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The Isolation, Characterization, and Sequence of the Pyruvate Kinase Gene of *Saccharomyces cerevisiae**

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The *Saccharomyces cerevisiae* gene encoding the glycolytic enzyme pyruvate kinase has been isolated by complementation of a pyk mutant with DNA from a wild type yeast genomic library. Pyruvate kinase enzyme activity is 20-fold higher in the transformant compared to the parental strain and is glucose inducible. The cloned gene has been localized by hybridization of DNA fragments to yeast poly(A$^+$) RNA and by complementation of the mutant defect with select subclones. A DNA sequence of 2885 nucleotides encoding a protein of 499 amino acids is reported. A polypeptide chain of 34 residues of the deduced yeast amino acid sequence closely resembles a peptide sequence at the ADP binding site of bovine muscle pyruvate kinase. The 5′ end of the pyruvate kinase mRNA has been mapped and starts within the DNA sequence CAAG at −38 to −27 nucleotides upstream from the first ATG. We note that the sequence PyAAPu in this region appears to be a common consensus site for yeast RNA polymerase II transcriptional starts.

Pyruvate kinase catalyzes the conversion of phosphoenolpyruvate to pyruvate and ADP to ATP, the final and net energy-producing reaction of glycolysis. The reaction is essentially irreversible *in vivo* and appears to be a control point for the regulation of glycolytic flux. In yeast, as well as in higher animals, the protein activity is modulated by many effectors including the positive allosteric effector fructose 1,6-diphosphate and the negative effectors ATP and citrate (Hunsley and Sueltor, 1969). In addition, in yeast, the enzyme activity is also apparently controlled at the level of transcription. Although pyruvate kinase is synthesized constitutively at a high basal level, the shift in growth conditions from a gluconeogenic to a glycolytic medium induces a 6- to 20-fold increase in activity (Hommes, 1966; Maitra and Lobo, 1971). Recently a yeast mutant has been described which reduces the level of pyruvate kinase and other glycolytic enzymes to 6–16% that of wild type depending on the carbon source in the medium (Clifton and Fraenkel, 1981). This mutant also shows reduced amounts of glycolytic enzyme specific mRNAs. Thus, pyruvate kinase represents a class of highly expressed and transcriptionally regulated yeast genes.

We are investigating factors which affect the level of gene expression in yeast in order to facilitate the use of this organism for the production of biologicals. Here we report the molecular cloning of the yeast pyruvate kinase gene isolated by functional complementation of a pyruvate kinase mutant. The nucleotide sequence and the amino acid sequence of the gene as well as about 1400 nucleotides of untranslated regions are presented. Gene expression from the cloned segment of pyruvate kinase is regulated by glucose. Since the pyk mutant has essentially no measurable activity, manipulation of this glucose-controlled gene should allow us to determine factors important for high level expression in yeast.

This gene has also been cloned recently by Kawasaki and Fraenkel (1982) using a similar strategy.

EXPERIMENTAL PROCEDURES

Materials—The *Escherichia coli* K12 strain HB101 (Boyer and Roulland-Dussoix, 1969) was used for bacterial transformation. The yeast strains S288C (*SUC2*, mal, *gal2*, *CUP1*) and pyk 1-5 (*a*, *ade1*, *leu1*, *met14*, *ura3*, *pyk 1-5*) were obtained from the Yeast Genetic Stock Center, Department of Biophysics, University of California, Berkeley. The selective minimal media used for the culture of yeast cells contained per liter, basal salts (6.68 g of yeast nitrogen base, 10 g of succinic acid, 6 g of NaOH), 0.5% casamino acids, 0.001% adenine, 0.001% methionine, and 2% glucose; nonselective minimal media contained, in addition to the above components, 0.001% uridine and 2% glycerol plus 2% ethanol substituted for the glucose. Rich media, designated as YEPD or YEPGE, contained per liter: 10 g of yeast extract, 20 g of bactopeptone, and either 2% glucose or 2% glycerol plus 2% ethanol as a carbon source. Yeast nitrogen base, casamino acids, yeast extract, bactopeptone, and agar were all obtained from Difco. Glusulase was obtained from Endo Laboratories, Inc. Yeast cell transformations were performed according to the method of Hinnen *et al.* (1978). Spheroplasts were prepared from 200 ml of cells grown to a density of 2.2×10^7 cells/ml by treating cells resuspended in 0.1 M Tris-HCl, pH 8, 0.1 M EDTA with 5 mM 2-mercaptoethanol for 10 min followed by digestion with 1% glusulase in 1 M sorbitol for 30 min. For plating transformants, both the bottom agar and the regeneration agar were supplemented with 1 M sorbitol.

The yeast genomic DNA bank used consists of a partial *Sau*3A restriction digest of total yeast DNA from the strain S288C cloned into the *Bam*HI site of the vector YEp24 (Fasiolo *et al.*, 1981). The plasmid "shuttle" vector, YEp24 (equivalent to pFL1, Chevallier *et al.*, 1980), consists of the plasmid pBR322 for selection and maintenance in bacteria, the yeast URA 3 gene for selection in yeast, and an *Eco*RI fragment of the 2 μ circle to ensure plasmid replication and segregation in yeast.

Isolation and Characterization of Yeast Plasmid DNA—DNA was prepared for bacterial cell transformation by resuspending a generous mound of yeast from a single colony patch in 0.3 ml of ice-cold 25% sucrose, 50 mM Tris-HCl (pH 7.5), 0.2 M NaCl. Glass beads, 0.6 g, (0.45–0.5-mm diameter, obtained from B. Braun Melsungen A.G.) were added along with 400 μl of phenol (saturated with 50 mM Tris-HCl, pH 7.5, 10 mM EDTA) and 400 μl of $CHCl_3$:isoamyl alcohol (24:1). The cells were disrupted by vortexing three times for 1-min intervals with cooling on ice in between. The tubes were centrifuged briefly and the supernatant transferred to a clean tube. The organic phase (containing the glass beads) was washed with an additional 200 μl of sucrose solution, and the aqueous phases were combined and extracted with an equal volume of $CHCl_3$:isoamyl alcohol (24:1). 20 μg of poly(A$^+$) carrier DNA (Collaborative Research) were added and the DNA was ethanol precipitated. The pellet was resuspended in 100 μl of water and used to transform bacteria directly. Bacterial transformation was performed essentially as described (Cohen *et al.*, 1972).

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Plasmid was prepared from bacteria on a small scale according to Holmes and Quigley (1981) or Birnboim and Doly (1979). For large scale preparation of plasmid DNA, *E. coli* was grown on M9 medium (Maniatis *et al.*, 1982) supplemented with 0.4% casamino acids, plasmids were amplified in the presence 200 μg/ml of chloramphenicol, and plasmid DNA was prepared using the cleared lysate technique (Clewell and Helinski, 1970). Restriction enzymes were obtained from Bethesda Research Laboratories and from New England Biolabs and used as directed.

