Mechanisms involved in transcriptional regulation of the osmotically controlled GPD1 gene in Saccharomyces cerevisiae were investigated by promoter analysis. The GPD1 gene encodes NAD\(^+\)-dependent glycerol-3-phosphate dehydrogenase, a key enzyme in the production of the compatible solute glycerol. By analysis of promoter deletions, we identified a region at nucleotides −478 to −324, in relation to start of translation, to be of great importance for both basal activity and osmotic induction of GPD1. Electrophoretic mobility shift and DNase I footprint analyses demonstrated protein binding to parts of this region that contain three consensus sequences for Rap1p (repressor activator protein 1)-binding sites. Actual binding of Rap1p to this region was confirmed by demonstrating enhanced electrophoretic mobility of the protein-DNA complex with extracts containing an N-terminally truncated version of Rap1p. The detected Rap1p-DNA interactions were not affected by changes in the osmolality of the growth medium. Specific inactivation of the Rap1p-binding sites by a C-to-A point mutation in the core of the consensus showed that this factor is a major determinant of GPD1 expression since mutations in all three putative binding sites for Rap1p strongly hampered osmotic induction and drastically lowered basal activity. We also show that the Rap1p-binding sites appear functionally distinct; the most distal site (core of the consensus at position −386) exhibited the highest affinity for Rap1p and was strictly required for low salt induction (≤0.6 M NaCl), but not for the response at higher salinities (≥0.8 M NaCl). This indicates that different molecular mechanisms might be operational for low and high salt responses of the GPD1 promoter.

Stress-activated signaling pathways in eukaryotic organisms are presently attracting much interest. The genetically tractable yeast Saccharomyces cerevisiae has proven particularly useful in identifying signal transduction components and in unraveling their function, which has generated a wealth of molecular information over the past few years (1–3). The HOG (high osmolarity glycerol response) mitogen-activated protein kinase pathway is a prominent signal transduction pathway responding to osmotic stress. The HOG1 and PBS2 genes, which code for a mitogen-activated protein kinase and its regulatory mitogen-activated protein kinase kinase, respectively, represent two central components of this pathway that were identified by analysis of yeast mutants sensitive to high salt concentrations (4). More recent work identified several upstream components of two distinct branches of the pathway affecting Hog1p phosphorylation (5–8). Another signaling pathway of general importance in modulating various cellular activities, i.e. the cAMP-dependent protein kinase A pathway, influences the expression of some of the stress-regulated genes (1, 9) by opposing the effects from signaling in the HOG pathway (10). There is, moreover, evidence for the existence of an osmostress-activated signaling pathway, involving calcineurin, that does not respond to general osmotic stress, but to high salt concentrations (11). In addition, phosphatidylinositol 3,5-bisphosphate rapidly accumulates in yeast cells during hyperosmotic stress (12, 13), suggesting the involvement of a so far uncharacterized phosphoinositide pathway in the yeast stress response. Clearly, yeast appears to coordinately activate various signal transduction pathways when confronted with osmotic challenges.

Central to the understanding of the overall cellular function of signaling pathways is the characterization of the regulatory elements of their target genes and the mechanism by which the transcription of these genes are controlled. However, only in a limited number of cases have the molecular details operational at the osmoregulated promoters been unraveled. It was recently shown that the ENA1 gene, encoding a P-type ATPase involved in the extrusion of Na\(^+\) from the yeast cytoplasm, is regulated by a derepression mechanism. The repressor that binds to the ENA1 promoter was identified as Sko1p (14), and the repression effect is dependent on the integrity of the Smn6p-Tup1p corepressor complex, which appears to be relieved by a HOG1-dependent mechanism. This corepressor has also been implicated in the control of the HAL1 gene and a number of other stress-regulated promoters, indicating derepression as a more general molecular mechanism for stress induction (15). The pentanucleotide element CCCCT, being the core consensus of the stress-responsive element (STRE),\(^1\) has been implicated in transcriptional activation of numerous genes during general stress conditions (1). This element responds to osmotic shock mediated via the HOG module. Two transcription factors, Msn2p and Msn4p, have been shown to bind to STRE and to be instrumental for STRE-activated transcription (16). The control of the Msn2p/4p-dependent gene activation by the HOG pathway appears to be influenced by the phosphorylation state

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\(^1\) The abbreviations used are: STRE, stress-responsive element; PCR, polymerase chain reaction; bp, base pair; kb, kilobase; CAT, chloramphenicol acetyltransferase; MOPS, 4-morpholinepropanesulfonic acid; EMSA, electrophoretic mobility shift assay.
of the transcription factors and their subsequent nuclear localization (17).

Elevated glycerol production is a prerequisite for the adaptation of *S. cerevisiae* to hyperosmotic stress, and several investigators have identified glycerol-3-phosphate dehydrogenase as the key enzyme in the glycerol synthesis pathway (18–21). The principal mechanism for increasing glycerol production is increased expression of the *GPD1* gene, and evidence has accumulated to indicate that *GPD1* promoter as a target for the diverse signaling pathways responding to cellular dehydration (22). Salt-induced expression is not dependent on a functional Sko1p,2 Msn2p/4p (23), or functional STREs. However, it was recently proposed that two putative transcription factors with slight sequence similarity to Gcr1p, an important regulatory factor of glycolytic gene expression, are involved in the hyperosmotic regulation of *GPD1* (23). The most prominent of these two candidates, Hot1p (high osmolarity-induced transcript), was shown to be essential for full level response to salt stress; the salt-induced transcriptional response in a hot1Δ mutant was −40% of the wild-type level. Simultaneous deletion of *MSN1*, encoding the other Gcr1p sequence homolog, resulted in a further reduction in salt-induced expression. However, irrespective of a combination of deletion of another gene knockout involving also Hog1p, Msn2p, and Msn4p, the *GPD1* gene remained salt-responsive, although to a much decreased extent. Thus, although Hot1p and Msn1p are involved in the osmostress-mediated transcriptional activation of *GPD1*, additional mechanisms are apparently in operation.

