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Efficient Expression of the *Saccharomyces cerevisiae PGK* Gene Depends on an Upstream Activation Sequence but Does Not Require TATA Sequences

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The *Saccharomyces cerevisiae PGK* (phosphoglycerate kinase) gene encodes one of the most abundant mRNA and protein species in the cell. To identify the promoter sequences required for the efficient expression of *PGK*, we undertook a detailed internal deletion analysis of the 5′ noncoding region of the gene. Our analysis revealed that *PGK* has an upstream activation sequence (UAS_{PGK}) located between 402 and 479 nucleotides upstream from the initiating ATG sequence which is required for full transcriptional activity. Deletion of this sequence caused a marked reduction in the levels of *PGK* transcription. We showed that *PGK* has no requirement for TATA sequences; deletion of one or both potential TATA sequences had no effect on either the levels of *PGK* expression or the accuracy of transcription initiation. We also showed that the UAS_{PGK} functions as efficiently when in the inverted orientation and that it can enhance transcription when placed upstream of a *TRP1-IFN* fusion gene comprising the promoter of *TRP1* fused to the coding region of human interferon α-2.

In the yeast *Saccharomyces cerevisiae* the *PGK* (phosphoglycerate kinase) gene encodes one of the most abundant mRNA and protein species in the cell, accounting for 1 to 5% of the total cellular mRNA and protein (22). The *PGK* gene has been cloned and characterized (11, 21), and the 5′ noncoding region has been found to contain sequences which are common to the promoters of all efficiently expressed yeast genes (11). When the *PGK* gene is present on a high-copy-number plasmid, phosphoglycerate kinase protein makes up approximately 50% of the total cell protein (31, 32). For this reason the promoter region of *PGK* has been used to construct some of the most powerful expression vectors available. These vectors have been used to direct the efficient synthesis of a number of heterologous gene products in yeasts (9, 31, 32, 49, 51). In these systems the promoter used comprises 1,500 base pairs (bp) upstream from the initiating ATG.

Previous studies on the promoters of yeast genes have revealed a number of control elements which are required for accurate and efficient transcription. One element, the TATA box, found in the 5′-flanking regions of virtually all protein-encoding eucaryotic genes (see reference 4 for a review), has been shown to be required for setting the site of transcription initiation (16, 17, 27a, 35, 41, 44) and, in some cases, maintaining levels of transcription (16, 44). A second element, the upstream activation sequence (UAS), has been found in the 5′ noncoding regions of a number of yeast genes, usually several hundred nucleotides upstream from the TATA box (14, 16, 26, 39, 44, 52). UASs have certain features in common. First, deletion of these sequences causes a marked reduction in levels of transcription. Second, in several cases the role of a UAS in activating transcription is subject to regulation dependent on the physiological condition of the cells (16, 50, 54). Third, hybrid gene constructions have shown that a UAS can drive transcription of a heterologous gene and that transcription is regulated in a UAS-dependent manner (15, 20, 45, 46). In addition to having elements that activate transcription, some yeast genes have negative regulatory regions which act by repressing transcription (25, 52, 53). To date, however, the precise role of upstream positive and negative regulatory regions in promoter function remains unclear.

We undertook a detailed internal deletion analysis of the promoter region of *PGK* to identify the control elements involved in efficient expression of the gene. Our results demonstrate that the *PGK* promoter contains a UAS which can function when inverted and which can direct transcription of a foreign gene. We also show that accurate transcription initiation of *PGK* does not depend on TATA sequences.

MATERIALS AND METHODS

Strains and media. Strains used were *Escherichia coli* AKEC28 (C600 *thrC leuB6 thyA trpC1117 hsdR hsdM*) and *S. cerevisiae* MD40/4c (α *ura2 trp1 leu2.3 leu2.112 his3.11 his3.15*) and AH22 (a *leu2.3 leu2.112 his4.519 can1*). *E. coli* cultures were grown on Luria broth (34) or on defined minimal medium without leucine (29). Ampicillin (Sigma Chemical Co., St. Louis, Mo.) was added to the medium at 50 μg/ml. Yeast cultures were grown at 30°C in YEPD (1% yeast extract, 2% peptone, 2% glucose) or defined minimal medium (18). Amino acids were added to the medium when necessary.

Enzymes and plasmid construction. Standard procedures were used for restriction digestion and plasmid construction (29). Restriction enzymes and T4 DNA ligase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were used according to the instructions of the supplier. Nonphosphorylated linkers were obtained from Collaborative Research Inc. (Waltham, Mass.). *Bal* 31 exonuclease (Bethesda Research Laboratories) was used as described previously (11) except that reactions were carried out at 25°C. *Bal* 31 deletion endpoints were sequenced by the dideoxy chain termination method of Sanger et al. (38). DNA fragments were purified from agarose or polyacrylamide gels as described by Tabak and Flavell (47) and Maniatis et al. (29), respectively.

