Cloning of cDNA for pyruvate, Pi dikinase from maize leaves

Donald R.Hague, Michael Uhler* and Pamela D.Collins

Departments of Biology and Chemistry, University of Oregon, Eugene, OR 97403, USA

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ABSTRACT

To obtain molecular probes for studies of gene regulation in photosynthetic tissues of maize, we have cloned DNA complementary to poly(A)+RNA extracted from green leaves by insertion into plasmid pBR322 and transformation of *E. coli*, strain RR1. Colonies were screened by sequential hybridization with ³²P-labeled single stranded cDNA synthesized from pooled aliquots of poly-(A)+RNA fractionated by sucrose density centrifugation. Among the clones bearing cDNA homologous to high molecular weight poly(A)+RNA, we identified one with an insert of 440 base pairs homologous to mRNA for pyruvate, Pi dikinase, a C-4 carbon cycle protein localized in mesophyll cells of the leaf. Our work indicates that the dikinase subunits are synthesized in the cytoplasm as precursors approximately 13,000 daltons larger than the mature peptide subunits. Leaves of seedlings illuminated during growth have higher levels of pyruvate, Pi dikinase mRNA than leaves of dark-grown plants.

INTRODUCTION

A number of species of higher plants have an auxiliary photosynthetic carbon pathway called the C-4 cycle. In such species, carbon-dioxide is fixed initially in the C-4 cycle and subsequently released for reduction in the Calvin cycle. Leaves of plants having a C-4 cycle exhibit greater net carbon fixation under specified environmental conditions than those of plants fixing solely through the Calvin cycle (reviewed in 1).

The sequence of reactions of the C-4 cycle occurs in two cell types of the leaf, mesophyll and bundle sheath cells. Enzymes carrying out specific reactions of the cycle are localized in one of these two cell types. It has been known for several years that levels of activity of enzymes of the C-4 cycle and the Calvin cycle are regulated by light (2, 3). We, and others, have more recently demonstrated that the concentrations of certain of these same enzymes are increased by light stimulation of dark-grown plants. The increases in protein concentrations are correlated with specific increases in levels of requisite template RNAs (poly(A)+RNAs) in the leaf tissues (4, 5, 6).

It appears likely from these results that specific structural genes of the C-4 cycle enzymes are differentially activated during development of mesophyll and bundle sheath cells in C-4 plants. Light acts through one or more photo-receptors (7) to increase mRNA levels of the activated genes by increasing transcription rates or changing rates of mRNA processing or degradation.

We are interested in the mechanisms of genetic regulation during differentiation of mesophyll and bundle sheath cells in the leaves of maize (Zea mays L.), and we are using the C-4 cycle enzymes of each cell type as molecular markers of the differentiation process. This report describes the identification of a cDNA clone homologous to the mRNA for Pyruvate, Pi Dikinase (EC 2.7.9.1), a C-4 cycle protein whose activity is localized in mesophyll cell chloroplasts (8).

MATERIALS AND METHODS

Plant Culture

Conditions for growth of sweet corn seedlings (Zea mays L., Golden Cross Bantam) have been described previously (9).

Isolation of Poly(A)+RNA

Total RNA was extracted from maize leaves by a modification of the guanidinium thiocyanate procedure (6, 10). Poly(A)+RNA was isolated on oligo(dT) cellulose according to Aviv and Leder (11). When required, poly(A)+RNA was fractionated by centrifugation on exponential sucrose gradients (6, 12).

Synthesis of Double-stranded cDNA

cDNA was prepared using total poly(A)+RNA from green leaves as template. First strand synthesis was carried out by modifying methods in the literature (13, 14). Poly(A)+RNA was heated at 70°C with oligo(dT) and Tris.Cl (pH 8.3) for 5 minutes and cooled for 5 minutes at room temperature before setting up the reaction. Final concentrations of reagents in the reaction mixture were: oligo(dT), 10 µg/ml; Tris.Cl (pH 8.3), 50 mM; KCl, 100 mM; MgCl₂, 10 mM; 2-mercaptoethanol, 28 mM; dATP, dGTP, dTTP, 300 µM; dCTP, 50 µM; and 100 µCi ³²P-α-dCTP (specific activity 800 Ci/mmol, New England Nuclear, Boston, MA.). Transcription was initiated by the addition of 10 units of reverse transcriptase (Life Sciences, Inc., St. Petersburg, FL.) per microgram of poly(A)+RNA, and the mixture was incubated at 42°C for 45 minutes.

