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Identification of regulatory proteins that might be involved in carbon catabolite repression of the aminopeptidase I gene of the yeast *Saccharomyces cerevisiae*

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Abstract Transcription of the vacuolar aminopeptidase yscI (*APE1*) gene in *Saccharomyces cerevisiae* has previously been suggested to require the participation of a *cis* upstream activation sequence (UAS) involved in carbon catabolite repression that responds to glucose. To determine the structure of the *APE1* UAS element, we used the 18-bp sequence 5′-ATGAATTAGTCAGCTTCT-3′ as the DNA-binding site. Using gel mobility shift assays, we have identified a 78 kDa protein from yeast that binds specifically to both single and double-stranded forms of the UAS DNA-binding site. We have also identified a 48 kDa heterodimer from yeast that binds specifically to the single-stranded form of the UAS and whose DNA binding activity is remarkably heat stable. Even though the *APE1* UAS contains a consensus sequence for the binding of the yeast activator protein yAP1, the two DNA–protein complexes could still be detected in a strain bearing a deletion in the *YAP1* gene.

Key words: Yeast; *Saccharomyces cerevisiae*; Catabolite repression; Aminopeptidase; Transcription regulation

1. Introduction

Levels of vacuolar aminopeptidase yscI, the product of the *APE1* gene in *Saccharomyces cerevisiae* [1], respond to both ethanol induction and carbon catabolite repression [2]. By using the cloned *APE1* gene as a probe, induction of *APE1* was shown to be accompanied by a large increase in steady state *APE1*-specific mRNA, while carbon catabolite repression was accompanied by a fast disappearance of *APE1*-specific mRNA [2]. By deletion analysis of *APE1-lacZ* fusions, a number of promoter regions were shown to be responsible for regulation of *APE1* transcription [2]. Based on those experiments, an upstream activating sequence (UAS) was hypothetically located between positions −368 and −406 upstream from the initiating ATG of the gene [2]. A consensus sequence for the binding of the yeast activator protein yAP1 [3] required for normal cadmium tolerance [4] was located within that region.

The purpose of this work was to define the boundaries of the putative UAS site, to ascertain whether protein binding to the site could be demonstrated and to establish the role of the yeast activator protein yAP1 [3,4] in transcriptional activation by glucose of the *APE1* gene.

The results reported here suggest that the *APE1* UAS consists of at most a 18-bp sequence which was observed by gel retardation assays to bind a protein of 78 kDa and a heterodimer of 48 kDa. Both complexes are formed in a strain bearing a deletion in the *yAP1* gene.

2. Materials and methods

2.1. Strains, growth conditions and enzyme assays

Saccharomyces cerevisiae strains YHH32 (MATα *his3–11,15 leu2–3,112 can*^{R} *ura3Δ5 pra1::URA3 prb1ΔAV*), kindly provided by Dr. D.H. Wolf (University of Stuttgart); and SM10 (MAT α *leu2 his3 lys2 trp1 ura3 yap1-Δ1::HIS3*) kindly provided by Dr. W.S. Moye-Rowley (University of Iowa) were grown on rich YPD or YPE media [2] to an $A_{600\ nm}$ of 1.00 . In growth-shift experiments yeast cells were grown on YPD medium to an $A_{600\ nm}$ of 1.00, washed and shifted to 2% glucose (D). The methods used for construction of the *APE1-lacZ* fusion plasmids, selection of single copy integrants, assay of β-galactosidase and protein determination have been previously described [2].

2.2. Gel mobility shift assays

Yeast protein extracts to be used for DNA-binding assays were made as described [5]. The protein–DNA binding reaction mixes were incubated for 20 min at room temperature in a final volume of 20 μl containing 20 μg of protein extract, 500 μg of polyd(I-C) and 10 ng of an oligonucleotide end labeled with ^{32}P and polynucleotide kinase in the extract buffer (4 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 20 mM sodium phosphate pH 7.0). The sequence of the single-stranded *APE1* UAS-binding site used in gel shifts is 5′-ATGAATTAGTCAGCTTCT-3′. The double-stranded *APE1* binding site used in the gel shifts was prepared by annealing the above oligonucleotide to its complementary oligonucleotide, 5′-AGAAGCTGACTAATTCAT-3′, at 90°C for 2 min, then cooling slowly to 25°C in a period of 30 min. The nonspecific oligo used in the competition gel shift is 5′-CAGCTGCAGCCAGTTGTTCA-3′. The protein–DNA complexes were resolved in 12% polyacrylamide gels in 1 × TBE (90 mM Tris-borate, 2 mM EDTA pH 8.3) for aproximately 3 h at 8 V/cm at room temperature. The gels were dried and autoradiographed on Kodak X-Omat films.

