4.1 Introduction:

Adaptation to weak acid stress, in particular due to acetic acid involves, modulation of expression of several genes belonging to diverse functional classes in *S. cerevisiae*. Transcriptional response to acetic acid stress involves interplay of several transcription factors, such as Haa1p, Rim101p and Msn2/4p. Each of these transcription factors activates set of target genes involved in adaptation to acetic acid stress and significant cross talk is also reported (Mira et al., 2010a). Haa1p is implicated in early adaptation of yeast cells to acetic acid and propionic acid stress, and *haa1Δ* strain is sensitive to acetic acid and propionic acid and marginally sensitive to benzoic acid (Fernandes et al., 2005). Haa1p regulates the expression of at least 80% of the acetic acid induced genes in *S. cerevisiae* that are induced immediately upon exposure to acetic acid (Mira et al., 2010a). Among Haa1p target genes, *TP02, TP03, YGP1, HRK1* and *SAP30* possibly play important role in adaptation to acetic acid since deletion of these genes render the cells sensitive to acetic acid (Fernandes et al., 2005; Mira et al., 2010a). Haa1p also upregulates the expression of *FLO11* and adhesive growth in acetic acid shocked yeast cells in Yak1p dependent manner (Malcher et al., 2011).

*HAA1* gene encodes a 694 amino acid protein having significant similarity to the N terminal region of copper regulated transcription factor Ace1p. However, the mechanism for activation of target genes by Haa1p is reported to be different from Ace1p (Keller et al., 2001). Thus, one of our objectives is to understand the molecular mechanism of Haa1p mediated acetic acid resistance in *S. cerevisiae*.

4.2 Haa1p is major regulator of acetic acid resistance in *S. cerevisiae*:

In our screen of multicopy yeast genomic library for genes conferring acetic acid resistance, 7 out of 26 resistant clones sequenced were found to have *HAA1* gene, validating the screen as well as the importance of Haa1p in acetic acid tolerance. Out of 7 clones having *HAA1* gene, four clones were sequenced from both ends of insert region. Clones named 3606-1 and 3606-3 carried full length *HAA1* gene with varying lengths of promoter region; clone 3606-1 begins from *REC8* gene situated upstream of *HAA1* gene and carries possibly all upstream regulatory elements, whereas clone 3606-3 has approximately 800 bp regulatory sequence upstream of *HAA1* coding sequence. In two other clones (3707-1 and 451406-2), upstream region was from *REC8* gene but they had incomplete *HAA1* coding sequence. Though the
Figure 4.1: Chromosome coordinates of four HAA1 carrying clones. Identified by screening of yeast transformants carrying genomic library cloned in multicopy vector for acetic acid resistance. Different lengths of HAA1 coding region and flanking regions are present in different clones, shown below the HAA1 region taken from SGD page for HAA1 (Position of HAA1 gene in respective clones is marked by vertical lines).
encoded Haa1p would be missing about 220 amino acids from the carboxyl terminus, still they were conferring acetic acid tolerance and thus were studied in depth (Figure-4.1).

4.3 HAA1 expression from truncated clones can complement acetic acid sensitivity of haa1Δ strain:

Wild type yeast strain FY3 transformed with different HAA1 overexpressing genomic library clones were checked for acetic acid tolerance by dilution spotting. The yeast strains carrying truncated clones were providing equal or even better acetic acid resistance as compared to strains overexpressing full length HAA1 gene (Figure-4.2 (a)). Strains deleted for HAA1 are impaired in acetic acid tolerance and also show increased lag phase of growth in media containing acetic acid (Fernandes et al., 2005). To check the ability of truncated HAA1 clones to complement HAA1 deletion, the haa1Δ strain was transformed with different HAA1 clones and checked for acetic acid resistance. The haa1Δ strain expressing HAA1 from library clones can grow on YPD plates containing 70 mM acetic acid at pH 3.0, whereas control strain is unable to grow at moderate 40 mM acetic acid concentration. More importantly clones with truncated copy of HAA1 were also able to complement the acetic acid sensitivity of haa1Δ strain (Figure-4.2 (b)), clearly indicating Haa1p protein deleted at the C terminus can complement for Haa1p function, at least for adaptation to acetic acid stress.

Expression of HAA1 mRNA was checked from HAA1 clones transformed in haa1Δ strain by northern blot analysis. PCR amplified 500 bp fragment of HAA1 gene from 5’ terminus of coding sequence was used to prepare the probe for detecting HAA1 mRNA. The haa1Δ strain transformed with pFL44L vector did not show any HAA1 transcripts, as expected. Clone 3606-1 which has the entire regulatory region present between HAA1 and upstream REC8 gene (Figure-4.1), was showing HAA1 mRNA of same size as wildtype strain (BY4741) but expression was significantly higher as it is expressed from a multicopy vector. Clones 3707-1 and 451406-2 with HAA1 gene truncated in the 3’ region were expressing shorter mRNA than full length HAA1 as expected. Surprisingly HAA1 expression from 3606-3 clone was aberrant as two transcripts were observed and expression was very high (Figure-4.3). Clone 3606-3 lacks part of regulatory regions upstream of HAA1 coding sequence, possibly resulting in increased transcription of HAA1. This finding also supports the possibility that HAA1 expression is regulated by repressors that bind to upstream sequences deleted in clone3606-3.
Figure 4.2: Acetic acid tolerance of HAA1 overexpressing clones. *S. cerevisiae* strains transformed with HAA1 overexpressing multicopy library clones and vector pFL44L were grown to exponential phase and dilution spotted on YPD agar plates containing indicated conc. of acetic acid at pH 3.0. (a) transformants of strain FY3 and (b) transformants of *haa1Δ* strain.