First strand synthesis was terminated by bringing the reaction mixture to 10 mM EDTA, 0.1% SDS, 0.3 M NaOH, and RNA hydrolysis was carried out by incubation of this solution at 37°C for 6 hours. After neutralization with 1M

HCl, labeled single-stranded cDNA was purified by chromatography over Sephadex G-100 equilibrated with 0.1 M NaCl. The second strand was synthesized according to Kurtz and Nicodemus (15) and synthesis was monitored by an S₁ nuclease protection assay (16). After S₁ digestion of the hairpin loop of double-stranded cDNA, analysis of the product in agarose gels showed that the double-stranded cDNA ranged from 300 to 2,000 bp in size.

Cloning

The complete double-stranded cDNA preparation was inserted in plasmid pBR322 by a double-linker technique described by Kurtz and Nicodemus (15) and modified by Uhler and Herbert (17). In brief, blunt-ended, double-stranded cDNA was ligated with a mixture of EcoRI and Hind III linkers (P.L. Biochemicals, Inc., Milwaukee, WI.). Ligated linkers were digested with EcoRI and Hind III restriction endonucleases (New England Biolabs, Inc., Beverly, MA.), and 25 ng of the purified, double-stranded cDNA was ligated with a five-fold molar excess of the large fragment prepared from pBR322 by digestion with the same two restriction enzymes. *E. coli*, strain RR1, was transformed with the ligated vector by the calcium chloride technique of Dagert and Ehrlich (18). Approximately 8,000 ampicillin resistant colonies resulted on plating of the transformed cells, and 5,000 of these were transferred to microtiter dishes containing L broth and ampicillin (50 µg/ml) for overnight growth.

Initial Screening

Colonies were transferred from microtiter dishes to cellulose nitrate membranes using a device described by Mangiarotti *et al.*, (19). The membranes were placed on the surface of petri plates containing L broth with ampicillin and solidified with agar. After 4 to 5 hours growth, colonies were visible, and the membranes were transferred to fresh agar plates containing chloramphenicol (10 µg/ml) for plasmid amplification (20 hrs). Finally, colonies were screened by hybridization according to the procedure of Grunstein and Hogness (20). Single-stranded cDNA of specific activity 8×10^7 cpm/µg, transcribed from sucrose-gradient fractionated poly(A)⁺RNA, was used as a hybridization probe as described in RESULTS.

Selection of mRNA by Hybridization to Selected Clones

Plasmid DNA containing selected inserts was isolated and 15 µg was applied to 4mm squares of cellulose nitrate membrane according to the procedure of Parnes *et al.* (21). Denatured DNA bound to the filters was submerged in 100 µl of a hybridization solution containing high molecular weight poly(A)⁺RNA enriched on sucrose density gradients (RNA concentration 150 µg/ml). After hybridization for 3 hours as described (21), the membranes were washed

thoroughly and then submerged in 300 μ l of water containing 20 μ g of bovine liver tRNA. The solution was heated at 100°C for one minute, then quick frozen in a dry ice-ethanol mixture and thawed. Cellulose nitrate membranes were removed and RNA was extracted with phenol-chloroform and precipitated with ethanol. RNA was pelleted after overnight storage at -70°C, and the pellet was rinsed twice with 70% ethanol, dried by aspiration and dissolved in 10 μ l sterile water for translation.

Large-Scale Plasmid DNA Preparations

One liter cultures were grown to $A_{600} = 0.7$ and plasmids were amplified by adding chloramphenicol (200 μ g/ml) and shaking for 16 hrs. Plasmid DNA was extracted according to Godson and Vapnek (22). After lysis and pelleting of debris, the supernatant was digested with heat-treated RNase A (25 μ g/ml) at 37°C for 30 minutes and then extracted with phenol-chloroform and finally with chloroform. DNA was precipitated with ethanol and pelleted. After redissolving in 2 ml of water and adding 0.5 ml of 50% glycerol and 3 drops of 10% bromophenol blue, DNA was purified by chromatography on Biogel A-50. The running buffer was 0.3M NaCl, 10 mM Tris.Cl (pH 7.6) and 0.01% sodium azide. The void volume was collected and precipitated with ethanol.

