

# Characterization of Soybean Choline Kinase cDNAs and Their Expression in Yeast and *Escherichia coli*

Dave E. Monks, John H. Goode, and Ralph E. Dewey\*

Department of Crop Science, North Carolina State University, Raleigh, North Carolina 27695–7620

---

An expressed sequence tag from *Arabidopsis* that displayed sequence homology to mammalian and yeast choline kinases was used to isolate choline kinase-like cDNAs from soybean (*Glycine max* L.). Two distinct cDNAs, designated GmCK1 and GmCK2, were recovered that possessed full-length reading frames, each sharing approximately 32% identity at the predicted amino acid level with the rat choline kinase. A third unique choline kinase-like cDNA, GmCK3, was also identified but was not full length. Heterologous expression of GmCK1 in yeast (*Saccharomyces cerevisiae*) and GmCK2 in both yeast and *Escherichia coli* demonstrated that each encodes choline kinase activity. In addition to choline, other potential substrates for the choline kinase enzyme include ethanolamine, monomethylethanolamine (MME), and dimethylethanolamine (DME). Both soybean choline kinase isoforms demonstrated negligible ethanolamine kinase activity. Competitive inhibition assays, however, revealed very distinct differences in their responses to DME and MME. DME effectively inhibited only the GmCK2-encoded choline kinase activity. Although MME failed to effectively inhibit either reaction, an unexpected enhancement of choline kinase activity was observed specifically with the GmCK1-encoded enzyme. These results show that choline kinase is encoded by a small, multigene family in soybean comprising two or more distinct isoforms that exhibit both similarities and differences with regard to substrate specificity.

---

PC is the most abundant phospholipid constituent of most eukaryotic cellular membranes (Ansell and Spanner, 1982). In animals and yeast, PC may be synthesized by either of two distinct metabolic pathways: (a) the methylation pathway, whereby PC is produced by the sequential methylation of phosphatidylethanolamine, or (b) the nucleotide pathway, a three-step enzymatic process in which free choline is incorporated into PC through a CDP-choline intermediate (Carman and Henry, 1989; Vance, 1991). In higher plants, however, recent studies of PC biosynthesis have provided compelling evidence suggesting that the majority of PC is produced through a single biosynthetic pathway possessing elements of both the traditional methylation and nucleotide pathways (reviewed by Kinney, 1993).

The initial step of the nucleotide pathway involves the ATP-dependent phosphorylation of choline catalyzed by choline kinase (EC 2.7.1.32). This reaction has been characterized in numerous plant species (Ramsarma and Wetter, 1957; Tanaka et al., 1966; Mellor et al., 1986; Kinney and Moore, 1988), and the enzyme has been partially purified

from soybean (*Glycine max*) (Wharfe and Harwood, 1979). The involvement of this enzyme in the synthesis of PC in plants appears to differ greatly depending on the availability and sources of free choline. Choline that is supplied exogenously is rapidly phosphorylated by choline kinase and quickly incorporated into PC (Kinney and Moore, 1987; Mudd and Datko, 1989; Che et al., 1990). Radiotracer studies designed to follow the endogenous synthesis of the choline head group in *Lemna*, soybean, and carrot, however, have shown that free choline is not produced in significant amounts and does not appear to be an intermediate in the synthesis of PC (Mudd and Datko, 1986; Datko and Mudd, 1988b). The choline that makes up the PC head group appears to be synthesized primarily at the phosphobase or phosphatidyl-base levels. Furthermore, in castor bean endosperm in which radiotracer studies did detect significant quantities of endogenously produced choline, this choline appeared to be sequestered in a pool that was largely unavailable for incorporation into PC (Kinney and Moore, 1987; Prud'homme and Moore, 1992).

Because of the inability to detect endogenously produced free choline as a major intermediate in PC biosynthesis, questions remain regarding the role of choline kinase in the absence of an external supply of substrate. An additional complication in our understanding of this reaction became apparent with the discovery by Kinney and Moore (1988) that germinating castor bean endosperm choline kinase activity is highly reversible. Under conditions of pH and substrate concentrations that were determined to be physiologically relevant, it was predicted that the reverse reaction would be greatly favored over the forward activity, leading to speculation that choline kinase may alternatively function as a means of producing choline in metabolic pathways leading away from PC.

To gain a greater understanding of the choline kinase reaction, how it is regulated, and its role in PC biosynthesis in higher plants, we have isolated and characterized two distinct soybean cDNAs that encode choline kinase activity. By expressing the soybean choline kinase cDNAs in yeast (*Saccharomyces cerevisiae*) and/or *Escherichia coli*, we were able to directly test for function and substrate specificities of the individual isoforms. We have also recovered an additional cDNA that displays significant predicted amino acid homology to known choline kinases, suggest-

---

\* Corresponding author; e-mail ralph\_dewey@ncsu.edu; fax 1-919-515-7959.

Abbreviations: DME, dimethylethanolamine; EST, expressed sequence tag; MME, monomethylethanolamine; PC, phosphatidylcholine.

ing that other isoforms of the enzyme are present in soybean.

## MATERIALS AND METHODS

### Construction and Screening of Soybean cDNA Library

Size-selected cDNA (>500 bp) was synthesized from 25-DAF developing soybean (*Glycine max* cv Dare) seed mRNA and ligated into phage vector  $\lambda$ ZAPII according to the manufacturer's instructions (Stratagene). Plaques were plated at a density of  $4 \times 10^4$  plaque-forming units per 150-mm Petri dish and transferred to nylon membranes as previously described (Sambrook et al., 1989).  $^{32}$ P-labeled insert DNA from a choline kinase-homologous Arabidopsis EST (clone No. 104E7T7) was hybridized to the library under low-stringency conditions (described below). Plaques demonstrating positive hybridization signals were purified to homogeneity and their corresponding pBluescript plasmids recovered as described in the  $\lambda$ ZAPII cloning protocol (Stratagene).

Nucleotide sequence information was obtained in both orientations for each of the selected cDNAs using the Sequenase version 2.0 kit (United States Biochemical). Pairwise comparisons between the predicted amino acid sequences of the soybean cDNAs and the rat and yeast choline kinase sequences were conducted using the GAP program of the University of Wisconsin Genetics Computing Group software package; multiple sequence alignments were performed using the PILEUP program (Genetics Computer Group, Madison, WI).