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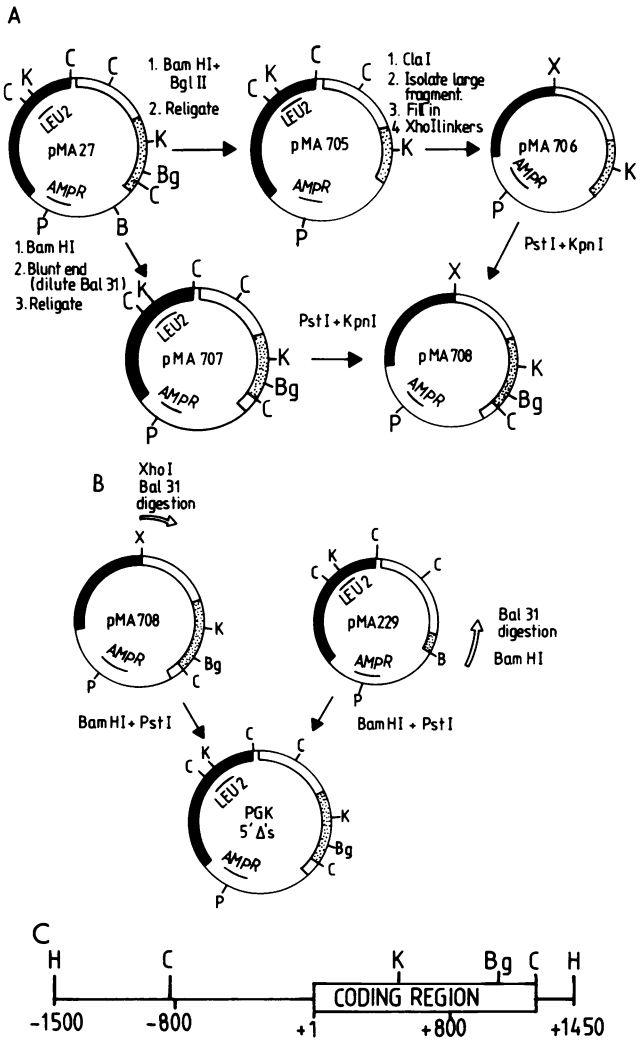

FIG. 1. Construction of internal deletions in the *PGK* 5′ noncoding region. (A) Steps in the construction of pMA708 from pMA27. pMA27 has been described eleswhere (32). (B) Construction of internal deletions in 5′ *PGK* from *Bal* 31-deleted derivatives of pMA708 and pMA229. pMA229 has been described previously (11). *Bal* 31 deletion endpoints were filled in and ligated with *Bam*HI linkers. Open line, *PGK* 5′ and 3′ noncoding regions; speckled line, *PGK* coding region; thin line, pBR322; solid line, 2μm-*LEU2* partial *Eco*RI fragment. (C) Restriction map of the 2.95-kilobase *PGK* fragment. Restriction sites are: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; K, *Kpn*II; P, *Pst*I; X, *Xho*I.

Construction of *PGK* promoter deletions. Internal deletions or "windows" in the 5′ noncoding region of *PGK* were constructed from *Bal* 31 deletion derivatives of pMA229 and pMA708. pMA229 is a member of the pMA22a deletion series which has been described elsewhere (11). This molecule contains the entire 5′ noncoding region of *PGK* but lacks all but the first 14 nucleotides of the *PGK* coding region (Fig. 1B). pMA708 was derived from pMA27, a high-copy-number plasmid carrying the 2.95-kilobase *PGK* fragment (Fig. 1) (32). pMA708 has a unique *Xho*I site replacing a *Cla*I site 820 bp upstream from the ATG of the *PGK* coding region (i.e., at −820), and it lacks both the *Bam*HI site in pBR322 sequences and the *LEU2* selectable marker. The construction of pMA708 from pMA27 is summarized in Fig. 1A.

One-sided deletions of the *PGK* promoter region in the 5′→3′ direction were generated by digesting pMA708 with *Xho*I followed by treatment with *Bal* 31 exonuclease. This produced a series of molecules which contained an intact *PGK* coding region and which retained between 91 and 540 bp of 5′ noncoding sequences. A second series of deletions removing sequences in the 3′→5′ direction was obtained by cutting pMA229 at the unique *Bam*HI site and by digesting with *Bal* 31. This generated a set of deletion molecules with endpoints from −129 to −595. The ends of the deletion molecules were filled in with the Klenow fragment of DNA polymerase (Bethesda Research Laboratories), ligated in the presence of excess *Bam*HI linkers, and transformed into AKEC28 selecting for ampicillin resistance. All the deletion endpoints were sequenced; the coordinate of each endpoint refers to the position of the last remaining base. A deletion endpoint 402 bp upstream from the ATG of the coding region is given as −402.

The construction of the *PGK* promoter windows followed the same basic pattern shown in Fig. 1B. The *Bam*HI-*Pst*I fragment containing the *PGK* coding region and pBR322 sequences from a pMA708 series deletion was ligated to the *Bam*HI-*Pst*I 2μm-*LEU2* fragment from a pMA229 deletion molecule. The resulting molecules have internal deletions or windows in the *PGK* 5′ region; the 5′ endpoint of each window is provided by a pMA229 series deletion, and the 3′ endpoints are provided by a pMA708 series deletion. These plasmids are identical to pMA27 except for deletions in the *PGK* 5′ noncoding region marked by a *Bam*HI site and the absence of the *Bam*HI site in pBR322 sequences.

Isolation of UAS_{PGK} and construction of a *PGK-TRP1* hybrid promoter. The UAS_{PGK} was isolated on a 136-bp *Bam*HI-*Hae*III fragment (coordinates −538 to −402) from pMA744, one of the pMA229 series deletion molecules which has a deletion endpoint at −402. The *Hae*III end of the fragment was converted to a *Bgl*II site by insertion into the polylinker of Sp46, a derivative of Sp64 (33) in which the *Hin*dIII site in the polylinker was converted to *Bgl*II. The UAS_{PGK} was isolated from Sp46 on a 161-bp *Bam*HI-*Bgl*II fragment (136 bp plus 25-bp linker sequence).

PGK-TRP1 hybrid promoters were constructed by inserting the UAS_{PGK} into the *Bam*HI site upstream of two *TRP1-IFN* gene fusions (pMA1557-10 and pMA1557-16) which contain the *TRP1* promoter fused to the coding region of the human interferon α-2 gene. The construction of the *TRP1-IFN* fusions is described elsewhere (27a). These molecules are identical except that pMA1557-10 contains the entire *TRP1* promoter while pMA1557-16 has a deletion in the *TRP1* promoter which removes the positive control element for the large *TRP1* transcript (27a). Plasmids pMA1557-10 and pMA1557-16 containing the UAS_{PGK} were designated pMA1610-10 and pMA1610-16, respectively (Fig. 2).