In this work, we report experimental evidence for the repressor/activator protein Rap1p (25) as an important determinant of both the basal and salt-induced transcriptional activities of the *GPD1* promoter. We also present evidence for the binding of Rap1p to neighboring binding sites that appear involved in mediating regulatory effects upon dehydration via different mechanisms, depending on the magnitude of stress. This is the first time that this multifunctional transcription factor being implicated in the osmostress response. It is hypothesized, based on the importance of Rap1p in the Gcr1p-mediated induction of glycolytic genes, that Rap1p might interact directly or indirectly with Hot1p and/or Msn1p and thereby facilitate their binding and subsequent activation of the *GPD1* promoter.

MATERIALS AND METHODS

**Strains and Growth Media**—The following strains of *S. cerevisiae* were used: YPH499 (MATα ura3-52 lys2-801 ade2-101 leu2-Δ1 his3-Δ200 trp1-Δ63) and YPH102a (MATα ura3-52 lys2-801 ade2-101 leu2-Δ1 his3-Δ200) (a gift from Mike Gustin, who had changed the mating type on the original YPH102) (26), W303-1A (MATα ade2-1 his3-11.15 leu2-3,112 trp1-1a ura3-1 can1-100 GAL SUC2) (27), and YD2S (ura his leu trp RAP1 and YPLS91 (YD2S with the RAP1 allele substituted for the RAP1Δα allele lacking 230 amino acids in the nonessential N terminus (Ura“ and LEU“)) (28). Cultures were grown in defined minimal medium (0.17% (w/v) yeast nitrogen base without amino acids and without ammonium sulfate (Difco) supplemented with 2% (w/v) glucose, 0.5% (w/v) (NH4)2SO4, and appropriate amino acids and nucleotides (120 mg/liter). All constituents, except the glucose, were sterilized by filtration. When indicated, strains were grown in complex yeast extract/peptone medium (1% (w/v) yeast extract and 2% (w/v) peptone, sterilized by filtration) supplemented with 2% (w/v) glucose and 120 mg/liter adenine.

Precultures (5 ml) were grown overnight in 15-ml Falcon tubes at 30 °C on a rotator, inoculated to an *A*610 of 0.1–0.2 in fresh medium, and grown to mid-exponential growth phase (*A*610 = 0.5–1) before harvest. Liquid cultures were incubated in E-flasks and with rotary agitation at 110 rpm at 30 °C. Plasmids were introduced to yeast by means of either electroporation (29) or the LiAc/polyethylene glycol method (30). Escherichia coli strain DH5α (recA1 endA1 thi-1 hsdR17 supE44 gyrA96 relA1 ΔlacZYA-argF-U169 (m80lacZAM15) was used for plasmid amplification and transformed by electroporation (31). Bacterial cultures were grown at 37 °C in LB medium.

**GPD1 Gene Promoter Constructs**—The plasmids constructed and the synthetic oligonucleotides (PCR primers) utilized in this work are listed in Table I. Oligonucleotides were purchased from Scandinavian Gene Synthesis AB (Koping, Sweden) and used without further purification. All basic recombinant DNA techniques were performed according to standard procedure (31) if not otherwise stated. Restriction enzyme-digested DNA was purified by agarose electrophoresis followed by phenol treatment (GELase F-centre Technologies, Germany, Roche Molecular Biochemicals, Mannheim, Germany) of the excised agarose-embedded DNA and subsequent ethanol precipitation.

The following procedure was applied in the construction of the plasmid pO54 (32). A 1448-bp *SphI*/*SapI* *GPD1* promoter fragment was isolated, ligated to a *BamHI* linker (CUGATCCG; Sigma catalog no. L6899), and inserted into the pEMHI site of the integrative CAT reporter plasmid YLP5-32cat (33). This procedure yielded a *GPD1* promoter-CAT reporter fusion (pGPD1-CAT) with the following junction sequence: 5′-3′(GPD1)CAATCCGATCCGAGGATCCGAAGGCTAAGGGAATTGGGACCATG-3′ (junction verified by sequencing; the *GPD1* promoter is in boldface, the *BamHI* linker is underlined, and the CAT gene is in italic with the coding sequence indicated in boldface). The *GPD1 promoter (−687 to −16) was constructed by inserting a 164-kb *SstI*/*XbaI* fragment from pO54, containing 670-bp upstream *GPD1* promoter fragment (nucleotides −687 to −16 upstream from the *GPD1* translational start codon) and the CAT gene, into *SmaI*/*BstXI*-cleaved pRS316 (26). The pPE110a plasmid is a pPE110 derivative with a XhoI/KpnI deletion in the multiple cloning site, pPE110b and pPE110c are pPE110 derivatives in which the *gpd1* promoter fragment has been exchanged for the corresponding fragments from pPE103a and pPE110b, respectively (introducing unique *KpnI* sites at positions −581 and −483 in the promoter).

