

# Chloride Homeostasis in *Saccharomyces cerevisiae*: High Affinity Influx, V-ATPase-dependent Sequestration, and Identification of a Candidate Cl<sup>-</sup> Sensor

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Chloride homeostasis in *Saccharomyces cerevisiae* has been characterized with the goal of identifying new Cl<sup>-</sup> transport and regulatory pathways. Steady-state cellular Cl<sup>-</sup> contents (~0.2 mEq/liter cell water) differ by less than threefold in yeast grown in media containing 0.003–5 mM Cl<sup>-</sup>. Therefore, yeast have a potent mechanism for maintaining constant cellular Cl<sup>-</sup> over a wide range of extracellular Cl<sup>-</sup>. The cell water:medium [Cl<sup>-</sup>] ratio is >20 in media containing 0.01 mM Cl<sup>-</sup> and results in part from sequestration of Cl<sup>-</sup> in organelles, as shown by the effect of deleting genes involved in vacuolar acidification. Organellar sequestration cannot account entirely for the Cl<sup>-</sup> accumulation, however, because the cell water:medium [Cl<sup>-</sup>] ratio in low Cl<sup>-</sup> medium is ~10 at extracellular pH 4.0 even in *vma1* yeast, which lack the vacuolar H<sup>+</sup>-ATPase. Cellular Cl<sup>-</sup> accumulation is ATP dependent in both wild type and *vma1* strains. The initial <sup>36</sup>Cl<sup>-</sup> influx is a saturable function of extracellular [<sup>36</sup>Cl<sup>-</sup>] with K<sub>1/2</sub> of 0.02 mM at pH 4.0 and >0.2 mM at pH 7, indicating the presence of a high affinity Cl<sup>-</sup> transporter in the plasma membrane. The transporter can exchange <sup>36</sup>Cl<sup>-</sup> for either Cl<sup>-</sup> or Br<sup>-</sup> far more rapidly than SO<sub>4</sub><sup>2-</sup>, phosphate, formate, HCO<sub>3</sub><sup>-</sup>, or NO<sub>3</sub><sup>-</sup>. High affinity Cl<sup>-</sup> influx is not affected by deletion of any of several genes for possible Cl<sup>-</sup> transporters. The high affinity Cl<sup>-</sup> transporter is activated over a period of ~45 min after shifting cells from high-Cl<sup>-</sup> to low-Cl<sup>-</sup> media. Deletion of ORF YHL008c (formate-nitrite transporter family) strongly reduces the rate of activation of the flux. Therefore, Yhl008cp may be part of a Cl<sup>-</sup>-sensing mechanism that activates the high affinity transporter in a low Cl<sup>-</sup> medium. This is the first example of a biological system that can regulate cellular Cl<sup>-</sup> at concentrations far below 1 mM.

## INTRODUCTION

The regulation of the cytosolic concentrations of inorganic ions is one of the fundamental functions of the plasma membranes of all cells. Many ion homeostatic mechanisms are reasonably well understood, especially with regard to Na<sup>+</sup>, K<sup>+</sup>, and Ca<sup>2+</sup>, in which the primary generators of ion gradients are ATP-driven pumps. The general principle of regulation of these cations is that the steady-state level of an ion depends on a balance between uphill and downhill transport processes (pump-leak paradigm) (Tosteson and Hoffman, 1960; Lew et al., 1982).

Regulation of cellular Cl<sup>-</sup> is less well understood. In general, the steady-state cytosolic Cl<sup>-</sup> concentration is determined by a combination of activities of cation-Cl<sup>-</sup> cotransporters (Haas and Forbush, 1998; Lu et al., 1999; Rivera et al., 1999), Cl<sup>-</sup>-HCO<sub>3</sub><sup>-</sup> (or other base) exchangers (Alper et al., 2002; Soleimani and Xu, 2006), and Cl<sup>-</sup> channels (Jentsch et al., 2002). In order for cells to regulate cytosolic Cl<sup>-</sup>, there must be mechanisms for sensing the Cl<sup>-</sup> concentration. One known mechanism is the inhibition of the Na<sup>+</sup>/K<sup>+</sup>/2Cl<sup>-</sup> cotransporter by increases in cytosolic [Cl<sup>-</sup>] (Lytle and

Forbush, 1996; Gillen and Forbush, 1999; Russell, 2000; Lytle and McManus, 2002), which limits net influx. Possible Cl<sup>-</sup>-sensing functions may also be associated with CFTR (Jiang et al., 1998) and other airway epithelial Cl<sup>-</sup> channels (Tarran et al., 2000), although the molecular identities of the actual Cl<sup>-</sup> sensors are not known.

The purpose of the work described here is to determine whether *Saccharomyces cerevisiae* is a useful system for the study of mechanisms of regulation of cellular Cl<sup>-</sup>. Very little is known about Cl<sup>-</sup> transport and regulation in yeast. Instead, attention has been focused on the wealth of K<sup>+</sup>, Na<sup>+</sup>, and H<sup>+</sup> transporters and channels available for study in this organism (Gustin et al., 1986; Nass and Rao, 1998, 1999; Bihler et al., 1999; Morsomme et al., 2000; Kuroda et al., 2004). In the early literature on ion transport in yeast, Cl<sup>-</sup> was considered an impermeant ion (Conway and Downey, 1950; Rothstein, 1974), because Cl<sup>-</sup> transport is far slower than that of many other ions. Most recently Coury et al. (1999) showed that

Abbreviations used in this paper: APG, arginine/phosphate/glucose medium; 2-DG, 2-deoxy-D-glucose; DNP, 2,4-dinitrophenol; LCAPG, low Cl<sup>-</sup> arginine/phosphate/glucose medium; LCYNB, low-Cl<sup>-</sup> yeast nitrogen base medium; V-ATPase, vacuolar H<sup>+</sup>-ATPase; YNB, yeast nitrogen base medium; YPGE, medium containing 1% yeast extract, 2% peptone, 2% glycerol, 2% ethanol.

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the influx of  $^{36}\text{Cl}^-$  into *S. cerevisiae* is almost negligible compared with that of  $^{86}\text{Rb}^+$  or phosphate.

Although the fluxes are small, there is evidence that  $\text{Cl}^-$  transport has physiological importance in *S. cerevisiae*. Gef1p is the sole member in *S. cerevisiae* of the CLC family (Jentsch et al., 1999), which includes mammalian  $\text{Cl}^-$  channels and as well as a bacterial protein of known structure (Dutzler et al., 2002; Dutzler et al., 2003) that acts as coupled exchanger of  $\text{H}^+$  for  $\text{Cl}^-$  (Accardi and Miller, 2004). The phenotype of *gef1* strains is slow growth at low  $\text{Fe}^{3+}$  concentrations, indicating a role for Gef1p in iron metabolism (Greene et al., 1993). Gef1p is expressed in Golgi or post-Golgi, prevacuolar vesicles (Schwappach et al., 1998; Gaxiola et al., 1999). The  $\text{Cl}^-$  ions transported into these vesicles by Gef1p have two functions. First, the  $\text{Cl}^-$  flux neutralizes the  $\text{H}^+$  pumped into the vesicle by the V-type  $\text{H}^+$ -ATPase (Wada and Ohsumi, 1992; Gaxiola et al., 1998). The second role of  $\text{Cl}^-$  is to act as a direct cofactor for  $\text{Cu}^{2+}$  loading on Fet3p (Davis-Kaplan et al., 1998). In addition to the role of Gef1p as an organellar  $\text{Cl}^-$  transport pathway, there is recent evidence that Gef1p is also expressed on the plasma membrane (López-Rodríguez et al., 2007).

Little is known about the distribution of  $\text{Cl}^-$  across the plasma membrane of *S. cerevisiae*. Yeast is expected to have a relatively low intracellular  $\text{Cl}^-$  concentration, because 100 mM  $\text{Cl}^-$  inhibits transcription in extracts of *S. cerevisiae* (Lue and Kornberg, 1987). From the steady-state accumulation of  $^{36}\text{Cl}^-$ , Coury et al. (1999) calculated that the intracellular  $[\text{Cl}^-]$  is  $\sim 0.15$  mM at extracellular pH 4.0 and extracellular  $[\text{Cl}^-]$  of 5 mM. This value of cellular  $[\text{Cl}^-]$  is of course an average over the whole cell, including cytosol, endoplasmic reticulum, Golgi, nucleus, mitochondria, endosomes, and vacuole(s). Although the most prominent ion channels in the vacuolar membrane of *S. cerevisiae* are cation selective (Bertl and Slayman, 1990), the membrane is permeable to  $\text{Cl}^-$  (Wada and Ohsumi, 1992), as expected given the role of  $\text{Cl}^-$  as a counterion for vacuolar acidification. Nothing is known about the steady-state distribution of  $\text{Cl}^-$  across the yeast vacuolar membrane.

The plasma membrane potential of *S. cerevisiae* is not known as accurately as that of *Neurospora crassa* (Ballarín-Denti et al., 1994), but it is probably in the range of  $-50$  to  $-120$  mV, with the magnitude depending on yeast strain and incubation conditions (Vacata et al., 1981). If the membrane potential is in this range, then a cytosolic  $[\text{Cl}^-]$  of 0.15 mM (Coury et al., 1999) in a medium containing 5 mM  $\text{Cl}^-$  is not far from that expected from a passive distribution. There are no published data on the cells:medium  $\text{Cl}^-$  distribution at either higher or lower  $[\text{Cl}^-]_o$ , except those of Conway and Downey (1950), in which intracellular  $\text{Cl}^-$  was difficult to distinguish from that trapped in the intercellular space.

There have been three published measurements of  $^{36}\text{Cl}^-$  fluxes in yeast. Groves et al. (1996) showed that

expression of the membrane domain of the erythrocyte anion exchanger AE1 (band 3) in yeast causes a large increase in the  $^{36}\text{Cl}^-$  influx, but the basal flux, though low, was measurable. Coury et al. (1999) measured the  $^{36}\text{Cl}^-$  influx (5 mM  $\text{Cl}^-$ ) in *S. cerevisiae* and found that it is dependent on extracellular pH (largest at pH 3) and is larger in the absence than in the presence of glucose, consistent with the idea that membrane depolarization increases the influx. The other  $^{36}\text{Cl}^-$  influx measurement was from this laboratory (Jennings et al., 2007) and indicated that there is a high affinity  $\text{Cl}^-$  transport pathway in yeast that does not involve the anion exchanger homologue Bor1p.

