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The UAS of the yeast PGK gene is composed of multiple functional elements

Alistair Chambers, Clive Stanway, Alan J.Kingsman and Susan M.Kingsman

Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, UK

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ABSTRACT

The UAS (upstream activator sequence) of the yeast PGK gene contains a transcriptional activator domain located between bases -479 and -402 upstream from the initiating ATG. This region of the UAS contains three direct repeats of the sequence 5'CTTCC3'. The roles in transcriptional activation of these repeats and other sequences within the activator domain were investigated. When short regions containing the repeats were removed PGK expression was considerably reduced, the magnitude of the effect depended upon which CTTCC block was absent. Sequences between -473 and -458 which did not contain a CTTCC block were also shown to be necessary for high levels of PGK expression. A DNA fragment containing activator sequences up to -473 was shown to interact specifically in vitro with a yeast nuclear protein extract. DNase I footprinting identified a protected region between -473 and -458 and single base changes in DNase I sensitivity at the CTTCC repeats.

INTRODUCTION

The PGK gene of Saccharomyces cerevisiae encodes the highly expressed protein phosphoglycerate kinase. This glycolytic enzyme comprises 1-5% of total cell protein and a correspondingly large amount of polyadenylated mRNA (1). When present on a high copy number plasmid the PGK gene can direct the production of PGK protein up to 50% of total cell protein (2, 3). The PGK promoter contains an upstream activator sequence (UAS), previously localised to a region between -538 and -402 bp upstream from the initiating ATG (4). The essential region required for transcriptional activation was shown by deletion analysis to reside between bases -479 and -402 (4). The UAS can function in either orientation and can drive expression from the heterologous promoter TRP1 (4).

The UAS was initially sub-divided into two fragments termed Y (-531 to -461) and Z (-460 to -402) (5). The Z fragment was shown to activate a heterologous promoter in the absence of other UAS sequences. Sequences located, at least in part, on the Y fragment were required to mediate carbon source dependent regulation of PGK expression. These observations suggested that the PGK UAS consists of at least two functionally distinct domains:- an activator domain located on the Z fragment and a modulator domain located either partly or completely on the Y fragment. The Y fragment was found to be the site of specific DNA:protein interactions with yeast nuclear protein extracts, however no DNA:protein interactions were found when the Z fragment was used in similar experiments (5).

The UASs of several other yeast genes are the binding sites for specific transcriptional activator proteins. In some cases the activator protein recognises a partially or completely palindromic sequence, for example the GAL4 protein recognises several copies of a seventeen base pair palindrome in the GAL1-10 UAS (6), the GCN4 protein recognises palindromic sequences in the HIS3 and HIS4 UAS (7, 8, 9) and the functional elements in the PHO5 UAS are also palindromic (10). However not all UASs contain palindromic sequences, for example the recognition sequence of the HAP1 protein in UAS1 of the CYC1 gene is not a palindrome (11, 12). A second property of UASs is that the crucial sequences are often repeated, for example the GAL4 recognition sequence occurs four times in the GAL1-10 UAS (6), multiple copies of the GCN4 recognition sequence are found in the HIS3 and HIS4 UASs (7) and the promoter of the yeast HO gene contains ten copies of the sequence CACGAAAA which can act as a cell type specific activator (13). The activator domain of the PGK UAS contains no obviously palindromic sequences however it does contain three direct repeats of the pentamer 5' CTTCC3', a sequence which occurs only once more in the whole of the PGK gene (at position +419 within the PGK coding region).

We have investigated the significance of the CTTCC repeats and other sequences within the UAS in transcriptional activation of the PGK gene.

MATERIALS AND METHODS

The following E.coli and S.cerevisiae strains were used :-
E.coli AKEC 28 (C6000, thrC, leuB6, thyA, trpCllll7, hsdRk, hsdMk)
S.cerevisiae MD40/4c (α, ura2, trp1, leu2.3, leu2.112, his3.11, his3.15).