Transformation of yeasts and *E. coli*. *E. coli* was transformed by standard methods (29). Yeasts were transformed as described by Hinnen et al. (19). Yeast strains containing plasmids are termed T, to designate transformant, followed by the pMA number of the particular plasmid; for example, a yeast strain transformed with pMA760 will be referred to as T760. For the analysis of *PGK* internal deletions, plasmids were transformed into MD40/4c; *TRP1-IFN* plasmids were analyzed in AH22.

DNA and RNA isolation. Plasmid DNA was isolated from *E. coli* by the methods of either Chinault and Carbon (6) or Birnboim and Doly (2) and for rapid analysis by the method of Holmes and Quigley (23). Total yeast DNA was prepared by the method of Cryer et al. (8). Total RNA was prepared

from yeast cells grown to a density of 4×10^6 cells per ml in minimal medium without leucine as previously described (10). Total yeast DNA for copy number determination and RNA for Northern analysis were always prepared from cells from the same culture.

Hybridization conditions and probes. Northern and Southern filters were hybridized under standard conditions (48). Probes were fragments labeled by nick translation (37) with [α-^{32}P]dTTP (Amersham International) to a specific activity of 4×10^7 to 6×10^7 cpm/μg. *PGK*, Ty, *IFN*, and ribosomal DNA (rDNA) fragments were purified from digests of pMA27 (32), pAT153-10 (10), pAT153-1 (31), and pY1RG12 (36), respectively.

Determination of plasmid copy number and RNA analysis. For determination of plasmid copy number in yeasts, total DNA from transformants was digested with *Eco*RI, electrophoresed on 1% agarose gels, and transferred to nitrocellulose filters as described by Southern (43). Southern filters were probed with *PGK*- and rDNA-specific fragments nick translated to the same specific activity. Comparison of the relative intensity of rDNA to *PGK* bands given an estimate of the number of plasmid copies in transformed cells relative to the number of repeats of rRNA genes (100 to 140 copies per haploid genome [36]). Total RNA isolated from transformants was electrophoresed on 6% formaldehyde–1% agarose gels, transferred to nitrocellulose filters (29), and probed either with *PGK*- and Ty- or with *PGK*- and *IFN*-specific probes.

RNA start site mapping. The mRNA start site of selected deletions was determined by the riboprobe mapping procedures described by Melton et al. (33) and Zinn et al. (55). A strand-specific *PGK* probe complementary to the mRNA was synthesized as follows. The *Bam*HI-*Cla*I fragment from pMA230 (49), which contains the entire *PGK* promoter plus 37 bp of *PGK* coding sequence, was inserted into the pSP65 polylinker at the *Bam*HI-*Acc*I sites (33). The template DNA was linearized and used to synthesize RNA transcripts labeled with [α-^{32}P]GTP (Amersham International) according to the transcription protocol of Melton et al. (33). Total yeast RNA (15 μg) was hybridized with excess probe (ca. 5×10^7 cpm) at 45°C for 11 to 12 h. The RNA-RNA hybrids were digested at 37°C for 30 min as described by Zinn et al. (55), and the protected fragments were resolved on an 8% polyacrylamide-urea sequencing gel.

RESULTS

The effects of deletions in the promoter on *PGK* expression were determined by examining the steady-state levels of *PGK* mRNA in total RNA extracts from yeast transformants by Northern blot analysis.

A preliminary one-sided deletion analysis of the *PGK* 5′ noncoding region demonstrated that deletion of sequences upstream of approximately −620 had no effect on the levels of *PGK* transcription (data not shown). One of the deletions, which removed all sequences upstream of approximately −350, caused a substantial reduction in *PGK* mRNA levels (data not shown). These preliminary data indicated that the region −620 to −350 contained an important promoter element for *PGK*, and therefore we focused on this region for the construction of the internal deletions.

The construction of internal deletions in the *PGK* 5′ noncoding region is described in the Materials and Methods. The coordinates of the deletions are given in Fig. 3. Figure 4 shows the full sequence of the *PGK* 5′ region; the sequencing of the *PGK* gene has been described elsewhere (11).

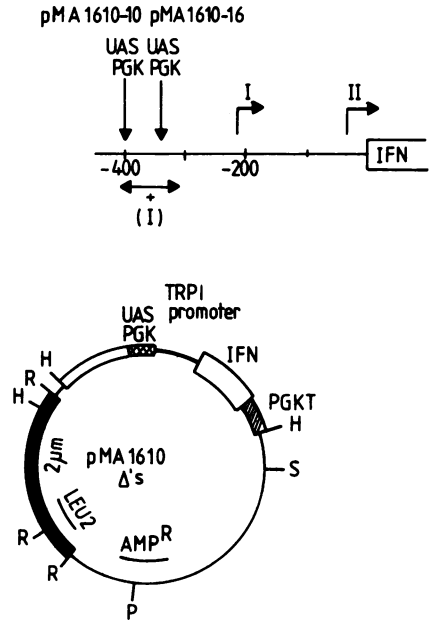

FIG. 2. General structure of the *PGK-TRP1* hybrid promoter. *PGK-TRP1* hybrid promoters were constructed by inserting the UAS_{PGK} upstream of *TRP1-IFN* gene fusions in vector pMA1557 which has been described elsewhere (27a). The gene fusion consists of the promoter of *TRP1* fused to the coding region of human interferon α-2. In pMA1610-10 the UAS_{PGK} is inserted upstream of the entire *TRP1* promoter, while in pMA1610-16 the UAS_{PGK} is substituted for one of the *TRP1* positive control elements. Open line, Region flanking *TRP1* promoter from pMA1557; PGKT, *PGK* terminator; thin line, pBR322; solid line, 2μm-*LEU2* fragment. Restriction sites are: H, *Hin*dIII; P, *Pst*I; R, *Eco*RI; S, *Sal*I.

