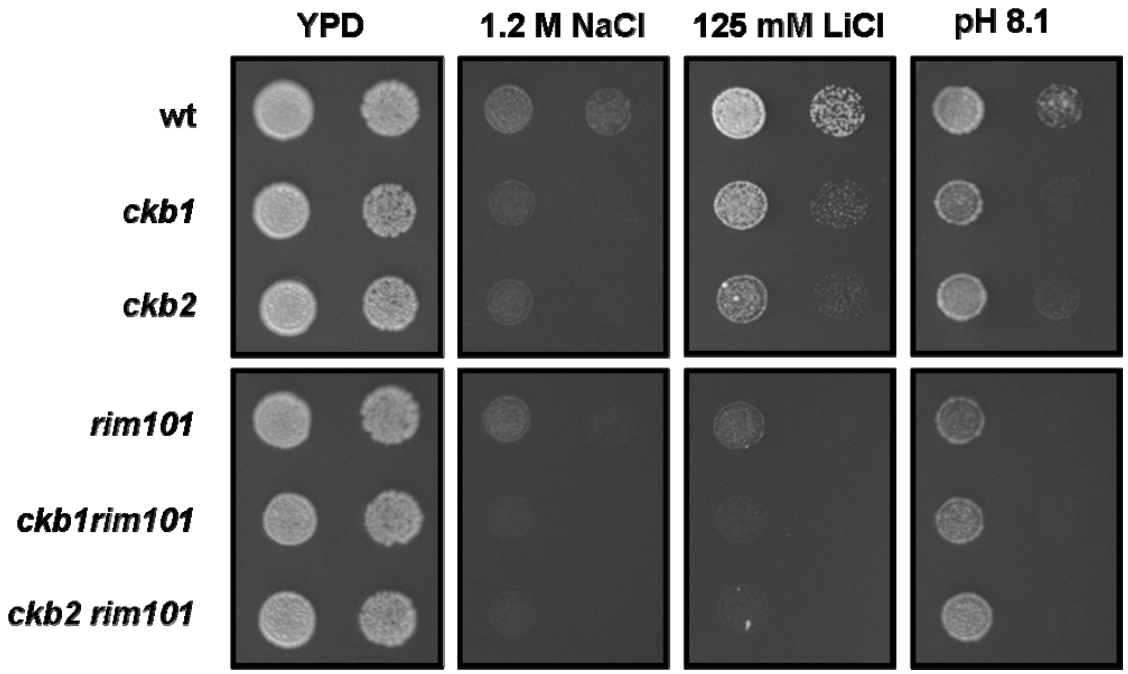

Figure 29. Effect of the Rim101 mutation in strains lacking the Ckb regulatory subunits of CK2 on high salt and alkaline pH tolerance. The indicated strains were grown to saturation, two consecutive dilutions of each culture were made to an A_{660} of 0.05 and 0.005, respectively, and 3 μl were dotted on YPD plates containing the indicated concentration of NaCl or LiCl. Alkaline tolerance was tested on YPD plates containing 50 mM TAPS and adjusted with KOH to pH 8.2. Growth was monitored after three days.

Our results demonstrate that Rim101 mediates expression of *ENA1* under lithium stress conditions and interestingly, that the reduction in *ENA1* expression resulting from *rim101* mutation after exposure to LiCl is similar to that observed after exposure to alkali. Although involvement of the Rim101 pathway in the alkaline adaptation mechanisms of yeast and other fungi such as *A. nidulans* and *C. albicans* is well established, its possible involvement in saline tolerance has not been given much attention. Lamb and collaborators observed that *rim101* mutants grow poorly in the

presence of NaCl and LiCl and attributed these phenotypes to reduced activity of Ena1, but they did not provide any experimental evidence (Lamb et al., 2001). Our results consolidate Rim101 as a positive effector of *ENA1* expression under lithium stress conditions and thus the Rim101 pathway as a saline responsive pathway.

Furthermore, the decrease in *ENA1* expression levels observed in a *rim101* strain is comparable to that of *ckb*, and more importantly, double *ckb rim101* mutants show only a minor additional loss of expression that could not be considered as an additive effect. These results are indicative that CK2 might be acting, at least in part, through the pathway mediated by Rim101. More evidence supporting such a theory comes from a study where the haploid deletion strain library was transformed with a Rim101-repressor reporter plasmid (made of four PacC sites fused to *LacZ)* to identify genes whose mutation results in impaired Rim101 activity. In wild type cells the reporter was expressed at low levels because Rim101 is active, thus repressing its expression. On the contrary, in *rim101 or rim13* cells, which contain no Rim101 or unprocessed protein, expression levels from this reporter were high, since no repression occurs. Interestingly, among the 40 genes found to be required for Rim101 activity this screen identified, in addition to known members of the Rim101 pathway such as *RIM8, RIM9, RIM13, RIM20* and *RIM21,* the CK2 holoenzyme components *CKA1* and *CKB2* (Barwell *et al.*, 2005). Thus the possibility that CK2 and Rim101 pathway share a common mechanism of action is reinforced.

Rim101 modulates expression of *ENA1* indirectly, through the Nrg1 repressor. Rim101 regulates Nrg1 transcriptionally, by binding to the 7-nucleotide motif that constitutes the PacC-binding site located on *NRG1* promoter. However, in a more recent study it was demonstrated that apart from its transcriptional regulation by Rim101, Nrg1 is also regulated by phosphorylation (Berkey *et al.*, 2006). More importantly, Nrg1 is phosphorylated in response to a variety of stresses, such as glucose-limiting conditions, alkaline pH, elevated NaCl and sorbitol concentrations, and the kinase identified as responsible for this phosphorylation is CK2. Nonetheless, the physiological role of this phosphorylation remains unknown. A possible scenario is that phosphorylation of Nrg1 by CK2 could be the key step for derepression of *ENA1*. If this would be the case, then in *ckb* mutants, where Nrg1 would not be phosphorylated, derepression due to relieve from Nrg1-binding would not be possible and expression levels of *ENA1* would be lower compared to that of a wild type strain.

To test the above hypothesis, we generated strains combining *ckb* and *nrg1* mutations, transformed them with the pKC201 plasmid and compared the expression levels of *ENA1.* As shown in Figure 30, expression from the entire *ENA1* promoter was increased in the *nrg1* mutant after exposure to alkali, in agreement with our previous results (see section 1 of *Results & Discussion*, Figure 13) using only the ARR2 region of the promoter. Comparable increases of the *ENA1* expression were also observed under lithium stress and in the absence of any stimuli in *nrg1* mutants, whereas in *ckb* mutants expression was decreased. Interestingly, expression levels of *ENA1,* in strains lacking both Nrg1 and Ckb, under alkaline and saline conditions were similar to those of a wild type strain.

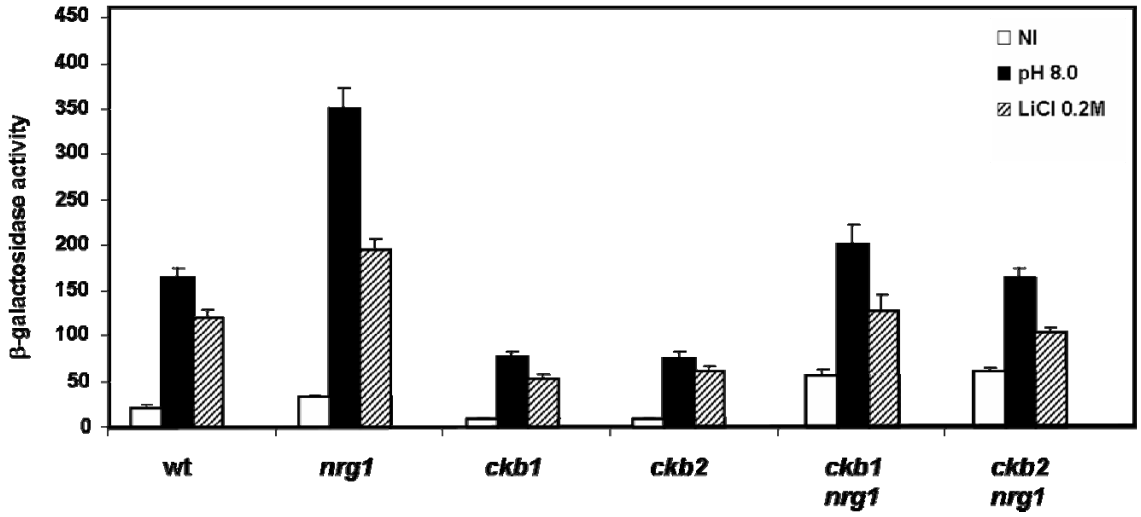

Figure 30. Effect of *NRG1* mutation on *ENA1* expression in strains lacking Ckb regulatory subunits. The indicated strains were transformed with the plasmid pKC201 and cells were processed as described in the legend of figure 24. Data are mean ± S.E. from at least six independent clones.

So far, our results support the idea that the regulatory subunits of CK2 are indispensable for full expression of *ENA1* under both alkaline and saline conditions. Moreover, they are in accordance with a possible role of Nrg1 downstream of CK2 in the regulatory mechanism by which the kinase affects *ENA1* expression for three reasons. First, deletion of *NRG1* in a *ckb* background substantially relieves transcriptional repression of *ENA1* observed in *ckb* strains (see Figure 30), suggesting that Nrg1 could possibly act downstream CK2. Second, mutation of *NRG1* confers to yeast cells tolerance to saline stress and partially counteracts the growth defect of *ckb1*

strains under saline stress conditions (Lamb *et al.*, 2001; Berkey *et al.*, 2006). Third, although there is evidence that this transcription factor is regulated by Snf1, no direct phosphorylation of Nrg1 by Snf1 has been demonstrated (Vyas *et al.*, 2001; Berkey *et al.*, 2004), so the regulatory mechanism responsible for Nrg1 repressor activity remains to been characterized. Remarkably, Nrg1 has been identified as a phosphorylation target of CK2 upon cell exposure to a variety of stresses (alkaline pH, high salt and low glucose conditions) and this phosphorylation is abrogated when Ckb1, Ckb2 or the catalytic subunit Cka1 are mutated (Berkey *et al.*, 2006). Although at the moment it is not known whether the phosphorylation state of Nrg1 affects its repressor activity, this possibility remains open. The repressor activity of many transcription factors is modulated by phosphorylation. For instance, phosphorylation of Mig1 by the Snf1 protein kinase is required for derepression of *ENA1* under glucose limiting conditions (see *Introduction* section 2.4). Therefore it is possible that phosphorylation of Nrg1 by CK2 could modulate its repressor activity and perhaps be required for the regulatory activity that Snf1 exerts through Nrg1. Nevertheless, in the absence of Nrg1, *ckb* mutation still results in repression of *ENA1* (compare expression levels between *nrg1* and *ckb nrg1* strains) suggesting the existence of additional CK2 target(s) relevant for regulation of *ENA1* promoter. A possible candidate would be the Mig1 transcription factor and its homologue Mig2, since they apparently modulate expression of *ENA1*, under basal and alkaline stress conditions (see Figure 10). However, in contrast to Nrg1 which is phosphorylated in response to saline, alkaline, oxidative and low glucose stress (Berkey *et al.*, 2006), it has been shown that Mig1 is phosphorylated under glucose limiting conditions by the protein kinase Snf1 (Treitel *et al.*, 1998), but not after exposure to saline stress (McCartney and Schmidt, 2001). However, it is still possible that Mig2 might act downstream CK2 in the regulation of *ENA1* expression. Such a role for Mig2 is supported by the fact that its mutation confers to yeast alkaline and saline tolerance (see Figure 11).

Our data do not exclude, though, the possibility that CK2 regulates *ENA1* transcription by a mechanism that is independent of Nrg1 repressor activity and that the expression levels of *ENA1* observed in *nrg1 ckb* mutants result from the additive affect of two distinct events: derepression caused by lack of Nrg1 and decrease in expression caused by lack of Ckb.

2.4 Interactions between the CK2 kinase and the Snf1 pathway.

It has been recently reported that the protein kinase Snf1 is activated in response to alkaline and saline stress by phosphorylation. However, the mechanism(s) that mediate regulation of Snf1 protein kinase activity by stress signals is not clear yet (Hong and Carlson, 2007). There are several lines of evidence suggesting that phosphorylation of Snf1 by the three upstream Snf1-activating kinases (Pak1, Tos3 and Elm1) is not the key step regulating its phosphorylation state (Hong *et al.*, 2005; Suter *et al.*, 2006; Sanders *et al.*, 2007).

Interestingly, early studies based on tandem affinity purification and mass spectrometry experiments at genomic scale (Gavin *et al.*, 2002) had identified CK2 subunits Cka1 and Ckb1 interacting with the Snf1 enzymatic complex (proteins Snf4, Gal83, Sip1 and Snf1) and other components related to the Snf1 pathway (Pak1, Reg1 and Bmh1). A subsequent, more specific study focused on the proteins associated with Pak1, has also come along with the CK2 catalytic subunits Cka1 and Cka2 (Elbing *et al.*, 2006). Moreover, alkaline and saline expression of *ENA1* is governed by both Snf1 and CK2 kinases (Alepuz *et al.*, 1997; Tenney *et al.*, 1999 and this work). Therefore, there are several evidences suggesting a possible interaction between the CK2 and Snf1 pathway.