2.3. UV cross-linking and SDS-polyacrylamide gel electrophoresis

The UV cross-linking of protein–DNA complexes was performed in the gel shift gels. After electrophoresis, the gel was exposed to an 8-W UV source of 254 nm wavelength for 10 min. The bands of interest were localized by exposure to X-Omat films, excised and extracted with 3 × gel slice volume of 1 × TBE. The extracts were then loaded in a SDS gel with a Tricine buffer system described previously [6]. The gels were run in duplicate. One-half was fixed and stained with Coomassie blue to visualize the molecular mass markers and the other half was subjected to autoradiography to visualize the proteins bound to the radioactive oligonucleotide.

3. Results and discussion

In a previous work we constructed several in-frame fusions of different 5′-truncated versions of the *APE1* promoter with the *E. coli lacZ* gene trying to identify *cis*-acting regulatory elements controling the transcription of the *APE1* gene [2]. Several upstream repressing sequences (URS) were found in

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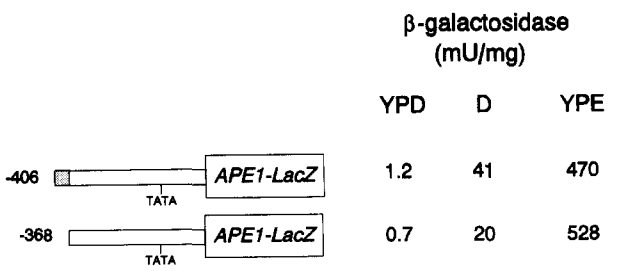

Fig. 1. 5′-deletions analysis of the *APE1* UAS region using *lacZ* as reporter gene. Deletions were performed as described in section 2. Coordinates are indicated relative to the start of translation. Cells were grown on rich YPD or YPE media to an $A_{600\ nm}$ of 1.0, prior to preparation of crude extracts. YPD grown cells were collected, washed and transferred for 4 h to a nitrogen-free medium containing glucose (D), prior to preparation of the crude extract. Dotted area = UAS for carbon catabolite repression.

contrast to a single upstream activating sequence (UAS) region hypothetically located between positions −406 and −368, whose removal did not affect significantly β-galactosidase activity in derepressing conditions, but decreased activity by 1.7-fold in glucose grown cells [2]. To further investigate the existence of such UAS, and taking into account that aminopeptidase yscI expression is not only under carbon catabolite repression but also subjected to a strong repression by the nitrogen source of the growth medium (Bordallo, J. and Suárez-Rendueles, P., manuscript in preparation) we measured β-galactosidase activity in yeast extracts prepared after shifting YPD grown cells to 2% glucose (D) for 4 h. From the results shown in Fig. 1 it can be deduced that vacuolar aminopeptidase yscI expression is indeed under a strong nitrogen regulation, as removal of the nitrogen source leads to an increase of about 30-fold in β-galactosidase expression. Nitrogen catabolite repression has also been found to be the main mechanism that regulates expression of another vacuolar peptidase from *Saccharomyces cerevisiae*, i.e. carboxypeptidase yscS [7,8]. Most interesting is the fact that the deletion from −406 to −368 decreased β-galactosidase expression by 2-fold in cells that have been transferred to glucose, thus confirming the existence of an upstream activating sequence UAS implicated in carbon catabolite repression in this region. It is worth recalling that a consensus binding site for the yeast activator protein yAP1 [3,4] also found in UAS regions implicated in catabolite repression of the *FBP1* and *PCK1* promoters [9], is located within this region.