Figure 4.3: Expression of HAA1 from HAA1 overexpressing multicopy library clones. The *haa1Δ* strain transformed with HAA1 overexpressing multicopy library clones and vector pFL44L were checked for HAA1 expression by northern blot analysis. Wildtype is the *BY4741* strain transformed with pFL44L. Total RNA was isolated, resolved on denaturing agarose gel, transferred to nylon membrane and hybridized with (α-32P-ATP) labelled HAA1 probe. mRNA level of IPP1 was detected and used as loading control.
4.4 HAA1 domain mapping and construction of deletion clones:

As truncated HAA1 gene was able to complement HAA1 function in haa1Δ strain, it was interesting to identify the exact regions or domains of HAA1 important for acetic acid adaptation in yeast. BLAST (Basic Local Alignment Search Tool) analysis of Haa1p showed significant similarity of N terminal 100 amino acid sequence both with other S. cerevisiae proteins as well as proteins encoded by other fungal species such as A. gossypii, D. hansenii, Z. rouxii, K. lactis etc. Haa1p sequence following this initial stretch of 100 amino acid residues shows no or very less similarity with other protein sequences. Moreover close homologs of Haa1p N terminal region Ace1p (225 aa) and Mac1p (417 aa) in S. cerevisiae, with N terminal DNA binding domain and C terminal activation domain, are quite smaller than Haa1p. Based on sequence similarity Haa1p has both Zn module and copper regulatory domain present in N terminal DNA binding domains of Ace1p and Mac1p. Activity of Ace1p and Mac1p is regulated by copper status of cell, whereas copper regulation of Haa1p is not found, although it is proposed that presence of tetracopper structure may stabilize the DNA binding domain of Haa1p (Jungmann et al., 1993; Keller et al., 2001). To understand the role of Haa1p in acetic acid adaptation in yeast, DNA binding ability of N terminal region of Haa1p and other important regulatory regions present in Haa1p need to be characterized. Thus, various deletions of Haa1p were characterized for their role in acetic acid adaptation of yeast.

Construction of HAA1 deletion clones to identify regions of importance for Haa1p function was based on (1) presence of DNA binding domain in the N terminal region and (2) our observation that truncated clone missing the C-terminal region from 466 to 685 aa was able to complement haa1Δ strain in acetic acid adaptation. To further delineate the function of C-terminal region, C-terminal deletions of HAA1 gene were made, encoding first 500 or 400 amino acids (further referred as H500 and H400), as around this length of truncated protein expressed from truncated clones were sufficient to complement Haa1p function in haa1Δ strain. C-terminal deletions encoding only the first 200 or 50 amino acid protein were also constructed as Haa1p homolog Ace1p has both DNA binding domain and activation domain in 225 aa sequence and initial 50 amino acids represent possible Zn domain in Haa1p (further referred as H200 and H50 respectively). Similarly N-terminal deletions lacking initial 50 or 100 residues were also constructed, missing N-terminal Zn domain (1-40 residues) and Zn domain along with tetracopper domain (41-80) respectively (further referred as N50 and
Later, based on phenotypic studies of these clones, two more deletions having both N and C terminal deletions were also constructed. These deletion clones have coding sequence for upto 400 residues and lack either 50 or 100 amino acid at N terminus (further referred as N504 and N1004 respectively) (Figure-4.4).

Haalp full-length gene and its deletions as designed above were PCR amplified, and cloned in yeast expression vectors under the control of strong and constitutive GPD or TEF promoter in both multicopy and single copy vectors. For these clonings, unique restriction sites Spel and EcoRI introduced at 5' and 3' ends of respective HAA1 constructs were used to generate compatible ends in vectors and insert. When these clones were transformed in haa1Δ strain, full-length HAA1 gene cloned either under GPD promoter or TEF promoter in multicopy vector yielded only few slow growing transformants. However deletions expressed from same vectors were growing normally and transformation efficiency was comparable to respective vectors. Upon expression of HAA1 gene from TEF promoter in a single copy vector growth was comparable to strains expressing HAA1 deletions. Thus in further studies yeast strains overexpressing full-length HAA1 and its deletions cloned in p416TEF were used.