Small-Scale Plasmid DNA Preparations

Small plasmid DNA preparations were made using the method of Birnboim and Doly (23).

Labeling of Plasmid DNA

Selected clones were labeled with ^{32}P - α -dCTP by a technique described by Whitfield *et al.* (24). Random primers, 8 to 12 nucleotides long, prepared from calf thymus DNA were boiled with plasmid DNA for 3 minutes then quenched. After addition of the Klenow fragment of DNA polymerase I and dNTPs with ^{32}P - α -dCTP, the mixture was incubated at room temperature for one hour. Labeled DNA was separated on a 5 ml column of Sephadex G-50 equilibrated with 2xSSC and 0.1% SDS. Specific activity was always in excess of 1.5×10^8 dpm/ μ g.

Agarose Gel Electrophoresis and Transfer of Nucleic Acids to Cellulose Nitrate Membrane

Double-stranded DNA was denatured with glyoxal and electrophoresed in 1.75% agarose according to the procedure of Carmichael and McMaster (25).

RNA was denatured in 50% formamide, 2.2M formaldehyde, 2 mM EDTA and 50 mM Hepes (pH 7.8) and separated in 1.5% agarose gels as described by Derman *et al.* (26). Hepes buffer (50 mM, pH 8.3) was substituted for borate buffer in sample denaturing and in electrode chamber buffers.

Plasmid fragments from restriction with Hind III and EcoRI were separated

on 5% polyacrylamide gels prepared in 40 mM Tris, 20 mM sodium acetate and 2 mM EDTA adjusted to pH 7.8 with HCl (27). The electrode buffer was of the same composition.

RNA and DNA were transferred to cellulose nitrate membrane and hybridized following the procedure of Thomas (28).

Labeling of Protein in vivo and Extraction of Leaves

Excised leaves were labeled with ^{35}S -methionine (specific activity $>800\text{ Ci}/\mu\text{mol}$, New England Nuclear, Boston, MA.) as described previously (6). Protein was extracted from leaves by a method described by Sugiyama and Laetsch (29).

Translation, Immunoprecipitation, and Polyacrylamide Gel Analysis

Poly(A)⁺RNA was translated in the presence of ^{35}S -methionine (specific activity $>800\text{ Ci}/\mu\text{mol}$, New England Nuclear, Boston, MA.) using a commercial rabbit reticulocyte translation kit (New England Nuclear). Rabbit antiserum to pyruvate, Pi dikinase was supplied to us by Dr. Tatsuo Sugiyama. Preparation of IgG fractions from serum, translations, immunoprecipitations and analysis of products on 5% to 15% polyacrylamide SDS gels have been described previously (4, 6, 9).

RESULTS

Our purpose in constructing a cDNA clone bank was to obtain probes to study the regulation of gene activity of C-4 cycle proteins in maize leaves. In an attempt to identify specific clones, we took advantage of the fact that two of the C-4 cycle enzymes are made up of large subunits and these, presumably, are synthesized from large mRNAs. These proteins are: (1) phosphoenolpyruvate carboxylase (PEP carboxylase), which is localized in the mesophyll cell cytoplasm, is composed of identical subunits of about 103,000 daltons and makes up 10% to 15% of the soluble leaf protein (1, 9) and (2) pyruvate, Pi dikinase (PPi dikinase), a mesophyll chloroplast protein which is made up of identical subunits of 94,000 to 97,000 (1, 30, 31) and comprises about 2% of the soluble protein (calculated from 30).

We fractionated poly(A)⁺RNA preparations by exponential sucrose gradient centrifugation and translated aliquots of fractions to identify the position of PEP carboxylase peptides in the gradient. Figure 1 shows that this peptide is synthesized on translation of the lowermost gradient fractions (identified as region II in the A₂₆₀ profile shown in Fig. 1B). As shown in the translation profile, however, this same region of the gradient is heavily contaminated with mRNAs of lower molecular weight proteins (Fig. 1A). To screen

Figure 1: Fractionation of poly(A)⁺RNA from green leaves by sucrose gradient centrifugation.

