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Identification of a Regulatory Region That Mediates Glucose-Dependent Induction of the *Saccharomyces cerevisiae* Enolase Gene *ENO2*

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There are two yeast enolase genes, designated *ENO1* and *ENO2*, which are expressed differentially in vegetative cells grown on glucose and in cells grown on gluconeogenic carbon sources. *ENO2* is induced more than 20-fold in cells grown on glucose, whereas *ENO1* expression is similar in cells grown on glucose and in cells grown on gluconeogenic carbon sources. Sequences within the 5′ flanking region of *ENO2* which are required for glucose-dependent induction were identified by deletion mapping analysis. These studies were carried out by using a fused gene containing the *ENO2* 5′ flanking sequences and the *ENO1* coding sequences. This fused gene undergoes glucose-dependent induction and is expressed at the same level as the resident *ENO2* gene in cells grown on glucose or gluconeogenic carbon sources. Expression of fused genes containing deletion mutations within the *ENO2* 5′ flanking region was monitored after integration at the *ENO1* locus of a strain carrying a deletion of the resident *ENO1* coding sequences. This analysis showed that there are two upstream activation sites located immediately upstream and downstream from a position 461 base pairs upstream from the transcriptional initiation site. Either one of these upstream activation sites is sufficient for glucose-dependent induction and normal gene expression in the presence of gluconeogenic carbon sources. Deletion of both regulatory regions results in a complete loss of gene expression. The regulatory regions function normally in both orientations relative to the coding sequences. Mutant fused genes containing small deletions within the regulatory regions were constructed; these genes were expressed normally in gluconeogenic carbon sources but were not induced in the presence of glucose. Based on this analysis, *ENO2* contains a *cis*-acting regulatory region which is required for gene expression and mediates glucose-dependent induction of gene expression.

There are two nontandemly repeated enolase structural genes per haploid yeast genome. These genes, designated *ENO1* and *ENO2*, are 95% homologous within their coding sequences, and they encode enolase polypeptides which differ by 20 of 436 amino acids (12). Both enolase genes are expressed during vegetative cell growth. Three enolase isozymes are resolved after DEAE-Sephadex chromatography of cell extracts, and these isozymes arise by random assortment of the two enolase polypeptides into catalytically active dimers (17). The steady-state concentrations of the enolase polypeptides are highly dependent on the carbon source used to propagate the cells (17). Cells grown in a medium containing glucose or glycerol plus lactate contain similar amounts of the enolase 1 polypeptide. In contrast, the steady-state concentration of the enolase 2 polypeptide is 20-fold higher in cells grown on glucose than in cells grown on glycerol plus lactate. The two enolase polypeptides are present at similar concentrations in cells grown on glycerol plus lactate. The differences in the steady-state concentrations of the enolase polypeptides as a function of carbon source are also reflected in the steady-state concentrations of the two mRNAs (17). These observations suggest that the two enolase genes are differently transcribed in glycolytic carbon sources compared with gluconeogenic carbon sources. A mutant strain which lacks a functional *ENO1* structural gene has been constructed. This strain contains only the enolase 2 homodimer, and it grows at wild-type rates in a medium containing either glucose or glycerol plus lactate (17).

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In this paper we describe the identification of a *cis*-acting regulatory region within the 5′ flanking sequences of *ENO2* which is required for gene expression in glycolytic or gluconeogenic carbon sources. We present evidence that this region also mediates glucose-dependent induction of *ENO2* expression.

MATERIALS AND METHODS

Materials. Restriction endonucleases were purchased from New England BioLabs, Inc., or Bethesda Research Laboratories, Inc. T4 DNA ligase was obtained from P-L Biochemicals, Inc. DNA polymerase I, the Klenow fragment, and bacterial alkaline phosphatase were obtained from Bethesda Research Laboratories. Exonuclease III was purchased from New England BioLabs. S1 nuclease was obtained from Boehringer Mannheim Biochemicals. *Sal*I restriction endonuclease linkers were purchased from Collaboratic Research, Inc. Oligonucleotide primers for sequencing deletion mutant endpoints were generously provided by Corey Levinson, Cetus Corp. [α-^{32}P]dCTP (400 Ci/mmol), [α-^{32}P]dATP (800 Ci/mmol), and [γ-^{32}P]ATP (2,000 to 3,000 Ci/mmol) were purchased from Amersham Corp. ^{125}I-labeled protein A was generously provided by John Hershey, University of California, Davis.

Strains and growth conditions. The bacterial strains and growth conditions used have been described previously (10). *Saccharomyces cerevisiae* S173-6B (α *leu2-3 leu2-112 his3-Δ1 ura3-52 trp1-289*) was provided by F. Sherman, University of Rochester. *S. cerevisiae* S173-LA (α *leu2-3 leu2-112 his3-Δ1 trp1-289 ura3-52 eno1-1*) was constructed from strain S173-6B as previously described (17). This latter strain carries a deletion of 90% of the *ENO1* coding sequences.

Yeast strains were grown at 30°C in YP medium (1% yeast extract, 2% Bacto-Peptone [Difco Laboratories]) or in a defined medium containing 0.67% yeast nitrogen base without amino acids (Difco) supplemented with 2 μg of uracil per ml, 2 μg of leucine per ml, and 2 μg of tryptophan per ml. Histidine (2 μg/ml) was also included for strains lacking a functional *HIS3* gene. The carbon sources used were 2% glucose or 2% glycerol plus 2% lactate.

Isolation of *peno*8-1. Yeast DNA was limit digested with *Bam*HI and ligated into the *Bam*HI site of pBR322. The ligation mixture was used to transform *Escherichia coli* RR101 to generate a yeast library of transformants. Transformants containing the *ENO1* or *ENO2* structural gene were identified by colony hybridization as previously described (11). A 0.75-kilobase (kb) *Kpn*I fragment from within the *ENO2* coding sequences was isolated from plasmid p*eno*8 (12), nick translated, and used as the hybridization probe. Plasmid DNA was isolated from positive transformants and subjected to restriction endonuclease mapping as previously described (12). A hybrid plasmid (p*eno*8-1) containing the *ENO2* structural gene was identified by comparing its restriction map with the cleavage map determined previously for p*eno*8 (12).

Construction of an *ENO2-ENO1* gene fusion. We constructed an *ENO2-ENO1* gene fusion which contained the *ENO2* 5′ flanking sequences joined to the *ENO1* coding sequences. Plasmid p*eno*46/*HIS3* contains a 4.6-kb segment of yeast genomic DNA which includes the entire *ENO1* structural gene ligated by the adenine-thymine tailing procedure into the *Eco*RI site of plasmid vector pSF2124 (12, 17). This plasmid also contains a functional yeast *HIS3* gene on a 1.7-kb *Bam*HI fragment ligated at the *Bam*HI site of pSF2124. Plasmid DNA was digested with *Hin*dIII in the presence of 150 μg of ethidium bromide per ml. Linear DNA molecules that were cleaved once by *Hin*dIII were isolated by preparative agarose gel electrophoresis and then limit digested with *Xba*I, which cleaves once at a site 800 base pairs (bp) upstream from the *ENO1* translation initiation codon. The largest linear molecules extending from the *Xba*I site to a *Hin*dIII cleavage site at codon 45 within *ENO1* were isolated by preparative agarose gel electrophoresis. These linear molecules were ligated to a 1.5-kb fragment isolated from p*eno*8-1 which extended from an *Xba*I site 1.3-kb upstream from the *ENO2* initiation codon to a *Hin*dIII cleavage site at codon 45 in *ENO2*. Following transformation of competent *E. coli* cells, a plasmid containing the *ENO2-ENO1* fusion at the homologous *Hin*dIII site at codon 45 was identified by restriction mapping and DNA sequence analysis. This plasmid was designated p*enoF*1.