In the work described here we have determined the steady-state cellular  $\text{Cl}^-$  contents in *S. cerevisiae* grown in synthetic medium derived from YNB (Sherman, 1991) and APG (Rodríguez-Navarro and Ramos, 1984; Nass et al., 1997) in which  $[\text{Cl}^-]$  was varied from 0.003 to 100 mM. We find that the cellular  $\text{Cl}^-$  content varies only slightly over a wide range of extracellular  $[\text{Cl}^-]$ . The effects of deletion of genes involved in vacuolar acidification indicates that a significant fraction of the cellular  $\text{Cl}^-$  is sequestered in the vacuole or in prevacuolar vesicles. Even in cells having no vacuolar  $\text{H}^+$ -ATPase, the cellular  $[\text{Cl}^-]$  in media containing very low  $[\text{Cl}^-]$  is much higher than expected for electrochemical equilibrium. The elevated cellular  $[\text{Cl}^-]$  is the consequence of a pH-dependent high affinity  $\text{Cl}^-$  transporter that is activated rapidly upon exposure of cells to low  $[\text{Cl}^-]$  media. The molecular identity of the high affinity transporter is not known, but the rapid activation of the transporter is dependent on the gene product of ORF YHL008c, which is homologous to formate-nitrite transporters. YHL008cp therefore may be a component of a  $\text{Cl}^-$ -sensing mechanism.

## MATERIALS AND METHODS

### Yeast Strains and Media

Salts and buffers were purchased from either Fisher Scientific or Sigma-Aldrich. Some of the  $^{36}\text{Cl}^-$  distribution studies were performed in *S. cerevisiae* strain FKY 282 (*MATa SRP40 pep4::LEU2 ura3-12 leu2-3,-112 his3-11,-15 trp01-1 ade2-1*, kanamycin resistant) (Groves et al., 1996). Most of the distribution and all the transport studies were performed on haploid strains obtained from the Euscarf Yeast Deletion Project via Invitrogen. Strains were all in the background of BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). Deletions used in this study, with Euroscarf identification numbers, are as follows: BOR1 (Y01169), GEF1 (Y06838), NHX1 (Y04290), SUL1 (Y07176), TRK1 (Y01296), TRK2 (Y05121), VMA1 (Y03883), YBR235w (Y03375), YHL008c (Y00955), and YPR003c (Y02830).

Low-Cl YNB medium (LCYNB) was prepared as follows. All concentrations of salts, vitamins, trace minerals, and amino acids are as listed by Sherman (1991) for YNB medium (with 2% glucose and 20 mg/liter uracil), with the following substitutions to remove  $\text{Cl}^-$ :  $\text{Ca}(\text{OH})_2$  for  $\text{Ca}(\text{Cl})_2$ ;  $\text{Na}_2\text{SO}_4$  for  $\text{NaCl}$ ;  $\text{Fe}(\text{NO}_3)_3$  for  $\text{Fe}(\text{Cl})_3$ ; arginine, lysine, and histidine-free bases instead of hydrochlorides. To make up for the base added with  $\text{Ca}(\text{OH})_2$ , the equivalent amount of  $\text{H}_2\text{SO}_4$  was added.

Low  $\text{Cl}^-$  medium based on APG (Rodriguez-Navarro and Ramos, 1984; Nass et al., 1997), designated here as LCAPG, was prepared using 10 mM arginine (free base), 8 mM  $\text{H}_3\text{PO}_4$ , 1 mM  $\text{KH}_2\text{PO}_4$ , 2 mM  $\text{MgSO}_4$ , 0.2 mM  $\text{Ca}(\text{OH})_2$ , and the other amino acids, vitamins, trace minerals, glucose, and uracil as in LCYNB described above. For the experiments examining the effect of extracellular  $[\text{K}^+]$ , the  $\text{KH}_2\text{PO}_4$  concentration was varied from 0.3 to 3 mM.

In most of the experiments the media included  $\text{Cl}^-$  present as pyridoxine HCl (2  $\mu\text{M}$ ) and thiamine HCl (1  $\mu\text{M}$ ). In addition, from the stated upper limits on the amounts of  $\text{Cl}^-$  in the salts used to prepare the media, we estimate that the final  $\text{Cl}^-$  concentration resulting from  $\text{Cl}^-$  in these salts is  $\sim 2 \mu\text{M}$ . For measurement of initial influx or steady-state distribution, the  $^{36}\text{Cl}^-$ -specific activity was calculated from the concentration of  $^{36}\text{Cl}^-$ , the specific activity of the  $\text{Na}^{36}\text{Cl}$  stock solution, and the isotope dilution caused by the presence of 5  $\mu\text{M}$  nonradioactive  $\text{Cl}^-$  (3  $\mu\text{M}$  from pyridoxine and thiamine if present, and 2  $\mu\text{M}$  as contaminants in other salts). In the  $^{36}\text{Cl}^-$  efflux experiments, in which it is important to minimize exchange of cellular  $^{36}\text{Cl}^-$  for nonradioactive  $\text{Cl}^-$ , and in the  $^{36}\text{Cl}^-$  distribution experiments at the lowest  $\text{Cl}^-$  concentrations, the  $\text{Cl}^-$  in the pyridoxine HCl and thiamine HCl stock solutions was removed with 50 mg Dowex 1 ( $\text{OH}^-$  form) per ml of stock solution. In the experiments with 2,4-dinitrophenol (DNP), possible contaminating  $\text{Cl}^-$  was removed by extracting DNP (Sigma-Aldrich) into ethyl ether from an aqueous solution that had been acidified to pH 2.0 with  $\text{H}_3\text{PO}_4$ .

#### Steady-State Cellular $\text{Cl}^-$

Cells from 1 ml of stationary culture were centrifuged, washed once in LCYNB or LCAPG medium, resuspended in 50 ml of the same medium containing 2% glucose and 20 mg/L uracil, and divided into aliquots.  $\text{Na}^{36}\text{Cl}$  (DuPont NEN) was added carrier-free to concentrations of between 1  $\mu\text{M}$  and 1 mM. (The half-life of  $^{36}\text{Cl}$  is so long [ $3 \times 10^5$  years] that a 1 mCi/ml stock solution contains  $\sim 2 \text{ M Na}^{36}\text{Cl}$ .) At total  $\text{Cl}^-$  concentrations  $> 0.3 \text{ mM}$ ,  $\text{Na}^{36}\text{Cl}$  was present at a concentration of 0.3 mM, and nonradioactive  $\text{Cl}^-$  was added as  $\text{NH}_4\text{Cl}$ ,  $\text{NaCl}$ , or  $\text{KCl}$ . Cultures were incubated aerobically overnight with rotary shaking at 225 RPM,  $30^\circ\text{C}$ . To determine the cellular  $\text{Cl}^-$  and the  $\text{Cl}^-$  distribution, cells were centrifuged 2 min at 4,000 RPM, and the  $^{36}\text{Cl}^-$  in aliquots of extracellular medium was determined by scintillation counting. Cells were immediately washed twice in ice-cold water and cellular  $^{36}\text{Cl}^-$  activity measured by liquid scintillation counting. Cellular  $\text{Cl}^-$  contents (mEq/liter cell water) were calculated from the extracellular specific activity, the amount of cell water present, and the total CPM in the cell pellet. The cell water (not including periplasm) was assumed to be 50% of the wet weight of the cell pellet, because 1 g (0.9 ml) of packed cells has total water of 0.78 g (Jennings et al., 2007), with 0.28 g ( $\sim 31\%$  of the pellet volume) of this water outside the plasma membrane (Conway and Downey, 1950). Cellular  $\text{K}^+$  was determined on lysates (following heating with 10 cell volumes of water for 15 min at  $100^\circ\text{C}$  and centrifuging cell solids) with a  $\text{K}^+$  selective electrode (Fisher Scientific).

In the experiments in Fig. 3, cells were grown overnight in LCAPG medium without  $^{36}\text{Cl}^-$ , centrifuged, and resuspended in fresh LCAPG medium, 2% glucose, plus 8  $\mu\text{M}$   $\text{Na}^{36}\text{Cl}$ . Media were buffered either with 5 mM K-citrate (initial pH 4.5), or with 5 mM K-citrate, 10 mM bistris, 20 mM tris (initial pH 7.6). After 2 h of aerobic incubation at  $30^\circ\text{C}$ , 5 ml aliquots of suspension were diluted in 40 ml of ice-cold water; centrifuged, the supernatant aspirated completely, and the cellular  $^{36}\text{Cl}^-$  determined by scintillation counting.

#### Unidirectional $^{36}\text{Cl}^-$ Influx and Efflux

Cells grown overnight in LCAPG were centrifuged and resuspended in fresh LCAPG, buffered as described in the figure legends. After incubating aerobically for 15 min at  $30^\circ\text{C}$ ,  $\text{Na}^{36}\text{Cl}$  was

added from a 10 mM stock solution to final total concentrations (including the small amount of nonradioactive  $\text{Cl}^-$  in the medium) of 9–133  $\mu\text{M}$ . Influx was allowed to proceed for various times (60 s for initial influx, longer times for total accumulation) before the suspension was diluted with 10 volumes of ice-cold water, centrifuged, and washed once with cold water. The  $^{36}\text{Cl}^-$  in the pellet was determined as described above. The efflux of  $^{36}\text{Cl}^-$  was measured by loading cells with  $^{36}\text{Cl}^-$  for at least 1 h,  $30^\circ\text{C}$ , in LCAPG, 5 mM K-citrate, pH 4.5, with 8  $\mu\text{M}$   $^{36}\text{Cl}^-$ . Cells were then centrifuged, resuspended in media containing no radioactivity, and incubated further as described in the figure legends. Cellular radioactivity was determined for most of the time points as the total activity minus the supernatant activity (following 1-min spin in a microfuge). At late time points, in which extracellular and total activity are similar, the cellular radioactivity was determined by counting the pellet directly.

Statistical analyses (paired  $t$  test) were performed using the statistical package in Sigma Plot (Systat).

