The protein factor which binds to the upstream activating sequence of *Saccharomyces cerevisiae* ENO1 gene

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ABSTRACT
    Using a gel retardation assay it was shown that the 87 bp DNA fragment (UAS_{87}) containing the upstream activating sequence (UAS) of S. cerevisiae ENO1 gene and a nuclear extract gave rise to three migration-retarded species specific to UAS_{87}. Heat- or proteinase-treatment of the nuclear extract revealed that these species were protein-DNA complexes. The precise binding region of the protein identified by DNaseI protection analysis was found to include a CCAAACA sequence which forms a dyad-symmetrical structure. The amount of one of the three migration-retarded species significantly increased when cells were grown in medium containing a gluconeogenic carbon source. The introduction of pGCR8, a multicopy plasmid containing GCR1 gene, a regulatory gene controling the expression of several glycolytic enzymes, showed no effect on the amount of three migration-retarded species.

INTRODUCTION

    The ENO1 gene of the yeast Saccharomyces cerevisiae encodes enolase [2-phospho-D-glycerate hydrolyase, 4.2.1.11], which catalyzes the reversible conversion of 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway. There are two enolase structural genes per haploid genome of yeast, ENO1 and ENO2. Both genes are expressed during the vegetative cell growth, but they are differentially regulated in the transcriptional level (1). Uemura et al. found that the region about 300 bp upstream from the transcription initiation site is responsible for the enhancement of the expression of ENO1 gene (2), and identified the nucleotide segment essential for the correct control of expression of the ENO1 gene within the 87 bp fragment (3). Recently, we also reported that the transcriptional level of PHO5 gene whose upstream activating sequence (UAS) was replaced with the 87 bp segment of ENO1 gene was also enhanced independently of the presence of inorganic phosphate in the medium (4). These results indicate that the UAS region of ENO1 gene is able to enhance the transcription of yeast other genes.

    The UAS of several genes have been reported (5-12) and it has been shown that genes regulated in an similar manner contain conserved UASs, while the

genes regulated independently do not (6,13). Trans-acting genes responsible
for the UAS-dependent regulation have been identified by genetic analysis of
mutant strains which lack normal expression of regulated genes (14-17) and
recently it was shown that the products of the GAL4 (18) and GCN4 (19), the
regulatory genes responsible for the transcription of GAL genes and genes of
the amino acid biosynthetic enzymes under general control, bind to the UAS
region of GAL1-10 gene (20) and HIS3 gene, (21), respectively. The UASs of
genes regulated in an identical manner contain a homologous sequence
responsible for the binding of the regulatory gene product (21,22).

   Kawasaki and Fraenkel have reported the isolation of the GCR1 gene which is
thought to be the positive regulatory gene for the expression of glycolytic
enzymes in yeast cells (23). We found that the transcriptional level of ENO1-
lacZ fused gene integrated in yeast chromosome was increased about 2-fold in
yeast cells transformed with a multicopy plasmid containing GCR1 gene (4).
Recently, Cohen et al. reported that the ENO1-UAS was responsible for the
positive regulation of ENO1 expression by glucose and that the upstream
repression sequence (URS) responsible for the negative regulation of ENO1
expression was also present downstream of the UAS (24). Glucose induction of
ENO2-UAS dependent expression has been observed and the two enolase UASs shows
functional similarity (24) in spite of the apparent constitutive expression of
ENO1 gene. In this paper, we report the detection of the specific binding of
proteins to the ENO1-UAS and its precise binding site. The effect of glucose
as a carbon source on the amount of the UAS_{87}-specific binding factor was also
examined. The structural relationship between ENO1-UAS and ENO2-UAS are
discussed in relation to one possible mechanism responsible for the glucose
induction.

MATERIALS AND METHODS
Yeast strains and media
   The S. cerevisiae strain DBY746 (MATα, his3, leu2-3, leu2-112, trp1-289,
ura3-52) was used to prepare the nuclear extract. Plasmid pGCR8 was a gift
from Dr. Fraenkel (23). Yeast cells were grown in YPD medium or in synthetic
complete media lacking leucine, if necessary, and containing 2% glucose (SD
medium) or 2% glycerol plus 2% sodium lactate (SGL medium) as a carbon source.
Preparation of nuclear extract from Saccharomyces cerevisiae
   Preparation of the nuclear extract was carried out using a slightly
modified procedure described by Celniker and Campbell (25). Cells were grown