For the Northern analysis, RNA was prepared from 100 ml of yeast grown to a cell density of 2.5–5.0 × 10^7 cells/ml by a scale up of the above procedure. Poly(A^+) RNA was fractionated from the total by passage over a 1-ml column of oligo(dT)-cellulose (obtained from Collaborative Research). The RNA was modified with glyoxal, separated on a 1% agarose gel (McMaster and Carmichael, 1977), and blotted onto a nitrocellulose membrane as described by Thomas (1980). The probe DNA fragments were labeled with [α-^{32}P]dNTP using the *E. coli* DNA polymerase Klenow fragment similar to a technique described by O'Farrell *et al.*, 1980.

For the labeling reaction, 1 μg of DNA was treated with 2.5 units of the Klenow polymerase fragment for 25 min at 37 °C in the absence of deoxynucleotide triphosphates in a 50-μl reaction volume containing 0.01 M Tris-HCl, pH 7.5, 0.05 M NaCl, 0.01 M $MgCl_2$, 1 mM dithiothreitol, and 100 μg/ml of nuclease-free bovine serum albumin. Then 50 μCi each of [α-^{32}P]dATP and [α-^{32}P]dCTP were added to the reaction along with 5 nmol each of dGTP plus dTTP, and the incubation continued for 25 min at 37 °C. Finally 5 nmol of unlabeled dATP and dCTP were added for 25 min at 37 °C. The reaction was stopped by the addition of EDTA to 25 mM and NaCl to 0.3 M and heating at 65 °C for 5 min. The product was separated from unincorporated isotope by rapid sedimentation through a 400 μl Sepharose CL-6B (Pharmacia) column by centrifugation, "spin dialysis" (Neal and Florini, 1973), and had a specific activity of greater than 3 × 10^7 cpm/μg. The Klenow polymerase was obtained from Bethesda Research Laboratories and the radiolabeled deoxyribonucleotides were from Amersham Corp. at a specific activity of 3000 Ci/mmol. For the hybridization approximately 1 × 10^6 cpm of heat-denatured probe DNA was added to 5 ml of solution containing 5 × SSC,[1] 1% Sarkosyl, 50% formamide, 0.1% sodium pyrophosphate, 10% dextran sulfate, 1 × Denhardt's solution (Maniatis *et al.*, 1982), 50 mM sodium phosphate, pH 6.8, and 100 μg/ml of denatured salmon testes DNA. The hybridization was for 16 h at 42 °C after which the filter was washed twice in 250 ml of 2 × SSC, 0.1% sodium dodecyl sulfate at 20 °C for 15 min by gentle shaking, twice in 250 ml of 0.1 × SSC, 0.1% sodium dodecyl sulfate at 50 °C for 30 min and twice with 250 ml of 3 mM Tris-HCl, pH 8, at 20 °C for 5 min. The filter was dried and exposed to x-ray film. The DNA blot transfer was performed as described by Southern (1975). Labeling and annealing conditions were as described above.

DNA was sequenced in the M13 vectors Mp8 and Mp9 (Messing *et al.*, 1981) using the dideoxy chain termination method of Sanger *et al.* (1977).

Pyruvate Kinase Assay—For the pyruvate kinase activity assay a 50-ml culture of yeast was grown to a cell density of about 7.5 × 10^7 cells/ml in minimal medium supplemented with the indicated carbon source. Cells were collected by centrifugation, washed with distilled water, and stored at −70 °C. Thawed cells were disrupted by vortexing in assay buffer (50 mM imidazole, pH 7.3, 50 mM KCl, 10 mM $MgCl_2$) with the addition of an equal volume of glass beads. The cell lysate was centrifuged for 10 min in an Eppendorf centrifuge at 4 °C, and the supernatant was used directly for enzyme assays. The enzyme assay was modified from that described by Bucher and Pfleiderer (1955). An aliquot of supernatant from the disrupted cells containing between 0.05 and 0.5 unit of activity was added to a solution containing 0.1 mM fructose 1,6-diphosphate, 0.1 mM phosphoenolpyruvate, 0.1 mM rADP, 2 mM NADH, and 4 units of lactate dehydrogenase. Activity was determined from the rate of absorbance decrease at 340 nm with 1 unit corresponding to the oxidation of 1 μmol of NADH per min at 25 °C.

Mapping the 5′ End of the Pyruvate Kinase Transcript—For the primer extension experiment, primer DNA fragments were isolated by electroelution from a 5% acrylamide-TBE (89 mM Tris base, 89 mM boric acid, 2.8 mM Na_3EDTA) gel and purified by spin dialysis through a 400-μl Sepharose CL-6B column. Two to 4 pmol of primer DNA were mixed with 30 μg of poly(A^+) RNA isolated from the pPYK 9.1 transformant, precipitated, and redissolved in freshly deionized formamide to a final RNA concentration of 600 μg/ml. As a control, a sample containing no primer was carried through the same operations. The samples were heat denatured by incubation for 5 min at 68 °C, adjusted to a final salt concentration of 10 mM 1,4-piperazinediethanesulfonic acid, pH 6.4, 0.4 M NaCl, and 1 mM EDTA and annealed for 3.5 h at 50 °C. Following ethanol precipitation, cDNA was synthesized in a 50-μl reaction containing 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 8 mM $MgCl_2$, 5 mM dithiothreitol, 0.2 mM of each dNTP, 100 μg/ml of bovine serum albumin, 50 μCi of [α-^{32}P]dCTP (3000 Ci/mmol), and 124 units of reverse transcriptase (Life Sciences, Inc.; specific activity, 3.6 × 10^5 units/mg). Synthesis was for 60 min at 45 °C. Samples were extracted with phenol and $CHCl_3$:isoamyl alcohol (24:1), spin dialyzed to remove unincorporated isotope, and ethanol precipitated. An aliquot of each sample was resuspended in 5 μl of 90% formamide, 1 mM Na_3EDTA, pH 8, and 0.05% each xylene cyanol and bromphenol blue, heated for 3 min at 90 °C, and run on a 5% acrylamide TBE-7 M urea 0.04-mm thick gel.

RESULTS

Isolation of the Pyruvate Kinase Gene by Complementation—Pyruvate kinase is encoded by a single gene in yeast. Mutants of this enzyme, characterized by their inability to grow on glucose as the sole carbon source, have been isolated by many groups (Maitra and Lobo, 1977b; Lam and Marmur, 1977; Clifton *et al.*, 1978; Sprague, 1977; and Ciriacy and Breitenbach, 1979). Due to the altered allosteric control of pyruvate kinase activity isolated from a revertant prototroph, Maitra and Lobo (1977a) postulated that their mutation was most likely in a structural rather than a regulatory gene. We have used a similar mutant, pyk 1-5, to provide a selection for the wild type gene from a recombinant YEp24 plasmid pool containing the entire yeast genome.

The frequency of a single reversion for the mutant strain to either pyk or to ura prototrophy alone was low, less than 1 per 10^8 cells. Thus the requirement for a simultaneous complementation of both the pyk defect by the wild type gene and the ura deficiency by the plasmid URA3 gene allowed us to select for a very low frequency event in the transformation. For this procedure, 3.5 × 10^9 yeast cells were treated with 30 μg of plasmid pool DNA. A total of five independent transformants were obtained which grew in the absence of uracil and in the presence of glucose. As shown in Table IA this result corresponds to a complementation frequency of approximately 1 pyk prototroph per 7000 ura^+ transformants or about one pyk^+ and ura^+ double transformant per 3.5 × 10^8 viable spheroplasts.