For the 5′-deletions, plasmids pPE111 (GPD1 (−377 to −16)) and pPE112 (GPD1 (−322 to −16)) were constructed by ligation of a 1.33-kb *SmaI*/*BstXI* fragment and a 1.28-kb *PstI*/*XbaI* fragment from pO54 to *SmaI*/*BstXI*-cleaved pRS316 (the PstI sticky ends were made blunt-ended before ligation). Plasmids pPE113 (GPD1 (−577 to −16)) and pPE114 (GPD1 (−478 to −16)) were made by adopting a PCR-mediated technique (31) utilizing Vent DNA polymerase (New England Biolabs Inc.). Primer pairs PCR1/PCR3 and PCR2/PCR3 were used for the amplification of a 560-bp and a 460-bp *GPD1* promoter fragment, respectively, using pO54 as template. PCR products were incubated with restriction enzymes *KpnI* and *BamHI* and ligated into pPE110 plasmid digested with the same enzymes (the promoter fragment of pPE110 resulting from the digestion was eliminated by gel electrophoresis prior to the ligation).

The internal deletions in the promoter were made by the following procedure where first some additional plasmid constructs were made. Plasmid pPE102 consists of a 1448-bp *SphI*/*SapI* *GPD1* promoter fragment and is digested with *KpnI* and *BamHI* linkers and is *BamHI*-cleaved vector pBSK+ (pBluescript II KS+, Stratagene). pPE102a is a modified pPE102 construct with the *KpnI*/*XhoI* fragment deleted in the multiple cloning site. pPE103 is a version of pPE102a with the distal 71 base pairs of the *GPD1* promoter deleted (down to nucleotide −678). The pPE104 plasmid (GPD1 (−687 to −16,Δ−580 to −479)) was constructed by PCR amplification of pPE105 using primers PCR2 and PCR4. This results in a *GPD1* promoter deletion between nucleotides −581 and −478 and introduction of a unique *KpnI* site. pPE105 (GPD1 (−687 to −16,Δ−377 to −333)) is a *GPD1* variant in which the fragment spanning nucleotides −377 (Smal site) to −333 (PstI site) has been deleted by digestion of pPE103 with PstI (5′-extension removed using Klenow fragment) and *SmaI*, followed by religation. The pPE106 (GPD1 (−687 to −16,Δ−580 to −323)) and pPE107 (GPD1 (−687 to −16,Δ−482 to −323)) plasmids were constructed using the same approach; pPE103a and pPE103b, respectively, were digested with PstI (5′-extension removed using Klenow fragment) and *KpnI* before religation. Plasmid pPE115 (GPD1 (−687 to −16,Δ−580 to −479)) is the result of swapping the promoter fragment (HindIII/*BamHI*) of pPE110a with the corresponding fragment in pPE104, pPE116 (GPD1 (−687 to −16,Δ−580 to −323)) and pPE117 (GPD1 (−687 to −16,Δ−377 to −333)) were constructed in the same manner, with the promoter part (HindIII/*BamHI*) in pPE110a swapped for the equivalent part of plasmids pPE106 and pPE107, respectively. Plasmids pPE119 (GPD1 (−687 to −16,Δ−580 to −379)) and pPE120
Rap1p-binding Sites in GPD1

(GPD1 (−687 to −61, 422 to −379)) are based on modifications of plasmids pPE110b and pPE110c, respectively. The unique Smal and Kpn1 sites in these plasmids were digested and made blunt-ended using T4 DNA polymerase, and the DNAAs were then subsequently religated, generating new cassettes and constructs designated PE110R1 and PE110R3 (−687 to −16) with a C-to-A exchange at nucleotide −386) was made by swapping the promoter fragment Clal/Xmol1 (nucleotides −687 to −378) from pPE110 with a PCR-generated fragment. Primer pair PCR9/PCR10 (utilizing pG54 as a substrate) was used to generate a 310-bp fragment that was digested with Clal/Xmol1 and ligated into pPE110. Plasmid pPE110R3 (−687 to −16) with a C-to-A exchange at nucleotides −386, −371, and −364 with the same strategy. An Xhol/BamHI promoter fragment (nucleotides −378 to −16) in pPE110R1 was swapped with a PCR-generated fragment (primer pair PCR18/PCR3) digested with the same enzymes. This generates three-point mutations in the presumed Rap1p-binding sites. The full GPD1 promoter sequence of all constructs was checked by sequencing using the sequencing kit BigDye (Perkin-Elmer catalog no. 4303149), and the PCR-generated products were analyzed by the Bio-Rad D1 protein assay with bovine serum albumin as the standard and indicated as parts/million.

Electrophoretic Mobility Shift Assays (EMASs)—Cells from a 0.5-liter culture (A500 = 1) were harvested by centrifugation (2600 x g, 10 min, 4 °C) and washed once with 15 ml of H-buffer (200 mM Tris-HCl (pH 8.6), 100 mM glycine, and 2 mM EDTA) and run at 13 V/cm for 2 h at 4 °C) and washed once with 15 ml of H-buffer (200 mM Tris-HCl (pH 8.6), 100 mM glycine, and 2 mM EDTA) and run at 13 V/cm for 2 h at 4 °C). The gels were dried, and PhosphorImager plates were exposed and subsequently scanned in a Molecular Dynamics PhosphorImager.

The images were processed with the aid of computer software from Adobe Systems Inc. (Photoshop Version 2.0.1) and Deneba Systems Inc. (Canvas Version 3.5).

