Mutations in the yeast Myb-like protein Bas1p resulting in discrimination between promoters in vivo but not in vitro

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ABSTRACT
Bas1p is a yeast transcription factor that activates expression of purine and histidine biosynthesis genes in response to extracellular purine limitation. The N-terminal part of Bas1p contains an Myb-like DNA binding domain composed of three tryptophan-rich imperfect repeats. We show that mutating the conserved tryptophan residues in the DNA binding domain of Bas1p severely impairs in vivo activation of target genes and in vitro DNA binding of Bas1p. We also found that two mutations (H34L and W42A) in the first repeat make Bas1p discriminate between promoters in vivo. These two BAS1 mutants are able to activate expression of an HIS4–lacZ fusion but not that of ADE1–lacZ or ADE17–lacZ fusions. Surprisingly, these mutant proteins bind equally well to the three promoters in vitro, suggesting that the mutations affect the interaction of Bas1p with some promoter-specific factor(s) in vivo. By mutating a potential nucleotide binding site in the DNA binding domain of Bas1p, we also show that this motif does not play a major role in purine regulation of Bas1p activity. Finally, using a green fluorescence protein (GFP)–Bas1p fusion, we establish the strict nuclear localization of Bas1p and show that it is not affected by extracellular adenine.

INTRODUCTION
The Bas1p and Bas2p transcription factors activate expression of genes in the histidine and purine biosynthesis pathways in response to exogenous adenine (1–4). While Bas1p apparently regulates these pathways specifically, Bas2p appears to be a more general factor.

Bas2p is a homeodomain protein which was initially characterized as Pho2p, a transcriptional activator of the PHO5 gene (5). Bas2p binds to several promoters in vitro (2,3,6,7) but no clear consensus binding site for Bas2p has been defined. While Bas2p binds to DNA in vitro, it has been shown in vivo to require other transcription factors for transcriptional activation.

For example, Bas1p and Bas2p bind to DNA independently in vitro, but in vivo the lack of BAS1, BAS2 or both leads to the same incapacity to activate expression of the target genes (2,3,6). Bas2p binds with Bas1p on several promoters (2–4,6,8), and this binding has been shown to be non-cooperative on the HIS4 promoter (6). The situation is different on the PHO5 or HO promoters, where Bas2p (Pho2p) cooperatively binds with Pho4p (9) or Swi5p (7) respectively.

The role of Bas1p seems to be more specific than that of Bas2p. We have recently shown that Bas1p activates a small number of genes, most of which encode purine or histidine biosynthesis enzymes (1). The structure of Bas1p is not known, but sequence comparison has revealed that Bas1p contains an Myb-like DNA binding domain located in the N-terminal part of the protein (6). This DNA binding domain is composed of three imperfect tandem repeats containing highly conserved tryptophan residues (Fig. 1). While for c-Myb the last two repeats are sufficient for DNA binding in vitro (10,11), for Bas1p all three repeats are required for proper DNA binding (12). Bas1p binds to a 5’-TGACTC-3’ consensus sequence as defined by DNaseI footprinting analysis and in vitro directed mutagenesis (3,6). Interference footprinting experiments have further documented the specific interaction of Bas1p with the TGACTC sequence and revealed downstream of the consensus sequence a 3 bp extension which could affect affinity of Bas1p binding (12). This TGACTC sequence is necessary but not sufficient for activation in the presence of BAS1. Indeed, expression of several genes carrying the TGACTC motif in their promoter is not affected by mutations in the BAS1 gene (1,8).

How do Bas1p and/or Bas2p respond to the presence of extracellular adenine? Two recent reports have provided preliminary answers to this question. First, we have shown that the regulatory signal is not adenine itself but rather ADP or a derivative of ADP (13). Second, Rolfes and co-workers have shown that a LexA–Bas1p fusion can activate transcription only in the presence of Bas2p and in an adenine-regulated fashion (14). Interestingly, a LexA–Bas2p fusion could activate transcription in the absence of Bas1p, but this activation was insensitive to extracellular adenine. These results suggest that a purine nucleotide can directly or
indirectly modify Bas1p ability to activate transcription, possibly by affecting its interaction with Bas2p. From these studies, it cannot be excluded that adenine regulation operates by excluding Bas1p from the nucleus. Interestingly, the other two Bas2p-interacting proteins (Pho4p and Swi5p) are regulated through their intracellular localization (15,16), i.e. they are excluded from the nucleus under certain conditions. Another interesting observation made by Arndt and co-workers (6) is that Bas1p contains a glycine-rich region in its DNA binding domain that is not found in any of the other Myb-like proteins (Fig. 1). This extra glycine-rich region has been suggested to be a potential nucleotide binding site (6), and it is therefore tempting to assume that this motif could play some role in the purine regulation process.