If the DNA fragment from −406 to −368, is indeed an activating factor binding site, it would be expected to bind one or more proteins from a yeast extract. For this purpose, we used a single stranded oligonucleotide extending from positions −387 to −370, designated *APE1* UAS and including the yAP1 binding site, in a gel shift assay (Fig. 2A). We identified two protein complexes, designated I and II (Fig. 2A, lane 2) that bind to the single-stranded form of the *APE1* UAS, that are not detected in the absence of cell extract (Fig. 2A, lane 1). Complex II seems to be the predominant one as it is always found in higher amounts than complex I. In lanes 3–6 of Fig. 2A we demonstrate the DNA binding specificity of both complexes. The DNA binding of complex I is specifically competed off by a ten-fold excess of unlabelled APE1 UAS, while the DNA binding of complex II is specifically competed off by a 20-fold excess of unlabelled *APE1* UAS. The DNA binding of any of the two complexes can not be competed off by a 50-fold excess of a single stranded oligonucleotide extending from positions −653 to −633 of the *APE1* promoter (Fig. 2A, lane 6). When we used the single-stranded complementary oligonucleotide only the complex II is detected (data not shown). When we used

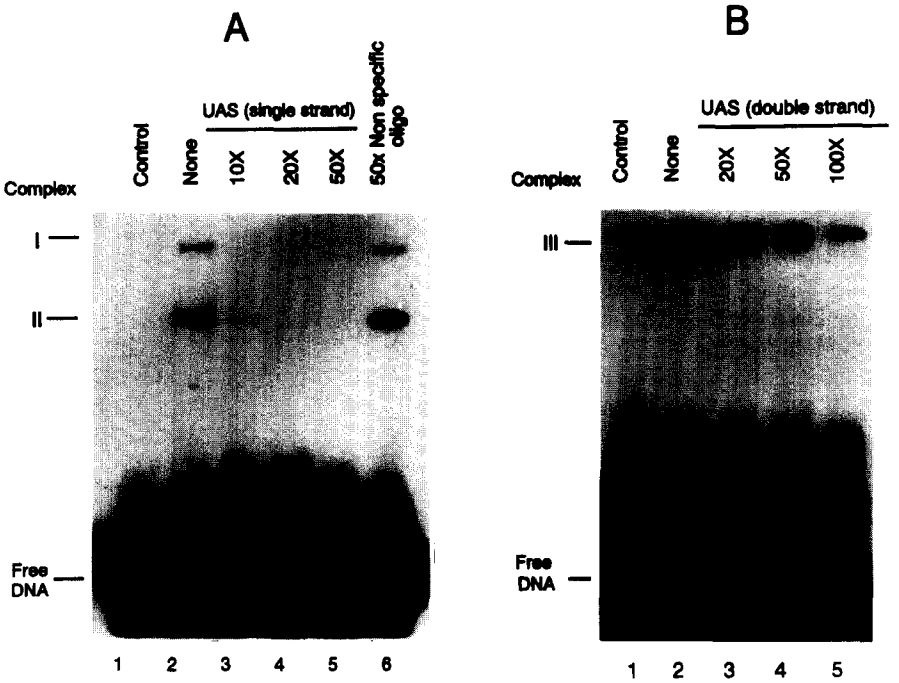

Fig. 2. Proteins binding to an oligonucleotide containing the *APE1* UAS element. (A) single-stranded oligonucleotide. (B) double-stranded oligonucleotide. Cell extracts were omitted from the reaction mixtures resolved in lanes 1A and 1B. The DNA binding of complexes I, II and III is competed with specific (UAS single strand; UAS double strand) or nonspecific DNA, as indicated at the top of the figure.