The ability of HAA1 deletions to complement acetic acid sensitivity of haa1Δ strain was checked by dilution spotting. It was observed that HAA1 deletions H500, H400, N50 and N100 increased the acetic acid tolerance of haa1Δ strain like full-length HAA1 gene. On the other hand, deletions H200, H50, N504 and N1004 did not rescue the acetic acid sensitivity of haa1Δ strain. Moreover C-terminal deletions H500 and H400 gave higher resistance to acetic acid compared to full-length HAA1 gene. H400 shows at least three dilutions growth benefit over full-length gene on 55 mM acetic acid plate, whereas growth of H400 was comparable to that of wild type HAA1on 40 mM acetic acid plate, indicating that H400 can provide additional 15 mM acetic acid. Expression of N50 can provide as much resistance as HAA1, but for N100 the resistance conferred is moderate compared to full-length HAA1 gene (Figure-4.5). The haa1Δ strain shows prolonged lag phase in acetic acid as a result of increased viability loss (Fernandes et al., 2005), the effect of reintroduction of full-length HAA1 and its deletions on lag phase was checked. When these strains were grown in YPD broth containing 40 mM acetic acid at pH 3.0, the vector control strain (lacking functional Haalp) showed lag phase of approximately 60 hours, the strain expressing full-length HAA1 showed a lag phase of approx 30 hours. Interestingly expression of H400 further reduced the
Figure 4.4: *HAA1* deletion clones. Deletions of *HAA1* constructed by PCR. The regions retained in truncated proteins are indicated by the amino acid coordinates.
Figure 4.5: Complementation of acetic acid sensitivity of *haalA* by expression of *HAA1* and its deletions under TEF promoter. Yeast strains grown up to exponential phase were serially 10-fold diluted and spotted on YPD plates with indicated conc. of acetic acid at pH 3.0.
lag phase to 15-20 hours, whereas the N100 expression showed a lag phase comparable to that of full-length \textit{HAA1} gene. The lag phase of N504 and N1004 deletion clones was comparable to that of \textit{haa1Δ} strain, consistent with comparable acetic acid sensitivity of these clones as seen in dilution spotting (Figure-4.6). These phenotypic studies show that presence of functional Haa1p is essential for adaptation of yeast cells to inhibitory concentrations of acetic acid. C-terminal deletions (H400 and H500) can fully complement whereas N-terminal deletions (N50 and N100) can partially complement the acetic acid sensitivity of \textit{haa1Δ} strain. The expression level of \textit{HAA1} and its deletions under TEF promoter were also checked by northern blotting. The size of the transcripts correlated with the length of \textit{HAA1} gene retained, as expected. However, the transcript level of N-terminal deletions was aberrantly high compared to full-length \textit{HAA1} or its C-terminal deletions (Figure-4.7). Moreover transcript level was found maximum in deletions lacking first 300 bp i.e. N100 and N1004, thus 5’ region of \textit{HAA1} may have some recognition sites targeting mRNA for degradation and regulating the transcript level. As overexpression of \textit{HAA1} leads to slow growth in yeast, such a mechanism controlling through mRNA turnover could be another level of regulation of Haa1p.

4.5 Effect of \textit{HAA1} deletions on expression of Haa1p target genes:

Since C-terminal \textit{HAA1} deletions were showing better acetic acid resistance than full-length \textit{HAA1} gene and N-terminal deletions were also complementing Haa1p function. The correlation between phenotype and ability to control the expression of Haa1p target genes was checked for \textit{HAA1} deletions. Upon propionic acid and acetic acid shock, Haa1p increases the expression of genes such as \textit{TPO2, TPO3, YGP1, PHM8} and \textit{YLR297w} etc and deletion of \textit{TPO2, TPO3} and \textit{YGP1} increases the sensitivity of yeast cells to acetic acid and other weak acids (Fernandes et al., 2005). So we selected these three genes as representative of Haa1p target genes and expression of these genes was checked under the control of \textit{HAA1} deletions by northern blot analysis.

So far we have assessed acetic acid sensitivity by dilution spotting, whereas for expression analysis yeast strains have to be grown in liquid broth. Addition of acetic acid to media at low pH results in extended lag phase of growth; moreover strains expressing different \textit{HAA1} deletions have varying duration of lag phase, and thus do not have comparable cell density at the time of sampling. Therefore, instead of growing in acetic acid containing media, appropriately grown yeast cells were further subjected to acetic acid shock. The \textit{haa1Δ} strains
Figure 4.6: Adaptation of *HAA1* and its deletions to acetic acid. The *haa1Δ* strains expressing *HAA1* and its deletions under TEF promoter were inoculated in YPD broth containing 40 mM acetic acid at pH 3.0 at 0.05 OD<sub>600</sub> and incubated at 30°C with shaking (200 rpm). Growth was monitored in term of increase in O.D<sub>600</sub> at intervals.

Figure 4.7: Abundance of transcripts of *HAA1* deletions expressed under TEF promoter in *haa1Δ* strain. The transcript length of *HAA1* corresponds to the size of different deletions. As loading control 25S and 18S RNA region of stained gel is shown in the lower panel.
expressing \textit{HAA1} deletions were grown to exponential phase in SD medium supplemented with auxotrophic supplements at 30°C. Cells were harvested at 0.6-0.8 OD$_{600}$ and incubated in YPD medium containing acetic acid (75, 100, 150 and 200 mM concentrations) at pH 3.0 at cell density corresponding to 1.0 OD$_{600}$ at 30°C. Viability of these shocked cells was checked at 0, 2, 4 and 6 hours of incubation by dilution spotting on SD medium plates. At 200 mM acetic acid conc. all strains were unviable after 2 hours and at 150 mM acetic acid yeast strains expressing \textit{HAA1} deletions N50, N100, N504 and N1004 were unviable after 4 hours and vector control strain was unviable after 2 hours. At 100 mM and 75 mM acetic acid conc. strains expressing \textit{HAA1} and its deletions were viable; moreover control strain lacking functional Haa1p (haa1\textit{Δ} strain transformed with vector alone) was significantly viable after acetic acid shock of 4 hours (Figure-4.8). Therefore acetic acid shock at 75 mM acetic acid for 4 hours was selected as sublethal condition for expression analysis of Haa1p target genes.