To further understand how Bas1p binds to DNA and how adenine regulation operates in yeast, we have addressed the following issues. Is Bas1p a typical Myb-like protein? Why is the first repeat of the Myb-like domain essential? What is the role of the extra glycine-rich region? Is Bas1p intracellular localization affected by extracellular adenine? In this paper we present some answers to these questions and describe mutations in the Bas1p DNA binding domain that allow this transcription factor to discriminate between promoters in vivo but not in vitro.

MATERIALS AND METHODS

Yeast strains and media

Yeast strains used in this study were Y329 (MATα ara3-52 leu2-3,112 gcn4-2 bas1-2), L3080 (MATα gcn4-2 ura3-2 bas1-2) and Y539 (MATα leu2-3,112 his2-201 trpl1::hisG ura3-52).

Yeast were grown in SD medium [2% (w/v) glucose, 0.17% (w/v) nitrogen base and 0.5% (w/v) ammonium sulfate], SD-CASA medium [SD supplemented with 0.2% (w/v) casamino acids; Difco Laboratories], or SC medium [SD supplemented with amino acids as described by Sherman et al. (17)]. Adenine was optionally added to a final concentration of 0.15 mM.

Oligonucleotides

The following oligonucleotides were used for site-directed mutagenesis: 70′-GAGGATGAGACGCTTCTTG-3′, 76′-TGTCGAAATAGTTAC-3′, 99′-TTCCTCGAGCCTCATTCTCTTAAATCTAATCTATCT-3′, 100′-TTCCTCGAGCCTACATTTCTTTCTTCTTCTTATC-3′, 101′-TTCCTCGAGCCTACATTTCTTTCTTCTTCTTATC-3′, 147′-CTTCCAGGTCCCAACAC-3′, 149′-TTGAAGAAAGGTAAAAGCTGAA-3′, 150′-GAGGA TGAGCAGCTCTTG-3′, 189′-CGTGAGTCACT-3′, 200′-GGTTAAAACGAGCGTAC-3′, 202′-GTTTAAACAGACCTGATAG-3′, 203′-GGTTTAAACAGACCTGATAG-3′, 204′-CAACCAGCTACGCAA TTTG-3′, 205′-TTA TCGTCGTCCTTGCT-3′.

Site-directed mutagenesis of BAS1

The plasmid used for Bas1p expression in yeast is named P79. This centromeric plasmid carrying the URA3 and BAS1 genes was constructed in two steps. A first plasmid (P75) was obtained by insertion of the Clal–SalI fragment containing the BAS1 gene from plasmid CB286 (a generous gift from K. Arndt) in a derivative of YCp50 in which the unique Xhol site had been destroyed (plasmid B836, kindly provided by G. Fink). P79 is a derivative of P75 in which the YCp50 BamHI site was filled with Klenow polymerase.

Several vectors derived from P79 were then constructed to facilitate in vitro manipulation of the BAS1 gene. P975 is a P79 derivative in which the YCp50 BsuI and HindIII sites were filled with Klenow polymerase. This plasmid contains unique BsuI and HindIII restriction sites in the BAS1 gene. Another derivative of P79, named P1016, corresponds to the replacement of the HindIII–Xhol fragment in the BAS1 gene of P975 by a HindIII–Xhol fragment from pL12 (18) containing a kanamycin resistance-conferring cassette. Similarly, P1035 and P1038 correspond to the replacement of the BamHI–HindIII and BamHI–Xhol fragments in the BAS1 gene of P975 by fragments containing a kanamycin resistance-conferring cassette from plasmids pJA50 (19) and pL12, respectively (18).