Construction of deletion mutations within the 5′ flanking region of the *ENO2-ENO1* gene fusion. We constructed deletion mutations within the *ENO2* 5′ flanking region of plasmid p*enoF*1 by using the linker scan method described by McKnight and Kingsbury (19). Two collections of deletion mutants were generated for these constructions. The first collection of deletion mutants was generated from the unique *Sal*I cleavage site in plasmid p*neoF*1 as shown in Fig. 1a. Plasmid p*enoF*1 was limit digested with *Sal*I, and the full-length linear molecules were isolated by preparative agarose gel electrophoresis. A 12-μg portion of linear plasmid DNA was digested at 25°C with 75 U of exonuclease III in a 30-μl reaction mixture containing 60 mM Tris-hydrochloride (pH 7.5), 5 mM $MgCl_2$, and 1 mM dithiothreitol. Under these conditions, exonuclease III removed approximately 100 nucleotides per min from the 3′ termini of the DNA. Samples of the reaction mixture (7.5 μl) were removed at varying times and mixed with 67.5 μl of a buffer containing 30 mM sodium acetate (pH 4.6), 300 mM NaCl, and 1 mM $ZnSO_4$. S1 nuclease (2.75 U/μg of DNA) was then added, and the reaction mixture was incubated at 25°C for 1 h to remove single-stranded 5′ terminal sequences. The reaction mixture was then adjusted to 100 mM Tris HCl (pH 8.0) and 12 mM EDTA, phenol extracted, and ethanol precipitated. The DNA was ligated in the presence of a 20-fold molar excess of phosphorylated *Sal*I linker molecules and used to transform competent *E. coli* cells. Plasmids containing deletion mutations and a *Sal*I linker were enriched in two steps. Initially, supercoiled plasmid DNA was isolated from the total population of *E. coli* transformants obtained for each exonuclease III digestion time point. Supercoiled plasmid DNA of the expected size was isolated by preparative agarose gel electrophoresis and used to transform competent *E. coli* cells. This first step eliminated plasmids which were grossly rearranged. Plasmid DNA was again isolated from the total transformants generated after the latter transformation and cleaved with *Sal*I. Full-length linear DNA molecules were isolated by preparative agarose gel electrophoresis, ligated again, and used to transform competent *E. coli* cells. This final step enriched for deletions containing a *Sal*I linker. Plasmid DNA was isolated from individual transformants, and the approximate size of the deletion mutation was estimated after cleavage with *Sal*I and *Hin*dIII. The exact endpoints of representative mutants were then determined by DNA sequence analysis.

A second collection of deletion mutants was constructed with a plasmid designated p*enoF*2 by using the strategy outlined in Fig. 1b. Plasmid p*enoF*2 was derived from plasmid p*enoF*1 by removing the 1.7-kb *Bam*HI fragment containing the yeast *HIS3* gene, creating a unique *Hin*dIII site by joining the distal *Hin*dIII sites at codon 45 and the termination codon with the *ENO1* coding region (12), and destroying the *Sal*I site at position −561 in the *ENO2* 5′ flanking sequences. The *Sal*I site at position −561 was destroyed by repairing the *Sal*I 5′ cohesive termini with the Klenow fragment. Deletions were generated from the unique *Hin*dIII site of p*enoF*2 by using exonuclease III and S1 nuclease as described above.

The final method used for construction of plasmids containing deletion-*Sal*I insertion mutations within the *ENO2* 5′ flanking sequences is shown in Fig. 1c. A DNA fragment isolated from a deletion mutant obtained from the first collection of mutants extending from the *Sal*I site downstream to the unique *Sma*I site in the vector was ligated to a DNA fragment isolated from a deletion mutant obtained from the second collection of mutants extending from the *Sal*I site upstream to the *Sma*I site in the vector. By choosing appropriate fragments from the two collections of deletion mutants described above, this latter *Sal*I-*Sma*I fusion produced deletion-*Sal*I insertion mutations at any position within the *ENO2* 5′ flanking sequences. The plasmids formed by this fusion also contained the *HIS3* gene needed to select for yeast transformants.

Analysis of the expression of deletion mutants. Plasmid p*enoF*1 and plasmids containing deletion-*Sal*I insertion mutations were used to transform *S. cerevisiae* S173-LA by using the procedure of Ito et al. (14). Transformants were cloned on a defined medium lacking histidine but containing 2% glycerol plus 2% lactate to insure respiration competence. We analyzed the expression of the fused gene in at least three independent transformants obtained with each deletion mutant. The steady-state concentration of the enolase 1 polypeptide synthesized from the *ENO2-ENO1*

