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Analysis and *in Vivo* Disruption of the Gene Coding for Adenylate Kinase (ADK1) in the Yeast *Saccharomyces cerevisiae**

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The gene (designated ADK1) encoding the so-called cytosolic adenylate kinase of the yeast *Saccharomyces cerevisiae* was isolated using a single mixed oligonucleotide hybridization probe designed from the published amino acid sequence. ADK1 was found to be identical to an adenylate kinase gene recently isolated by an approach entirely different from ours (Magdolen, V., Oechsner, U., and Bandlow, W. (1987) *Curr. Genet.* 12, 405–411). The gene resides on yeast chromosome IV adjacent to the histone gene H2A-1. Southern blot analysis revealed only one copy of the gene, and no other related yeast DNA sequences were detected. By gene disruption it is shown that the ADK1 gene is needed for normal cell proliferation but is not essential for cell viability. Immunological studies confirmed the absence of the ADK1 gene product in mutant cells; in extracts of total cellular protein, however, there were still about 10% of the wild-type enzymatic activity present. This indicates the existence of two or more adenylate kinase isozymes in yeast. From preliminary ^{31}P NMR measurements on suspensions of yeast cells, a significant decrease in the level of nucleoside triphosphates was found in the mutant strain carrying the disrupted and partially deleted ADK1 locus.

Adenylate kinases (NTP:AMP phosphotransferases; N, adenine or guanine) are relatively small, monomeric, intracellular enzymes (molecular mass 21 to 27 kDa) which catalyze the interconversion of nucleotides according to the equation $Mg^{2+}NTP + AMP \rightleftharpoons Mg^{2+}NDP + ADP$. The enzyme is ubiquitous and particularly plentiful in tissues where the turnover of energy from adenine nucleotides is high (1); it has an important role in maintaining the energy charge of the adenylate pool (2, 3). In mammalian tissues, three isoenzymes have been identified and their amino acid sequences determined: AK1, the cytosolic type, and AK2 and AK3 which are localized in the mitochondrial intermembraneous space and in the mitochondrial matrix, respectively. Functional and structural similarities among the various adenylate kinases justify their classification as a protein family (4).

Recently, this enzyme has gained more general interest as a nucleoside triphosphate-binding protein having short segments of amino acid sequence that are homologous to sequences found in many other nucleotide-binding proteins, including ras p21 and several protein kinases (5), for which detailed structural data are not available yet. Although numerous studies on the molecular properties of the isoenzyme proteins have been carried out, little is known about adenylate kinase genes. The only prokaryotic gene that has been cloned and sequenced is that of *Escherichia coli* (6). In this case, it was known from previous studies on thermosensitive mutants of *E. coli* that adenylate kinase is a key enzyme in controlling the rate of cell growth (7). cDNAs have recently been reported for chicken muscle (8), bovine mitochondrial (9), and yeast cytosolic adenylate kinase (10).

As a step towards understanding the physiological role of this protein in a eukaryotic organism, we isolated and characterized the gene coding for the so-called cytosolic adenylate kinase of the budding yeast *Saccharomyces cerevisiae*. While this work was in progress Magdolen *et al.* (11) reported the nucleotide sequence of a yeast adenylate kinase gene isolated by an approach entirely different from ours; as the two sequences turned out to be identical we merely give a short description of the gene isolation procedure and do not present the sequence data.[1] We show that the gene, designated ADK1, is present in one copy per haploid genome and is located on yeast chromosome IV, separated by a relatively short gap from the gene coding for histone H2A-1. Furthermore, by gene disruption, we demonstrate that ADK1 is needed for normal cell growth but is not essential for cell viability.

MATERIALS AND METHODS

General Methods of DNA Manipulation—Standard methods for recombinant DNAs were followed as described by Maniatis *et al.* (12). DNA were cloned in commercially available (Pharmacia LKB Biotechnology Inc.) vectors pUC-8 and pTZ18R in *E. coli* RR1. For screening of cloned recombinant DNA by *in situ* hybridization, the thermosensitive *E. coli* strain 1398 (13) was used. Oligonucleotides were synthesized using an Applied Biosystems oligonucleotide synthesizer. Sequence analysis was done on overlapping restriction fragments according to Maxam and Gilbert (14).

Yeast Strains and Media—The diploid *S. cerevisiae* strain DAH2215 (Mata/Matα leu2/leu2 his3/His3 his4/His4) (15) was used for yeast manipulations. Laboratory strains of *Schizosaccharomyces pombe* and *Kluyveromyces marxianus* were used for preparing protein extracts for immunoblotting. Methods of yeast growth, sporulation, and tetrad analysis were as described by Sherman *et al.* (16). All yeast transformations used the lithium acetate procedure described by Ito *et al.* (17). To follow cell growth, the absorbance of dilute cell suspensions was measured in 1-cm plastic cuvettes at 600 nm. An optical density of 1 corresponded to $(1 \pm 0.5) \times 10^7$ viable cells/ml as determined by plating aliquots on glucose-containing media.

