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Yeast adenylate kinase is transcribed constitutively from a promoter in the short intergenic region to the histone H2A-1 gene

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Yeast mitochondrial adenylate kinase (high molecular mass form, gene locus: AKY2) is encoded on chromosome IV of the same DNA strand as histone H2A-1. The nontranslated intergenic region spans 560 bp, the nontranscribed spacer can be estimated to comprise at most 300 bp. The TATA-box sequence is contained in a striking environment consisting of 20 alternating pyrimidines and purines. The AKY2 transcript is made constitutively: (i) the cellular mRNA concentration does not vary significantly with either growth conditions or elapse of the cell cycle; (ii) β-galactosidase activity is about constant in yeast cells grown on various carbon sources after transformation with AKY2-promoter/lacZ fusions; (iii) primer elongation analysis shows that utilization of 5 initiation sites is qualitatively and quantitatively independent of the growth conditions and the carbon source used; (iv) Western blot analysis and adenylate kinase activity measurements indicate the absence of post-transciptional controls as well.

Mitochondrial adenylate kinase; Constitutive promoter; Histone H2A1 intergenic region; Promoter/lacZ fusion; Nucleotide sequence; (*Saccharomyces cerevisiae*)

1. INTRODUCTION

The yeast genome is much more densely packed with transcribed genetic information than that of vertebrates [1–3] and frequently encodes, in close vicinity, enzymes of metabolically unrelated function (e.g. [4–6]). To prevent uneconomic simultaneous expression of these genes in spite of sometimes very short intergenic distances in yeast, distinct mechanisms must exist which guarantee individual gene control. In recent years much work has been done on the elucidation of various types of regulated gene activation. The majority of the yeast genes is, however, transcribed constitutively [2,3]. Little is known about sequences and proteins involved in the expression of these genes. Only a limited number of promoters of constitutive genes (e.g. those of PET56, HIS3 and DED1 [6–9]) have been analyzed in greater detail as yet.

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The genes for the histones H2A-1 and H2B-1 (HTA1 and HTB1, respectively) are clustered on chromosome IV of the yeast genome [10,11]. Their transcription is correlated with the progression of the cell cycle and turned on for only a short period of time at the beginning of the S-phase [12–15]. Here we report that the gene for the mitochondrial isozyme of adenylate kinase (AKY2) is arranged in tandem 3′ of the HTA1 gene at the rather short distance of 560 bp. The nucleotide sequence of AKY2 [16] and the subcellular location of the encoded protein [17] have been reported recently. The AKY2 transcript is present in the cell at a concentration comparable to that of each of the histone messengers. Its synthesis is, however, neither influenced by the cell cycle nor by the carbon source offered in the growth medium. A regulation of the latter kind would be expected if the enzyme were engaged preferentially in the oxidative metabolism. We were able to show by Northern blotting, by β-galactosidase assays of yeast transformants harbouring AKY2 promoter/lacZ fusions and by fragment elongation using murine

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reverse transcriptase and total RNA from differently grown cells as template, that yeast adenylate kinase (AKY2) is transcribed constitutively from one single TATA sequence using 5 main initiation sites.

2. MATERIALS AND METHODS

2.1. *Strains, growth conditions, cellular subfractionation and enzyme assays*

For preparation of cellular subfractions, cells of the haploid wild-type strain D273-10B (ATCC 25657) were grown in a medium (1% yeast extract) containing either 6% glucose, 2% galactose, 2% glycerol plus 0.5% ethanol, or 2% lactate, respectively, until a titer of 2×10^7 cells/ml was reached. Preparation of subcellular fractions from lysed spheroplasts and further purification of mitochondria by centrifugation on 28% Percoll gradients (Pharmacia, Freiburg) have been described. Mutual contamination of the fractions could be excluded as judged from routinely assayed marker enzymes [17,18]. The adenylate kinase reverse reaction was measured by assaying Ap5A-sensitive formation of ADP as described [17]. Adenylate kinase and hexokinase protein was detected after SDS-polyacrylamide gel electrophoresis by electroblotting and immune decoration [19]. To measure β-galactosidase activity in yeast transformants, the cells were homogenized with glass beads, incubated with the chromogenic substrate *o*-nitrophenyl β-galactoside and centrifuged before recording optical density as reported by Guarente and Ptashne [20]. Protein was determined by the BCA reagent (Atlanta/Pierce Biochemicals, Heidelberg) using the protocol of the manufacturer.