1. Construction of promoter deletions.

The method used was essentially as previously described (4). Two one-sided Bal31 deletion series were generated from the molecules pMA746 and pMA722 (4) to give a range of deletion end-points within the PGK UAS. 0.5 units of Bal31 exonuclease (BRL) was used to digest 0.5 µg of DNA at 22°C for times ranging from ten to ninety seconds. Deletion molecules were ligated in the presence of BamHI linkers (BRL). These molecules were sequenced (14) and the position of the deletion end-point numbered relative to the initiating ATG. In each case the number of the last remaining base present was given as the end-point number. Deletion windows were created by digestion of two molecules (one from each series) at their unique BamHI sites followed by ligation.

2. Yeast transformation and analysis of PGK expression.

The resulting high copy number, leucine selectable, plasmids containing a PGK promoter deletion followed by the intact PGK gene were transformed into S.cerevisiae MD40/4c by standard procedures (15). Two transformants were streaked out on yeast minimal plates minus leucine (16). Transformants were grown at 30°C in yeast minimal media with glucose as a carbon source to a cell density of 4-6 x 10^6 cells per ml. Cultures were harvested and cells from the same culture were used to prepare RNA to analyse PGK expression and DNA to check the plasmid copy number (17, 18). 4-8 µg of RNA from each culture was electrophoresed on a 1% formaldehyde agarose gel and transferred onto nitrocellulose by Northern blotting (19, 3). DNA prepared from yeast transformants was digested with EcoRI (BRL) before electrophoresis on a 1% agarose gel followed by Southern blotting (20). Both Northern and Southern filters were probed using a mixture of PGK and ribosomal DNA specific probes nick translated to a specific activity of 1 x 10^8 cpm/µg.

3. Gel retardation assays.

The procedure followed was based on methods described by Fried and Crothers (21) and Garner and Revzin (22). The DNA fragments from the PGK UAS used in this study were as follows:- Y fragment = -531 to -461, Z fragment = -460 to -402, Z+ fragment = -473 to - 409.

These fragments were sub-cloned into pSP46, a derivative of pSP64 (23) in which the HindIII polylinker site has been converted to a BglII site (4). The fragments were isolated from the vector and end-labelled using α^{32}P dCTP (Amersham 3000Ci/mmole) and Klenow polymerase (Amersham).

The preparation of nuclear protein extracts from yeast cultures has been previously described (5). Crude extracts were further purified using heparin-sepharose and oligo-affinity columns (24, 25). Binding reactions were set up as previously described (5) except that in addition each reaction contained 20mM KCl. Each binding reaction also contained calf thymus DNA plus or minus Sau3A digested pSP46 DNA as a non-specific competitor. Sau3A digested pSP46-Z+ DNA was used as a specific competitor. The amounts of competitor DNA added to each binding reaction is described in the figure legends. After a thirty minute incubation at 22°C DNA:protein complexes were resolved by electrophoresis on a 5% polyacrylamide gel containing 0.5 x TBE.

4. DNase I footprinting.

The basic method of Galas and Schmitz (26) was followed with the modification of Singh et al. (27). The DNase I treated binding reaction was first electrophoresed on a retardation gel. The complexed and free fragments were isolated from the gel, phenol extracted and ethanol precipitated and resolved on a 8% denaturing polyacrylamide gel.

RESULTS

1. A high resolution deletion analysis through the PGK UAS.

A deletion analysis was carried out to investigate which sequences within the PGK UAS are necessary for transcriptional activation. Deletion windows in the PGK promoter were constructed as previously described (4). In each case a defined region of the PGK promoter was removed and replaced with a BamHI linker (see

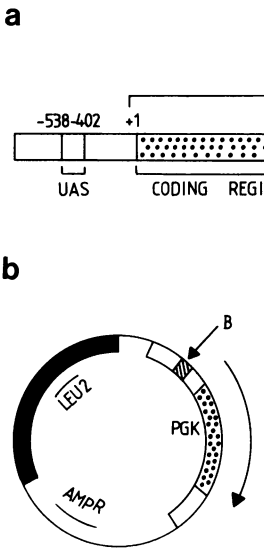

Figure 1a. The yeast PGK gene showing the position of the UAS within the promoter. Co-ordinates are numbered relative to the initiating ATG. **1b.** The yeast high copy number plasmid carrying the PGK gene. B marks the position in the promoter of the deletion window replaced by a BamHI linker.