in 2 l of YPD medium to an A_{590} value of 6 and harvested by centrifugation. Cells were suspended in 50 ml of 100 mM tris-HCl (pH 8.0) containing 10 mM EDTA and 0.5% ß-mercaptoethanol, incubated on ice for 30 minutes, washed with 20 mM sodium phosphate buffer (pH 6.5) containing 1 M sorbitol and 1 mM phenylmethylsulfonyl fluoride (PMSF). After resuspension in 12 ml of the same buffer, cells were treated with 1 mg/ml Zymolyase 100T at $30^{0}C$ for 60 minutes. Spheroplasts were collected by centrifugation at 900 xg for 5 minutes and burst into 10 mM tris-HCl (pH 8.0) containing 1.5 mM $MgCl_2$, 15 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol (DTT) and 1 mM PMSF. Nuclei were collected by centrifugation at 12,000 xg for 15 minutes, suspended in 30 ml of the same buffer and burst by addition of 7.6 ml of 4 M ammonium sulfate. Debris was precipitated by centrifugation at 100,000 xg for 60 minutes and the supernatant was diluted with the same buffer to a whole volume of 48 ml. The crude nuclear extract was applied to a DE52 column (12 x 30 mm) equilibrated with the same buffer containing 0.3 M KCl. Solid ammonium sulfate was added to the fraction from the DE52 column to a final concentration of 60% saturation and incubated on ice for 30 minutes. The precipitate was collected by centrifugation, suspended with the same buffer and dialyzed against 50 mM tris-HCl (pH 8.0) containing 0.1 mM EDTA, 0.5 mM DTT, 50 mM ammonium sulfate and 10% glycerol. The nuclear extract was then frozen in liquid nitrogen and stored at $-80^{0}C$.

The nuclear extract for the analyses of the effect of the higher dose of the GCR1 gene and the effect of carbon source was prepared in the same way except that the cells were grown in SD or SGL medium and harvested at an A_{590} value of 1.

Preparation of plasmid DNA

Plasmid DNA was prepared from ampicillin-resistant E. coli by the method of Birnboim and Doly (26).

Preparation of oligonucleotides

Generally the oligonucleotides were prepared from enzymatically digested plasmids, separated on 8% polyacrylamide gel electrophoresis and eluated from the corresponding bands by electrophoresis. UAS_{87}, the 87 bp Sau3AI-SmaI fragment which contains the complete activity of ENO1-UAS (Fig. 1) was prepared from pHU38003 (3). ORF_{86}, the 86 bp Sau3AI-HincII fragment containing a part of the coding region of E.coli ß-lactamase (Fig. 1) was prepared from pBR322. UAS_{368}, the 368 bp EcoRI-AvaII fragment containing ENO1-UAS was prepared from pACYC184-ENO62 (2).
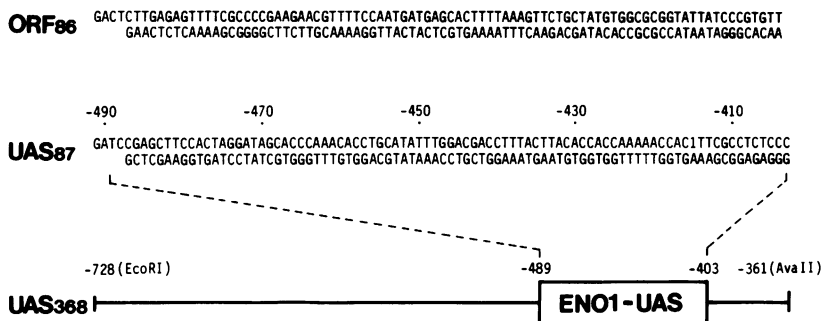
ORF86 GACTCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTGTT
        GAACTCTCAAAAGCGGGGCTTCTTGCAAAAGGTTACTACTCGTGAAAATTTCAAGACGATACACCGCGCCATAATAGGGCACAA

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          -490            -470            -450            -430            -410
           .               .               .               .               .
UAS87  GATCCGAGCTTCCACTAGGATAGCACCCAAACACCTGCATATTTGGACGACCTTTACTTACACCACCAAAAACCACITTCGCCTCTCCC
       GCTCGAAGGTGATCCTATCGTGGGTTTGTGGACGTATAAACCTGCTGGAAATGAATGTGGTGGTTTTTGGTGAAAGCGGAGAGGG
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       -728(EcoRI)                                    -489         -403   -361(AvaII)
UAS368  ├─────────────────────────────────────────┤  ENO1-UAS  ├─────────────────────┤
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Figure 1   Sequence of oligonucleotides used in the gel retardation assay and
DNaseI protection analysis. UAS87 (87 bp length of Sau3AI-SmaI fragment),
ORF86 (86 bp length of Sau3AI-HincII fragment), and UAS368 (368 bp length of
EcoRI-AvaII fragment) were prepared from pHU38003, pBR322 and pACYC184-ENO62,
respectively, by an enzymatic digestion followed by the recovery from a
polyacrylamide gel. UAS87 and UAS368 which contain the complete UAS region of
ENO1 gene are located from -489 to -403 and from -728 to -361, respectively,
when the translation initiation site of ENO1 gene defined as +1.

For gel retardation assays chemically synthesized UAS_{87} was also used.
Single stranded deoxyoligonucleotides of 16-19 bases were synthesized by DNA-
synthesizer (Applied Biosystems, 380A), and then phosphorylated by T4-
polynucleotide kinase. The synthetic deoxyoligonucleotides complementary to
each other were mixed, annealed and ligated by T4-DNA ligase to make a double
stranded DNA. The sequence of the ligated DNA was confirmed by dideoxy chain-
termination method of Sanger et al. (27) after cloning in M13-mp19 plasmid.