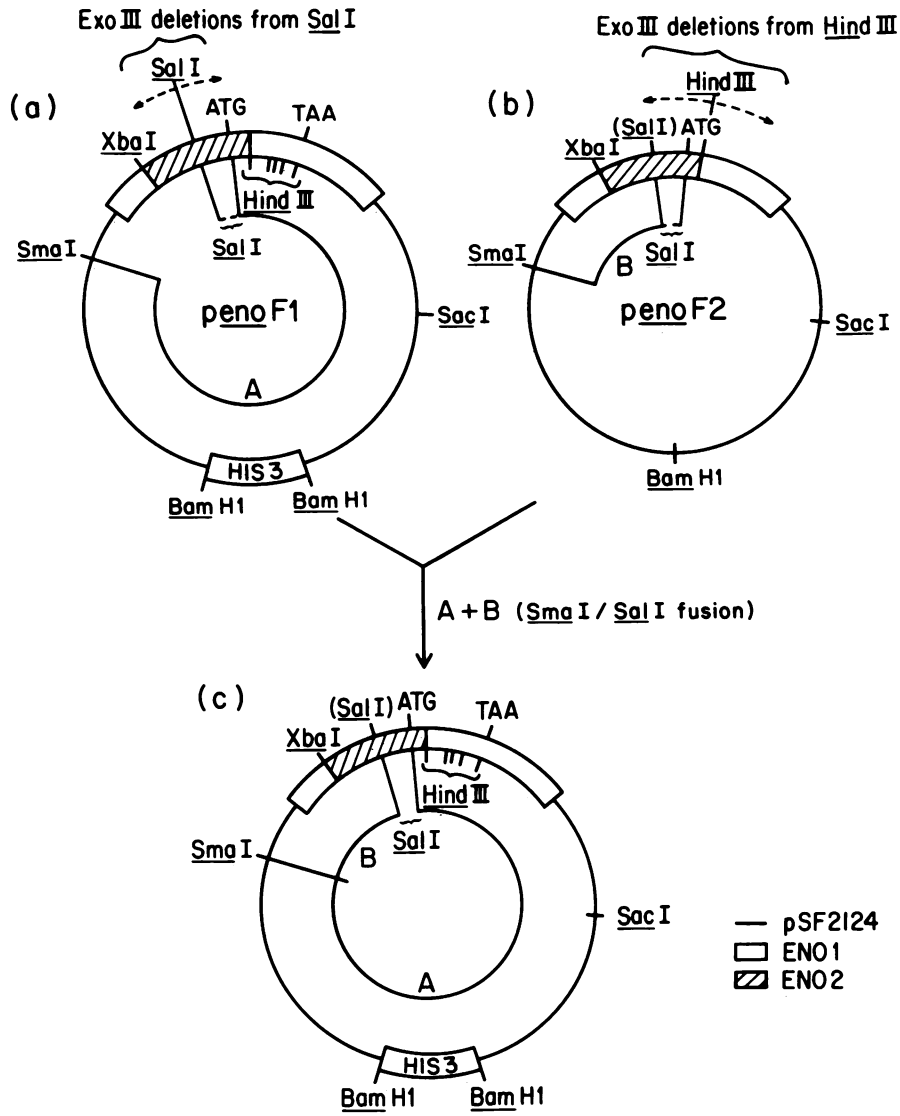

FIG. 1. Construction of deletion-*Sal*I insertion mutations within the 5′ flanking sequences of the *ENO2-ENO1* gene fusion. Plasmid p*enoF*1 contains the *ENO2-ENO1* gene fusion and a yeast *HIS3* gene on plasmid vector p5F2124. Plasmid p*enoF*2 was generated from p*enoF*1 by joining the distal *Hin*dIII sites in the *ENO1* coding sequences to create a unique *Hin*dIII site, removing the *HIS3* gene, and destroying of the *Sal*I site at position −561 in the *ENO2* 5′ flanking sequences. Deletion mutants were generated by cleavage of p*enoF*1 with *Sal*I and digestion with exonuclease III and S1 nuclease (a). Deletion mutations were generated in p*enoF*2 by the same procedure following digestion with *Hin*dIII (b). In both cases, the exonuclease III-S1 nuclease-treated plasmid DNA was ligated in the presence of *Sal*I linkers to generate a unique *Sal*I cleavage site at each deletion endpoint. The endpoints of representative deletion mutations in p*enoF*1 and p*enoF*2 were determined by DNA sequencing. Plasmids containing deletion-*Sal*I insertion mutations within the 5′ flanking sequences of the fused gene were constructed by ligating a fragment extending from the *Sal*I site of a deletion mutant constructed in p*enoF*1 downstream to the *Sma*I site in pSF2124 (fragment A) to a fragment extending from the *Sal*I site of a deletion mutant constructed in p*enoF*2 upstream to the *Sma*I site in pSF2124 (fragment B). The resulting plasmid that was generated by this *Sma*I-*Sal*I fusion contained a deletion-*Sal*I insertion within the 5′ flanking region of the fused gene and the *HIS3* selectable marker (c).

gene fusion was determined relative to the steady-state concentration of the enolase 2 polypeptide synthesized from the resident *ENO2* gene after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of whole-cell extracts as previously described (17). A Western blot analysis was carried out according to the procedure of Howe and Hershey (13), using a rabbit anti-yeast enolase polyclonal antibody and ^{125}I-labeled *Staphylococcus aureus* protein A. The copy number and relative orientation of the mutant fused gene relative to the resident *ENO1* deletion after integration at the *ENO1* locus were determined by a Southern blot analysis as previously described (17).

Intracellular *ENO1* and *ENO2-ENO1* mRNAs were analyzed by Northern blotting, using total cellular RNAs isolated from strains S173-6B and S173-LA and cells carrying *ENO2-ENO1* gene fusions containing deletion mutations within the *ENO2* 5′ flanking sequences. Cells were harvested from 10-ml log-phase cultures and were disrupted by vortexing with glass beads at 60°C in the presence of a 1:1 mixture of phenol and buffer containing 50 mM sodium acetate (pH

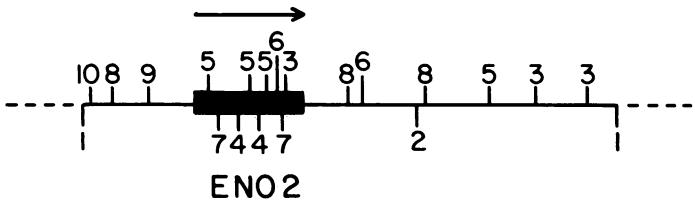

FIG. 2. Restriction endonuclease cleavage map of p*eno*8-1. The solid bar indicates the location of the *ENO2* coding sequences within the 6.6-kb *Bam*HI fragment of yeast genomic DNA. The arrow indicates the direction of *ENO2* transcription. The dashed lines indicate pBR322 vector sequences. Restriction enzyme sites: 1, *Bam*HI; 2, *Bgl*I; 3, *Bgl*II; 4, *Eco*RI; 5, *Hin*dIII; 6, *Hpa*I; 7, *Kpn*I; 8, *Pvu*II; 9, *Sal*I; 10, *Xba*I.

5.0), 0.1 M NaCl, 10 mM EDTA, and 0.5% SDS. Samples (5 μg) of total cellular RNA were electrophoresed on 1% agarose gels in the presence of formamide and transferred to GeneScreen membranes according to the procedure recommended by the manufacturer (New England Nuclear Corp.). The hybridization conditions used were those recommended by New England Nuclear Corp. A 1.4-kb *Hin*dIII-*Eco*RI fragment isolated from p*eno*46 (12) was nick translated and used as the hybridization probe. This fragment extended from a *Hin*dIII site at the translational termination codon of *ENO1* to an *Eco*RI site in the 3′ flanking region of the gene. This probe hybridized to *ENO1* 3′ untranslated mRNA sequences and did not show significant cross-hybridization with *ENO2* mRNA.

Analysis of the orientation dependence of the *ENO2* upstream activation region. The orientation dependence of the *ENO2* upstream activation region for expression of the fused gene was analyzed by first constructing a plasmid containing *Sal*I cleavage sites upstream and downstream from the activation region, using the method of Chang et al. (1). Plasmid p*enoF*1 containing a *Sal*I site at position −561 was cleaved at the unique *Sac*I site in the pSF2124 vector. A second plasmid containing a *Sal*I cleavage site at coordinates −351/−229 was cleaved at the unique *Sma*I site in the pSF2124 vector. Equimolar amounts of the two linear plasmids were mixed, denatured, and reannealed to form heteroduplexes by using the method of Chang et al. (1). The reannealed DNA was used to transform competent *E. coli* cells. Plasmid DNA was isolated from individual transformants and analyzed by restriction endonuclease mapping. Approximately 20% of the plasmids analyzed contained *Sal*I cleavage sites at positions −561 and −351/−229. A plasmid containing both *Sal*I cleavage sites was digested with *Sal*I, religated, and used to transform competent *E. coli* cells. Plasmids containing both orientations of the upstream activation region were identified by restriction mapping and DNA sequence analysis. Expression of the fused gene in these plasmids was analyzed as described above.

DNA sequencing. The DNA sequence of the *ENO2* 5′ flanking region between position −561 and the initiation codon was determined on both strands by the method of Maxam and Gilbert (16). The endpoints of deletion mutants were routinely determined by the dideoxy sequencing method (20), using several oligonucleotide primers complementary to sequences within the *ENO2* 5′ flanking region.

RESULTS

Construction and expression of an *ENO2-ENO1* gene fusion. A segment of yeast genomic DNA corresponding to the *ENO2* structural gene was previously cloned and characterized (12). This plasmid, designated p*eno*8, contains the entire *ENO2* coding region but lacks a complete 5′ flanking region. A second clone, designated p*eno*8-1, containing the *ENO2* structural gene on a 6.6-kb *Bam*HI fragment of yeast genomic DNA, was isolated. A restriction endonuclease cleavage map of this 6.6-kb fragment is shown in Fig. 2. The

