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Open reading frames in the antisense strands of genes coding for glycolytic enzymes in *Saccharomyces cerevisiae*

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Abstract Open reading frames longer than 300 bases were observed in the antisense strands of the genes coding for the glycolytic enzymes phosphoglucose isomerase, phosphoglycerate mutase, pyruvate kinase and alcohol dehydrogenase I. The open reading frames on both strands are in codon register. It has been suggested that proteins coded in codon register by complementary DNA strands can bind to each other. Consequently, it was interesting to investigate whether the open reading frames in the antisense strands of glycolytic enzyme genes are functional. We used oligonucleotide-directed mutagenesis of the *PGI1* phosphoglucose isomerase gene to introduce pairs of closely spaced base substitutions that resulted in stop codons in one strand and only silent replacements in the other. Introduction of the two stop codons into the *PGI1* sense strand caused the same physiological defects as already observed for *pgi1* deletion mutants. No detectable effects were caused by the two stop codons in the antisense strand. A deletion that removed a section from − 31 bp to + 109 bp of the *PGI1* gene but left 83 bases of the 3′ region beyond the antisense open reading frame had the same phenotype as a deletion removing both reading frames. A similar pair of deletions of the *PYK1* gene and its antisense reading frame showed identical defects. Our own Northern experiments and those reported by other authors using double-stranded probes detected only one transcript for each gene. These observations indicate that the antisense reading frames are not functional. On the other hand, evidence is provided to show that the rather long reading frames in the antisense strands of these glycolytic enzyme genes could arise from the strongly selective codon usage in highly expressed yeast genes, which reduces the frequency of stop codons in the antisense strand.

Key words *Saccharomyces cerevisiae* · Glycolysis · Phosphoglucose isomerase · Antisense · Double-strand coding

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Introduction

Usually, only one of the two complementary strands of DNA is transcribed into an mRNA, which in turn serves as a template in protein synthesis. The transcribed DNA strand is complementary to the mRNA. It has become customary to call the non-transcribed strand the sense or coding strand. Consequently, a gene contains one sense strand with an open reading frame (ORF) delineated by an ATG initiation codon and one of the three stop codons. The complementary antisense strand usually does not contain any ORF of more than 100 bp.

Nevertheless, the genomes of mitochondria and a number of DNA viruses contain overlapping genes on one and the same and also on both DNA strands. Overlapping genes have also been reported for nuclear genes of *Saccharomyces cerevisiae*. Hahn et al. (1988) showed that the *HAP3* locus encodes divergent overlapping RNAs. Moreover, Thompson-Jäger and Domdey (1990) demonstrated an antisense transcript that partially overlaps the adjacent genes *YPT1* and *ACT1*.

Blalock and Smith (1984) proposed that proteins encoded by complementary DNA strands in the same reading frame specifically interact with each other owing to a molecular complementarity of amino acids represented by complementary nucleic acid codons. In fact, Bost et al. (1985) synthesized a 24 amino acid "antipeptide" of corticotropin (ACTH) as predicted by the nucleotide sequence of derived antisense mRNA of

the ACTH coding region. The natural sense peptide and its complementary antisense peptide bound each other with an affinity comparable to that with which ACTH binds to its natural low-affinity receptor. Moreover, antibodies to this antisense peptide recognized the ACTH receptor. Mulchahey et al. (1986) made similar observations for the luteinizing hormone-releasing hormone (LHRH). Knutson (1988) synthesized an antisense hexapeptide that binds insulin at high affinity. Zull and Smith (1990) argued that high affinity binding is due to the retention of protein structural information in both strands of DNA because in most cases the antisense codons code for amino acids that show the same secondary structure tendencies as the amino acids coded by the sense codons. Recently, Konecny et al. (1993) concluded on the basis of an information-theoretical analysis of the genetic code that coevolution of proteins coded by complementary DNA strands is a distinct possibility.

Zimmermann (1992) and Démolis et al. (1993) reported that the yeast *PGI1* phosphoglucose isomerase (PGI) gene overlaps an ORF of 840 bp in the antisense strand, which uses the same codon register. So far, all the deletions of the *PGI1* gene have included the antisense reading frame and caused metabolic defects that cannot easily be explained by the mere absence of this enzyme activity (Aguilera 1986, 1987; Boles and Zimmermann 1993a). Therefore, Zimmermann (1992) speculated on a possible role of the putative antisense protein. Such an antisense protein could serve to couple PGI to the cytoskeleton or to integrate PGI into a glycolytic multienzyme complex. Interactions with actin filaments have been demonstrated for the glycolytic enzyme aldolase (Pagliaro and Taylor 1992). Multienzyme complexes are considered to be important for the function of several metabolic pathways (reviewed by Srere 1987; Ovádi 1988). Brand and Heinickel (1991) discussed such an organizing function in a glycolytic multienzyme complex for the 11.5 kDa rat parathymosin, which strongly binds several glycolytic enzymes during affinity chromatography.