## RESULTS

### Steady-State Cellular $\text{Cl}^-$ Contents

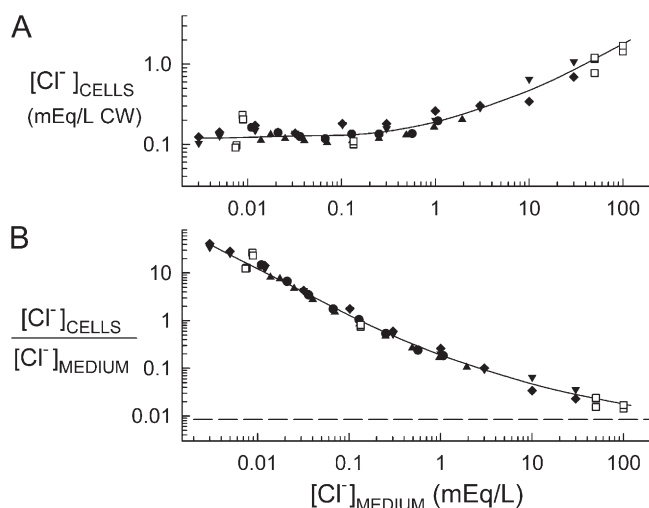
Fig. 1 A shows the cellular  $\text{Cl}^-$  contents (mEq/liter cell water) as a function of extracellular  $[\text{Cl}^-]$  in overnight cultures of haploid yeast strains grown aerobically in media containing 0.003–100 mM  $\text{Cl}^-$ . At  $\text{Cl}^-$  concentrations below 4 mM, the  $\text{Cl}^-$  normally present in YNB or APG was replaced by  $\text{SO}_4^{2-}$ . At higher concentrations,  $\text{Cl}^-$  was added as  $\text{NH}_4\text{Cl}$ ,  $\text{KCl}$ , or  $\text{NaCl}$ , with indistinguishable results.

Over a range of extracellular  $[\text{Cl}^-]$  from 0.003 to 5 mM, the cellular  $\text{Cl}^-$  contents are remarkably constant:  $0.2 \pm 0.1$  mEq/liter cell water, and the cell water:medium  $\text{Cl}^-$  distribution ratio varies over several orders of magnitude (Fig. 1 B). The dashed line represents the expected distribution if  $\text{Cl}^-$  were passively distributed across the plasma membrane, with a membrane potential of  $-120$  mV and  $\text{Cl}^-$  uniformly distributed in all cell water. If the actual membrane potential is closer to  $-180$  mV, as is true for *Neurospora crassa* (Ballarin-Denti et al., 1994), then the  $\text{Cl}^-$  ratio for a passive distribution would be 10-fold lower. It is clear that the observed  $\text{Cl}^-$  distribution is reasonably close to a passive distribution only at extracellular  $[\text{Cl}^-] > 10 \text{ mM}$ . At very low extracellular  $[\text{Cl}^-] (< 0.1 \text{ mM})$  the cellular  $\text{Cl}^-$  contents are orders of magnitude higher than those expected for a passive distribution.

### Effect of $\text{K}^+$ Gradient on $\text{Cl}^-$ Accumulation

It is known that  $\text{K}^+$  starvation hyperpolarizes the *S. cerevisiae* plasma membrane (Madrid et al., 1998); therefore, the membrane potential, though not known precisely, should be more negative in media of very low  $\text{K}^+$  concentration. To try to determine whether plasma membrane potential has any effect on the accumulation of cellular  $\text{Cl}^-$  at very low extracellular  $\text{Cl}^-$  concentrations, cells were grown in media with low  $\text{K}^+$  concentrations. The strain chosen for this experiment has a deletion for a gene, *SUL1*, that is not expressed at the methionine concentrations used here



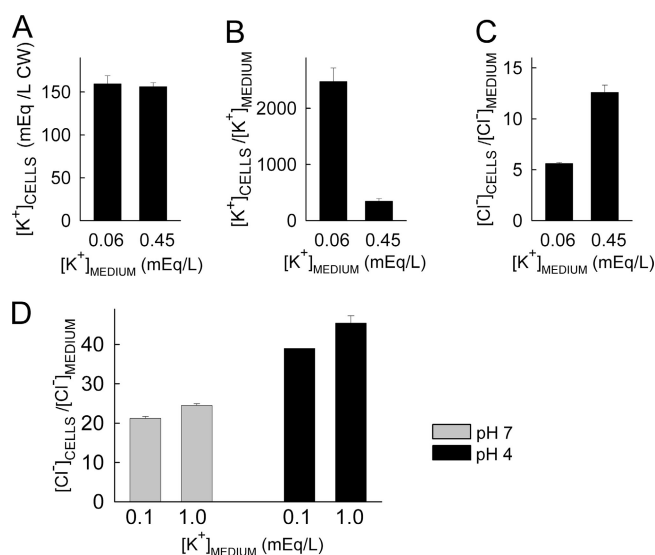


**Figure 1.**  $\text{Cl}^-$  contents (A) and distribution ratios (B) for *S. cerevisiae* grown in media containing varying concentrations of  $\text{Cl}^-$ . Solid symbols: cells were washed and suspended at  $A_{600}$  of 0.05 in media in which all but  $\sim 5 \mu\text{M}$   $\text{Cl}^-$  had been replaced by  $\text{SO}_4^{2-}$ , and  $\text{Cl}^-$  was added back as  $\text{Na}^{36}\text{Cl}$  (0.001–0.3 mM), plus, for higher concentrations, 0.7–100 mM  $\text{NH}_4\text{Cl}$ ,  $\text{KCl}$ , or  $\text{NaCl}$ . Suspensions were incubated aerobically 16–20 h at  $30^\circ\text{C}$ , and the cellular  $^{36}\text{Cl}^-$  contents were determined as described in Materials and methods. Unless otherwise indicated the final extracellular pH was  $3.5 \pm 0.5$ . Open symbols: cells were grown in APG, washed, and incubated 2 h at  $30^\circ\text{C}$  in APG containing various concentrations of  $^{36}\text{Cl}^-$  before determining cellular  $^{36}\text{Cl}^-$  contents. The dashed line in B is the distribution ratio predicted from a Nernst distribution, with plasma membrane potential of  $-120$  mV. The solid curves through the data have no theoretical significance.  $\blacklozenge$ : strain BY4741, YNB, 25 mM K-citrate buffer, final pH 4.4.  $\blacktriangledown$ ,  $\blacktriangle$ : strain BY4741, YNB.  $\bullet$ : strain FKY282, YNB.  $\square$ : SUL1 deletant (BY4741 background), 2 h, APG.

(Cherest et al., 1997); this strain is used for comparisons with other deletants in the same background (BY4741) in further experiments below.

Fig. 2 C shows the cells:medium  $[\text{Cl}^-]$  ratio in cultures grown 16 h in LCAPG medium containing  $5 \mu\text{M}$   $^{36}\text{Cl}^-$  (plus  $\sim 5 \mu\text{M}$  nonradioactive  $\text{Cl}^-$ ) and (initially) either 0.3 mM or 1 mM  $\text{K}^+$ . (We found, in agreement with other laboratories [Rodriguez-Navarro and Ramos, 1984; Hess et al., 2006], that similar experiments could not be performed in YNB medium because of the toxicity of  $\text{NH}_4^+$  in low  $\text{K}^+$  media). In medium initially containing 0.3 mM  $\text{K}^+$ , the final cell density is only about half that in 1 mM  $\text{K}^+$ , but the cellular  $\text{K}^+$  ( $\sim 150$  mEq/liter cell water) is the same in both media (Fig. 2 A). The cells:medium  $^{36}\text{Cl}^-$  ratio is lower in cells grown in low  $\text{K}^+$  medium. In four measurements on two separate cell preparations, the cells:medium  $^{36}\text{Cl}^-$  ratio in media with 0.06 mM  $\text{K}^+$  is less than half that in the medium with 0.45 mM  $\text{K}^+$  (Fig. 2 C).

The effect of the  $\text{K}^+$  gradient on the  $\text{Cl}^-$  distribution is much smaller if the cells are grown in LCAPG medium containing 2 mM  $\text{K}^+$  and then incubated for 2 h in LCAPG medium containing  $8 \mu\text{M}$   $^{36}\text{Cl}^-$  and either

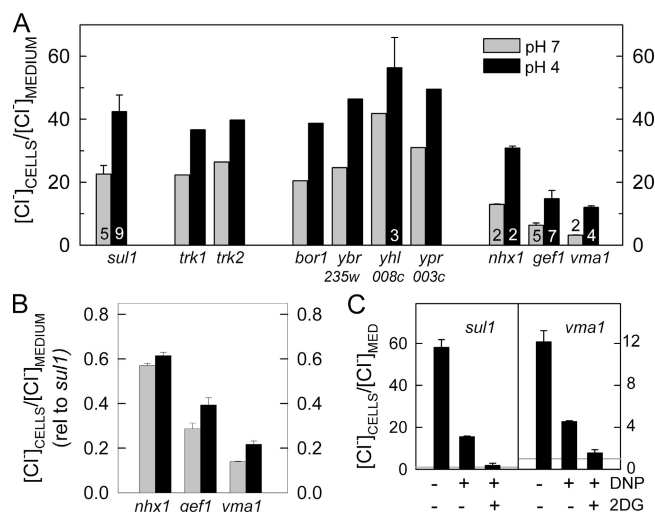


**Figure 2.** (A–C) Effect of low extracellular  $\text{K}^+$  on the cells:medium  $^{36}\text{Cl}^-$  ratio of cells grown in low  $\text{Cl}^-$  medium. Cells (*sul1* in BY4741 background) were grown 16 h in LCAPG, 2% glucose, and  $^{36}\text{Cl}^-$  (total of  $8 \mu\text{M}$   $\text{Cl}^-$ ). The medium initially contained either 1 mM  $\text{K}^+$  (normal APG) or 0.3 mM  $\text{K}^+$ . After aerobic growth at  $30^\circ\text{C}$  for 16 h, cells were centrifuged, washed once in cold water, and the  $[\text{K}^+]$  (B) and  $[\text{Cl}^-]$  (C) distribution ratios were measured. The final extracellular  $\text{K}^+$  concentrations were 0.45 mM and 0.06 mM, respectively, for the media that had initially contained 1 mM and 0.3 mM  $\text{K}^+$ . The total cellular  $\text{K}^+$  contents were the same in the two media (A). (D) Effect of low extracellular  $\text{K}^+$  on the cells:medium  $^{36}\text{Cl}^-$  ratio of cells following a 2-h incubation. Cells (*sul1*) were grown 16 h in LCAPG medium containing 2 mM  $\text{K}^+$  and then incubated 2 h in fresh LCAPG medium containing  $8 \mu\text{M}$   $^{36}\text{Cl}^-$  and either 0.1 mM or 1 mM  $\text{K}^+$ . The media were buffered either at initial pH 4.5 with 5 mM Na-citrate or initial pH 7.6 with K-citrate, 10 mM bistris, 20 mM tris.