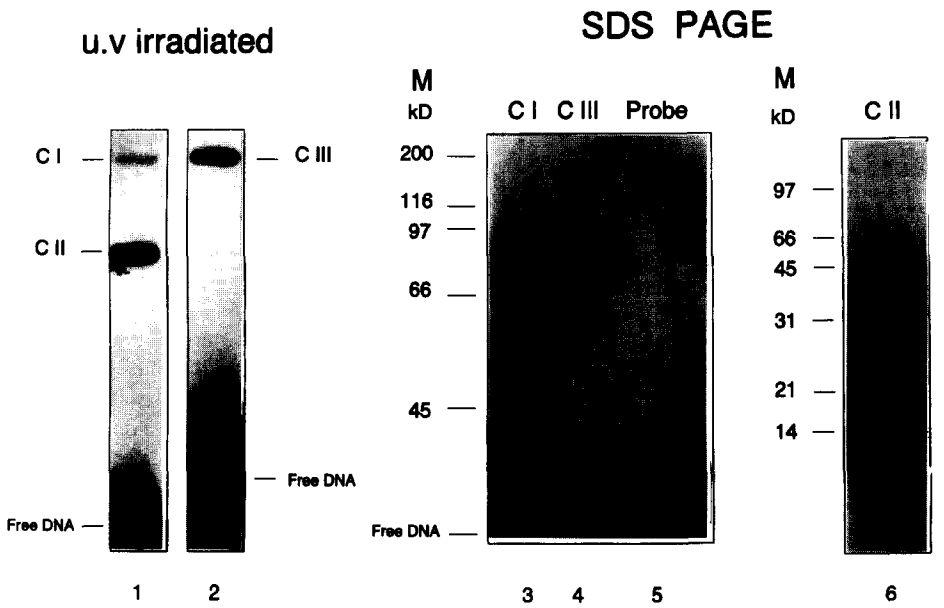

Fig. 3. Identification of the proteins that bind to the *APE1* UAS. The DNA–protein complexes (CI, CII and CIII) of yeast crude extracts with the *APE1* UAS were cross-linked in the gel shifts (lanes 1 and 2). The bands were excised, extracted, resolved in either a 8% (lanes 3–5) or 12% SDS gel (lane 6) and autoradiographed. Labelled double strand oligonucleotide (lane 5).

the double-stranded form of the oligonucleotide corresponding to the *APE1* UAS (Fig. 2B) we identified a single specific protein complex, designated complex III (Fig 2B, lane 2), whose DNA binding is specifically competed off by an excess of unlabelled double-stranded *APE1* UAS (Fig. 2B, lanes 3–5). The DNA binding of complex III can not be competed off by an excess of a nonspecific oligonucleotide (data not shown).

Complexes I, II and III were cross-linked in situ in the gels (Fig. 3, UV irradiated, lanes 1 and 2), the bands excised and the cross-linked products resolved in SDS gels (Fig. 3, SDS-PAGE, lanes 3–6). Complex I and complex III revealed each a 78-kDa protein (Fig. 3, lanes 3 and 4), while complex II seems to be a heterodimer composed by two proteins of 34-kDa and 15-kDa (Fig. 3, lane 6). The fast moving radioactive band in lanes 3 and 4 corresponds to the labelled probe (see the control included in lane 5) not cross-linked by the UV treatment. We interpret the faint cross-linked upper protein band of 48-kDa as being a small amount of the heterodimer not denatured by the SDS treatment. If the two 78 kDa proteins found in complexes I and III (Fig. 3, lanes 3 and 4) are identical, they would be expected to compete for DNA binding. The results shown in Fig. 4 suggest that this indeed the case, as DNA binding of complex I is specifically competed off by an excess of unlabelled single- and double-stranded *APE1 UAS* (lanes 3 and 4), while DNA binding of complex II is only specifically competed off by an excess of unlabelled single-stranded *APE1* UAS (lane 3). Therefore, the 78 kDa protein is able to bind both, single- and double-stranded versions of the *APE1* UAS, while the hetero-