### Northern and Southern Blot Analyses

Genomic DNA and mRNAs were isolated from soybean and transferred to nylon membranes as previously described (Dewey et al., 1994).  $^{32}$ P-labeled DNA probes were synthesized using random primers according to the method of Feinberg and Vogelstein (1983). All nucleic acid blots were incubated overnight with labeled probe in a hybridization solution containing  $6\times$  SSPE,  $10\times$  Denhardt's solution, 1% SDS, and  $100 \text{ mg mL}^{-1}$  denatured herring sperm DNA. Hybridization under standard conditions was conducted at an incubation temperature of  $65^\circ\text{C}$  followed by washing the blot twice at room temperature in  $2\times$  SSC, 0.1% SDS for 15 min; once at  $65^\circ\text{C}$  in  $1\times$  SSC, 0.1% SDS for 15 min; and once at  $65^\circ\text{C}$  in 0.2% SSC, 0.1% SDS for 15 min. The low-stringency hybridization for screening the soybean cDNA library was conducted at an incubation temperature of  $50^\circ\text{C}$  and by washing twice at room temperature in  $2\times$  SSC, 0.1% SDS for 15 min, and once at  $50^\circ\text{C}$  in  $1\times$  SSC, 0.1% SDS for 15 min. Autoradiograms were produced by exposing the washed blots to Kodak XAR-5 film.

### Expression of Soybean Choline Kinase cDNAs in Yeast

To test clones GmCK1 and GmCK2 for functional activity in yeast (*Saccharomyces cerevisiae*), the cDNA inserts were excised from their original pBluescript vectors and cloned into the multiple cloning site of vector pIC-20H. Because

the pIC-20H polylinker is flanked by two *Hind*III restriction sites, subsequent digestion of these constructs with this enzyme permitted the cloning of the GmCK inserts into the *Hind*III cloning site of yeast expression vector pDB20 (Becker et al., 1991). Restriction mapping of the GmCK inserts within pDB20 enabled the selection of individuals that were oriented correctly with respect to the *ADH1* promoter that drives the constitutive expression of transgenes cloned into this vector. Designated GmCK1.DB20 and GmCK2.DB20, the respective constructs were transformed into yeast strain CTY392 (*cki-*, McGee et al., 1994) using the procedure of Gietz et al. (1992).

Transformed yeast colonies were inoculated into 50 mL of synthetic minimal medium minus uracil (Guthrie and Fink, 1991) and grown at  $30^\circ\text{C}$  to an  $A_{600}$  of 1.0 to 1.2. After the cells were harvested by centrifugation at  $3,000g$  for 5 min, the pellet was washed with water and resuspended in 1 mL of 20% glycerol, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA. The amount of 0.5 mL of 0.5-mm glass beads was added to each preparation, and the cells were disrupted with two 20-s pulses at 5000 rpm using a mini-beadbeater (Biospec Products, Inc., Bartlesville, OK). The homogenate was centrifuged at  $10,000g$  for 20 min at  $4^\circ\text{C}$ , and the resulting supernatant was assayed for choline kinase activity.

### Expression of GmCK2 in *Escherichia coli*

To facilitate the cloning of the GmCK2 cDNA into an *E. coli* expression vector, a PCR-mediated site-directed mutagenesis strategy was used to introduce unique restriction sites at the initiator Met codon and in the 3' flanking region of the soybean clone. Mutagenic primers 5'-TTGACCATG-GCCATAAAGGC-3' and 5'-GTTGGGAGCTCACCT-AC-3' were used to amplify a GmCK2 sequence (using GmCK2 in pBluescript as the template) possessing an *Nco*I site at the initiator codon and an *Sst*I site in the 3' flanking region of the cDNA. Nucleotides shown in bold type correspond to the sequences in the primers that differ from the wild-type sequence. The newly created restriction sites are underlined; the initiator ATG codon is italicized. PCR reactions were conducted using the standard conditions described in the GeneAmp PCR kit (Perkin-Elmer Cetus).

PCR-amplified products were digested with the appropriate restriction endonucleases (*Nco*I and *Sst*I) and cloned into *E. coli* expression vector pUC120. pUC120 is a derivative of pUC118 that possesses a unique *Nco*I restriction site at the *lacZ* start codon to facilitate isopropyl- $\beta$ -D-thiogalactopyranoside-inducible expression of cloned inserts (Messing, 1991). This construct was designated GmCK2.Nco. An expression cassette was created, by engineering an *Nco*I site at the start codon and using this site to clone into pUC120, that allowed synthesis of the entire plant protein without any additional amino acids derived from vector sequence.

*E. coli* cells (strain MV1190) transformed with GmCK2.Nco were cultured in 250 mL of Luria broth medium (containing ampicillin) to an  $A_{550}$  of 0.4 to 0.6 and then induced by the addition of isopropyl- $\beta$ -D-thiogalactopyranoside to a final concentration of 0.75 mM. After an additional 2 h of culturing, cells were harvested and resus-

ended in 2.0 mL of 20% glycerol, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA. After a 5-min incubation with lysozyme (2.5 mg mL<sup>-1</sup>), cells were disrupted using a mini-beadbeater at 5,000 rpm for 80 s. The homogenate was centrifuged at 10,000g for 20 min at 4°C, and the resulting supernatant was assayed for choline kinase activity. Protein concentrations for both the yeast and *E. coli* extracts were determined by the method of Lowry et al. (1951) using bovine  $\gamma$ -globulin as the standard.