0.1 mM or 1 mM  $\text{K}^+$  (Fig. 2 D). There is slightly less  $\text{Cl}^-$  in the cells incubated in 0.1 mM  $\text{K}^+$ , but the difference is smaller than that observed following a 16-h incubation. Therefore, the effect of extracellular  $\text{K}^+$  observed following a 16-h incubation does not appear to be a direct effect of the  $\text{K}^+$  gradient. Instead, adjustments in gene expression that allow the cells to grow in very low  $\text{K}^+$  may have indirect effects on the accumulation of  $\text{Cl}^-$ . Because there is little acute effect of the  $\text{K}^+$  gradient on cellular  $\text{Cl}^-$ , we can draw no conclusions about the effect of membrane potential on the cells:medium  $^{36}\text{Cl}^-$  ratio in low  $\text{Cl}^-$  medium.

#### Effect of Extracellular pH

To test the effect of extracellular pH on  $\text{Cl}^-$  distribution, cells were grown overnight in LCAPG medium ( $\sim 5 \mu\text{M}$   $\text{Cl}^-$ ) and then incubated at least 2 h in fresh medium containing  $8 \mu\text{M}$   $\text{Na}^{36}\text{Cl}^-$ , buffered at either pH 4.0 or pH 7. In all strains tested, the steady-state  $\text{Cl}^-$  contents are substantially higher at extracellular pH 4.0 than at pH 7.0 (Fig. 2 D and Fig. 3 A), suggesting a possible  $\text{H}^+$ -coupled  $\text{Cl}^-$  influx.



**Figure 3.** (A) Cell water:medium distribution ratio of  $^{36}\text{Cl}^-$  in various deletant strains. Cells were grown overnight in LCAPG and then incubated 2 h at 30°C in fresh medium plus 8  $\mu\text{M}$   $\text{Na}^{36}\text{Cl}$ . Cellular  $^{36}\text{Cl}^-$  contents at the end of the incubation were determined as described in Materials and methods. Media were buffered with 5 mM K-citrate (initial pH 4.5, final pH 4.1; black bars), or 5 mM K-citrate, 10 mM bistris, 20 mM tris (initial pH 7.6, final pH 6.7; gray bars). Strains with deletions of the indicated genes were all in the background of BY4741. Bars labeled *yhl*, *ybr*, and *ypr* refer respectively to strains deleted in YHL008c, YBR235w, and YPR003c. Data represent single determinations except for bars labeled to indicate the number of determinations. Error bars are SEM. (B) Cellular  $\text{Cl}^-$  contents in low  $\text{Cl}^-$  media in paired experiments with control strain *sul1*. Cells were incubated in LCAPG media containing 8  $\mu\text{M}$   $^{36}\text{Cl}^-$ , and cellular  $\text{Cl}^-$  contents were determined after sufficient time to reach a steady-state distribution (>1.5 h). Bars represent mean  $\pm$  SEM of the ratio between cellular  $[\text{Cl}^-]$  in the test strain (*nhx1*, *gef1*, or *vma1*) and control strain *sul1* at either pH 4.0 (black bars) or pH 7.0 (gray bars). Results of paired Student *t* tests (pH 4 and pH 7.0 combined): *sul1* vs. *nhx1*,  $P < 0.02$  ( $n = 4$ ); *sul1* vs. *gef1*,  $P < 10^{-5}$ ,  $n = 12$ ; *sul1* vs. *vma1*,  $P < 0.002$ ,  $n = 6$ . (C) Cells:medium  $^{36}\text{Cl}^-$  distribution ratio for *sul1* and *vma1* cells incubated as in A and B but with 0.2 mM DNP or DNP and 20 mM 2-deoxyglucose replacing 2% glucose during the 2 h incubation with  $^{36}\text{Cl}^-$ . The gray horizontal lines indicate a cells:medium ratio of unity.

#### Genes for Possible Plasma Membrane $\text{Cl}^-$ Transporters Have No Effect on $\text{Cl}^-$ Distribution

Deletion of any of several genes with homology to plasma membrane  $\text{Cl}^-$  transporters has no effect on  $\text{Cl}^-$  accumulation at either pH 4.0 or pH 7.0 (Fig. 3 A). For example, BOR1, the single ORF in the family of bicarbonate transporters (Alper et al., 2002; Romero et al., 2004), is known to catalyze boric acid efflux (Takano et al., 2002; Takano et al., 2007), probably as exchange with  $\text{H}^+$  (Jennings et al., 2007). Deletion of BOR1 does not affect  $^{36}\text{Cl}^-$  accumulation in a low  $\text{Cl}^-$  medium, in agreement with our earlier conclusion that Bor1p does not transport  $\text{Cl}^-$  (Jennings et al., 2007). Similarly, deletion of YBR235w, the single *S. cerevisiae* member of the  $\text{Cl}^-$ -coupled cation transporter family (André and Scherens, 1995; Gamba, 2005), also has no effect on  $^{36}\text{Cl}^-$  distribution. The same

is true of YPR003c, a member of the SCL26 family, which in humans includes several  $\text{Cl}^-$  transporters (Soleimani and Xu, 2006).

The effect of deletion of the genes for the  $\text{K}^+$  transporters Trk1p and Trk2p (Ko and Gaber, 1991) was also tested, because, under some conditions, these proteins can mediate a  $\text{Cl}^-$  current (Kuroda et al., 2004). There is no effect of deletion of either of these genes on  $\text{Cl}^-$  accumulation in a low  $\text{Cl}^-$  medium. This finding is consistent with the work of Kuroda et al. (2004), who showed that Trk1p and Trk2p do not mediate significant  $\text{Cl}^-$  currents when the cytosolic  $\text{Cl}^-$  concentration is  $<1$  mM. Another yeast  $\text{Cl}^-$  channel is the voltage-dependent anion channel in the mitochondrial outer membrane (Forte et al., 1987); this channel was not tested because mitochondria, with a matrix-negative inner membrane potential, should not be a major cellular  $\text{Cl}^-$  compartment.

In one strain, *yhl008c*, the data suggest that there is slightly more  $\text{Cl}^-$  than in any of the other strains (Fig. 3 A), although the difference between this strain and the control strain *sul1* is small and of questionable statistical significance ( $0.1 > P > 0.05$ ). The YHL008c ORF is homologous to formate-nitrite transporters (Suppmann and Sawers, 1994; Deloménie et al., 2007). This strain is examined further below in reference to regulation of  $\text{Cl}^-$  transport.

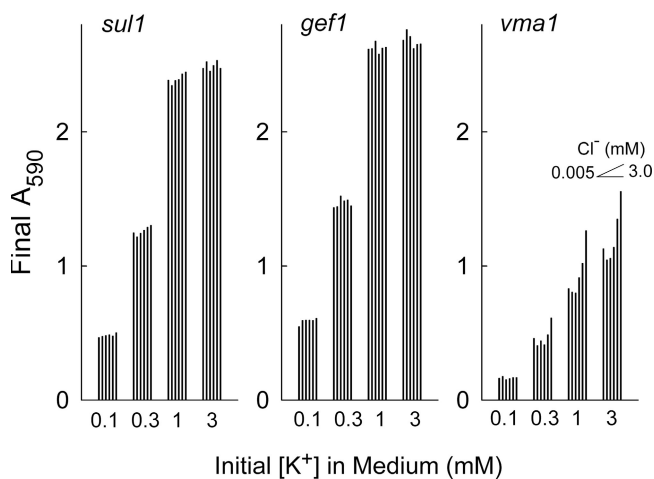
#### Genes Involved in Organellar Acidification Affect $\text{Cl}^-$ Distribution

The only gene deletions that were found to reduce steady-state  $\text{Cl}^-$  accumulation in low  $\text{Cl}^-$  medium are those involved in organellar acidification: the genes code for the subcellular  $\text{Na}^+\text{-H}^+$  exchanger Nhxl1p (Nass and Rao, 1998), the CLC family member Gef1p (Greene et al., 1993; Schwappach et al., 1998; Flis et al., 2002), and the vacuolar  $\text{H}^+$ -ATPase catalytic subunit Vma1p (Kane, 2006). Fig. 3 B shows the results of paired comparisons between each of these deletions and the control deletion *sul1*. In the *nhx1* strain the amount of cellular  $^{36}\text{Cl}^-$  is  $\sim 60\%$  that of the control strain at either pH 4.0 or pH 7. The effect of deletion of GEF1 is larger; cellular  $\text{Cl}^-$  is  $\sim 35\%$  of control. Deletion of VMA1 has a very large effect; cellular  $\text{Cl}^-$  in *vma1* cells is only  $\sim 14\%$  that of the control strain at pH 7, and 20% at pH 4.

The effects of deletion of NHX1, GEF1, or VMA1 are consistent with the idea that  $\text{Cl}^-$  moves through Gef1p into a subcellular compartment (post-Golgi, prevacuolar vesicles) as a counterion for the  $\text{H}^+$  pumped by the ATPase (Davis-Kaplan et al., 1998; Gaxiola et al., 1998), and that Nhxl1p facilitates the accumulation of  $\text{Cl}^-$  by exchanging cations for  $\text{H}^+$  in prevacuolar compartments (Nass and Rao, 1998, 1999).

#### Effect of ATP Depletion

Depletion of ATP has a major effect on the accumulation of  $^{36}\text{Cl}^-$  in low  $\text{Cl}^-$  media (Fig. 3 C). Cells from control

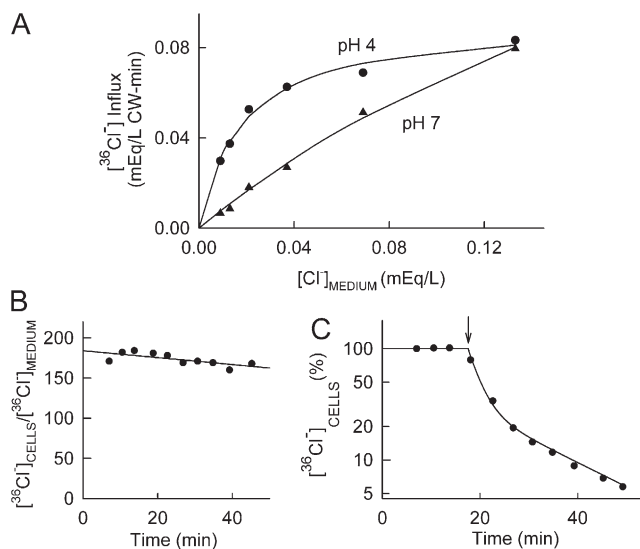


**Figure 4.** Effect of low  $K^+$  and  $Cl^-$  concentrations on growth of *sul1*, *gef1*, and *vma1* strains of *S. cerevisiae*. Cells from each strain were suspended at initial  $A_{590}$  of 0.1 and incubated aerobically at 30°C with rotary shaking for 24 h. The medium was derived from LCAPG, with increasing amounts of  $Cl^-$  added as arginine  $Cl^-$ . The initial concentrations of  $Cl^-$  in the media were ~0.005, 0.035, 0.10, 0.30, 1.0, and 3.0 mM (left to right) in each group of six bars. The initial concentrations of  $K^+$  (added as  $KH_2PO_4$ ) were 0.1, 0.3, 1.0, and 3.0 mM.