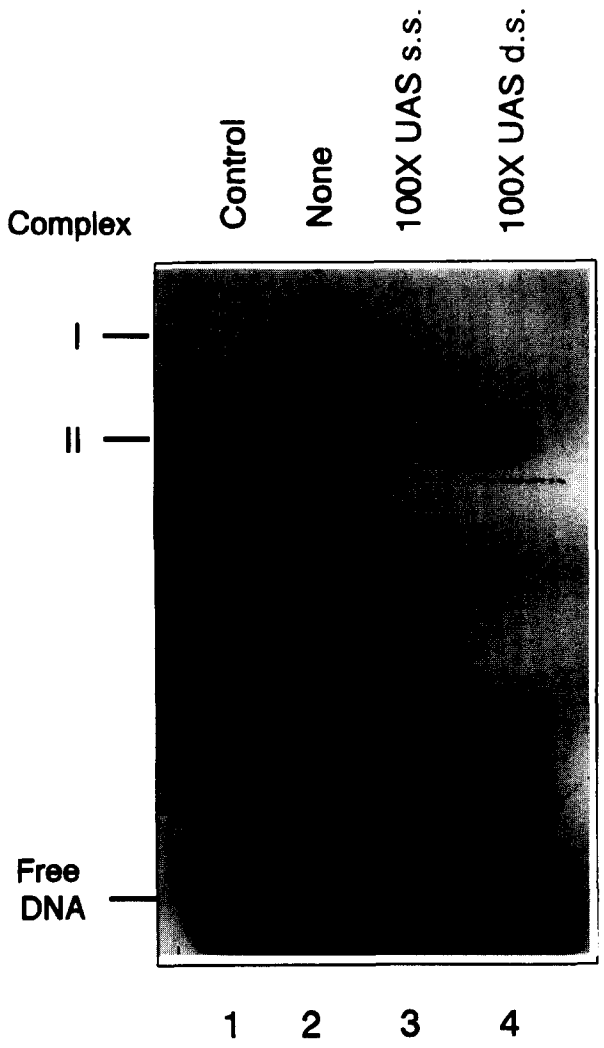

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Fig. 4. Effect of nonradioactive competitor DNA on the ability of the single-stranded oligonucleotide containing the *APE1* UAS to form protein–DNA complexes. Lane 1 = reaction mixture that did not contain cell extract; lane 2 = labelled *APE1* UAS single-stranded oligonucleotide in the absence of competitor; lane 3 = 100-fold excess of unlabelled single stranded oligonucleotide corresponding to the *APE1* UAS; lane 4 = 100-fold excess of unlabelled double-stranded oligonucleotide corresponding to the *APE1* UAS.

dimer present in complex II is only able to bind the single-stranded *APE1* UAS.

The heat stability of both complex I and complex II was increased by DNA binding (Fig. 5). When crude yeast extract was heated to 55°C or higher for 10 min in the absence of the *APE1* UAS, the DNA binding activity of complex I was destroyed (Fig. 5, compare lanes 2 and 3 with lanes 5, 7 and 9), while that of complex II was only destroyed after heating to 65°C (Fig. 5, compare lanes 2, 3 and 5 with lane 7). But when yeast extract was incubated with DNA before heating, the DNA binding activity of complex I was retained up to 55°C (lane 6), and that of complex II was retained up to 75°C (lane 10) in contrast to the other complex. It is worth noting that the DNA binding activity of the 78 kDa protein (complex I) increases when the temperature rises from room temperature (lane 2) to 45°C (lane3), while the opposite effect is seen with the heterodimer of complex II, where a temperature rise in the absence of DNA leads to a decrease in DNA binding activity (Fig. 5, compare lanes 2 and 3) probably due to the fact that dimer formation is disturbed by a higher temperature. The results shown in Fig. 5 suggest that the DNA binding activity of the heterodimer is unusually stable, being resistant to very high temperature, implying that the interactions between DNA and the heterodimer are DNA base- and amino acid-specific. We are exploiting this property to purify the heterodimer in order to identify its constituents.