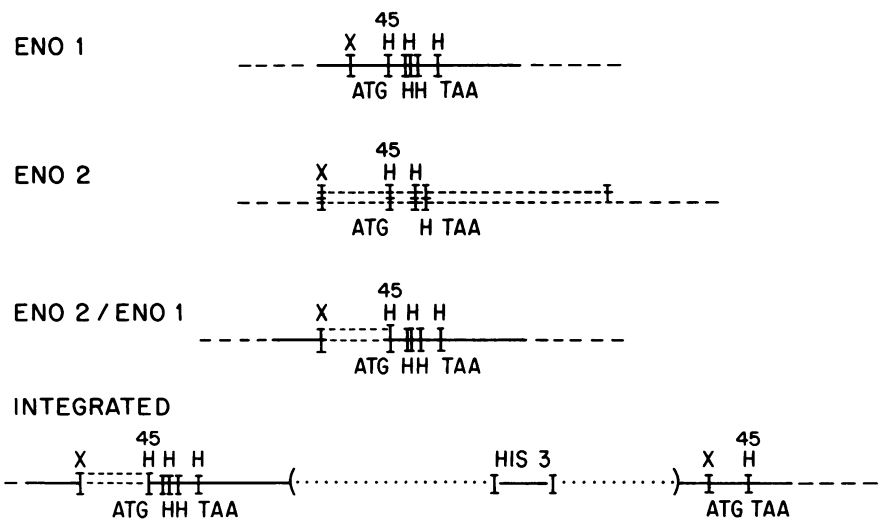

FIG. 3. Construction of an *ENO2-ENO1* gene fusion. A restriction fragment extending from an *Xba*I site 760 bp upstream from the *ENO1* transcription initiation site to a *Hin*dIII site at codon 45 in plasmid p*eno*46/*HIS3* was replaced with a fragment isolated from p*eno*8-1 which extends from an *Xba*I site 1.3 kb upstream from the *ENO2* transcription initiation site to a homologous *Hin*dIII site at codon 45. The solid horizontal lines indicate *ENO1* genomic sequences, and the double dashed horizontal lines indicate *ENO2* genomic sequences. The dashed lines indicate plasmid vector sequences. A plasmid designated p*enoF*1 containing the *ENO2-ENO1* gene fusion and a selectable yeast *HIS3* gene was used to transform yeast strain S173-LA, which contains a deletion of 90% of the coding sequences in the resident *ENO1* gene. A restrictive map of p*enoF*1 integrated in tandem with the resident *ENO1* deletion was determined by a Southern blot analysis. H, *Hin*dIII; X, *Xba*I.

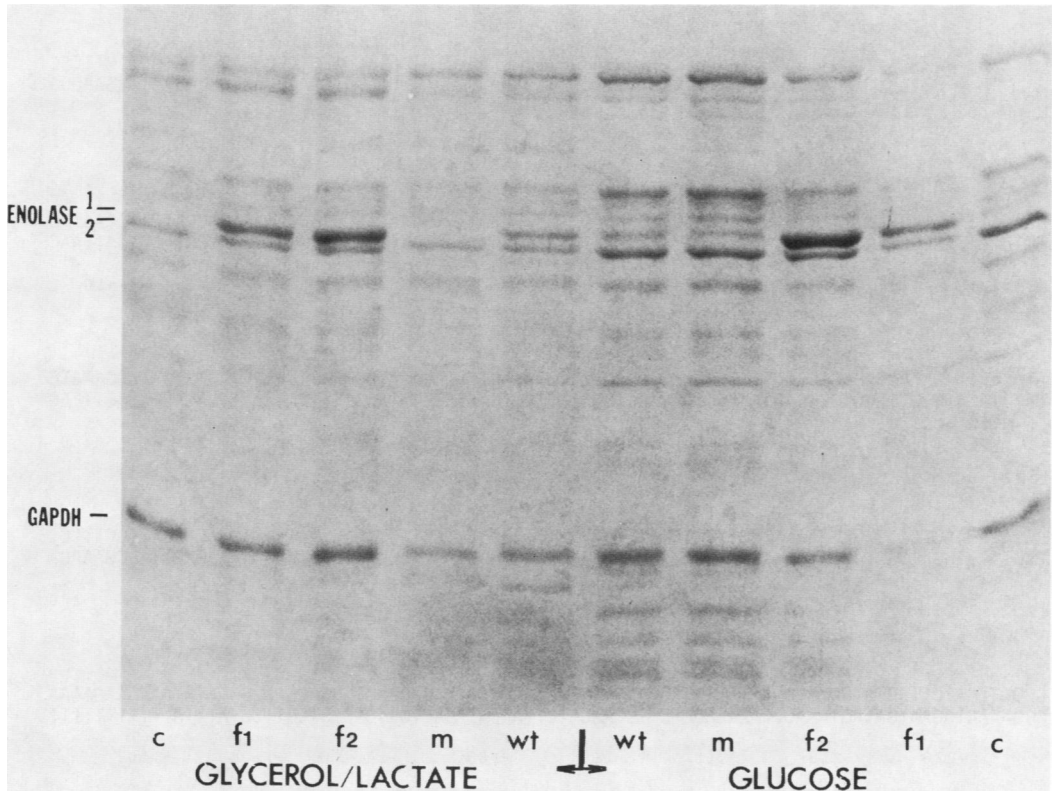

FIG. 4. Expression of the *ENO2-ENO1* gene fusion. Expression of plasmids carrying the *ENO2-ENO1* gene fusion was monitored after transformation of yeast strain S173-LA. Cloned transformants were grown in a defined medium containing either glucose or glycerol plus lactate as carbon sources. The total cell protein was isolated from log-phase cultures and analyzed after SDS-polyacrylamide gel electrophoresis and Coomassie blue staining. Lanes wt, Protein extracts from strain S173-6B, which carries the wild-type *ENO1* structural gene; lanes m, protein extracts from strain S173-LA, which carries a deletion of 90% of the coding sequences of the resident *ENO1* gene; lanes f1, a transformant containing one copy of a plasmid carrying the *ENO2-ENO1* gene fusion (p*enoF*1) integrated at the *ENO1* locus of strain S173--LA; lanes f2, a transformant containing two copies of a plasmid carrying the *ENO2-ENO1* gene fusion (p*enoF*1) integrated at the *ENO1* locus of strain S173-LA; lanes c, a transformant containing an integrated copy of a plasmid carrying an *ENO2-ENO1* gene fusion in which the *Hin*dIII site at codon 45 in *ENO2* is joined to the *Hin*dIII site at codon 182 in *ENO1*. The locations of the enolase 1, enolase 2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) polypeptides on the electrophoretogram are indicated.

fragment contains all of the yeast DNA sequences on p*eno*8, as well as additional sequences upstream and downstream from the *ENO2* coding region. Introduction of the 6.6-kb fragment into yeast cells on an autonomously replicating multicopy plasmid results in overproduction of the enolase 2 polypeptide when cells are grown in a medium containing glucose or glycerol plus lactate (data not shown). From this analysis we concluded that the 6.6-kb fragment contains a complete *ENO2* structural gene.

To study the structure and function of the *ENO2* 5′ flanking sequences, an *ENO2-ENO1* gene fusion was constructed. The fusion was made at a homologous *Hin*dIII site present at codon 45 in *ENO2* and *ENO1* (12). A fragment extending from the *Hin*dIII site at codon 45 to an *Xba*I site 760 bp upstream from the transcriptional initiation site in *ENO1* was replaced with a fragment from *ENO2* extending from the homologous *Hin*dIII site to an *Xba*I site 1.3 kb upstream from the *ENO2* transcriptional site. The construction of plasmid p*enoF*1 containing the *ENO2-ENO1* gene fusion is outlined in Fig. 3.

A yeast recipient strain (*eno1 his3*) was transformed with plasmid p*enoF*1 in order to monitor expression of the fused gene. This strain carries a deletion of 90% of the *ENO1* coding sequences (17). The plasmid does not contain a yeast origin of replication, and the vast majority of the transformants contain the entire plasmid integrated at the *ENO1* locus. The orientation of the integrated plasmid relative to the resident *ENO1* deletion mutation was determined by Southern blotting (Fig. 3). There is no growth phenotype associated with the absence of a functional *ENO1* gene in cells grown in the presence of glucose or glycerol plus lactate (17). The amino acid sequences of the enolase 1 and enolase 2 polypeptides are identical through residue 45 (12). Therefore, the fused gene encodes a native enolase 1 polypeptide. The two enolase polypeptides are completely resolved after one-dimensional SDS-polyacrylamide gel electrophoresis of yeast whole-cell extracts (17).