All the mutants described below were constructed in the P79 vector or derivatives and were verified by sequencing. W42A Bas1p mutant was obtained by the megaprimer method (20). Synthetic oligonucleotides used for site-directed mutagenesis are listed above. The first PCR amplification was done with oligonucleotides 76 and 148 and the second PCR with oligonucleotide 147. For both PCR amplifications, P975 was used as a matrix. The product of the second PCR was cut with BamHI and HindIII and inserted in P1035 linearized with BamHI and HindIII. The resulting plasmid was named P1083. The W119S mutant was obtained by a gap-repair method (21). First, a DNA fragment containing the mutation was amplified by PCR with oligonucleotides 147 and 149 with P79 as a matrix (the mutation was contained in oligonucleotide 149). Yeast strain L3080 was then transformed with 1 μg of the PCR fragment and 0.2 μg of P975 linearized with HindIII. Transformants, corresponding to recircularization of the plasmid either by ligation or recombination with the PCR product, were selected on SD-CASA medium supplemented with tryptophan and adenine and replica-plated on SC medium lacking uracil and supplemented or not with histidine. Plasmid DNA from clones unable to grow in the absence of histidine were extracted from yeast by the method of Robzyk (22), then amplified in Escherichia coli and sequenced. The resulting plasmid was named P1005. The G164E mutant (plasmid P526) was constructed by replacing the P79 BamHI–Xhol fragment by a PCR amplification fragment (obtained on P79 as a matrix with oligonucleotide 76 and oligonucleotide 100 which contains the mutation) cut with BamHI and Xhol. The double mutant W172L–T173A (plasmid P528) was obtained by substitution of the P79 BamHI–Xhol fragment by a PCR amplification fragment (obtained on P79 as a matrix with oligonucleotide 76 and oligonucleotide 101 which contains the two mutations) cut with BamHI and Xhol. The G167D mutant was constructed by replacing the P79 BamHI–Xhol fragment by a PCR amplification fragment (obtained on P79 as a matrix with oligonucleotide 76 and oligonucleotide 101 which contains the two mutations) cut with BamHI and Xhol. Sequencing of the resulting plasmid (P530) revealed a double mutation in the BAS1 gene. The single G167D mutant was then obtained by replacement of the HindIII–Xhol fragment of P1016 by the HindIII–Xhol fragment of P530 resulting in plasmid P1052. Four other mutations described in this paper were unexpected mutations resulting from polymerase errors during PCR amplification. These mutations were separated from other mutations in BAS1 as
follows: the P1078 and P1080 plasmids were obtained by replacing the BamHI–HindIII fragment from P1035 by the BamHI–HindIII fragment from plasmids P530 and P531 containing the L128S and H34L mutations, respectively. The plasmid P1077 was constructed by replacement of the BamHI–XhoI fragment from P1038 by the BamHI–XhoI fragment from plasmid P1042 containing the S142P mutation. Finally, W210C was obtained as an His− mutant in a gap repair experiment done on P79. A single mutation W210C was found by sequencing. The resulting plasmid was named P933.

GST–HA–Bas1p fusions construction

The plasmid used to construct the GST–HA–Bas1p fusion is named pDC40 (a generous gift from D. Chao). This plasmid carries the LacI gene and a fusion between the GST gene and the HA epitope under the control of Ptac promoter. The plasmid P841 carrying GST–HA–BAS1 fusion was obtained by inserting a BamHI–SspI fragment (corresponding to amino acids 17–811 of Bas1p) from P79 in pDC40 linearized with BamHI and Smal. To facilitate construction of the various GST–HA–Bas1p mutants, a derivative of P841 (plasmid P870) was obtained by replacement of the BamHI–XhoI fragment in the BAS1 gene of P841 by a BamHI–XhoI fragment from pluU12 (18) containing a kanamycin resistance-conferring cassette. Plasmids carrying the various mutant versions of the GST–HA–Bas1p fusion for H34L, W42A, W119S, L128S, S142P, G164E, G167D and W172L–T173A mutants were obtained by replacement of the BamHI–XhoI fragment of P870 by the BamHI–XhoI fragment from P1080, P1083, P1005, P1078, P1077, P526, P1052 and P528, respectively. Finally, GST–HA–Bas1p fusion for W210C was obtained by replacement of the BamHI–BglI fragment from P870 by the BamHI–BglI fragment from P933.