Gel retardation assay

Oligonucleotides (UAS_{87} or ORF_{86}) were 5'-labeled with $[\gamma-^{32}P]ATP$ by T4-
polynucleotide kinase or filled with dATP, dGTP, dTTP and $[\alpha-^{32}P]dCTP$ at
Sau3AI site by Klenow fragment to a specific activity of 0.1-0.2 nCi/fmol DNA.
^{32}P-Labeled oligonucleotide (20-50 fmol) was incubated for 15 minutes at
$30\,^{\circ}C$ together with the nuclear extract (0.3-3 μg protein) in 20 μl of 25 mM
sodium N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate (Hepes) buffer (pH 7.5)
containing 1 mM EDTA, 5 mM DTT, 0.5 mM PMSF, 100 mM NaCl and 10% glycerol.
The reaction mixture was subsequently subjected to electrophoresis on 8%
polyacrylamide gel (acrylamide/bisacrylamide = 29:1) in 50 mM tris-384 mM
glycine buffer (pH 8.5) containing 4 mM EDTA. Following electrophoresis, gels
were briefly soaked in 5% glycerol, dried and autoradiographed.

DNaseI protection analysis

Oligonucleotide (UAS_{368}) was labeled with $[\alpha-^{32}P]dGTP$ at AvaII site by

Klenow fragment to a specific activity of about 1 nCi/fmol. ^{32}P-labeled oligonucleotide (1-3 nCi) was incubated with the nuclear extract (10-150 µg protein) for 30 minutes at 25°C in 50 ul of 25 mM sodium Hepes buffer (pH7.8) containing 0.05 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 50 mM KCl and 5% glycerol, and after the addition of 2 µl of 0.1 M MgCl$_2$, the cleavage reaction was performed by 0.3-3 units of DNaseI for 60 seconds at room temperature. The reaction mixture was treated with 100 µl of 100 mM tris-HCl buffer (pH 8.0) containing 1% sarkosyl, 10 mM EDTA, 100 µg/ml proteinase K, 25 µg/µl salmon testes DNA and 100 mM NaCl, incubated for 15 minutes at 37°C and heated for 2 minutes at 95°C. After phenol extraction, DNA was precipitated with ethanol, dried, and treated at 95°C for 1 minute in 0.1 x TBE buffer (1 x TBE buffer ; 89 mM Tris, 89 mM borate, 2 mM EDTA) containing 80% formamide, 0.15% xylene cyanol and 0.15% bromophenol blue and quickly chilled on ice. The denatured DNA was electrophoresed on 6% polyacrylamide gel in 1 x TBE buffer containing 6 M urea. The gels were dried and autoradiographed.

RESULTS

Detection of the protein factor(s) specifically bound to UAS of ENO1 gene

^{32}P-Labeled oligonucleotides were mixed with the nuclear extract from S. cerevisiae and electrophoresed on 8% polyacrylamide gels. Using ^{32}P-labeled UAS$_{87}$, an 87 bp fragment containing the complete ENO1-UAS, several slow-migrating species were detected in the presence of plasmid DNA pACYC184 as a nonspecific competitor. Of eight species three slow-migrating species (S1-S3) were judged to be specific to UAS$_{87}$ as seen by comparing the migration patterns obtained from UAS$_{87}$ and ORF$_{86}$ (Fig. 2A). All of the specific and nonspecific species disappeared when the nuclear extract was heat- and/or Proteinase K-treated before mixing with the labeled DNA (data not shown), indicating that the slow-migrating species were formed by the binding of protein factors to DNA.