Effects of internal deletions on *PGK* transcription. The *PGK* mRNA levels of yeasts transformed with the internal deletion plasmids were compared with those of the untransformed strain MD40/4c (i.e., chromosomal *PGK*) and of T27, a transformant containing pMA27 which is a high-copy-number plasmid carrying the entire *PGK* gene (32). The chromosomal levels of *PGK* in MD40/4c are approximately 50-fold less than those of T27 (Fig. 5A, lanes a and b).

To check that differences in *PGK* mRNA levels were not due to discrepancies in the loading of gels, all Northern filters were probed with Ty-specific, in addition to *PGK*-specific, nick-translated fragments. Furthermore, the plasmid copy number of each transformant was determined relative to the number of repeats of the rRNA genes (see Materials and Methods). Southern blot analysis of *Eco*RI-digested total DNA from transformants showed that the relative intensity of plasmid-specific *PGK* bands to rDNA bands was similar for virtually all transformants (Fig. 5B), indicating that plasmid copy number did not differ significantly.

Northern blot analysis of RNA prepared from transformants showed that the deletions could be separated into two classes depending on their effects on *PGK* transcription (Fig. 5A). These were: class 1, deletions which caused a marked reduction in the levels of *PGK* transcription giving levels that were intermediate between those of T27 and chromosomal *PGK* (represented by + or +/− in Fig. 3; chromosomal *PGK* is represented by −); class 2, deletions which had no effect on *PGK* transcription giving the high levels characteristic of T27 (represented by ++ in Fig. 3).

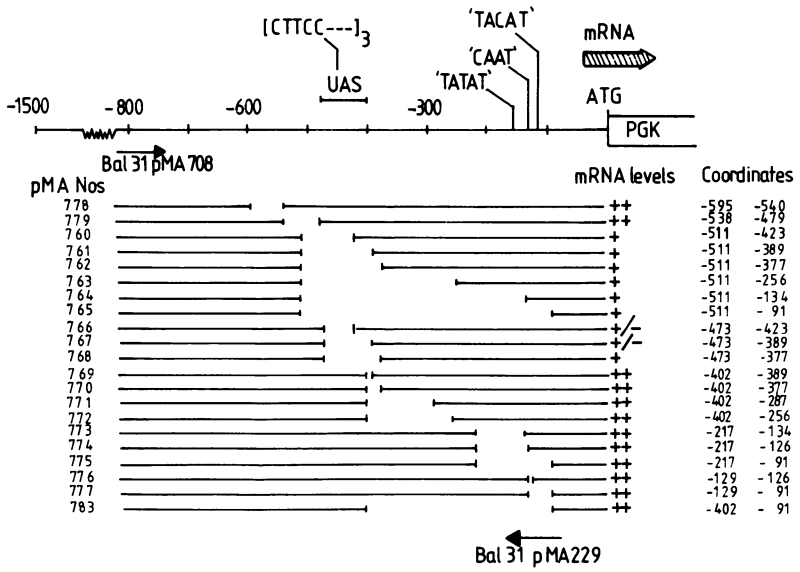

FIG. 3. Diagrammatic representation of the *PGK* 5′ noncoding region showing the internal deletions and their effects on *PGK* transcription. Internal deletions in the *PGK* 5′ region were constructed from *Bal* 31-deleted derivatives of pMA229 and pMA708 (see Materials and Methods). *PGK* mRNA levels are: ++, high levels characteristic of the undeleted promoter, i.e., pMA27; +, levels intermediate between those produced by pMA27 and by the chromosomal *PGK* gene; +/−, low levels of *PGK* mRNA; −, chromosomal *PGK*. All deletions contain the region −820 to −1500 of the promoter. Coordinates of deletion endpoints are given relative to the ATG of the coding region.

Class 1 deletions. Class 1 deletions define sequences in the *PGK* 5′ region that are required for maximal transcriptional activity. Removal of sequences in pMA760 to pMA768 caused a marked reduction in *PGK* transcription, although significant levels of mRNA are still observed; typical mRNA levels for class 1 deletions, i.e., pMA762, pMA764, pMA768, are shown in Fig. 5A (lanes d, e, and g). Examination of the coordinates of these deletions indicates that an essential part of the *PGK* promoter is centered around the regions deleted in pMA760, pMA766, pMA767, and pMA768 (i.e., pMA511 to pMA377 [Fig. 3]). It seems likely that these sequences harbor a UAS for *PGK*, which is designated UAS_{PGK}.

An important observation for class 1 deletions is that they produce substantial levels of *PGK* mRNA in the absence of the UAS_{PGK}. Most notable are deletions pMA764 (−511 to −134) and pMA765 (−511 to −91) which remove large regions of the *PGK* promoter, including the UAS_{PGK}, and yet retain considerable levels of transcription (Fig. 3 and 5A, lane e). Thus, there must be additional sequences outside the range of our deletion series, either upstream of −595 (the most 5′ deletion endpoint) or downstream of −91 (the most 3′ endpoint), that can direct transcription (see Discussion).

Class 2 deletions. The *PGK* mRNA levels of transformants containing class 2 deletions were indistinguishable from those of T27 (Fig. 5A, lanes b, c, h, and i), indicating that the deleted sequences are not required for high levels of *PGK* expression. We found that up to 311 bp of the *PGK* promoter could be deleted without affecting transcriptional levels. All the deletions of this type retain the UAS_{PGK}, as defined above.

Three main observations can be made from class 2 deletions. First, the precise location and extent of the UAS_{PGK} is confirmed by data for pMA779 (−538 to −479) and deletions which have −402 as their 5′ endpoint (i.e., pMA769, pMA770, pMA771, pMA772, pMA783 [Fig. 3]). These transformants have T27 mRNA levels and have deletions located on each side of the pMA766 window which abolishes UAS_{PGK} activity. Thus, the 3′ endpoint of the pMA779 window (−479) and the 5′ endpoint of windows pMA769 to pMA772 and pMA783 (−402) delimit the 5′ and 3′ boundaries of the UAS_{PGK}; respectively. This confirms that the UAS_{PGK} is located within a 77-bp region 402 to 479 bp upstream from the ATG.