### Enzyme Assays

Choline kinase activity was assayed as described by Uchida and Yamashita (1992) with minor modifications. Except where otherwise stated, reaction mixtures comprised 100 mM Glyc-NaOH, pH 7.5, 10 mM ATP, 10 mM MgCl<sub>2</sub>, 0.18 mM [<sup>14</sup>C]choline chloride (54 mCi mmol<sup>-1</sup>, NEN-DuPont), and 5 to 30  $\mu$ g of yeast or *E. coli* extract protein in a total volume of 10  $\mu$ L. After incubation at 30°C for 10 min, the reactions were stopped by transferring the tubes to a boiling water bath for 3 min. Under these assay conditions the incorporation of radioactivity into choline phosphate was linear with time (data not shown). Assays designed to measure a potential reverse reaction were conducted using the same reaction conditions with the exception that 10 mM ADP was used instead of ATP and the choline radiolabel was replaced with 0.2 mM [<sup>14</sup>C]choline phosphate (50 mCi mmol<sup>-1</sup>, NEN-DuPont). The ethanolamine kinase assays were conducted in the same reaction mixture described for choline kinase except that 1.6 mM [<sup>14</sup>C]ethanolamine chloride (2.5 mCi mmol<sup>-1</sup>, NEN-DuPont) was used as the radiolabel. The enzyme kinetic data for GmCK2p were obtained from a 3-min incubation of a 100- $\mu$ L reaction containing 1.5  $\mu$ g of *E. coli* extract protein in combination with the various concentrations of choline, DME, and ATP specified in Figure 6. The reaction mixture used to assay for the DME kinase activity of GmCK2p was identical with that described for choline kinase assays with the exception that DME was added to a concentration of 0.2 mM, and 1 mM [ $\gamma$ -<sup>32</sup>P]ATP (1 Ci mmol<sup>-1</sup>, NEN-DuPont) was used as the source of radiolabel. The reaction products for each of the above assays were spotted onto silica gel 60 plates and resolved by TLC using a developing solvent of 95% ethanol, 2% NH<sub>4</sub>OH (1:1, v/v). Radiolabeled substrates and products were visualized by autoradiography and quantitated by scraping the labeled spots and measuring counts using liquid scintillation spectrometry.

## RESULTS

### Isolation of Choline Kinase cDNAs from Soybean

The generation of ESTs through the Arabidopsis genome initiative is proving to be an invaluable resource as a means of identifying genes in higher plants that have previously been isolated from only nonplant sources (Newman et al., 1994). To determine whether an EST possessing homology to a known choline kinase had been identified in a higher plant, data bases harboring

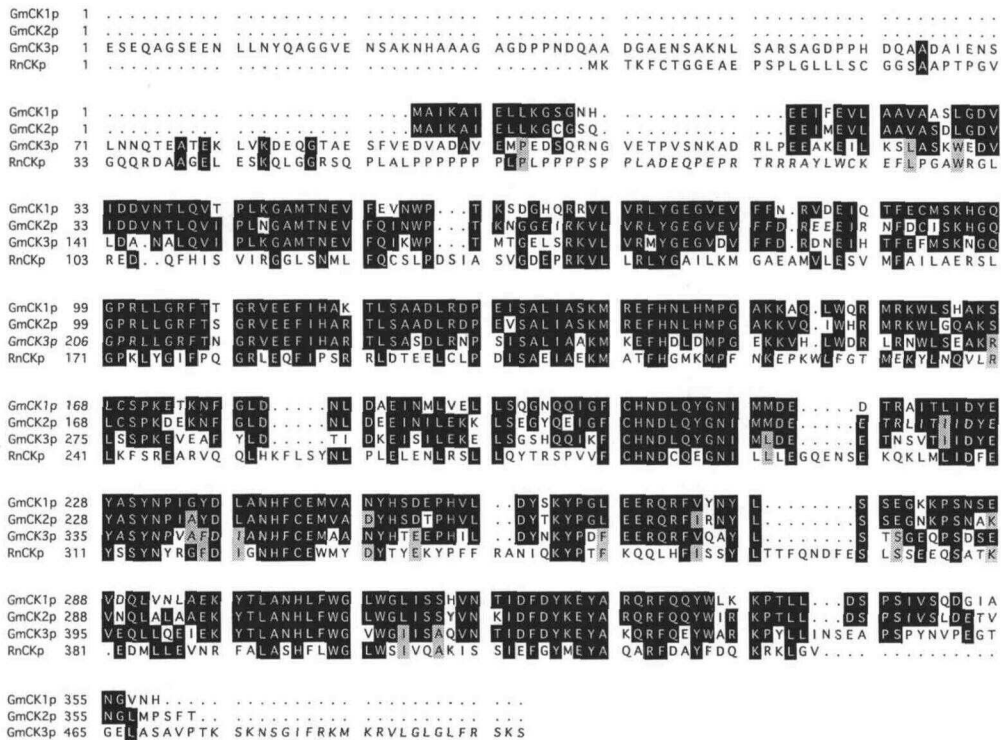
EST information were searched using the predicted amino acid sequence of yeast choline kinase as the query sequence. This search revealed a single Arabidopsis EST (GenBank accession No. T22378) that displayed significant homology to the yeast sequence (BLAST score = 96). The cDNA corresponding to this EST (clone No. 104E7T7) was obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus) using the Arabidopsis Information Management System.

To isolate potential choline kinase cDNAs from soybean, clone 104E7T7 was used as a heterologous hybridization probe against a  $\lambda$ ZAPII cDNA library generated from 25-DAF developing soybean seed mRNAs. After screening approximately 240,000 plaques under conditions of low hybridization stringency, four positive plaques were identified and purified to homogeneity and their corresponding plasmids were recovered. Restriction enzyme assays and limited DNA sequence analysis demonstrated that two of the sequences corresponded to the same gene. The larger of the two was selected for further analysis and designated GmCK1. The remaining two unique cDNAs were designated GmCK2 and GmCK3.

The complete nucleotide sequence was determined for all three clones, and their predicted amino acid sequences are shown in Figure 1. The sizes of the three cDNA inserts are 1417 bp (GmCK1), 1456 bp (GmCK2), and 1646 bp (GmCK3). Based on the presence of in-frame stop codons upstream of the proposed Met start codons, it was concluded that GmCK1 and GmCK2 possess complete reading frames capable of encoding proteins of 359 amino acids (designated GmCK1p) and 362 amino acids (designated GmCK2p), respectively. Although GmCK3 is considerably longer than GmCK1 and GmCK2, it does not appear to be full length as judged by the continuance of the open reading frame through the 5' end of the cDNA. The GmCK3-encoded polypeptide is predicted to be greater than 497 amino acids in size (Fig. 1).