Characterization of the Transforming DNA—To facilitate these characterizations, plasmids were first transferred from

TABLE I

Efficiency of pyruvate kinase complementation by yeast transformation

A

	Selection		Ratio
	ura^+	ura^+ & pyk^+	ura^+ & pyk^+/ura^+
Transformants/viable spheroplast	2.7 × 10^{-4}	3.5 × 10^{-8}	1.29 × 10^{-4}
Transformants/μg plasmid DNA	7000	0.9	1.29 × 10^{-4}

B

	Selection			Ratio
	ura^+	pyk^+	ura^+ & pyk^+	ura^+/pyk^+
Transformants/viable spheroplast	3.7 × 10^{-5}	1.2 × 10^{-6}	1.9 × 10^{-6}	20
Transformants/μg plasmid DNA	1097	56	36	20

[1] The abbreviations used are: SSC, 0.015 M Na citrate, 0.15 M NaCl; kb, kilobase pairs; bp, base pairs; EDTA, ethylenediaminetetraacetic acid.

the original yeast isolates to bacteria by transformation. The plasmids isolated from bacteria were than analyzed. To prove that the phenotypic change in yeast was plasmid mediated, we first determined that all five transformants contained both the YEp24 plasmid and an insert of sufficient size to encode pyruvate kinase, a protein with a monomer molecular weight of 57,500 (Aust and Suelter, 1978). Preliminary restriction enzyme mapping of these plasmids showed that the yeast DNA inserts shared some common restriction sites indicating all 5 plasmids contained overlapping segments of the same yeast chromosomal DNA region (data not shown). The transforming plasmid which contained the smallest DNA insert, 7.0 kb, was selected for further characterization. This plasmid was called pPYK 9.1 to designate it as a particular isolate, 9.1, of a wild type, dominant pyruvate kinase yeast gene.

The plasmid DNA pPYK 9.1 was used to retransform the yeast pyk auxotroph so that the frequency of transformation to pyk$^+$ could be compared to that of ura$^+$. Transformation to pyk$^+$ occurred 1/20–1/50 as often as that to ura$^+$; all pyk$^+$ transformants were also ura prototrophs (Table IB). Although this frequency was lower than expected, it is still 100-fold higher than the frequency of $1/10^4$ obtained in the initial transformation (Table IA). The lower frequency of transformation to pyk$^+$ compared to ura$^+$ may reflect a lag in expression of the pyruvate kinase gene relative to that of URA3 and a subsequent loss of the transformed colony under selective pressure.

As an additional test of identity for pPYK 9.1 we measured pyruvate kinase enzyme activity directly in the mutant, the transformant, and the parental wild type yeast strain. As shown in Table II, the mutant has a very low level of activity, 2% that of the wild type strain. This level was increased more than 1000-fold in the transformant compared to the mutant. In fact the mutant which harbors pPYK 9.1 has a significantly higher level of enzyme activity (20-fold) than the parental wild type strain consistent with the presence of the gene on a multicopy plasmid.

The level of expression of pyruvate kinase is regulated by glucose in yeast (Maitra and Lobo, 1971). In order to test if the pPYK 9.1 cloned gene segment included this glucose control region, we assayed these cultures for enzyme activity when grown under inducing (glucose as a carbon source) and noninducing (glycerol/ethanol) conditions. The transformant strains exhibit a 4.4-fold increase in pyruvate kinase activity when grown on a glycolytic carbon source (Table II) while the parent shows only a 2-fold increment. This level of induction suggests that the entire glucose regulatory region is contained on the pPYK 9.1 plasmid.

Localization of the Pyruvate Kinase Gene—The plasmid

TABLE II

Pyruvate kinase activity of wild type, mutant, and transformed yeast strains

Yeast strain	Carbon source	Pyruvate kinase activity[a]	Glucose induction	Level of expression[b]
		units/mg protein	*-fold*	
Wild type S288C	Glucose	5.1	1.9	1
	Glycerol/ethanol	2.7		
Mutant pyk 1-5	Glycerol/ethanol	0.06		0.02
Transformant pyk 9.1	Glucose	99.4	4.4	19.6
	Glycerol/ethanol	22.5		

[a] One unit of pyruvate kinase activity corresponds to the oxidation of 1 μmol of NADH per min at 25 °C.

[b] Pyruvate kinase activity/activity of wild type strain.

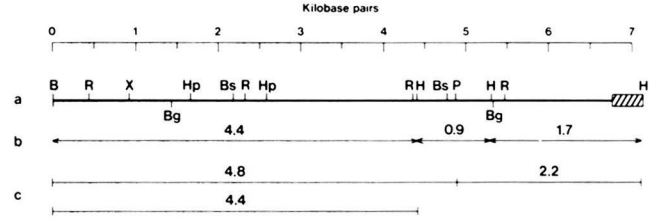


FIG. 1. **Characterization of the transforming plasmid pPYK 9.1.** *a*, partial restriction map of the yeast DNA insert in the plasmid pPYK 9.1. Restriction enzymes shown include: B, *Bam*HI; *Bg*, *Bgl*II; *Bs*, *Bst*EII; *R*, *Eco*RI; *H*, *Hind*III; *Hp*, *Hpa*I; *P*, *Pvu*II; *X*, *Xba*I. The *hatched region* designates DNA originating from the plasmid pBR322 DNA which spans the *Bam*HI site at nucleotide 375 to the *Hind*III site of nucleotide 29 (Sutcliffe, 1979). This *Bam* site was lost upon insertion of a *Sau*3A fragment into the YEp24 vector. *b*, yeast DNA fragments used to probe a Northern blot of yeast poly(A+) RNA. These fragments were generated by a *Bam*HI plus *Hind*III restriction digest of the insert DNA. *c*, DNA fragments used to test complementation of a yeast pyk mutant.