Figure 2B shows the inhibition of the migration-retardation with the unlabeled oligonucleotides. The three specific species disappeared by the addition of increasing amount of unlabeled UAS$_{87}$ whereas the addition of unlabeled ORF$_{86}$ had no effect. These results confirm the sequence specific binding of the three species, S1-S3. Among the nonspecific species (n1-n5), n3 also disappeared by the addition of unlabeled UAS$_{87}$ and not by the addition of unlabeled ORF$_{86}$, while, in contrast, the nonspecific species, n4 and n5, disappeared by the addition of ORF$_{86}$ and not by the addition of UAS$_{87}$. Probably because the oligonucleotides, UAS$_{87}$ and ORF$_{86}$, are so short and
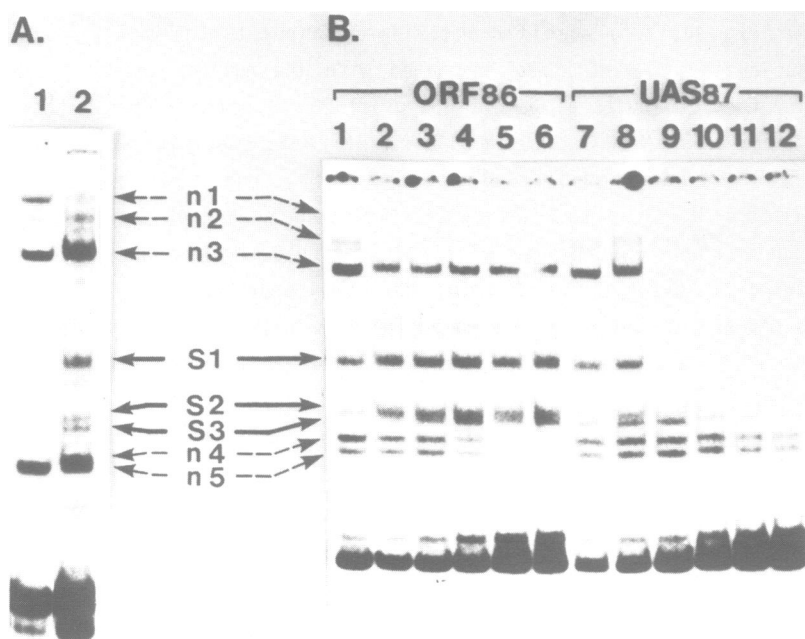
<u>Figure 2</u>   Detection of the binding factor specific to UAS_{87} and competition of the migration patterns with unlabeled oligonucleotides. (A) 20 fmol of the oligonucleotide ORF_{86} (lane 1) or UAS_{87} (lane 2) labeled with $[\alpha-^{32}P]dCTP$ by the filling with Klenow fragment was incubated with the nuclear extract containing 3 μg of protein in the presence of 200 ng of pACYC184 and electrophoresed on an 8% polyacrylamide native gel. Radioactive bands were identified by autoradiography. The positions of the UAS_{87}-specific species (S1-S3) and nonspecific species (n1-n5) are indicated by arrows. (B) Each assay was performed with 50 fmol of UAS_{87} 5'-labeled with $[\gamma-^{32}P]ATP$ by T4-polynucleotide kinase and with the nuclear extract containing 0.9 μg of protein. Increasing amounts of unlabeled ORF_{86} or unlabeled UAS_{87} were added before the addition of labeled UAS_{87}. Lanes 1 to 6 represent the addition of unlabeled ORF_{86}; the relative molar ratio of unlabeled ORF_{86} to labeled UAS_{87} was 0, 0.07, 0.2, 0.7, 2 and 7, respectively. Lanes 7 to 12 represent addition of unlabeled UAS_{87}; the relative molar ratio of unlabeled UAS_{87} to labeled UAS_{87} was equivalent to lanes 1 to 6.

consequently do not represent a random sequence, some nonspecific binding factor may prefer to bind to either of the two oligonucleotides.

The oligonucleotides labeled by filling with Klenow fragment were used for the comparison between two migration patterns obtained from UAS_{87} and ORF_{86} (Fig. 2A). The oligonucleotides labeled by T4-polynucleotide kinase were also used in the other gel retardation assays (Fig. 2B). No remarkable changes of the migration patterns were observed by two different labeling methods (Fig.
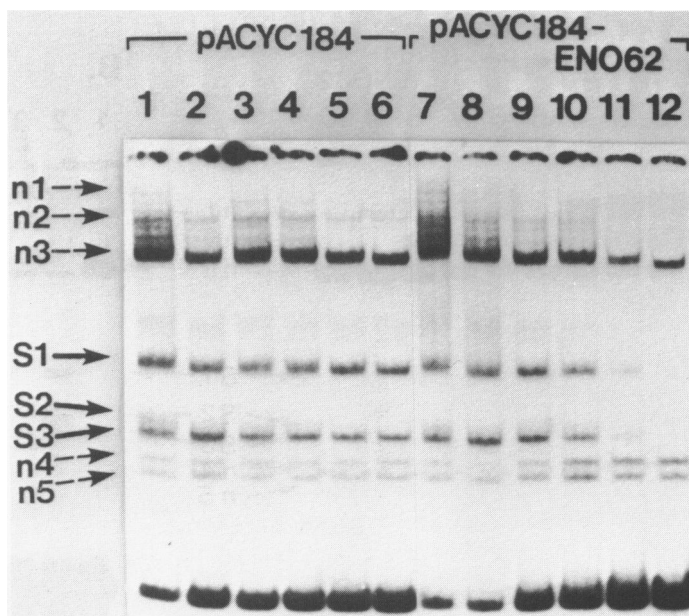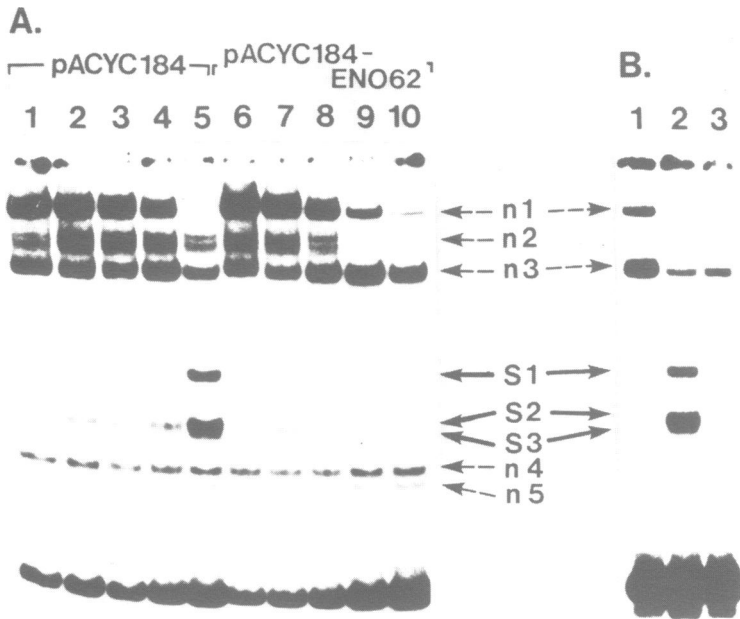
Figure 3   Competition of the migration patterns with plasmid DNAs either containing the ENO1 promoter region or not.   The gel retardation assay was performed with 50 fmol of 5'-labeled UAS_{87} and the nuclear extract containing 0.9 μg of protein.   Plasmid DNA was added before the addition of labeled UAS_{87}.   Lanes 1 to 6 represent the addition of 0, 2, 5, 10, 20 and 50 ng of pACYC184, respectively, and lanes 7 to 12 represent the addition of the equivalent amount of pACYC184-ENO62.