(*sul1*) or *vma1* strains were incubated 2 h in LCAPG containing 8  $\mu M$   $^{36}Cl^-$  as Fig. 3 A. The medium contained either 2% glucose, 0.2 mM 2,4-dinitrophenol (DNP), or DNP plus 20 mM 2-deoxoglucose (2-DG), which is known to strongly deplete ATP in *S. cerevisiae* (Chuang et al., 2005). As in Fig. 3 A, the cells:medium  $^{36}Cl^-$  ratio in the *vma1* strain in glucose medium is only ~20% of that in the *sul1* strain. DNP alone causes a decrease in the accumulation of  $^{36}Cl^-$ , but the cellular  $^{36}Cl^-$  in both strains is still higher than that in the medium. DNP plus 2DG reduces cellular  $^{36}Cl^-$  to levels that are not distinguishable from that in the medium. The fact that ATP depletion has a strong effect on  $^{36}Cl^-$  accumulation even in the *vma1* strain indicates that *S. cerevisiae* cells are capable of concentrating  $Cl^-$  by a mechanism that does not require the V-ATPase but that is dependent on cellular energy metabolism. Therefore, the cellular  $Cl^-$  in *vma1* cells is not likely to be bound passively to the cell wall or outer surface of the plasma membrane.

#### Minimal Effect of Extracellular $Cl^-$ on Growth

Despite the fact that *gef1* and *vma1* cells have significantly lower cellular  $Cl^-$  in LCAPG than other strains, there are at most very minor effects of low  $Cl^-$  on cell growth. Fig. 4 shows the final cell density of *sul1*, *gef1*, and *vma1* strains grown 24 h in APG media (final pH ~3.5) having various concentrations of  $Cl^-$  and  $K^+$ . Each of the four groups of six bars represents cells grown at a given initial  $K^+$  concentration with 0.005, 0.03, 0.1, 0.3, 1, and 3 mM  $Cl^-$ . As in Fig. 2, a low  $K^+$  concentration results in a lower final cell density, and the *vma1* strain, as expected (Kane, 2006), grows to a lower density than

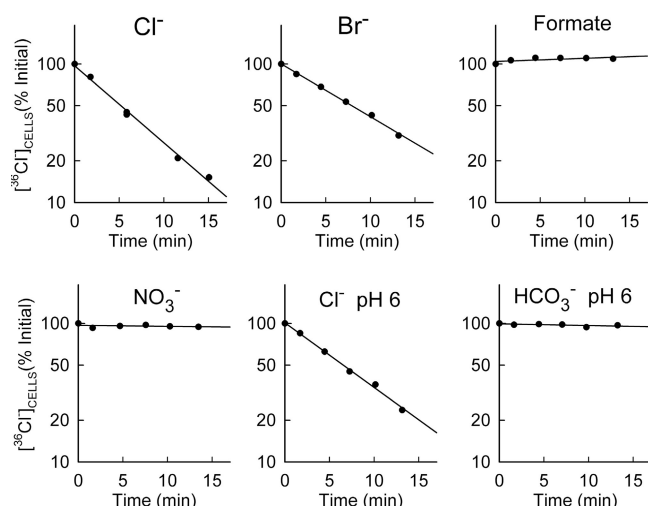


**Figure 5.** (A) Initial  $^{36}Cl^-$  influx as a function of concentration. Cells (*sul1*) were grown overnight in LCAPG and then incubated in fresh medium at either pH 4.0 or pH 7. Media were buffered with 5 mM K-citrate (initial pH 4.5, final pH 4) or 5 mM K-citrate, 10 mM bistris, 10 mM Tris (initial pH 7.6, final pH 6.8). The initial (1 min) influx of  $^{36}Cl^-$  was measured at 30°C in LCAPG with the indicated concentration of total  $Cl^-$  (0.005 mM nonradioactive  $Cl^-$  plus various added amounts of  $^{36}Cl^-$ ). The solid curves through the data are rectangular hyperbolae with  $K_{1/2} = 0.018$  mM,  $J_{Max} = 0.092$  mEq/L cell water-min (pH 4), and  $K_{1/2} = 0.30$  mM,  $J_{Max} = 0.261$  mEq/L cell water-min (pH 7). (B) Very slow efflux of  $^{36}Cl^-$  following resuspension of  $^{36}Cl^-$ -loaded cells (*sul1*) in LCAPG medium. Overnight culture of *sul1* cells in LCAPG was incubated in fresh LCAPG, 5 mM citrate, pH 4.5, and 8  $\mu M$   $^{36}Cl^-$ . After 2 h at 30°C, cells were centrifuged and resuspended in fresh medium containing no  $^{36}Cl^-$  and incubated at 30°C. (C) Rapid efflux of  $^{36}Cl^-$  following addition of extracellular  $Cl^-$ . Overnight culture of *sul1* cells in LCAPG was prepared and incubated as in B, except that 2 mM KCl was added at the arrow. Vertical axis is logarithmic; the curve through the data represents the sum of two exponentials with 70% of the tracer efflux with rate constant 0.41/min and the remaining 30% with rate constant 0.050/min.

the other strains. At a given  $K^+$  concentration, varying the  $Cl^-$  has little effect on final cell density. In the *vma1* strain the data suggest an effect of  $Cl^-$  on final cell density, but there is no effect of  $Cl^-$  on growth rate of exponential cultures (unpublished data). We conclude that there are at most very small effects of  $Cl^-$  on growth, even in the *vma1* strain, which has a lower total cellular  $Cl^-$  than the other strains.

#### High Affinity $Cl^-$ Transporter

Fig. 5 shows that cells grown in a low  $Cl^-$  medium have a high affinity pathway for initial  $^{36}Cl^-$  influx, and that the apparent  $Cl^-$  affinity is increased by low extracellular pH. Cells (*sul1*) were grown in LCAPG medium, and the initial  $^{36}Cl^-$  influx was measured in LCAPG plus various concentrations of  $^{36}Cl^-$ . At extracellular pH 4, the influx vs. extracellular  $Cl^-$  is described reasonably well by a hyperbola, with  $K_{1/2} \sim 0.018$  mM, similar to that observed previously in low  $Cl^-$  YNB medium (Jennings et al., 2007).



**Figure 6.** Exchange of cellular  $^{36}\text{Cl}^-$  for extracellular  $\text{Cl}^-$  or  $\text{Br}^-$ , but not for  $\text{NO}_3^-$ ,  $\text{HCO}_3^-$ , or formate. Cells (*sul1*) were grown in LCAPG and incubated 2 h,  $30^\circ\text{C}$ , in fresh LCAPG +  $8\ \mu\text{M}$   $\text{Na}^{36}\text{Cl}$ . Cells were centrifuged and resuspended at  $30^\circ\text{C}$  in LCAPG plus 2 mM KCl, KBr, K-formate,  $\text{KNO}_3$ , or  $\text{KHCO}_3$ , as indicated. Cellular  $^{36}\text{Cl}^-$  as a percent of the value at the first time point is plotted on a logarithmic scale as a function of time. Unless otherwise indicated, the effluxes were performed in media buffered at pH 4.5 with 5 mM K-citrate. The two experiments at pH 6.0 were in media buffered with 5 mM K-citrate and 10 mM bistris. The semi-log plots are fit with straight lines because the time points are too early to reflect the slow component shown in Fig. 5.

At extracellular pH 7, the  $K_{1/2}$  is over 10-fold higher than at pH 4.0 (Fig. 5 A). This finding suggests that  $\text{H}^+$  is co-transported with  $\text{Cl}^-$ . However, the  $V_{\text{max}}$  of the influx is also higher at pH 7.0 than at pH 4, indicating that the effects of pH on the kinetics of  $\text{Cl}^-$  influx are not simple. Irrespective of the detailed kinetics, the  $^{36}\text{Cl}^-$  influx at very low extracellular  $[\text{Cl}^-]$  is lower at pH 7.0 than at pH 4. This is consistent with the fact that the steady-

state distribution ratio is also lower at pH 7.0 (Fig. 2 D and Fig. 3 A).