Yeast strains expressing different \textit{HAA1} deletions were grown to exponential phase in minimal medium, cells were harvested for immediate RNA isolation as well as incubated in YPD medium with 75 mM acetic acid at pH 3.0 for 4 hours and RNA was isolated by hot phenol method (Mannan et al., 2009). The mRNA level of Haa1p target genes \textit{TPO2, TPO3} and \textit{YGP1} along with \textit{IPP1} as loading control was checked by northern blotting. In exponentially grown cells, \textit{HAA1} is necessary for expression of \textit{TPO2, TPO3} and \textit{YGP1} and upon acetic acid shock expression of \textit{TPO2} and to some extent \textit{YGP1} was further enhanced as earlier reported. In control strain lacking functional Haa1p, transcripts of these genes were not detectable in any condition tested. Haa1p C-terminal deletions H500 and H400 were comparable to full-length \textit{HAA1} in regulation of target genes, whereas N-terminal deletions N50 and N100 can support the expression of \textit{TPO3} and \textit{YGP1} but no \textit{TPO2} transcript was detected with or without shocked cells. Interestingly acetic acid induced increase in \textit{TPO2} expression was maximum with H400, as faint transcript of \textit{TPO2} was seen in untreated cells, however upon acetic acid shock expression of \textit{TPO2} was comparable to full-length \textit{HAA1} gene. In N50 and N100 deletions, \textit{YGP1} transcript level was less in acetic acid treated cells compared to untreated as well as expression level in \textit{HAA1} and C-terminal deletions (Figure-4.9). Simultaneous deletion from both N and C termini results in possibly nonfunctional Haa1p protein, since none of Haa1p target genes was expressed in strains with N504 and N1004 deletion clones (Data not shown).
Figure 4.8: Survival time course of HAA1 and its deletions in acetic acid at pH 3.0. Exponentially grown cells were harvested and resuspended at 1 OD$_{600}$ into YPD broth at pH 3.0 containing (a) 75, (b) 100, (c) 150 and (d) 200 mM acetic acid. Treated cells were dilution spotted on SD plates with auxotrophic supplements, after 0, 2, 4 and 6 hours of incubation.
Figure 4.9: Differential regulation of Haa1p target genes by HAA1 deletions. The haalΔ strain transformed with HAA1, its deletions under TEF promoter (in p416TEF) were grown to exponential phase and further subjected to acetic acid shock (YPD, pH 3.0 with 75 mM acetic acid) for 4 hours. The expression of YGP1, TPO2 and TPO3 in untreated (a & b) or acetic acid shocked cells (c & d) was checked by northern blot. The positions of transcripts of TPO2, TPO3 and YGP1 are indicated. Expression of IPP1 was used as internal loading control.
4.6 Expression and purification of Haa1p and H400p from E. coli:

Since HAA1 deletions can induce expression of Haa1p target genes, the potential DNA binding ability of Haa1p and its deletions to possible promoter regions upstream of Haa1p target genes was checked. To study DNA binding ability purified protein or cell lysate having functional protein is required. Thus, HAA1 full-length gene and H400 deletion construct were cloned in pET28c (+) at Ndel and EcoRI restriction sites, thereby adding N-terminal His tag. These clones were transformed into E. coli expression strain BL21-CodonPlus and protein expression was induced at 25°C with 0.25 mM IPTG at a cell density of OD600 = 1.0 to 1.25 (Figure-4.10 (a)). Recombinant proteins thus produced were bound to Ni-NTA affinity matrix and eluted in buffer having 300 mM NaCl, 50 mM Tris-HCl (pH 8.0), 10% glycerol and 200 mM imidazole. To check purity, eluted proteins were run on SDS-PAGE and stained with Coomassie brilliant blue R-250. Full-length Haa1p of 87 kDa and H400 protein of 47 kDa were seen, along with significant number of bands of low molecular weight that can result from poor purification or degradation of purified protein (Figure-4.10 (b)). So purity of eluted proteins were further checked by western blotting using anti His monoclonal antibody, which showed that the majority of bands were result of Haa1p degradation (Figure-4.10 (c)).

To minimize protein degradation various parameters affecting protein expression and purification were tried. IPTG induction was done at different temperatures; at 37°C induced protein mainly goes to insoluble pellet fraction after sonication, whereas at temperatures below 20°C no induction was seen. Various protease inhibitor cocktails were used in lysis buffer and elution buffer but no improvement in the quality of eluted protein was seen. Buffers at various pH ranges were also tried such as MES buffer, pH 6.0 (Figure-4.11 (b) & 4.11 (c)), carbonate buffer, Tris-HCl (pH 9.0) and phosphate buffer. No improvement was seen in the quality of protein; moreover upon dialysis significant aggregation was seen for Haa1p and H400p (Figure-4.11 (a)). In an attempt to further purify partially purified protein, we performed gel filtration chromatography of dialyzed protein on superdex S200 column. SDS-PAGE analysis of eluted fractions of gel filtration showed consistent degradation of both Haa1p and H400p (Figure-4.12). Thus Haa1p and H400p seem to highly unstable proteins as consistent pattern of degradation was seen. Purification of Haa1p and its deletion proteins from native host S. cerevisiae were also tried; C-terminally His tagged proteins were expressed from constitutive GPD promoter or inducible GAL1 promoter. The expressed
Figure 4.10: Expression and purification of Haalp and H400p. *HAA1* and H400 were expressed from pET28c (+) in *E. coli* expression strain Codon+. (a) induction of proteins, with 0.5 mM IPTG for 2 and 4 hours at 37°C, UI (uninduced) and I (induced). (b) Purification profile of H400p and Haalp eluted by Ni-NTA chromatography, MW (molecular weight marker), FT (flow through), Wash, E1 (elution 1) and E2 (elution 2) (c) Western blot of purified recombinant Haalp. His tagged protein was probed with anti His mouse antibody and followed by HRP conjugated anti mouse IgG, signal was detected by Millipore chemiluminescence detection kit. FT (flow through), Wash E1 (elution 1), E2 (elution 2) and Control (Duglp-His).
Figure 4.11: Purification of Haa1p and H400p from *E. coli*. *HAA1* and H400 were expressed in *E. coli* strain Codon+ from pET28c (+) and proteins were purified by NiNTA affinity chromatography. (a) Protein purified from Tris-HCl pH 8.0 based buffer and dialyzed and run on SDS-PAGE. (b) Haa1p purified from MES pH 6.0 based buffer (c) H400p purified from MES pH 6.0, SUP (supernatant), FT (flow through), Wash, E1 (elution 1) and E2 (elution 2).