2.2. *Preparation of RNA and blotting procedures*

RNA was prepared from cells harvested at a titer of 2×10^7 cells/ml. Cells were homogenized by hand-shaking with glass beads in the presence of 4 M guanidinium isothiocyanate. Debris and nuclei were removed (45 000 × *g*, 30 min) and the RNA purified by centrifugation through a cushion of CsCl ($n = 1.408$) [21]. Primer elongation was performed by annealing a denatured, end-labeled 45 bp *Hin*fI/*Rsa*I fragment (position 16 to 60 of the AKY2 coding sequence [16]) to total RNA and elongating the 3'-end of the DNA primer by murine reverse transcriptase (Pharmacia-PL, Freiburg) as described [22].

2.2.1. Northern blotting

After agarose (1.5%) gel electrophoresis the glyoxylated RNA was transferred to Gene Screen (New England Nuclear, Dreieich) by capillary blotting [23] and hybridized to a nick-translated, denatured 355 bp *Hin*dII fragment in the presence of 50% deionized formamide, 5 × Denhardt's, 0.2% SDS and 5 × SSC at 42°C for 16 h. The probe contained exclusively coding sequences. For estimating the approximate position of the 3'-end of the H2A-1 transcript, both a 247 bp *Nsi*I/*Taq*I and a 380 bp *Aha*III/*Taq*I fragment were used as probes in Northern blot hybridization. The relevant restriction sites are indicated in fig.3.

2.3. *Construction of lacZ fusions*

A 985 bp *Pvu*II/*Rsa*I fragment derived from pUHC3-12 [16], containing 3'-terminal sequences from the HTA1 gene, the entire HTA1/AKY2 intergenic region and the first 60 bp of the AKY2 gene, was inserted, in frame with the lacZ gene, into the unique *Sma*I site of plasmid pMC1871 lacking a promoter and a start codon [24] yielding plasmid pAKY/lacZ. In the transition region, the correct reading frame was verified by DNA sequencing. For yeast transformation [25] the construct was excised with *Sal*I, inserted into the unique *Xho*I site of the shuttle vector YEp13 and transferred into the yeast strains AH215 *MATa, leu2-112, his3* or TO2 *MATa, leu2-112, hem1-1* [21]. DNA sequences were determined by the chain termination procedure using supercoiled double-stranded template DNA, as described by Chen and Seeburg [26].

3. RESULTS

The nucleotide sequence of the yeast adenylate kinase gene (high molecular mass isozyme, gene locus AKY2) has been published recently [16]. Subsequently, the encoded protein was found to occur in the active form both in mitochondria and in the cytoplasm. Haploid disruption mutants were shown to lack the protein simultaneously in both compartments and to have a pet phenotype (no growth on nonfermentable carbon sources) [17] implying that the encoded gene product plays a vital role in the oxidative metabolism. Therefore one may suspect that the expression of this enzyme is regulated, in co-ordination with respiratory functions in a carbon source-dependent manner. In order to test this hypothesis, two sets of experiments were performed. In the first approach, RNA was isolated from cells grown on various carbon sources and analyzed on a Northern blot as indicated in fig.1A. The figure shows an AKY2-specific transcript of about 1000 bases in RNA isolated from wild-type cells grown on lactate (fig.1A, lane 6), on glucose (lane 5), on galactose, anaerobically (lane 4) or aerobically (lane 3). The results indicate that the cellular pool of mRNA from the AKY2 gene is not significantly influenced by the carbon source. No gross differences in transcript concentration were observed also when the RNA was prepared from heme-deficient DczH1-1B cells, grown on galactose in either the absence (lanes 1 in fig.1A and B) or the presence (lanes 2) of deuteroporphyrin IX, an analogue of the heme precursor protoporphyrin IX. On the one hand, this substance cannot be attached covalently to hemoproteins and, consequently, prevents the formation of respiratory competent cells. On the other hand, this effector is capable of inducing transcription of heme-controlled genes [27]. In