Data base searches of the predicted amino acid sequences of the three soybean clones against the major protein data banks revealed extensive homologies only to known choline kinases from mammals and yeast. The calculated identities between the soybean polypeptides with each other and with the rat and yeast choline kinase sequences are presented in Table I. Although GmCK1p and GmCK2p are more closely related to each other than to the predicted GmCK3 protein, all three soybean sequences display much higher identities with each other than with the rat or yeast sequences.

Southern and northern blots using the GmCK1 insert as a hybridization probe are shown in Figure 2. The hybridization pattern revealed by the Southern blot is consistent with that of a gene family comprising three or more members. Northern blot assays using mRNA from young leaf tissue consistently revealed two distinct hybridizing bands: a small band estimated to be 1.5 kb in size and a larger band of approximately 2.1 kb. The lower band is similar in size to the GmCK1 and GmCK2 cDNA inserts and, therefore, is likely to represent mature transcripts corresponding to these two genes. Because the incomplete GmCK3



**Figure 1.** Alignment of deduced amino acid sequences of three choline kinase-like cDNAs from soybean and the rat choline kinase polypeptide. Identical amino acid residues are shown on a black background. Where two separate matches occur within the same column, one pair is shown with a black background and the other pair with a gray background. The sequences deduced from soybean cDNAs GmCK1, GmCK2, and GmCK3 are represented as GmCK1p, GmCK2p, and GmCK3p, respectively. The sequence from rat (*Rattus norvegicus*) is designated RnCKp (Uchida and Yamashita, 1992).

clone is considerably longer than the GmCK1 and GmCK2 inserts that possess complete reading frames, it is tempting to speculate that the 2.1-kb mRNA represents the mature GmCK3 message. However, the isolation of a full-length GmCK3 cDNA and the use of gene-specific probes will be necessary to definitively establish the size and expression pattern of the individual GmCK cDNAs.

**Expression of GmCK cDNAs in Yeast and *E. coli***

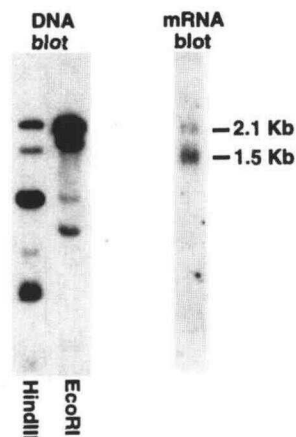
Although the use of ESTs and heterologous hybridization strategies can be a powerful tool in accelerating the gene isolation process, such approaches must be accompanied by some assay to confirm protein function. Yeast strain CTY392 possesses a knockout mutation in its endogenous choline kinase gene (McGee et al., 1994). To determine whether GmCK1 and/or GmCK2 encode choline ki-

nases, the ability to restore this activity to the mutant yeast strain by expressing the soybean cDNAs was tested. Yeast expression vector pDB20 is designed to express cloned inserts using the constitutive *ADH1* promoter (Becker et al., 1991). The GmCK1 and GmCK2 cDNA inserts were cloned into pDB20 (designated GmCK1.DB20 and GmCK2.DB20),

**Table I.** Predicted amino acid sequence identities between choline kinases from soybean, rat, and yeast

The yeast choline kinase sequence is referred to as ScCKp. The nomenclature of the other polypeptides is the same as in Figure 1.

	GmCK1p	GmCK2p	GmCK3p	RnCKp
GmCK2p	81			
GmCK3p	63	63		
RnCKp	32	32	32	
ScCKp	31	32	27	26



**Figure 2.** DNA and mRNA blot analyses of soybean using a GmCK1 probe. For the Southern blot, each lane represents 10 µg of genomic soybean DNA digested with the indicated restriction enzyme. The northern blot represents 5 µg of poly(A)<sup>+</sup> RNA that was isolated from young soybean leaves. The sizes of the hybridizing mRNAs were estimated using RNA size markers.

transformed into CTY392, and assayed for choline kinase activity.

In yeast it is possible to assay for the restoration of choline kinase activity both *in vivo* and *in vitro*. The *in vivo* assay involves adding radiolabeled choline to growing yeast cultures and measuring incorporation of radiolabel into PC; *in vitro* assays are conducted using cellular extracts as described in "Materials and Methods." Hosaka et al. (1989) reported that their yeast mutant containing a choline kinase disruption displayed residual activity *in vivo* but virtually no activity *in vitro*. This *in vivo* activity was presumed to be the result of a small amount of cross-activity from the closely related ethanolamine kinase. We have made similar observations using strain CTY392 (data not shown) and have, therefore, chosen to conduct the choline kinase assays using the *in vitro* assay that is essentially free of background activity.

The results of the choline kinase assays of CTY392 transformed with GmCK1.DB20 and GmCK2.DB20 are shown in Figure 3. Yeast extracts generated from strains expressing either soybean cDNA clearly possessed substantial choline kinase activity (lanes 3 and 4). In fact, the choline kinase activity from extracts of CTY392 transformed with GmCK1.DB20 was greater than 70 times that observed from CTY182 (lane 1), the wild-type yeast strain from which the CTY392 mutant was derived (McGee et al., 1994). Although the choline kinase activity attributable to the GmCK2.DB20 construct was considerably less than GmCK1.DB20, it was still approximately 5 times the levels of the wild-type CTY182 extract (lane 4 versus lane 1). Because of the unavailability of an antibody directed against soybean choline kinase, we cannot determine whether the lesser activity derived from GmCK2.DB20 re-

lative to GmCK1.DB20 is a result of the differential expression of the two constructs in yeast versus the alternative explanation that the GmCK2p enzyme is less active than GmCK1p *per se*.