A. In vitro translations of fractionated poly(A)⁺RNA. Lane numbers correspond to fraction numbers in Fig. 1B. T = translations of total poly(A)⁺RNA. P110 = prominent peptide of approximately 110,000 daltons (6). PEPC = subunit of phosphoenolpyruvate carboxylase (6).

B. A₂₆₀ profile of fractionated poly(A)⁺RNA. Arrows indicate division into "high" and "low" molecular weight regions pooled for cDNA synthesis.

for clones homologous to mRNAs of high molecular weight proteins, we hybridized colony blots first with ³²P-labeled single-stranded cDNA prepared by reverse transcription of mRNA from the pooled fractions of region I of the gradient. After autoradiography, the same blots were rehybridized with ³²P-labeled cDNA prepared from the pooled fractions of gradient region II and autoradiographed a second time. By this procedure we identified eight colonies, from a total of 480 examined, that appeared to contain DNA homologous to poly(A)⁺RNA from region II of the sucrose gradient. Plasmid DNA

Figure 2: "Northern" blot of poly(A)+RNA electrophoresed in 1.5% agarose gel and hybridized with ^{32}P -labeled 12-C-6 (specific activity 2.2×10^8 cpm/ μg). Lane 1: "Low" molecular weight poly(A)+RNA fraction (see Fig. 1B). Lane 2: "High" molecular weight poly(A)+RNA fraction. Five micrograms RNA applied in each lane.

was prepared from several of these colonies. Restriction of the preparation from colony 12-C-6 with Hind III and EcoRI endonucleases and subsequent polyacrylamide gel electrophoresis revealed a single cDNA insert of approximately 440 bp in length (data not shown).

To confirm that cDNA of 12-C-6 was homologous to the high molecular weight poly(A)+RNA of maize leaves, we hybridized ^{32}P -labeled 12-C-6 to "Northern" blots of RNA from regions I and II of the gradient fractionated RNA (Fig. 1). The autoradiogram (Fig. 2) shows that 12-C-6 contains DNA homologous to high molecular weight RNA and is devoid of sequences homologous to RNA from other regions of the gradient. Molecular weight markers on the RNA gel indicate that the cDNA probe hybridizes to an mRNA species that migrates more rapidly than 25S plant cytosolic rRNA but less rapidly than 23S bacterial rRNA. Therefore, the size of the mRNA hybridized by 12-C-6 is greater than 2900 nucleotides (23S rRNA) and less than 3700 nucleotides (25S rRNA) (32, 33).

To complete the identification of clone 12-C-6, we selected mRNA homologous to the clone by hybridization of the high molecular weight poly(A)+RNA fraction to denatured 12-C-6 immobilized on cellulose nitrate membrane and then translated the selected species. Because PEP carboxylase makes up a large part of the protein of green leaves, and the mRNA for this protein is a large component of the high molecular weight messenger RNA fraction (4), we first immunoprecipitated the hybridization-selected mRNA translation products with immunoglobulin G prepared against this carboxylase. Figure 3 shows that 12-C-6 does not select the mRNA for the carboxylase. Instead, it selects an mRNA that translates to yield a higher molecular weight peptide, P110 (Fig. 3, lane 5). Comparison of lane 5 with lanes 4 and 6 of Figure 3 shows that P110

Figure 3: In vitro translation of hybridization-selected mRNA and immunoprecipitation with PEPC antiserum. Lanes 1, 4, and 6: Translations of total poly(A)+RNA. Lane 2: Immunoprecipitation of products of total poly(A)+RNA translation with anti-PEPC IgG. Lane 3: Immunoprecipitation of products of hybridization-selected mRNA translation with anti-PEPC IgG. Lane 5: Total products of translation of hybridization-selected mRNA. Fluorogram exposed for 20 hours. PEPC and P110 as in Fig. 1.

is the largest peptide observed on analysis of products from in vitro translation of total poly(A)+RNA from green leaves. This large peptide (approximately 110,000 daltons) is not present in soluble protein extracts of green plants (6) (also see Fig. 4, lane 9).