DNAse I Footprint Experiments—For the DNAse I footprint experiments, plasmid pPE130 (GPD1 (−478 to −16)) was cleaved with HindIII, labeled (Klenow fill-in reaction) with [α-32P]dATP, purified with the Wizard DNA clean-up system, cleaved with BamHI, and ethanol-precipitated, and the resulting fragments were electrophoretically separated on a 1.5% agarose gel. The fragment of interest was electroblotted onto a DEAE membrane (Schleicher & Schuell) and eluted according to a standard procedure (31). 1 x 10^6 cpm (Cerenkov) of the probe were used for each binding reaction and for a Maxam-Gilbert sequencing reaction (31). The probe was mixed with binding buffer (supplemented with 2 mM MgCl2 and 10 mM CaCl2), 4 μg of poly(dd-dC), and 20 μg of protein extract (except for the control sample) to a final volume of 20 μl. The mixture was incubated for 15 min at 30 °C before the addition of 2 μl of DNAse I (5120 units/ml; 2560 units/ml for the control sample without protein). After a 120-s incubation at 30 °C, the reaction was stopped by adding 2 μl of 0.5 x EDTA and 2 μg of poly(dd-dC), and the samples were put on ice. A 1-min extraction with a phenol/chloroform/soyamyl alcohol mixture (25:24:1; v/v) followed; the DNA was ethanol-precipitated twice; and the pellet was washed once with 70% ethanol. The DNA was vigorously resuspended in 3 μl of 1 x binding buffer before the addition of 20 μl of formamide dye solution were added; and the samples were frozen for later use. A 6% denaturing polyacrylamide gel (31:1 acrylamide/bisacrylamide) was prepared and prerun for 1 h, and the samples were applied after a 5-min denaturation step at 90 °C in a water bath. The gels were run at 90 watts for ~2 h at ~50 °C, dried, and exposed to a PhosphorImager plate for 11 days. After scanning of the plate in the PhosphorImager, the image was analyzed by the following computer software: PDQuest (background subtraction, Protein Data Bases Inc.), Photoshop Version 2.0.1, and Canvas Version 3.5.

RESULTS

Promoter Regions of Importance in the Osmotic Induction of the GPD1 Gene—Transcription of the GPD1 gene is enhanced by increased osmolarity, instigated by either NaCl or sorbitol additions, as shown previously either by Northern analysis or by the use of a GPD1 promoter-CAT reporter fusion (22, 32). To identify the element(s) of the GPD1 promoter responsible for the osmotically controlled transcriptional activation, a series of 5′-promoter deletions were constructed starting at nucleotide −687 in relation to the start of translation. The promoter constructs were fused to a CAT reporter on a YCP vector plasmid (pPE110–114) (Table I) and introduced into strains YPH499 and W303-1A. This reporter construct reflects well the salt response of the normal chromosomal GPD1 gene and thus appears to contain all relevant regulatory sequences. Transforms were cultured to the mid-exponential growth phase at different salinities (0, 0.5, and 1.0 M NaCl), and the resulting amount of CAT protein was measured in crude protein extracts by an immunooassay (Fig. 1). Extensive 5′-deletions of the promoter only slightly affected the transcriptional activity during growth in basal medium; ~75% of the GPD1 promoter activity remained (7.1 to 5.2 ppm) even for the deletion to position −322 (construct pPE112). Cells growing exponentially in 1 M NaCl medium exhibited for the full-length promoter (pPE110, nucleotides −687 to −16) almost 3-fold higher levels of transcriptional activity than when cultured without salt addition, and ~50% of this salt-induced level remained for the most extensive deletion (19.0 to 9.0 ppm). The greatest reduction in salt-induced levels was observed for deletions beyond position −478; however, the promoter fragment containing only the most proximal 322 nucleotides still displayed salt stimulation. Thus, these 5′-deletion studies indicate that the GPD1 promoter does not contain one exclusive element involved in the osmosic responsiveness.

To further substantiate which regions are implicated in the salt-induced regulation of the promoter, internal deletions were made (pPE115–120) (Table I), and the transcriptional
activity was again analyzed during exponential growth (Fig. 1). It was apparent that constructs harboring internal deletions exhibited more severe promoter activity defects than the 5'-deletions. For example 5'-deletion down to nucleotide 322 only slightly reduced the basal activity (75% remaining), whereas the constructs encompassing internal deletions of the region proximal to nucleotide 478 displayed at the most only ~20% of the activity of the full-length promoter. It was also evident that deletions of region 377 to 322 strongly affected the general level of activity of the promoter while still responding to an osmotic upshift, in particular to low salinity (0.5 M NaCl). This was also seen for the more distal region 482 to 378, although the response to salt for this construct was less significant. Most strikingly, when both these regions (pPE118, nucleotides 482 to 322) were deleted, the GPD1 promoter appeared almost completely inactive under any growth condition. It was also notable that more extensive deletions, nucleotides 580 to 323 or 687 to 323, regained some of the activity for the promoter. It thus appears as if the region upstream from nucleotide 478 might harbor repressor(s), whereas region 478 to 324 might be the site for binding of protein(s) acting as a repressor of the upstream repressor(s). However, we cannot exclude position effects by bringing the vector DNA closer to the transcriptional initiation site in these deletion constructs.

Protein-DNA Interactions at the GPD1 Promoter—The promoter region spanning nucleotides 687 to 16 was analyzed for protein-DNA interactions utilizing different parts of the GPD1 promoter in an EMSA. Protein extracts from the two strains YPH499 and W303-1A were tested for all indicated probes, yielding indistinguishable results. Three major interactions were detected along the examined promoter region (Fig. 2), and all these protein-DNA interactions were clearly specific since they could be competed out with an excess of nonradioactive competitor fragments covering the site of interaction. The fragment encompassing nucleotides 687 to 708 (probe I) revealed one interaction (designated I*) (Fig. 2A). This binding could be out-competed with an excess of unlabeled fragment covering the same range of the promoter, but not by a fragment spanning nucleotides 577 to 377 (probe II) (Fig. 2B). A less prominent interaction (designated III*) (Fig. 2C) was located at positions 377 to 324. This latter localization was accomplished using