We found that not only the structural gene of *S. cerevisiae* coding for PGI but also those coding for phosphoglycerate mutase, pyruvate kinase and alcohol dehydrogenase I include ORFs in the antisense strands. Extensive computer analysis revealed that the antisense reading frames are the consequence of the strongly selective codon usage in the highly expressed genes coding for glycolytic enzymes. This observation and other experimental data presented here suggest that the antisense reading frames have no function.

Materials and methods

Yeast strains

Strain ENY.WA-1A (*MATα, ura3-52, leu2-3, 112, trpl-289, his3-Δ1 MAL2-8c, MAL3, SUC3*) was kindly provided by K.-D. Entian (University of Frankfurt, Germany). Strains EBY*23* (*pgilΔ*) (Boles and Zimmermann 1993a) and EBY55 (*pyk1Δ*) (Boles and Zimmermann 1993b) were derived from this strain. Growth conditions were as described previously (Boles and Zimmermann 1993a, b).

Molecular biological techniques

Molecular biological techniques were performed essentially according to the procedures described in Sambrook et al. (1989). Specific yeast techniques are described by Guthrie and Fink (1991). DNA was sequenced by the method of Sanger et al. (1977) using the T7 sequencing kit of Pharmacia (Freiburg, Germany).

For oligonucleotide-directed mutagenesis, the T7-Gen in vitro mutagenesis kit from USB (Bad Homburg, Germany) was used (Vandeyar et al. 1988). A 1.2 kb *Eco*RI-*Hin*dIII fragment containing the *PGI1* coding region from + 109 bp to + 1328 bp (Green et al. 1988; Tekamp-Olson et al. 1988) was cloned into M13mp19 and M13mp18, resulting in MPGI-19 and MPGI-18, respectively. Oligonucleotide 1 (5′ GCATTGAGAAACTGAGCTAACTAGCCAATG 3′) changes codons 115 and 118 of the *PGI1* gene ORF in MPGI-19 from AGA (Arg) to TGA (stop) and AAG (Lys) to TAG (stop). These substitutions lead to synonymous replacements of a serine and a leucine codon in the antisense ORF (Fig. 1). Oligonucleotide 2 (5′ GTACCAGACTGACAATAAACCACCCAAC 3′) replaces codon 333 of the *PGI1* ORF in MPGI-18 with another serine codon (TCA) and codon 331 with another leucine codon (TTA). It changes codon 13 of the antisense strand ORF from AGA (Arg) to TGA (stop) and codon 15 from CAA (Gln) to TAA (stop) (Fig. 1). The nucleotides representing the base substitutions in the oligonucleotides are underlined. The substitutions in MPGI-19 and MPGI-18 were verified by sequencing using the M13 sequencing primer. For each pair of substitutions, the 1.2 kb *Eco*RI-*Hin*dIII fragment of pEB2 (*PGI1* in pUC19) (Boles and Zimmermann 1993a) containing part of the original *PGI1* coding sequence was replaced by the corresponding mutagenized DNA fragments from recombinant bacteriophages, resulting in $pPGI_{Stop}$ and $pASP_{Stop}$. In both cases, correct replacement was verified by double-strand sequencing of the regions containing the mutations, using the other oligonucleotides as a primer. The 2.7 kb DNA inserts of plasmids $pPGI_{Stop}$ and $pASP_{Stop}$ containing the complete but mutated *PGI1* genes were then each recloned into YCplac111 and YEplac181 of Gietz and Sugino (1988).