We have previously observed that subunits of NADP malic enzyme, a C-4 cycle enzyme of bundle sheath chloroplasts, are synthesized, in vitro, in the form of precursors approximately 12,000 daltons in excess of the mature peptide subunits (6). In the present study, the absence of a peptide band in extracts of plant protein analogous to the 110,000 dalton peptide synthesized in vitro in response to the hybrid-selected mRNA led us to speculate that P-110 might be a precursor that is processed in intact cells to yield a lower molecular weight peptide. PPi dikinase activity is localized in mesophyll cell chloroplasts (8). If this protein is translated from poly(A)+RNA, its subunits will be synthesized as precursor peptides. The published molecular weight

Figure 4: Immunoprecipitations of pyruvate Pi dikinase synthesized *in vivo* and *in vitro*. Lanes 1 and 5: Translation products of total poly(A)+RNA. Lane 2: Translation products of reticulocyte system without exogenous mRNA. Lane 3: Total products of translation of hybridization-selected mRNA. Lane 4: Products immunoprecipitated with anti-PPiD IgG after translation of hybridization-selected mRNA. Lane 6: Supernatant after immunoprecipitation with anti-PPiD IgG of total poly(A)+RNA translation of total poly(A)+RNA. Lane 7: Products immunoprecipitated with anti-PPiD IgG after translation of total poly(A)+RNA. Lane 8: Immunoprecipitation with anti-PPiD IgG of protein labeled *in vivo*. Lane 9: Protein labeled *in vivo*. Fluorogram exposed for 24 hours. PPiDP = pyruvate, Pi dikinase precursor, PPiD = pyruvate, Pi dikinase subunit, PEPC = phosphoenolpyruvate carboxylase subunit.

of PPi dikinase subunits is 94,000 to 97,000 daltons (30, 31). Addition of a "transit" sequence similar in size to that of NADP malic enzyme would cause PPi dikinase precursors to migrate in the same region of the gel as P-110.

To test this hypothesis we translated the hybridization-selected mRNA and immunoprecipitated the products with immunoglobulin G prepared from PPi dikinase antiserum furnished by Dr. Tatsuo Sugiyama. Data of Figure 4 confirm that the single product of translation of this selected mRNA is immunoprecipitated with the monospecific antibody (lane 4). Immunoprecipitation of total poly(A)+RNA translation products specifically removes the identical peptide (lanes 5, 6, 7). When total protein labeled *in vivo* is reacted with dikinase IgG preparation, a peptide band migrating more rapidly than the product synthesized *in vitro* is immunoprecipitated (lane 8). We estimate the molecular weight of the cell synthesized product to be about 97,000 daltons in our gels,

Figure 5: "Northern" blot of poly(A)+RNAs electrophoresed in 1.5% agarose and hybridized with ^{32}P -labeled clone 12-C-6 (specific activity 1.8×10^8 cpm/g). Lane 1: poly(A)+RNA from dark-grown corn leaves. Lane 2: poly(A)+RNA from corn leaves. Three micrograms of poly(A)+RNA were applied to each lane. Film was exposed for 24 hours with an intensifying screen at -70°C .

and we assume this to be the mature form of the PPI dikinase subunit peptide.

Activities of the C-4 cycle enzymes increase in maize tissues during illumination of dark grown plants. We have shown previously that the increases in malic enzyme and PEP carboxylase activity are the result of new protein synthesis and that messenger RNA levels of both these proteins increase several fold during greening (4, 6). Measurements of relative levels of PPI dikinase mRNA in dark-grown and light-stimulated tissues are shown in the "Northern" blot in Figure 5. We consistently observe a four- to five-fold increase in messenger RNA levels for this protein during greening of dark-grown tissue. It is likely that the increase in PPI dikinase activity during greening (1) is primarily the result of synthesis of dikinase protein utilizing the high levels of mRNA resulting from light stimulation.