Expression of the fused gene was monitored in cells containing one or two copies of the fused gene integrated at the *ENO1* locus of the *eno1* recipient strain. As shown in Fig. 4, cells carrying a single integrated copy of the fused gene (lanes f1) contained similar amounts of the enolase 1 and enolase 2 polypeptides when they were grown in a medium containing glycerol plus lactate or glucose. Cells carrying two tandemly integrated copies of the fused gene (lanes f2) contained proportionately more enolase 1 polypeptide when they were grown on either carbon source. Cells carrying an integrated copy of a control fusion (lanes c) in which the *Hin*dIII site at codon 45 in *ENO2* was joined at the *Hin*dIII site at codon 182 of *ENO1* did not contain the

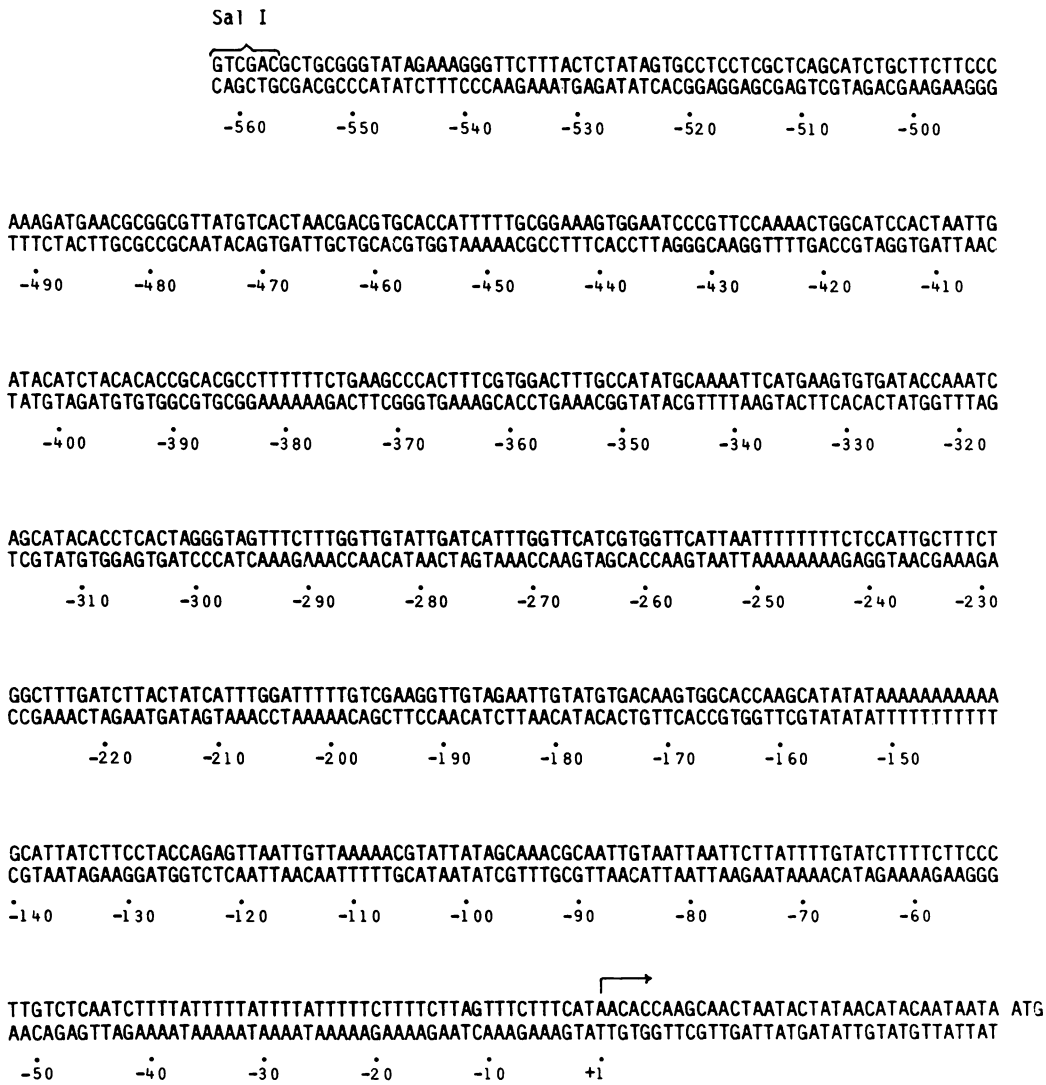

FIG. 5. Nucleotide sequence of the *ENO2* 5′ flanking region. The nucleotide sequence of the *ENO2* 5′ flanking region extending from a *Sal*I cleavage site 561 bp upstream from the transcriptional initiation site to the translational initiation codon was determined as described in Materials and Methods. The nucleotide sequence is numbered relative to the transcriptional initiation site (arrow) 36 bp upstream from the translational initiation codon.

enolase 1 polypeptide. From these data we concluded that the fused gene directs the synthesis of the enolase 1 polypeptide. Since the steady-state concentration of the fused gene-encoded enolase 1 polypeptide was similar to the concentration of the resident *ENO2* gene-encoded enolase 2 polypeptide in cells grown in the presence of glucose, we also concluded that sequences which mediate glucose-dependent induction of *ENO2* reside within the 5′ flanking sequences of the *ENO2* gene.

Identification of *cis*-acting regulatory sequences within the 5′ flanking region of the *ENO2* structural gene. To locate *cis*-acting sequences which regulate expression of the fused gene, we constructed deletion mutations within the *ENO2* 5′ flanking sequences as described in Materials and Methods (Fig. 1). Initially, sequences between the *Xba*I site and the *Sal*I site (700 bp) were deleted on plasmid p*eno*F1 (Fig. 1a). The mutant fused gene was expressed at the same levels as the resident *ENO2* gene when cells were grown in a medium containing glucose or glycerol plus lactate (data not shown). These data position the *ENO2* regulatory sequence between the *Sal*I cleavage site and the initiation codon. The nucleotide sequence of the latter region is shown in Fig. 5. The sequence is numbered from the first nucleotide of the transcript synthesized in vivo from the *ENO2* gene (9), 36 bases upstream from the initiation codon.

To map the apparent 5′ boundary of the regulatory sequences within the *ENO2* 5′ flanking region of the fused gene, we analyzed the expression of a series of deletion mutants extending from the *Sal*I site at position −561 for variable distances toward the initiation codon. The endpoints of these deletion mutations are indicated in Fig. 6. Fused genes with deletions extending from position −561 up to position −461 were expressed at levels similar to the level of the resident *ENO2* gene in cells grown on either glucose or glycerol plus lactate as carbon sources (Fig. 7). Fused genes containing deletions extending to position −433 and beyond were not expressed at detectable levels in cells grown on either carbon source. These data place the apparent 5′