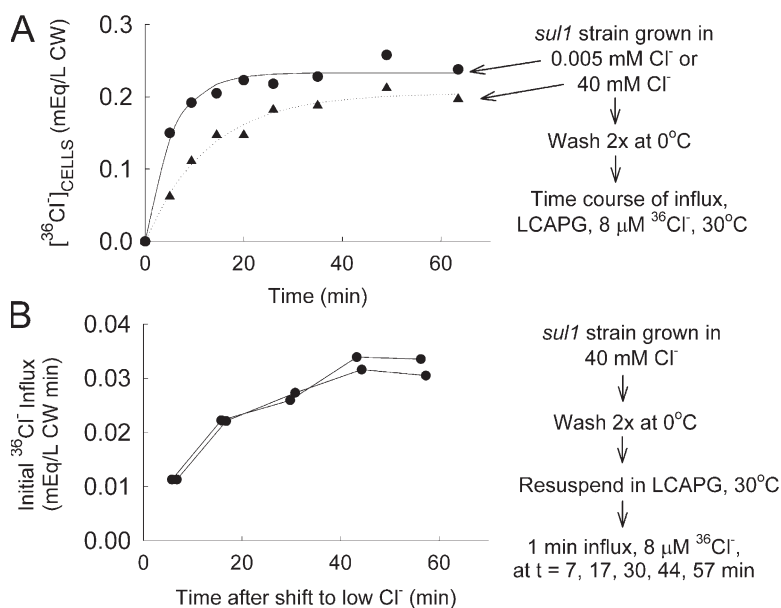
#### Exchange of $^{36}\text{Cl}^-$ for Nonradioactive $\text{Cl}^-$

Cells loaded with  $^{36}\text{Cl}^-$  in media containing  $8\ \mu\text{M}$   $^{36}\text{Cl}^-$  and then resuspended in LCAPG containing no added  $\text{Cl}^-$  can maintain a very high cells:medium  $^{36}\text{Cl}^-$  ratio for many minutes (Fig. 5 B). There is only very slow loss of  $^{36}\text{Cl}^-$  despite the fact that the ratio of intracellular to extracellular  $[\text{Cl}^-]$  is over 100. This gradient does not represent a true steady state and cannot be generated except by resuspending preloaded cells in tracer-free medium. The experiment shows, however, that there is little efflux of  $^{36}\text{Cl}^-$  in a medium containing  $\text{SO}_4^{2-}$  (2 mM),  $\text{H}_2\text{PO}_4^-$  (8 mM), and traces of iodide (0.6  $\mu\text{M}$ ), molybdate (1  $\mu\text{M}$ ), and  $\text{NO}_3^-$  (4  $\mu\text{M}$ ). If 2 mM KCl is added (Fig. 5 C),  $^{36}\text{Cl}^-$  leaves the cells rapidly, indicating that the cells have a mechanism for  $^{36}\text{Cl}^-$ – $\text{Cl}^-$  exchange. The first  $\sim 70$ – $80\%$  of the  $^{36}\text{Cl}^-$  efflux has a time course that is close to a single exponential, but the remaining  $^{36}\text{Cl}^-$  efflux is slower (Fig. 5 C).

#### Exchange of $^{36}\text{Cl}^-$ for Anions other than $\text{Cl}^-$

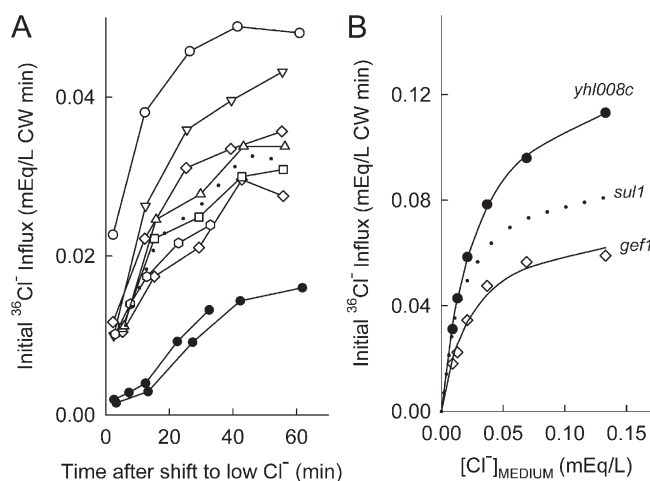
Several anions other than  $\text{Cl}^-$  were tested as possible exchange partners for  $^{36}\text{Cl}^-$  (Fig. 6). Cells were loaded in LCAPG ( $8\ \mu\text{M}$   $^{36}\text{Cl}^-$ ) as in Fig. 3 and resuspended in LCAPG containing various anions. The efflux of  $^{36}\text{Cl}^-$  is almost as rapid in the presence of 2 mM  $\text{Br}^-$  as in  $\text{Cl}^-$ . In contrast, the presence of 2 mM  $\text{NO}_3^-$  or formate does not stimulate efflux of  $^{36}\text{Cl}^-$ .

Because of the ubiquitous presence of  $\text{HCO}_3^-$ , it is of interest to determine whether  $\text{HCO}_3^-$  is an exchange partner for  $\text{Cl}^-$ . It is difficult to study  $\text{HCO}_3^-$  transport at pH 4.5 because  $\text{HCO}_3^-$  is converted mainly to  $\text{CO}_2$  at this pH. Instead, bicarbonate was added as 4 mM  $\text{KHCO}_3$  in



**Figure 7.** Activation of high affinity  $\text{Cl}^-$  transport following shift from 40 mM  $\text{Cl}^-$  to low  $\text{Cl}^-$  medium. (A) Cells (*sul1*) were grown overnight in LCAPG with no added  $\text{Cl}^-$  ( $\sim 0.005\ \text{mM}$ ;  $\bullet$ ) or 40 mM added  $\text{NaCl}$  ( $\blacktriangle$ ), and then washed twice in cold LCAPG, resuspended in LCAPG, pH 4.5,  $30^\circ\text{C}$ , with  $8\ \mu\text{M}$   $\text{Na}^{36}\text{Cl}$ , and the accumulation of  $^{36}\text{Cl}^-$  measured over the next 50 min. (B) Cells (*sul1*) were grown in APG + 40 mM  $\text{NaCl}$ , washed twice in cold LCAPG, and resuspended at  $t = 0$  in LCAPG, pH 4.5,  $30^\circ\text{C}$ . At the indicated times,  $8\ \mu\text{M}$   $\text{Na}^{36}\text{Cl}$  was added and the tracer influx measured for 1 min. Data are from two separate cell preparations.





**Figure 8.** (A) Activation of high affinity  $\text{Cl}^-$  transport in *yh1008c* strain compared with other deletant strains. Cells were grown in APG containing 40 mM NaCl as in Fig. 5 A, washed in cold LCAPG, and resuspended at  $t = 0$  in LCAPG, pH 4.5, 30°C. At the indicated times, 8  $\mu\text{M}$   $\text{Na}^{36}\text{Cl}$  was added and the tracer influx measured for 1 min. Deletant strains are *trk1* ( $\circ$ ), *trk2* ( $\nabla$ ), *gef1* ( $\diamond$ ), *ypr003c* ( $\triangle$ ), *bor1* ( $\square$ ), and *yh1008c* ( $\bullet$ ). The dotted line represents the *sul1* data in Fig. 5 A. (B) High affinity  $\text{Cl}^-$  flux in strains *yh1008c* ( $\bullet$ ) and *gef1* ( $\diamond$ ) after overnight growth in low  $\text{Cl}^-$  medium. Cells were prepared and initial influx was measured at pH 4.0 as a function of extracellular  $\text{Cl}^-$ , as in Fig. 3 A. For comparison, the dotted curve represents the data from Fig. 3 A for control strain *sul1*.

medium buffered at pH 6. At this pH, about half the  $\text{HCO}_3^-$  is quickly converted to  $\text{CO}_2$  (raising the pH slightly in the buffered medium), leaving  $\sim 2$  mM  $\text{HCO}_3^-$ . Addition of  $\text{HCO}_3^-$  does not stimulate efflux of  $^{36}\text{Cl}^-$  (Fig. 6, bottom right). In this experiment the  $\text{pCO}_2$  in the atmosphere above the suspension was not controlled. In separate experiments conducted in a 70%  $\text{CO}_2$ , 30%  $\text{O}_2$  atmosphere, the presence of  $\text{HCO}_3^-$  again did not stimulate efflux of  $^{36}\text{Cl}^-$ , indicating that there is no evidence for a  $^{36}\text{Cl}^-$ – $\text{HCO}_3^-$  exchange process in *S. cerevisiae*. At pH 6.0 the exchange of  $^{36}\text{Cl}^-$  for extracellular  $\text{Cl}^-$  is slightly slower than at pH 4.5, but the  $\text{Cl}^-$ – $\text{Cl}^-$  exchange mode of the transporter is clearly functional at extracellular pH 6, and  $\text{Cl}^-$ – $\text{HCO}_3^-$  exchange under the same conditions is undetectable.

#### Rapid Activation of High Affinity $\text{Cl}^-$ Influx following a Shift from High $\text{Cl}^-$ to Low $\text{Cl}^-$ Medium

In media containing  $>1$  mM  $\text{Cl}^-$ , *S. cerevisiae* should have no need for a high affinity  $\text{Cl}^-$  transporter, because the cells exclude rather than accumulate  $\text{Cl}^-$  (Fig. 1). To determine whether the high affinity  $\text{Cl}^-$  influx is regulated, cells (*sul1*) were grown in either LCAPG medium or APG containing 40 mM KCl. Cells were then washed in cold LCAPG medium to remove extracellular  $\text{Cl}^-$ , resuspended in LCAPG plus 8  $\mu\text{M}$   $^{36}\text{Cl}^-$  at 30°C, and the accumulation of  $^{36}\text{Cl}^-$  measured over the next 30 min. As shown in Fig. 7 A, the initial  $^{36}\text{Cl}^-$  influx is lower in

cells grown in 40 mM  $\text{Cl}^-$ , but the final accumulation of  $^{36}\text{Cl}^-$  is nearly independent of whether the cells had been grown in high or low  $\text{Cl}^-$ . This suggests that the high affinity  $\text{Cl}^-$  influx activates rapidly following exposure of cells to low  $\text{Cl}^-$  medium.

To examine the time course of activation of the high affinity  $\text{Cl}^-$  flux in more detail, cells were grown in APG plus 40 mM  $\text{Cl}^-$ , washed in cold LCAPG, and resuspended at 30°C in LCAPG containing no  $^{36}\text{Cl}^-$ . At various times, 8  $\mu\text{M}$   $^{36}\text{Cl}^-$  was added to aliquots of the suspension, and the initial (1 min) flux was measured (Fig. 7 B). The flux is low at early times but activates to a steady-state level with a half time of  $\sim 20$  min.

The high affinity  $\text{Cl}^-$  influx activates with roughly the same time course following a shift to LCAPG in all the deletant strains tested except for *yh1008c* (Fig. 8 A). In this strain the 1-min  $^{36}\text{Cl}^-$  influx is initially much lower than in the other strains and also activates more slowly (Fig. 8 A, solid symbols). In the same strain incubated in low  $\text{Cl}^-$  medium for several hours, the flux eventually becomes slightly higher than that in the control strain (Fig. 8 B). Therefore, Yhl008cp, an uncharacterized protein with homology to bacterial and *Euglena* formate or nitrite transporters (Suppmann and Sawers, 1994; Deloménie et al., 2007), is definitely not the high affinity  $\text{Cl}^-$  transporter, but it may have a role in activation of the transporter following a shift to low  $\text{Cl}^-$  media.