Second, the distance between the UAS_{PGK} and the *PGK* coding region can be reduced by removing from 13 bp (pMA769) to 311 bp (pMA783) of DNA, thus bringing the UAS_{PGK} closer to the transcription initiation site without having any deleterious effects on the levels of transcription (i.e., deletions pMA769 to pMA777 and pMA783).

Third, deletion of one (pMA773, pMA774, pMA777) or both (pMA775) of the potential TATA sequences, TATATATAAA (−154) and TACATA (−117), which were previously identified from sequence data (11), had no effect on the levels of *PGK* transcription (Fig. 3 and 5A, lane i). We also found that deletion of the CAAT sequence at −129 (pMA776), which bears some resemblance to the CAAT box of higher eucaryotes (11), does not affect *PGK* transcription (Fig. 3).

Accurate initiation of *PGK* mRNA does not require TATA sequences. The results from the class 2 deletions described above show that the two potential TATA sequences in the *PGK* promoter (TATATATAAA at −154 and TACATA at −117) are not required for maintaining high levels of transcription. Since TATA sequences have been shown to be involved in setting the mRNA initiation site in yeasts and in higher eucaryotes, we investigated the role of the two potential TATA sequences of *PGK* in transcription initiation. It should be noted that the only other sequence in the *PGK* 5′ region (i.e., between −40 and −820) that resembles the TATA consensus is located at −739. As this sequence is located so far upstream from the *PGK* coding region, we doubt that it plays a role in transcription initiation.

The mRNA start sites of a number of *PGK* promoter deletions and of T27 and the untransformed strain MD40/4c were determined by the riboprobe RNase mapping proce-

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-820
*
TTAATTTTTT TTTCTTTCCT CTTTTTATTA ACCTTAATTT TTATTTTAGA

-770
*
TTCCTGACTT CAACTCAAGA CGCACAGATA TTATAACATC TGCATAATAG

-720
*
GCATTTGCAA GAATTACTCG TGAGTAAGGA AAGAGTGAGG AACTATCGCA

-670
*
TACCTGCATT TAAAGATGCC GATTTGGGCG CGAATCCTTT ATTTTGGCTT

-620
*
CACCCTCATA CTATTATCAG GGCCAGAAAA AGGAAGTGTT TCCCTCCTTC

-570
*
TTGAATTGAT GTTACCCTCA TAAAGCACGT GGCCTCTTAT CGAGAAAGAA

-520
*
ATTACCGTCG CTCGTGATTT GTTTGCAAAA AGAACAAAAC TGAAAAAACC

-470
*
CAGACACGCT CGACTTCCTG TCTTCCTATT GATTGCAGCT TCCAATTTCG

-420
*
TCACACAACA AGGTCCTAGC GACGGCTCAC AGGTTTTGTA ACAAGCAATC

-370
*
GAAGGTTCTG GAATGGCGGG AAAGGGTTTA GTACCACATG CTATGATGCC

-320
*
CACTGTGATC TCCAGAGCAA AGTTCGTTCG ATCGTACTGT TACTCTCTCT

-270
*
CTTTCAAACA GAATTGTCCG AATCGTGTGA CAACAACAGC CTGTTCTCAC

-220
*
ACACTCTTTT CTTCTAACCA AGGGGGTGGT TTAGTTTAGT AGAACCTCGT

-170
*
GAAACTTACA TTTACATATA TATAAACTTG CATAAATTGG TCAATGCAAG

-120
*
AAATACATAT TTGGTCTTTT CTAATTCGTA GTTTTTCAAG TTCTTAGATG

-70
*
CTTTCTTTTT CTCTTTTTTA CAGATCATCA AGGAAGTAAT TATCTACTTT

-20
*
TTACAACAAA TATAAAAACA ATG TCT TTA TCT TCA AAG TTG TCT GTC CAA
                    MET SER LEU SER SER LYS LEU SER VAL GLN
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UAS PGK

FIG. 4. Nucleotide sequence of the 5′ noncoding region of *PGK*. The sequencing of the *PGK* gene has been described previously (11). Potential TATA sequences are underlined. Arrows mark the region containing the UAS_{PGK}.

dure. The internal deletions used are shown in Table 1. The results (Fig. 6) show that all the transformants have the same mRNA initiation site and that this does not differ from the initiation site of T27 or MD40/4c. This demonstrates that deletion of either or both of the potential TATA sequences, with or without the UAS_{PGK}, has no effect on transcription initiation in *PGK*. The size of the protected fragments agrees with previously published data except that with this tech-