We decided then to test a possible role of the Snf1 pathway in the mechanism by which CK2 regulates *ENA1* expression. To this end we generated strain *ckb1 ckb2 snf1*, lacking both regulatory subunits of the enzyme, and the protein kinase Snf1 and transformed yeast cells with the pKC201 reporter. As observed in Figure 31 A, the effect of the *snf1* mutation is rather strong and results in a loss of *ENA1* response that is quantitatively more important than that produced by mutation of *ckb* subunits. Surprisingly, combination of both mutations results in a response of the *ENA1* promoter that is similar to that observed in the *ckb1 ckb2* strain.

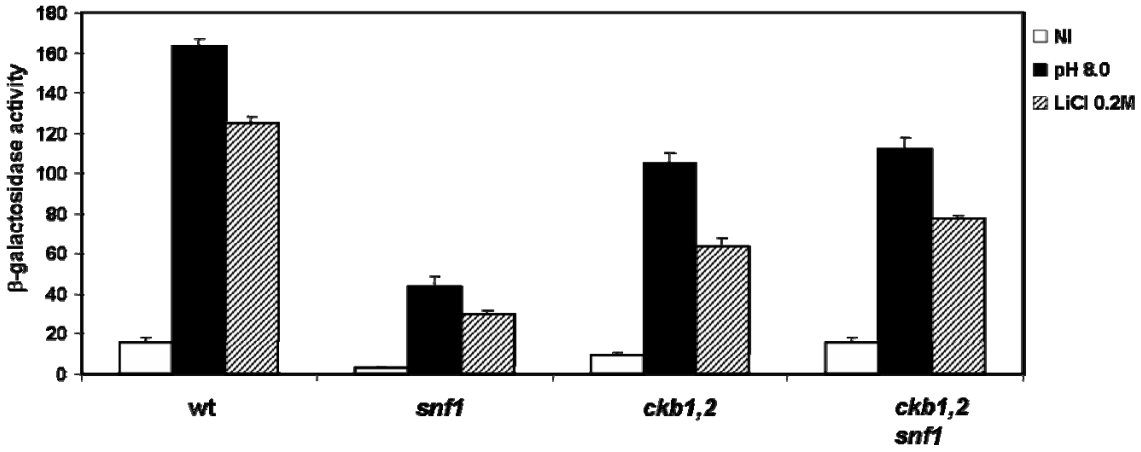

Figure 31 A. Mutation of *CKB1* and *CKB2* partially restores impaired expression of *ENA1* under alkaline and saline stress observed in cells lacking the Snf1 kinase. The indicated strains were transformed with the plasmid pKC201 and cells were processed as described in the legend of figure 19. Data are mean ± S.E. from at least six independent clones.

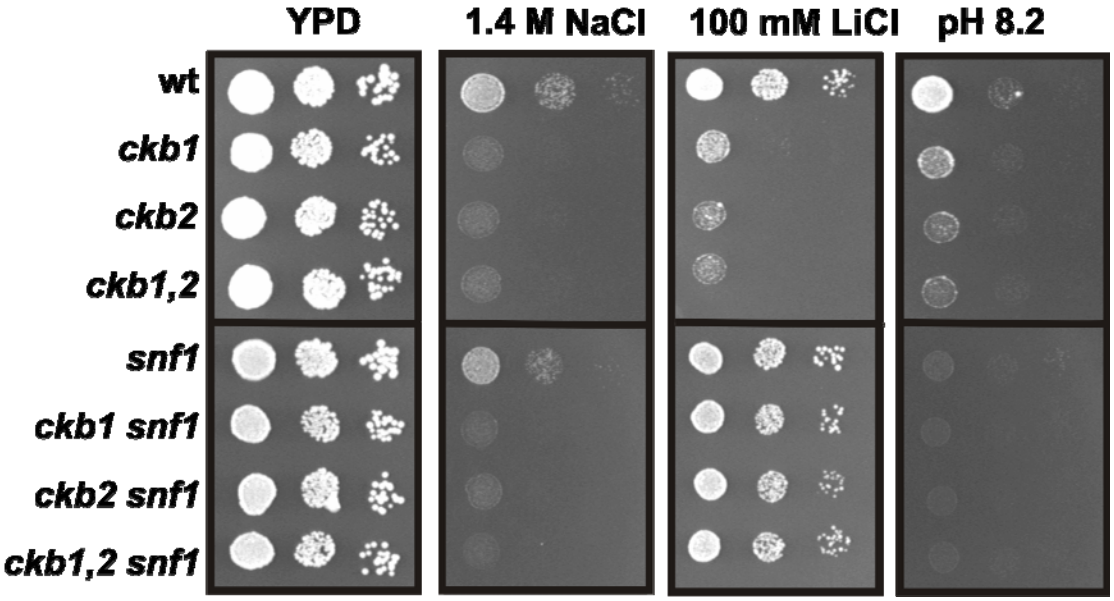

Figure 31 B. Effect of mutation of the *SNF1* gene in strains lacking the Ckb regulatory subunits of CK2 on high salt and alkaline pH tolerance. The indicated strains were grown to saturation, three consecutive dilutions of each culture were made to an A_{660} of 0.05, 0.001 and 0.002, respectively, and 3 μl were dotted on YPD plates containing the indicated concentration of NaCl or LiCl. Alkaline tolerance was tested on YPD plates containing 50 mM TAPS and adjusted with KOH to pH 8.2. Growth was monitored after two days.

Alkaline and saline tolerance of strains lacking Snf1 and/or Ckb subunits was also monitored (see Figure 31 B). Remarkably, tolerance to LiCl is not significantly altered by lack of Snf1, but the growth defect of *ckb* mutants is improved when *SNF1* is deleted. In contrast, *ckb snf1* cells are as sensitive as *ckb* mutants to NaCl, suggesting that the positive effect of *SNF1* mutation on saline tolerance of *ckb* strains is specific

for lithium stress. Alkaline sensitivity of *ckb* strains, as observed in figure 31 B, was somewhat aggravated by deletion of *SNF1* gene.

Our results indicate that *ckb* and *snf1* mutations display genetic interactions as far as *ENA1* expression is concerned. As observed in Figure 31 A, mutation of *CKB* genes partially suppresses the defect of *snf1* mutant with respect to *ENA1* expression at alkaline pH and high LiCl. These results are in sharp contrast with the scenario mentioned above, supporting a positive regulatory role for CK2 in *ENA1* expression under saline and alkaline stress conditions through phosphorylation of Nrg1 and Mig2 transcription factors. Therefore, a much more complex interaction between CK2 and the components of Ena1 regulatory mechanism must exist.

The phenotypic assays shown in Figure 31 B, suggest that mutation of *SNF1* restores the sensitivity of a *ckb* mutant to LiCl, but not that to NaCl or to alkaline pH, giving evidence for differential regulation depending on the stress. Although sodium and lithium are considered as analogous ions, they are not fully identical. Specific targets for lithium have been described, such as phosphoglucomutase (Masuda *et al.*, 2001), an enzyme that is required for yeast growth on galactose. Interestingly, it was shown that overexpression of the *PGM2* gene, encoding the major enzyme isoform, confers lithium tolerance to yeast cells (Masuda *et al.*, 2001). Therefore, it is possible that the lithium tolerance observed in *ckb snf1* strains could be the result of the increased expression levels of a gene (i.e. P*GM2*) encoding a specific lithium target.

As mentioned in the introduction (section 2.4), the protein kinase Snf1 is required for the transcriptional activation of glucose-repressed genes, such as *ENA1,* upon glucose starvation or in the presence of non-fermentable carbon sources. To gain insight into the molecular basis of the functional interaction of CK2 with Snf1, we decided to test whether lack of Ckb subunits also affects transcriptional responses under low glucose conditions. To this end, we decided to test expression levels of the *SUC2* gene, encoding the sucrose hydrolyzing enzyme invertase under normal and low glucose conditions. *SUC2* is a glucose-repressible gene that under low-glucose conditions is derepressed through a mechanism mediated by the kinase Snf1, the Mig1, Mig2 and Nrg1 repressors and the Ssn6-Tup1 co-repressor complex. *SUC2* has a quite simple promoter compared to that of *ENA1* and the main regulatory input that it receives comes from the Snf1 pathway (Gancedo, 1998; Carlson, 1999). Therefore, to define any possible role of the Ckb proteins in the signaling pathway governing derepression of this gene, we transformed the strains *ckb1,2, snf1* and *ckb1,2 snf1* with

a reporter construct that contains the entire *SUC2* promoter fused to the *lacZ* gene and shifted the cells for three hours from high glucose (2 %) to low glucose (0.05 %).

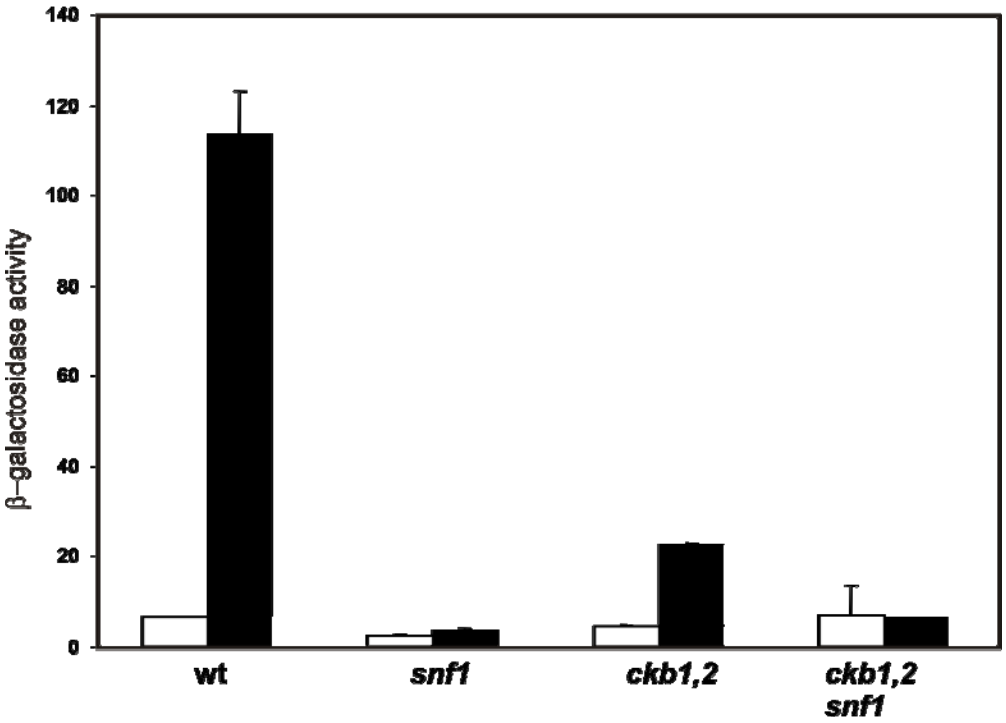

Figure 32. Derepression of *SUC2* under low glucose conditions is severely impaired in strains lacking the regulatory subunits of CK2. The indicated strains were transformed with the plasmid p*SUC2* that contains the entire *SUC2* promoter fused to *LacZ* and β–galactosidase activity was tested in cells grown at YPD (*empty bars*) and YP 0.05 % glucose (*full bars*) for 3 hours. Data are mean ± S.E. from at least six independent clones.

Our data shows the potent induction of *SUC2* expression when glucose is limited (see Figure 32) and the complete abolishment of such induction in a *snf1* strain. Interestingly, induction was also severely decreased in *ckb1 ckb2* cells, whereas expression in of triple mutant *ckb1 ckb2 snf1* was similar to that of *snf1 strain*. This finding was surprising because there are no previous reports suggesting any intervention of the CK2 kinase in the regulation of *SUC2.* However, in the triple *ckb1,2 snf1* mutant, and in contrast to what occurs with *ENA1,* we did not observe any transcriptional activation of the *SUC2* gene.

To corroborate these results, invertase activity was also measured in the above strains under repressing (normal glucose) and derepressing (low glucose) conditions.

As shown in Figure 33, growth of the wild-type cells under glucose-limited conditions resulted in high invertase activity, which was completely abolished in strains lacking Snf1, as it would be expected. Strains lacking Ckb subunits of CK2 lost an important part of this activity (from 272 mU mg^{-1} to 75 mU mg^{-1}), whereas the triple mutant strain *ckb1,2 snf1* showed activity similar to that of a *snf1* mutant. Thus, results from invertase activity assays correlated with the activity levels of the *SUC2* promoter measured by β–galactosidase activity.

