

Communication

Cell-Specific Photosynthetic Gene Expression in Maize Determined Using Cell Separation Techniques and Hybridization *in Situ*¹

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ABSTRACT

Bundle sheath strands and mesophyll cell extracts have been isolated from maize (*Zea mays L.*) leaves using a mechanical disruption-filtration technique. Northern blot analysis showed that phosphoenolpyruvate carboxylase (PEPCase; EC 4.1.1.31) mRNA accumulates only in mesophyll cells. The mechanisms regulating the cell-specific expression of this gene must, therefore, be at either the level of RNA transcription or that of mRNA turnover. The first successful application of hybridization to mRNA molecules in photosynthetic plant tissue sections is described. Results obtained from this *in situ* study corroborate our finding that PEPCase mRNA accumulates only in mesophyll cells as well as the previously reported (Link, G, DM Coen, L Bogorad 1978 Cell 15: 725-731) finding that the accumulation of ribulose 1,5-bisphosphate carboxylase (RuBPCase; EC 4.1.1.39) large subunit mRNA is restricted to bundle sheath cells. Demonstrating the differential accumulation of PEPCase mRNA and RuBPCase mRNA by utilizing the *in situ* hybridization technique paves the way for its use as a powerful tool in relating cellular differentiation to gene expression during plant development.

photosynthetic plant cells, is described. While the technique is utilized here to corroborate information obtained through Northern blot analysis, its real utility will be in studies of differentiating cells. In such cells, gene expression cannot be studied using cell separation methods due to the absence of morphological distinctions.

MATERIALS AND METHODS

Plant Material. Mature, field-grown maize leaf tissue (inbred B73, gift of Pioneer Hi-bred International, Johnston, IA) was harvested early, preferably on foggy mornings, to minimize the amount of starch in the chloroplasts. Such leaf tissue also contains relatively abundant amounts of RuBPCase and PEPCase mRNAs, roughly 1,000 to 10,000 mRNA molecules per cell (based on estimates made for mature regions of maize seedling leaves) (18), ensuring that the sensitivity of the mRNA detection methods employed would not be limiting. Leaf blades used for the isolation of mesophyll cell extracts and bundle sheath cell strands were deribbed, separated into thin lengthwise strips and quickly placed into liquid N₂. Once frozen, the leaf material was chopped into approximately 0.5 cm long pieces by vigorous hand-blending. This leaf material was then stored at -70°C until used.

Leaf tissue used for *in situ* hybridization experiments was cut into pieces 2 to 3 mm on a side and placed immediately into Farmer's fluid fixative (3 parts anhydrous ethyl alcohol: 1 part glacial acetic acid) (4) for 30 min. The tissue was then dehydrated using a tertiary butyl alcohol series (4) and embedded in paraplast. Transverse sections 10 μm thick were cut on a Spencer '280' microtome and mounted on 'subbed' slides (9). Slides were stored away from heat, dust, and moisture for up to several months.

Isolation of Cell-Specific mRNAs and Northern Blot Analysis. A modification, previously described (23), of the method used by Ghiradi and Melis (10) to separate mesophyll from bundle sheath cells for thylakoid membrane and protein studies was used. Successful separation was ascertained by light microscopic examination of the cell fractions (data not shown). RNA isolation from mesophyll cell extracts and bundle sheath cell strands was carried out using a previously described modification (18) of the protocol of Schmidt *et al.* (22). Oligo(dT) cellulose column-fractionated RNA (3) aliquots of approximately 2 μg each were separated on 1.0% agarose gels in the presence of formaldehyde (16) and transferred to nitrocellulose (24). Oligo(dT) columns were washed under stringent conditions (18). (For a discussion of possible explanations for chloroplast encoded LSU mRNA binding to oligo(dT) cellulose, see Ref. 18). The blotted RNAs

Studies of the specialized process whereby maize and other C₄ plants convert solar energy into usable chemical energy can provide valuable information about both photosynthesis itself and mechanisms of cell-specific gene expression. These plants possess two carboxylase enzymes, PEPCase⁴ and RuBPCase, which are compartmentalized in different photosynthetically active leaf cell types in maize. The cellular localization of the proteins (13, 20) and enzymic activities (15) of PEPCase and RuBPCase in maize has been well documented.