Investigations of mammalian choline kinases have shown that this enzyme may be effectively expressed in both yeast and *E. coli* (Hosaka et al., 1992; Uchida and Yamashita, 1992). Because *E. coli* neither synthesizes PC nor possesses any of the enzymes of the nucleotide pathway, it provides a background for expressing the soybean choline kinase that is likely to be devoid of potential regulatory proteins that could interact with or influence the activity of a foreign choline kinase, as may be present in a system such as yeast that has an active nucleotide pathway. To provide an additional background in which the function and various properties (such as substrate specificities) of a soybean choline kinase may be assayed, the cDNA insert of GmCK2 was tested for its ability to express choline kinase activity in *E. coli*. Using a PCR-based strategy, we subcloned the cDNA insert of GmCK2 into expression vector pUC120 (designated GmCK2.Nco) and transformed *E. coli* strain MV1190. Enzyme assays of these *E. coli* extracts again verified that GmCK2 encodes an active choline kinase function. The choline kinase activity per milligram of cellular extract protein was approximately 10 times greater for GmCK2 expressed in *E. coli* than when expressed in CTY392 (Fig. 3, lanes 4 and 5).

Kinney and Moore (1988) reported that the castor bean endosperm choline kinase was highly reversible and could readily catalyze the formation of choline and ATP, given choline phosphate and ADP as substrates. When extracts from yeast expressing the two soybean choline kinase cDNAs and the extract from *E. coli* expressing GmCK2.Nco were tested for their ability to catalyze the reverse reaction, this activity could not be detected (Fig. 3, lanes 6–8). The very minor amounts of [<sup>14</sup>C]choline (less than 0.1% of input radiolabel) that appeared in these assays was presumed to be the result of a low level phosphatase activity present in the extracts, since similar levels of this product were also observed from vector-only control extracts (data not shown).

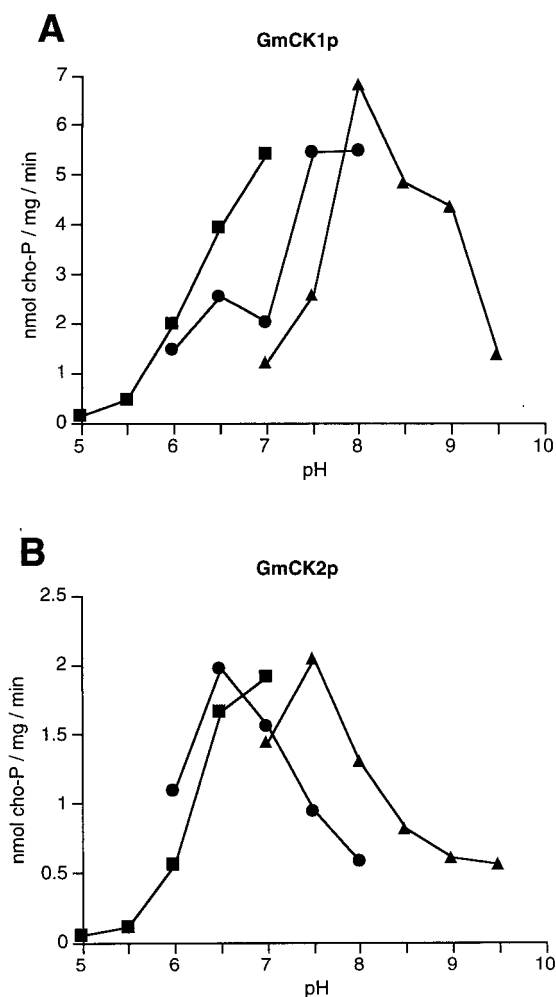
Previous studies of choline kinases from both plant and nonplant sources have shown that the enzyme is typically most active at alkaline pHs when assayed *in vitro* (Wittenberg and Kornberg, 1953; Tanaka et al., 1966; Uchida and Yamashita, 1990). The pH profiles of the two soybean choline kinase isoforms are shown in Figure 4. Surprisingly, both soybean enzymes demonstrated optimal activity in the neutral pH range (6.5–8.0). For GmCK2p, the pH profile was identical regardless of whether the enzyme was synthesized in the yeast (data not shown) or *E. coli* systems. A significant buffer effect was also observed in these experiments; the relative enzyme activities varied depending on whether the assays were conducted in Gly-NaOH, Hepes-NaOH, or Mes-NaOH.

#### Substrate Specificities of the Soybean Choline Kinase Isoforms

To determine whether GmCK1p or GmCK2p may also utilize ethanolamine as a substrate, extracts from yeast and



**Figure 3.** Expression of GmCK1 and GmCK2 cDNAs in yeast and *E. coli*. Extracts of yeast or *E. coli* cells expressing GmCK1, GmCK2, or control vectors were assayed for choline kinase activity (lanes 1–5), the reverse reaction (lanes 6–8), and ethanolamine kinase activity (lanes 9–12). The reaction products were separated using TLC and visualized by autoradiography. Lane 1, Wild-type yeast strain CTY182 transformed with pDB20. Lanes 2 and 9, Yeast strain CTY392 transformed with pDB20. Lanes 3, 6, and 10, CTY392 transformed with GmCK1.DB20. Lanes 4, 7, and 11, CTY392 transformed with GmCK2.DB20. Lanes 5, 8, and 12, *E. coli* strain MV1190 transformed with GmCK2.Nco. The locations of choline (CHO), choline phosphate (CHO-P), ethanolamine (ETN), and ethanolamine phosphate (ETN-P) are indicated. The autoradiogram was overexposed to allow visualization of the minor amounts of ETN-P observed in lanes 10 and 12.