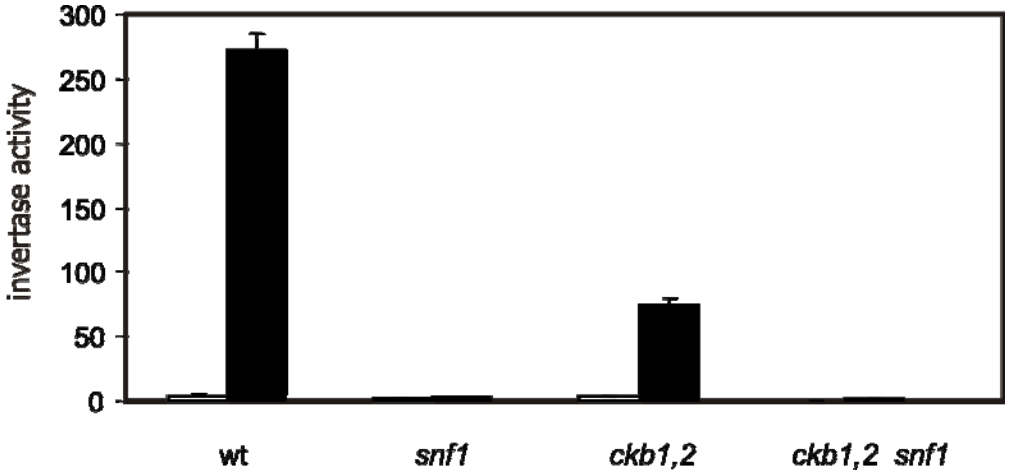

Figure 33. Effect of lack of the Ckb subunits on the induction of the *SUC2* gene by low glucose. Isogenic strains with the indicated genotype were grown in YPD (YP plus 2 % glucose) to the exponential phase and then resuspended in YP with 0.05 % glucose (*filled bars*). After 3 hours of incubation invertase activity was measured as described in *Materials & Methods*. Data are duplicates from one experiment.

Our findings reveal a role for CK2 in the regulation of the glucose repression pathway. The effect of the Ckb mutation on the expression of *SUC2* under glucose-limiting conditions as evaluated by measurements of β–galactosidase or invertase activity, suggests that CK2 plays a major role in the transcriptional response of this gene. Since the main pathway regulating transcriptional activation of *SUC2* is Snf1, these findings would support our notion about an interaction between the Snf1 pathway and the kinase CK2. We should underline the fact though, that in contrast to what happens with *ENA1* expression, loss of the *SUC2* transcriptional induction observed in a *snf1* mutant is not restored by additional mutation of Snf1 (see Figures 31 A and 32). Therefore, the effect of Ckb mutation on the expression levels of *ENA1* observed in a *snf1* strain seems to be specific.

Both *ENA1* and *SUC2* genes under non-limiting glucose conditions are repressed by a common mechanism that involves the repressors Mig1, Mig2 and Nrg1 and the co-repressor complex Tup1–Ssn6. When glucose is scarce or in the presence of non-fermentable carbon sources, expression of *SUC2* and *ENA1* is derepressed by a mechanism that requires the Snf1 kinase complex and leads to release from the Mig1, Mig2 and Nrg1 repressor activity (Treitel *et al.*, 1995; Lutfiyya *et al.*, 1996; Alepuz *et al.*, 1997; Proft *et al.*, 1999; Park *et al.*, 1999; Zhou *et al.*, 2001; Vyas *et al.*, 2001). Since we show that CK2 is required for full activity of both gene promoters, a reasonable possibility would be that CK2 could be required for fine regulation of this common repressor mechanism. The fact that Nrg1 was found to be phosphorylated by CK2 in response to a variety of stresses also points to this direction. Interestingly, the molecular basis for the regulation of Mig2 has not been deciphered. Although both Nrg1 and Mig2 seem to act downstream Snf1 (Vyas *et al.*, 2003; Berkey *et al.*, 2004 and this work) their phosphorylation by Snf1 *in vitro* has not been demonstrated. Therefore, a possible scenario would be that phosphorylation of Nrg1 and/or Mig2 by CK2 contributes to the release from repression of the genes that are under the control of these repressors. Phosphorylation of Nrg1 and/or Mig2 by CK2 could regulate their repression activity by affecting their localization, binding to the promoter, interaction with the co-repressor complex Tup1-Ssn6 or even modulating its regulation by Snf1. This hypothesis is represented by the scheme in Figure 34. The possibility that a hierarchy of phosphorylation events by the CK2 and Snf1 kinases would regulate repressor activity of Nrg1 and/or Mig2 is denoted.

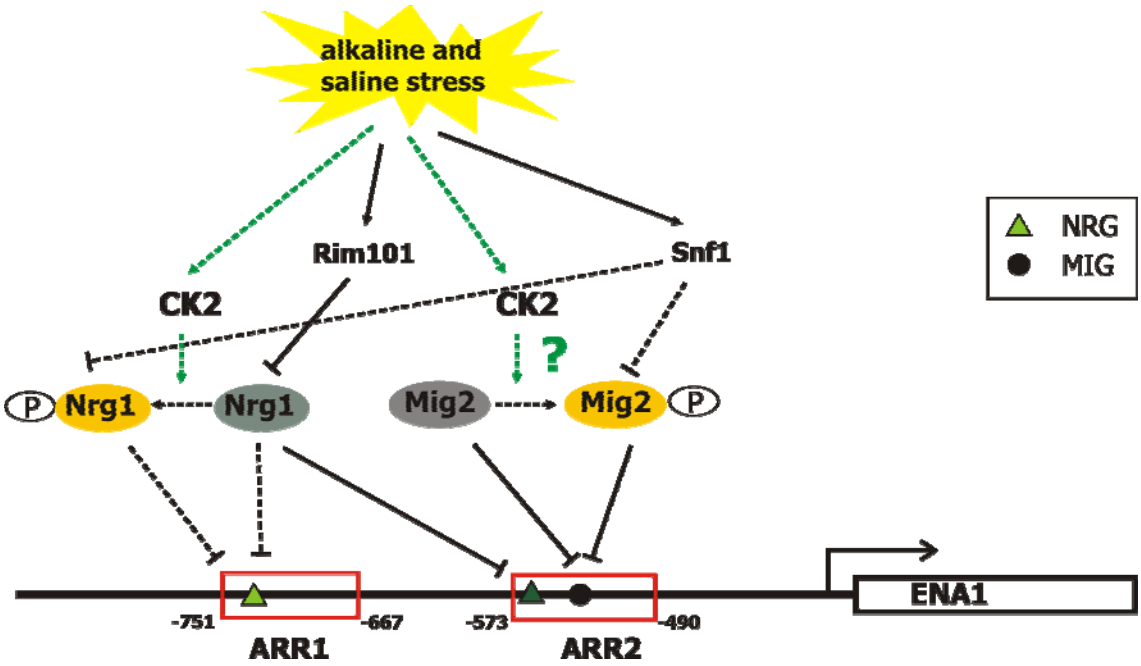

Figure 34. Schematic model for the signaling that mediates *ENA1* response to saline and alkaline stress, regulated by the kinase CK2. Only the MIG and the NRG regulatory elements of the promoter (which is not drawn to scale) are depicted schematically. Phosphorylation of Nrg1 and/or Mig2 by CK2 is suggested to be a requirement for Snf1 regulation of these transcription factors and hence *ENA1* full derepression under alkaline and saline stress conditions. *Discontinuous lines* represent hypothetical interactions.

The hypothesis that CK2 may control *ENA1* expression through Nrg1 and/or Mig2 implies that *ckb* mutations should affect expression driven from both ARR1 and ARR2 regions of the *ENA1* promoter (see Figure 34). To test this possibility we transformed *ckb* cells with the reporter plasmids pMRK212 and pMRK213, which contain the *ENA1* promoter fragments included in ARR1 and ARR2, respectively (see *Materials & Methods* section 4 and Serrano *et al.*, 2002).

A shown in Figure 35, expression levels from both regions was decreased in *ckb* strains under alkaline pH conditions. Remarkably, the decrease for ARR2 was stronger than that observed for ARR1. This result would fit with our hypothesis, since expression of the ARR2 region is regulated by both Nrg1 and Mig2, as we showed previously (see section 1 of *Results & Discussion*), whereas expression from ARR1 is largely calcineurin-dependent.

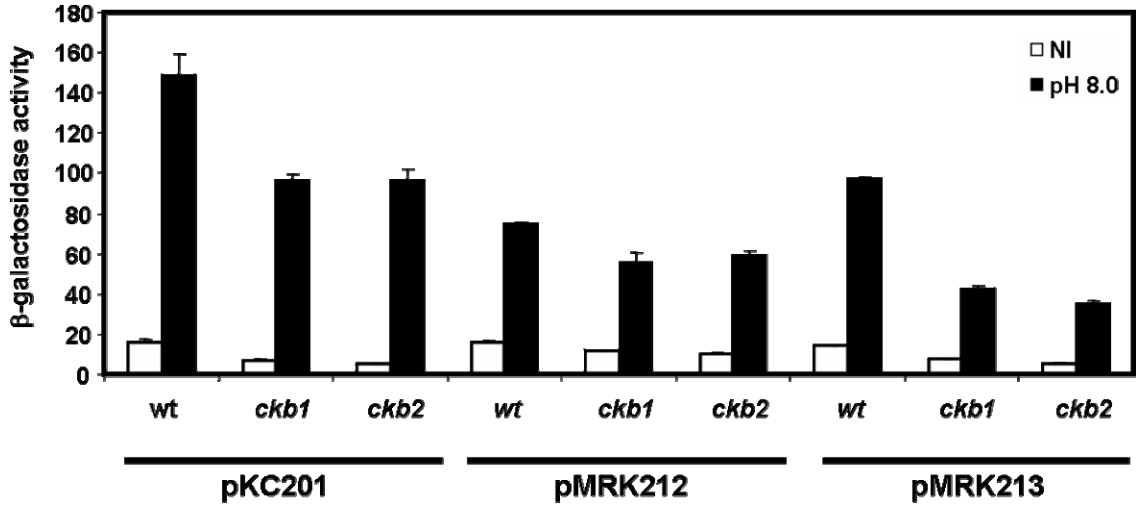

Figure 35. Evidence for CK2-dependent activation of both ARR1 and ARR2 regions of the *ENA1* promoter under alkaline stress conditions. The indicated strains were transformed with the reporter plasmid pKC201 (contains the entire *ENA1* promoter) or pMRK212 (contains ARR1) or the pMRK213 (contains ARR2) and cells were processed as described in the legend of figure 9. Data are mean ± S.E. from at least six independent clones.

Nonetheless, such a scenario is not sufficient to justify the increase of the *ENA1* expression observed in the *ckb1,2 snf1* strains, a result that suggest that the functional interaction between CK2 and Snf1, as far as regulation of *ENA1* expression is concerned, cannot be described in these relatively simple grounds. It must be noted that CK2 is a ubiquitous and pleiotropic protein and it is likely that, apart from modulating activity of Nrg1 and/or Mig2, could have other targets among the transcriptional machinery of *ENA1* gene. It has been reported previously that many CK2-regulated genes encode proteins involved in chromatin remodeling and modification (Barz *et al.*, 2003). Interestingly, it was shown that in mammalian cells CK2 is responsible for phosphorylation of HDAC1 and HDAC2, which are histone deacetylase enzymes required for transcriptional repression (Tsai *et al.*, 2002). Moreover, in a more recent study (Pluemsampant *et al.*, 2008) it has been reported that CK2 is a key activator of mammalian HDAC enzymes after exposure of cells to hypoxia stress. Both HDAC1 and HDAC2 are homologues to the Rpd3 histone deacetylase enzyme of *S. cerevisiae*. Remarkably, Rpd3 was found to be necessary for full repression of *ENA1* (Wu *et al.*, 2001). In addition, Rpd3 was found to interact with Cka1 and Ckb2 in a global tandem affinity purification assay performed by Gavin & collaborators (Gavin *et al.*, 2002). Thus a possible scenario would be that, similarly with what occurs in mammals, CK2 in *S. cerevisiae* could phosphorylate Rpd3, and this process would be necessary for the repressor role of the histone deacetylase.

Therefore, CK2 would have a dual and opposite effect on *ENA1* expression: a positive one, through regulation of the Nrg1 (and perhaps Mig2) repressors, and a negative one, mediated by Rpd3. In the presence of an intact Snf1-mediated signaling pathway, the positive effect would predominate and hence mutation of CK2 would lead to impaired expression from the *ENA1* promoter. In the absence of Snf1 the tight repression mediated by Nrg1, Mig1 and Mig2 would be alleviated by the impaired function of Rpd3 caused by lack of CK2.

V. CONCLUSIONS

1. The transcriptional response of the yeast sodium ATPase *ENA1* gene to alkaline stress involves three main signaling pathways.

1.1. The calcineurin independent responsive region of the *ENA1* promoter was mapped to a small segment of 42 nucleotides (nt -573/-531), denominated MCIR (Minimum Calcineurin Independent Region).