Isolation and Analysis of Yeast DNA and RNA—Genomic yeast DNA, prepared essentially as described by Winston *et al.* (18), was digested with various restriction endonucleases and DNA fragments were separated on 1% agarose gels in a Tris acetate electrophoresis buffer (12). The DNA was denatured *in situ* and, instead of being transferred to nitrocellulose paper, the gel itself was in most cases dried and used for direct gel hybridization (19). Nitrocellulose blots

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[1] The sequence of ADK1 and surrounding regions, as shown in Fig. 1, can be obtained through the GenBank data library (accession number M18455), or directly from the author.

containing yeast chromosomes separated by orthogonal field alternation-gel electrophoresis were a generous gift of Dr. P. Philippsen, Giessen, Federal Republic of Germany. Total cellular RNA was isolated as described previously (20) and denatured by glyoxylation (21). Hybridization using suitable DNA fragments as probes were performed at 42 °C for 15 h under the conditions given in Ref. 21.

Preparation of Yeast Extracts and Enzymatic Activity Assays—Published procedures were used to prepare yeast cell extracts (15) and to assay protein concentration (22) and adenylate kinase activity (23). Concentrations of auxiliary enzymes in coupled enzyme systems were always sufficient for not being rate-limiting. When studying the inhibition of the adenylate kinase reaction by P^1,P^5-di(adenosine-5′)-pentaphosphate (Ap_5A,[2] kindly provided by Dr. R. S. Goody, Heidelberg), purified adenylate kinase or protein extracts were preincubated with the inhibitor for ½ h at room temperature.

ADK1 Antibodies and Immunoblot Analysis—Preparative amounts of pure baker's yeast enzyme were obtained essentially as described by Ito *et al.* (24). Antibodies against electrophoretically pure ADK1 protein were raised in rabbits following standard immunization schemes (25). IgG was purified from the sera as described by Broome and Gilbert (26). Hybridomas were produced by fusion of splenocytes from immunized BALB/c mice and mouse myeloma cells (hypoxanthine-guanine phosphoribosyltransferase non-producer line "PAI," kindly provided by Prof. K. Unsicker, Marburg), followed by published selection and subcloning procedures (27). Clones selected by standard enzyme-linked immunosorbent assay techniques and by enzyme inhibition studies were expanded and grown as ascitic tumors in pristane-primed BALB/c mice. For immunoblot analysis, 50 to 100 μg of total cellular protein were separated on 12.5% polyacrylamide sodium dodecyl sulfate gels (28) and transferred to nitrocellulose filters (manufacturers' protocol, Schleicher & Schüll). Transfer efficiency was routinely checked by reversibly staining the membranes with Ponceau red (29). Protein blots were treated either with polyclonal rabbit antibodies or with the monoclonal antibody AKY-5G9.B2.E2, subclass IgG1, and stained with ^{125}I-labeled anti-rabbit Ig or anti-mouse Ig (Amersham Corp.), respectively.

Generation and Identification of Clones Containing ADK1 DNA—Using the information derived from the authentic yeast protein of known primary structure (30), an oligonucleotide probe was prepared which corresponded to the amino acid sequence from position 190 to 197 (numbering according to Ref. 30); it was designed as a degenerate 24-mer taking into account the pattern of codon usage in yeast (31). Genomic yeast DNA was digested with several restriction endonucleases and separated on 1% agarose gels. DNA of the 12-kb hybridization band seen in the *Eco*RI digest was ligated to the *Eco*RI-cleaved pUC-8 vector. Colony hybridization (13) was performed with the labeled mixed oligonucleotide probe (about 5×10^8 cpm/μg) for 15 h at 40 °C in 50 mM Tris-Cl, pH 7.5, 0.6 M NaCl, 1 mM EDTA, 0.2% sodium dodecyl sulfate, 10 μg/ml *E. coli* tRNA.

Gene Disruption—For intrachromosomal ADK1 gene disruption, a linear 4.3-kb *Apa*LI fragment, harboring the partially deleted ADK1 gene and the yeast LEU2 insertion, was used. It was obtained in the following way (Fig. 3*A*). The 1683-bp *Apa*LI fragment, containing the entire ADK1 coding region (Fig. 1), was excised from the recombinant plasmid pUC-8.AKY(*Eco*12) (pUC-8 with the 12-kb *Eco*RI yeast DNA fragment on which the ADK1 gene had first been localized), and the *Apa*LI sites were filled in by using the Klenow enzyme. The plasmid pTZ18R was cleaved with *Sph*I, and the protruding ends were removed by T4 DNA polymerase. The *Apa*LI fragment was inserted into this vector by blunt-end ligation thereby generating two additional *Apa*LI sites. The resultant plasmid was cleaved with *Bgl*II thereby removing 375 bp of the ADK1 coding region, and then ligated with a 3.0-kb LEU2-carrying *Bgl*II fragment that had been excised from the recombinant plasmid YEp13 (32). *Apa*LI cleavage of this novel plasmid generated the linear 4.3-kb *Apa*LI fragment which contained the LEU2 gene as selectable marker and the interrupted, partially deleted ADK1 gene that was then used for transforming the recipient strain DAH2215.

^{31}P NMR Studies—Yeast wild-type and mutant strains obtained by tetrad analysis (see above) were grown aerobically at 30 °C. Cells were harvested at mid-exponential phase by slow-speed centrifugation at 4 °C and washed three times in the ice-cold resuspension medium (50 mM Tris-Cl, pH 7.0, 150 mM NaCl, 5 mM $MgCl_2$). The cells were resuspended and examined by NMR at densities of 40% wet weight. Perchloric acid extracts were prepared as outlined by den Hollander *et al.* (33). During NMR measurements, the temperature was regulated to 10 ± 1 °C. Spectra were recorded on a Bruker MSL-300 NMR spectrometer, operating in the Fourier transform mode at 121.5 MHz. The spectra were accumulated by using 30 ° pulses and 0.4-s repetition time. The samples consisted of 2.5 ml of the cell suspensions or the extracts, respectively, in NMR sample tubes of 10-mm outer diameter.