Figure 1). Molecules consisting of promoter deletions followed by the intact PGK gene, on a high copy number plasmid, were transformed into yeast strain MD40/4c. PGK expression in transformants grown in glucose minimal media was analysed by Northern blotting (10) (Figure 2). As a control to demonstrate that observed differences in PGK mRNA levels were not simply due to differences in gel loading the Northern filters were also probed with a ribosomal DNA probe, this gave a constant signal in all the gel tracks. In each experiment plasmid copy numbers were determined from culture aliquots, no significant differences were observed between any of the plasmids described (data not shown). Initially experiments were performed to investigate the contribution to transcriptional activation of the three CTTCC blocks (Figure 2.a). For convenience these blocks are referred to as 1 (-428 to -432), 2 (-445 to -449) and 3 (-453 to -457). Deletions were made which removed individual blocks, pairs of blocks or all three blocks and different amounts of flanking

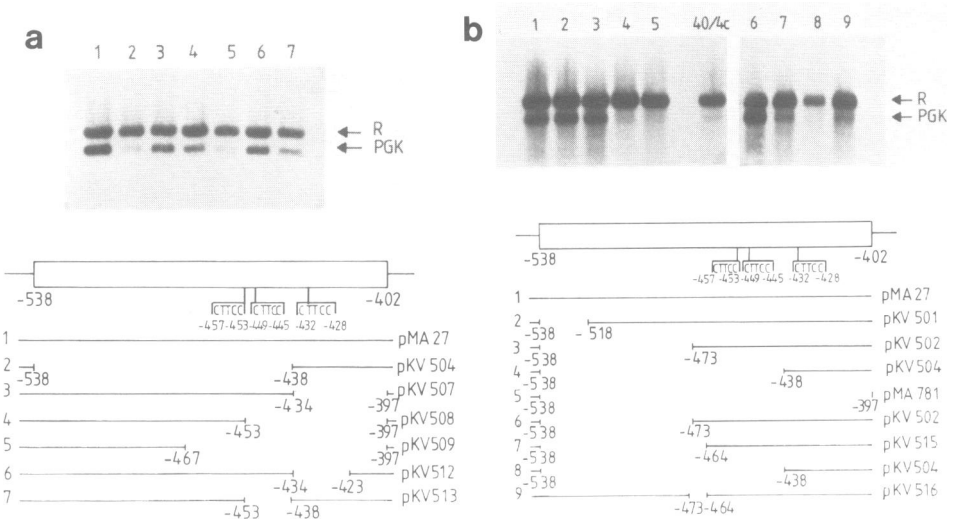

Figure 2. Northern analysis showing the effect of UAS deletions on PGK mRNA levels. R indicates the ribosomal RNA probed as a loading control. PGK indicates the position of the PGK mRNA. The structures of the deletion molecules analysed are shown. 40/4c shows the chromosomal PGK mRNA level in the untransformed yeast strain.

sequences. The CTTCC blocks were found to make different contributions to transcriptional activation. When block 1 alone was removed (pKV507 and pKV512) PGK expression was reduced by approximately 50% (Figure 2.a, tracks 3 and 6). This indicated that CTTCC block 1 forms part or all of an element necessary for full expression of the PGK gene. When UAS sequences upstream of block 1 were removed leaving block 1 intact (pKV504) PGK expression was reduced to less than 10% of wild type levels (Figure 2.a, track 2). This indicated that CTTCC block 1 alone is not sufficient to activate transcription, it only functions as an activator when other sequences within the UAS are also present.

The removal of block 2 alone (pKV513) reduced PGK expression by about 75% (Figure 2.a, track 7). This indicated that CTTCC block 2 also forms part or all of an element necessary for full activation. Block 2 appears to be more significant than block 1 because removal of block 2 caused a greater reduction in PGK expression than the removal of block 1.