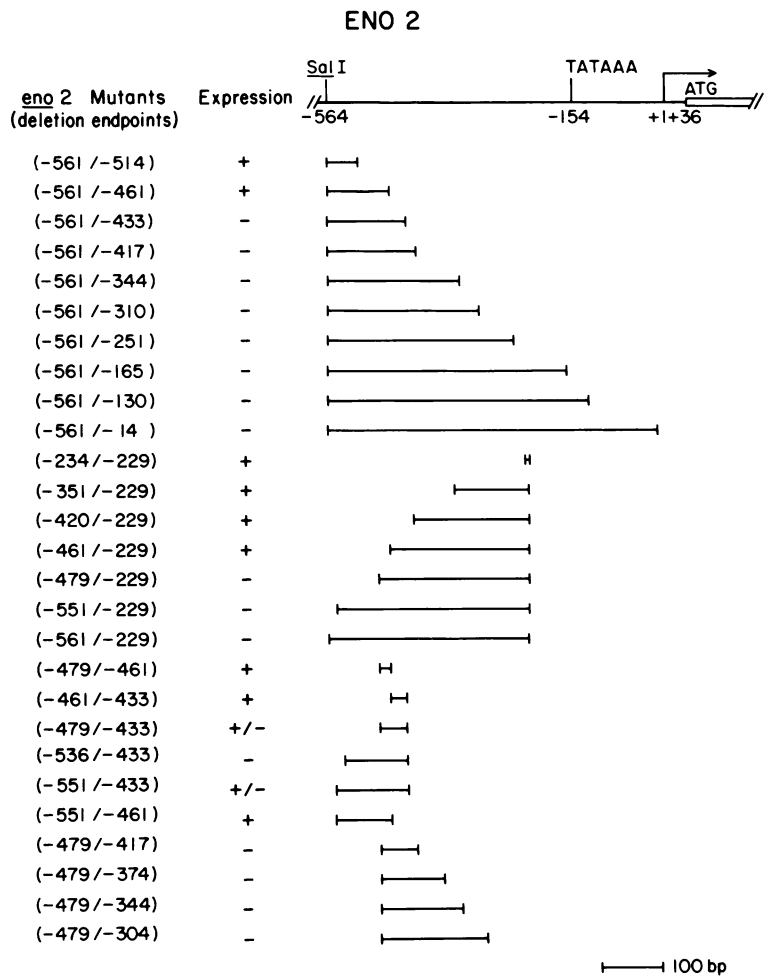

FIG. 6. Deletion mutants within the 5′ flanking region of the *ENO2-ENO1* gene fusion. Plasmids containing the deletion-*Sal*I insertion mutation within the 5′ flanking sequence of the *ENO2-ENO1* gene fusion were constructed as described in Materials and Methods. The coordinates of the deletion endpoints are numbered relative to the last *ENO2* nucleotide remaining at each endpoint. Each mutant contained a *Sal*I linker sequence between these endpoints. Expression of each mutant gene was monitored after integration of a plasmid containing the mutation at the *ENO1* locus of strain S173-LA. Expression was scored as follows: +, the mutant gene was expressed at normal levels relative to the unaltered fused gene in cells grown on glucose or glycerol plus lactate; −, the mutant gene was not expressed at detectable levels in cells grown on glucose or glycerol plus lactate; and +/−, the mutant gene was expressed normally in cells grown on glycerol plus lactate but was not induced in cells grown on glucose.

boundary of a *cis*-acting regulatory region between positions −461 and −433.

The apparent 3′ boundary of the *ENO2 cis*-acting regulatory region was mapped by analyzing the expression of a series of mutant fused genes containing deletion mutations extending from position −229 for variable distances toward position −561 (Fig. 6). As illustrated in Fig. 8, fused genes containing deletions extending from position −229 up to position −461 directed the synthesis of the enolase 1 polypeptide at levels comparable to the levels of the endogenous enolase 2 polypeptide when cells were grown on glucose or glycerol plus lactate. Fused genes containing deletions extending from position 229 to position −479 and beyond were not expressed at detectable levels in either carbon source. These results place the apparent 3′ boundary of the regulatory region between positions −461 and −479.

Paradoxically, the apparent 5′ and 3′ boundaries of the regulatory region map to the same position. The simplest interpretation of these data is that they are functionally similar regulatory sequences upstream and downstream from position −461. To test this hypothesis, we analyzed the expression of fused genes containing deletion-insertion mutations within the regulatory region. The endpoints of these deletion mutations are indicated in Fig. 6. The relative steady-state concentrations of the enolase 1 polypeptide synthesized from these mutant fused genes are shown in Fig. 9.

As expected, mutant fused genes containing deletions between positions −479 and −461, as well as positions −461 and −433, were expressed normally in cells grown on both carbon sources. Surprisingly, a fused gene containing a deletion between positions −479 and −433 was expressed normally in cells grown on glycerol plus lactate, but was not induced when cells were grown on glucose. A similar phenotype was observed for a deletion extending from position −433 to position −551. In contrast, mutant genes containing deletions extending from position −433 to positions −536 and −561 were not expressed in cells grown on either carbon

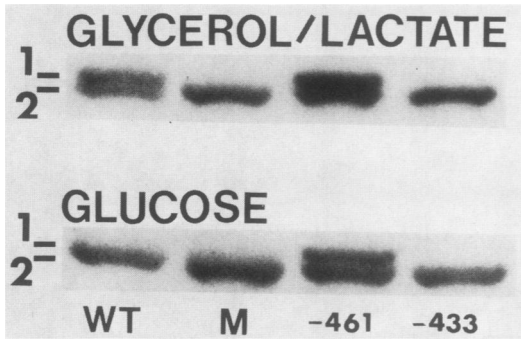

FIG. 7. Mapping of the apparent 5′ boundary of *cis*-acting sequences required for expression of the *ENO2-ENO1* gene fusion. Plasmids containing a deletion within the 5′ flanking sequences of the *ENO2-ENO1* gene fusion extending from position −561 variable distances toward the translational initiation codon were integrated at the *ENO1* locus of strain S173-LA. The expression of each mutant fused gene in cloned transformants was monitored in log-phase cells grown on glucose or glycerol plus lactate after SDS-polyacrylamide gel electrophoresis of whole-cell extracts. Western blots of whole-cell extracts obtained from transformants carrying mutant fused genes containing deletions extending from position −561 to positions −461 and −433 are shown. Extracts obtained from strain S173-6B (WT) and strain S173-LA (M) served as controls. The position of the enolase 1 (1) and enolase 2 (2) polypeptides on the autoradiogram are indicated.

source. The simplest interpretation of these data is that there are regulatory sequences downstream from position −433 whose function is, in some way, influenced by the nucleotide sequences placed upstream from position −433. This hypothesis is supported by the observation that fused genes containing deletions extending from position −479 to position −417 and beyond are not expressed in the presence of either carbon source. These data show that glucose-dependent induction of some mutant fused genes can be uncoupled from normal expression in cells grown on glycerol plus lactate, strongly suggesting that this regulatory region mediates glucose-dependent induction of gene expression.

Upstream regulatory region of *ENO2* regulates transcription of the *ENO2-ENO1* fused gene. To determine whether the upstream regulatory sites within the 5′ flanking sequences of *ENO2* regulate transcription of the fused gene, a Northern blot analysis was carried out with total cellular RNA isolated from log-phase cultures of strains S173-6B and S17-LA and strains carrying *ENO2-ENO1* gene fusions containing deletion mutations within the *ENO2* 5′ flanking sequences. A hybridization probe which is complementary to the 3′ untranslated portion of *ENO1* mRNA was used for this analysis. This probe hybridized with *ENO1* mRNA and mRNA synthesized from the *ENO2-ENO1* gene fusion. As shown in Fig. 10, the steady-state concentrations of *ENO1* mRNA in parental strain S173-6B were similar in cells grown on glucose and cells grown on glycerol plus lactate. Trace amounts of mRNA were detected in strain S173-LA, which carries a deletion of the coding sequences in the resident *ENO1* gene. We assumed that the small amount of hybridization observed for strain S173-LA was due to cross-hybridization with *ENO2* mRNA. We observed glucose-dependent induction of enolase mRNA for a strain carrying an *ENO2-ENO1* gene fusion containing a deletion extending from position −561 to position −461. A strain carrying a deletion from position −561 to −433 contained very little hybridizable enolase mRNA when it was grown in either carbon source. These observations are consistent with the protein expression data shown in Fig. 6 and demonstrate that the upstream regulatory region of *ENO2* regulates transcription of the fused gene.