[2] The abbreviations used are: Ap_5A, P^1,P^5-di(adenosine 5′)-pentaphosphate; Kb, kilobase pair; bp, basepair.

RESULTS

ADK1 Residues on Chromosome IV Adjacent to the Histone Gene H2A-1—A restriction endonuclease cleavage map of the subcloned overlapping *Hin*dIII (1.40 kb) and *Hin*fI (1.43 kb) fragments, representing a total of 2367 nucleotides, is shown in Fig. 1. The nucleotide sequence of the 666-bp open reading frame is identical to that reported by Magdolen *et al.* (11). From Northern blot analysis, a message size of about 0.9 kb is predicted (Fig. 2*B*). When probing a blot of yeast chromosomes separated by orthogonal field alternation-gel electrophoresis with a labeled DNA fragment of the ADK1 coding region, only one band was seen (Fig. 2*C*); it corresponds to chromosome IV which is the second largest of the yeast chromosomes (34). At both the 5′-end (*Hin*dIII site) and the 3′-end (*Hin*fI site) of the 2.37-kb fragment shown in Fig. 1, open reading frames of more than 350 bp each were identified. When comparing the former to sequences available through the GenBank nucleic acid data base, complete identity was found with the gene of yeast histone H2A-1 whose structure had been determined several years ago (35). The stop codon of this histone gene is separated by 557 bp from the translation initiation codon of ADK1. The open reading frame beginning 360 bp after the two ADK1 stop codons has a relatively high A + T content (62%); no hybridization band was seen in Northern blots using as probe the 403-bp *Hin*dIII/*Hin*fI fragment of this open reading frame.

Southern blots of total yeast DNA were probed at relatively low stringency (52 °C; 0.6 M NaCl) with the 695-bp *Hin*fI/*Dra*I fragment encompassing almost all residues of the ADK1 protein coding region. In each digest the genomic fragments observed were identical to those predicted from the restriction map (Fig. 1) of the cloned segments; no additional hybridization signals were seen (Fig. 2*A*). These findings indicate that ADK1 is encoded by a single copy gene in the yeast genome and there are no other ADK1-related sequences.

Disruption of the ADK1 Gene Is Not Lethal—As adenylate kinase is purported to be involved in essential functions that regulate the interconversion of the constituents of the adenine nucleotide pool we decided to determine whether adenylate kinase is essential for cell growth. The yeast ADK1 gene was therefore disrupted *in vitro* and reintroduced into the genome by homologous recombination (36) (Fig. 3*A*). Cells that had stably integrated the disrupted ADK1 gene were selected by their ability to grow in the absence of leucine. Genomic DNA from five independent Leu$^+$ transformants was analyzed to

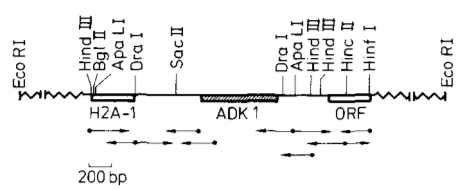

FIG. 1. **Restriction sites in the sequenced 2.36-kb yeast DNA fragment and sequencing strategy.** The *arrows* indicate direction and extent of each sequencing analysis. The *hatched box* stands for the protein coding region of the ADK1 gene, the *dotted box* represents part of the histone H2A-1 gene, and the *open box* stands for an open reading frame (*ORF*; over 350 bases long) of unknown identity. The fragment is part of the 12-kb yeast DNA segment cloned into the *Eco*RI site of pUC-8. *Wavy lines* indicate flanking regions that were not further studied.