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**Table I**

| Plasmids | Description | Ref. or source |
|----------|-------------|----------------|
| p054     | 1.4-kb *SspI/SspI* GPD1 fragment fused to CAT gene in YIp5-32cat | 32 |
| pPE102   | GPD1(-1449/-16) subcloned in pBSKS+ | This work |
| pPE102a  | GPD1(-1449/-16) subcloned in pBSKS+ | This work |
| pPE102a  | ΔXhoI/KpnI in MCS | This work |
| pPE103a  | pPE103, KpnI site at -581/-577 | This work |
| pPE103a  | pPE103, KpnI site at -483/-478 | This work |
| pPE104   | GPD1(-377/-322) in GPD1 | This work |
| pPE105   | GPD1(-377/-322) in GPD1 | This work |
| pPE106   | GPD1(-377/-322) in GPD1 | This work |
| pPE107   | GPD1(-377/-322) in GPD1 | This work |
| pPE110   | GPD1(-687/-16)-CAT | This work |
| pPE110a  | pPE110, ΔXhoI/KpnI in MCS | This work |
| pPE110b  | pPE110a, GPD1 fragment from pPE103a | This work |
| pPE110c  | pPE110a, GPD1 fragment from pPE103b | This work |
| pPE110R1 | pPE110, nt -386, C-to-A exchange | This work |
| pPE110R3 | pPE110, nt -386, -371, -358, C-to-A exchange | This work |
| pPE111   | GPD1(-377/-16)-CAT | This work |
| pPE112   | GPD1(-377/-322)-CAT | This work |
| pPE113   | GPD1(-377/-16)-CAT | This work |
| pPE114   | GPD1(-478/-16)-CAT | This work |
| pPE115   | GPD1(-687/-16,Δ-580/-479)-CAT | This work |
| pPE116   | GPD1(-687/-16,Δ-377/-323)-CAT | This work |
| pPE117   | GPD1(-687/-16,Δ-580/-323)-CAT | This work |
| pPE118   | GPD1(-687/-16,Δ-482/-323)-CAT | This work |
| pPE119   | GPD1(-687/-16,Δ-580/-378)-CAT | This work |
| pPE120   | GPD1(-687/-16,Δ-482/-378)-CAT | This work |
| pPE130   | GPD1(-478/-16) subcloned in pBSKS+ | This work |

| Oligonucleotides | Description | Ref. or source |
|------------------|-------------|----------------|
| PCR1 (-577/-557) | 5’-gg ggtacc ATTCACATACGTTTGG-3’ | This work |
| PCR2 (-478/-458) | 5’-gg ggtacc AACAGCGGTTCAATAGGC-3’ | This work |
| PCR3 (-16/-28) | 5’-gg GATCC GATTGGTTGGTTG-3’ | This work |
| PCR4 (-581/-601) | 5’-gg ggtacc GTTTTTCCAGTTAGAGTAG-3’ | This work |
| PCR5 (-483/-504) | 5’-gg gcc ggtacc GTTGGCTTCTCTTCTGGTTC-3’ | This work |
| PCR9 (-687/-669) | 5’-cc atcgat CTCTCTCTGGGTTGAG-3’ | This work |
| PCR10 (-370/-399) | 5’-gg TGCCGGGGG GGGGGTGTTTGTTGATGTT-3’ | This work |
| PCR18 (-384/-344) | 5’-ACAC CCCGGGG CACACAAAGTCCCCCAGCACCACACCAATAC-3’ | This work |

*a* MCS, multiple cloning site; nt, nucleotide(s).

*b* Sequences from the GPD1 promoter are given in uppercase letters. Lowercase letters indicate introduced nucleotides. Restriction sites are in boldface. Point mutations in PCR10 and PCR18, in Rap1p-binding sites, are underlined.

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**Listing of plasmids, constructs, and oligonucleotides (PCR primers) used**

- **Plasmids**
  - p054: 1.4-kb *SspI/SspI* GPD1 fragment fused to CAT gene in YIp5-32cat
  - pPE102: GPD1(-1449/-16) subcloned in pBSKS+
  - pPE102a: ΔXhoI/KpnI in MCS
  - pPE103a: pPE103, KpnI site at -581/-577
  - pPE103b: pPE103, KpnI site at -483/-478
  - GPD1(-377/-322) in GPD1
  - GPD1(-377/-322) in GPD1
  - GPD1(-377/-322) in GPD1
  - GPD1(-377/-322) in GPD1
  - GPD1(-377/-322) in GPD1
  - GPD1(-687/-16)-CAT
  - pPE110, ΔXhoI/KpnI in MCS
  - pPE110a, GPD1 fragment from pPE103a
  - pPE110b, GPD1 fragment from pPE103b
  - pPE110R1, nt -386, C-to-A exchange
  - pPE110R3, nt -386, -371, -358, C-to-A exchange
  - GPD1(-377/-16)-CAT
  - GPD1(-377/-322)-CAT
  - GPD1(-377/-16)-CAT
  - GPD1(-478/-16)-CAT
  - GPD1(-687/-16,Δ-580/-479)-CAT
  - GPD1(-687/-16,Δ-377/-323)-CAT
  - GPD1(-687/-16,Δ-580/-323)-CAT
  - GPD1(-687/-16,Δ-482/-323)-CAT
  - GPD1(-687/-16,Δ-580/-378)-CAT
  - GPD1(-687/-16,Δ-482/-378)-CAT
  - GPD1(-478/-16) subcloned in pBSKS+