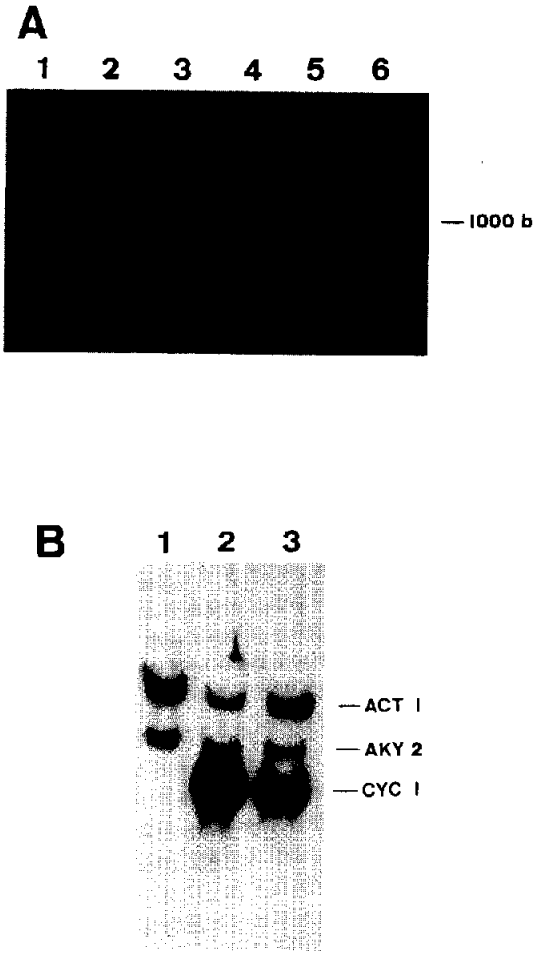

Fig. 1. Autoradiograms of blots of total RNA isolated from yeast cells grown under various conditions. (A) RNA probes with an AKY2-specific 355 bp *Hin*dII fragment. Lanes:1, strain DczH1-1B heml-1, grown on galactose in the absence of heme; 2, same strain as in lane 1, 3 h after supplementation with 10 μM deuteroporphyrin IX; 3, AH215 wild-type grown aerobically on galactose; 4, same strain as in lane 3, grown anaerobically; 5, same strain as in lane 3 grown on glucose; 6, same strain as in lane 3 grown on lactate. (B) RNA hybridized simultaneously with probes from AKY2, actin 1 and iso-1-cytochrome *c*. Lanes: 1, strain DczH1-1B heml-1, grown on galactose in the absence of heme; 2, DczH1-1B heml-1, 3 h after supplementation with 10 μM deuteroporphyrin IX; 3, RNA from AH215 wild-type cells grown on lactate. RNA sizes are indicated.

fig.1B, mRNA concentrations are standardized by internal controls. In addition to an AKY2-specific nick-translated fragment, in this experiment the blot was hybridized simultaneously to probes derived from the actin gene (as a constitutive gene) and from the iso-1-cytochrome *c* gene (as a heme-controlled gene). No significant variation in the concentration of AKY2-specific transcripts relative to the constitutively transcribed actin message could be detected, whereas the mRNA of the CYC1 gene is below the level of detection in heme-free cells (fig.1B, lane 1), but present in heme-proficient (lane 2) and oxidatively grown cells (lane 3). Fig.1A and B suggests that the transcription of AKY2 is independent of the carbon source offered in the growth medium and, at most, marginally influenced by effectors like heme and oxygen.