2), suggesting that the terminal end structure of DNA has no effect on protein binding.

A further competition experiment with closed circular plasmid DNAs, pACYC184 and pACYC184-ENO62, was carried out to confirm the specificity of DNA binding.   pACYC184-ENO62 harboring a 1.6 kb fragment of ENO1 gene with the complete promoter sequence might exert a stronger and more specific competition than UAS_{87} since the ENO1-UAS is present in a complete promoter region and thus may form three-dimensional structure, not displayed by a short DNA fragment.   As shown in Fig. 3, 50 ng of pACYC184-ENO62 effectively inhibited the formation of three slow-migrating species specific to UAS_{87} (S1-S3) but the same amount of pACYC184 did not.   On the other hand, the addition of either pACYC184 or pACYC184-ENO62 had almost no effect on the formation of the other major species (n3-n5).   These results indicate that the three slow-migrating species (S1-S3), are produced by the specific binding of factors to

Figure 4 Effect of the migration patterns with plasmid DNAs in the presence of 250 mM NaCl or in the presence of 100 mM NaCl plus 15 mM $MgCl_2$. Each assay was performed with 20 fmol of 5'-labeled UAS_{87} and nuclear extract containing 0.3 µg of protein. (A) Binding reaction was carried out in the presence of 250 mM NaCl and plasmid DNA was added before the addition of labeled UAS_{87}. Lanes 1 to 5 represent the addition of 0, 2, 20, 60 and 200 ng of pACYC184, respectively. Lanes 6 to 10 represent the addition of the equivalent amount of pACYC184-ENO62. (B) Binding reaction was carried out in the presence of 100 mM NaCl and 15 mM $MgCl_2$. Lanes 1 did not contain any additional plasmid DNA in the binding reaction while lanes 2 and 3 represent the addition of 200 ng of pACYC184 and the same amount of pACYC184-ENO62, respectively.

the ENO1-UAS and confirm the nonspecificity of the other major species (n3-n5). The amounts of pACYC184-ENO62 and UAS_{87} for the effective competition were similar (15-20 fmol) while the total length of plasmid DNAs was about 60 times longer than that of UAS_{87}. Therefore, it would seem that the regions around the UAS and a closed circular structure of DNA did not affect the binding of the specific factor although nonspecific factors seem to have a lower affinity to closed circular DNA than to linear DNA.

The effects of NaCl and $MgCl_2$ on the binding of the specific factors to UAS

In the presence of higher concentrations of NaCl or $MgCl_2$ all specific species disappeared and the intensity of the slower-migrating species, n1, was remarkably enhanced (Fig. 4, lane 1 and 6). Since the slower-migrating

species, n1, was also observed at the same position when labeled ORF_{86} was used (Fig. 2A). This species, n1, is therefore nonspecific to ENO1-UAS. However, since the appearance of the slower-migrating species seems to increase as the three specific species (S1-S3) decrease, it may be possible that the high concentration of NaCl or $MgCl_2$ causes some conformational change of specific binding factors that results in a change of migration. Alternatively, it is possible that the formation of specific protein-DNA complexes was inhibited by the binding of nonspecific DNA-binding proteins like histones whose binding increased at high salt concentrations (28). Thus, a competition experiment in the presence of either 250 mM NaCl or 100 mM NaCl plus 15 mM $MgCl_2$ was carried out with the plasmid DNAs which were found to exert more specific competition and were expected to have a larger capacity for the binding of nonspecific-proteins than fragmented DNA .