A strain carrying a deletion from position −479 to position −433 in the *ENO2-ENO1* fused gene contained similar amounts of enolase mRNA when it was grown on glucose and when it was grown on glycerol plus lactate. Again, the data are consistent with the protein expression data shown in Fig. 9 which indicate that this mutation blocks glucose-dependent induction of the fused gene but does not affect expression in cells grown on glycerol plus lactate. Glucose-dependent induction of enolase mRNA synthesis was observed for a strain carrying an *ENO2-ENO1* gene deletion mutation extending from position −229 to position −461; however, the extent of induction was reduced. A strain carrying a deletion extending from position −229 to position −479 did not contain significant hybridizable enolase mRNA when it was grown in either carbon source. These latter observations are consistent with those shown in Fig. 8. In summary, the Northern blot analysis confirmed that the upstream regulatory region of *ENO2* regulates transcription of the fused gene.

Upstream regulatory region of *ENO2* functions in both orientations. To test the effect of reversing the orientation of the regulatory region on expression of the fused gene, we constructed a deletion-insertion mutant at coordinates −351/−229. A plasmid containing this mutation was cleaved with *Sma*I in the vector sequences and then heteroduplexed

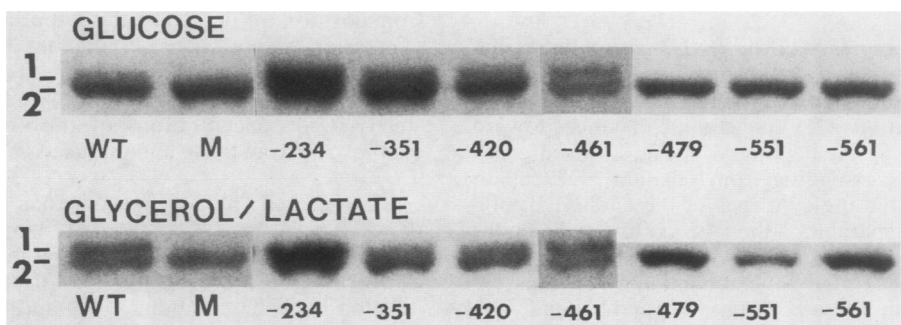

FIG 8. Mapping of the apparent 3′ boundary of *cis*-acting sequences required for expression of the *ENO2-ENO1* gene fusion. Plasmids containing deletions within the 5′ flanking region of the *ENO2-ENO1* gene fusion extending from position −229 variable distances toward position −561 were integrated at the *ENO1* locus of strain S173-LA. The expression of each mutant fused gene in cloned transformants was monitored in log-phase cells grown on glucose or glyceral plus lactate after Western blotting of whole-cell extracts. The deletion mutations extended from position −229 to the positions indicated below each autoradiogram. Extracts from strain S173-6B (WT) and strain S173-LA (M) were analyzed as controls. The positions of the enolase 1 (1) and enolase 2 (2) polypeptides on the autoradiogram are indicated.

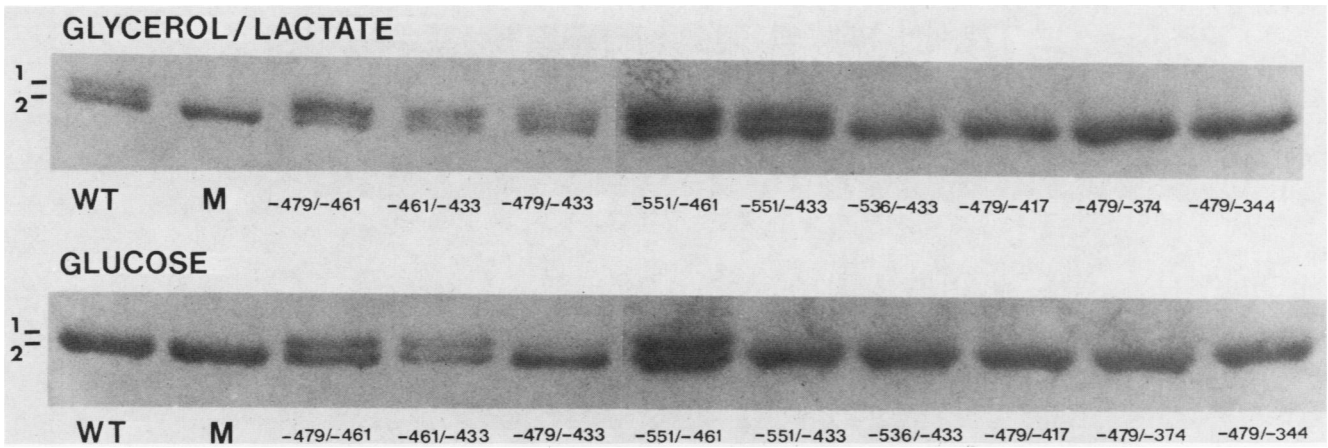

FIG. 9. Expression of *ENO2-ENO1* fused genes containing deletion mutations within the upstream regulatory region. Plasmids containing deletions within the upstream regulatory region were integrated at the *ENO1* locus of strain S173-LA. The expression of each mutant fused gene was monitored in log-phase cells grown on glucose or glycerol plus lactate after Western blotting of whole-cell extracts. The coordinates of the deletion mutations are indicated below each autoradiogram. Extracts from stain S173-6B (WT) and strain S173-LA (M) served as controls. The positions of the enolase 1 (1) and enolase 2 (2) polypeptides on the autoradiograms are indicated.

with *Sac*I linear plasmid p*enoF*1 containing the original fused gene and the natural *Sal*I site at position −561. Following transformation of *E. coli*, we identified plasmids which contained *Sal*I cleavage sites at positions −561 and −351/−229. A restriction map of a representative plasmid is shown in Fig. 11. To reverse the orientation of the regulatory region, this plasmid was digested with *Sal*I and religated. Plasmids which contained the regulatory region in the opposite orientation were identified and used to transform the *eno1* recipient strain. As shown in Fig. 11, fused genes containing the regulatory region in both orientations were expressed at levels similar to the level of the resident *ENO2* gene in cells grown in glucose or glycerol plus lactate. These results demonstrate that the regulatory region can function in an orientation-independent manner.

DISCUSSION

The *ENO2-ENO1* fused gene described in this paper directs the synthesis of the enolase 1 polypeptide at levels comparable to the levels of the *ENO2*-encoded enolase 2 polypeptide in cells grown on glucose or glycerol plus lactate. This observation is consistent with our earlier observations (17) and strongly suggests that differential expression of the two yeast enolase genes is regulated primarily at the level of transcription. The deletion mapping studies establish that the upstream regulatory region of *ENO2* contains fully functional upstream activation sites (UAS) immediately upstream and downstream from position −461. The results position the 5′ boundary of the downstream UAS and the 3′ boundary of the upstream UAS. More detailed identification of the precise dimensions of the *cis*-acting regulatory sequences in each UAS will require additional deletion mapping studies on genes containing a single regulatory region. There are some conserved sequences within the two UAS regions; however, in the absence of additional information it is not possible to make any clear interpretations regarding their possible role in regulation of gene expression. It is also not clear that the two UAS regions are functionally identical. Multiple upstream activation sequences have been reported for other yeast genes, including *HIS3* (21), *HIS4* (4), *CYC1* (8), and *GAL1* (5, 22). As is the case for *ENO2*, the effects of multiple regulatory regions versus a single regulatory region on gene expression in *CYC1* and *GAL1* are small or at best additive (5, 8). Our results with the *ENO2* upstream regulatory region are consistent with the observations that the upstream regulatory regions of *CYC1* (7) and *GAL1* (22) function in both orientations relative to their respective coding sequences.