The second approach to examine whether AKY2 expression is regulated involved promoter/lacZ fusions and yeast transformation. The AKY2 upstream region, including the 20 N-terminal codons of the gene, was ligated in frame with the coding sequence of the lacZ gene from *E. coli* which lacked the authentic N-terminus. When yeast cells, transformed with this construct, pAKY/lacZ, were grown in media supplemented with various carbon sources and then assayed for β-galactosidase, the activities differed from one metabolic condition to the other by a factor of less than 2 (table 1A). Also no significant influence of oxygen (not shown) or heme could be detected. The latter was examined in the heme-deficient mutant strain TO2 after transformation with the same fusion plasmid without or with supplementation by a heme analogue or heme precursor (table 1B). The apparent slight induction observed, which is much less than found with genes controlled by heme, is likely to be caused by a general metabolic activation after the supplementation with δ-aminolevulinate resulting, e.g., also in an enhanced expression of genes like *LEU2* and

Table 1

β-Galactosidase activity of yeast transformed with AKY2 promoter/lacZ fusions (units per ml [20])

A	Glucose	Galactose	Glycerol
AH215-AKY/lacZ	215.6	237.3	233.5
B	**Galactose -DIX**	**Galactose +DIX**	**Galactose +ALA**
TO2-AKY/lacZ	148.1	294.2	244.0

A, wild-type transformants grown on various carbon sources; B, transformants of the heme-deficient strain T02 heml-1, grown in the absence or presence of inducer

URA3 (not shown). The increase with deuteroporphyrin IX is due to its interference of the colour of this substance with the β-galactosidase test. It is concluded from the experiments that lacZ expression from the AKY2 promoter is constitutive.

The finding that displacement of the gene for AKY2 by a nonfunctional allele resulted in a viable haploid progeny which was incapable of utilizing nonfermentable carbon sources and, thus, had a pet phenotype despite their ability to respire [17] suggested an essential role of the gene product in nonfermentative growth. Since no control of AKY2 expression by glucose repression could be detected, the existence of post-transcriptional controls of the amount and of the activity of the enzyme were examined by immune decoration (fig.2) and by enzyme activity tests (table 2), respectively. The Western blot experiments with mitochondrial (odd-numbered lanes in fig.2) and cytoplasmic (even-numbered lanes) protein from differently grown cells reveal: (i) adenylate kinase occurs in mitochondria and, the bulk, in the cytoplasmic fraction. The two differently located forms (molecular mass of about 24 kDa) exhibit electrophoretic mobilities which cannot be distinguished by SDS-polyacrylamide gel electrophoresis. (ii) Also the concentration of cross-reacting adenylate kinase protein does not vary significantly in either the cytoplasmic or mitochondrial fractions in a carbon source-dependent manner. (iii) The ratio of the imported to the nonimported material is nearly independent of the carbon source indicating that also the import into mitochondria of adenylate kinase is not influenced by the growth conditions. The slightly fainter signal in mitochondria from glucose-grown cells is likely to be due to a higher fragility of the organelles under these conditions. The blot was simultaneously decorated with an antibody against hexokinase. The absence of a signal for hexokinase from mitochondria proves that the signal for the high molecular mass adenylate kinase in mitochondria is not caused by a cytoplasmic contaminant.