The importance of a region containing block 3 and upstream

sequences was demonstrated by a deletion which removed all three CTTCC boxes and ten bases of upstream sequence (pKV509). This deletion reduced PGK expression to less than 10% of wild type, a considerably greater effect than was caused by the removal of only blocks 1 and 2 (Figure 2.a, compare tracks 5 and 4).

Further deletions were analysed in order to investigate the importance of sequences upstream of CTTCC block 3. A deletion which removed the region between -538 and -473 (pKV502) had no effect on PGK expression (Figure 2.b, tracks 3 and 6). When this deletion was extended in the downstream direction to -464 (pKV515) PGK expression was reduced to below 20% of the wild type level (Figure 2.b, track 7). This indicated that the region between -473 and -464 contains part or all of a further element required for full activation of PGK expression. This result was confirmed by a deletion which removed only the sequence between -473 and -464 (pKV516). This deletion again produced PGK mRNA at less than 20% of the wild type level (Figure 2.b, track 9).

The deletions analysed in this study have demonstrated that multiple sequences within the PGK UAS are necessary for full transcriptional activation. The direct repeats of the sequence CTTCC are important elements. CTTCC block 2 appears to be more important than CTTCC block 1. A sequence element upstream of CTTCC block 3 is also of considerable importance, deletion of a region between -473 and -464 caused an 80% reduction in PGK expression even though all three CTTCC boxes were still present. We have called the essential element upstream of block 3, extending from -473 to -458, the activator core (AC) sequence.

2. DNA protein interactions at the PGK UAS.

We previously performed experiments to investigate DNA:protein interactions at the PGK UAS using two sub - fragments of the UAS termed Y and Z (5). These fragments contained sequences from -531 to -461 and from -460 to -402 respectively. The Y fragment, containing sequences necessary for the carbon source dependent regulation of PGK expression, was found to be a site of strong DNA:protein interactions in vitro. The major complex formed between the Y fragment and proteins from a yeast extract was localised by DNase I footprinting to a region from -523 to -496. Despite using numerous different binding conditions no specific

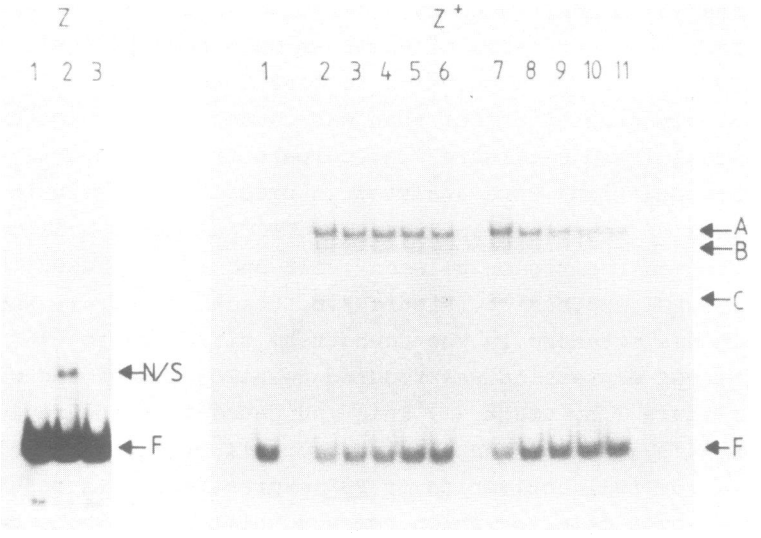

Figure 3. Gel retardation assay using Z and Z+ fragments with a yeast nuclear protein extract. Z panel:- F=free fragment, N/S=non-specific complex, lane 1=unbound fragment, lane 2=fragment plus extract plus 50ng calf thymus DNA, lane 3=fragment plus extract plus 100ng calf thymus DNA. Z+ panel:- F=free fragment, A,B and C=DNA:protein complexes, lane 1=unbound fragment, lanes 2-6=fragment plus extract plus 50ng calf thymus DNA plus increasing amounts of Sau3A cut pSP46 DNA as follows, lane 2=0ng, lane 3=50ng, lane 4=100ng, lane 5=150ng, lane 6=200ng. Lanes 7-11 as lanes 2-6 using Sau3A cut pSP46-Z+ DNA as competitor.