In general, little effect on expression is observed when upstream activation sequences of yeast genes are brought

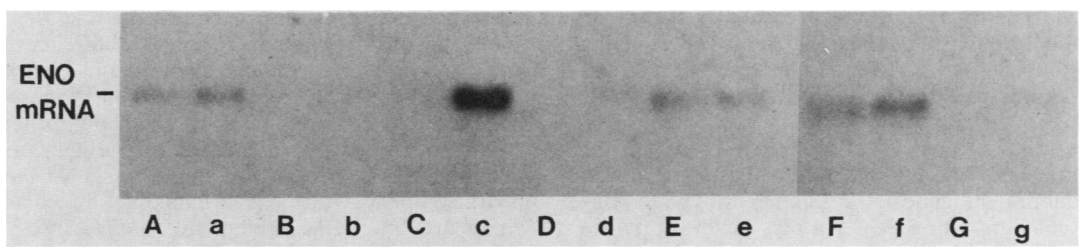

FIG. 10. Northern blot analysis of strains carrying deletion mutations within the 5′ flanking sequences of the *ENO2-ENO1* gene fusion. Northern blotting was carried out by using 5-μg portions of total cellular RNAs isolated from strains grown on glucose or glycerol plus lactate. The hybridization probe was complementary to the 3′ untranslated portion of *ENO1* mRNA and hybridized with mRNA encoded by the *ENO2-ENO1* gene fusion. Upper-case letters indicate RNAs isolated from strains grown on glycerol plus lactate. Lower case letters indicate RNAs isolated from strains grown on glucose. Lanes A and a, Strain S173-6B; lanes B and b, strain S173-LA; lanes C and c, *ENO2-ENO1* (−561/−461); lanes D and d, *ENO2-ENO1* (−561/−433); lanes E and e, *ENO2-ENO1* (−479/−433); lanes F and f, *ENO2-ENO1* (−461/−229); lanes G and g, *ENO2-ENO1* (−479/−229).

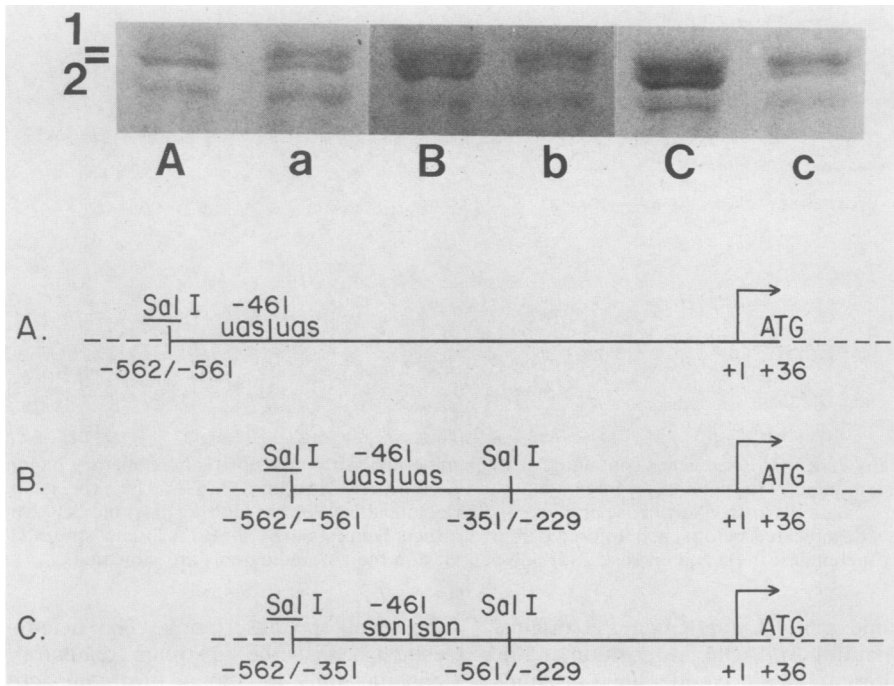

FIG. 11. Orientation independence of the *ENO2* upstream activation region. A plasmid containing *SALI* cleavage sites at coordinates −562/−561 and −351/229 was isolated as described in Materials and Methods. The expression of the fused gene contained on this plasmid (B) and a plasmid in which the orientation of the sequences between these two *Sal*I cleavage sites was reversed (C) was compared with the unaltered *ENO2-ENO1* gene fusion(A) after SDS-polyacrylamide gel electrophoresis of whole-cell extracts. Electrophoretograms for extracts prepared from transformants grown on glucose are indicated by upper-case letters, and electrophoretograms for extracts prepared from cells grown on glycerol plus lactate are indicated by lower-case letters. The positions of the enolase 1 (1) and enolase 2 (2) polypeptides on the electrophoretograms are indicated.

closer to the transcriptional initiation site (6). In the case of *ENO2*, there is no effect on expression when the upstream regulatory region is moved closer to the transcriptional initiation site when cells are grown in glycerol plus lactate. In contrast, we have reproducibly observed spacing effects on expression of the gene in cells grown on glucose. Mutant genes containing deletions extending from position −229 to positions −351, −420, and −461, for example, are expressed at lower levels in glucose than the unaltered fused gene or a gene carrying a deletion from position −229 to position −234. These effects could be due to spacing alterations between the UAS regions and the TATA box or to the removal of important sequences. Our data do not distinguish between these possibilities. Interestingly, none of the mutant genes that we have analyzed is expressed at levels which are higher than the level of the unaltered fused gene when cells are grown on glucose or glycerol plus lactate. These data do not exclude the possible existence of negative *cis*-acting regulatory regions; however, they argue against the existence of such regions within the 5′ flanking region of *ENO2* analyzed in this investigation.

The observation that small deletion mutations within the upstream regulatory region of *ENO2* abolish glucose-dependent induction of gene expression without affecting expression in cells grown on glycerol plus lactate provides compelling evidence that the regulatory region mediates glucose-dependent induction of gene expression. This observation raises interesting questions about the relationship between the upstream regulatory sites of *ENO2* and the putative regulatory regions of other yeast glycolytic genes. There is genetic evidence that yeast glycolytic gene expression is coordinately controlled (2, 3), suggesting that these genes may share functionally similar regulatory regions. Yeast strains carrying the *gcr1* mutation contain 20- to 50-fold lower specific activities of most glycolytic enzymes when they are grown in the presence of gluconeogenic carbon sources. Interestingly, the specific activities of many of these glycolytic enzymes increase significantly when *gcr1* strains are grown on glucose. This latter effect is not observed in the wild-type parental strain (2). These results suggest that many glycolytic genes are capable of glucose-dependent induction. We have analyzed expression of the yeast *ENO1* gene (8) and the three glyceraldehyde-3-phosphate dehydrogenase genes (18) and have found that these genes are not induced when cells are grown on glucose. In contrast, Maitra and Lobo (15) have reported glucose-dependent increases in the specific activities of several glycolytic enzymes, including glyceraldehyde-3-phosphate dehydrogenase. It is likely that the differences among these observations relate to differences among the stains and growth conditions employed in each study. Nevertheless, these observations suggest that glucose-dependent induction may not be peculiar to *ENO2* but rather that many glycolytic genes have the capacity for glucose-dependent induction. In support of this hypothesis, we have recently identified UAS within the 5′ flanking sequences of *ENO1* which are functionally identical to the *ENO2* regulatory region described here (unpublished data). In the case of *ENO1*, a second regulatory region represses glucose-dependent induction of the wild-type gene (unpublished data). Therefore, it is possible that the regulatory regions described for *ENO2* also mediate coordinate control of expression with other yeast glycolytic genes. Additional studies will be necessary to test this hypothesis.

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