- **Oligonucleotides**
  - PCR1 (-577/-557)
  - PCR2 (-478/-458)
  - PCR3 (-16/-28)
  - PCR4 (-581/-601)
  - PCR5 (-483/-504)
  - PCR9 (-687/-669)
  - PCR10 (-370/-399)
  - PCR18 (-384/-344)
FIG. 1. Deletion analysis of the GPD1 promoter and the influence on osmotic stress induction. The indicated promoter deletions (5'- or internal deletions) were fused to the coding region of bacterial CAT on a YCp shuttle vector and transformed into yeast (data for strain YPH499 is displayed; 5'-deletions were also tested in the W303-1A background, yielding identical results). Nucleotide positions were assigned relative to the translational start site (designated +1). Arrows above the schematic GPD1-CAT construct (upper left) indicate restriction enzyme sites utilized in making the deletion constructs (arrows without any restriction enzyme designation indicate the positions for the introduced Kan sites), and numbers indicated below the drawing give the end point nucleotide in the 5'-deletions. The first and last nucleotides removed in the deletions are listed as the deletion end points. The transcriptional importance of different promoter fragments was measured for exponentially growing cells in minimal medium (yeast nitrogen base) without the addition of salt (white bars) or supplemented with 0.5 M (checked bars) or 1.0 M (black bars) NaCl. The amount of CAT protein was recorded by an immunoassay, normalized to the total amount of protein in the extract, and expressed as parts/million. Data represent means ± S.D. (error bars) of three independent experiments.

probe −377 to −16 (probe III) in combination with competition with either fragment −377 to −16 (competitor III) or fragment −324 to −16 (competitor V) (Fig. 2C, lanes 4 and 6, respectively), where the latter competitor could not rival the III+ interaction. However, a competitor DNA fragment containing sequence −478 to −377 (competitor II) totally abolished this III+ interaction, indicating overlapping DNA element specificity for the protein(s) binding to probes II and III (Fig. 2C, compare lanes 2 and 7). These two sites, apparently harboring binding potential for the same protein(s), exhibited clearly different binding affinities for this factor. Thus, the region of the GPD1 promoter exhibiting the greatest impact on both basal and salt stress-induced promoter activities, region −478 to −324 (Fig. 1), exhibited two strong protein-DNA interactions, potentially resulting from binding of the same protein(s).

No Salt-dependent Binding to the GPD1 Promoter Can Be Detected—The detection of protein-DNA interactions prompted gel shift investigations for any salt-dependent binding with either fragment −377 to −16 (competitor III) or fragment −324 to −16 (competitor V) (Fig. 2C, lanes 4 and 6, respectively), where the latter competitor could not rival the III+ interaction. However, a competitor DNA fragment containing sequence −478 to −377 (competitor II) totally abolished this III+ interaction, indicating overlapping DNA element specificity for the protein(s) binding to probes II and III (Fig. 2C, compare lanes 2 and 7). These two sites, apparently harboring binding potential for the same protein(s), exhibited clearly different binding affinities for this factor. Thus, the region of the GPD1 promoter exhibiting the greatest impact on both basal and salt stress-induced promoter activities, region −478 to −324 (Fig. 1), exhibited two strong protein-DNA interactions, potentially resulting from binding of the same protein(s).

Identification of Rap1p as the Major Binding Activity—To locate more precisely the sites of the most proximal protein-DNA interactions detected by the EMSA analysis, region −478 to −16 of the GPD1 promoter was analyzed in a DNase I footprint assay. The only interaction that was experimentally observed was positioned between nucleotides −394 and −374 (Fig. 3). This fragment contains a sequence element well in agreement with the complement of the published Rap1p-binding consensus sequence (5'-T(A/G)T(A/G)CACCCANNC(C/A)CC-3') (35). To establish whether the binding activity of the protein detected in the footprint assay varied depending on the osmotic stress situation, extracts from cells grown in media with different salinities were analyzed. A slight difference in the 5'-portion of the resulting footprint could be seen (Fig. 3) when comparing extracts from cultures grown in 0, 0.7, and 1.4 M NaCl. However, this difference could not be verified in later experiments. To exclude that the detected footprint was caused by a Rap1p-related protein with the same binding specificity, we utilized protein extracts from a strain with the wild-type RAP1 gene exchanged for a truncated gene. This curtailing results in a functional Rap1p with a 230-amino acid deletion in the nonessential N terminus (Δ230-Rap1p) (28). When using extracts from the Δ230-Rap1p strain, the major II+ interaction between nucleotides −478 and −323 was shifted to a faster mobility (Fig. 4A). Similarly, the mobility of the complex formed from the interaction in region −377 to −16 was altered (Fig. 4B). These results clearly indicate that the protein-DNA interactions occurred at nucleotides −394 to −374 (interaction II+) and −377 to −324 (interaction III+) both involve binding of Rap1p. Interaction I+ between nucleotides −687 to −577 was, however, not affected in the Δ230-Rap1p strain (Fig. 4C), proving this protein-DNA interaction to be distinct from the other two more proximal interactions and not involving Rap1p. This
distal factor will here be designated GUP for GPD1 promoter upstream binding protein(s).

The Rap1p-binding Sites are Functional in Vivo and Are Differentially Involved in Low and High Salt Response Mechanisms—To provide in vivo evidence for the functionality of the high affinity Rap1p interaction (interaction II*) between positions −394 and −374 (indicated as RAP1a in Fig. 5A), one essential nucleotide in the center of the Rap1p-binding consensus sequence, C−386, was exchanged for an A (Fig. 5A). This nucleotide at the core of the consensus sequence is vital for Rap1p binding (35). In accordance, the in vitro Rap1p interaction with the mutated binding site, RAP1a (C386A), was abolished (Fig. 5B). In initial in vivo experiments, it was evident that the GPD1 promoter containing the mutated RAP1a (C386A) site was responding to high (but not low) concentrations of NaCl. Therefore, to more precisely locate the NaCl threshold value for this RAP1a site-dependent induction of GPD1, the effect of stepwise increase in salinity was monitored (Fig. 5C). It was evident that the wild-type promoter responded almost linearly to increases in NaCl over the whole range of concentrations tested, whereas the GPD1 promoter containing the mutated RAP1a (C386A) site exhibited no salt-enhanced activity until ≥0.8 M NaCl. Apparently, there is a clear mechanistic threshold between 0.6 and 0.8 M NaCl, and a functional Rap1p site positioned around nucleotide −386 is a prerequisite for the stress control of the promoter at low salt concentrations.