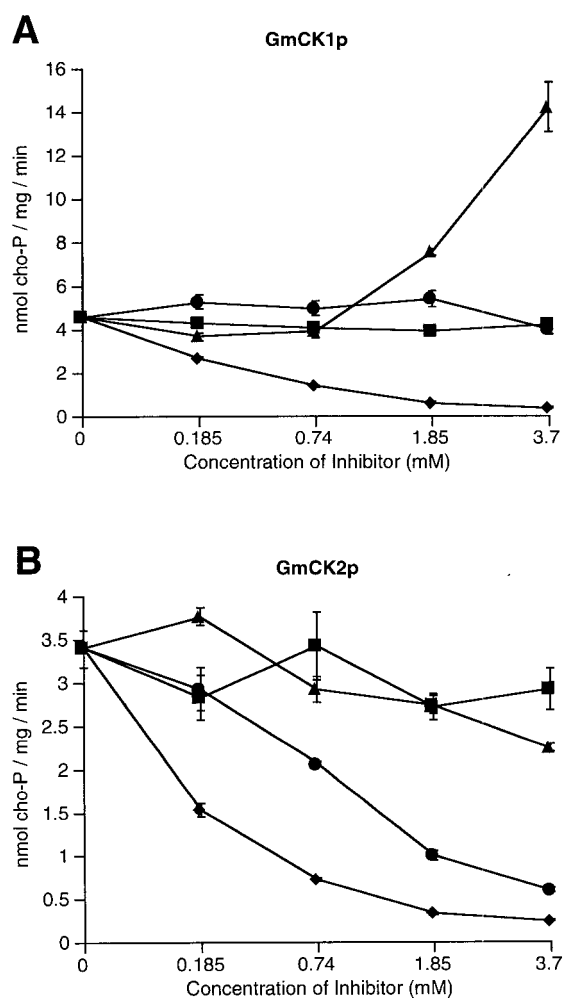


**Figure 4.** pH profiles of soybean choline kinases. Enzyme activity, expressed in terms of nanomoles of radiolabeled choline phosphate produced per milligram of extract protein per minute (nmol cho-P/mg/min), was assayed as described in "Materials and Methods" except that the indicated buffers of varying pH were used. A, pH profile of the soybean GmCK1.DB20 construct expressed in CTY392 (GmCK1p). B, pH profile of the soybean GmCK2.Nco clone expressed in *E. coli* (GmCK2p). The buffers used were Gly-NaOH (▲), Hepes-NaOH (●), and Mes-NaOH (■).

*E. coli* expressing the soybean cDNAs were tested for ethanolamine kinase activity. Because the majority of the ethanolamine kinase activity in yeast results from the cross-activity of the choline kinase enzyme (Hosaka et al., 1989), strain CTY392 demonstrates greatly reduced ethanolamine kinase activity. Under the *in vitro* assay conditions of Figure 3, the synthesis of radiolabeled ethanolamine phosphate using extracts of CTY392 (vector only control) was nondetectable by autoradiography (lane 9) and could barely be measured by scintillation counting. This lack of significant ethanolamine kinase activity makes CTY392 a suitable background for testing the soybean cDNAs for this function. Only a minimal amount of ethanolamine kinase activity could be detected using extracts of CTY392 transformed with GmCK1.DB20 or GmCK2.DB20 (Fig. 3, lanes

10 and 11). Because *E. coli* possesses no ethanolamine kinase activity, the extracts from the MV1190 cells expressing GmCK2.Nco can also be used effectively to test whether ethanolamine can act as a substrate. Similar to the results using the yeast extracts, the synthesis of [<sup>14</sup>C]ethanolamine phosphate was almost nondetectable from GmCK2p produced in *E. coli* (Fig. 3, lane 12). For all three extracts, the same results were observed regardless of whether the assays were conducted at pH 7.5 or 9.5 (data not shown).

In addition to ethanolamine and choline, the mono- and dimethylated ethanolamine bases (MME and DME, respectively) are potential substrates for the choline kinase reaction. To determine whether either of these compounds can potentially act as a substrate for the GmCK1 or GmCK2 gene products, their ability to inhibit the choline kinase activity of the two soybean enzymes was tested (Fig. 5). Although both soybean choline kinases responded simi-

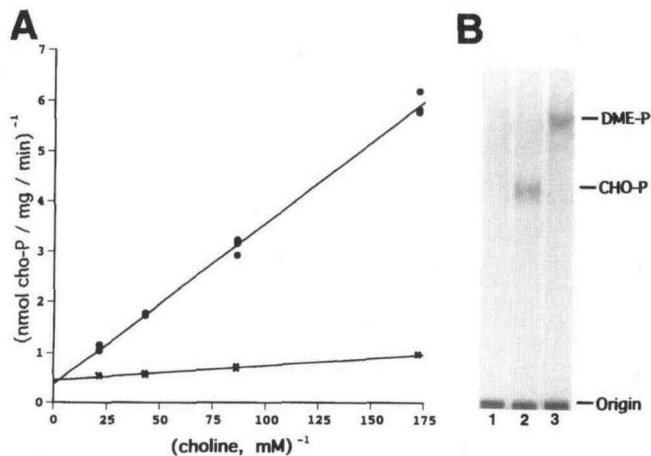


**Figure 5.** Effect of choline (◆), MME (▲), DME (●), and ethanolamine (■) on the phosphorylation of [<sup>14</sup>C]choline by GmCK1p (A) and GmCK2p (B). GmCK1p activity was tested using extracts of yeast strain CTY392 expressing GmCK1.DB20. GmCK2p activity was measured using extracts of *E. coli* expressing GmCK2.Nco. An identical profile, however, was also observed using extracts of CTY392 transformed with GmCK2.DB20 (data not shown). Error bars denote the SE of three replications.



larly to increasing concentrations of unlabeled choline and ethanolamine in a manner consistent with the results of the direct enzyme assays, both MME and DME gave very different inhibition profiles with respect to each isoform. MME failed to inhibit the activity of GmCK2p but unexpectedly stimulated the choline kinase activity of GmCK1p at the higher MME concentrations. As increasing amounts of MME were added to the reaction mixture beyond that shown in Figure 5, GmCK1p activity remained at a plateau of approximately 3 times that observed with no MME (data not shown). DME inhibited the incorporation of [ $^{14}$ C]choline into [ $^{14}$ C]choline phosphate for GmCK2p but failed to inhibit the choline kinase activity of GmCK1p.

To establish whether the observed inhibition of GmCK2p by DME is competitive, double-reciprocal plots of initial velocity against choline concentration were determined in the presence and absence of DME (Fig. 6A). Addition of DME to the reaction clearly shows the kinetics of a competitive inhibitor. The apparent  $K_m$  of GmCK2p for choline was determined to be  $6.7 \mu\text{M}$ . This estimate correlates well with the  $K_m$  observed for the rat choline kinase reaction (Uchida and Yamashita, 1990) but is notably lower than previous measurements reported for plant choline kinases (Tanaka et al., 1966; Kinney and Moore, 1988). To confirm that DME is a viable substrate for the GmCK2-encoded enzyme, direct DME kinase assays were also conducted (Fig. 6B). The phosphorylation of DME was demonstrated from *E. coli* extracts expressing GmCK2 using [ $\gamma$ - $^{32}$ P]ATP as the radiolabeled substrate.