Bas1p expression and purification

Bacterial strain ER2267 (Biolabs) transformed with the various GST constructs was grown overnight at 37°C in LB supplemented with ampicillin (100 µg/ml). Bacteria were then diluted to 1:100 in 500 ml of fresh medium containing ampicillin and were grown at 37°C to an OD600 of 1. Cells were harvested by centrifugation, the pellet was frozen in liquid nitrogen and stored at –80°C. The pellet was thawed at room temperature and resuspended in 6 ml of Buffer A [25 mM HEPES buffer (pH 7.7) containing 50 mM KCl, 0.5 mM EDTA, 10% (w/v) glycerol, 10 mM 2-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride] to which 500 µl of bacterial protease inhibitor cocktail (from Sigma) were added. Cells were lysed by sonication with four 15 s pulses at maximum intensity (ultrason-Annemasse type 75T20K) on ice followed by three cycles of freezing in liquid nitrogen and thawing by immersion of the tube in room temperature water. Cellular debris were removed by centrifugation at 20 000 g for 20 min at 4°C. Batch purification of the fusion protein was done as follows: supernatant was incubated for 1 h at room temperature with 650 µl of glutathione–Sepharose 4B resin (Pharmacia) equilibrated by three washes with 10 ml of buffer A. Resin was harvested by centrifugation at 1000 g for 1 min and was washed three times with 10 ml of Buffer A. The GST fusion proteins were then eluted from resin by a 20 min incubation in 500 µl of Buffer A containing 10 mM of glutathione and by a centrifugation at 1000 g for 1 min. The protein eluate was supplemented with 100 µl of glycerol, then aliquoted, frozen in liquid nitrogen and stored at –80°C.

Western blot analysis

Western blot analysis of GST–HA–Bas1p fusion was done as described (23) with anti-HA (12CA5, Boehringer) as primary antibody (diluted at 0.5 µg/ml) and peroxidase-conjugated anti-mouse IgG (Pierce) as secondary antibody (diluted 1:2500).

Electrophoresis mobility shift assays

Electrophoresis mobility shift assays were performed as described (6) with 100 fmol of each radiolabelled probe and 2 µl (0.5 µg proteins) of purified GST or GST–Bas1p wild-type or mutant. Radiolabelled probes were obtained by PCR on yeast genomic DNA from S288C strain in the presence of 10 µl of [α-32P]dATP (400 Ci/mmol, 10 mCi/ml). The following pairs of oligonucleotides were used for PCR amplification of the probes: ADE1 (oligonucleotides 190 and 200), ADE17 proximal (oligonucleotides 190 and 191), ADE17 distal (oligonucleotides 201 and 202), HIS4 (oligonucleotides 188 and 189). The oligonucleotide sequence is given in the oligonucleotides section of Materials and Methods.

β-galactosidase activity

For βGal assays, Y329 cells were co-transformed with a plasmid carrying the lacZ fusion and with the centromeric plasmid carrying the various BAS1 mutants. Six clones for each transformation were grown overnight in SC medium and then diluted at 0.1 OD600 in the same medium supplemented or not with 0.15 mM adenine. After 6 h at 30°C, the βGal assays were performed on these exponentially growing cells. Assays were done using the method of Kippert (24) with N-lauroyl sarcosine as a permeabilization agent. Units of β-galactosidase activity were calculated by the formula (1000 × reaction mix OD420)/(culture OD600 × vol of culture × min of assay). In each experiment, two independent βGal assays were performed, and each assay was done on six independent transformants. Inter-assay variation was <20%.

Green fluorescence protein (GFP)–Bas1p fusion

An Nhel–Smal DNA fragment containing the entire BAS1 open reading frame was amplified with the following oligonucleotides: BAS1NHE 5’–TCGAGAGCTAGCAA TA TAAGTACCAAAG-3’ and BAS1SMAL 5’–TCCCCCGGGATTCAGTGGCAGGACT-3’ and was cloned in the pGFP-N-FUS vector (25) linearized by SpeI and Smal. The resulting plasmid carrying the GFP–BAS1 fusion was named P972.

Propidium iodide staining

Yeast cells from strain Y539 transformed with the P972 plasmid carrying the GFP–BAS1 fusion were fixed by addition of 110 µl formaldehyde 37.5% (v/v) to 1 ml of culture in exponential phase. After incubation at room temperature for 10 min, cells were harvested by centrifugation and washed twice with 1 ml of 50 mM sodium citrate, pH 7 (Buffer B). Pellets were resuspended in 500 µl of buffer B supplemented with RNase A at a final concentration of 0.25 mg/ml. Cells were then incubated at 50°C for 1 h. Next, 500 µl of buffer B containing 50 µg/ml propidium iodide solution were added. After incubation for 30 min in the dark at room temperature, cells were harvested by centrifugation.
and resuspended in 50 μl of buffer B. This suspension was placed on a microscope slide, covered by a coverslip and analyzed by microscopy.

Microscopy imaging

Conventional epifluorescence microscopy was performed on a Leica DMRXA microscope using a 100 × immersion objective. For GFP studies, fluorescence was visualized using a FITC filter cube (Leica): 450–490 nm excitation, 510 nm dichromatic mirror, 515–560 nm emission. Propidium iodide fluorescence was visualized with a Texas-red filter cube (Leica): 530–595 nm excitation, 600 nm dichromatic mirror, LP 615 nm emission. Images were acquired and digitized directly with a cooled CCD camera MicroMax (Princeton Instruments) controlled and analyzed by the Metamorph 3.0 software (Universal Imaging Corporation).