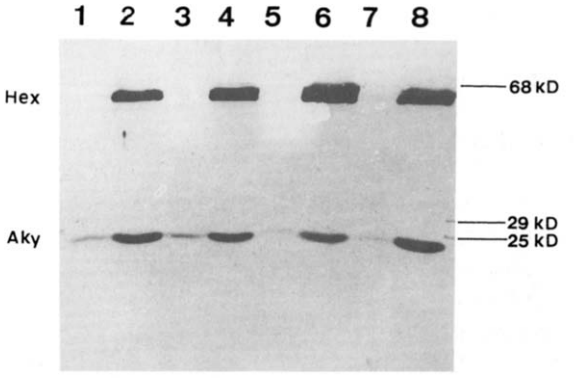

Fig. 2. Western blot analysis of subcellular fractions from cells grown on various carbon sources. Odd-numbered lanes show mitochondrial proteins, even-numbered lanes cytoplasmic proteins. Cells analyzed in lanes 1 and 2 were previously grown on lactate, in lanes 3 and 4 on glycerol, in lanes 5 and 6 on galactose and in 7 and 8 on 6% glucose. The blot was reacted simultaneously with antisera directed against hexokinase and mitochondrial adenylate kinase, immune decorated with peroxidase-coupled protein A and visualized by the peroxidase reaction. Molecular mass standards used were bovine serum albumin (67 kDa), carbonic anhydrase (28 kDa) and chymotrypsinogen (25 kDa). Their positions together with those of hexokinase (Hex) and adenylate kinase (Aky) are marked.

The enzymic activity of adenylate kinase was assayed in subcellular fractions from the same cells as above. The results, summarized in table 2, show that also the specific activity of the enzyme (measured as Ap5A-sensitive formation of ADP) does not vary strongly in the different fractions with relation to the carbon source. This indicates that the mitochondrial adenylate kinase isozyme is also not regulated at the enzyme level.

As a first approach to analyze the constitutive AKY2 promoter the 5'-upstream region of the gene

Table 2

Adenylate kinase activity of wild-type yeast grown on various carbon sources (μmol/mg · min)

	Glucose	Galactose	Raffinose	Glycerol	Lactate
Cytoplasm	3.01	2.29	2.19	2.93	2.62
	(75.9)	(49.8)	(45.4)	(41.6)	(78.0)
Mitochondria	0.41	0.34	0.40	0.39	0.38

In parentheses, total adenylate kinase activity (μmol/min · g wet wt cells)

was sequenced. The results revealed that the gene is preceded, at the relatively short distance of 560 bp, by the histone H2A-1 gene (fig.3). The sequence of the latter (up to position −503 in fig.3) [28] and that of AKY2 (3′ of position −162) [16] have been published. Before defining *cis*-activating regions of AKY2 it was essential to identify the 3′-end of the H2A message. On the 3′-side of the HTA1 gene a canonic termination signal [16] is found 142 bp downstream of the stop codon. To approximate the position of the 3′-end of the HTA1 transcript two Northern blots of total yeast RNA were hybridized to two different downstream DNA fragments. One of these, the positive control, extended from the *Aha*III site shortly 3′ of the HTA1 stop codon to the *Taq*I site, 5′ of the AKY2 TATA box (fig.3). The other contained sequences spanning the DNA from 3′ of the *Nsi*I site at the termination signal to the same *Taq*I site as above. As both fragments hybridize to the H2A1 messenger (not shown), it is evident that the 3′-end of the transcript extends considerably beyond the termination signal. It has been reported for the transcripts of several yeast genes, e.g. AKY2, PFY, ACT1 and CYC1, that their 3′-untranslated ends are quite long and may extend even more than 150 bases beyond the termination signals [22,30–32].

To narrow down further the region potentially containing sequences required for the permanent activation of the AKY2 gene, the 5′-ends of AKY mRNAs were determined. Apart from two separate TATA-like sequences upstream of AKY, no obvious promoter elements could be detected on the basis of sequence homology with known upstream control sites like those used to mediate cell cycle control, heme-dependence, galactose-induction or glucose catabolite repression of transcription [33]. Fig.3. depicts five major transcription initiation sites which were deduced from an experiment shown in fig.4. All major start sites are constituted of A residues. The most upstream of these is found at position −60, a minor one even at −70. Together with the presumed approximate position of the 3′-end of the HTA1 mRNA this reduces the nontranscribed intergenic region to less than 300 bp which are likely to harbour all signals necessary for transcription initiation of the AKY2 gene. None of the initiation sites coincides with the 5′-end of the cDNA sequence of adenylate kinase which was reported recently [30]. Since both the 5′-