As stated above, the *APE1* UAS contains the sequence TTAGTCAG related to the AP-1 response element (ARE) [3], recognized by the transcriptional regulatory protein yAP1, a biochemical homologue of the mammalian AP-1 DNA binding activity [10], that belongs to the yeast family of basic region-leucine zipper (bZip) transcription factors [4,11–13]. To test possible involvement of the *YAP1* gene product [3] in DNA binding to the *APE1 UAS*, we checked the ability of a cell extract from a yeast strain with a deletion in the *YAP1* gene that abolishes its DNA-binding activty [4] to bind the single (Fig. 6A) and double-stranded (Fig. 6B) forms of the *APE1* UAS oligonucleotide. The results found demonstrate that yeast cell extracts devoid of the yAP1 regulator protein (Fig. 6A, lane 2; Fig. 6B, lane 2) alter the mobility of the oligonucleotides giving rise to protein–DNA complexes that are indistinguishable from complexes I, II and III detected with a wild-type yeast extract (Fig. 6A, compare lanes 1 and 2; Fig. 6B, compare lanes 1 and 2). These data demonstrate that the protein encoded by the *YAP1* gene does not seem to be any of the *trans*-acting elements that bind to the *APE1* UAS, and so there must be some other transcriptional activator protein(s) with a basic region-leucine zipper domain similar to that of yAP1 responsible for the activation of *APE1* transcription.

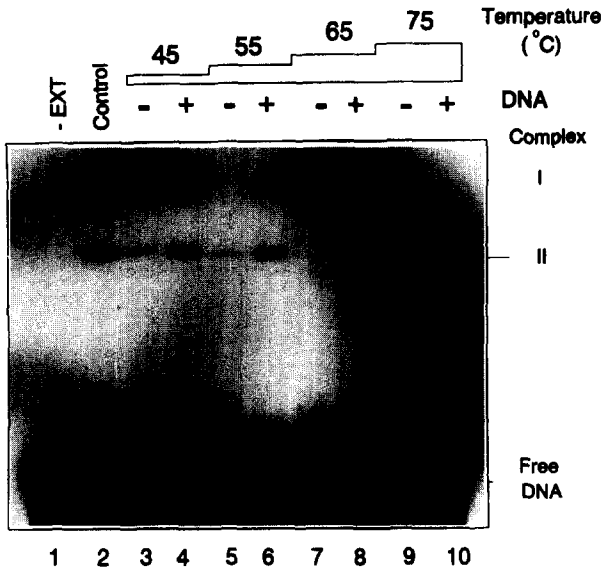

Fig. 5. Heat stability of the protein–DNA complexes. Yeast extract was omitted from the reaction mixture in lane 1. Yeast extracts were heated to the temperature indicated in the figure, either before (−) or after (+) *APE1* UAS single-stranded oligonucleotide was added.

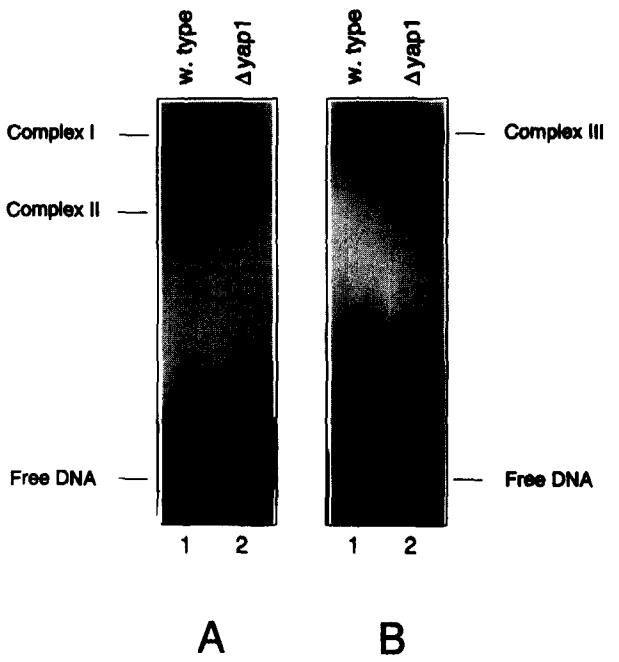

Fig. 6. Protein binding to the *APE1* UAS is not affected by the functional status of the *YAP1* allele. (A) single-stranded *APE1* UAS oligonucleotide. (B) Double-strand *APE1* UAS oligonucleotide. Lanes 1A and 1B = cell extracts from a *YAP1* yeast strain; lanes 2A and 2B = cell extracts from the Δyap1 strain SM10.

Further work is in progress to identify these transcriptional activator proteins involved in carbon regulation of the *APE1* gene expression.

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