Construction of deletion strains EBY44 (*pgi1Δ*) and EBY56 (*pyk1Δ*)

The 0.14 kb *Cla*I-*Eco*RI fragment of plasmid pEB2 (Boles and Zimmermann 1993a) containing positions −31 bp to + 109 bp of the *PGI1* gene (Green et al. 1988; Tekamp-Olson et al. 1988) was replaced by a 1.1 kb *Cla*I-*Eco*RI fragment bearing the *URA3* gene of pURA3 (Boles and Zimmermann 1993a), resulting in pEB44. The 0.2 kb *Xba*I-*Acc*I fragment of plasmid pEB5 (Boles and Zimmermann 1993b) containing parts of the *PYK1* coding region from positions + 4 bp to + 228 bp (Burke et al. 1983) was replaced by a 2.3 kb *Xba*I-*Acc*I fragment carrying the *LEU2* gene, originally cloned into pUC19, resulting in pEB56. The deletion mutants were constructed following the one-step gene replacement procedure of Rothstein (1983). Deletion plasmid pEB44 was digested with *Dra*I and *Bam*HI and transformed into the haploid strain ENY.WA-1A. Selection for uracil prototrophy on a medium with 2% fructose and 0.1% glucose resulted in isolation of strain EBY44 (*pgi1Δ::URA3*). Deletion plasmid pEB56 was digested with *Bam*HI and *Hin*dIII and transformed into strain ENY.WA-1A. Selection for leucine prototrophy on a medium supplemented with 3% ethanol and 3% glycerol, resulted in strain EBY56 (*pyk1Δ::LEU2*). The deletions were verified by Southern blot analysis.

Enzyme assays

Enzyme activities were determined as described before (Boles and Zimmermann 1993a, b; Boles et al. 1993). However, pyruvate kinase (PYK) activity was determined in a coupled assay with hexokinase and glucose-6-phosphate dehydrogenase, measuring the formation of ATP via reduction of NADP. The assay mixture, in a 1 ml assay volume, contained 50 mM imidazole buffer, pH 7.0, 10 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, 0.3 mM NADP, 1 mM fructose-1,6-bisphosphate, 2 mM ADP, 3 mM glucose, 2 mM phosphoenolpyruvate (used to start the reaction), 0.5 U hexokinase and 0.5 U glucose-6-phosphate dehydrogenase (Boehringer-Mannheim, Germany). The standard pyruvate kinase assay which measures formation of pyruvate with lactate dehydrogenase, was unsatisfactory because it was not sufficiently specific and did not show a total lack of activity in a *pyk1* deletion mutant. Phosphoenolpyruvate carboxykinase was measured according to Hansen et al. (1976). Specific activities were computed as units per milligram protein determined according to the microbiuret method (Zamenhoff 1957) using bovine serum albumin as a standard. Ethanol production was determined enzymatically using a kit from Boehringer-Mannheim (Germany).

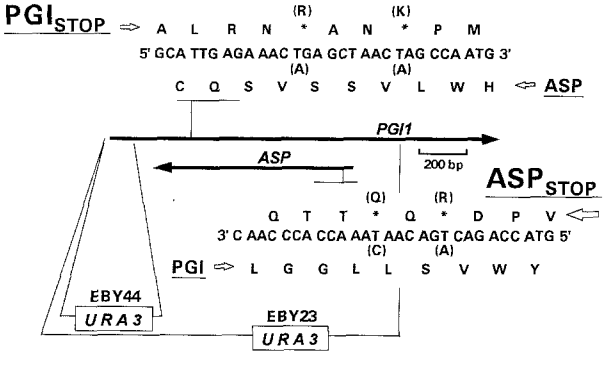
Computer assisted sequence analysis

The DNASIS/PROSIS program (Pharmacia, Freiburg, Germany) was used for DNA and protein analysis. Comparisons were made by using CD-ROM and the HIBIO Gene/Protein Sequence Database (Hitachi).

Results

We prepared by in vitro mutagenesis a mutant allele with two closely spaced stop codons in the *PGI1* ORF (PGI_{Stop}), and another mutant allele with two nonsense codons in the antisense ORF (ASP_{Stop}) (Fig. 1). All four nonsense mutations caused only silent base substitutions in the opposite DNA strands. The wild-type PGI, the PGI_{Stop} and the ASP_{Stop} alleles were ligated into centromeric plasmids (e.g. YCpPGI) and multicopy plasmids (e.g. YEpPGI). These constructs were transformed into the *pgi1* deletion strain EBY23.