1.2. The Snf1 protein kinase is required for full induction from MCIR. Activation of this kinase alleviates the repression of the *ENA1* promoter by regulating Mig2 function. Mig1 also cooperates in repressing *ENA1*, but it is not regulated by Snf1 under alkaline pH stress.

1.3. The Rim101 pathway also regulates the alkaline response from MCIR, through the Nrg1 transcription factor. Under normal growth conditions, Nrg1 binds directly to a regulatory element located at the 5′-end of this promoter segment (nt -573 to -561), which contains a likely Nrg1 target sequence (AGACCCT). Upon exposure to alkaline stress binding is abrogated, thus releasing *ENA1* from repression. Nrg1 function on *ENA1* expression is also modulated by the Snf1 protein kinase.

1.4. The alkaline response of *ENA1* is under the tight regulation of three pathways: the calcineurin pathway through Crz1, the Rim101 pathway through Nrg1 and the Snf1 pathway through the Nrg1 and Mig2 transcription factors. The relative relevance of these pathways is Snf1>calcineurin>Rim101.

2. The alkaline and saline response of *ENA1* is regulated by the protein kinase CK2.

2.1. Expression of *ENA1* under saline and alkaline stress conditions is decreased in mutants lacking the β regulatory subunits of the protein kinase CK2. *ckb* strains show increased sensitivity, not only to sodium and lithium cations as previously described, but also to alkaline pH stress.

2.2. Inhibition of the calcineurin pathway in *ckb* mutants results in further decrease of expression of the ATPase gene, indicating that CK2 and calcineurin mechanisms on *ENA1* expression are independent.

2.3. The Rim101 pathway and CK2 may share a common mechanism in *ENA1* regulation under alkaline and saline stress conditions. The Nrg1 transcriptional repressor, which is phosphorylated by CK2 in response to saline and alkaline stress, possibly mediates the effect of CK2 on *ENA1* expression under these conditions.

2.4. CK2 may interact with Snf1 to regulate *ENA1* expression at high lithium and alkaline pH. Mutation of Ckb in *snf1* cells substantially restores *ENA1* expression and confers tolerance to lithium cations, thus revealing a complex interaction between CK2 and the Snf1 pathway. This effect is not observed for *SUC2* expression, a well known Snf1-regulated gene.

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VII. APPENDIX

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 48, pp. 36632–36642, December 1, 2006

The Transcriptional Response of the Yeast Na^+-ATPase *ENA1* Gene to Alkaline Stress Involves Three Main Signaling Pathways*[S]

Received for publication, July 7, 2006, and in revised form, September 22, 2006 Published, JBC Papers in Press, October 24, 2006, DOI 10.1074/jbc.M606483200

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Adaptive response of the yeast *Saccharomyces cerevisiae* to environmental alkalinization results in remodeling of gene expression. A key target is the gene *ENA1*, encoding a Na^+-ATPase, whose induction by alkaline pH has been shown to involve calcineurin and the Rim101/Nrg1 pathway. Previous functional analysis of the *ENA1* promoter revealed a calcineurin-independent pH responsive region (ARR2, 83 nucleotides). We restrict here this response to a small (42 nucleotides) ARR2 5′-region, named MCIR (minimum calcineurin independent response), which contains a MIG element, able to bind Mig1,2 repressors. High pH-induced response driven from this region was largely abolished in *snf1* cells and moderately reduced in a *rim101* strain. Cells lacking Mig1 or Mig2 repressors had a near wild type response, but the double mutant presented a high level of expression upon alkaline stress. Deletion of *NRG1* (but not of *NRG2*) resulted in increased expression. Induction from the MCIR region was marginal in a quadruple mutant lacking Nrg1,2 and Mig1,2 repressors. *In vitro* band shift experiments demonstrated binding of Nrg1 to the 5′ end of the ARR2 region. Furthermore, we show that Nrg1 binds *in vivo* around the MCIR region under standard growth conditions, and that binding is largely abolished after high pH stress. Therefore, the calcineurin-independent response of the *ENA1* gene is under the regulation of Rim101 (through Nrg1) and Snf1 (through Nrg1 and Mig2). Accordingly, induction by alkaline stress of the entire *ENA1* promoter in a *snf1 rim101* mutant in the presence of the calcineurin inhibitor FK506 is completely abolished. Thus, the transcriptional response to alkaline stress of the *ENA1* gene integrates three different signaling pathways.

Environmental changes force cells to adapt to the new situation to survive. The budding yeast *Saccharomyces cerevisiae* grows better at acidic pH and for this microorganism, sudden alkalinization of the medium represents a stress situation because it interferes with the proton gradient essential for uptake of many solutes from the medium, such as nutrients or diverse cations (1, 2). Growth at neutral or alkaline external pH requires two cation pumps: the vacuolar membrane H^+-ATPase, which is composed of multiple subunits and is essential for acidification of the vacuole (see Ref. 3 for a recent review), and the Na^+-ATPase encoded by the *ENA1*/*PMR2* gene (4, 5). Consequently, null mutants in many components of the vacuolar ATPase complex or strains lacking a functional *ENA1* gene are unable to proliferate even under mild alkaline conditions (6, 7).

The Ena1 P-type ATPase also represents a major element in the detoxification of sodium and lithium cations and *ena1* mutants are largely defective in cation efflux and extremely sensitive to these toxic ions. *ENA1* is the first member of a cluster composed by four to five genes encoding very similar proteins (*ENA1–ENA5*) (4, 5, 7) and, although early work suggested post-transcriptional regulation of the ATPase (5), compelling evidence indicates that regulation of the Na^+-ATPase function in the cell is mainly based on the control of the expression of the gene. *ENA1* is hardly expressed under standard growth conditions, but its expression is rapidly induced after exposure to saline, osmotic or alkaline stress. The complex regulation of *ENA1* expression after saline stress (8) has been characterized in some detail in the past. The presence of glucose in the medium represses *ENA1* expression by a mechanism that involves the Snf1 kinase and the repressor complex Mig1-Ssn6-Tup1, which targets to a MIG element located at position −534/−544 in the *ENA1* promoter (9, 10). In addition to the release from Mig1-mediated repression, induction of *ENA1* by high salt involves at least three different pathways. Activation of the HOG pathway in response to high osmolarity results in phosphorylation and activation of the Hog1 mitogen-activated protein kinase, which migrates to the nucleus and phosphorylates the Sko1 repressor (9). Phosphorylation of Sko1 prevents its binding to the cAMP response element present in the promoter at position −502/−509. Activation of the *ENA1* promoter by high salt is also greatly influenced by the calcineurin pathway (5, 8, 11–14). Acti-

* This work was supported in part by Grants BMC2002-04011-C05-04 and BFU2005-06388-C4-04-BMC (to J. A.) and BFU2004-02855-C0202 (to F. M.) from the Ministerio de Educación y Ciencia, Spain, and Fondo Europeo de Desarrollo Regional. 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.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

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vation of calcineurin increases *ENA1* transcription through dephosphorylation and activation of the Crz1/Tcn1/Hal8 transcription factor (15–17), which binds to specific DNA sequences (calcineurin-dependent response elements). In the case of the *ENA1* promoter, two such elements are present at positions −813/−821 and −719/−727, the downstream element being more important for the transcriptional response under saline stress (18). Finally, a role of the TOR pathway in the regulation of *ENA1* expression has been documented. In this case, saline stress would inhibit the TOR pathway, so the Gln3 and Gat1 transcription factors would no longer be retained in the cytoplasm and, upon entry into the nucleus, would activate *ENA1* by (presumably) binding to the diverse GATA sequences present in the ATPase gene promoter (19).

In contrast to the fairly well defined response of *ENA1* to high salt, the mechanisms underlying its activation by alkaline stress are not fully understood. Current evidence suggests that high pH stress triggers a burst of cytoplasmic calcium that results in activation of calcineurin and subsequent Crz1-dependent induction of *ENA1* expression (20, 21). Functional mapping of the promoter revealed that the calcineurin-dependent pH-responsive region could be restricted to a region, named ARR1, which contains the calcineurin-dependent response element at −719/−727, whereas a second pH-responsive but calcium-independent region (ARR2) was located downstream ARR1, spanning from position −490 to −573 (21). Induction of *ENA1* by high pH is also influenced by the Rim101 pathway. Rim101, a transcription factor initially identified as a positive regulator of gene expression in meiosis and sporulation (22), is a homolog of the *Aspergillus nidulans* PacC pH response regulator (23). *S. cerevisiae rim101* mutants display sensitivity to high pH and decreased expression of several genes induced by high pH (24). However, in contrast to what is known in *A. nidulans*, there is evidence that Rim101 acts *in vivo* as a repressor that targets to, at least, two previously known transcriptional repressors: Smp1 and Nrg1 (25). It has been proposed that activation of Rim101 would block the expression of Nrg1, thus releasing the *ENA1* promoter from the negative control exerted by the repressor. Consistent with this model, alkaline induction of *ENA1* is impaired in a *rim101* mutant (21, 24, 25) and this effect is suppressed by deletion of Nrg1 (25).

The *ENA1* promoter contains two sequences that were recognized as consensus binding sites for Nrg1 (CCCTC and CCCCT), at positions −725/−729 and −647/−651, respectively (25). The former is located in ARR1 and, in fact, overlaps with the downstream calcineurin-dependent response element sequence found in this region. The latter is located between ARR1 and ARR2, and is probably not functional because this segment of the promoter is not responsive to high pH (21). In contrast, whereas functional mapping of the *ENA1* promoter revealed that the alkaline response from both ARR1 and ARR2 regions was clearly reduced by mutation of *RIM101*, no evident Nrg1 site was identified in ARR2 (21). Furthermore, there is evidence that blocking the calcineurin-mediated response in a *rim101* mutant does not fully abolish the transcriptional response of *ENA1* to alkaline stress and that the ARR2 region of this promoter must contain regulatory elements other than those controlled by Rim101 (21). All these evidence definitely indicates that still unknown, Rim101- and calcineurin-independent regulatory components of the *ENA1* response to high pH stress must exist. In this work we characterize a third regulatory pathway relevant in this response and provide a working model that integrates our current knowledge in this field.

TABLE 1
***Saccharomyces cerevisiae* strains used in this study**

Name	Relevant genotype	Source/Ref.
DBY746	MAT*a*, *ura*3–52 *leu*2-3112 *his*3-Δ1 *trp1*-Δ239	D. Botstein
RSC10	DBY746 *snf1*::*LEU2*	50
RSC13	DBY746 *mig1*::*LEU2*	This work
RSC21	DBY746 *rim101*::*kanMX4*	21
RH16.6	DBY746 *ena1–4*::*LEU2*	7
MAR15	DBY746 *cnb1*::*KanMX*	21
MP005	DBY746 *rim101*::*kanMX snf1*:*LEU2*	This work
MP008	DBY746 *nrg1*::*kanMX*	This work
MP009	DBY746 *mig1*::*LEU2 nrg1*::*kanMX4*	This work
MP010	DBY746 *mig2*::*TRP1*	This work
MP011	DBY746 *mig2*::*TRP1 nrg1*::*kanMX4*	This work
MP012	DBY746 *mig1*::*LEU2 mig2*::*TRP1*	This work
MP013	DBY746 *mig1*::*LEU2 mig2*::*TRP1 nrg1*::*kanMX4*	This work
MP014	DBY746 *snf1*::*LEU2 mig1*::*kanMX4*	This work
MP015	DBY746 *snf1*::*LEU2 mig2*::*TRP1*	This work
MP016	DBY746 *snf1*::*LEU2 mig2*::*TRP1 mig1*::*kanMX4*	This work
MP017	DBY746 *nrg2*::*k anMX4*	This work
MP018	DBY746 *nrg2*::*TRP1*	This work
MP019	DBY746 *nrg1*::*kanMX4 nrg2*::*TRP1*	This work
MP020	DBY746 *snf1*::*LEU2 nrg1*::*kanMX4*	This work
MP021	DBY746 *snf1*::*LEU2 nrg2*::*kanMX4*	This work
MP022	DBY746 *nrg1*::*kanMX4 nrg2*::*TRP1 snf1*::*LEU2*	This work
MAR194	DBY746 *mig1*::*LEU2 mig2*::*TRP1 nrg2*::*KAN*	This work
MAR195	DBY746 *nrg2*::*TRP1 rim101*::*KAN*	This work
MAR198	DBY746 *mig1*::*LEU2 mig2*::*TRP1 nrg1*::*nat1*	This work
MAR199	DBY746 *mig1*::*LEU2 mig2*::*TRP1 nrg2*::*KAN nrg1*::*nat1*	This work
MAR200	DBY746 *nrg2*::*TRP1 rim101*::*KAN nrg1*::*nat1*	This work
MAR206	DBY746 *rim101*:*kanMX nrg1*::*nat1*	This work

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Media—The *S. cerevisiae* strains used throughout this work are derived from the DBY746 haploid wild type strain and are listed in Table 1. Yeast cultures were grown at 28 °C in YPD medium (10 g/liter yeast extract, 20 g/liter peptone, 20 g/liter dextrose) or complete minimal medium. When indicated, synthetic complete medium lacking appropriate supplements was used to maintain selections for plasmids (26).