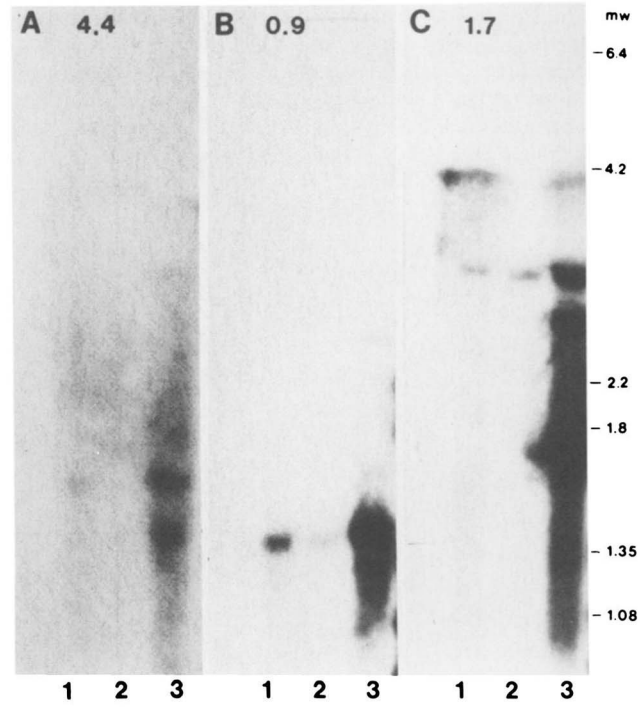


FIG. 2. **A Northern blot of yeast poly(A$^+$) RNA probed with specific fragments of the plasmid pPYK 9.1 yeast DNA insert.** The probe fragments employed are indicated in Fig. 1 and consist of a 4.4-kb *Bam*HI-*Hind*III segment, a 0.9-kb *Hind*III-*Hind*III segment, and a 1.7-kb *Hind*III-*Hind*III segment. The probes were radiolabeled to comparable specific activity. Poly(A$^+$) RNA was isolated from the yeast strains: *1*, S288C, a pyruvate kinase prototroph; *2*, pyk 1-5, a pyruvate kinase auxotroph; and *3*, pyk 9.1, the pyruvate kinase auxotroph pyk 1-5 transformed with a plamid containing the wild type gene obtained from the strain S288C. 2.5 μg of RNA were loaded on each lane of 1.0% agarose gel.

pPYK 9.1 contains a 7.0-kb yeast DNA insert. A partial restriction map of this DNA fragment is given in Fig. 1. To localize the pyruvate kinase gene we divided this insert into 3 DNA segments of 4.4 kb, 0.9 kb, and 1.7 kb determined by convenient *Hind*III restriction sites (see Fig. 1). These three segments were used as probes to a Northern blot of total poly(A$^+$) RNA from a wild type yeast strain. As shown in Fig. 2, *lanes 1*, the 4.4-kb region encodes an abundant message of 1.7 kb as well as part of a message of lower abundance (1.4 kb) which also hybridizes with the adjacent 0.9-kb region. The 1.7-kb region hybridizes to a 3.0-kb RNA. When these same

DNA fragments are used to probe poly(A$^+$) RNA isolated from the pyruvate kinase transformant, the same transcripts are observed in greater abundance, consistent with their origin from a multicopy plasmid. In addition, several other RNA species hybridized to the 1.7-kb region. These transcripts apparently originate from the plasmid copy rather than the chromosomal copy of the 1.7-kb region since they are detected only in the transformed yeast strain.

The size and relative abundance of poly(A$^+$) mRNAs homologous to the 4.4-kb fragment suggested this DNA encoded all or part of the pyruvate kinase gene. To verify this assumption the 4.4-kb *Bam*HI-*Hind*III fragment was subcloned into the YEp24 vector to test its ability to complement the pyk mutant. Two additional DNA fragments, which split the 7.0-kb region into pieces of 4.8 kb (encompassing the 4.4-kb segment plus one-half of the 0.9-kb segment) and 2.2 kb (the 1.7-kb region plus the remaining one-half of the 0.9-kb region), were also tested (see Fig. 1). As shown in Table III, the 4.4-kb fragment and the larger 4.8-kb fragment both complemented the pyk mutation with equal efficiencies; these complementation frequencies were the same as that obtained with the intact pPYK 9.1. In contrast, the 2.2-kb fragment yielded no pyruvate kinase transformants, even though the transformation efficiency (as detected by ura$^+$ complementation) was high for this DNA segment. These results, combined with the Northern analysis, indicate that the pyruvate kinase gene is encoded by a 1.7-kb abundant poly(A$^+$) RNA contained entirely within the 4.4-kb DNA fragment.

TABLE III

Efficiency of pyruvate kinase complementation by subclones of pPYK 9.1

The DNA fragments, 4.8-kb *Bam*HI-*Pvu*II, 4.4-kb *Bam*HI-*Hind*III, and 2.3-kb *Pvu*II-*Hind*III, all obtained from restriction digestion of the 7.0-kb intact insert DNA (see Fig. 1), were subcloned into the *Bam*HI site of YEp24. These plasmids were used to transform the pyk 1-5 yeast mutant. Transformants were screened separately for ura or pyk complementation.

DNA fragment	Selection	
	ura$^+$	pyk$^+$
kb	*transformants/μg DNA*	
2.2	6000	0
7.0	2952	40
4.8	88	12
4.4	1538	25

A detailed restriction map of the 4.4-kb region is displayed in Fig. 3. To locate the pyruvate kinase gene more precisely, three DNA fragments from this region, obtained from a double *Bam*HI/*Eco*RI digest (see Fig. 3), were used as probes to a Northern blot of poly(A$^+$) mRNA obtained from a wild type yeast strain. As shown in Fig. 4, the 1.8-kb internal RI DNA fragment hybridized strongly to a 1.7-kb message. The adjacent 2.2-kb fragment gave about a 10-fold attenuated signal for the 1.7-kb message and, as expected, also hybridized to the lower abundance 1.4-kb message. No homology was observed with the 0.4-kb left-hand fragment. We conclude that the pyruvate kinase mRNA is almost entirely encoded by the internal 1.8-kb DNA segment with a small overlap into the adjacent 2.2-kb RI fragment.

A DNA filter blotting analysis was performed to establish that the region around the cloned pyruvate kinase gene had not undergone rearrangement. Genomic DNA from the parental strain was digested with several different restriction enzymes, separated on a 1% agarose gel, transferred to a nitrocellulose membrane, and hybridized with α-^{32}P-labeled 4.4-kb probe. As shown in Fig. 5, predicted chromosomal DNA fragments hybridized to the probe DNA. For example, the 4.4-kb probe was complementary to the expected 1.8-kb and 2.2-kb *Eco*RI fragments as well as a large fragment which flanks the leftmost *Eco*RI site. The analysis was extended to three additional restriction enzyme digests as shown. This experiment shows that 4.8 kb of the cloned pyruvate kinase DNA, from the leftmost *Eco*RI site of the 4.4-kb fragment to the *Bgl*II site of the 1.7-kb fragment, is congruent with genomic DNA. It also establishes by direct molecular analysis that pyruvate kinase is a single copy gene in yeast *Saccharomyces cerevisiae*, a fact previously inferred from genetic experiments.