Fig. 1 Schematic representation of the selectively mutagenized *PGI1* gene and construction of the two different *pgi1* deletion mutants (see Materials and methods for details)



Pgi1 deletion mutants cannot grow on glucose as the sole carbon source but only on a mixture of fructose and small amounts of glucose (Aguilera 1986, 1987). *pgi1* deletion mutants transformed with any plasmid containing a functional *PGI1* ORF showed exactly the same growth rates, irrespective of the copy number or the functionality of the antisense ORF [doubling time 160 min on synthetic complete (SC) medium without uracil and leucine and with 2% glucose, or 450 min with 3% ethanol]. On the other hand, transformants with plasmids $YCpPGI_{Stop}$ and $YEpPGI_{Stop}$, like the untransformed *pgi1* deletion strain, did not grow during 3 days of incubation at 28°C either on synthetic medium with 0.1% or 2% glucose or with 3% ethanol. Growth of *pgi1* mutants on 2% fructose medium is impaired by glucose concentrations higher than 0.2% (Aguilera 1986). This kind of glucose sensitivity could not be overcome by transformation with plasmids $YCpPGI_{Stop}$ or $YEpPGI_{Stop}$ which contain the functional antisense ORF.

We could not confirm the results of Aguilera (1987) who observed complete cessation of fermentation when *pgi*1 deletion mutant cells were transferred from permissive medium with 2% fructose and 0.1% glucose to pure fructose medium. We determined the yields of ethanol from cells pregrown on YEP medium supplemented with 2% fructose and 0.1% glucose, 23.5 h after transfer to 2% fructose synthetic medium without glucose. A value of 1.183 g/l per OD_{600} was obtained for ENY.WA-1A (wild type) and 0.740 g/l per OD_{600} for EBY23 (*pgi1Δ*). Ethanol production could also be observed under starvation for essential amino acids where, in contrast to the conditions above, neither wild-type nor mutant cells could grow (ethanol yields after 9 h: 0.615 g/l per OD_{600} for ENY.WA-1A and 0.373 g/l per OD_{600} for EBY23).

Measurement of PGI specific activities showed no obvious differences between various *pgi*1 transformants. After growth on synthetic medium with 2% glucose and 3% ethanol, respectively, PGI activities were determined to be 16.3 and 14.4 U/mg for YEpPGI, 17.9 and 18.4 U/mg for $YEpASP_{Stop}$, 1.9 and 0.9 U/mg for YCpPGI and 1.7 and 0.7 U/mg for $YCpASP_{Stop}$ containing transformants. On the other hand, PGI activities of transformants with $YEpPGI_{Stop}$ and $YCpPGI_{Stop}$ were below the limits of detection (< 1 mU/mg) after growth on a mixture of 2% fructose and 0.1% glucose.

Pyruvate decarboxylase is not completely induced with glucose or fructose in a *pgi1* deletion mutant and fructose-1,6-bisphosphatase is not inactivated (Boles and Zimmermann 1993a). Transformation into strain EBY23 (*pgi1Δ*) of the wild-type *PGI1* gene or the mutant allele containing the antisense ORF with the two stop codons either on centromeric or multicopy plasmids completely corrected these regulatory defects (Fig. 2).

The *PGI1* gene was deleted from − 31 bp to + 109 bp in strain EBY44 (Fig. 1). This deletion did not

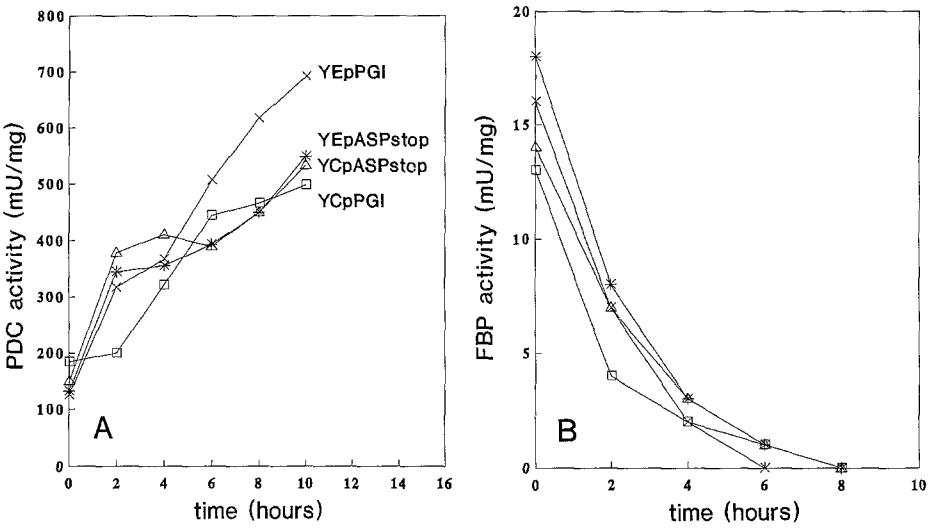


Fig. 2A, B Pyruvate decarboxylase activity (**A**) and fructose-1,6-bisphosphatase activity (**B**) in cells of the *pgi1* deletion strain EBY23 transformed with plasmids YCpPGI (□), $YCpASP_{Stop}$ (△), YEpPGI () or $YEpASP_{Stop}$ (*) after the addition of 2% glucose to ethanol-grown cultures