RESULTS

To investigate functionally the Myb-like DNA binding domain of the Bas1p protein, we produced mutations affecting conserved residues. We initially aimed to focus on substitutions of the tryptophan residues which are typical of the Myb-family of transcription factors, but several unexpected mutations resulting from polymerase errors during the PCR amplification process were also isolated and studied (Materials and Methods). The positions of the mutated residues in the Bas1p protein together with a sequence comparison with the chicken c-Myb protein are presented in Figure 1. Each of the bas1 mutants results from a unique mutation, except W172L-T173A which is a double substitution.

Mutations in the first repeat make Bas1p discriminate between promoters in vivo but not in vitro

Mutations in the three Myb-like repeats of the Bas1p DNA binding domain were obtained by site-directed mutagenesis. The effect of these mutations was evaluated in vivo in the gcn4 bas1 double mutant strain Y329. In this strain, expression of the HIS4 gene is limiting for growth in the absence of histidine (2). Transformation of such a strain with a centromeric vector carrying the wild-type BAS1 gene is sufficient to restore prototrophy for histidine. We took advantage of this convenient phenotype to evaluate the effect of the mutations in the Bas1p DNA binding domain. Each mutant carried on a centromeric vector was transformed in the Y329 yeast strain and the transformants were tested for their ability to grow on minimal medium lacking histidine. The results presented in Figure 2 show that three mutants (H34L, W42A and L128S) grew as well as the wild-type, while three others (W119S, S142P and the double mutant W172L-T173A) did not grow at all. Finally, one mutant (W210C) behaved in an intermediary way.

To get a more quantitative insight into the effect of the various mutations, we evaluated the capacity of these mutants to activate expression of three lacZ fusions (ADE1–lacZ, ADE17–lacZ and HIS4–lacZ) that are known to require Bas1p for optimal expression (1–3). Transformation of such a strain with a plasmid carrying the lacZ fusion and with the centromeric plasmid carrying the various BAS1 mutants. Expression of the fusion was then monitored after growth in the presence or absence of adenine. The results presented in Figure 3A confirm that the

Figure 1. Sequence alignment of Bas1p and chicken c-Myb DNA binding domains. Optimal sequence alignment of Bas1p and chicken c-Myb proposed in (12). Identical amino acids are marked by an asterisk. Introduced mutations in Bas1p DNA binding domain are mentioned at the tip of arrows. Numbers in brackets refer to the position of mutated amino acid residues in the Bas1p sequence.
mutants that were unable to grow in the absence of histidine are indeed unable to activate expression of the HIS4–lacZ fusion. More surprisingly, two mutants (H34L and W42A) behaved very differently depending on the fusions tested. While these mutations abolished the capacity to activate expression of the ADE1–lacZ and ADE17–lacZ, they had almost no effect on HIS4–lacZ expression (and consistently on growth in the absence of histidine, Fig. 2). This result suggests that mutations in the first repeat could lead to a promoter discrimination by the Bas1p factor. This could be due either to a discrimination by the Bas1p protein itself as a function of the promoter sequence or to its incapacity to interact in vivo with other factor(s) required for transcription activation. To distinguish between these two possibilities, we monitored the ability of the various mutants to bind in vitro to the promoters of the ADE1, ADE17 and HIS4 genes.

The wild-type Bas1p and each of the mutant proteins were purified as a GST–HA–Bas1p fusion (Materials and Methods). The amount of purified protein was estimated by western blot with monoclonal antibodies raised against the HA epitope. Figure 3B shows that approximately the same amount of protein was
purified for each construct. In each lane, the higher band corresponds to the full-length fusion protein while the lower band corresponds to a degradation product. When these purified proteins were used for electrophoretic mobility shift assays (EMSA), we routinely observed two very close band shifts, suggesting that both full-length and proteolyzed fusion proteins are able to bind DNA. We have never observed conditions where these two bands behave differently, so we assume that this partial proteolysis does not interfere with DNA binding. Similar results were obtained by others using a slightly different GST–Bas1p fusion (26).