**Figure 6.** Double-reciprocal plots and DME kinase activity of GmCK2p. A, GmCK2p activity at constant ATP concentration (5 mM) and various choline concentrations in the presence (●) and absence (×) of saturating DME ( $1.6 \mu\text{M}$ ). B, DME kinase (lanes 1 and 3) and choline kinase (lane 2) assays using [ $\gamma$ - $^{32}$ P]ATP as the radiolabeled substrate. Reactions were conducted using *E. coli* extracts expressing GmCK2.Nco (lanes 2 and 3) or a vector-only control (lane 1). For all assays, radiolabeled compounds were observed that did not migrate beyond the origin; unincorporated [ $\gamma$ - $^{32}$ P]ATP was observed to migrate just below the solvent front (not shown). The locations of choline phosphate (CHO-P) and DME phosphate (DME-P) are indicated.

## DISCUSSION

Utilizing resources that have become available as a result of the Arabidopsis genome project, we have isolated three distinct soybean cDNAs that are homologous to known choline kinases from yeast and animals. By expressing in yeast or *E. coli* the two isoforms for which full-length cDNAs were recovered (GmCK1 and GmCK2), we established that each encodes choline kinase activity. Although function has yet to be demonstrated for the GmCK3 cDNA, it shows considerably less homology to GmCK1 or GmCK2 than the latter pair shares with each other and is predicted to encode a much larger protein. All three soybean sequences, however, share similar homologies to the yeast and animal choline kinases (Table I).

Our characterization of the GmCK cDNAs and their predicted protein products may help resolve an apparent discrepancy in the literature regarding the nature of this enzyme in soybean. Two previous studies describing choline kinases from soybean resulted in very different molecular weight estimations. The partially purified enzyme from germinating seeds reported by Wharfe and Harwood (1979) had a molecular mass of 36 kD. Mellor et al. (1986), in contrast, described two soybean choline kinase isoforms from roots of 58 and 60 kD (one of which was detected only upon infection with *Rhizobium japonicum*). Assuming no posttranslational modification, the predicted molecular masses of the GmCK1 and GmCK2 gene products are 40,941 and 41,590 D, respectively; these estimations are close in size to that observed in germinating seeds. Should GmCK3 also prove to encode a functional choline kinase activity, it may represent one of the isoforms identified in root preparations, since it is predicted to be greater than 56,273 D in size (the molecular mass calculated from the 497 amino acids shown in Fig. 1). Once a function for GmCK3 has been established, it will be of interest to utilize gene-specific probes to determine whether a specific isoform does in fact demonstrate tissue-specific expression. In this respect, however, it must be presumed that all three GmCK gene products are active to some degree in developing seeds, since all three cDNAs were obtained from a library generated from immature seed mRNA.

Our failure to detect a reverse enzyme activity for either GmCK1p or GmCK2p is consistent with the observations of Tanaka et al. (1966), who reported the lack of such an activity in a partially purified extract from spinach, but differs from the results obtained from castor bean endosperm preparations (Kinney and Moore, 1988). This fact, together with our observations of lower pH optima for GmCK1p and GmCK2p than have been previously observed, raises some intriguing questions regarding the nature of the choline kinase reaction in higher plants. The isolation of choline kinase genes from other plant species and a characterization of their encoded protein products would be of great interest in establishing whether these observed differences are the result of distinct classes of the choline kinase enzyme per se or the result of interactions with other proteins or cellular cofactors that may be

present in plant cells and absent in the heterologous yeast or *E. coli* systems.

Animal and yeast choline kinases have also been shown to function effectively as ethanolamine kinases (Hosaka et al., 1989; Ishidate, 1989). Studies from a variety of plant systems, however, suggest that plant choline kinases are fundamentally different in this regard and do not utilize ethanolamine as a substrate (Kinney, 1993). Our current results support this conclusion. Ethanolamine served as a very poor substrate for both GmCK1p and GmCK2p (Fig. 3) and failed to effectively inhibit the choline kinase reaction even at concentrations 20 times of that of the choline substrate (Fig. 5). Although the substrate specificities of the two soybean choline kinase isoforms were very similar with respect to ethanolamine and choline, very distinct differences were observed in their response to MME and DME. The inhibition studies suggested that DME is a viable substrate for GmCK2p but not for GmCK1p. This observation was confirmed by the direct demonstration of DME phosphorylation by GmCK2p. MME failed to inhibit the choline kinase activity of GmCK2p but unexpectedly enhanced the activity of GmCK1p at concentrations above 1 mM. At MME concentrations of 3.7 mM and higher, GmCK1p activity was maintained at a level 3 times that observed in the absence of MME. Because it has been shown that soybeans do not appear to accumulate significant amounts of MME (Datko and Mudd, 1988a), the physiological relevance of this result is unclear and merits further investigation.

In conclusion, the use of heterologous expression systems has proven to be very effective in verifying function and defining substrate specificities of two soybean choline kinase isoforms. Once a full-length cDNA for GmCK3 has been obtained, these systems should also be of value in determining whether this cDNA represents yet another choline kinase isoform or, alternatively, encodes a closely related function such as ethanolamine kinase. The availability of genes encoding choline kinase, together with those encoding cholinephosphotransferase (Dewey et al., 1994) and cholinephosphate cytidylyltransferase (R.E. Dewey and D.E. Monks, unpublished results), should accelerate the study of PC biosynthesis in higher plants and help elucidate the processes by which plants regulate the production of this important phospholipid at both the molecular and cellular levels.

#### ACKNOWLEDGMENTS

We are very grateful to Dr. Vytas Bankaitis for providing yeast strains CTY392 and CTY182 and to the Arabidopsis Biological Resource Center for providing Arabidopsis clone 104E7T7. We also thank Rich Wilson, Bill Novitsky, and Stephanie Monks for invaluable consultation, as well as Cinthia Williford for technical assistance.

Received September 27, 1995; accepted January 5, 1996.

Copyright Clearance Center: 0032-0889/96/110/1197/09.