Since increasing amounts of plasmid DNA effectively inhibited the formation of the slower-migrating species, n1, even in the absence of the ENO1 gene (Fig. 4A), the slower-migrating species, n1, is clearly not specific to ENO1-UAS as suggested earlier.   The three migration-retarded species specific to UAS_{87} (S1-S3) were just detectable in the presence of 2-60 ng of pACYC184 or pACYC184-ENO62.   However, with 200 ng of pACYC184 increased these three specific species but they almost disappeared when 200 ng of pACYC184-ENO62 was used.   In the presence of 100 mM NaCl and 15 mM $MgCl_2$, only two slower-migrating species, n1 and n3, were observed and the specific species (S1-S3) were not; again the addition of 200 ng of pACYC184 stimulated the formation of the specific species (S1-S3) while the addition of pACYC184-ENO62 did not (Fig. 4B).   These results indicate that the presence of large amount of nonspecific DNA-binding factors either in high salt concentration or in the presence of $MgCl_2$ makes it difficult to detect the specific DNA-binding factor, and suggest that a tight binding of the nonspecific factor to the competitor DNA resulted in the decrease of the interference against the binding of the specific factor.   Furthermore, the magnesium ion may stabilize the binding activity or the conformation of the DNA-binding proteins since the migration pattern was simplified by the disappearance of the faintly visible species in the presence of 15 mM $MgCl_2$ (Fig. 4B).

Determination of the DNA binding site of the ENO1-UAS specific protein

The 368 bp DNA fragment containing the UAS region of ENO1 gene was end-labeled with $[\alpha-^{32}P]dGTP$ at AvaII site, and treated with nuclear extract under similar conditions as the gel retardation assay.   After brief DNaseI digestion, the protection pattern against DNaseI was analyzed by gel
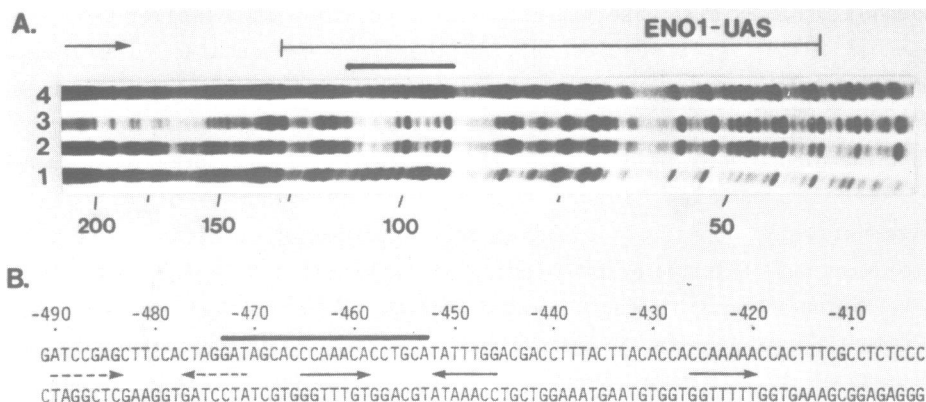
Figure 5 Binding site of nuclear factor on ENO1-UAS. (A) DNaseI protection analysis was performed as described in MATERIALS AND METHODS. Lane 1 represents the absence of the nuclear extract while lanes 2 to 4 represent the presence of the nuclear extract. Fragmented salmon testes DNA (lanes 1 and 2), pACYC184 (lane 3) or pACYC184-ENO62 (lane 4) was added to the reaction mixture before the addition of labeled oligonucleotide. Thin line represents the region corresponding the ENO1-UAS region and solid black line indicates the protected region against DNaseI digestion. DNA lengths in nucleotides indicated blow are based on the pattern of products of sequencing reaction run in the same gel. The arrow denotes the direction of electrophoresis. (B) The binding site of the nuclear factor to ENO1-UAS is shown. The sequence data of ENO1-UAS are from Uemura et al. (3). Solid black line indicates the protected region against DNaseI digestion. The arrows and broken arrows denote the two dyad-symmetrical sequences, and the homologous direct repeat, respectively.

electrophoresis under denaturing conditions. In the presence of sheared salmon testes DNA or pACYC184 as a nonspecific competitor, the region from 92 bp to 112 bp away from AvaII site was protected against DNaseI digestion (Fig. 5A, lane 2 and 3) and the protected region was entirely contained in ENO1-UAS, i.e. in UAS_{87} segment (Fig. 5B). The region protected against DNaseI digestion almost disappeared in the presence of pACYC184-ENO62 but not in the presence of pACYC184 when the ratios of plasmid DNA to nuclear extract were similar to those used in the gel retardation assay. Considering the similarity of the competition effects by plasmid DNAs observed in the DNaseI protection analysis and in the gel retardation assay, it is very likely that the binding factor(s) detected by DNaseI protection analysis was identical to that detected by gel retardation assay. The 21 bp protected against DNaseI digestion is a reasonable length to be protected by a single protein.

Effects of GCR1 gene and glucose on the amount of the UAS_{87}-specific binding factor

It has been reported that the expression of ENO1 gene is regulated by

Table I   Effects of <u>GCR1</u> gene and carbon source on the amount of UAS_{87}-specific binding factors.