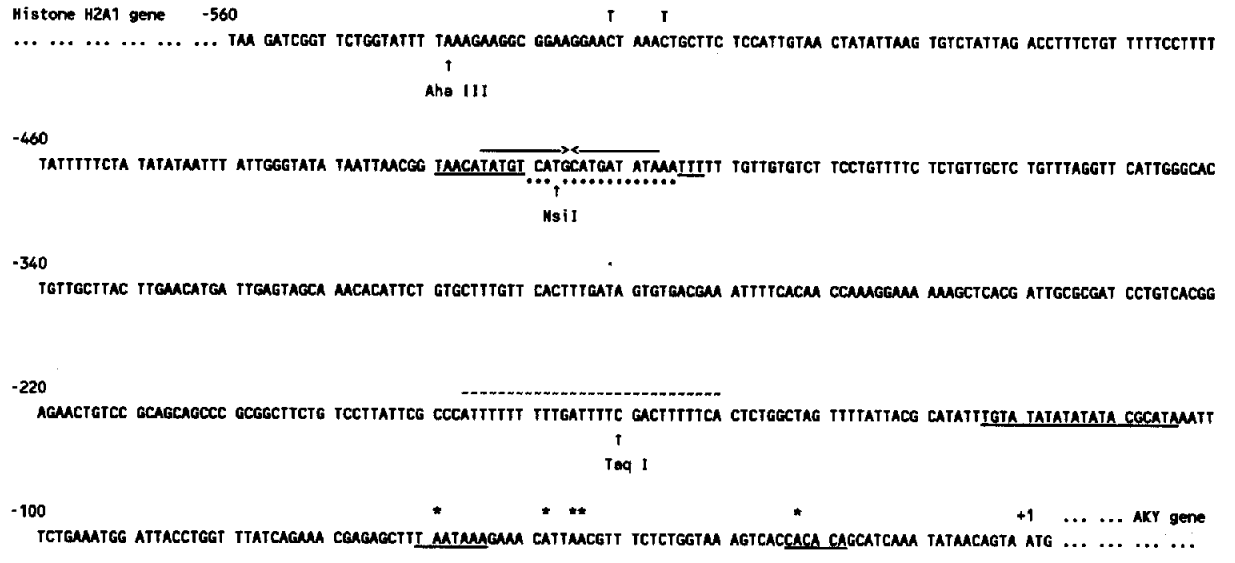

Fig. 3. Nucleotide sequence of the noncoding intergenic region between the histone H2A1 and the AKY2 genes. Two deviations from sequences published from the histone 3′-flank are marked. A possible termination signal for histone H2A1 mRNA, two TATA-like sequences and a CAPyACA box found in the leaders of frequently expressed genes [29] are underlined. Horizontal inverted arrows mark a palindromic sequence, and a wavy line emphasizes a T-rich sequence. Initiation sites for AKY2 transcription (*) and restriction nuclease cleavage sites used (↑) are indicated.

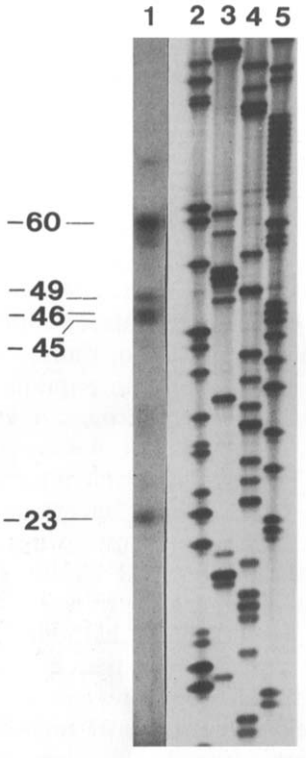

Fig. 4. Fragment elongation to map 5'-ends of AKY2-specific transcripts. An end-labeled denatured 45 bp *HinfI/RsaI* fragment was annealed to total RNA from lactate-grown yeast cells and elongated by murine reverse transcriptase (lane 1). Lanes 2–5, ladder of known nucleotide sequence. 5'-ends are indicated.

and the 3'-end sequences of the cDNA diverge from the genomic sequence [16], they are unlikely to represent genuine ends of an AKY transcript.