In the experiment in Fig. 8 B, the concentration dependence of  $^{36}\text{Cl}^-$  influx was also measured in the *gef1* strain, demonstrating that these cells have a high affinity  $\text{Cl}^-$  influx that is very nearly as large as that of the control strain. Therefore, even though the *gef1* strain has 65% lower steady-state  $\text{Cl}^-$  contents in LCAPG (Fig. 3), the initial  $^{36}\text{Cl}^-$  influxes are quite similar to those of the control strain, suggesting that the plasma membrane  $\text{Cl}^-$  influx in the *gef1* strain is normal and that the lower steady-state levels are the result of reduced organellar sequestration of  $\text{Cl}^-$ .

## DISCUSSION

The work described here has shown that *S. cerevisiae* maintains total cellular  $\text{Cl}^-$  within a narrow range even if extracellular  $[\text{Cl}^-]$  is varied  $>10,000$  fold. The  $\text{Cl}^-$  distributions and fluxes indicate the existence of a  $\text{Cl}^-$  transporter with a very high apparent affinity for  $\text{Cl}^-$  at acid extracellular pH. The activation of this transporter depends on a  $\text{Cl}^-$ -sensing mechanism, one component of which may be the formate-nitrite transporter homologue Yhl008cp. The experiments also provide evidence that there are steep  $\text{Cl}^-$  gradients across the membranes of vacuoles or prevacuolar vesicles in *S. cerevisiae* grown in low  $\text{Cl}^-$  media. These gradients are dependent on the vacuolar  $\text{H}^+$ -ATPase, the yeast CLC Gef1p, and, to a lesser extent, the cation– $\text{H}^+$  exchanger Nhxl p. A model based on these findings is in Fig. 9.



## Two Sites of Concentrative $\text{Cl}^-$ Transport: Plasma Membrane and Vacuole/Prevacuole

The  $\text{Cl}^-$  distribution data in Fig. 3 indicate that, in a very low  $\text{Cl}^-$  medium, there are two sites of uphill  $\text{Cl}^-$  influx in *S. cerevisiae*: the plasma membrane and subcellular membranes of compartments that are acidified by the vacuolar  $\text{H}^+$ -ATPase. There is clearly a very large effect of deleting VMA1 on the cells:medium  $[\text{Cl}^-]$  ratio, indicating that a substantial fraction of the cellular  $\text{Cl}^-$  is sequestered in V-ATPase-dependent compartments. However, even in *vma1* cells, the cells:medium  $\text{Cl}^-$  ratio is far higher than that expected for electrochemical equilibrium (Fig. 3). These experiments were performed in medium containing arginine rather than  $\text{NH}_3/\text{NH}_4^+$  as the main nitrogen source. Therefore, the  $\text{NH}_3$ -dependent vacuolar acidification mechanism (Plant et al., 1999) in cells lacking the vacuolar  $\text{H}^+$ -ATPase should not be operative. Accordingly, in a low  $\text{Cl}^-$  medium, the plasma membrane itself is capable of maintaining a cytosolic  $\text{Cl}^-$  concentration that is much higher than that in the medium.

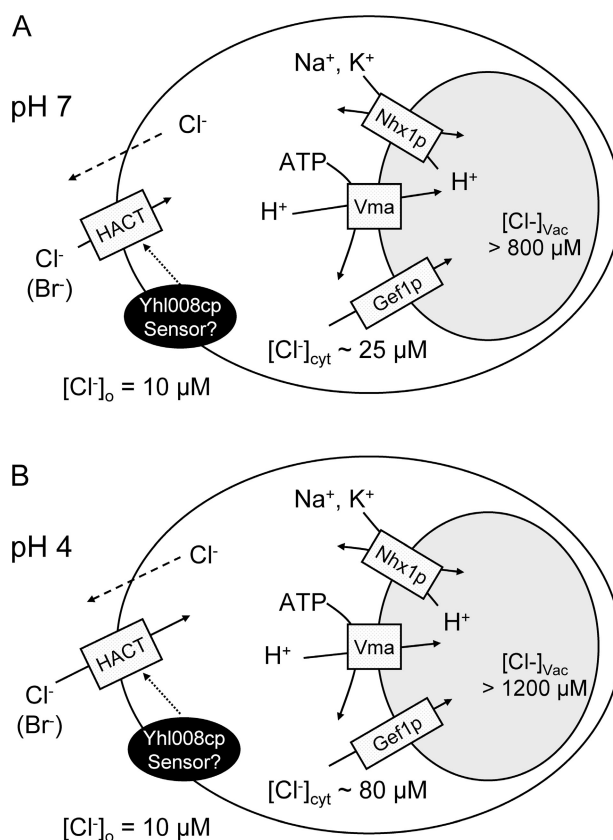
## ATP Dependence

ATP depletion strongly reduces the cellular  $\text{Cl}^-$  contents of both control and *vma1* strains (Fig. 3 C). Therefore, the concentrative transport of  $\text{Cl}^-$  across vacuolar (or prevacuolar) membranes and across the plasma membrane is ATP dependent. The ATP dependence of  $\text{Cl}^-$  accumulation in organelles is clearly expected if the transport requires luminal acidification by the V-ATPase. The fact that ATP depletion also prevents  $\text{Cl}^-$  accumulation in the *vma1* strain is consistent with the idea that plasma membrane  $\text{Cl}^-$  influx takes place by  $\text{H}^+$ - $\text{Cl}^-$  co-transport driven by the  $\text{H}^+$  gradient.

At extracellular pH 7, the cells:medium  $[\text{Cl}^-]$  ratio is  $\sim 2$  in the *vma1* strain in low  $\text{Cl}^-$  media (Fig. 3). A plasma membrane 1:1  $\text{H}^+$ / $\text{Cl}^-$  cotransporter could account for this accumulation if the cytosolic pH were at least 7.3. Alternatively, if there is a 2:1  $\text{H}^+$ / $\text{Cl}^-$  cotransporter, the inward electrochemical  $\text{H}^+$  gradient could easily drive cytosolic  $\text{Cl}^-$  far above the extracellular concentration, because the membrane potential would help drive the influx. Yeast have  $\text{SO}_4^{2-}$  transporters that cotransport at least two  $\text{H}^+$  ions with  $\text{SO}_4^{2-}$  (Roomans et al., 1979), and it is possible that there is a similar transporter for  $\text{Cl}^-$ . (It is clear that the high affinity  $\text{Cl}^-$  transporter is not a  $\text{SO}_4^{2-}$  transporter, because  $\text{Cl}^-$  does not exchange for  $\text{SO}_4^{2-}$ , and the high  $\text{SO}_4^{2-}$  concentration in YNB medium does not compete with  $\text{Cl}^-$  for high affinity transport sites.)

## A Regulated Plasma Membrane $\text{Cl}^-$ Transport Process with Very High Apparent Affinity

The initial flux of  $^{36}\text{Cl}^-$  into *S. cerevisiae* that have been incubated in low  $\text{Cl}^-$  media is a saturable function of the extracellular  $[\text{Cl}^-]$ , with a  $K_{1/2}$  of  $\sim 0.02$  mM (Fig. 5 A). The true  $\text{Cl}^-$  affinity of the transporter cannot be in-



**Figure 9.** Possible transport mechanisms accounting for the cellular accumulation of  $\text{Cl}^-$  in pH 7.0 (A) or pH 4.0 (B) media containing  $10 \mu\text{M}$   $\text{Cl}^-$ . The elevated cellular  $\text{Cl}^-$  content is proposed to be a consequence of two processes. (1) Influx across the plasma membrane via a high affinity  $\text{Cl}^-$  transporter (HACT), which is regulated by a mechanism that includes Yhl008c (depicted here on the plasma membrane, but the actual cellular location is not known). The  $\text{Cl}^-$  gradient across the plasma membrane is higher at extracellular pH 4.0 than at pH 7, consistent with  $\text{H}^+$ - $\text{Cl}^-$  co-transport across the plasma membrane. The dashed arrow represents downhill efflux of  $\text{Cl}^-$  through a pathway that is unknown but must be very slow in a low  $\text{Cl}^-$  medium (Fig. 5 B). (2) Sequestration of  $\text{Cl}^-$  in the vacuole or prevacuolar compartment by a process that is powered by the V-ATPase (Vma), with  $\text{Cl}^-$  transport (probably as  $\text{Cl}^-/\text{H}^+$  exchange; see text) through Gef1p, and the pH gradient modulated by Nhx1p.

ferred from flux measurements (e.g., Fröhlich and Gunn, 1986), but the apparent affinity for  $\text{Cl}^-$  is at least 100-fold higher than those of mammalian anion exchangers (Gunn et al., 1973; Fröhlich and Gunn, 1986) or cotransporters (Dunham et al., 1980; Isenring et al., 1998). The high affinity  $\text{Cl}^-$  transporter, denoted HACT in Fig. 9, exhibits a robust  $\text{Cl}^-$ - $\text{Cl}^-$  and  $\text{Cl}^-$ - $\text{Br}^-$  exchange activity, but it does not transport formate, bicarbonate, nitrate, phosphate, or sulfate. The fact that the apparent  $\text{Cl}^-$  affinity (Fig. 5 A) as well as the steady-state  $\text{Cl}^-$  distribution ratio (Fig. 3 A) depend on pH suggests that the high affinity  $\text{Cl}^-$  influx is mediated by a  $\text{H}^+$ - $\text{Cl}^-$  cotransporter (or  $\text{OH}^-/\text{Cl}^-$  exchanger). Whatever the nature of the transport process, it must be able to produce steady-state

cytosolic  $\text{Cl}^-$  levels that are higher than those in the extracellular medium despite the presence of a very negative membrane potential.

We do not know the molecular identity of the high affinity  $\text{Cl}^-$  transporter. Several candidate transporters have been ruled out on the basis of the lack of effect of gene deletion on  $\text{Cl}^-$  accumulation in low  $\text{Cl}^-$  media: Bor1p (anion exchanger homologue now known to be a  $\text{H}_3\text{BO}_3$  transporter [Takano et al., 2002; Jennings et al., 2007]), YBR235w ( $\text{Cl}^-$ -coupled cotransporter homologue), and YPR003c (Pendrin, DRA homologue). We did additional studies on YBR235w, including transport measurements in the deletant strain, which showed that the high affinity flux is present, as well as overexpression of the protein, without an increase in  $\text{Cl}^-$  transport (unpublished data). The functions of Ybr235wp and Ypr003cp remain unknown.