Figure 4.12: Gel filtration analysis of Haa1p and H400p, Ni-NTA purified proteins, dialyzed against 300 mM NaCl, 50 mM Tris-HCl pH 8.0 and 10% glycerol, were, passed through superdex S200 column. Different gel filtration fractions were run on SDS-PAGE to detect homogeneity of eluted protein. (a) gel filtration fractions of Haa1p and (b) gel filtration fractions of H400p.
protein complemented the acid sensitivity of *haalΔ* strain (Data not shown), however these proteins could not be detected by western blotting or purified.

4.7 **Haa1p binds upstream DNA sequences of *YGP1* and *TPO2* in metal dependent manner:**

As discussed earlier, Haa1p functions as transcription factor in yeast and has a possible N terminal DNA binding domain. Very recently Haa1p responsive element (HRE) has been identified in upstream region of *TPO3* located between nucleotides -590 and -690. Purified Haa1p DBD (N’ terminal 127 amino acids) interacts with sequence 5’-(G/C)(A/C)GG(G/C)G-3’ in promoter region of Haa1p target genes (Mira et al., 2011). Simultaneously we have also tried to prove DNA binding ability of Haa1p to upstream regulatory regions of *YGP1* and *TPO2* coding sequences using gel shift experiment with recombinant Haa1p and H400p. The gel shift or electrophoretic mobility shift assay (EMSA) is a rapid and sensitive method to detect protein-nucleic acid interactions. It is based on the observation that the electrophoretic mobility of a protein-nucleic acid complex is typically less than that of the free nucleic acid. Generally a radioactively end labeled nucleic acid probe is incubated with protein of choice, and run on native polyacrylamide gel to study mobility of DNA-protein complexes with respect to free probe.

To study the DNA binding ability of Haa1p and H400p, upstream sequences of *TPO2* and *YGP1* coding region were PCR amplified in 3 separate overlapping fragments covering 1kb region as shown in Figure-4.13. PCR amplified DNA fragments were purified by gel extraction and used for 5’ end radiolabelling using 32P-γ-ATP in a reaction catalyzed by poly nucleotide kinase (PNK). After purification yield for H400p was higher than full-length Haa1p and percentage of undegraded protein was more in H400p. Therefore, initially H400p was incubated with 20 f mole probe DNA of *YGP1* upstream regions at 20°C for 30 min to study DNA binding of Haa1p. Protein DNA complexes thus formed were electrophoresed on 5% polyacrylamide native gel and analyzed for mobility shift relative to free probe molecules. With increasing concentration of H400p, mobility shift in *YGP1*-A, B and C probes were seen, similar mobility shift was also observed with *TPO2*-A, B and C probe DNA (Figure-4.14 and 4.15). DNA binding was also seen for recombinant Haa1p in mobility shift assay with *TPO2* and *YGP1* probes (Figure-4.16). Thus these preliminary observations indicated that Haa1p has direct DNA binding ability in upstream region of Haa1p target genes.
Figure 4.13: *YGP1* and *TPO2* upstream regions used as end labeled probes in DNA binding assays. 3 overlapping DNA fragments covering 1 KB upstream region were PCR amplified, end labeled with $\gamma^{32}$P and used in DNA binding experiments with Haa1 and H400. (a) YGP1 probes, YGP1-A (-1 to -390), YGP1-B (-301 to -680) and YGP1-C (-573 to -980) and (b) TPO-2 probes, TPO2-A (-1 to -405), TPO2-B (-331 to -640) and TPO2-C (-561 to -1000).
Figure 4.14: DNA binding assay of H400p with YGP1 probes. End labeled YGP1 probes were mixed with H400p proteins and incubated at 20°C for 30 minutes, as described (section 2.2.15). Samples were run on native PAGE, after which gel was dried and possible protein DNA complexes were analyzed on phosphorimager. The probe and protein amount used in reaction is indicated above the lane. One μl of protein is equivalent to 0.94 μg.

Figure 4.15: DNA binding assay of H400p with YGP1 and TPO2 probes. End labeled probes were mixed with H400p proteins, incubated at 20°C for 30 minutes and DNA protein complexes were analyzed. The probe and protein amount used in reaction is indicated above the lane. One μl of protein is equivalent to 0.42 μg.
Haa1p DNA binding domain has a Zn stability domain and Cu regulatory domain, and thus it is likely that these metal ions play significant role in DNA binding property of Haa1p. Although metalloregulation of Haa1p is not reported, but it has been proposed that poly copper thiolate structure may stabilize DNA binding domain of Haa1p (Keller et al., 2001). Thus, effect of chelation of metal ions present in Haa1p DNA binding domain on its DNA binding ability was checked. Recombinant Haa1p and H400p protein were either incubated with or without 1 mM EDTA at 30°C for 30 min; these protein samples were separately dialyzed and used for DNA binding experiment. Both Haa1p and H400p, once incubated with EDTA loses their ability to bind YGPJ or TPO2 probe molecules, as no gel shift was observed, however proteins similarly incubated without EDTA showed gel shift. These results indicate that presence of these metal ions is essential for DNA binding ability of Haa1p (Figure-4.16 and 4.17). Specificity of DNA binding ability of Haa1p with YGP1 or TPO2 probes were confirmed as similar set of protein did not show any binding with nonspecific Mycobacterial probe DNA used in DNA binding experiment (Figure-4.16).