Mapping the 5′ Terminus of Pyruvate Kinase mRNA—The results of the Northern analyses localized the pyruvate kinase mRNA. To define the direction of transcription and then to determine the mRNA start site(s), we used the technique of primer extension mapping (Proudfoot *et al.*, 1980; Hagenbuchle *et al.*, 1980). Two small restriction fragments from opposite ends of the gene were chosen as primers, a 140-bp *Bst*EII-*Eco*RI fragment from the right and a 286-bp *Acc*I-BglII left-hand fragment (see Fig. 3). These were isolated and annealed to poly(A$^+$) mRNA prepared from the pPYK 9.1 yeast transformant. As a control, poly(A$^+$) RNA was annealed in the absence of primer DNA. Labeled cDNA was synthesized

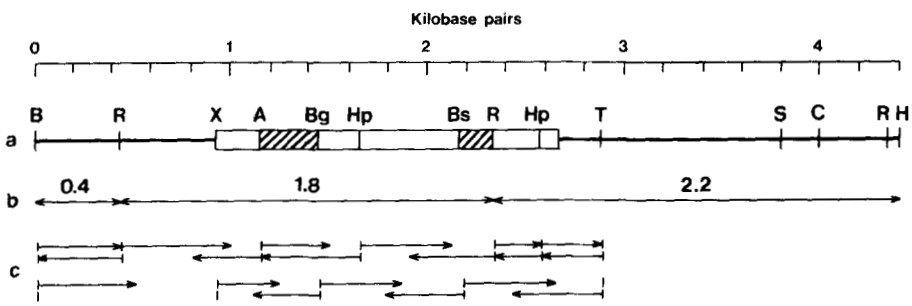
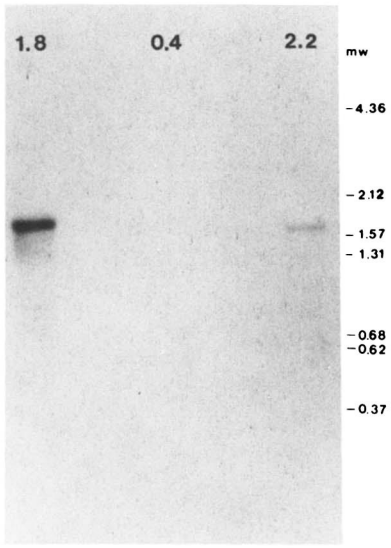
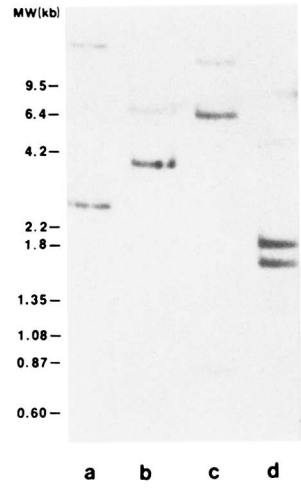


FIG. 3. **Characterization of the pyruvate kinase gene.** *a*, a partial restriction map of the 4.4-kb *Bam*HI-*Hind*III yeast DNA fragment which encodes pyruvate kinase. Restriction enzymes shown include: *A*, *Acc*I; *B*, *Bam*HI; *Bg*, *Bgl*II; *Bs*, *Bst*EII; *R*, *Eco*RI; *C*, *Cla*I; *H*, *Hind*III; *Hp*, *Hpa*I; *S*, *Sst*I; *T*, *Tha*I; *X*; *Xba*I. The actual protein coding region is enclosed in a *box*. The *hatched regions* designate those fragments used to map the 5′ end of the gene by primer extension with reverse transcriptase. *b*, DNA fragments used to probe a Northern blot of poly(A$^+$) yeast RNA. *c*, strategy used to sequence the pyruvate kinase gene. The 10 restriction fragments shown were each isolated and cloned into the M13 bacteriophage derivative plasmids pMP8 and pMP9 for sequence analysis using the method of dideoxy chain termination. 82% of the reported total of 2885 nucleotides (shown in Fig. 7) was determined from both DNA strands as indicated by the *overlapping arrows*. Those regions determined from a single DNA strand include nucleotides −431 to −113 in the 5′ untranslated region, nucleotides 740–859 within the coding region inclusive of amino acids 246–287, and nucleotides 1189 to 1266 in the coding region covering amino acids 396–422 for a total of 517 nucleotides (refer to Fig. 7 for exact locations).





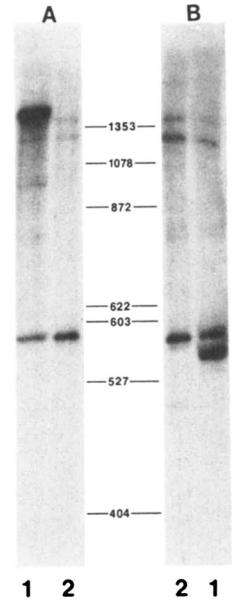


FIG. 4 (*left*). **A Northern blot of yeast poly(A$^+$) RNA probed with specific fragments of the cloned pyruvate kinase gene segment.** The DNA probes employed are derived from the 4.4-kb *Bam*HI-*Hind*III fragment and are indicated in Fig. 3. They were of comparable specific activity. The poly(A$^+$) RNA was isolated from the yeast strain S288C, a pyruvate kinase prototroph.

FIG. 5 (*center*). **Hybridization of yeast genomic DNA digested with restriction enzymes to a pyruvate kinase 4.4-kb *Bam*HI-*Hind*III DNA probe.** Genomic DNA isolated from the strain S288C was digested with: *a*, *Bst*EII; *b*, *Bgl*II; *c*, *Hpa*I; and *d*, *Eco*RI restriction endonucleases, electrophoresed on a 1% agarose gel and transferred to nitrocellulose membrane by the method of Southern (1975). The blot was probed with the 4.4-kb *Bam*HI-*Hind*III fragment of pPYK 9.1.

FIG. 6 (*right*). **Mapping the 5′ end of the pyruvate kinase mRNA by primer extension.** A 140-bp *Bst*EII-*Eco*RI fragment (*lane A1*) or a 285-bp *Acc*I-*Bgl*II fragment (*lane B1*) was used to prime pyruvate kinase cDNA synthesis catalyzed by reverse transcriptase. The products were analyzed as described under "Experimental Procedures." Control reactions containing no primer DNA (*lanes A2* and *B2*) were performed and analyzed simultaneously. Marker DNAs of the indicated size (bp) were run in an adjacent lane.

from each template by reverse transcriptase-catalyzed primer extension, and the products were sized on a polyacrylamide urea gel.

The extension of the 140-bp *Bst*EII-EcoRI fragment with reverse transcriptase yielded a predominant cDNA of 1450 ± 20 bp (Fig. 6*A*, *lane 1*); thus this primer fragment maps in the 3′ end of the gene. The 5′ end of the pyruvate kinase mRNA was mapped more precisely by using the 285-bp *Acc*I-BglII fragment as a primer (Fig. 6*B*, *lane 1*). The predominant cDNA was 545 ± 5 bp long. When no primer DNA was present, minor bands up to 1400 bp long were seen (Fig. 6, *lanes 2*). These bands could represent cDNA synthesis primed by hairpins in the RNA. These experiments indicate that there is a single major pyruvate kinase mRNA and map its 5′ terminus to position −38 to −27 (see Fig. 7).

The Nucleotide Sequence of the Yeast Pyruvate Kinase Gene—We have sequenced a total of 2885 nucleotide base pairs encompassing the yeast pyruvate kinase gene. The nucleotide sequence and the deduced protein sequence are shown in Fig. 7. The gene encodes a protein of 499 amino acids in a single open reading frame for a monomer molecular mass of 54,608 including the NH_2-terminal methionine. This size agrees very well with the value of 57,500 daltons previously determined (Aust and Suelter, 1978). The amino acid composition calculated from translation of the nucleotide sequence also corresponds closely with that measured for the isolated protein as shown in Table IV.