Fig.4, lane 1, shows the mRNA start sites used in derepressed cells. No gross qualitative or quantitative differences can be detected in comparison to the sites used for mRNA initiation in glucose-grown cells (not shown). This confirms the results shown in fig.1 and tables 1 and 2 and underlines that, although being an enzyme that occurs in mitochondria, AKY2 is transcribed to a similar extent under both repressed and derepressed conditions.

4. DISCUSSION

Information about *cis*- and *trans*-acting elements involved in constitutive expression is scarce as compared to regulated promoters (see e.g. [7–9]), although the majority of yeast genes is not regulated. It has been noted by Struhl [7] that, in a variety of aspects, constitutive promoters differ from inducible ones. For example the TATA element responsible for constitutive background transcription of HIS3 as well as the PET56 TATA box were found to deviate from the canonic sequence, TATAAA, of regulated genes in one position (usually the fifth base). A sequence satisfying these prerequisites is also found in front of AKY2. In this case it is particularly remarkable, since it consists of 20 alternating pyrimidines and purines with an AT-rich core, $(AT)_6$. The fact that all AKY2-specific mRNAs start at sites which agree with the consensus distance [8] to the most upstream TATA box, but are too close to the downstream one, supports the conclusion that only the upstream TATA-like element, consisting of alternating purines and pyrimidines, is active in transcription initiation.

Apart from the TATA sequence, the untranslated intergenic region of 560 bp appears to contain additional elements required for efficient transcription initiation. Among these is a block of poly(dA-dT) sequences (position −153 to −177; fig.3). Similar elements are found in front of other constitutive genes such as PET56 [7] and DED1 [5].

It is unexpected to find that yeast mitochondrial adenylate kinase is expressed constitutively for two reasons: (i) oxidative phosphorylation occurs at high rates only in derepressed yeast mitochondria. No obvious need for phosphorylation of AMP by mitochondrially made ATP is apparent in glucose-repressed and anaerobic cells, because the cytoplasmic isozyme [17] (Schricker, R., unpublished) is expected to fulfill this task under these conditions. (ii) It was observed earlier [17] that displacement of the genomic copy of the AKY2 gene by a nonfunctional allele led to a respiratory deficient (pet) phenotype. This indicated that the enzymic function of AKY2 is dispensable in fermenting yeast cells, but plays an essential role in the utilization of ATP generated oxidatively inside mitochondria. These two observations underline the greater importance of AKY2 for oxidative than for fermentative metabolism and leads to the expectation that AKY2 expression is regulated in a carbon source-dependent fashion. However, such controls were found to be absent both at the transcriptional and post-transcriptional levels.

Hereford et al. [12,13] had used an unassigned transcript of about 1000 bases, which hybridized to the same DNA fragment as the histone H2A/H2B messengers, as a constitutively expressed internal standard in their experiments to demonstrate cell cycle control of histone gene transcription. It is clear now that this RNA species is the messenger of AKY2, formerly termed protein 1 [13]. Their results further showed that transcription of protein 1/AKY2 is not controlled by the cell cycle.

Since the mRNAs from the HTA1 gene (650 bases) and from the AKY2 gene (1000 bases) can be probed with the same restriction fragment, it can be judged from the signal intensities in a Northern blot (not shown) that their intracellular concentrations are about equal during the logarithmic growth phase. This agrees with the notion that AKY2 is a moderate highly expressed gene and is consistent with the codon bias indices calculated for these genes (0.66 for AKY2, 0.63 for HTA1 and 0.71 for HTB1) [34,35]. This high expression of adenylate kinase verifies that a deviation of the TATA-box sequence from the consensus is not in conflict with a high expression of the 3'-succeeding gene. So it appears tempting to speculate that this type of TATA box specifically mediates constitutive expression and uses a different set of *cis*-acting proteins for transcription activation [9]. Such a mechanism would provide a means for preventing the transmission of regulatory signals from a neighbouring inducible promoter to an unregulated gene.

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