Recombinant DNA Techniques—Escherichia coli DH5α was used as a host for DNA cloning experiments. Bacterial cells were grown at 37 °C in LB medium containing, when needed, 50 μg/ml ampicillin for plasmid selection. *E. coli* cells were transformed by standard treatment with calcium chloride (27). *S. cerevisiae* cells were transformed by a modification of the lithium acetate method (28). Restriction mapping, DNA ligations, and other recombinant DNA techniques were carried out by standard methods (27). Purification of DNA fragments from complex mixtures, including PCRs and restriction nuclease digests was performed by agarose gel electrophoresis. The appropriate bands were recovered from agarose gel slices using the Agarose Gel DNA Extraction Kit (Roche).

Deletion Cassettes and Gene Disruptions—The *mig2*::*TRP1* cassette was constructed as follows. A 1.76-kbp[6] region of the

[6] The abbreviations used are: kbp, kilo base pair(s); TAPS, *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid; MCIR, minimum calcineurin independent response; GST, glutathione *S*-transferase; HA, hemagglutinin.

MIG2 genomic locus, spanning from nucleotides −809 to +1149 from the start codon, was amplified by PCR using primers mig2_5′_SalI and mig2_3′_PstI, which contain artificial SalI and PstI restriction sites (the sequence of oligonucleotides used in this work can be found in supplemental Table 1). The amplification fragment was cloned into the SalI/PstI sites of plasmid pBluescript-SK, to give pBS-MIG2. A 387-bp SnaBI/NheI fragment of the open reading frame was replaced by a 1.0-kbp *TRP1* marker recovered from plasmid YDp-W by digestion with SmaI/NheI, yielding plasmid pBS-MIG2::*TRP1*. This plasmid was digested with StuI/AfeI and the 2.0-kbp fragment obtained was used to transform the yeast cells. The *nrg1*::*kanMX4* disruption cassette was amplified using oligonucleotides 5′-nrg1_disr and 3′-nrg1_disr from genomic DNA obtained from a *nrg1*::*kanMX4* mutant in the BY4741 background, in which the entire *NRG1* open reading frame was replaced by the heterologous marker (29). A similar strategy, using the appropriate deletion mutants, was used to generate the *nrg2*::*kanMX4* (oligonucleotides 5′-nrg2_disr and 3′-nrg2_disr) and *mig1*::*kanMX4* (5′-mig1 _disr and 3′-mig1_disr). Disruption of *NRG1* with the *nat1* marker was accomplished as follows. A 2.1-kbp DNA fragment encompassing the *NRG1* gene was amplified from genomic DNA by PCR using oligonucleotides 5′-nrg1_comp and 3′-nrg1_disr. The fragment was digested with XbaI and PmlI, yielding a 1.7-kbp fragment that was cloned into the XbaI and HincII sites of pBluescript-SK to generate pBS-NRG1. This construct was digested with EcoRI and HincII and the 0.8-kbp fragment replaced by the 1.27-kbp *nat1* gene fragment recovered from plasmid pAG25 (30) by digestion with EcoRI and PvuII. The resulting construct, pBS-*NRG1*::*nat1*, was then digested with XbaI and XhoI and the 2.15-kbp fragment was released used to transform yeast cells. Disruption of the *NRG2* gene by the marker *TRP1* was generated as follows. A 2.0-kbp region of the *NRG2* locus was amplified by PCR from genomic DNA, prepared from the DBY746 wild type strain, using oligonucleotides *5′-nrg2_disr*, which contains an artificial restriction EcoRI site, and *3′-nrg2_disr*, which contains an artificial PstI site. This fragment was cloned into the same sites of plasmid pBluescript-SK to give plasmid pBS-NRG2, which was digested with NheI/HincII to remove a 652-bp internal region. This region was replaced by a 1.0-kbp *TRP1* marker recovered from plasmid YDp-W by digestion with SmaI/NheI, to yield plasmid pBS-NRG2::*TRP1*. This plasmid was digested with EcoRI/SmaI and the 2.2-kbp fragment obtained was used to transform the appropriate yeast strain.

Strain MP005 (*rim101 snf1*) was generated by transformation of strain RSC21 (*rim101*) with the *snf1*::*LEU2* cassette recovered from plasmid pCC107::*LEU2* (31) after digesting with restriction enzymes BamHI and HindIII. Strains MP012 (*mig1 mig2*) and MP015 (*snf1 mig2*) were made by introducing the *mig2*::*TRP1* cassette into strains RSC13 (*mig1*) and RSC10 (*snf1*), respectively. Strains MP009 (*mig1 nrg1*) and MP011 (*mig2 nrg1*) were constructed by transformation of strains RSC13 (*mig1*) and MP010 (*mig2*) with the *nrg1*::*kanMX4* cassette. Strains MP013 (*mig1 mig2 nrg1*) and MP020 (*snf1 nrg1*) were generated by introducing the cassette *nrg1*::*kanMX4* into strains MP012 and RSC10, respectively. Strains MP014 (*snf1 mig1*) and MP016 (*snf1 mig2 mig1*) were generated by transformation of strains RSC10 and MP015, respectively, with the cassette *mig1*::*kanMX4*. Strain MP019 (*nrg1 nrg2*) was constructed by transforming strain MP008 (*nrg1*) with the *nrg2*::*TRP1* disruption cassette. Strains MP021 (*snf1 nrg2*) and MAR194 (*mig1 mig2 nrg2*) were obtained by transformation of strains RSC10 and MP012 with the *nrg2*::*kanMX4* disruption cassette. Strain MP022 (*nrg1 nrg2 snf1*) was generated by introducing the *snf1*::*LEU2* cassette into MP019 strain. Strain MAR195 (*nrg2 rim101*) was obtained by transformation of strain MP018 (*nrg2*::*TRP1*) with the *rim101*::*kanMX4* disruption cassette (21). Strains MAR198 (*mig1 mig2 nrg1*), MAR199 (*mig1 mig2 nrg2 nrg1*), MAR200 (*nrg2 rim101 nrg1*), and MAR206 (*rim101 nrg1*) were constructed by introducing the cassette *nrg1*::*nat1* in strains MP012, MAR194, MAR195, and RSC21, respectively.

Construction of β-Galactosidase Reporters—The following reporter plasmids, containing diverse regions of the *ENA1* promoter, were used. Construction of pMRK213 and pKC201 was described in Refs. 21 and 10, respectively. Plasmids pMRK505, pMRK525, and pMRK564 were constructed as follows: the corresponding *ENA1* promoter region was amplified by standard PCR using the appropriate primers (see supplemental data) and plasmid pKC201, which contains the entire promoter, as a template. The PCR products were digested by restriction enzymes XhoI and SalI and cloned into the XhoI restriction site of pSLFΔ-178K. This plasmid is a *CYC1* promoter-lacZ fusion from which the *CYC1* UAS elements have been deleted (32). All inserts were verified by sequencing using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems).

β-Galactosidase Activity Assays—Yeast cells were grown to saturation in the appropriate drop-out media overnight and then inoculated in YPD at A_{660} 0.2. Growth was resumed until A_{660} of 0.5 to 0.7 and cultures were then distributed into 1.0-ml aliquots and centrifuged for 5 min at 1620 × *g*. The supernatant was discarded and cultures were resuspended in the appropriate media. Unless otherwise stated, alkaline stress was provoked by resuspending cells in YPD containing 50 mM TAPS adjusted to pH 8.5 by addition of potassium hydroxide (autoclaving decreases the pH of the medium up to pH 8.0 approximately). Non-induced cells were resuspended in YPD medium adjusted to pH 6.3 after autoclaving. Cell were grown for 60 min and β-galactosidase activity assayed as described (33). Saline stress was induced by addition of 0.4 M NaCl. Growth was resumed and cells were collected after 60 min.

Real-time Reverse Transcriptase-PCR—Saturated cultures of the appropriate strains were diluted to A_{660} of 0.2 in YPD and grown up to A_{660} of 0.5–0.7. Then, two 10-ml aliquots of each culture were centrifuged during 5 min at 1620 × *g* at room temperature and pellets were resuspended in 10 ml of fresh YPD containing 50 mM TAPS buffered at pH 8.0 (alkaline induction) or YPD (no induction), respectively. After 10 min, cells were harvested by centrifugation for 2 min at 1620 × *g* at 4 °C, washed once with cold water, and centrifuged again to obtain cell pellets that were immediately frozen and stored at −80 °C until RNA purification. When inhibition of calcineurin was desired, FK506 (final concentration of 1.5 μg/ml) was added to the medium 1 h prior initiation of the alkaline treatment. The drug was also present in final resuspension media during stress.

Total RNA was purified using the RiboPure-Yeast kit (Ambion) following the manufacturer's instructions. RNA quality was assessed by denaturing 0.8% agarose gel electrophoresis and RNA quantification was performed by measuring A_{260} in a BioPhotometer (Eppendorf). Real time PCR was performed in a SmartCycler (Cepheid) apparatus, using the QuantiTect SYBR Green reverse transcriptase-PCR kit (Qiagen) and 10 ng of total RNA. For *ENA1* amplification, oligonucleotides ENA1fw and ENA1rev were used. Control experiments were carried out by amplifying a fragment of the *PHO84* gene using oligonucleotides PHO84fw and PHO84rev. In this case, due to the slower induction of this gene after alkaline stress, cell samples were taken after 30 min of induction. Reverse transcription was performed for 30 min at 50 °C, followed by incubation at 95 °C for 15 min. Finally, 45 PCR cycles (15 s at 94 °C, 30 s at 50 °C, and 30 s at 72 °C) were carried out.

Expression in E. coli of S. cerevisiae Nrg1—For expression in *E. coli*, the *NRG1* open reading frame (approximately 0.7 kbp) was amplified by PCR using oligonucleotides 5′-Nrg1 and 3′-Nrg1. The amplification fragment was cleaved with BamHI and XhoI restriction enzymes and cloned into the BamHI-XhoI site of pGEX-4T-1 (Amersham Biosciences) to yield plasmid pGEX-4T1-Nrg1. *E. coli* BL21-Codon Plus (DE3)-RIL cells (Stratagene) were transformed with plasmid pGEX-4T1-Nrg1 and grown overnight at 37 °C in LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. The culture was then diluted 1/10 in the same medium and cultures were grown to an A_{660} of 0.6–0.8. At this point isopropyl β-D-thiogalactopyranoside was added to the medium to a final concentration of 1 mM and cells were induced for 3 h at 37 °C. Cells were centrifuged at 1620 × *g* at 4 °C, washed twice, and resuspended in ice-cold lysis buffer (20 ml/liter of culture) that contained 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM dithiothreitol, 10% glycerol, and 0.1% Triton X-100, plus a protease inhibitor mixture (0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine, 1 μg/ml pepstatin A, 1 μg/ml leupeptin). Cells were harvested by sonication (3 × 15 s), centrifuged at 800 × *g* at 4 °C for 5 min, and the supernatant collected. The GST-Nrg1 fusion protein was purified by incubating the bacterial crude lysate with glutathione-Sepharose 4B beads (Amersham Biosciences), allowing gentle shaking for 1 h at 4 °C. GST-Nrg1 was eluted with a 10 mM glutathione solution (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM dithiothreitol).

Electrophoresis Mobility Shift Assays—Probes were labeled with ^{32}P by PCR using [α-^{32}P]dCTP and the appropriate primers, and purified using S-200 HR microspin columns (Amersham Biosciences). Various amounts of GST-Nrg1 were incubated with 10 ng of α-^{32}P-labeled probe (~10,000 cpm) and 1 μg of poly(dI-dC) for 30 min at 30 °C in a reaction volume of 20 μl containing 20 mM HEPES (pH 7.6), 1 mM $MgCl_2$, 60 mM KCl, 12% glycerol, 6 μg of bovine serine albumin, 10 μM $ZnCl_2$, and 1 mM dithiothreitol. Probe 578, which was added as a nonspecific competitor where indicated, was obtained by amplification from genomic DNA of the −578/−640 region of the *ENA1* promoter, using primers ena1_pr_5′_640 and ena1_pr_3′_578. The reaction samples were loaded onto 5% non-denaturing polyacrylamide gels and electrophoresed at 150 V for 3 h in a 22.5 mM Tris base, 22.5 mM boric acid, and 0.63 mM disodium EDTA buffer, adjusted to pH 8.0. The gel was dried and subjected to autoradiography.