Since presence of metal ions (Cu⁺ and Zn²⁺) in DNA binding domain of Haa1p was found essential for its DNA binding ability, as EDTA inactivated proteins fail to bind target DNA probes. We tried to regenerate functional protein by incubating apoprotein (Haa1p or H400p inactivated with EDTA) with ZnSO₄ and CuSO₄. Divalent Copper salt was selected as monovalent copper salts are insoluble in aqueous medium. Moreover to find out functional significance of any other metal ion in Haa1p function, MgSO₄ and FeSO₄ were also tested. Apo H400p incubated with increasing concentration of different metal salts (1 µM, 10 µM, 100 µM and 1mM) was used for DNA binding experiment with YGP1-C probe. DNA binding ability of apoH400p was not regained after incubation with any of the metal ion tested (Figure-4.18). Furthermore combinations of Cu²⁺ and Zn²⁺ were also tested as possibly Haa1p DBD binds both of these metal ions. Hence apoH400p was simultaneously incubated with gradually increasing concentration of both ZnSO₄ and CuSO₄ together, and DNA binding ability was checked, but activity was not regained (Figure-4.19). These results indicate that DNA binding ability of Haa1p is metal dependent and DBD of Haa1p is stabilized by presence of metal ion.

Mac1p is a copper responsive transcription factor in S. cerevisiae; upon copper starvation it induces the expression FRE1, CTR1, CTR3 and FRE7 (Jungmann et al., 1993). Mac1p shares homology with N-terminal DBD of Haa1p and Ace1p, having Copper regulatory domain and
Figure 4.16: Metal ion dependent DNA binding assay of Haa1p and H400p with TPO2 and nonspecific probes. Purified Haa1p and H400p were incubated with or without 10 mM EDTA, dialyzed and further used for DNA binding assay. End labeled probes were mixed with Haa1 and H400p proteins, incubated at 20°C for 30 minutes and DNA protein complexes were analyzed. X-DNA is non specific Mycobacterial DNA used as probe. Proteins were used at final conc. of 1 μM.

Figure 4.17: Metal ion dependent DNA binding assay of Haa1p and H400p with YGPl probes. Purified Haa1p and H400p were incubated with or without 10 mM EDTA, proteins were dialyzed and further used for DNA binding assay. End labeled probes were mixed with these Haa1 and H400p proteins, incubated at 20°C for 30 minutes and DNA protein complexes were analyzed. Proteins were used at final conc. of 1 μM.
Figure 4.18: Effect of metal ions on DNA binding ability of EDTA inactivated H400p. H400p incubated with 10 mM EDTA, dialyzed and further used for DNA binding assay after incubation with ZnSO₄, CuSO₄, MgCl₂ or FeSO₄. End labeled probes were mixed with H400p, incubated at 20°C for 30 minutes and DNA protein complexes were analyzed. Lane 2 is untreated and lane 3 is EDTA treated H400p. Proteins were used at final conc. of 1 μM.

Figure 4.19: Effect of CuSO₄ and ZnSO₄ on DNA binding ability of EDTA inhibited H400p. H400p incubated with 10 mM EDTA, dialyzed and further used for DNA binding assay after incubation with ZnSO₄ and CuSO₄. End labeled probes were mixed with H400p incubated at 20°C for 30 minutes and DNA protein complexes were analyzed. Lane 2 is untreated and lane 3 is EDTA treated H400p. Proteins were used at final conc. of 1 μM.
zinc stability domain. It has been shown that when Mac1p is expressed from *E. coli* strain BL21 (DE3), addition of 1 mM ZnSO$_4$ after IPTG induction improves the protein purity (Jensen et al., 1998). Thus we checked the effect of ZnSO$_4$ on protein expression and purification of Haa1p and H400p. After 30 minutes of IPTG induction of *E. coli* strain expressing Haa1p and H400p, ZnSO$_4$ was added (0.25, 0.5 and 1.0 mM) and the cells were grown for additional 5 hrs. It was observed that addition of increasing concentration of ZnSO$_4$ significantly improves the purity of protein of both Haa1p and H400p, but yield decreases (Figure-4.20 (a-c)). Thus proteins purified after addition of 0.5 mM ZnSO$_4$ were further used for DNA binding experiments. However, proteins expressed in the presence of additional Zn$^{2+}$ ions lack DNA binding ability as no mobility shift in *YGP1* probes was seen (Figure-4.20 (d)).

4.8 Promoter mapping of *YGP1* and *TPO2*:

Our previous northern blot experiments have shown that Haa1p regulates the expression of *YGP1* and *TPO2* differently. Subsequent DNA binding experiments of Haa1p/H400p with *YGP1* and *TPO2* upstream regions indicate the possibility of more than one binding sites. Based on DNA binding experiments, *YGP1* and *TPO2* genes were cloned with promoter having 400 bp to 1000 bp of upstream region in p315ADH replacing ADH promoter (Fig-21 (a) and 22 (a)). *YGP1* clones thus constructed were named as YS1, YS2 and YS3 (containing -390 bp, -579 bp and -1000 bp upstream region respectively) were transformed into *ygp1Δ* strain. Similarly *TPO2* clones named TS1, TS2 and TS3 (containing -405 bp, -640 bp and -1000 bp upstream region respectively) were transformed into *tpo2Δ* strain. Expression of *TPO2* was checked in respective strains with or without acetic acid shock by northern blotting. *TPO2* expression was seen at all the promoter lengths tested, in unshocked as well as acetic acid shocked cells, although *TPO2* expression increases upon acetic acid shock. Thus essential regulatory regions required for *TPO2* expression could be present within 405 nucleotides upstream of its coding sequences (Fig-21 (b)). To identify the regulatory elements important for *TPO2* expression clones having -304 bp (TS4) and -214 bp (TS5) upstream regions were also constructed and transformed in *tpo2Δ* strain. Expression of *TPO2* was checked from these clones as well as original clones. It was observed that 405 bp upstream region is required for *TPO2* expression in acetic acid shocked or unshocked cells as TS4 and TS5 shows no expression of *TPO2* (Fig-21 (c)). *TPO2* seems to have a single regulatory element located between nucleotide -405 and -304 and it is sufficient for *TPO2*
Figure 4.20: Effect of ZnSO₄ addition after IPTG induction on protein purification and DNA binding ability of Haalp and H400p. After 30 min of IPTG induction of E. coli strain codon² carrying pET28c+HAAl or +H400, ZnSO₄ was added to induced culture and protein was purified after 5 hours of induction. Elutions of Haalp and H400p purified from cultures added with (a) 1 mM ZnSO₄, (b) 0.5 mM ZnSO₄ and (c) 0.25 mM ZnSO₄. (d) DNA binding assays of Haalp and H400p purified after addition of 0.5 mM ZnSO₄ with YGP1 probes. Lane 1-7 YGP1-A, 8-14 YGP1-B and YGP1-C.
expression in with or without acetic acid shock. However, based on previous reports and our own observation that TPO2 expression increases upon acetic acid shock, we called this element as Acetic Acid Response Element (AARE) for TPO2.