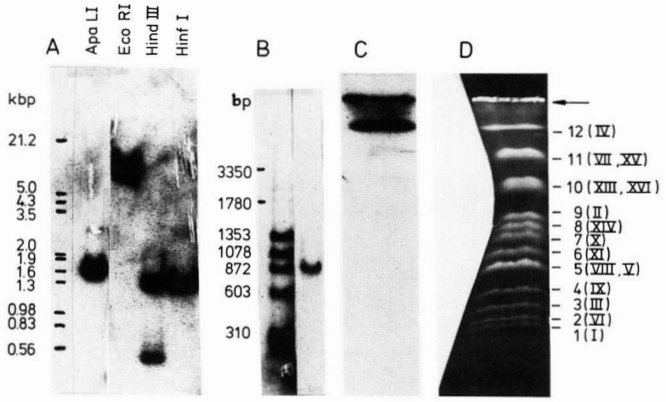

FIG. 2. **Hybridization analysis of the yeast adenylate kinase gene ADK1.** *A*, adenylate kinase is encoded by a unique, single-copy gene. A Southern blot of total yeast DNA (10 μg/lane) cut with the restriction enzymes *Apa*LI, *Eco*RI, *Hin*dIII, and *Hin*fI was hybridized at relatively low stringency (52 °C; 0.6 M NaCl) to the labeled 695-bp *Hin*fI/*Dra*I fragment containing almost all residues of the ADK1 protein coding region. *B*, analysis of yeast RNA. Ten micrograms of total yeast RNA (strain DAH 2215) were treated with 1 M glyoxal, run through a 1.5% agarose gel, and transferred to a nitrocellulose filter (21). This filter was probed with the ^{32}P-labeled 355-bp *Hin*cII fragment from the ADK1 coding region. ^{32}P-end-labeled *Hae*III restriction fragments of φX174 replicative form DNA were glyoxal-denatured (21) and run in the adjacent lane. Their sizes (in bp) as well as the positions of the 18 S (1.78 kb) and 25 S (3.35 kb) yeast ribosomal RNA visualized after staining with ethidium bromide are indicated at the left of the autoradiogram. *C* and *D*, assignment of ADK1 to yeast chromosome IV. Ethidium bromide-stained agarose gel on which the chromosomal DNA molecules of yeast have been separated by orthogonal field alternation-gel electrophoresis (*D*). The assignment of chromosomes to individual bands is according to Ref. 44. After blotting onto nitrocellulose, hybridization was performed with a specific probe containing part of the ADK1 protein coding region (355-bp *Hin*cII fragment). The ADK1 gene has thus been located on the uppermost electrophoretic band which corresponds to chromosome IV (*C*). The *long arrow* indicates the position of the sample well.

confirm that the disrupted ADK1 gene had integrated at the ADK1 locus. All five heterozygous diploid cells had one wild-type copy of the ADK1 gene and one disrupted allele (data not shown). About 20 tetrads taken from three different transformants were dissected; all tetrads analyzed gave rise to four viable spores, two of which formed wild-type sized colonies and two formed small colonies, the difference in colony size being more pronounced at 37 than at 30 °C. When examined under the light microscope, wild-type and mutant cells were morphologically indistinguishable. Characterization of these tetrads indicated that large colonies were composed of Leu$^-$ cells and that small colonies were formed by Leu$^+$ cells. DNA blot analysis of sibling spores confirmed that the Leu$^-$ cells contained the 1.7-kb *Apa*LI fragment corresponding to the wild-type version of ADK1, whereas the size of the hybridization band seen in the slow-growing Leu$^+$ spores was 4.3 kb corresponding to the *Apa*LI fragment that had been used for transformation (Fig. 3*B*). Growth rates of wild-type (Leu$^-$ADK1$^+$) and mutant (Leu$^+$ADK1$^-$) spores of two tetrads were determined in suspension cultures under aerobic conditions, over a period of 16 h. During the exponential phase of cell proliferation, wild-type cells exhibited a generation time of 1.3 h at 30 °C and 1.5 h at 37 °C, whereas for mutant cells these values were 2.3 and 3.7 h, respectively. Cell densities reached in the stationary phase were nearly the same in all cases (A_{600} from 15 to 20). When glucose in the media was replaced by glycerol and ethanol, either cell type grew more slowly, resulting in doubling times of 2.2 h for the wild-type and 3.3 h for the mutant, at 30 °C.

Immunological Studies—The observation that ADK1-deficient mutants are viable could be explained by the assumption that other enzymes (*e.g.* isozymes) can replace the ADK1 gene product in catalyzing the adenylate kinase reaction. In attempting to detect ADK1-related proteins, monoclonal and polyclonal antibodies raised against the highly purified ADK1 gene product were used for analyzing protein extracts from *S. cerevisiae* wild-type and mutant cells as well as for showing eventual cross-reactivities of these antibodies with adenylate kinases from other sources. When assayed with the monoclonal antibody AKY-5G9.B2.E2, strains carrying the disrupted ADK1 gene (Fig. 4*B*, *lanes 10* and *11*) are obviously devoid of the adenylate kinase band seen in the wild type (Fig. 4*B*, *lanes 8* and *9*). There is, however, relatively strong cross-reactivity, both in wild-type and mutant cells, with two unidentified proteins of approximate molecular mass 16 and 52 kDa which are also present in extracts from the yeast *K. marxianus* (*lane 5*). This antibody shows no reaction at all with partially purified *S. pombe* adenylate kinase (*lane 6*) and with commercially obtained adenylate kinases from chicken, porcine, and rabbit muscle (*lanes 1, 2,* and *3*). There is no cross-reactivity with any *E. coli* protein, and in *K. marxianus* a strong band is seen at the position where the *S. cerevisiae* ADK1 protein is located (*lane 5*). From the pattern produced by this monoclonal antibody it may thus be concluded that adenylate kinase proteins from *S. cerevisiae* and *K. marxianus* have an epitope in common that is not present in the homologous proteins from *S. pombe, E. coli,* and vertebrate muscle.

With polyclonal antibodies, a somewhat different picture emerged from the analysis of an immunoblot which contained the same amounts of proteins and protein extracts in the same order as the former one. *S. cerevisiae* mutants (Fig. 4*A*, *lanes 10* and *11*) lack again the adenylate kinase band, and background reactivity is relatively low. In wild-type cells (Fig. 4*A*, *lanes 8* and *9*), a strong signal is seen at the expected position of the 24-kDa ADK1 gene product, and several minor bands (M_r < 18,000) are present which very likely originate from degradation products of the ADK1 protein since they are missing in the mutant cells, but are also observed in electrophoretically pure (>95% pure when judged from Coomassie Blue stained gels) *S. cerevisiae* adenylate kinase. In contrast to monoclonal antibodies, these polyclonal antibodies can detect ADK1-related proteins from all other sources tested, except *E. coli*, and notably from vertebrate muscle, too. In *S. cerevisiae* mutant cells, however, no additional band became apparent in the range from 21 to 27 kDa which is characteristic of adenylate kinases studied so far (4). One can therefore assume that, in *S. cerevisiae*, there is no other protein detectable that is immunologically related to the ADK1 gene product.