Chromatin Immunoprecipitation Assay—The *NRG1-HA* strain was obtained by transformation of the W303-1A wild type strain with a modification module containing as selectable marker the *E. coli kan*^{r} gene. The resulting strain has the 3′ end of *NRG1* tagged with 3 hemagglutinin epitope (HA) sequences. The C-terminal modification module was obtained by PCR using as template plasmid pFA6a-3HA-kanMX6 (34) and primers containing the *NRG1*-specific sequences of the forward primer (O5) chosen to end just upstream of the stop codon, preserving the reading frame of the tag, whereas those of the reverse primer (O6) to end just downstream of the stop codon. To test the Nrg1-HA construct we used immunoblot analysis. Twenty to 40 μg of proteins from a *NRG1-HA* yeast extract were separated by SDS-12% polyacrylamide gel electrophoresis and transferred to enhanced chemiluminescence nitrocellulose membranes (Amersham Biosciences) by electroblotting, which were then incubated with an anti-HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase-conjugated protein A was used as secondary reactant. The complex was detected by the ECL detection system (Amersham Biosciences).

Chromatin immunoprecipitation assays were performed essentially as described previously (35, 36) with the following modifications. Yeasts cells were grown in 100 ml of low pH (pH 6.2) YPD medium at 28 °C to an $A_{600\ nm}$ of 1.0. The cells from one-half (50 ml) of the culture were shifted from low to high pH (pH 8.0) YPD medium for 30 min. Each sample was treated with formaldehyde (final concentration 1%) for 60 min at 20 °C, and 2.5 ml of 2.5 M glycine was added to stop the cross-linking reaction. Cells were harvested and disrupted by vortexing in the presence of glass beads, and the lysate was sonicated to generate DNA fragments that ranged in size from 300 to 500 bp. To immunoprecipitate HA-tagged proteins, we incubated the extract overnight at 4 °C with anti-HA antibodies, and the extract/antibody mixture was incubated for 3–4 h with protein A-Sepharose beads (Amersham Biosciences). Immunoprecipitates were washed 4-fold with 1 ml each of lysis buffer (50 mM HEPES, pH 7.5, 250 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A). The DNA was eluted with elution buffer (100 mM sodium bicarbonate and 1% SDS). After reversal of the formaldehyde-induced cross-links, 1/5000 of input DNA and 1/45 of each immunoprecipitated DNA were used as templates for amplification by PCR (35 cycles), using primers O1 (sense), O2 (sense), O3 (sense), and O4 (antisense). Band intensities were quantified using the 1D Image Analysis Software (Kodak Digital Science).

Growth Tests—Sensitivity of yeast cells to high pH was evaluated by growth on liquid cultures performed in 96-well plates. Two hundred fifty-μl cultures at an initial A_{660} of 0.01 were grown for 14–20 h at 28 °C in YPD containing 50 mM TAPS buffered at the indicated pH values. Growth was monitored in an iEMS Reader MF apparatus (Labsystems) at 620 nm.

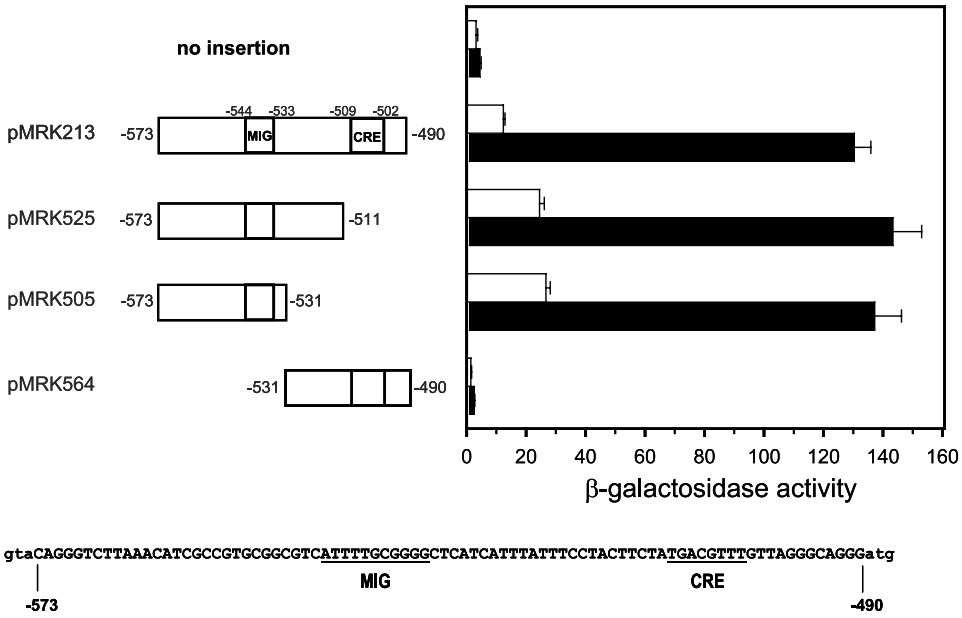

FIGURE 1. **Functional mapping of the ARR2 region of the *ENA1* promoter in response to alkaline stress.** Wild type DBY746 cells were transformed with the indicated constructs and cultures were grown for 60 min at pH 6.3 (*empty bars*) or 8.0 (*filled bars*). Cells were collected and β-galactosidase activity measured as described under "Experimental Procedures." Data are mean ± S.E. from at least six independent clones. The segments of the *ENA1* promoter included in each plasmid are denoted by *boxes*, and their relative position is indicated by *numbers* (nucleotide positions from the initial Met codon) flanking each box. Relevant known regulatory elements are depicted schematically and placed on the sequence of the ARR2 fragment (*capital letters*) at the *bottom*. Details on the constructions of plasmids can be found in the text.

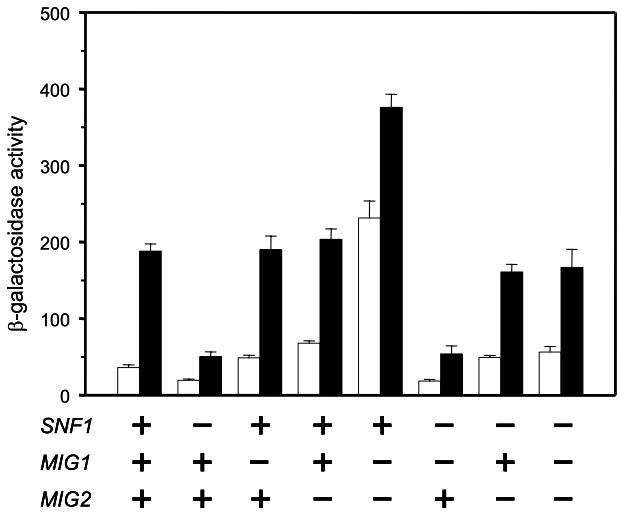

FIGURE 2. **Evidence for Snf1- and Mig1/2-dependent activation of the *ENA1* promoter MCIR region under alkaline stress.** The indicated strains were transformed with plasmid pMRK505 and β-galactosidase activity was tested in cells grown at pH 6.3 (*empty bars*) or 8.0 (*filled bars*). Data are mean ± S.E. from at least six independent clones.

RESULTS

Functional Mapping of the Calcineurin Independent Response of the ENA1 Promoter—We previously demonstrated that the region spanning between nucleotides −490/−573 of the *ENA1* promoter, named ARR2, was able to elicit a transcriptional response that was not affected by blockage of the calcineurin pathway (21). To further characterize this region, we constructed several reporter plasmids containing different portions of the ARR2 region. As can be observed in Fig. 1, full response to alkaline stress was observed in a construct (pMRK505) containing the −531/−573 region. However, the region from −490/−531 (pMRK564), which roughly represents the 3′-half of ARR2, was completely unable to sustain a transcriptional response to alkaline stress. Therefore, the calcineurin independent response of the *ENA1* promoter could be mapped to the 43-nucleotide fragment contained in pMRK505. This region was named MCIR (for minimum calcineurin independent response) and, consequently, the pMRK505 reporter plasmid was used for further characterization of the response.

Effect of Lack of the Snf1 Protein Kinase and the Mig1,2 Transcriptional Repressors—Because of the presence of a MIG element in the MCIR region, we considered it necessary to evaluate the possible role of the Mig1 and Mig2 repressors, which are able to bind to the MIG element. As shown in Fig. 2, mutation of Mig1 or Mig2 barely affects the response to high pH, although slightly higher basal levels (that is, in the absence of stress) are observed. In contrast, mutation of both genes results in markedly increased basal levels and enhanced response to alkaline stress. Interestingly, mutation of the Snf1 protein kinase, which is known to inhibit Mig1, results in a dramatic decrease in response, suggesting that this kinase plays an important role in controlling activation of the *ENA1* promoter by acting on the ARR2 region. We also observed that the expression from the MCIR region in a strain lacking both Snf1 and Mig1 is identical to that found in the *snf1* mutant. In contrast, the response in the *snf1 mig2* mutant was almost as strong as in the *mig2* strain. The fact that a strain lacking both Mig1 and Mig2 proteins was still able to promote induction from the MCIR after alkaline shock suggests that additional regulatory elements must be present in this region. Interestingly, mutation of *SNF1* markedly decreased the very high expression characteristic of the *mig1 mig2* strain. This could be explained if one assumes that Snf1 is controlling an additional negative regulator of *ENA1* expression located within the ARR2 region.

Influence of the Rim101 Pathway in the Alkaline Induction from the MCIR—We had previously observed that mutation of *RIM101* decreased the response from the ARR2 region of the *ENA1* promoter (21). Therefore, it was reasonable to assume that the Rim101 pathway could provide the missing regulatory mechanisms mentioned above. To test this, we constructed strains lacking the *RIM101* gene, as well as the Nrg1 and Nrg2 repressors that have been shown to be functionally related to Rim101, and tested the expression in these cells from the reporter plasmid pMRK505. As it can be observed in Fig. 3, cells lacking Rim101 presented a decreased response, although the

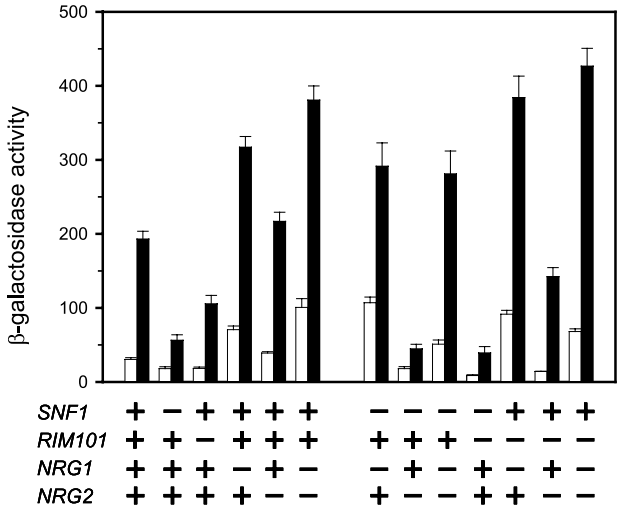

FIGURE 3. **Both Snf1 and Rim101 pathways participate in the alkaline response of the MCIR region by regulating the activity of Nrg repressors.** The indicated strains were transformed with the plasmid pMRK505 and cells processed as described in the legend to Fig. 2. Data are mean ± S.E. from at least six independent clones.

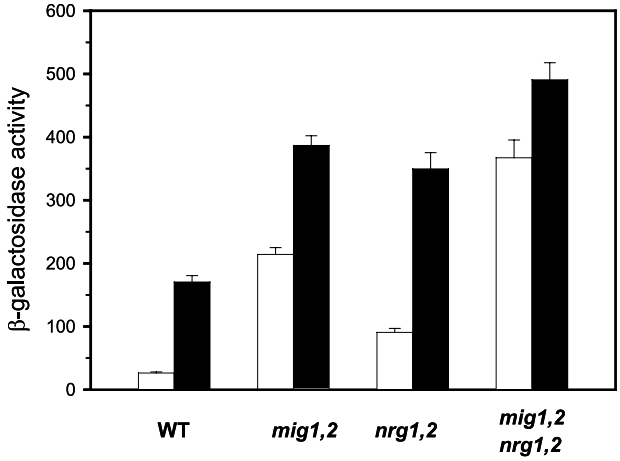

FIGURE 4. **Response of the MCIR region to alkaline stress in cells lacking repressors Mig and Nrg.** The indicated strains were transformed with plasmid pMRK505 and cells processed as described in the legend to Fig. 2. Data are mean ± S.E. from at least six independent clones.

decrease was not as strong as in the case of *snf1* cells. The absence of Nrg1 resulted in increased basal expression and response to high pH, whereas mutation of *NRG2* had little effect and the expression level in the double mutant was only slightly higher than in the *nrg1* strain. These results were indicative that Nrg1 was playing a repressor role in the alkaline response from the MCIR region. We observed that mutation of *NRG1* strongly increases the basal levels and alkaline response from plasmid pMRK505 in the *rim101* mutant, whereas mutation of *NRG2* has almost no effect. Therefore, lack of Nrg1, but not Nrg2, abolishes the decrease in response from the MCIR to the high pH observed in the *rim101* mutant. Because Nrg1 and Nrg2 are also known to be under regulation of the Snf1 kinase, we constructed cells deficient in all possible combinations of these genes and tested expression from pMRK505. As shown in Fig. 3, mutation of *NRG1* drastically counteracts the effect of the *snf1* mutation, whereas lack of Nrg2 has no effect. Taken together, these results suggested that both Snf1 and Rim101 may act by controlling the repressor effect of Nrg1, being the role of Nrg2 virtually negligible.