The amino acid sequence of yeast pyruvate kinase is specified by a highly restricted set of codons. Only 35 of the possible 61 codons are utilized and 97% of these amino acids are specified by a subset of only 26 codons (data not shown). This biased codon utilization has been observed for other highly expressed yeast genes including the two glyceraldehyde 3-phosphate dehydrogenase genes (Holland and Holland, 1979, 1980), the two enolase genes (Holland *et al.*, 1981), the triose phosphate isomerase gene (Alber and Kawasaki, 1982), and the alcohol dehydrogenase gene (Bennetzen and Hall, 1982a).

TABLE IV

Comparison of amino acid composition of yeast pyruvate kinase

Amino acid	Number of residues		Per cent of total	
	Found[a]	Reported[b]	Found	Reported
Val	47	40	9.4	8.9
Ala	40	36	8.0	8.1
Thr	38	37	7.6	8.3
Lys	37	34	7.4	7.6
Ile	37	36	7.4	8.1
Leu	36	36	7.2	8.1
Gly	34	30	6.8	6.7
Asp	32	49[c]	6.4	11.0[c]
Asn	26	49[c]	5.2	11.0[c]
Glu	28	34[c]	5.6	7.6[c]
Gln	10	34[c]	2.0	7.6[c]
Ser	27	25	5.4	5.6
Pro	26	23	5.2	5.1
Arg	25	21	5.0	4.7
Tyr	15	14	3.0	3.1
Phe	15	13	3.0	2.9
Met	11	7	2.2	1.6
His	7	6	1.4	1.3
Cys	7	5	1.4	1.1
Trp	1	1	0.2	0.2

[a] Number deduced from the nucleotide sequence reported here for a protein of 54,608 daltons.

[b] Number determined from isolated yeast protein by Yun *et al.* (1976) based on a molecular mass of 52,500 daltons.

[c] Sum of acid and amide forms.

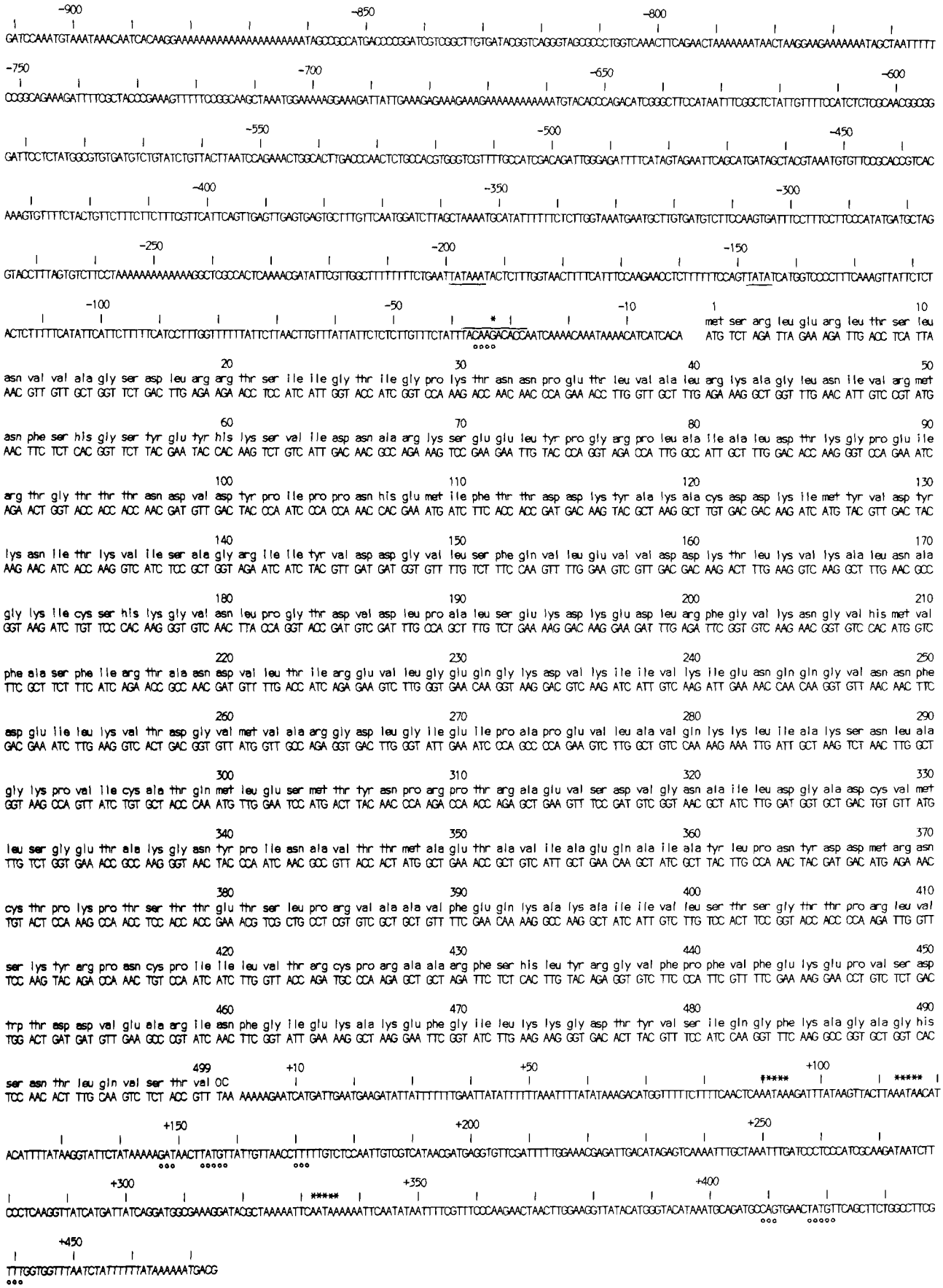


FIG. 7

The 5′ and 3′ Noncoding Regions of the Yeast Pyruvate Kinase Gene—The 911 nucleotides 5′ to the pyruvate kinase initiation codon show an overall A+T composition of 64% and contain many purine (predominantly A) and pyrimidine tracts of varying length. A-rich purine tracts of 26, 26, 18, 27, and 15 bases are found at positions −886, −790, −708, −685, and −256 (Fig. 7). The 2-14-base region proximal to the initiating ATG may be divided into two domains. The region from −214 to −38 is very pyrimidine rich (74%), and of these nucleotides 73% are T, usually arranged in short (less than 10 bases) T tracts. The 37-base domain immediately adjacent to the initiating ATG is very A rich (59%) and contains only a single G. An A is found at position −3 as has been seen so far in all sequenced yeast genes (Kozak, 1981; Dobson *et al.*, 1982). A TATA sequence, believed to be important in positioning transcription initiation sites by RNA polymerase II (Grosschedl and Birnstiel, 1980; Fay *et al.*, 1981; McKnight and Kingsbury, 1982), is located at −199 and at −148. The PuCACACA sequence found in several yeast genes about 20 bp upstream of the translation start site (Dobson *et al.*, 1982) is not found in the pyruvate kinase gene. The first ATG upstream of the proposed pyruvate kinase initiation codon is found at −142. Dobson *et al.* (1982) postulated that a CT block larger than 20 nucleotides followed by the sequence CAAG within 15 bases is characteristic of yeast genes that encode abundant mRNAs. The CAAG found at −36 is 1 base away from the very long CT-rich domain described above. The related sequence CAAAG is found at −130 in the same domain. CAAG is present 3 other times in the 5′ untranslated region (at −309, −714, and −887) but is unassociated with a CT-rich region (more than 15 bases away) or is associated with a very short (less than 10 bases) CT tract.