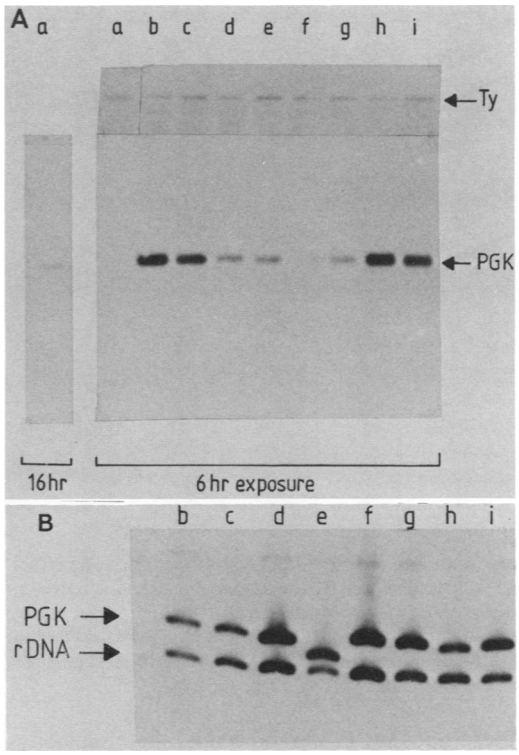

FIG. 5. Effects of promoter deletions on *PGK* transcription. (A) Total RNA (10 μg) from transformants was analyzed by Northern blotting. Northern filters were probed with *PGK*- and Ty-specific fragments. The 5.7-kilobase Ty transcript was used as an internal loading control. Levels of *PGK* mRNA (1.45 kilobases) were quantitated by densitometer tracing and adjusted when necessary to allow for variation in the Ty loading control. *PGK* mRNA levels of the deletions were determined relative to the level of T27, the wild-type *PGK* gene on a high-copy-number plasmid, which was given an abitrary value of 50. Lanes and relative *PGK* mRNA values (in parentheses) are: a, MD40/4c (not detectable); b, T27 (50.0); c, T778 (47.0); d, T762 (11.1); e, T764 (10.8); f, T766 (3.8); g, T768 (9.4); h, T772 (49.4); i, T774 (45.2). (B) Copy number of *PGK* 5′ deletion plasmids was determined by Southern blot analysis of *Eco*RI-digested total DNA from transformants. Southern filters were probed with *PGK*- and rDNA-specific fragments labeled to the same specific activity. The relative intensity of plasmid (*PGK*) to rDNA bands gave an estimate of plasmid copy number. The fainter uppermost band on the gel is chromosomal *PGK*. Lanes are as for the Northern blot in panel A except there is no MD40/4c lane on the Southern blot.

TABLE 1. Internal deletions used for mRNA start site mapping

Plasmid pMA no.	Sequences present		
	UAS_{PGK}	TATATAA[a]	TACATA[b]
764	−	−	+
765	−	−	−
774	+	−	+
775	+	−	−
776[c]	+	+	+
777	+	+	−
27[d]	+	+	+
MD40/4c[d]	+	+	+

[a] Starts at −154.
[b] Starts at −117.
[c] Only deletes CAAT sequence.
[d] pMA27 and MD40/4c represent the undeleted promoter.

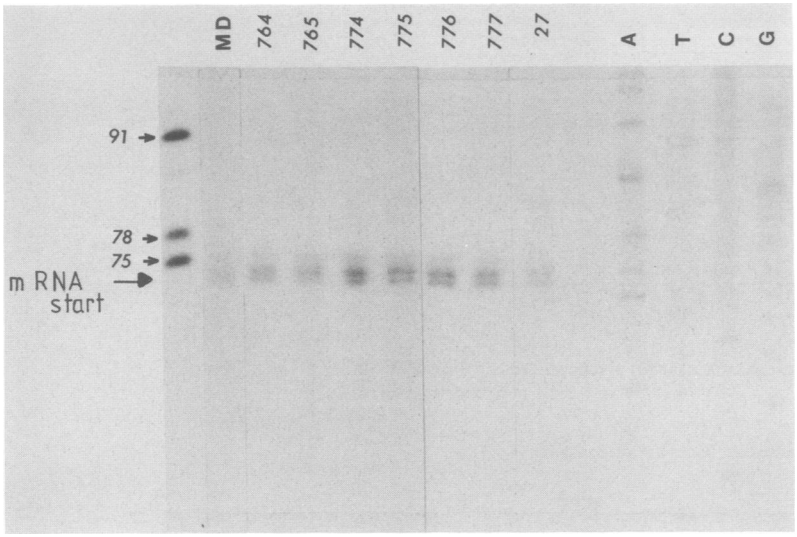

FIG. 6. Mapping of the *PGK* mRNA start site. The effect of deletion of one or both of the potential TATA sequences, with or without the UAS_{PGK}, was determined by the riboprobe mapping technique. Protected fragments were resolved on a polyacrylamide–8 M urea sequencing gel. Lanes from left are: end-labeled pBR322 digested with *Sau*3A (DNA fragment sizes are given in base pairs); MD40/4c and deletions as labeled; ladder of known DNA sequence. The MD40/4c lane is a 3-day exposure; all other lanes are overnight exposures. Coordinates and properties of deletions analyzed for *PGK* mRNA start site can be found in Table 1.

nique there appears to be some microheterogeneity in the mRNA start (Fig. 6).

UAS_{PGK} can activate transcription when inserted in pMA766. To verify that sequences between −402 and −479 harbored the UAS_{PGK}, we inserted a 162-bp fragment containing sequences from −402 to −538 into the *Bam*HI site of pMA766 in both orientations (see Materials and Methods).

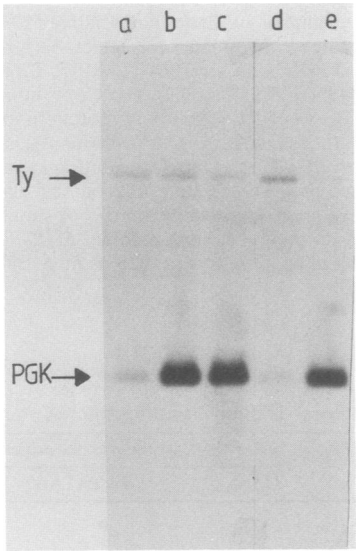

FIG. 7. *PGK* mRNA levels of deletion pMA766 containing the UAS_{PGK} in both orientations. The UAS_{PGK} was isolated on a 161-bp fragment and inserted into the pMA766 window in the correct (pMA766-UAS) and in the inverted (pMA766-UAS_{INV}) orientation. RNA from transformants was analyzed and quantitated as described in the legend to Fig. 5. Lanes and *PGK* mRNA levels are: a, T766 (5.0); b, T766-UAS_{INV} (43.7); c, T766-UAS (45.2); d, MD40/4c (1.5); e, T27 (50.0). The slight overloading of the MD40/4c lane and underloading of the T27 lane were taken into account when quantitating *PGK* mRNA levels.