Table 1 Analysis of the four putative glycolytic antisense genes and their regulatory sequences. Antisense sequences were analysed for possible TATA box motives (Struhl 1986), a CAAG box motif (Dobson et al. 1982; Burke et al. 1983) and a transcription termination consensus sequence (Zaret and Sherman 1982); (' > ' indicates the position of the first nucleotide of the consensus sequence behind the stop codon). The codon bias index was determined according to Bennetzen and Hall (1982a)

Antisense gene of	Length of antisense ORF (bp)	Position relative to the glycolytic gene (bp)	TATA box	CAAG box	Termination consensus	Codon bias index
PGI1	840	+ 192–1032	–	− 27/ − 48	> 2	0.274
GPM1	507	+ 276–42 downstream	− 70/ − 252	− 34/ − 129	> 80	0.274
PYK1	969	+ 540–12 downstream	−51/−118/−373	− 26 (CAAA)	> 110	0.356
ADH1	327	+ 186–513	–	− 81	> 4	0.295

affect the antisense ORF although possible 3′ regulatory regions involved in transcriptional termination and polyadenylation were deleted. The physiological defects of this strain were identical to those of EBY23 (Boles and Zimmermann 1993a) in which both the sense and the antisense ORFs were deleted.

Homologies to sequence motifs often found in the upstream and downstream regions of genes could be found for the antisense ORF (Table 1). However, Northern hybridization experiments with polyadenylated mRNA of yeast wild-type cells and [^{35}S]dATP labelled double-stranded DNA as a probe identified only the 1.8 kb transcript of the *PGI1* gene, but not a transcript corresponding to the antisense ORF of about 1 kb (data not shown).

Computer analysis of all genes of *S. cerevisiae* coding for glycolytic enzymes revealed some interesting features. There are antisense ORFs on the opposite strands of the genes coding for phosphoglycerate mutase (*GPM1*) (White and Fothergill-Gilmore 1988; Heinisch et al. 1991), pyruvate kinase (*PYK1*) (Burke et al. 1983) and alcohol dehydrogenase I (*ADH1*) (Bennetzen and Hall 1982b), which are all in the same codon register as their sense counterparts. Features of these antisense genes are listed in Table 1. Moreover, an antisense ORF of 645 bp not in the same codon register could be identified on the opposite strand of the *PGK1* gene (Hitzeman et al. 1982), which codes for phosphoglycerate kinase.

The *PYK1* gene was deleted from + 4 bp to + 228 bp in strain EBY56 without affecting the integrity of the antisense ORF but possibly altering its 3′ flanking region. Strain EBY55 (Boles and Zimmermann 1993b) carries a deletion of both the *PYK1* gene and the complete antisense coding region. Both mutant strains grew well on medium with 3% ethanol and both failed to grow on 2% glucose medium. Pyruvate kinase activities were below the limits of detection (< 1 mU/mg) in both mutant strains. Furthermore, no obvious differences could be observed between the two strains regarding induction of pyruvate decarboxylase or inactivation of fructose-1,6-bisphosphatase and phosphoenolpyruvate carboxykinase (data not shown).

Further analysis showed a reduced incidence of stop codons in the inverse complement codon register of several of the genes coding for glycolytic enzymes. Glycolytic genes are known to be highly expressed and have high codon biases (Bennetzen and Hall 1982a). We systematically analysed the inverse complement codon registers of all glycolytic genes and several other yeast genes and compared the incidence of inverse stop codons with the codon bias indices of the sense ORFs