Adenylate Kinase Activity Detectable in ADK1-deficient Cells—Due to the lack of substrate specificity of NTP:NMP phosphotransferases (37), ADK1-related enzymes could catalyze the interconversion of adenine mono-, di-, and triphosphates, although most likely in a less efficient manner than adenylate kinase and with different kinetic parameters. If adenylate kinase isozymes exist that are not recognized by our monoclonal or polyclonal antibodies it should be possible to demonstrate enzymatic activity in protein extracts from ADK1-deficient cells. In order to discriminate between authentic adenylate kinase activity and substrate turnover that might be caused by other enzymes, various substrate pairs were assayed (Table I) and the influence of the synthetic, highly specific substrate inhibitor Ap_5A on adenylate kinase catalyzed reactions was studied. Surprisingly, mutant cells

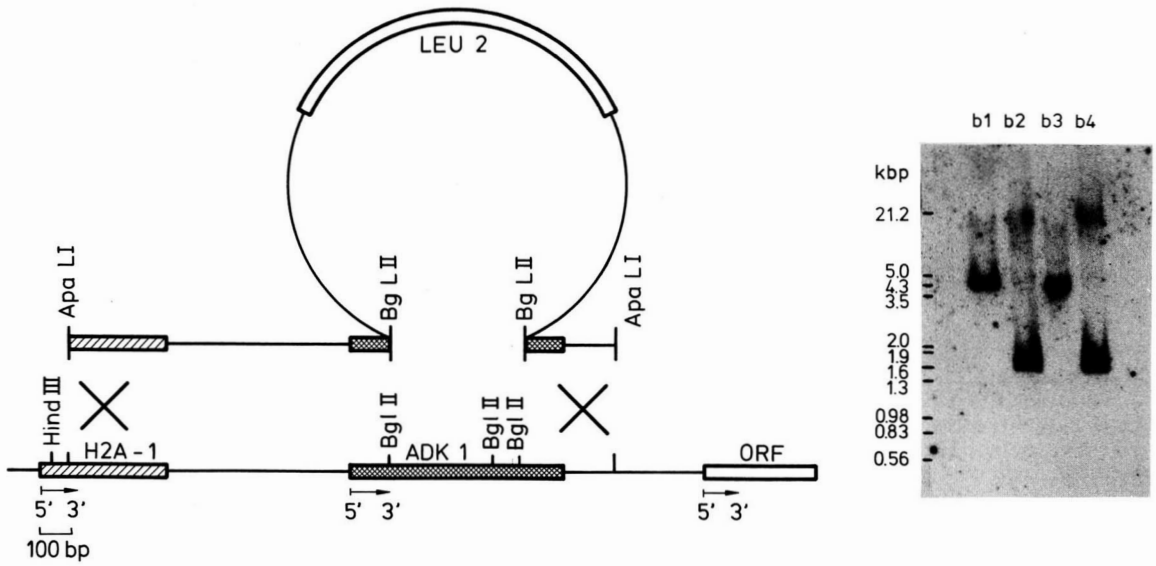

FIG. 3. **Construction of a diploid yeast strain with disrupted ADK1.** *A*, schematic representation of the ADK1 gene disruption on yeast chromosome IV. Shown is the chromosomal arrangement of the genes for histone H2A-1, adenylate kinase ADK1, and an unidentified open reading frame (*ORF*). From the ADK1 gene lying on a 1.68-kb *Apa*LI fragment two *Bgl*II-fragments of 375 bp total length were first deleted and then a 3.0-kb *Bgl*II fragment carrying the yeast LEU2 gene as a selectable marker was introduced. The linear 4.3-kb *Apa*LI fragment was then used to transform a diploid Leu$^-$ strain. *Crossed bars* indicate regions of homologous recombination between the fragment ends and chromosomal DNA. *B*, Southern analysis to demonstrate the substitution of the ADK1 locus by the construction introduced. *Apa*LI digests of DNA of each tetrad spore (named b1, b2, b3, and b4) revealed the expected sizes of bands that hybridized with the labeled 695-bp *Hin*fI/*Dra*I fragment. The wild-type strains (*lanes b2* and *b4*) showed only one band of 1.68-kb size each, whereas the mutant strains (*lanes b1* and *b3*) showed only one band each about 2.6 kb longer than the former which equals the size of the LEU2 insert (3.0 kb) minus 375 bp (deleted *Bgl*II fragments of the ADK1 coding region). The relatively faint bands seen in the 20- to 22-kb region are due to hybridization of the probe to undigested genomic yeast DNA.