Downstream of the TAA translation termination codon an additional 474 bp have been sequenced. Like the 5′ untranslated region, this region is also A-T rich (77% A+T). The sequence AATAAA has been postulated to specify a site for polyadenylation in higher eucaryotes (Proudfoot and Brownlee, 1976; Fitzgerald and Shenk, 1981); many of the sequenced yeast genes show a similar sequence (Bennetzen and Hall, 1982a). In the yeast pyruvate kinase gene the sequences AAATAAA, AAATAA, and AATAAA are found at +89, +112, and +332, respectively. The sequence TAG....TATGT or TAGT..(A-T rich)..TTT... has been postulated by Zaret and Sherman (1982) to be a signal for transcription termination/polyadenylation. Two sequences similar to this consensus sequence are found at positions 147 GAT....TATGT....11 bases....TTT and +410 CAG....TATGT...17 bases...TTT of the 3′ untranslated region of the pyruvate kinase gene.

A computer search has been made for inverted repeats and direct repeats in the 5′ and 3′ untranslated regions of the pyruvate kinase gene. Using 10 bases as a minimum length and eliminating those elements which arise due to the biased base composition of these regions, only one was considered to be of possible significance. This 10-base direct repeat, TGTGATGTCT, is found at −578 and −321 in the 5′ untranslated region.

DISCUSSION

The yeast pyruvate kinase gene has been isolated by complementation of a pyk mutant, cloned, and sequenced. The isolated gene is pyruvate kinase based on: 1) functional complementation of the pyruvate kinase deficiency by the intact yeast DNA plasmid and select subclones; 2) elevated pyruvate kinase activity in the transformant consistent with the expression of the pyruvate kinase gene from a multicopy plasmid; 3) good agreement between the previously reported amino acid composition and molecular weight for the yeast enzyme with that deduced here from the nucleotide sequence, 4) the occurrence of a carboxyl-terminal valine residue as reported; 5) biased codon usage characteristic of highly expressed yeast glycolytic genes; and 6) correspondence between the amino acid sequence of a peptide at the active site of bovine pyruvate kinase and a region of the yeast enzyme.

Comparisons of Protein Sequence for Pyruvate Kinases—Previous workers have determined that yeast pyruvate kinase has an acetylated NH_2 terminus and a valine residue at the carboxyl terminus (Hess and Sossinka, 1974); no other protein sequence data for the yeast enzyme is available. However, a peptide residue from rat liver pyruvate kinase, composed of 20 amino acids at the phosphorylation site of a cyclic 3′,5′-AMP-dependent protein kinase, has been sequenced (Edlund *et al.*, 1975). These residues include a corresponding smaller hexapeptide isolated from the pig liver enzyme, Leu-Arg-Arg-Ala-Ser-Leu (Hjelmquist *et al.*, 1974). The array Leu-Arg-Arg-Thr-Ser-Ile, which occurs at amino acids 18–23 in the yeast enzyme, may represent a similar conserved phosphorylation site. The remaining 14 residues at this site show no correspondence. An additional comparison can be made for an 8-residue polypeptide chain from rabbit muscle pyruvate kinase where the sequence Ile-Ile-Gly-Arg-Cys-Asn-Arg-Ala was determined (Anderson and Randall, 1975). The sequence Arg-Cys-Pro-Arg-Ala located at amino acids 424–428 for the yeast enzyme resembles this rabbit enzyme sequence.

A 34-residue peptide surrounding an essential lysine has been sequenced from bovine muscle pyruvate kinase (Johnson *et al.*, 1979). Trinitrophenylation of the amino group of this lysine which is located at or near the ADP-binding site results in inactivation of enzyme activity (Johnson *et al.*, 1979). The sequence reported, Ala-Glu-Gly-Ser-Asp-Val-Ala-Asn-Ala-Val-Leu-Asp-Gly-Ala-Asp-Cys-Ileu-Met-Leu-Ser-Gly-Glu-Thr-Ala-Lys-Gly-Asp-Tyr-Pro-Leu-Glu-Ala-Val-Arg, corresponds impressively closely to a 34-residue stretch of the yeast enzyme from amino acids 313–346 which reads Ala-Glu-Val*-Ser-Asp-Val-Gly*-Asn-Ala- Ile*-Leu-Asp-Gly-Ala-Asp-Cys-Val*-Met-Leu-Ser- Gly-Glu-Thr-Ala-Lys-Gly-Asn†-Tyr-Pro-Ile*-Asn†-Ala-Val-Thr†. Of these 34 residues, 26 are identical, 5 are conservative substitutions (marked with asterisks), and 3 are nonconservative changes (marked with daggers). Thus, this catalytically important region is highly conserved between the two very divergent species. Although an x-ray crystallographic analysis of cat muscle pyruvate kinase reveals structure to 2.6 resolution and shows a monomer chain length of 500 amino acids (Levine *et al.*, 1978), no protein sequence data for this enzyme exists. In view of the ADP-binding site homology reported here between the yeast and bovine enzymes, the protein sequence derived from the yeast gene should aid in an analysis of the x-ray data and an elucidation of the catalytic mechanism for pyruvate kinase.

The yeast pyruvate kinase gene shows a strong bias for the 25 "preferred codons" derived from an analysis of the codon

FIG. 7. **The DNA sequence of the yeast pyruvate kinase gene.** The complete sequence of the pyruvate kinase structural gene as well as 1385 nucleotides of flanking DNA is presented. The sequence is shown only for the coding strand of the gene. Specific features of the 5′ flanking sequence are noted as follows: the "TATA" consensus sequences are *underlined*; the mapped pyruvate kinase mRNA start site is indicated by an *, the *overline* reflects the experimental error; a "CAAG" sequence in indicated by *underdots*. Specific features of the 3′ flanking sequence are noted as follows: the proposed consensus polyadenylation signals "AATAA" are denoted with *asterisks*; the proposed consensus polyadenylation/termination signals "TAG...TATGT...TTT" are denoted by *underdots*.

usage of 7 sequenced yeast genes (Bennetzen and Hall, 1982b). The 25 preferred codons are known, in most instances, to complement the anticodons of the major isoaccepting tRNA species. A positive correlation has been noted between yeast protein abundance (mRNA abundance) and degree of bias in codon usage (Bennetzen and Hall, 1982b). Whether a highly favorable codon bias is a necessary condition for high level gene expression in yeast remains to be determined. We note that we were greatly aided with our early analysis of the location of the pyruvate kinase gene on the DNA sequence by the fact that highly expressed yeast genes utilize such a restricted set of codons. We found that the probability that a given open reading frame across a DNA segment was correct could be readily evaluated based on codon preference.