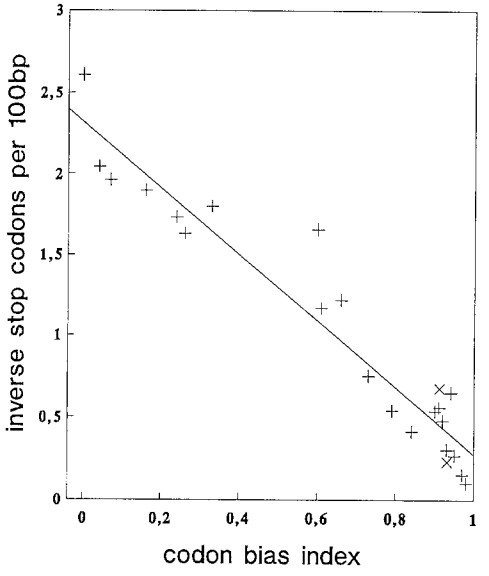


Fig. 3 Incidence of inverse stop codons per 100 bp of the complementary codon register of several yeast genes as a function of their codon bias indices. The genes selected for this analysis included all the genes coding for glycolytic enzymes and, additionally, some other genes involved in carbohydrate metabolism (*GAL2*, *GAL4*, *GCR1*, *FBP1*, *SNF1*, *SNF3*, *PDC1*, *PDC5* and *ADH1*) (the codon bias indices of all known genes of *Saccharomyces cerevisiae* have recently been summarized by Mossé et al. 1993)

(summarized by Mossé et al. 1993). Interestingly, an inverse correlation could be found between the incidence of stop codons in the complementary codon register and the codon bias index of the translation products of the sense strand (Fig. 3).

Discussion

Statistically, one would expect to find only short ORFs in the antisense strands of genes, as is the case in DNA of random sequence. An ORF on the antisense strand that is in codon register with the ORF of the sense strand requires that the inverse complements of the amber, ochre and opal stop codons are absent from and the inverse complement of the start codon is present at the corresponding segment of the sense strand. These codons are, respectively, CTA (Leu), TTA (Leu), TCA (Ser) and CAT (His). Interestingly, they are rarely used in highly expressed yeast genes like those coding for glycolytic enzymes because they are not among the codons highly homologous to the anticodons of the major yeast isoacceptor tRNA species (Bennetzen and Hall 1982a). On the other hand, they are more frequently used in poorly expressed yeast genes. Therefore, we observed that the incidence of in register stop codons in the antisense strands of several yeast genes shows a very strong inverse correlation with their codon bias index as defined by Bennetzen and Hall (1982a). This seems to be the reason why most of the antisense strands in register to the genes coding for glycolytic enzymes contain stretches of hundreds of bases without a stop codon. On the other hand, most of them do not contain an appropriate ATG codon and therefore cannot be considered to be ORFs. Only in the case of the *PGI1*, *GPM1*, *PYK1* and *ADH1* genes are complete antisense ORFs starting with an ATG codon present. Ikehara and Okazawa (1993) observed that in *Flavobacterium* sp. several genes encoding enzyme proteins also had long stretches without stop codons on their antisense strands. The authors concluded that these nonstop frames on the antisense strands were due to a strong codon usage bias on the sense strands.

We investigated whether or not the antisense strand of the *PGI1* gene is transcribed into an mRNA and codes for a protein involved in the metabolism of sugars. The introduction of two closely spaced stop codons in the antisense ORF of the *PGI1* gene that caused only silent base substitutions in the *PGI1* coding region did not cause any detectable effect. Also, selective deletions affecting only the coding regions of the *PGI1* and *PYK1* genes showed effects indistinguishable from the defect caused by deletions removing both the sense and the antisense ORFs. Northern analysis using a [^{35}S]dATP-labelled, double-stranded probe of very high specific activity revealed only the typical *PGI1* but not the much shorter transcript that could be coded by the antisense ORF. Northern analyses for the other glycolytic genes using double-stranded probes had also shown only one transcript per gene (Rodicio and Heinisch 1987; Burke et al. 1983; Bennetzen and Hall 1982b; Denis et al. 1983).

From these data we conclude that the presence of long antisense ORFs in glycolytic enzyme genes is fortuitous. Zull and Smith (1990) proposed that antisense strands, instead of being transcribed represent an evolutionary source of information that could provide DNA sequences important for binding of the complementary proteins after, e.g., translocation or gene duplication. We performed extensive database homology searches with the putative antisense sequences of all yeast glycolytic enzymes but were unable to find any significant homologies to published sequences. Furthermore, we could not find sequence similarities to glycolytic enzymes in the putative antisense sequence of the 11.5 kDa rat parathymosin (Trompeter et al. 1989; Brand and Heinickel 1991).

Extensive genome sequencing leads to the detection of many ORFs of unknown function. It can be expected that more examples of overlapping ORFs on opposite DNA strands will be found. We would predict that in most cases only one of the two ORFs will be active, as illustrated by investigations of Kreike et al. (1987) for the *MRS1* gene, Estruch and Carlson (1990) for the *SNF6* gene and our own investigations.

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