that do no longer produce the ADK1 gene product still show 10 to 15% of the adenylate kinase activity found in wild-type cells. Purified ADK1 enzyme exhibits a high preference for adenine nucleotides, as was reported before (24), ATP being a more than 10-fold better phosphate donor than GTP, and GMP being a very poor phosphate acceptor (Table I). Adenylate kinase activities in the presence and absence of Ap_5A were measured in the reverse reaction (ADP as substrate) with the same concentration of ADK1 enzyme or protein extract. At 25 °C and pH 7.5, in the presence of 0.2 mM ADP, 1 μM Ap_5A caused 30% inhibition and 25 μM Ap_5A led to 90% inhibition of adenylate kinase activity of either purified enzyme or protein extract from wild-type cells, the percent inhibition being defined as $100 \times (v - v_i)v$, where v and v_i are initial velocities in the absence and presence of inhibitor, respectively. When choosing even higher Ap_5A concentrations, 100% inhibition was achieved in all cases. Activities in mutant cells were inhibited to a similar degree although exact determination was less accurate due to relatively low turnover rates/mg of protein. Taken together, these data make the existence of an ATP:AMP phosphotransferase isozyme very likely and exclude the possibility that residual activities detectable in mutant cells have to be predominantly ascribed to GTP:AMP or ATP:GMP phosphotransferases.

A rough estimate of the magnitude of the inhibition constant for Ap_5A can be obtained in the following way. As Ap_5A was shown formerly (23, 38) to be a competitive inhibitor with respect to all three nucleotide substrates of adenylate kinase it is easily demonstrated, by applying the rules for steady state kinetics, that $K_i = K_m \times I/(K_m + S)(v/v_i - 1)$, where K_i stands for inhibition constant, K_m for Michaelis constant, S and I for free concentrations of substrate and inhibitor, respectively, and v and v_i defined as above. Assuming $K_m = 23$ μM in the case of ADP (24), and taking into account the experimental data given above, a value of 0.28 μM for K_i is calculated which is about 10 times lower than the inhibition constant for mitochondrial adenylate kinase (about 3 μM; see Ref. 31), but about 10 times higher than the value found for muscle cytosolic adenylate kinase (38).

In Vivo ^{31}P NMR Studies—Although we have shown in this report that the ADK1 gene product is not essential for *S. cerevisiae* cell growth, there is an 8- to 10-fold reduction of adenylate kinase activity in ADK1-deficient cells which might be reflected in significantly altered levels of adenine nucleotides. A suitable means for testing this assumption is *in vivo* ^{31}P NMR spectroscopy (see Ref. 39 for review). We decided to apply this noninvasive technique in order to tackle the question of whether ADK1-deficient *S. cerevisiae* cells exhibit reduced levels of nucleoside di- and triphosphates.

Fig. 5 shows representative ^{31}P NMR spectra of resting wild-type (*a*) and ADK1-deficient mutant (*b*) cells. The wild-type spectrum is qualitatively similar to that obtained by other workers using different yeast strains (40). The two spectra look very much alike, having two prominent signals that arise from intracellular inorganic phosphate (around −1 ppm) and from the inner peaks of longer polyphosphates (around 23 ppm). The latter is the most abundant phosphate-containing compound in *S. cerevisiae* that can accumulate to more than 10% of total dry weight of yeast cells (42). In cell spectra, the terminal and α-phosphates of ATP and ADP are not resolved; the β-phosphate peak of ATP is close to polyphosphate resonances, and thus mostly obscured (39). In the present experiments we were therefore unable to see the ATP and ADP signals separately. The α-phosphate peaks, however,

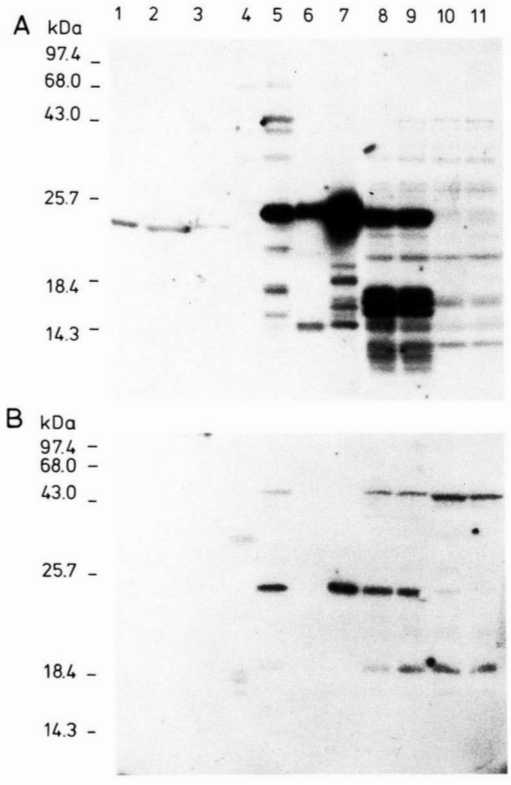

FIG. 4. **Immunoblot analysis of adenylate kinases using antibodies directed against yeast ADK1.** Purified adenylate kinases from several sources and protein extracts from yeast and *E. coli* cells were electrophoresed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and analyzed by immunoblotting for reactivity with polyclonal rabbit anti-ADK1 antibodies (*A*) or with the monoclonal mouse anti-ADK1 antibody AKY-5G9.B2.E2 (*B*): chicken muscle adenylate kinase, 1 μg (*lane 1*); porcine muscle adenylate kinase, 1 μg (*2*); rabbit muscle adenylate kinase, 1 μg (*3*); *E. coli* total protein, about 50 μg (*4*); *K. marxianus* protein extract, about 50 μg (*5*); *S. pombe* adenylate kinase, partially purified, 2 μg (*6*); *S. cerevisiae* adenylate kinase, 0.5 μg (*7*); extracts from wild-type (*8, 9*) and mutant (*10, 11*) cells of one *S. cerevisiae* tetrad, about 50 μg each. Molecular weight markers (Gibco-Bethesda Research Laboratories) were electrophoresed in parallel and visualized by Coomassie Blue staining; their positions are indicated on the left.