3′ and 5′ Untranslated Sequences of Yeast Pyruvate Kinase Gene—The 3′ and 5′ untranslated regions of the gene for yeast pyruvate kinase shows common sequence signals previously defined by comparisons among sequenced yeast and higher eucaryotic genes. The proposed polyadenylation signal AATAA(A) found 20–30 nucleotides upstream of the poly(A) addition site in yeast genes and in higher eucaryotic genes (Bennetzen and Hall, 1982a; Proudfoot and Brownlee, 1976) is repeated three times in the yeast pyruvate kinase 3′ flanking sequence. The consensus sequence TAG...TAGT or TATGT..(A-T rich)...TTT found in 3′ flanking region of several yeast genes has been postulated (Zaret and Sherman, 1982) to be important in transcription termination and polyadenylation. Two related sequences are found in the pyruvate kinase 3′ flanking sequence at positions 147–172 and 410–441. Since the 3′ flanking sequences of many yeast genes show both of these consensus sequences, further work is required to define the role of each in transcription termination and polyadenylation.

The TATA sequence found in the 5′ untranslated region of eucaryotic genes is believed to function in positioning mRNA start sites and may mediate the efficiency of transcription initiation as well (Grosschedl and Bernstiel, 1980; Faye *et al.*, 1981; McKnight and Kingsbury, 1982). Two such sequences TATAAA and TATAT are found in the yeast pyruvate kinase gene about 160 and 110 bp, respectively, upstream of the mapped pyruvate kinase mRNA start site. A positive correlation has been noted between the level of expression of yeast genes and the pyrimidine content of sequences between the TATA sequence and the A-rich region immediately adjacent to the initiation ATG (Montgomery *et al.*, 1980). It has been proposed that the presence in this region of a pyrimidine block and the sequence CAAG about 20 bp downstream correlated with a high level of expression (Dobson *et al.*, 1982). The pyruvate kinase gene sequence has a CAAG downstream of a CT block in this region. We observe that the single pyruvate kinase mRNA start site maps to this CAAG sequence. A compilation of the mapped 5′ ends for several yeast genes is shown in Fig. 8. For the highly expressed glycolytic genes in particular, the mRNA start maps to the sequence CAAG. Considering the 5′ ends of other mapped yeast mRNAs as well, we can define a consensus sequence PyAAPu at or very near to transcription start sites. This sequence may act in concert with the TATA in fixing mRNA start sites. The pyrimidine block may function in mediating the level of transcription as previously suggested (Montgomery *et al.*, 1980; Dobson *et al.*, 1982) and not interact directly with PyAAPu). In this regard, the mating type, MAT α2 gene, has 2 mRNA starts, each of which maps to a sequence PyAAG, but has no C-T block within 70 nucleotides. It should be noted that the mRNA start for the actin gene does not map to a PyAAG (Gallwitz *et al.*, 1981). Rather, it has been mapped to a C in the middle of a CT block 20 nucleotides upstream of a CAAG.

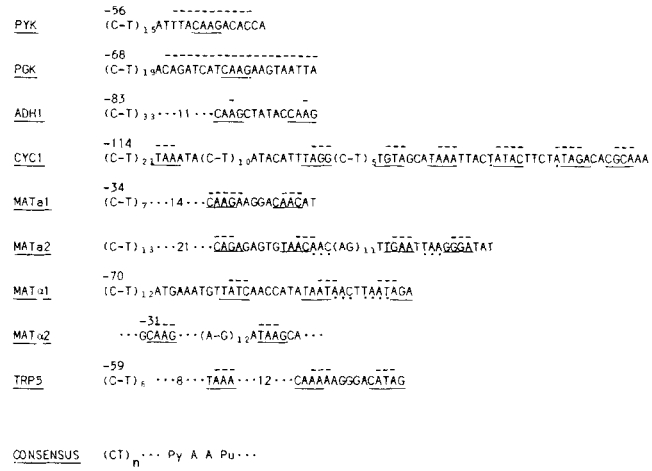


FIG. 8. **A comparison of mRNA start site(s) for several yeast genes and a postulated consensus sequence.** The sequences presented are all for the coding strand of DNA; numbering is upstream from the initiating ATG. *Dashed lines* above the sequence represent mapped mRNA start site(s); their length reflects reported error. *Solid underlining* represents sequences used to determine the consensus sequence; *underdots* represent sequences which further increase the significance of the postulated consensus sequence. The 5′ end of pyruvate kinase mRNA was determined by sizing of reverse transcriptase extended fragment. The 5′ ends of the PGK (Dobson *et al.*, 1982), ADH1 (Bennetzen and Hall, 1982a), CYC1 (Faye *et al.*, 1981), MAT (Nasmyth *et al.*, 1981), and TRP5 (Zalkin and Yanofsky, 1982) mRNAs were determined by sizing of S1-protected fragments.

This mRNA may be unusual; most yeast mRNAs initiate with a purine.

The role of 3′ and 5′ flanking sequences in modulating the level of gene expression in yeast is as yet not well understood. There is little evidence that 3′ flanking sequences play a major role in determining the level of expression. A *cyc1* mutant with a 38-bp deletion in the 3′ flanking sequence makes 10% as much iso-1-cytochrome *c* mRNA as does the wild type. It is not clear if this reduction in the level of expression of this gene is a consequence of faulty termination or is the result of convergent transcription from an adjacent opposing gene (Zaret and Sherman, 1982). Complete substitution of the 3′ flanking region does not result in large differences in the efficiency of expression of at least one yeast gene (Guarente and Ptashne, 1981). Also, yeast genes, which differ 500-fold in levels of expression do not show obvious differences in their 3′ flanking sequences.

The importance of 5′ flanking sequences in modulating gene expression at the level of transcription is more established (Rose *et al.*, 1981; Guarente and Ptashne, 1981; Faye *et al.*, 1981; Struhl, 1981). However, here too the relationship between structure and the level of transcription is poorly understood. Experiments in both higher eucaryotes and yeast suggest that 5′ regions upstream of the TATA are important in modulating the level of transcription (Benoist and Chambon, 1981; Faye *et al.*, 1981; Montgomery *et al.*, 1982). Generally, the 5′ flanking regions of yeast genes are A-T rich and organized in alternating purine-pyrimidine rich stretches. The glycolytic genes including the pyruvate kinase gene are extreme in this biased sequence organization. Furthermore, for the pyruvate kinase gene this biased sequence organization, in particular the presence of long A-rich purine tracts, persists throughout the 922 bases 5′ to the initiating ATG. The involvement of such distal sequences in chromatin organization, regulation, and level of transcription is unknown.

It is clear that more work is needed to define precisely the role of 5′ and 3′ flanking sequences, codon usage, and other

factors in mediating the level of gene expression in yeast. The cloned pyruvate kinase gene system is suitable for further investigation of these questions.

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The isolation, characterization, and sequence of the pyruvate kinase gene of Saccharomyces cerevisiae.

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