DNA:protein interactions were found to occur using the Z fragment (unpublished data and see Figure 3). The new results reported here, showing that at least part of the activator core sequence is located in the region between -473 and -461, which is not present in the Z fragment, suggested a possible explanation for this observation. A fragment extending from -473 to - 409, containing all of the newly defined activator core sequence was therefore tested for DNA:protein binding. This extended Z fragment was termed the Z+ fragment. Radioactively labelled Z+ fragment was incubated with crude and heparin sepharose purified yeast protein extracts. DNA:protein complexes were identified using the gel retardation technique (21, 22) (Figure 3). Three complexes A,B and C were observed. Complex A was the most

abundant and had the lowest gel mobility. These complexes were shown to be the result of specific DNA:protein interactions by competition experiments. Adding 200 ng of unlabelled, Sau3A digested, pSP46 DNA (23, 4) had little effect on the amount of the three complexes (tracks 2 to 6). Adding the same amount of Sau3A digested pSP46-Z+ (the pSP46 plasmid containing the Z+ fragment) considerably reduced the amounts of complexes A, B and C (tracks 7 to 11). The two faster migrating complexes (B and C) probably result from partial proteolysis of the protein(s) responsible for complex A because increasing the amount of protein extract in the binding reaction did not alter the ratio of complexes B and C to complex A (data not shown). If B and C had been intermediates in the formation of A increasing the amount of protein would probably have reduced the amounts of B and C and increased the amount of A. The results demonstrate that the Z+ fragment is subject to specific DNA:protein interactions in vitro but the Z fragment is not. The Z fragment contains all three CTTCC blocks but not the entire AC sequence whereas the Z+ fragment contains the whole of the AC sequence as well as the three CTTCC blocks. The most likely explanation for the difference in protein binding observed between these two fragments is that a protein required for complex formation recognises the extra sequence present in the Z+ fragment, the activator core sequence.

The activator domain of the PGK UAS is therefore subject to specific DNA:protein interactions in vitro but only as long as the activator core sequence is present. The DNase I protection technique (26, 27) was then used to investigate the DNA:protein interactions involved in complex A (Figure 4). The version of the method used here involved DNase I treatment of a binding reaction followed by electrophoresis through a standard retardation gel. The retarded and free fragments were then eluted from the gel, purified and resolved on a denaturing polyacrylamide gel. Changes in DNase I sensitivity were observed for both strands of the Z+ fragment, however these changes were not identical on the two strands. The non-coding strand showed a region of protection extending from -473 to -455 interupted by a DNase I cut at about -466. This protected region includes the AC