We have used two independent techniques to examine PEPCase and RuBPCase gene expression in specific photosynthetic cells. The *in situ* hybridization technique employed, the first demonstration of such a technique to mRNA molecules in

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⁴ Abbreviations: PEPCase, phosphoenolpyruvate carboxylase; RuBPCase, ribulose 1,5-bisphosphate carboxylase; LSU, large subunit; SSU, small subunit.

were hybridized, using hybridization conditions described elsewhere (18), with nick-translated cDNA probes for PEPCase (12), RuBPCase SSu (L McIntosh, E Bell, J Fitchen, N Dawn, T Nelson, J Yamaguchi, WC Taylor, unpublished data) and a cloned fragment of maize chloroplast DNA containing RuBPCase LSu (7).

In Situ Hybridization. Slides carrying leaf tissue sections prepared as described above were deparaffinized in xylene and then hydrated by passing them through an ethyl alcohol hydration series (100, 95, 85, 70, 50, and 30%) and then through distilled H₂O twice, 5 min in each solution. The slides were next incubated in a solution consisting of 100 mM Tris-HCl (pH 8.0), 50 mM EDTA and 1 μ g/ml proteinase K (E. Merck, Darmstadt, Germany) at 37°C for 30 min, washed briefly in distilled H₂O, and dipped in 100 mM triethanolamine-HCl (pH 8.0) at room temperature (8). Maximal hybridization probe penetration with minimal deterioration of tissue morphology was observed under these conditions. (The proteinase K step appears to be crucial in making cytoplasmic RNAs accessible to the hybridization probes. While we do not have an estimate for the depth of probe penetration, the proteinase K conditions described provide for uniform labeling of maize leaf sections fairly consistently.) Positive charge on the sections and slides was then neutralized by treatment with acetic anhydride as described by Hayashi *et al.* (14). The slides were then washed briefly in a solution of 2 \times SSC (1 \times SSC consists of 0.15 M NaCl and 15 mM Na citrate). The leaf sections were then dehydrated by passing the slides (in reverse order) through the ethanol series described above and air dried.

Hybridization probes were prepared using the Sp6 transcription vector system (Promega Biotec). A 660 base pair restriction endonuclease *Pst*I fragment isolated from a maize PEPCase cDNA clone (pPC2) (12) and a 580 base pair *Pst*I fragment containing only coding region for RuBPCase LSu and isolated from a piece of maize chloroplast DNA (*Bam* 9 fragment of maize chloroplast DNA) (17) were subcloned, in both orientations, into *Pst*I-digested pSP65 vector DNA. Radioactively labeled RNA transcripts were prepared from these pSP65 vector constructions following the manufacturer's protocol. In the experiments reported here, RuBPCase LSu transcripts were pre-

pared using [³H]rUTP (33.7 Ci/mmol, New England Nuclear) and PEPCase probes were labeled using [³²P]rGTP (410 Ci/mmol, Amersham). The transcripts were hydrolyzed to lengths of approximately 100 to 150 base pairs and hybridizations carried out in 50% deionized formamide, 0.3 M NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA, 0.02% BSA, 0.02% Ficoll, 0.02% PVP, 500 μ g/ml yeast tRNA, 10% dextran sulfate, and 500 μ g/ml poly(A) as described by Cox *et al.* (8). Thirty μ l of hybridization probe (1 \times 10⁶dpm) was added to each slide, two 22 mm siliconized coverslips were carefully placed over 15 μ l aliquots of the hybridization solution. The coverslips were sealed with rubber cement and the slides placed in moist chambers (9). Hybridization was allowed to proceed at 42 to 46°C for 36 to 48 h.

Following the incubation period, the rubber cement and the coverslips were removed from the slides by gently pulling them off with a pair of forceps. The slides were then treated with RNase A and washed extensively to remove non-specifically bound probe. The post-hybridization protocol of Angerer and Angerer (1) was used for these procedures. The slides were then dehydrated through an ethyl alcohol series, 5 min in each: 30, 50, 70, 85, and 90%. To prevent denaturation of the nucleic acid hybrids, the ethanol solutions contained 300 mM ammonium acetate (5). The slides were then passed through two changes of 100% ethanol and air dried.