The data collected so far suggest that expression from the MCIR region in response to alkaline pH is mostly controlled through the release from the negative effect of the Mig1,2 and Nrg1 repressors. If so, one would expect that cells lacking all four repressors, Nrg1,2 and Mig1,2, would show a very high basal activity from the pMRK505 reporter and, in addition, further increase induced by alkaline shock would be either eliminated or severely impaired. In agreement with this hypothesis, it can be observed (Fig. 4) that in a quadruple *mig1,2 nrg1,2* mutant, expression from the MCIR give rises to higher basal levels than in *mig1,2* or *nrg1,2* cells and that its activity after alkaline stress increases only marginally (1.3-fold, in comparison with 8.5-fold in the wild type strain). It is worth noting that lack of Mig1,2 results in basal expression levels from pMRK505 that almost double those observed in *nrg1,2* cells, suggesting that the Mig1,2 repressors exert a stronger repressor control on this promoter region.

Identification of Nrg1 Binding to the ARR2 Region—In contrast with the evidence described above, canonical sequences previously defined for Nrg1 (25, 37) were not identifiable within the ARR2 region. Therefore, we considered it necessary to directly test whether or not Nrg1 was able to interact with this region and the possible functional relevance of such an interaction. To this end, we expressed in *E. coli* a GST-Nrg1 fusion protein and tested its ability to bind *in vitro* to the ARR2 region. As can be observed in Fig. 5*A*, the presence of GST-Nrg1 in the mixture is able to shift the electrophoretic mobility of a radioactively labeled ARR2 fragment and this fragment can be competitively displaced by unlabeled ARR2. To further map the interaction region, we generated additional fragments either fully outside (5′) the ARR2 region (fragment 578) or overlapping its 5′ end (fragments 541, 551, and 561) and tested their ability to bind Nrg1. Our results (Fig. 5*B*) indicate that the 578 fragment does not bind at all Nrg1, but fragment 561 still does. This suggests that the Nrg1 binding region is located at the 5′ end of ARR2 and can be mapped between nucleotides −561/−574.

To test the biological relevance of the binding of Nrg1 to the ARR2 region, we carried out chromatin immunoprecipitation experiments. For this purpose, we replaced in the wild type strain W303-1A the native *NRG1* open reading frame with a HA-tagged version and immunoprecipitated, using anti-HA antibodies, fragmented genomic DNA prepared from this strain. As shown in Fig. 6, from the immunoprecipitated DNA we could specifically amplify fragments surrounding the ARR2 region, indicating that binding was occurring *in vivo* under standard culture conditions. More importantly, when the same experiment was made in cells subjected to alkaline shock for 30 min, the amount of amplified material was substantially reduced. It is worth noting that this does not appear to be a general, nonspecific effect because in a parallel experiment, using HA-tagged Med8 (which binds to the *HXK2* gene), binding was not altered in cells exposed to high pH (not shown). These results provide evidence that Nrg1 binds *in vivo* to the ARR2 region and that this binding is altered as a result of sudden environmental alkalinization.

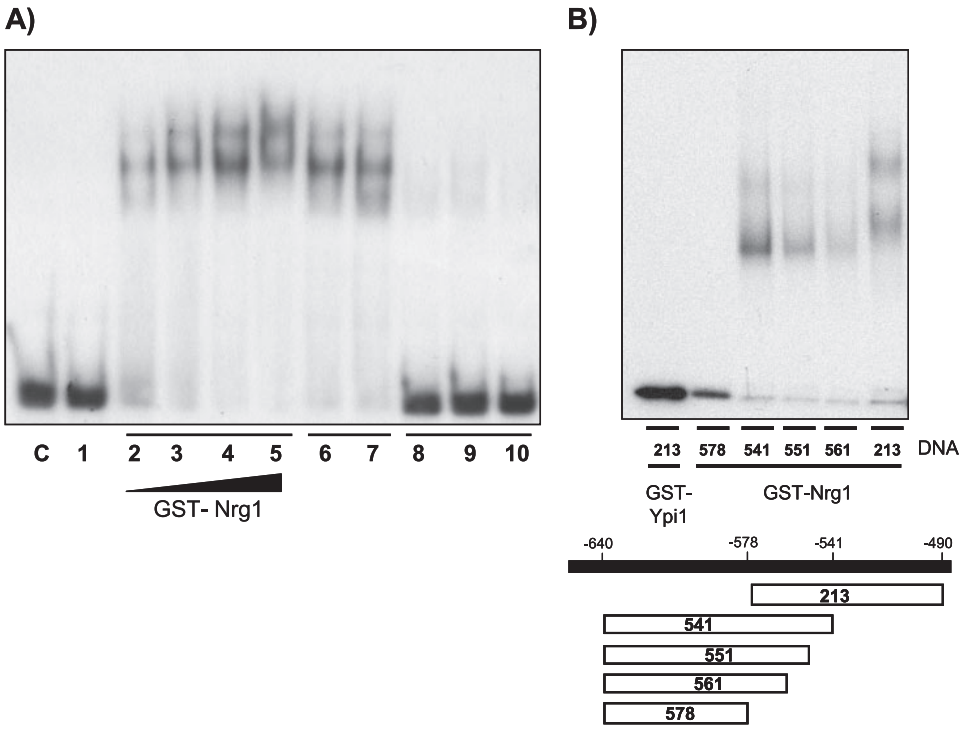

FIGURE 5. **Nrg1 binds *in vitro* to the ARR2 region of the *ENA1* promoter.** *A*, electrophoretic mobility shift assays were performed with purified protein GST-Nrg1 and ^{32}P-labeled probe 213, which corresponds to the ARR2 region. *C*, no added protein; *1*, GST (0.05 μg); *2–5*, increasing amounts of GST-Nrg1 (0.025, 0.05, 0.1, and 0.2 μg); *6* and *7*, 0.05 μg of GST-Nrg1 plus increasing amount of nonspecific competitor (16- and 32-fold molar excess of probe 578); *8–10*, 0.05 μg of GST-Nrg1 plus unlabeled specific competitor (16-, 32-, and 64-fold molar excess of probe 213). *B*, electrophoretic mobility shift assays were performed with 0.15 μg of GST-Nrg1 and different ^{32}P-labeled fragments that include segments of the ARR2 region as depicted. GST-Ypi1 (0.15 μg) was included as negative binding control. The labeled 213 probe plus 0.15 μg of GST-Nrg1 is included as a positive control. Fragments of the *ENA1* promoter included in each probe are denoted by *boxes* and named (except for 213) after the corresponding relative position of their 3′ extreme (nucleotide positions from the initiating Met codon).

Induction of the ENA1 Promoter by Ambient Alkalinization Involves Three Main Signaling Pathways—The results obtained indicated that the calcineurin-independent activation in response to high pH of the ARR2 region of the *ENA1* promoter was controlled through the Snf1 and Rim101 pathways. Therefore, calcineurin, Snf1, and Rim101 would define the main elements governing the transcriptional response of the *ENA1* gene to ambient alkalinization. In this case, one would expect that blockage of these three pathways would result in the incapacity of the *ENA1* promoter to respond to alkaline stress. To test this hypothesis, we combined the *snf1* and *rim101* mutations and tested expression from the reporter plasmid pKC201, which contains the entire *ENA1* promoter, in the presence of the calcineurin inhibitor FK506. As shown in Fig. 7*A*, systematic blockage of each signaling component results in additive loss of transcriptional response, whereas loss of all three elements results in a fully insensitive promoter. Therefore, calcineurin, Snf1, and Rim101 define three pathways that account for virtually every positive input to the *ENA1* promoter in response to extracellular alkalinization. However, the relevance of the different pathways in control of the *ENA1* promoter is not the same and, under this specific experimental condition, a sequence Snf1 > calcineurin > Rim101 can be established (Fig. 7*A*). These results were substantiated by direct analysis of the *ENA1* mRNA levels by real-time reverse transcriptase-PCR in different mutants after exposure to alkaline stress (Fig. 7*A*, *inset*). As observed using the β-galactosidase reporter, inhibition of calcineurin in a strain lacking Snf1 and Rim101 fully blocks the high pH-induced expression of the *ENA1* gene. In contrast, induction of the *PHO84* gene was unaltered in this strain when compared with wild type cells (not shown).

As mentioned in the Introduction, *ENA1* expression can be induced by saline as well as by alkaline stress. We wanted to compare the relative relevance of the pathways studied in this work in response to both types of stress. Cells were exposed to pH 7.9 or 0.4 M NaCl, because these treatments provoke an *ENA1* response of similar potency and timing. Under these conditions (Fig. 7*B*), the induction of *ENA1* by saline stress and high pH was similarly decreased in a calcineurin mutant. In contrast, lack of Snf1 had a strong effect on the alkali-induced response but produced only a moderate decrease in expression in cells exposed to NaCl. Mutation of *RIM101* barely affected the response to saline stress.

We have also compared how blockage of the three signaling pathways affects cell tolerance to high pH. As shown in Fig. 8, chemical inhibition of the calcineurin pathway and mutation of *SNF1* decrease similarly the tolerance to high pH, whereas mutation of *RIM101* has a lesser effect. In all cases, the phenotype observed was less intense than that produced by the absence of the ATPase gene. However, cells lacking both Snf1 and Rim101 grown in the presence of the calcineurin inhibitor FK506 were clearly more sensitive to high pH than *ena1–4* cells.

DISCUSSION

The promoter of the *ENA1* ATPase gene can integrate a large number of signals produced in response to changes in the ionic composition of the environment. Previous work has defined in some detail how the calcineurin pathway regulates the expression of *ENA1* under alkaline pH stress (20, 21) and there is also compelling evidence that activation of the Rim101 pathway can result in increased *ENA1* expression by acting on the Nrg1 repressor (21, 24, 25), although in this case the target elements in the *ENA1* promoter were still undefined. It was evident that activation of the calcineurin and Rim101 pathways could not fully explain the response of the ATPase gene under alkaline conditions (21), indicating that additional mechanisms must be involved.

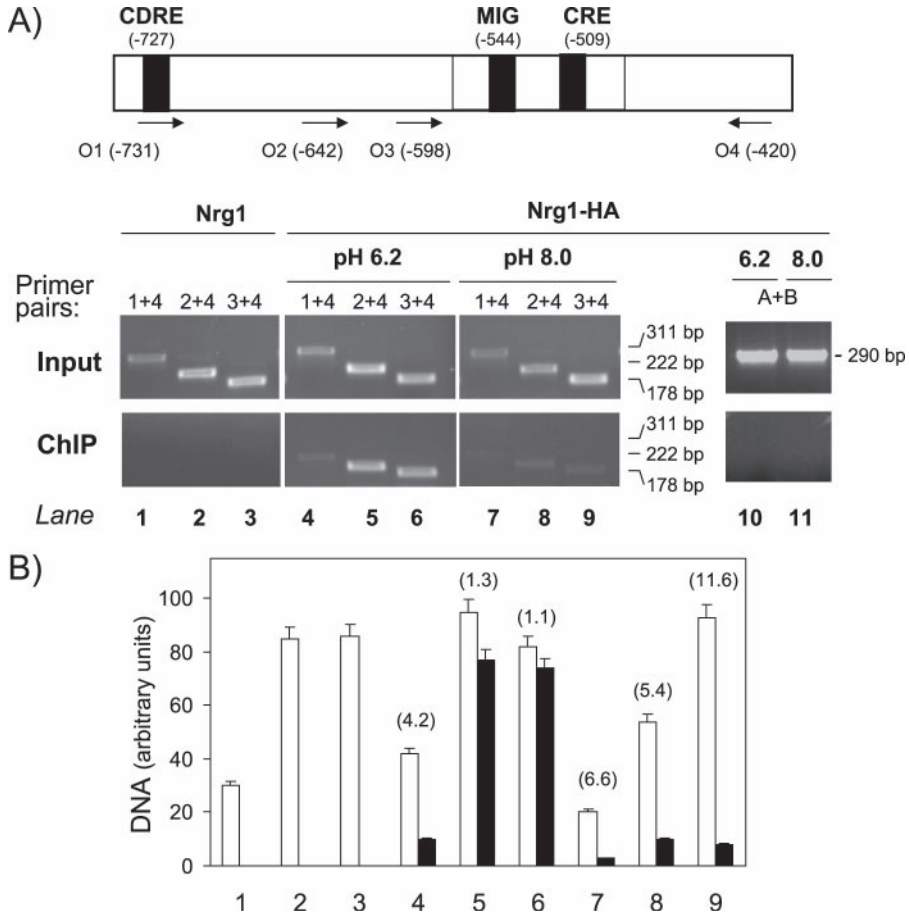

FIGURE 6. ***In vivo* binding of Nrg1 to the ARR2 region of the *ENA1* promoter.** *A*, schematic depiction of the region of the *ENA1* promoter amplified. The *dashed box* denotes the ARR2 region. Wild type strain W303-1A containing the native (*Nrg1*) or the HA-tagged version of Nrg1 (*Nrg1-HA*) were grown on YPD medium and switched at the indicated pH values for 30 min before cells were collected. Fragmented DNA was obtained and immunoprecipitated as described under "Experimental Procedures" and amplified by PCR using the indicated combination of oligonucleotides. *Lanes 10* and *11* show the amplification of a region of the *HXK2* promoter with oligos A (ACTACGAGTTTTCTGAACCTCC) and B (TAATTTCGTGGATCTCGCAATC). This region contains an Rgt1 binding site but is devoid of Nrg1 binding sequences. The amplification fragments were resolved by agarose electrophoresis. Migration of standard markers is indicated on the *right*. *B*, the intensity of the amplification DNA band from the input sample (*empty bars*) and the chromatin immunoprecipitate (*filled bars*) obtained for each condition was integrated and the ratio calculated when possible (*numbers in parentheses*). Data represent the mean ± S.D. of three experiments in duplicate.