The accession numbers for the sequences reported in this article are U43838 (GmCK1), U43839 (GmCK2), and U43840 (GmCK3).

#### LITERATURE CITED

- Ansell GB, Spanner S** (1982) Phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine. In A Neuberger, LLM van Deenen, eds, *New Comprehensive Biochemistry*, Vol 4. Elsevier, New York, pp 1–50
- Becker DM, Fikes JD, Guarente L** (1991) A cDNA encoding a human CCAAT-binding protein cloned by functional complementation in yeast. *Proc Natl Acad Sci USA* **88**: 1968–1972
- Carman GM, Henry SA** (1989) Phospholipid biosynthesis in yeast. *Annu Rev Biochem* **58**: 635–669
- Che F-S, Cho C, Hyeon S-B, Isogai A, Suzuki A** (1990) Metabolism of choline chloride and its analogs in wheat seedlings. *Plant Cell Physiol* **31**: 45–50
- Datko AH, Mudd SH** (1988a) Phosphatidylcholine synthesis: differing patterns in soybean and carrot. *Plant Physiol* **88**: 854–861
- Datko AH, Mudd SH** (1988b) Enzymes of phosphatidylcholine synthesis in *Lemma*, soybean, and carrot. *Plant Physiol* **88**: 1338–1348
- Dewey RE, Wilson RF, Novitzky WP, Goode JH** (1994) The *AAPT1* gene of soybean complements a cholinephosphotransferase-deficient mutant of yeast. *Plant Cell* **6**: 1495–1507
- Feinberg AP, Vogelstein B** (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **132**: 6–13
- Gietz D, St Jean A, Woods RA, Schiestl RH** (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res* **20**: 1425
- Guthrie C, Fink GR, eds** (1991) *Guide to Yeast Genetics and Molecular Biology: Methods in Enzymology*, Vol 194. Academic Press, New York
- Hosaka K, Kodaki T, Yamashita S** (1989) Cloning and characterization of the yeast *CKI* gene encoding choline kinase and its expression in *Escherichia coli*. *J Biol Chem* **264**: 2053–2059
- Hosaka K, Tanaka S, Nikawa J-I, Yamashita S** (1992) Cloning of a human choline kinase cDNA by complementation of the yeast *cki* mutation. *FEBS Lett* **304**: 229–232
- Ishidate K** (1989) Choline transport and choline kinase. In DE Vance, ed, *Phosphatidylcholine Metabolism*. CRC Press, Boca Raton, FL, pp 9–32
- Kinney AJ** (1993) Phospholipid head groups. In TS Moore, ed, *Lipid Metabolism in Plants*. CRC Press, Boca Raton, FL, pp 259–284
- Kinney AJ, Moore TS** (1987) Phosphatidylcholine synthesis in castor bean endosperm. *Metabolism of L-serine*. *Plant Physiol* **84**: 78–81
- Kinney AJ, Moore TS** (1988) Phosphatidylcholine synthesis in castor bean endosperm: characteristics and reversibility of the choline kinase reaction. *Arch Biochem Biophys* **260**: 102–108
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ** (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275
- McGee TP, Skinner HB, Bankaitis VA** (1994) Functional redundancy of CDP-ethanolamine and CDP-choline pathway enzymes in phospholipid biosynthesis: ethanolamine-dependent effects on steady-state membrane phospholipid composition in *Saccharomyces cerevisiae*. *J Bacteriol* **176**: 6861–6868
- Mellor RB, Christensen TMIE, Werner D** (1986) Choline kinase II is present only in nodules that synthesize stable peribacteroid membranes. *Proc Natl Acad Sci USA* **83**: 659–663
- Messing J** (1991) Cloning in M13 phage or how to use biology at its best. *Gene* **100**: 3–12
- Mudd SH, Datko AH** (1986) Phosphoethanolamine bases as intermediates in phosphatidylcholine synthesis by *Lemma*. *Plant Physiol* **82**: 126–135
- Mudd SH, Datko AH** (1989) Synthesis of methylated ethanolamine moieties. Regulation by choline in soybean and carrot. *Plant Physiol* **90**: 306–310
- Newman T, de Bruijn FJ, Green P, Keegstra K, Kende H, McIntosh L, Ohlrogge J, Raikel N, Somerville S, Thomashow M, Retzel E, Somerville C** (1994) Genes galore. A summary of methods for accessing results from large-scale partial sequencing of anonymous *Arabidopsis* cDNA clones. *Plant Physiol* **106**: 1241–1255



- Prud'homme M-P, Moore TS** (1992) Phosphatidylcholine synthesis in castor bean endosperm. Free bases as intermediates. *Plant Physiol* **100**: 1527–1535
- Ramsarma T, Wetter LR** (1957) Choline kinase of rapeseed (*Brassica campestris*, L.). *Can J Biochem Physiol* **35**: 853–863
- Sambrook J, Fritsch EF, Maniatis T** (1989) *Molecular Cloning: A Laboratory Manual*, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Tanaka K, Tolbert NE, Gohlke AF** (1966) Choline kinase and phosphorylcholine phosphatase in plants. *Plant Physiol* **41**: 307–312
- Uchida T, Yamashita S** (1990) Purification and properties of choline kinase from rat brain. *Biochim Biophys Acta* **1043**: 281–288
- Uchida T, Yamashita S** (1992) Molecular cloning, characterization, and expression in *Escherichia coli* of a cDNA encoding mammalian choline kinase. *J Biol Chem* **267**: 10156–10162
- Vance DE** (1991) Phospholipid metabolism and cell signalling in eukaryotes. *In* DE Vance, J Vance, eds, *Biochemistry of Lipids, Lipoproteins and Membranes*. Elsevier, Amsterdam, The Netherlands, pp 205–240
- Wharfe J, Harwood JL** (1979) Purification of choline kinase from soya bean. *In* LA Appelqvist, C Liljenberg, eds, *Advances in the Biochemistry and Physiology of Plant Lipids*. Elsevier, Amsterdam, The Netherlands, pp 443–447
- Wittenberg J, Kornberg A** (1953) Choline phosphokinase. *J Biol Chem* **202**: 431–443