When expression of YGP1 was checked from YS1, YS2 and YS3 clone, it was detected at all the promoter lengths tested in unshocked cells but upon acetic acid shock YS1 clone having first 390 bps of YGP1 upstream region was defective in YGP1 expression (Fig-22 (b)). These observations indicated that YGP1 expression is regulated differently in unshocked and acetic acid shocked cells, as different promoter elements seems to be required for YGP1 expression in these conditions. To further scale down the essential regulatory elements present upstream of YGP1 and required for acetic acid induced and constitutively induced YGP1 expression, clones having -579 bp (YS4), -479 bp (YS5) and -290 bp (YS6) upstream region were constructed and transformed in ygp1Δ strain. Expression of YGP1 was checked from these clones as well as original clones in with and without acetic acid shock. It was observed that 390 bp promoter region present in YS1 is minimum region required for YGP1 expression in unshocked cells as in YS6 clone with 290 bp upstream region no expression is seen. In acetic acid shocked cells 579 bp promoter region present in YS4 is minimum region required for YGP1 expression, as clones YS5 and YS1 with shorter promoter regions were defective in acetic acid induced YGP1 expression (Fig-22 (C)). This upstream regulatory element present between nucleotide -579 and -479 is referred as Acetic Acid Response Element (AARE) for acetic acid induced YGP1 expression, whereas upstream elements present between nucleotide -390 and -290 is referred to as Non Acetic acid Response Element (NARE) for YGP1 expression.

4.9 Discussion:

Haa1p is a transcriptional activator involved in early adaptation to acetic acid stress in yeast. In our screening for genes conferring acetic acid resistance upon overexpression, we found multiple clones expressing HAA1. However, among HAA1 clones few were expressing shortened gene product, as confirmed by sequencing and northern blot analysis. Truncated HAA1 clones were acetic acid resistant in screening and were able to complement the acetic acid sensitivity of haalΔ strain. Such a finding was interesting as possibly C-terminal part of Haa1p either has no function in Haa1p mediated acetic acid tolerance or it plays some regulatory roles which can be bypassed upon encountering acetic acid shock.
Figure 4.21: Promoter mapping of TPO2. (a) Schematic representation of TPO2 promoter deletions. TPO2 gene with 5' deletions of upstream regulatory region were cloned in pRS315, covering maximum 1000 bp and progressively reduced to 214 bp of upstream region. (b) The TPO2 expression was initially checked in the tpo2Δ strain transformed with TS1, TS2, TS3 and (c) TS1 to TS5 in untreated cells and after acetic acid shock (YPD with 75 mM acetic acid at pH 3.0), VC is vector control.
Figure 4.22: Promoter mapping of YGP1. (a) Schematic representation of YGP1 promoter deletions. YGP1 gene with 5' deletions of upstream regulatory region were cloned in pRS315, covering maximum 1000 bp and progressively reduced to 290 bp of upstream region. (b) The YGP1 expression was initially checked in the ygp1Δ strain transformed with YS1, YS2, YS3 and (c) YS4, YS5, YS6 in untreated cells and after acetic acid shock (in YPD with 75 mM acetic acid at pH 3.0), VC is vector control.
To map domains or regions of Haa1p important for acetic acid adaptation in yeast, deletions of *HAA1* lacking either C-terminal or N-terminal regions or both were generated and expressed under TEF promoter. C-terminal deletions H500 and H400 can complement acetic acid sensitivity of *haalΔ* strain and provide tolerance to acetic acid stress better than full-length *HAA1*. Like full-length *HAA1*, expression of H400 and H500 can induce expression of Haa1p target genes *TPO2*, *TPO3* and *YGP1*. Haa1p mediated regulation of *YGP1* and *TPO3* was relatively similar in yeast cells treated or untreated with acetic acid, whereas expression of *TPO2* increases upon acetic acid shock. Relative upregulation of *TPO2* upon acetic acid shock was highest under H400, although *TPO2* transcript level is not higher than that seen in full-length *HAA1* or H500. Moreover H400 provide higher resistance than full-length or any other deletions tested on acetic acid containing plates and causes least hours of lag phase in acetic acid containing broth. Thus there is a possibility that C-terminal region of Haa1p beyond first 400 or 500 amino acids has some regulatory region that represses Haa1p activity. Here an intriguing possibility is that C-terminal region of Haa1p is subject to repression via other repressor proteins in normal growth conditions and upon acetic acid shock Haa1p is released from repression to perform its function. Thus C-terminal deletion clones H400 and H500 lacking Haa1p domains or regions subjected to repression are more active in providing acetic acid resistance in yeast cell. Expression of full-length *HAA1* from GPD promoter results into slow growth phenotype, whereas slow growth is not seen upon expression of C-terminal deletions from GPD promoter. Thus it is possible that full-length Haa1p regulates the expression of additional genes, not regulated by H400 and H500, resulting in growth defect. Thus, C-terminal deletions can be preferably used in strain improvement as they provide more acetic acid tolerance without causing growth defect upon overexpression.