Purified Bas1p was used for EMSA on the promoters of the three genes studied with lacZ fusions (ADE1, ADE17 and HIS4). The Bas1p binding site on the HIS4 promoter has been precisely defined by DNaseI footprinting analysis (6). For ADE1 and ADE17 the situation is less clear. Both genes are known to be less expressed in a bas1 mutant strain (1,3) but the precise Bas1p binding site(s) on these promoters have never been mapped. Sequence analysis revealed that both promoters contain two consensus Bas1p binding motifs, 5′-TGACTC-3′. In the ADE1 promoter, these two motifs are very close to each other (positions –228 and –219 relative to the ATG) and the probe that we used contained the two potential binding sites. For ADE17, the distal and proximal Bas1p binding sites are found at positions –307 and –177 upstream of the ATG, respectively. We therefore designed two probes, named distal and proximal hereafter, each centered on the respective potential Bas1p binding motifs. The four probes, HIS4, ADE1, ADE17 proximal and ADE17 distal, were used for EMSA assays with wild-type and mutant Bas1p purified proteins. Figure 3C shows that the wild-type purified Bas1p binds to all four probes, thus establishing the direct binding of Bas1p in vitro to the ADE1 and ADE17 promoters. As expected for ADE1, two bands were detected by EMSA, the higher shift being most probably due to the binding of two Bas1p molecules to the probe. Bas1p was able to bind to both proximal and distal ADE17 probes with similar efficiency. Surprisingly, EMSA on the distal probe revealed a second band shift, suggesting that a second Bas1p molecule could bind to the probe despite the fact that only one TGACTC motif was found in the probe sequence.

Very similar binding patterns were obtained on the three promoters with the various DNA binding domain mutants in Bas1p. The L128S mutation did not affect binding of the protein to the three promoters, in good agreement with the lack of effect of this substitution in vivo (see Figs 2 and 3A). Two mutations (W119S and W172L-T173A) totally abolished binding of Bas1p to DNA. Finally, four mutant proteins (H34L, W42A, S142P and W210C) were still able to bind DNA but less efficiently than the wild-type. It should be noted that for S142P, no binding was detected on the HIS4 probe while a weak binding was observed on the three other probes. Together, these results show that H34L, W42A and W210C are able to bind to the ADE1 and ADE17 promoters in vitro (Fig. 3C) but are unable to activate the expression of these genes in vivo (Fig. 3A). The same mutants bound to the HIS4 promoter in vitro and activated transcription of the HIS4 gene in vivo. Binding of W210C on the HIS4 probe, which is hardly visible in Figure 3C, was demonstrated by overexposure of the gel (data not shown).

The relative affinity of Bas1p for the different promoters was further assayed by competition experiments. Binding of wild-type Bas1p and H34L mutant protein on either HIS4 or ADE1 promoter sequences was competed with unlabelled HIS4 or ADE1 competitor DNA. The results clearly showed that ADE1 or HIS4 promoter sequences compete equally well for binding of the wild type or mutant transcription factor on either probe (data not shown). We therefore conclude that affinity for these promoters is not strongly affected by the mutation and cannot account for the strong differential effect observed in vivo.

**Mutations in the glycine-rich region do not affect regulation by adenine in vivo**

The glycine residues G164 and G167 were mutated and the corresponding plasmids were transformed in the Y329 strain. Growth of the transformants was then monitored on minimal medium lacking histidine. Figure 4 clearly shows that both mutants grew like the wild-type control. These results were confirmed by assaying the ability of Bas1p in these mutants to activate expression of the HIS4–lacZ fusion. The βGal activity in the mutants was ~80% of that observed in the wild-type (Fig. 5A). Interestingly these mutations had no effect on the repression by adenine (Fig. 5A), which strongly suggests that the glycine-rich region is not involved in the adenine repression process. We further tested these mutants using two other lacZ fusions (ADE1–lacZ and ADE17–lacZ). The two mutants behaved very differently. G164E was unaffected for ADE1–lacZ activation and ADE17–lacZ expression in this mutant was 75% of that of the wild-type (Fig. 5A). For G167D, it could activate ADE1–lacZ expression only up to 38% of the wild-type activation and for ADE17–lacZ it was even more affected (12% of the wild-type level). In all cases, repression by adenine was maintained, thus confirming the results obtained with the HIS4–lacZ fusion.