The slides were dipped in Kodak NTB-2 photographic emulsion, diluted 1:1 with water, dried and stored in light tight boxes at 4°C. All photographic manipulations were carried out in absolute darkness. Exposure times varied but were generally 14 to 30 d for ³H-labeled probes and 4 to 10 d for ³²P-labeled probes. (Preliminary experiments conducted using ³⁵S-labeled probes required exposure times of 48 to 72 h. However, nonspecific background hybridization has been problematic.) Slides were developed in D-19 developer for 5 min, rinsed briefly in distilled H₂O and fixed in Kodak Fixer for 10 min. Developing slides at 15°C was found to greatly decrease the number of background grains observed (K Fechtel, personal communication). Some slides were stained for 5 min in 0.05% toluidine blue O (21), briefly destained in distilled H₂O and allowed to air dry. The slides were then permanently mounted with Permout (Sigma), viewed through a Leitz microscope and photographed.

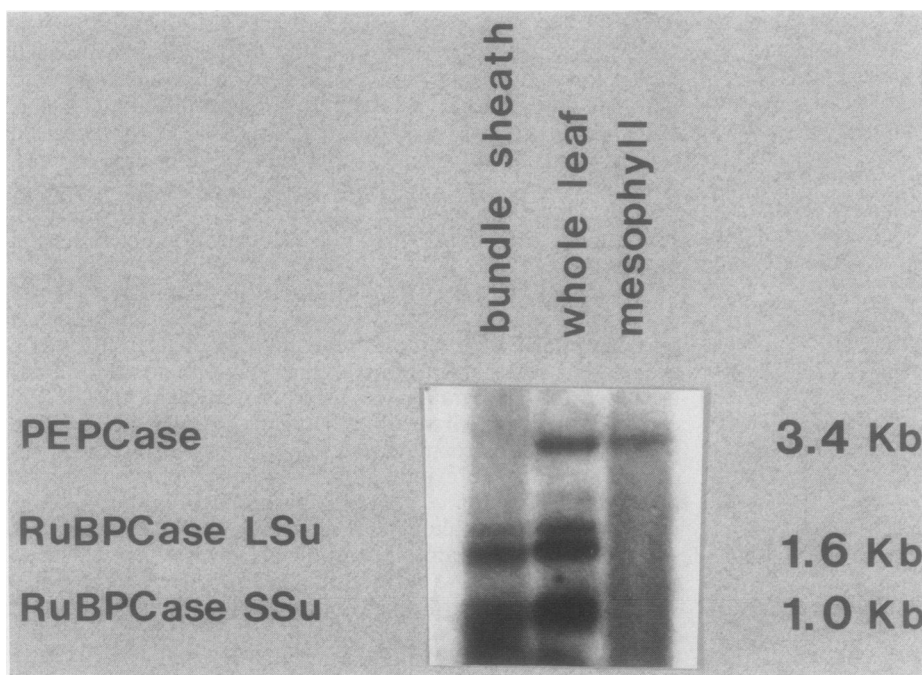


FIG. 1. Cell-specific mRNA accumulation. Northern blot of gel-fractionated poly(A) RNA isolated from bundle sheath strands, mesophyll cell extracts and whole leaf tissue and hybridized with nick-translated probes for PEPCase, RuBPCase LSu, and RuBPCase SSu mRNAs. Equal amounts of the three probes, nick-translated to the same specific activities, were used for the hybridization.