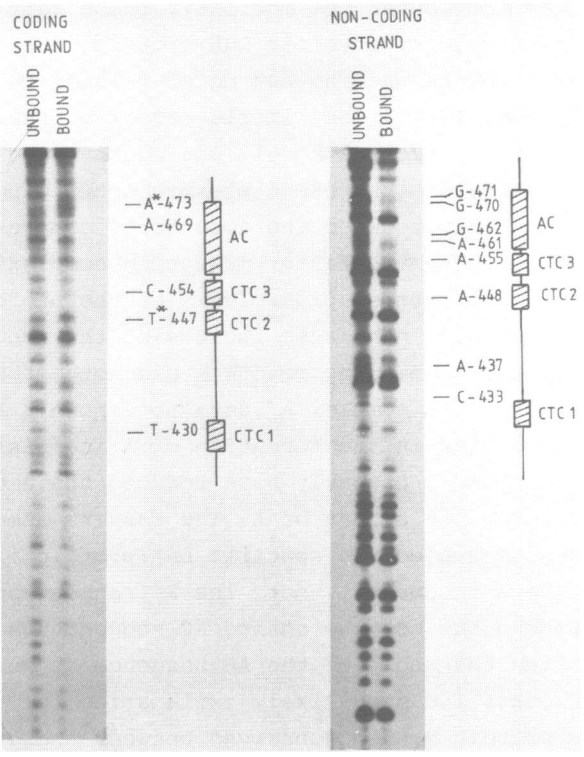

Figure 4. DNase I footprinting of both the coding and non-coding strands of the PGK Z+ fragment. Decreases in band intensities in the bound relative to the unbound track are arrowed. Increases in band intensity are marked*. The activator control sequences identified by deletion analysis are indicated in the line diagrams alongside each panel, AC=activator core sequence, CTC=5' CTTCC3'.

sequence and part of CTTCC block 3. Other smaller protected regions at CTTCC block 2 and close to CTTCC block 1 were also observed. The coding strand showed smaller changes in DNase I sensitivity. Slight protections of one or two nucleotides were observed in the AC sequence and CTTCC blocks 3 and 1 and slight enhancements of cleavage were observed at the upstream end of the AC sequence and in CTTCC block 2. These reproducible effects in the DNase I digests of the two DNA strands suggests a possible assymetry in DNA:protein binding.

The gel retardation and DNase I cleavage results strongly

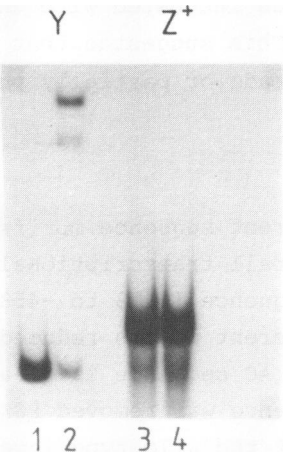

Figure 5. The Y and Z+ binding proteins are not identical. A purified protein fraction containing the Y binding protein was incubated with Y and Z+ fragments. DNA:protein complexes were identified by gel retardation. lanes 1 and 3 = free fragment, lanes 2 and 4 = fragment plus Y binding protein plus 100ng calf thymus DNA.

suggest that one region involved in the DNA:protein binding reaction in vitro is the AC sequence, a region demonstrated by the deletion analysis to be essential for full activation in vivo. The three CTTCC blocks which are also essential elements for full activation in vivo also showed changes in DNase I sensitivity indicating that they may also be involved in DNA:protein contacts.

3. The Y fragment binding protein does not form complexes with the Z+ fragment.

The Y fragment, from the PGK UAS, interacts strongly with proteins from yeast extracts to form a number of complexes (5). The protein which forms the most abundant complex protects a region of Y extending from -523 to -496 in DNase I footprinting experiments. This Y binding protein was purified from yeast cell extracts using heparin sepharose and oligo-affinity columns (24, 25 and Stanway et al., unpublished data). The purified Y protein was tested for it's ability to form complexes in vitro with the Z+ fragment (Figure 5). The purified Y protein formed specific complexes with the Y fragment however no complexes were observed

when the Z+ fragment was incubated with the Y protein under identical conditions. This suggested that the Y protein is not the protein which in crude or partially purified extracts binds to the Z+ fragment.

DISCUSSION

There are two different sequence motifs within the PGK UAS that contribute to overall transcriptional activation, the activator core (AC) sequence (-473 to -458) and the CTTCC blocks. Deletion of these different motifs reduced PGK transcription by different amounts. The AC sequence is clearly very important because when this sequence was removed PGK transcription was reduced to below 20% of the wild type level. The three CTTCC blocks appear to contribute differently to transcriptional activation, deletion of block 1 (-432 to -428) had less effect on PGK transcription than deletion of block 2 (-449 to -445). Maximum expression of the PGK gene requires the AC sequence and all three CTTCC blocks.