TABLE I

Enzymatic activities of purified ADK1 protein and protein extracts in the presence of various substrate pairs

Activities were determined by spectrophotometric assays in 100 mM Tris-Cl, 100 mM KCl, 5 mM $MgCl_2$, pH 7.5, and 25 °C in the presence of 2 mM nucleoside triphosphate (ATP or GTP) and 2 mM nucleoside monophosphate (AMP or GMP) in the forward reaction, and in the presence of 2 mM ADP in the reverse reaction. 0.1 μg of purified ADK1 protein and about 50 μg of protein extracts from wild-type (a1, a2) and mutant (a3, a4) cells of tetrad spores (shown are mean activity values for either pair of cell types) were used for adenine-containing nucleotides in 0.5-ml reaction mixtures; when guanine nucleotides were assayed, 5 to 10 times more enzyme was used. One unit of the enzymatic activity is defined as the consumption (forward reaction) or the production (reverse reaction) of 1 μmol of nucleoside triphosphate/min at 25 °C.

Source of enzymatic activity	Substrate			
	ATP + AMP	ATP + GMP	GTP + AMP	ADP
	units/mg of protein			
ADK1	1150	35	95	650
a1/a2	4.3	0.18	0.45	0.95
a3/a4	0.6	<0.05	0.09	0.16

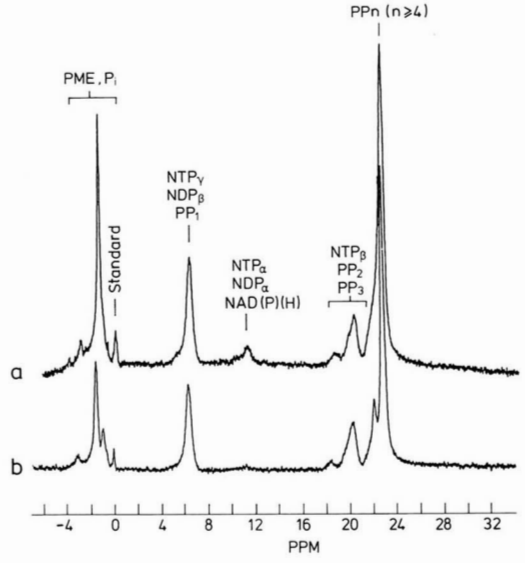

FIG. 5. **^{31}P NMR spectra of suspensions of *S. cerevisiae* wild-type (*a*) and ADK1-deficient mutant (*b*) cells.** Yeast cells were suspended at densities of 40% wet weight. Sample conditions and data acquisition procedures are described under "Materials and Methods." For either spectrum, the number of scans was 6400. Chemical shifts are reported as parts/million relative to glycerophosphorylcholine. Peaks were assigned according to the findings of den Hollander *et al.* (41) and by comparison to spectra of standard solutions of AMP, ADP, ATP, GDP, GTP, NAD, NADP (obtained from Boehringer Mannheim), and the sodium salts of inorganic phosphate, pyrophosphate, tripolyphosphate, and tetrapolyphosphate (obtained from Sigma). *PME*, phosphomonoesters (sugar phosphate; nucleoside monophosphates); NDP_α, NDP_β, primary and terminal phosphate of nucleoside diphosphates, respectively; NTP_α, NTP_β, NTP_α, primary, middle, and terminal phosphate of nucleoside triphosphates. PP_1 represents the sum of several terminal phosphate resonances from phosphate chains, including pyrophosphate ($P_2O_7^{4-}$); peaks PP_2 and PP_3 are penultimate phosphates from polyphosphate chains, including the middle phosphate of tripolyphosphate ($P_3O_{10}^{5-}$); PP_n comes from the inner peaks of longer polyphosphates.

which occur close to 12 ppm, can be used to measure the sum of intracellular ATP and ADP concentrations, which is typically in the range of 5 to 10 mM (43). The most striking difference between wild-type and mutant spectrum is indeed seen around 12 ppm. This peak, which is well separated from surrounding resonances and contains, among others, the overlapping α-phosphate signals of nucleoside di- and triphosphates, almost disappears in mutant cells. It seems justified to interpret this difference as a significant drop in the ATP concentration since ATP was shown to be the predominant nucleoside triphosphate in the yeast cell (40). A reliable estimate of relative changes in intracellular levels of ATP and ADP can be obtained by comparing the area of the peak at 12 ppm to the integral of the whole spectrum, *i.e.* to the total phosphate contained in the cells. It then turns out that in wild-type cells, this peak represents 1.5 to 2% of the total spectrum, whereas in mutant cells it contributes less than 0.2%, indicating that the intracellular ATP + ADP level in the mutant strain probably is about 10-fold lower than in the wild-type strain. These findings are qualitatively similar to those reported for *E. coli* cells harboring a thermolabile adenylate kinase (7, 43).