This gave plasmids pMA766-UAS and pMA766-UAS_{INV}. Northern analysis of RNA from transformants containing these plasmids (T766-UAS and T766-UAS_{INV}) showed that the *PGK* mRNA levels were comparable with those of T27 (Fig. 7, lanes b, c, and e). Plasmid copy numbers in T766-UAS and T766-UAS_{INV} were the same as in T27 (data not shown). These results confirm the location of the UAS_{PGK} and demonstrate that the activating sequences can still function efficiently when inverted relative to the *PGK* coding region.

UAS_{PGK} can activate transcription of a *TRP1-IFN* fusion gene. One of the features of yeast UASs is that they can activate transcription of other yeast genes when correctly positioned (15, 20, 45, 46). To determine whether this is also true for the UAS_{PGK}, we examined its effects on transcription of *TRP1-IFN* gene fusions (27a). The *TRP1* promoter generates two groups of mRNA species, designated transcript I and transcript II, which have separate control domains and which differ in size by approximately 150 bp, transcript I being the larger of the two (27a).

The effects of the UAS_{PGK} on transcription of *TRP1* were examined by inserting the UAS_{PGK} fragment upstream of two *TRP1-IFN* gene fusions and by assessing levels of transcription by Northern analysis (Fig. 8). Yeast transformants containing pMA1557-10, which has the entire *TRP1* promoter, produced transcripts I and II in a ratio of 1:2. Insertion of the UAS_{PGK} upstream of the positive control element for the *TRP1* transcript I (i.e., in pMA1610-10 [Fig. 2]) resulted in an increase in the levels of transcript I so that the transcript I/transcript II ratio increased from 1:2 to 5:1 (Fig. 8, lanes a and b). It is interesting to note that an increase in levels of transcript I was accompanied by a marked reduction in levels of transcript II such that the total level of transcription (i.e., transcript I and II) remained the same. This reciprocal exchange of *TRP1* transcripts has been observed previously (27a).

Similar results were obtained when the UAS_{PGK} was inserted into pMA1557-16. In T1557-16 strains the levels of transcript I were approximately 30% less than those pro-

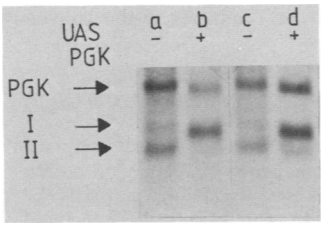

FIG. 8. Effect of the UAS_{PGK} inserted in the *TRP1* promoter. The UAS_{PGK} was inserted upstream of two different *TRP1-IFN* gene fusions (see text); pMA1557-10 has the entire *TRP1* promoter, while pMA1557-16 has a deletion removing the positive control element for the large *TRP1* transcript. Insertion of the UAS_{PGK} upstream of the two gene fusions gave pMA1610-10 and pMA1610-16, respectively. RNA from transformants was anlayzed by Northern blotting. The filter was probed with *IFN*- and *PGK*-specific fragments; chromosomal *PGK* mRNA was used as the internal loading control. I and II are transcripts I and II, the two major transcript groups directed by the *TRP1* promoter. Levels of transcripts I and II were quantitated by densitometer tracing and adjusted relative to the level of *PGK* mRNA and then to the level of transcript I of T1557-10 (wild-type promoter) which was given an arbitrary value of 1. The corrected ratios of transcript I/transcript II are given in parentheses. Lanes: a, T1557-10 (without UAS_{PGK}) (1:2.1); b, T1610-10 (with UAS_{PGK}) (4.8:0.9); c, T1557-16 (without UAS_{PGK}) (0.3:1.9); d, T1610-16 (with UAS_{PGK}) (3.8:1).

duced by the nondeleted promoter. Insertion of the UAS_{PGK} in place of the transcript I positive element (i.e., in pMA1610-16 [Fig. 2]) resulted in an increase in levels of transcript I, altering the transcript I/transcript II ratio to approximately 4:1 (Fig. 8, lanes c and d). Southern blot analysis of total DNA from the four *TRP1-IFN* transformants showed that there were no differences in plasmid copy number (data not shown).

In summary, the data for pMA1610-10 and pMA1610-16 demonstrate that the UAS_{PGK} can enhance the transcriptional levels of a heterologous gene.

DISCUSSION

This paper describes an internal deletion analysis of the 5′ noncoding region of the yeast *PGK* gene which reveals a number of interesting features in the promoter. Maximal expression of *PGK* requires a UAS which is orientation independent and which can enhance transcription of a heterologous gene. We also showed that the *PGK* gene has no requirement for TATA sequences either for maintaining levels of transcription or for the accuracy of mRNA initiation.

The UAS of the *PGK* gene (UAS_{PGK}) is located within a 77-bp region ca. 402 to 479 bp upstream from the initiating ATG sequence. Insertion of a fragment containing sequences from −402 to −538 into the pMA766 window restored transcriptional levels to those of the intact promoter, confirming that this region is necessary for maximum transcriptional activity. The functional extent of the UAS_{PGK} is determined by deletions which are located on each side of the region essential for maximum transcription (defined by pMA760 to pMA762 and pMA766 to pMA768) but which do not affect *PGK* transcriptional levels themselves (i.e., upstream by deletion pMA779 and downstream by deletions which have −402 as their 5′ endpoint [Fig. 3]). It is important to note that deletions removing the UAS_{PGK} (class 1 deletions) did not abolish transcription completely but in fact retained significant levels of mRNA (Fig. 5). This indicates that *PGK* must contain an additional element that can direct transcription. A second positive element must be located outside the range of our deletion series, i.e., upstream of −595 or downstream of −91. We have no direct evidence to suggest the precise location of a second activating element. However, data from our one-sided deletion series demonstrate that sequences upstream of −620 are not required for maintaining high levels of *PGK* transcription. Furthermore, a one-sided deletion extending to −350, although causing a marked reduction in transcription, had *PGK* mRNA levels that were significantly higher than those of chromosomal *PGK*. This points to sequences downstream of −91 harboring the second positive element. Two possible candidates for an element of this type are either the sequences in the region of the mRNA initiation site or sequences within the *PGK* coding region. Previous work in this laboratory indicates that the *PGK* coding region does contain an element that directs transcription (31; J. Mellor, M. J. Dobson, N. A. Roberts, A. Kingsman, and S. Kingsman, manuscript in preparation). However, at this stage we cannot rule out the possibility that sequences around the transcriptional start influence the frequency of mRNA initiation.