The promoter fragment containing the mutated RAP1a site never reached wild-type levels of induction at higher salinities; however, the level of induction (~4-fold during growth in 1.4 M NaCl medium) was of roughly the same magnitude as for the wild-type promoter (Fig. 5C).

The exact position of the weak Rap1p site between nucleotides −377 and −324 (interaction III*) remains to be determined since there are two theoretical locations (indicated as RAP1b and RAP1c in Fig. 5A). The in vivo importance of these more proximal binding sites (RAP1b and RAP1c) was thus examined in combination by totally abolishing Rap1p binding by C-to-A mutations in all three putative core regions (RAP1a (C386A), RAP1b (C371A), and RAP1c (C358A)) (Fig. 5A). A functional role was confirmed by the fact that the triple binding site-mutated GPD1 promoter exhibited low basal activity and only slight salt induction, even during growth in 1.4 M NaCl medium (Fig. 5C). Apparently, binding of Rap1p to the RAP1b and/or RAP1c sites is a prerequisite for induction at high salinity, at least in the construct where binding to the strong RAP1a site is abolished.

The salt-induced response of the GPD1 promoter at salinities below 0.6 M NaCl is exclusively dependent on the most distal and strongest Rap1p-binding site. However, at higher salinities, one (or both) of the weaker and more proximal Rap1p-binding sites can partially compensate for the loss of the strong RAP1a site. Furthermore, the data support the existence of a
threshold value in the range 0.6–0.8 M NaCl that distinguishes two different induction mechanisms at this promoter during saline growth.

**DISCUSSION**

**Complex Regulation of the GPD1 Promoter—**Insights into the mechanisms involved in the control of the GPD1 promoter will provide a major key to our general understanding of hyposomatic stress-induced gene regulation. Mechanisms in operation at this promoter have the potential of being dehydration-specific since other types of stresses such as heat, starvation, etc., that strikingly affect the expression of many other osmostress-controlled genes like **CIT1**, **HSP12**, and **HSP104** (10, 36) do not strongly influence the expression of **GPD1** (37, 38). Here we provide evidence that a number of different promoter elements are involved in the transcriptional regulation of **GPD1** during saline conditions, our main contribution being the identification and functional characterization of **Rap1p**-binding sites. **Rap1p** binds to at least two sites in the region −400 to −350 nucleotides upstream from the translational start site, and functional **Rap1p**-binding sites appear to be a prerequisite for proper osmotic control of **GPD1** transcription.

We also provide data regarding the importance of some other regions of the promoter under basal growth conditions and high salinity stress. The presence of repressor element(s) whose function is counteracted by binding of **Rap1p** to the promoter is suggested by the finding that internal deletion of nucleotides −478 to −322 almost completely abolished promoter activity under any growth condition, whereas a 5′-deletion to position −322 only marginally influenced the promoter activity. Further support that **Rap1p** binding *per se* might negatively influence the activity of the putative repressor elements comes from the observation that the single and triple **Rap1p** site mutations give a low basal activity of the promoter (Fig. 5C). Apparently, **Rap1p** will repress the activity of these elements when bound to the promoter. It has been suggested that a number of osmostress-induced genes, including **GPD1**, are controlled by an **SSN6/TUP1**-mediated repression mechanism that is lifted in the presence of osmotic stress (15). This mechanism was shown to be operational for the **HAL1** gene through a defined upstream repressing sequence element (15), whereas for the **ENA1** gene, it was demonstrated that Sko1p apparently tethers the **Ssn6p-Tup1p** corepressor to the promoter (14). The **GPD1** promoter does not appear to be controlled by such a salt-induced derepression mechanism since none of the 5′- or internal promoter deletions exhibited any substantially increased basal activity. This lack of evidence for salt-induced derepression of the **GPD1** promoter via **Ssn6p-Tup1p** is at variance with earlier results (15), but in accordance with a more recent report (23).

Proximal to the **Rap1p**-binding sites are three elements identical to the pentanucleotide **STRE**, having the central C of the core sequence **CCCT** positioned at nucleotides −328, −284, and −32. **STRE**s are found in many promoters and have been shown to be required for the general stress regulation of a number of genes (1). It was recently demonstrated by the use of **GPD1** promoter constructs in which all three **STRE**s had been deleted, that the **GPD1** promoter does not appear to be controlled by such a salt-induced derepression mechanism since none of the 5′- or internal promoter deletions exhibited any substantially increased basal activity. This lack of evidence for salt-induced derepression of the **GPD1** promoter via **Ssn6p-Tup1p** is at variance with earlier results (15), but in accordance with a more recent report (23).

**Figure 3.** DNase I footprint assay with a probe encompassing nucleotides −478 to −16 of the promoter, potentially identifying **Rap1p** as one of the proteins responsible for the strong protein-DNA interaction (complex II*; see Fig. 2) of the **GPD1** promoter. The reversed **Rap1p**-binding consensus sequence is *boxed*, and the location is given by comparing the footprints with the Maxam-Gilbert G + A promoter sequence. Protein addition to the binding mixture is indicated as −P (no protein extract added) and +P (20 µg of protein extract added), and the concentrations of NaCl (0, 0.7, and 1.4 M) used to supplement the growth medium are indicated above lanes 2–4.