The ability of these mutant proteins to bind DNA in vitro was estimated by EMSA. Clearly the two glycine mutants were able to bind the four probes (Fig. 5C). Because this glycine-rich region has been proposed to be a potential nucleotide binding motif (6) and because ADP or a derivative of ADP is responsible for regulation by adenine in yeast (13), we also tested whether addition of ADP or ATP might affect binding to DNA of the wild-type and mutant Bas1p proteins. Figure 5C demonstrates that addition of these nucleotides does not affect binding of Bas1p to DNA. Interestingly, the G164E mutant apparently bound more efficiently than the wild-type Bas1p protein to the probes. This effect was observed on all the probes but was more apparent on the ADE1 and ADE17 distal probes where it induced a higher shift. This extra band was also observed with the wild-type protein when larger amounts of protein were used in the EMSA assay (Fig. 3C and data not shown).
**Figure 5.** *In vivo* and *in vitro* effect of glycine mutations in Bas1p. (A) Effect of G164E and G167D mutations on Bas1p activity in *vivo*. Y329 cells were co-transformed with a plasmid carrying the lacZ fusion and with the centromeric plasmid B836 carrying the various BAS1 glycine mutants. Experiments were performed as in Figure 3A. (B) Western blot analysis of wild-type and glycine mutants of Bas1p. Purified GST–HA (control lane) or GST–HA–Bas1p wild-type and glycine mutant fusions were purified as described in Materials and Methods. Electrophoresis, electroblotting and western blotting were done as described in Figure 3B. (C) Effect of ADP and ATP on *in vitro* DNA binding activity of Bas1p. Electrophoretic mobility shift assay was performed with GST–HA (control lane) or GST–HA–Bas1p fusion as described in Figure 3C in the presence or absence of 1 µM of ADP and ATP.

### Nuclear localization of Bas1p is unaffected by exogenous adenine

Another hypothesis to account for adenine regulation in yeast is that under adenine repression conditions, Bas1p could be excluded from the nucleus. This mode of regulation has been documented for several transcription activators in yeast, two of which being the Bas2p interacting proteins Swi5p and Pho4p (15,16). To test this hypothesis, we constructed a fusion between BAS1 and the GFP gene. This fusion was shown to fully complement the histidine auxotrophy of the L3080 strain (data not shown), so the fusion protein is probably at least partially correctly localized within the cell. Yeast cells expressing the GFP–Bas1p construct or GFP alone were observed by fluorescence microscopy. The GFP–Bas1p fluorescence was found as a spot that co-localized with propidium iodide staining of nuclear DNA (Fig. 6A). This typical nuclear localization was expected for a transcription factor and was in good agreement with the complementation data. With the GFP expressing vector, we observed the previously described uniform staining of the cell with vacuolar exclusion (25; Fig. 6B). As expected, this localization was unaffected by addition of adenine to the medium (Fig. 6B). The localization of the GFP–Bas1p fusion was compared in cells grown in the presence or absence of adenine. Figure 6C shows that GFP–Bas1p fusion was still detected in the nucleus, even under conditions where Bas1p is less active as a transcription factor, i.e. in the presence of adenine. In conclusion, these data show that Bas1p is a nuclear localized protein and that extracellular adenine does not affect this localization.
DISCUSSION

Importance of tryptophan residues in Bas1p repeats

The Bas1p protein carries three tryptophan-rich repeats characteristic of the Myb protein DNA binding domain. We have investigated the role of four of these tryptophan residues by site-directed mutagenesis. Indeed, replacement of any of these tryptophan residues results in a defective transcription factor both in vivo and in vitro. This result confirms the important role of the tryptophans for binding of Myb-like proteins to DNA. It also shows that these tryptophan residues do not have redundant functions. Nevertheless, mutations in the four tryptophans does not result in the same phenotype, thus suggesting that each of the three repeats plays a specific role. It has been proposed that repeats 2 and 3 physically interact with DNA and are responsible for specific binding site recognition (27). Repeat 1 is not required for c-Myb binding to DNA in vitro but is required for Bas1p binding (12). Two possible alignments have been proposed between Bas1p and c-Myb repeat 1 (6,12), one of which suggests that the tryptophan at position 42 might be part of the first repeat and could therefore participate in DNA binding. Our results obtained with mutant W42A (Fig. 3) clearly show that this mutation impairs in vitro binding of Bas1p to DNA and severely affects the ability of Bas1p to activate transcription in vivo on certain promoters. In addition, a mutation further upstream in the protein (H34L) leads to the same defects as W42A both in vivo and in vitro. These results suggest that compared to other Myb-like proteins, Bas1p has an extra loop in repeat 1 as proposed previously (12). Whether this extra sequence plays some role in Bas1p function remains to be established.