	Glucose		Glycerol+Lactate	
	pGCR8(-)	pGCR8(+)	pGCR8(-)	pGCR8(+)
S1	0.09	0.07	0.48	0.43
S2+S3	0.91	0.89	1.06	1.45
Total	1.00	0.96	1.54	1.88

Cells were grown in a synthetic complete medium containing either 2% glucose or 2% glycerol plus 2% sodium lactate as a carbon source. The preparation of the nuclear extract from the cell and the assay condition for gel retardation were described in MATERIALS AND METHODS. The binding reaction in the gel retardation assay was performed in the presence of 100 mM NaCl, 15 mM $MgCl_2$, 50 ng pACYC184 and nuclear extract (1.5-2.0 µg protein). The amounts of the migration-retarded species were measured by densitometric method and normalized by the amount of total protein in nuclear extract. The migration-retarded species, S1-S3, were referred to Fig.2-5. Since S2 and S3 species were too closely migrated to be measured separately, the sum of the amounts of S2 and S3 species are shown. The total amount of the three specific species (S1-S3) is shown by "Total". The relative values are expressed as compared with the amount of the total specific species in nuclear extract obtained from DBY746 cells which did not contain a plasmid pGCR8 and were grown on glucose.

trans-acting <u>GCR1</u> gene (4) and that the <u>ENO1</u>-UAS dependent expression is controlled by glucose in the medium (24). Therefore, we examined whether the UAS-specific binding factor identified by gel retardation assay were associated with these regulations or not.

S. cerevisiae, DBY746 and DBY746 harboring a multicopy plasmid carrying GCR1 gene, pGCR8, were grown in SD or SGL medium and the nuclear extracts were prepared from each strain. The UAS_{87}-specific binding was analyzed by gel retardation assay in the presence of 100 mM NaCl plus 15 mM $MgCl_2$, then the amount of the UAS_{87}-specific species were measured by densitometric method. The UAS_{87}-specific binding activity was normalized against total proteins in the nuclear extract.   Table I shows the effects of both the introduction of pGCR8 and a carbon source (glucose or glycerol plus lactate) on the amount of the UAS-specific binding activities.   The presence of pGCR8 in host cells showed no significant effects on the amount of the UAS_{87}-specific protein whether the cells were grown on glucose or glycerol plus lactate (Table I). No effects of pGCR8 were also observed on the overall migrating pattern in the gel retardation assay (data not shown).   These results indicate that the enhancement of <u>ENO1</u> gene expression by <u>GCR1</u> gene is not mediated by the UAS-

specific binding factor. On the other hand, the amount of the specific species, S1, significantly increased when cells were grown on glycerol plus lactate as compared with that grown on glucose (Table I). This agrees with the result that ENO1-UAS dependent expression is enhanced by glucose in the medium (24).

DISCUSSION

   Several sequences of the UAS region of yeast genes have been reported (5-12), and sequences crucial for the binding of nuclear factor have been identified in the case of GAL gene family, HIS3 gene and PGK gene by DNaseI protection analysis (20,21,29). Dyad-symmetrical structures and/or directly repeated sequences are found in all UASs of yeast gene reported so far (5-12), and it has been noted that the dyad-symmetrical structures and directly repeated sequences are essential for the binding of GAL4 protein (22) and GCN4 protein (21), respectively. The DNaseI protection analysis revealed that the binding factor specific to ENO1-UAS binds to one of the two dyad-symmetrical structure (Fig. 5B). It is possible that the nuclear factor recognizes the CCAAACA sequence positioned at -465 to -459 because the sequence is able to form a stem-and-loop structure with the TATTTGG sequence positioned at -452 to -446 and is also able to form a homologous direct repeat with the CCAAAAA sequence positioned at -426 to -420. If the dyad-symmetrical sequence forms a stem-and-loop structure, the binding of the factor is not able to protect the CCAAACA region against DNaseI digestion without any change of DNaseI sensitivity on the TATTTGG which forms a double stranded stem with CCAAACA. But since the DNaseI-digestion pattern at the TATTTGG region is not affected by the addition of nuclear extract (Fig. 5A), we suggest that this region does not form a stem-and-loop structure during the binding of the specific factor. The nuclear factor binding might indeed destroy a stem-and-loop structure in this region and these conformational changes could be important for the transcription enhancement of ENO1 gene.

   In the 5'-flanking region of ENO2 gene the sequences homologous to CCAAACA of ENO1 are also observed within 40 bp apart from center of two UASs (30); CCAAAGA from -530 to -524 (upstream from the center) and CCAAAAC from -463 to -457 (downstream from the center) when the translation initiation site defined to +1. The upstream CCAAACA sequence in ENO1 and the upstream CCAAAGA sequence in ENO2 contains 6 out of 7 identical bases, and furthermore, the downstream CCAAAAA sequence which is tandemly repeated in ENO1 and the downstream CCAAAAC sequence in ENO2 also contains 6 out of 7 identical bases.