**Figure 4.** Identification of **Rap1p** as the protein responsible for the formation of complexes II* and III* in the **GPD1** promoter, but not of complex I* (see Fig. 2). EMSA was carried out using an extract from mutant cells with truncated **Rap1p** that is deleted of its most N-terminal 230 amino acids, YLS91 (Rt), which was compared with the corresponding wild-type full-length **Rap1p**, YDS2 (wt). A, probe VI, nucleotides −477 to −323; B, probe III, nucleotides −377 to −16; C, probe I, nucleotides −687 to −478 (see Fig. 2). **Rap1p** and Δ**Rap1p** indicate the **Rap1p**-DNA complexes with the wild type and the truncated mutant, respectively. GUP stands for **GPD1** promoter upstream binding protein. For further explanations, see legend to Fig. 2. comp., competitor; FP, free probe.
inactivated by mutations\(^5\) that the response of the promoter to either salt stress or heat treatment was STRE-independent. Apparently, the presence of STREs is not a strict requirement for stress-induced expression, as is further supported by the lack of STREs in the salt-responsive DAK1 gene (9).

**Rap1p Acts as a Major Activator in the Transcription of GPD1**—We have identified the multifunctional DNA-binding protein Rap1p as an important activator of GPD1 expression under basal and saline growth conditions and shown that at least two nearby Rap1p-binding sites are occupied \textit{in vitro} and are functional \textit{in vivo}. The most distal site (core of consensus sequence at position –386) exhibited the highest affinity for Rap1p. The exact position of the weak Rap1p site between nucleotides –377 and –324 remains to be determined since there are two theoretical locations (indicated as RAP1b and RAP1c in Fig. 5A). However, the relative proximity of the RAP1b site to the strong RAP1a site makes RAP1b a less likely candidate. The distance between the central parts of the two core consensus sequences is only 14 nucleotides, which is in the range of the published minimum distance (39). Based on recent studies of the crystal structure of the DNA-binding domain of Rap1p in complex with telomeric DNA (40), it was concluded that the protein contains two similar DNA-binding domains recognizing a tandemly repeated DNA sequence. The repeated sequence in telomeric DNA responsible for the high affinity binding of Rap1p is ACACC with the intermediate sequenceCAC. When comparing these recognition sequences with the nucleotide sequence of the three theoretical Rap1p-binding sites of the GPD1 promoter, it is clear that the RAP1a site has the best homology, followed by RAP1c. The RAP1b site almost completely lacks the second ACACC motif. The interaction(s) identified as Rap1p binding to site b or c were efficiently out-competed by fragments containing the RAP1a site (Fig. 2C, compare lane 7 with lane 3). This is in agreement with the finding that the RAP1a site is the most conserved in relation to the consensus sequence and thus promotes, at least \textit{in vitro}, a stronger binding than the RAP1b or RAP1c site.

Rap1p is an essential and ubiquitous DNA-binding protein involved in a wide variety of cellular activities such as (i) silencing of mating-type genes, (ii) ensuring telomere function and structure, (iii) stimulating meiotic recombination, (iv) binding to the nuclear scaffold protein complex, and (v) regulating the transcription of an array of genes (25). Rap1p has been experimentally linked to the transcriptional regulation of genes involved in different cellular contexts (25), and homologies to a binding site consensus sequence have been found in >100 yeast promoters (35). Among the experimentally well verified examples are genes coding for glycolytic enzymes, where a heteromeric complex consisting of the glycolytic regulator Gcr1p and Rap1p is required to mediate an efficient transcriptional activation. Gcr1p was shown to bind a so-called CT box found in several promoters of glycolytic genes (41), and it was later concluded that the Gcr1p-mediated activity of the glycolytic ADH1 promoter was dependent on a functional Rap1p-binding upstream activating sequence element (42). The conclusion drawn from these experiments was that Rap1p increases the possibility of Gcr1p-specific transcriptional activation by physical interaction, thereby attracting the regulator to the promoter.

Since functional Rap1p-binding sites in promoters are often found close to additional elements that control transcription in a specific manner, one might hypothesize that Rap1p recruits osmoregulatory factors in a GPD1 promoter context. The ability of Rap1p to bind and perturb the chromatin structure of promoter regions has been proposed to be a prerequisite for binding of other transcription factors with low element affinity (43). Using a two-hybrid screen, Rep et al. (23) identified a protein named Hot1p as interacting with the mitogen-activated protein kinase, Hog1p. Hot1p displays sequence similarity to the DNA-binding C-terminal domain of Gcr1p, as well as to Msn1p, and deletion of either HOT1 or MSN1 reduced the magnitude of induction of GPD1 by salt stress. Since Gcr1p is dependent on Rap1p for proper control of certain glycolytic genes (44), the Hot1p and Msn1p members of the Gcr1p family might depend on binding of this general transcription factor in their partial control of the GPD1 gene.

**Distinct Low and High Salt Mechanisms**—This report provides the first documentation of a role for Rap1p in the regulation of a gene responsive to osmotic stress. In addition, we also demonstrated that the binding sites for Rap1p appear functionally distinct since a mutation in the distal high affinity
site leads to a nonresponsive promoter at low salinity while leaving the high salinity response more or less intact. Indications of distinct low and high salt mechanisms for gene activation are also apparent from studies on the differential induction of ALD2 (low salt) and DDR48 (high salt) (45). The protein phosphatase calcineurin has been implicated in high salt activation of ENA1 (11). However, it is less likely that this signaling pathway is responsible for the observed high salt response of the GDP1 promoter since only marginal effects on the salt induction of normal chromosomal GDP1 were apparent in the calcineurin-deficient strain cna1cna2Δ (46). The high salt response mechanism appeared in the present study to require terminal truncated Rap1p were kindly provided by H.-J. Schüller.

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