DISCUSSION

We have cloned a cDNA sequence homologous to a region of the messenger RNA for pyruvate, Pi dikinase. This protein is localized in mesophyll cell chloroplasts of maize leaves where it functions to complete the C-4 pathway by forming phosphoenolpyruvate, the acceptor of atmospheric CO_2 . PPI dikinase is a large protein and is encoded in a large mRNA. This permitted us to select a small number of cDNA clones from which we isolated and identified a single clone 12-C-6, homologous to PPI dikinase mRNA.

We estimate that the mRNA for PPI dikinase is 2900 to 3700 nucleotides in length. The molecular weight of the subunits of the dikinase precursor peptides is about 110,000 daltons. The approximate mean molecular weight of the amino acids making up PPI dikinase is 130 daltons (calculated from 34). There-

fore, 2540 nucleotides are required to code the information for the amino acid sequence of the protein. If one assumes a 3' polyadenylic acid sequence of 100 nucleotides on the dikinase mRNA, then the length of both the 3' and 5' untranslated regions of the mRNA is 360 to 1060 nucleotides. It is uncertain, from this consideration, that the insert cloned in 12-C-6 extends significantly into the region of the mRNA coding for the structural sequence of the protein. In addition, on further screening of the eight colonies selected by the sequential blot hybridization procedure, we found two additional clones with inserts specific to PPI dikinase mRNA and of approximately the same size (440 bp) as that of 12-C-6. The similarity in size of the inserts in these three clones may be the result of restriction sites for EcoRI or Hind III in dikinase cDNA, leading to the accumulation of the 400 bp fragments on cleavage of leader sequences during our cloning procedure. To obtain longer stretches of cDNA for sequencing, we are screening a second cDNA library prepared from corn leaf mRNA by G-C tailing techniques.

Estimates in the literature of the molecular weight of mature subunits of PPI dikinase vary from 94,000 to 97,000 (30, 31). We estimate the molecular weight of the precursor to this protein as 110,000 daltons, and we infer from these data that a "transit" sequence of 13,000 daltons to 16,000 daltons is excised from the precursor on transport into mesophyll cell chloroplasts. Our previous studies show that NADP malic enzyme, localized in bundle sheath chloroplasts, is composed of subunits synthesized from poly(A)⁺RNA as precursors with a "transit" sequence of 12,000 daltons (6). The similarity in size of the large excised sequence of these cytoplasmically-synthesized, chloroplast-active proteins of the two different cell types is intriguing. These are the largest "transit" sequences yet reported for proteins of the chloroplast stroma, but sequences of similar size have been reported for proteins of chloroplast membranes synthesized in the cytoplasm (35).

We have shown that levels of mRNA for three C-4 cycle proteins, NADP malic enzyme, PEP carboxylase, and PPI dikinase increase on light stimulation of dark grown plants (4, 6 and unpublished results). PEP carboxylase and PPI dikinase are localized in mesophyll cells, while NADP malic enzyme is a bundle sheath cell protein. Are mRNA levels in the two cell types regulated coordinately, possibly through a common mechanism? As cDNA probes for malic enzyme and PEP carboxylase are discovered, we will quantitate changes in mRNA levels during light stimulation of dark grown seedlings to determine their relative rates of increase. The plant pigment phytochrome has been implicated in the light regulation of mRNA levels of several photosynthetic proteins (7) and may be

the photoreceptor required for the regulation of the C-4 protein mRNAs also.

Although mRNA levels for the three C-4 proteins are enhanced by light, we consistently observe low levels of the same mRNAs in leaves of dark grown plants (Fig. 5 and References 4 and 6). We are hybridizing "Northern" blots prepared from root tissue RNA with the dikinase probe to see if the "dark" level in the leaves is tissue specific. In addition, we are separating mesophyll cells and bundle sheath cells for RNA for "Northern" blots to determine if the dikinase mRNA in leaf tissue is limited to mesophyll cells, the site of dikinase enzymatic activity. Our working hypothesis is that differentiation in the dark grown tissue results in activation of PPI dikinase genes in the mesophyll cells and subsequent activation of these cells enhances the accumulation of products from these genes.

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*Present address: Department of Genetics of Medicine, Stanford University Medical School, Stanford, CA 94305, USA

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