Therefore the tandemly repeated sequences are found to be conserved in both ENO1-UAS and ENO2-UAS. ENO2-UAS is known as a regulatory region for the glucose induction of ENO2 gene (30), and recently, ENO1-UAS is also found to be responsible for the glucose induction by deleting the URS region of ENO1 promoter (24). The result that the amount of the ENO1-UAS binding protein which forms the migration-retarded species, S1, is regulated by the presence of glucose in the medium (Table I), together with the finding that the glucose induction is responsible for the ENO1-UAS, indicates that the binding factor forming S1 species is either a repressor protein against the ENO1-UAS mediated transcription or an activator whose function is repressed either by modification, such as phosphorylation, or by an association of another modulating protein. Concomitantly it is also suggested that CCAAACA and/or tandemly repeated CCAAA sequence may be responsible for the glucose induction.

Stanway et al. investigated the protein binding to the UAS of S. cerevisiae PGK gene which encodes phosphoglycerate kinase, and reported that the binding site of 28 bp length was found in the modulating region which is responsible for the regulation by glucose in the medium. The whole sequences of the protein binding site showed a 50% homology between ENO1-UAS and PGK-UAS, but CCAAACA which was thought to be a core sequence in case of ENO1-UAS was not found in the binding site of PGK-UAS. In addition, the amount of the ENO1-UAS binding protein is negatively regulated by the addition of glucose into the medium, while the amount of the PGK-UAS binding protein is positively regulated. Probably, the regulation mechanism by glucose may be different between the two glycolytic genes, ENO1 and PGK. Furthermore, if the protein binding site present in the modulating region is a site of primary protein-DNA contact as Stanway et al. discussed, it may be still possible that CCAGACA in the activating region of the PGK-UAS (-471 to -465) which resembles to CCAAACA of ENO1-UAS and shows a short dyad-symmetry with TGTCT (-452 to -448) is essential for the glucose regulation. In this case, the binding protein which forms the migration-retarded species, S1, detected with the ENO1-UAS fragment may be identical to the binding protein not to the modulating region but to the activating region of PGK-UAS.

Kawasaki and Fraenkel isolated the GCR1 gene, which regulates the expression of several glycolytic enzymes of S. cerevisiae including enolase (23). Uemura et al. found that the introduction of a multicopy plasmid containing GCR1 gene into the cell caused an about 2-fold enhanced expression of the ENO1-lacZ fused gene integrated in the chromosome (4). There are two regions which are thought to be responsible for the regulation of ENO1 gene

expression; one is the UAS described here and the other is the upstream repression sequence (URS), a second regulatory region of ENO1 gene, which is found downstream from the UAS by Cohen et al. (24). There are three possible mechanisms by which the protein factor could regulate gene expression by binding to the cis-acting regulatory region; the regulation could be achieved by the change in 1) the amount of the binding factor, 2) the binding constant of the factor to DNA or 3) the activity of the bound factor to enhance transcription. In the case of 1) and 2), the amount of the binding factor detected by gel retardation assay should change with the expression level of the regulated gene. In the case of 3), since the factor which directly binds to DNA is either associated with or modified by the second factor which does not bind to DNA, the number and the amount of the migration retardation species should also change with the expression of the regulated gene. In our experiments, the introduction of multicopy GCR1 genes to the cell caused no significant changes in the amount of ENO1-UAS specific binding factor (Table I). This supports that the product of the GCR1 gene, which is found to encode a 94 kd protein (31), does not bind directly to the UAS region of ENO1 gene. It is also unlikely that the GCR1 product interacts with or modifies the ENO1-UAS binding factor. The GCR1 product may interact with the ENO1-URS, instead of the ENO1-UAS. However, if the ENO1-UAS binding factor is either associated with or modified by the GCR1 product so weakly as to observe mobility changes in the gel retardation assay, we cannot detect the dosage effect of the multiple GCR1 genes and cannot eliminate the above possibility completely.

We estimate that there should be at least two specific binding factors by the following reasons; 1) in the gel retardation assay, only the amount of S1 varied without any changes of the amounts of S2 and S3 when the cells are grown on either glucose or glycerol plus lactate as a carbon source (Table I), and 2) on the anion exchange chromatography, the binding activity to form the migration-retarded species, S1, was separated from the species, S2 and S3 (data not shown). However, it is reasonable to assume that the migration-retarded species, S2 and S3, are derived from a single protein and the separation of these will be caused by the conformational change or the subunit-subunit interaction of the binding protein during the migration since the binding activities forming the species, S2 and S3, are coeluated as described above. It may be possible that all of the specific factors to form S1-S3 may have a common binding region, or alternatively, a single factor may be able to bind directly to DNA and the other factors may interact with this DNA-binding factor and not bind directly to DNA.

The purification of the UAS_{87}-specific binding factor is in progress. Studies on the purified factor will reveal the mechanism of the UAS-dependent transcription of ENO1 gene which will add to our understanding the general mechanism of transcriptional enhancement of UASs in yeast and perhaps other organisms.

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