As with other UASs we have confirmed that activation of PGK is probably mediated by the specific binding of transcriptional activator proteins. A DNA fragment termed the Z+ fragment (-473 to -409) was shown to interact specifically *in vitro* with yeast nuclear protein extracts. The Z+ fragment contains the AC sequence and all three CTTCC blocks. The AC sequence appears to be critical for this interaction because a similar fragment (Z) which contains all three CTTCC blocks but not the AC sequence did not show specific binding. DNaseI footprinting of the most abundant complex formed by the Z+ fragment showed that the AC sequence underwent changes in DNase I sensitivity on protein binding. This suggested that this sequence is a site of DNA:protein contact. Other minor changes in DNase I sensitivity were also found to occur at, or proximal to, the CTTCC blocks. These sequences may also be the sites for weak DNA:protein interactions *in vitro*. The simplest explanation for these observations is that protein binding at the AC sequence stimulates the interactions at the CTTCC blocks.

The AC sequence defined by our deletion analysis contains a ten base pair sequence with a high degree of homology to the RPG-box,

a known activator sequence found upstream of yeast ribosomal protein genes which is the binding site for the protein factor TUF (28, 29, 30). The consensus RPG-box sequence ACACCCATACAT is very similar to the sequence ACCCAGACAC found between -473 and -464 in the AC sequence of the PGK promoter. This sequence is also highly homologous to the recognition sequences of the proposed yeast general regulatory protein RAP1/GRF1 (31, 32). The consensus RAP1/GRF1 binding site ACCCANNCAT/CT closely ressembles both the AC sequence and the TUF recognition sequence . The RAP1 protein binds in vitro to the UASs of the MATα gene, the elongation factor gene TEF1 and the ribosomal protein gene RP51. It also binds to the silencer elements at the HML and HMR loci and sequences within the ($C_{1-3}A$) repeat region at yeast telomeres. It has been suggested that RAP1/GRF1 may play a role in both activation and repression of transcription depending on the context of its binding site. RAP1 and TUF are apparently not the same protein factor as both have been purified and have different molecular weights (31, 33). The DNase I footprint of the TUF protein on RPG-box B of the ribosomal protein gene L25 is a strong protection of 23 b.p (29), considerably different to the footprint we observe on the AC sequence. RAP1 was also found to produce a larger footprint on the HMR silencer than was seen for the AC binding protein on the PGK UAS. However when purified from a sequence specific column RAP1 did appear to protect less strongly a region in the centre of the binding site (31) a similar effect to that seen on the AC sequence. The protein which binds to the AC sequence may be TUF, RAP1/GRF1 or a different protein which recognises a similar sequence. Interestingly it has recently been shown that the UAS from another yeast glycolytic gene ENO1 interacts specifically with yeast nuclear proteins in vitro (34). The region found to be protected in DNase I footprinting experiments contained the sequence ACCCAAACAC which is a good match to the AC sequence and the TUF and RAP1 binding sites. The footprint obtained on the ENO1 UAS also closely ressembles the footprint on the PGK AC sequence.

Little is known about carbon source regulation of the glycolytic genes. Regulated expression of almost all of these genes depends on the product of the gene GCR1 (35, 36). We have

previously implicated a protein (Y protein), that binds between -523 and -496 in the PGK UAS, in carbon source regulation of PGK (5). Our previous study demonstrated that a DNA fragment that contained the Y protein binding site confered carbon source regulation on a heterologous promoter. We now know that this fragment also contained the newly defined AC sequence and therefore this sequence could also be involved in carbon source regulation. Recently a RPG-box consensus oligomer derived from the L25 ribosomal protein gene was shown to be partly capable of confering carbon source regulation on a heterologous promoter (37). This supports the proposal that the AC binding protein may be involved in regulation as well as activation. At present we do not know if any of the protein interactions at the PGK UAS directly involve the GCR1 protein.

The results reported here have clearly shown that the PGK UAS is not a simple on/off switch, it has a complex organisation which gives it the potential to activate transcription to varying degrees. It is our aim to isolate and characterise the genes that encode the protein components of the PGK UAS activator complex and define their mode of action.

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