DISCUSSION

The structural gene ADK1 encoding the so-called cytosolic adenylate kinase from baker's yeast was isolated by oligonucleotide screening of a *S. cerevisiae* genomic library. Blot hybridization using the cloned sequence as a probe showed

that ADK1 is a single copy gene and is transcribed into an 0.9-kb message. Surprisingly, when sequencing regions in the direct neighborhood of ADK1, we detected the histone gene H2A-1, lying ahead of ADK1 and being transcribed in the same direction as ADK1. Thus, the protein of unknown function (named protein 1; 30 kDa apparent molecular mass) which had been found by Hereford and co-workers (45) when studying the organization of histone gene loci in *S. cerevisiae* has been identified as adenylate kinase. Whereas H2A-1 was found to be periodically transcribed, reaching a peak in mid S-phase of the cell cycle, the ADK1 transcript remains at an almost constant level (46, 47). By hybridization of appropriate probes to electrophoretically separated chromosomes, we demonstrate that the ADK1 gene resides on chromosome IV confirming recent genetic mapping of the tandem H2A-H2B gene pair (48).

The ADK1 gene product had been called a cytosolic enzyme (24, 49). However, its subcellular location remains to be studied, since other authors reported yeast adenylate kinase to be a marker enzyme of the outer mitochondrial compartment (50), similar to the situation found in mammalian tissues (isoenzyme AK2). On the other hand, it resembles the AK3 isozyme in possessing tryptophan which is absent in all the other adenylate kinases studied so far. Vertebrate AK1 and AK2 species were also classified into two independent groups by considering differences in antigenicity (51), and it was claimed that antibodies against the yeast enzyme highly cross-reacted with the rat liver isozyme (AK2), but not with the rat muscle isozyme (AK1) (52), suggesting that the yeast enzyme should be a closer relative of the mitochondrial isozyme subgroup. Our polyclonal antibodies show cross-reactivity with muscle enzymes from different sources when assayed on Western blots, but do not recognize *E. coli* adenylate kinase whose primary structure is more similar to the yeast enzyme than it is to the muscle enzymes (4).

From a detailed comparison of the primary structures of various adenylate kinases (4) it emerged that the muscle AK1 species are the only members of the adenylate kinase family that lack the continuous, relatively hydrophobic stretch of 31 residues located nearly in the center of the primary structure of all the other isozymes (AK2, AK3, *E. coli, S. cerevisiae*). As the amino terminus of the primary translation product deduced from the ADK1 open reading frame does not extend beyond the amino-terminal end of the sequenced protein, one might speculate that internal sequences contain the structural motifs that function as sorting signals to determine the isozyme locations in the mitochondria. This is an attractive idea (53) which is strengthened by the recent observations of Hurt and Schatz (54) who concluded from their experiments on dihydrofolate reductase that potential mitochondrial targeting sequences may be hidden in many cytosolic proteins. In addition, in the case of the *E. coli* enzyme, there are indications that adenylate kinase is located both in the periplasmic and the cytoplasmic compartment (55).[3] In the present report we have shown that mutant strains carrying the disrupted ADK1 gene are viable, growing two to three times more slowly than normal; and our biochemical studies have provided evidence for the existence of an ATP:AMP phosphotransferase in the mutant strain. Our data are thus in line with the assumption that the budding yeast possesses at least two adenylate kinase isozymes, and that this eukaryotic cell can dispense with one of them. The two adenylate kinases appear to be encoded by fairly divergent genes since low stringency hybridization analysis of chromosomal DNA and immunoblot analysis using polyclonal antibodies failed to detect additional bands. Strong cross-reactivity between antibodies directed against the ADK1 enzyme and an adenylate kinase from *S. pombe* has enabled us to isolate the corresponding fission yeast gene.[4] That there is a nonessential gene for adenylate kinase may not be too surprising, however, since there are estimates indicating that only about 12% of the yeast genome are essential for cell viability under standard laboratory conditions, about 14% are required for optimal cell growth (56). In the simplest case, an enzyme defect may be compensated for by the activity of an isozyme.

The importance of the adenine nucleotides as major regulatory factors in controlling metabolic processes is well established (57), and as a useful expression to describe the energy state of the cell the "energy charge" was proposed (3), defined by the relation (ATP + 0.5 ADP)/(ATP + ADP + AMP); this parameter can range in value from 1 (all ATP) to 0 (all AMP). As pointed out (2, 57), adenylate kinase performs the important function of catalyzing the rapid return of the adenine nucleotide pool to equilibrium following a change in any one of its constituents, without altering the energy charge, however. *E. coli* mutants with a thermolabile adenylate kinase are not viable at a nonpermissive temperature, and *S. cerevisiae* mutants lacking the ADK1 gene product, which contributes about 90% to the total adenylate kinase activity detectable in yeast cells, are considerably impaired in growth. The energy charge of most cells is around 0.9 (57), and in *E. coli*, cell viability is severely affected when this value drops below about 0.5 (58), in marked contrast to results obtained with yeast cells that remained viable at energy charge values below 0.1 (59). This finding might partially explain why the growth rate of the ADK1 deletion mutant was not as drastically reduced as would have been expected beforehand. In future studies, ^{31}P NMR saturation transfer may be a suitable technique for monitoring *in vivo* activity of adenylate kinase. We hope to gain further insight into the biological role and significance of yeast adenylate kinases from the characterization of the existing isozyme(s), in conjunction with random and site-specific mutagenesis of the corresponding genes. This combined approach will eventually allow for a cell biological interpretation of the concept of energy charge.

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