#### Regulation of the High Affinity $\text{Cl}^-$ Influx by a Mechanism Involving Yhl008cp

The high affinity  $\text{Cl}^-$  influx is activated relatively rapidly following a shift from a high  $\text{Cl}^-$  to a low  $\text{Cl}^-$  medium (Figs. 7 and 8). It is not clear whether the change in extracellular  $\text{Cl}^-$  is sensed directly, or whether the shift from high to low  $\text{Cl}^-$  medium causes a drop in cytosolic  $\text{Cl}^-$  that then triggers the activation of  $\text{Cl}^-$  influx. In either case, Yhl008cp may be involved in either sensing low  $\text{Cl}^-$  or transmitting the signal that eventually activates the transporter. It is not known to what extent transcriptional, translational, and/or posttranslational mechanisms contribute to the activation of the transporter.

The sequence of Yhl008cp is homologous to that of bacterial formate transporters, but the protein has been shown not to be a monocarboxylate transporter (Makuc et al., 2001). The only other experimental information on its properties is that the GFP fusion with Yhl008cp gives a low signal, possibly localized to the vacuole (Huh et al., 2003). Yhl008cp may be another example of a protein that is in a family of transporters but is actually a sensor (Forsberg and Ljungdahl, 2001); however, its true role in  $\text{Cl}^-$  regulation remains to be determined.

#### Evidence for Large Subcellular Transmembrane $\text{Cl}^-$ Gradients

The fact that deletion of VMA1, GEF1, or NHX1 causes a decrease in cellular  $\text{Cl}^-$  is strong evidence that, at least in low  $\text{Cl}^-$  media, a substantial fraction of cellular  $\text{Cl}^-$  is in a subcellular compartment that is acidified by the V-type  $\text{H}^+$ -ATPase. Although it is widely accepted that organellar acidification takes place by way of an ATP-driven  $\text{H}^+$  influx and a parallel influx of  $\text{Cl}^-$  (Wada and Ohsumi, 1992; Forgac, 1999; Jentsch et al., 2002), there have been few estimates of the sizes of  $\text{Cl}^-$  gradients across organellar membranes in intact cells. The cells:medium  $^{36}\text{Cl}^-$  distribution ratios we have determined in

control and *vma1* strains allow a rough estimate of the  $\text{Cl}^-$  distribution between vacuole and cytosol. At extracellular pH 7.0 in media containing 10  $\mu\text{M}$   $\text{Cl}^-$ , the cells:medium  $\text{Cl}^-$  ratio is 20 in the control strain and  $\sim 2$  in the *vma1* strain (Fig. 3). The simplest interpretation of this finding is that in the control strain under these conditions,  $\sim 90\%$  of the cellular  $\text{Cl}^-$  at pH 7.0 is in a compartment that is dependent on Vma1p (Fig. 9). If this compartment is the vacuole, which occupies  $\sim 25\%$  of cell volume (Wiemken and Dürr, 1974), then the concentration of  $\text{Cl}^-$  in the vacuole must be nearly 30 times as high as that in the cytosol. If  $\text{Cl}^-$  is actually concentrated more in prevacuolar vesicles than in the vacuole itself, then the concentration of  $\text{Cl}^-$  in the vesicles must be even higher, because these vesicles occupy a smaller fraction of the cell volume. A 30-fold  $\text{Cl}^-$  gradient across the vacuolar membrane is much steeper than has been reported for the alga *Eremosphaera viridis*, where there is an approximately threefold gradient (6.2 to 2.2 mM) of  $\text{Cl}^-$  between the vacuole and the cytosol (Bethmann et al., 1995). However, high inorganic anion gradients across vacuolar membranes are not unprecedented; there is a 30-fold  $\text{NO}_3^-$  gradient across the *Arabidopsis thaliana* vacuolar membrane (Cookson et al., 2005). In this context it should be noted that, given the complexity of vacuolar contents, our estimate of total subcellular  $\text{Cl}^-$  does not allow an estimate of electrochemical activity gradients across subcellular membranes.

#### Is Gef1p a Conductive Channel or a Stoichiometric $\text{Cl}^-/\text{H}^+$ Exchanger?

Gef1p forms conductive channels when reconstituted in lipid bilayers (Flis et al., 2002). However, these channels were observed at  $\text{Cl}^-$  concentrations roughly 1,000-fold higher than those in yeast cytosol under the present conditions, and it is possible that Gef1p behaves differently in high vs. low  $\text{Cl}^-$  solutions. The vacuolar membrane potential in higher plants and in *Neurospora crassa* is roughly 30 mV (lumen positive) (Bertl and Slayman, 1990; De Angeli et al., 2006). If the vacuolar membrane potential is of this magnitude in *S. cerevisiae*, then  $\text{Cl}^-$  transport through a conductive channel can account for only an approximately threefold ratio of vacuolar to cytosolic  $[\text{Cl}^-]$ .

On the basis of the presence of a glutamate residue in the position of Glu203 in bacterial CLC-ec1, Gef1p is expected to be a stoichiometric  $\text{Cl}^-/\text{H}^+$  exchanger rather than a conductive  $\text{Cl}^-$  channel (Accardi et al., 2005). The equilibrium condition for  $\text{Cl}^-/\text{H}^+$  exchange across a membrane with a membrane potential of  $-30$  mV (cytoplasm negative relative to lumen; see Bertl et al., 1992) is

$$[\text{Cl}^-]_{\text{Cyt}}^n / [\text{Cl}^-]_{\text{Vac}}^n = ([\text{H}^+]_{\text{Cyt}} / [\text{H}^+]_{\text{Vac}}) * e^{[(n+1)*(-30F/RT)]}, \quad (1)$$

where  $n$  is the number of  $\text{Cl}^-$  ions transported per  $\text{H}^+$  ion, the subscripts refer to cytosolic and vacuolar

concentrations, and F, R, and T have their usual meanings. If the vacuolar luminal pH is 6.2 (Preston et al., 1989) and the cytosolic pH is 7.2, then a  $\text{Cl}^-$  gradient of  $\sim 20$ -fold could be generated by a 2:1  $\text{Cl}^-/\text{H}^+$  exchanger. A 1:1 exchanger could produce an  $\sim 100$ -fold gradient under these conditions. Our data do not allow a distinction to be made between a 2:1 and 1:1 exchanger, but our data are much more consistent with Gef1p acting as a  $\text{Cl}^-/\text{H}^+$  exchanger than as a  $\text{Cl}^-$  channel.

A recent paper (López-Rodríguez et al., 2007) concluded that, in addition to being an organellar  $\text{Cl}^-$  transporter, Gef1p also mediates  $\text{Cl}^-$  efflux across the plasma membrane in yeast grown in YPG medium. The  $\text{Cl}^-$  concentration in medium containing 1% yeast extract and 2% peptone is 350 mg/liter, or 10 mM, according to Dionex Application Note 123 ([http://www1.dionex.com/en-us/webdocs/4082\\_AN123\\_07May07\\_LPN1030\\_2.pdf](http://www1.dionex.com/en-us/webdocs/4082_AN123_07May07_LPN1030_2.pdf)). Our data do not address the possibility that Gef1p mediates plasma membrane  $\text{Cl}^-$  efflux in this medium. However, in the low- $\text{Cl}^-$  media in which all the transport experiments described here were performed, if Gef1p (either as a conductive channel or a  $\text{Cl}^-/\text{H}^+$  exchanger) mediates a  $\text{Cl}^-$  efflux across the plasma membrane, the efflux is overridden by the high affinity, concentrative  $\text{Cl}^-$  influx that we have described. Certainly Gef1p is not responsible for the high affinity  $\text{Cl}^-$  influx.

#### Nonexponential Time Course of $^{36}\text{Cl}^-$ - $\text{Cl}^-$ Exchange

The time course of  $^{36}\text{Cl}^-$ - $\text{Cl}^-$  exchange in a medium containing 2 mM  $\text{Cl}^-$  is not distinguishable from a single exponential for the first 70–80% of the efflux (Fig. 5 C and Fig. 6), but the remainder of the efflux is slower (Fig. 5 C). This slower component could represent exchange with a subcellular compartment, or it could be the result of progressive inactivation of the transporter following exposure to 2 mM  $\text{Cl}^-$ . These possibilities are difficult to distinguish, and a systematic study of the time course was not performed.

#### Plasma Membrane Conductive $\text{Cl}^-$ Permeability

Fig. 2 C shows that, in the long-term presence of very high outward  $\text{K}^+$  gradients, the ability of *S. cerevisiae* to accumulate  $\text{Cl}^-$  in a low  $\text{Cl}^-$  medium is diminished. However, the  $\text{K}^+$  gradient does not have a major effect on  $\text{Cl}^-$  accumulation during a 2-h incubation (Fig. 2 D). Therefore, in a low  $\text{Cl}^-$  medium, the conductive  $\text{Cl}^-$  flux must not be very large; otherwise, the cells would not have a higher  $\text{Cl}^-$  concentration than the medium. Nonetheless, there must be a finite conductive  $\text{Cl}^-$  permeability in the plasma membrane, and this conductive permeability may become a major determinant of the cellular  $\text{Cl}^-$  distribution at high extracellular  $\text{Cl}^-$ , when there is a large inward gradient (Fig. 1). It is not known whether Gef1p (López-Rodríguez et al., 2007), Trk1p, Trk2p (Rivetta et al., 2005), or some other protein is responsible for the conductive  $\text{Cl}^-$  flux.

#### Could Yeast Live and Grow without Any $\text{Cl}^-$ At All?

The finding that yeast grown in low  $\text{Cl}^-$  media retain  $\text{Cl}^-$  at concentrations much higher than those in the medium suggests that yeast require  $\text{Cl}^-$  for growth. However, it is possible that the  $\text{Cl}^-$  regulation that we have observed serves a nonessential purpose (at least under the conditions tested thus far) and that yeast do not have an absolute  $\text{Cl}^-$  requirement for growth. Even in media that are nominally  $\text{Cl}^-$  free, there are traces of  $\text{Cl}^-$ , and we have not tried to lower the  $\text{Cl}^-$  concentrations of media below  $\sim 2 \mu\text{M}$ . The purpose of the growth studies was to see whether there was an obvious effect of  $\text{Cl}^-$  on growth in the range of  $\text{Cl}^-$  concentrations in which we measured the  $\text{Cl}^-$  distribution, and not to perform a comprehensive study of the effects of extremely low  $\text{Cl}^-$  on yeast growth. Such a study would require systematic variations in the carbon source, iron, copper,  $\text{Cl}^-$ , and pH, as well as deletion and overexpression of genes involved in iron and copper metabolism (Davis-Kaplan et al., 1998).

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