As expected, the conserved tryptophan residues in repeats 2 and 3 are important for Bas1p function. Two other mutations in repeat 2 were studied (L128S and S142P). The L128S mutation did not result in any major phenotype, suggesting that the presence of a hydrophobic residue at position 128 in the second repeat is not critical for DNA binding and transcription activation. The replacement of the serine residue at position 142 by a proline led to a Bas1p protein that was not functional in vivo and only poorly bound DNA in vitro (Fig. 3). In c-Myb, the A119 residue, equivalent to S142 in Bas1p, is located in the second \( \alpha \)-helix of repeat 2 (28). Amino acid residues (W115 and S116) of this second \( \alpha \)-helix have been shown to interact directly with DNA via hydrogen bonds with phosphate backbones (28). As proline residues are known to act as ’\( \alpha \)-helix breaker’, the S142P substitution in Bas1p could severely alter the formation of the second \( \alpha \)-helix in the second repeat and hence severely impair formation of the Bas1p–DNA complex.

Neither the glycine-rich region in Bas1p nor nuclear localization of Bas1p are responsible for regulation by adenine in yeast

An attractive hypothesis to explain how Bas1p might respond to purine availability is that it could interact directly with some...
purine compound and thus sense a purine pool. A good candidate site for such an interaction was the glycine-rich motif in the DNA binding domain of Bas1p that was proposed by Arndt and co-workers as a potential purine nucleotide binding site (6). We tested this hypothesis by mutating two of the glycine residues in this motif which is located in the ‘linker’ region between repeats 2 and 3. Clearly these mutations do not abolish repression by adenine (Fig. 5A), thus strongly suggesting that the glycine-rich region does not play a role in purine sensing.

In this paper, we have also tested the possibility that in the presence of adenine, Bas1p could be excluded from the nucleus. Our data clearly establish that Bas1p is a nuclear protein and that this subcellular localization is unaffected by extracellular adenine.

We conclude that neither the glycine-rich region nor the nuclear localization of Bas1p are responsible for regulation by adenine in yeast. At this stage, the most likely mechanism for adenine regulation remains that a purine nucleotide (ADP or a derivative of ADP) (13) directly or indirectly affects interaction between Bas1p and Bas2p (14).

**Promoter discrimination by Bas1p mutants**

In the course of this work, we have observed that several mutants (H34L, W42A and G167D) in the Bas1p DNA binding domain discriminate in vivo between the promoters while they bind equally well in vitro to the same promoters. A somewhat similar promoter discrimination in vivo was observed with mutants deleted in the Bas1p N-terminal by Gabrielsen and co-workers (unpublished results). Interestingly, mutants in the homeodomain of Pho2p (Bas2p) that discriminate between promoters have been described recently (29). These mutants can activate aADE1–lacZ but not a HIS4–lacZ fusion in vivo. It should be noted that this is exactly the opposite of what we observed with our mutants in the DNA binding domain of Bas1p.

How does the in vivo discrimination occur? It is known that despite the fact that Bas1p or Bas2p can bind to DNA on their own in vitro, they cannot activate transcription in the absence of each other in vivo (2). Whether Bas1p and Bas2p can bind independently to DNA in vivo is not known, but the fact that Bas2p is unable to activate transcription in the absence of Bas1p while aLexA–Bas2p fusion can (14) strongly suggests that, in vivo, Bas2p does not bind to its natural target promoters in the absence of Bas1p. Therefore, the difference between the in vivo and in vitro results could indicate that these Bas1p mutants might not be able to bind in vivo to certain promoters.

What is the difference between the wild-type and mutant Bas1p that enable the latter to be active on some promoters in vivo? Two main reasons could account for this observation. Either the mutation in BAS1 affects a domain required for promoter specific interaction in vivo, or the mutation reduces the amount of Bas1p within the cell and the remaining protein is less efficiently recruited to some promoters in vivo. Western blot analysis on yeast protein extracts did not reveal any major difference in the relative amount of wild-type and mutant Bas1p proteins (data not shown). Therefore, what is the difference between the HIS4 and the ADE1 or ADE17 promoters that makes the former less sensitive to mutations in the DNA binding domain of Bas1p? It could be that binding of Bas1p and Bas2p to the ADE promoters operates cooperatively while this is not so on the HIS4 promoter [as shown by others (6)]. Our attempt to show cooperative binding of Bas1p and Bas2p on the ADE1 and ADE17 promoters failed. Indeed, we found the same non-cooperative binding of the transcription factors on the HIS4 and ADE promoters (data not shown). The most likely hypothesis to account for in vivo discrimination by the Bas1p mutants is that these mutations affect an interaction of Bas1p with some factor that is required for specific binding on some but not all Bas1p responsive promoters. The nature of this factor remains to be determined.

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