In this work we delineate the three main regulatory pathways that would account for the full response of *ENA1* under alkaline stress and their possible interactions (Fig. 9). As previously reported, calcineurin would control *ENA1* by binding of the calcineurin-activated transcription factor Crz1 to the calcineurin-dependent response element present in the ARR1 region. The calcineurin-independent response would be under the control of two additional pathways. One of them would involve the MIG binding site at nucleotides −534/−544, previously defined as important for response to glucose starvation (9). Our results suggest that both Mig1 and Mig2 act on the ARR2 region, as expression from the MCIR is stronger in the *mig1 mig2* strain than in each single mutant, but that probably only Mig2 is under the control of Snf1 (Fig. 2). This would explain the early observation by Alepuz and co-workers (10) that a *snf1* mutant displays decreased *ENA1* expression at pH 8.5 when compared with a wild type strain. The Snf1 kinase is a key regulator of the yeast response to decreased glucose availability (38) and the activation of this kinase in response to alkaline stress suggests that exposure to high pH could mimic a situation of glucose starvation. This notion would be supported by the observation that severe alkaline stress induces the expression of many genes that are also induced by low glucose availability (20). The third pathway would be defined by Rim101. Our data support the previous observation that the impaired expression of *ENA1* in a *rim101* mutant is overridden by deletion of Nrg1 (25). Interestingly, we observe that Nrg2 does not seem to play a role in repression of *ENA1* under high pH stress. This could be considered unexpected, because Nrg1 and Nrg2 are closely related proteins and a recent genomic survey revealed that both proteins exert a similar control over most Nrg-regulated promoters (39).

Our results provide the basis to understand how Nrg1 regulates *ENA1* expression. Previous results indicated that alkaline-induced expression from both ARR1 and ARR2 regions were sensitive to the absence of Rim101 (21). It has been suggested that Nrg1 could bind to two sequences (CCCCT and CCCTC) that occur in the *ENA1* promoter at positions −650 and −725, respectively (25). However, whereas the latter may well account for the decreased response from the ARR1 region in a *rim101* strain, the former occurs in a region between ARR1 and ARR2 that has been shown to be insensitive to high pH (21). In addition, this location would not explain the presence of a Rim101-sensitive component within the ARR2 region (21). In this work we present evidence that the Nrg1 binding element must be located at the 5′-end of ARR2, comprising nucleotides −561/−573. Interestingly, this short region does contain, in the non-coding strand, a AGACCCT sequence that is somewhat different from the initially proposed sequence for recognition by Nrg1 (37) but that closely matches the consensus sequence ggACCCT, identified in very recent studies as a very likely Nrg1 binding element (40, 41). Therefore, we propose that this sequence is responsible for the control exerted by the Rim101 pathway on the ARR2 region of *ENA1*. The fact that we detect *in vivo* binding of Nrg1 to the ARR2 region and that this binding is substantially decreased upon exposure of the cells to high pH stress provides further support to our hypothesis and suggest that Nrg1 binding to this region has physiological relevance.

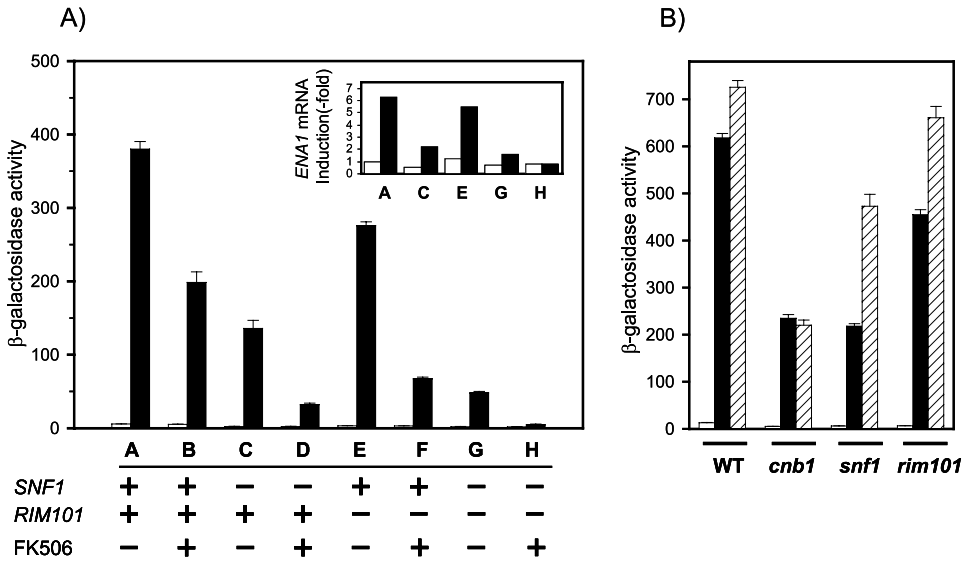

FIGURE 7. **Activation of three signaling pathways, calcineurin, Snf1, and Rim101, accounts for full response of the *ENA1* promoter to alkaline stress.** *A*, the indicated strains were transformed with the pKC201 plasmid that contains the entire promoter of *ENA1* and cells were subjected to alkaline stress in the presence or absence of FK506 (1.5 μg ml^{-1}). *Empty bars* denote β-galactosidase activity under non-stressing conditions (pH 6.3) and *filled bars* under stressing conditions (pH 8.0). Data are mean ± S.E. from at least six independent clones. The *inset* shows the induction of the *ENA1* gene evaluated by real-time PCR in the indicated strains or conditions. The expression level of the non-induced (pH 6.3, *empty bars*) wild type strain was considered as the unit. *Filled bars* denote cells subjected to alkaline stress (pH 8.0) for 10 min. Data represent the mean of two independent determinations. *B*, the indicated strains were transformed with the pKC201 plasmid and subjected to alkaline (pH 7.9, *filled bars*) or saline stress (0.4 M NaCl, *hatched bars*) for 60 min before cells were collected and β-galactosidase activity measured. Data represent the mean ± S.E. of six experiments.

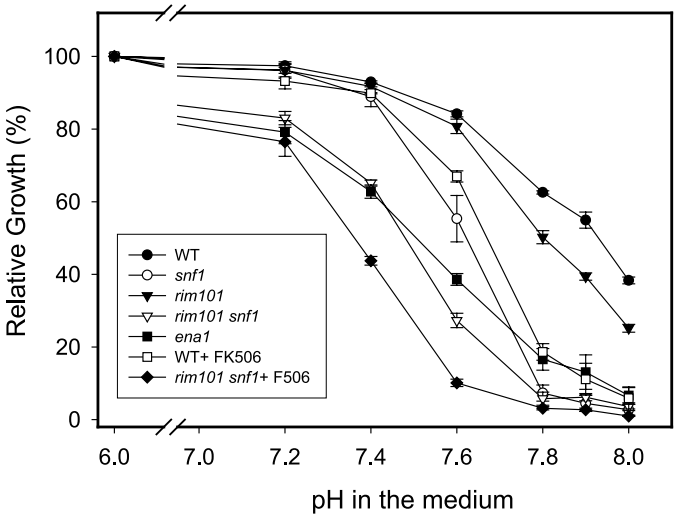

FIGURE 8. **Effect of blockage of calcineurin, Snf1, and Rim101 pathways on high pH tolerance.** The indicated strains were inoculated at an initial A_{660} of 0.01 and in YPD medium buffered at the indicated pH values. FK506 was used at 1.5 μg ml^{-1}. Growth is represented as the percentage of cell density at a given pH relative to cells growing at pH 6.0. Data correspond to the mean ± S.E. of three experiments.

We observe that mutation of *NRG1* largely counteracts the decrease in response from the MCIR of both the *rim101* and the *snf1* mutants, suggesting that the ability of Nrg1 to interact and repress the *ENA1* promoter would be under the dual regulation of Rim101 and Snf1, as depicted in Fig. 9. Therefore, Snf1 would control two different repressors, Mig2 and Nrg1. This would fit with the observation that derepression under alkaline stress is somewhat stronger in a *rim101 nrg1* mutant than in a *snf1 nrg1* strain and would explain why the absence of Snf1 causes a stronger effect on *ENA1* expression than that of Rim101 (Figs. 3 and 7*A*). The possibility of Snf1 controlling the activity of Nrg1 on a given promoter is not surprising, as previous evidence indicates that Nrg1 interacts with the Snf1 kinase (42) and has a role in regulating diverse glucose-repressed genes (37, 42, 43).

On the basis of previously reported data and the experiments presented here it is clear that the mechanisms for controlling *ENA1* expression in response to saline and alkaline stress are somewhat overlapping but not identical. Whereas the calcineurin pathway plays a relevant role in both saline and alkaline stress, mapping of the ARR2 region clearly shows that the cAMP response element responsible for derepression of the *ENA1* promoter in response to osmotic stress (9, 44) is irrelevant under alkaline stress. This result allows explaining the previous observations that a *hog1* mutant has unaltered *ENA1* transcriptional response (21) and does not display increased sensitivity under high pH conditions. Our observation that blocking calcineurin, Snf1, and Rim101 pathways fully abolishes the response from the *ENA1* promoter suggests that, in contrast to what has been described for saline stress (19), the TOR pathway and the Gln3/Gat1 GATA transcription factors are not relevant for *ENA1* regulation under alkaline stress. This is remarkable, because Ure2, Gln3, and Gat1 have been found to be required for normal high pH tolerance (45–47). Therefore, the pH-related phenotypes of these mutant strains cannot be explained through a role for the corresponding gene products in the control of *ENA1* expression.

The Ena1 ATPase plays an important role in the adaptation to high pH conditions, as evidenced by the intense pH-sensitive phenotype of a strain defective in the ATPase function. However, we observe (Fig. 8) that simultaneous blockage of the three main pathways governing *ENA1* expression under alkaline stress results in a phenotype substantially stronger than that observed for the ATPase-deficient strain. This clearly indicates that one or more of these pathways play role(s) relevant for high pH tolerance that are independent of the ATPase function. Although this investigation is out of the scope of the present work, possible alternatives can be envisaged. For instance, it is suggestive that the calcineurin pathway was found to be relevant in the maintenance of cell integrity in front of cell wall damaging conditions (48, 49), whereas exposure to high pH has

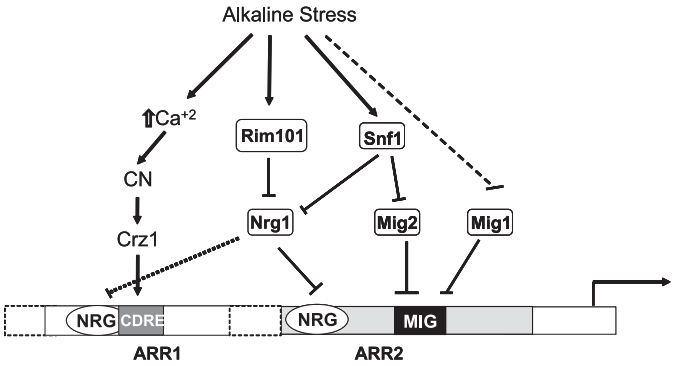

FIGURE 9. **Schematic model for the signaling pathways mediating response of the *ENA1* promoter to alkaline stress.** Regulatory elements in the promoter are depicted schematically. *Dotted lines* denote still uncharacterized processes. The effect of the calcineurin (*CN*) pathway on *ENA1* expression was reported previously (20, 21). The regulation exerted by Nrg1 on the ARR1 region is presumed from data reported previously using *rim101* mutants and from the identification of a putative Nrg1 binding sequence (21, 25).

been recently proposed as a circumstance able to induce cell wall damage (20).[7]

Acknowledgments—We thank A. Friedrich and F. Pérez-Bermejo (Astellas Pharma, formerly Fujisawa Co.) for kindly supplying the calcineurin inhibitor FK506. The excellent technical assistance of Anna Vilalta and María Jesús Álvarez is acknowledged.

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