Deletions lacking N-terminal 50 (N50) or 100 (N100) amino acids of Haa1p can partially complement the *haalΔ* strain for acetic acid sensitivity and induction of Haa1p target genes. N-terminal deletions can regulate the expression of *YGP1* and *TPO3*, but they seem unable to activate expression of *TPO2*. N-terminal deletions lack possible DNA binding region of Haa1p transcription factor, consisting of Zn module responsible for stability and Cu regulatory domain. Such DNA binding regions are present in copper regulated transcription factors Ace1p and Mac1p, responding to copper status of the cell. But it is known that expression of Haa1p target genes is not affected by copper status of the yeast cell and DNA binding of Haa1p is copper independent (Keller et al., 2001). A recent report shows that Haa1p DBD (1-127 aa) can interact with Haa1p responsive elements present in *TPO3*.
upstream region (Mira et al., 2011). Our results show that N-terminal 50 or 100 amino acids are essential for upregulation of \textit{TPO2} expression. Earlier it is shown that N-terminal minimal DNA binding region of Haa1p (1-124 amino acids) fused with VP16 activation domain can induce expression of \textit{TPO2}. Thus, these results indicate that N-terminal region of \textit{HAA1} has DNA binding region for \textit{TPO2}. On the other hand N-terminal deletions are able to induce expression of \textit{TPO3} and \textit{YGP1}, thus N-terminal region is not essential for \textit{TPO3} and \textit{YGP1} expression. Moreover clones having simultaneous deletion at N and at C termini of \textit{HAA1}, N504 and N1004 are unable to activate expression of any Haa1p target gene tested and did not complement the \textit{haa1\Delta}. These results indicate that Haa1p regulates the expression of \textit{TPO3} and \textit{YGP1} differently than it does for \textit{TPO2}. A Haa1p region beyond first 100 amino acids may have a DNA binding region that in association with C-terminal region beyond 400 amino acids can alternatively activate the expression of \textit{TPO3} and \textit{YGP1} in N-terminal deletions. Haa1p mediated upregulation of \textit{TPO3} and \textit{YGP1} may represent general response of Haa1p, whereas \textit{TPO2} expression is induced mainly upon acetic acid shock. Haa1p may have dual regulatory regions; one responding to generalized and other responding to acetic acid induced expression of Haa1p target genes. N-terminal region of Haa1p may function as acetic acid induced DNA binding domain, as it is essential for \textit{TPO2} expression. There could also be proteins that differentially bind to Haa1p and H400, thereby modulating their function at different promoters.

The histidine tagged Haa1p and its truncated proteins purified from heterologous \textit{E. coli} host were consistently degraded in similar manner. Possibly Haa1p has intrinsic instability associated with its structure in heterologous host, moreover we were unable to purify Haa1p or its deletion proteins from native host. DNA binding of partially pure Haa1p and H400 with upstream sequences of \textit{YGP1} and \textit{TPO2} coding region were seen. Haa1p protein showed DNA binding with three separate overlapping regions within 1 kb upstream region of \textit{TPO2} and \textit{YGP1} coding sequences. On the other hand Haa1p does not show any DNA binding with non specific Mycobacterial probe DNA used. These results show that Haa1p specifically binds to multiple binding sites in \textit{TPO2} and \textit{YGP1} regulatory regions. Exact Haa1p binding sites were not mapped as protein was partially purified and possibility of multiple binding sites is still to be validated fully. N terminal DNA binding domain in Haa1p similarly have Cu and Zn ions associated with it, as shown by atomic force spectroscopy data (Data not shown). Haa1p chelated with EDTA loses the metal ions associated with it and also DNA binding ability. Moreover loss of DNA binding ability of Haa1p is not restored upon
incubation of EDTA inactivated Haa1p with copper or zinc salt either alone or together. These results show that Haa1p DNA binding domain is stabilized by Zn and Cu ions and presence of Zn$^{2+}$ and Cu$^+$ ion is essential for DNA binding ability of Haa1p. Like Haa1p, H400p also shows similar metal dependent DNA binding with YGP1 and TPO2 upstream probes. Thus H400p has all DNA binding regions present in Haa1p that are required for activation of TPO2 and YGP1 expression.

Promoter mapping of YGP1 and TPO2 to identify regulatory regions required for Haa1p mediated expression, revealed that both genes are regulated quite differently. YGP1 has two regulatory elements in upstream region, one responding to acetic acid called, Acetic Acid Response Element (AARE) and another responding in untreated cells which is not required for acetic acid induced expression, referred to as Non Acetic acid Response Element (NARE). On the other hand TPO2 has single regulatory element in upstream region, mainly responding to acetic acid shock, thus referred as AARE. Since Haa1p response element has been identified in upstream region in one of the Haa1p target gene TPO3 and DNA binding has been established (Mira et al., 2011), we propose that Haa1p has two possible binding sites in upstream region of YGP1 in AARE and NARE respectively, whereas TPO2 has single Haa1p binding in its AARE. Since HAA1 C-terminal deletions H400 and H500 as well as N-terminal deletions N100 and N50 can induce expression of YGP1 independently. It is possible that Haa1p has two DNA binding domain and earlier shown N-terminal DBD preferably recognizes to AARE like in TPO3 and as it can be proposed for TPO2, whereas NARE can be recognized by additionally or alternatively by separate Haa1p domain.