It should be noted that two deletions, pMA766 and pMA767, gave significantly lower levels of *PGK* mRNA than other class 1 deletions (represented by +/− in Fig. 3; Fig. 5, lane f). This is perhaps surprising in view of their similarlity to other class 1 deletions; for example, pMA768 and pMA760, which remove the same essential region of the promoter in addition to a further 12 bp downstream from the 3′ endpoint of pMA767 and 28 bp upstream from the 5′ endpoints of both pMA766 and pMA767, respectively (Fig. 3). There appear to be two possible explanations for this observation. First, the novel arrangement of sequences caused by the juxtaposition of deletion endpoints in pMA766 and pMA767 might create an unsuitable promoter environment thereby reducing levels of transcription. Second, the anomalous behavior of the two constructions may point to a more complex set of promoter control elements. If the sequences flanking the pMA766 and pMA767 windows contained a negative element we would expect to see an increase in transcription when these regions are deleted. Indeed, this is the case for deletions pMA760 to pMA765 and pMA768 which delete regions flanking the pMA766 window and the UAS_{PGK}. However, the fact that deletions removing these regions but leaving the UAS_{PGK} intact (pMA778, pMA779, pMA769, to pMA773) have T27 levels and not increased levels of transcription argues against the existence of a negative element. Thus, we favor the former explanation, that of an unusual promoter configuration, to account for transcriptional levels of pMA766 and pMA767.

A most interesting feature of the *PGK* promoter is that it has no requirement for TATA sequences. Deletion of one or both of the potential TATA sequences (TACATA at −117 and TATATATAAA at −154) does not affect either the levels of transcription or the site of mRNA initiation. This observation is unique among yeast genes. Deletion of functional TATA elements in other yeast genes results in either a reduction in levels of transcription (16, 44) or alterations in the site of transcription initiation such that the mRNA start sites become dispersed along the promoter (16, 27a, 41). The observation that *PGK* has no requirement for TATA sequences is not only novel among yeast genes but is also in contrast to the majority of higher eucaryotic genes (1, 13; reviewed in reference 4) in which the importance of the TATA box has been reiterated on numerous occasions. However, it is interesting to note that a few higher eucary-

otic genes, including the human X-linked *PGK* gene (42), do not contain TATA sequences. Recent reports on a number of yeast genes demonstrate that the TATA box plays a functional, yet flexible, role in setting the transcriptional start and that sequences in the region of the mRNA initiation site itself determine the precise point of initiation (5, 17, 30, 35). In *PGK* the accuracy of transcription initiation must involve a different or altered mechanism. We presume that correct initiation depends on sequences downstream of our most 3′ deletion endpoint, i.e., −91; indeed, in *PGK* the mRNA initiation site may be all that is required for setting the transcriptional start.

The UAS_{PGK} shares several general characteristics with the UASs of other yeast genes. First, data for class 2 deletions show that the UAS_{PGK} can be brought closer to the transcriptional start without affecting the levels of transcription, as observed for other yeast genes (14, 16, 44, 54). We showed that the position of the UAS_{PGK} relative to the site of transcription initiation is remarkably flexible since the UAS_{PGK} functions as efficiently with only approximately 50 bp, compared with the normal 360 bp, upstream from the mRNA start site.

Second, the UAS_{PGK} functions as effectively when inverted. To date a number of UASs have been shown to function in an orientation-independent manner: the UAS_{GAL} of the *GAL10-GAL1* divergent promoter (45) and the UASs of *GAL7* (28), *CYC1* (15), and *SUC2* (40), although the last two function less efficiently when inverted. The orientation independence of yeast UASs shows that they bear some resemblance to the enhancer elements of higher eucaryotes (see reference 27 for a review).

A third property of the UAS_{PGK} that appears to be a general feature of UASs is the ability to activate transcription of a heterologous gene. The UAS_{PGK} enhances transcription of a *TRP1-IFN* gene fusion comprising the promoter of yeast *TRP1* fused to the human interferon α-2 coding region (27a). Insertion of the UAS_{PGK} into the *TRP1* promoter upstream of the *TRP1* promoter control elements (pMA1610-10) and also when substituted for one of the *TRP1* positive control elements (pMA1610-16) resulted in increased levels of the larger of the two transcripts (Fig. 8).

An interesting feature of the region containing the UAS_{PGK} is that it contains three repeats of the sequence CTTCC located between −427 and −467. These repeats are not arranged in tandem but are interspersed by 3 and 11 bp, respectively. A search of the entire *PGK* gene shows that the CTTCC sequence occurs only once outside this region and that is at +418 in the coding region. Recent studies have identified repeated or related sequences in the upstream control elements of two yeast genes, the UAS_{GAL} (3, 12) and *HIS3* (24), which are binding sites for regulatory proteins. It is tempting to speculate that repeated sequences (e.g., CTTCC) in the UAS_{PGK} are also involved in the recognition or binding of a regulatory protein. To date only one gene, *GCR*, is known to affect *PGK* transcription, with *gcr* mutant strains having reduced levels of the majority of the glycolytic enzymes, including PGK (7). The role of the *GCR* product in the regulation or control or both of *PGK* expression remains unclear. Further analysis of the UAS_{PGK} should reveal whether this sequence contains a regulatory protein-binding site and whether the CTTCC repeats are involved in this role.

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