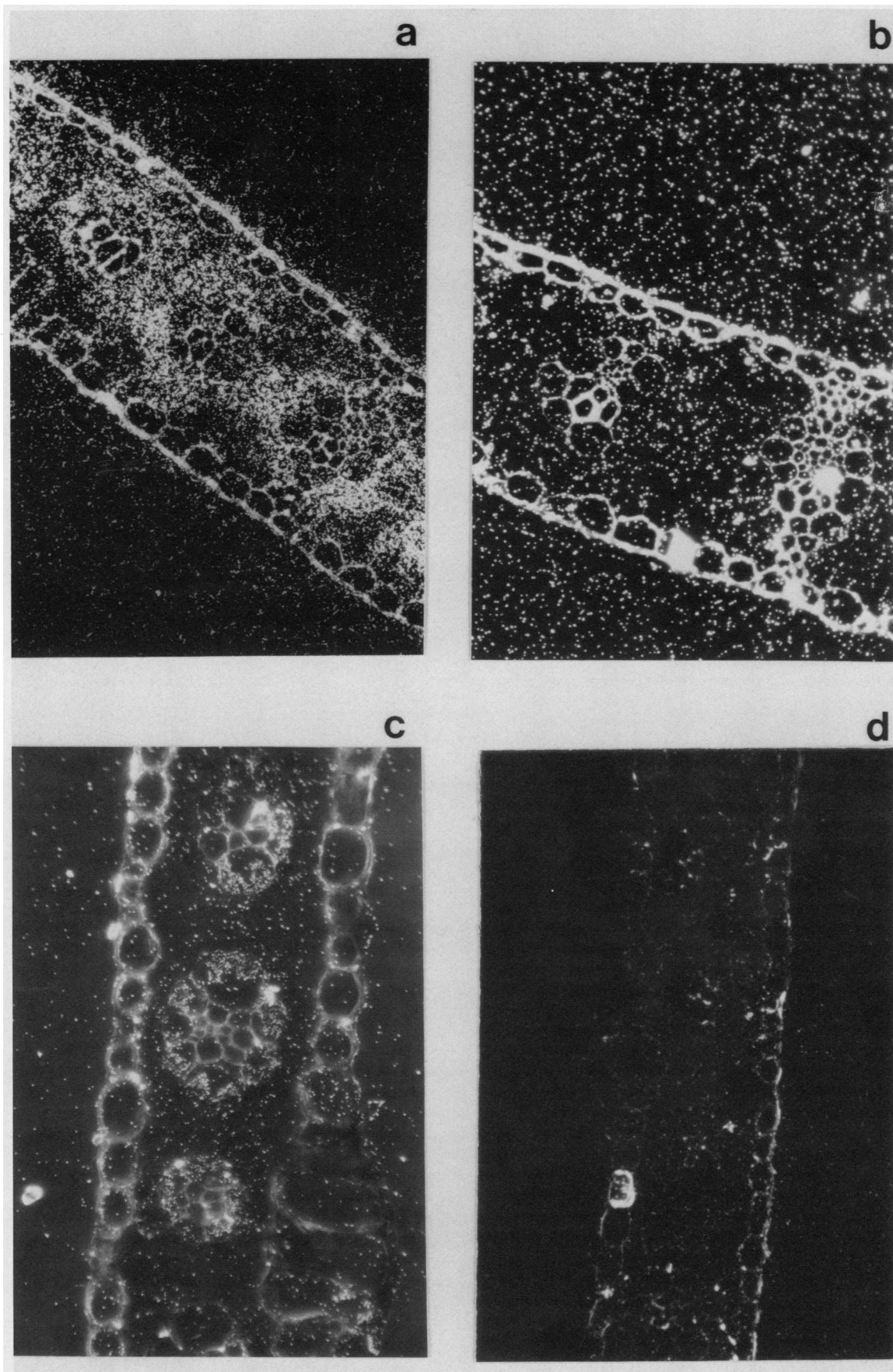


FIG. 2. Distribution of PEPCase and RuBPCase mRNAs in maize leaf sections. Hybridization to mesophyll cell layers (Fig. 2a) and bundle sheath cells (Fig. 2c) is observed with PEPCase and RuBPCase LSu antisense probes, respectively. No specific hybridization can be observed in control section incubated with PEPCase sense (Fig. 2b) and RuBPCase LSu sense (Fig. 2d) probes. The PEPCase probes were labeled to a specific activity of approximately 102 Ci/mmol using [32 P]-rGTP and the RuBPCase LSu probes were labeled to a specific activity of approximately 8.4 Ci/mmol using [3 H]rUTP (see "Materials and Methods") (magnification approximately $\times 500$).

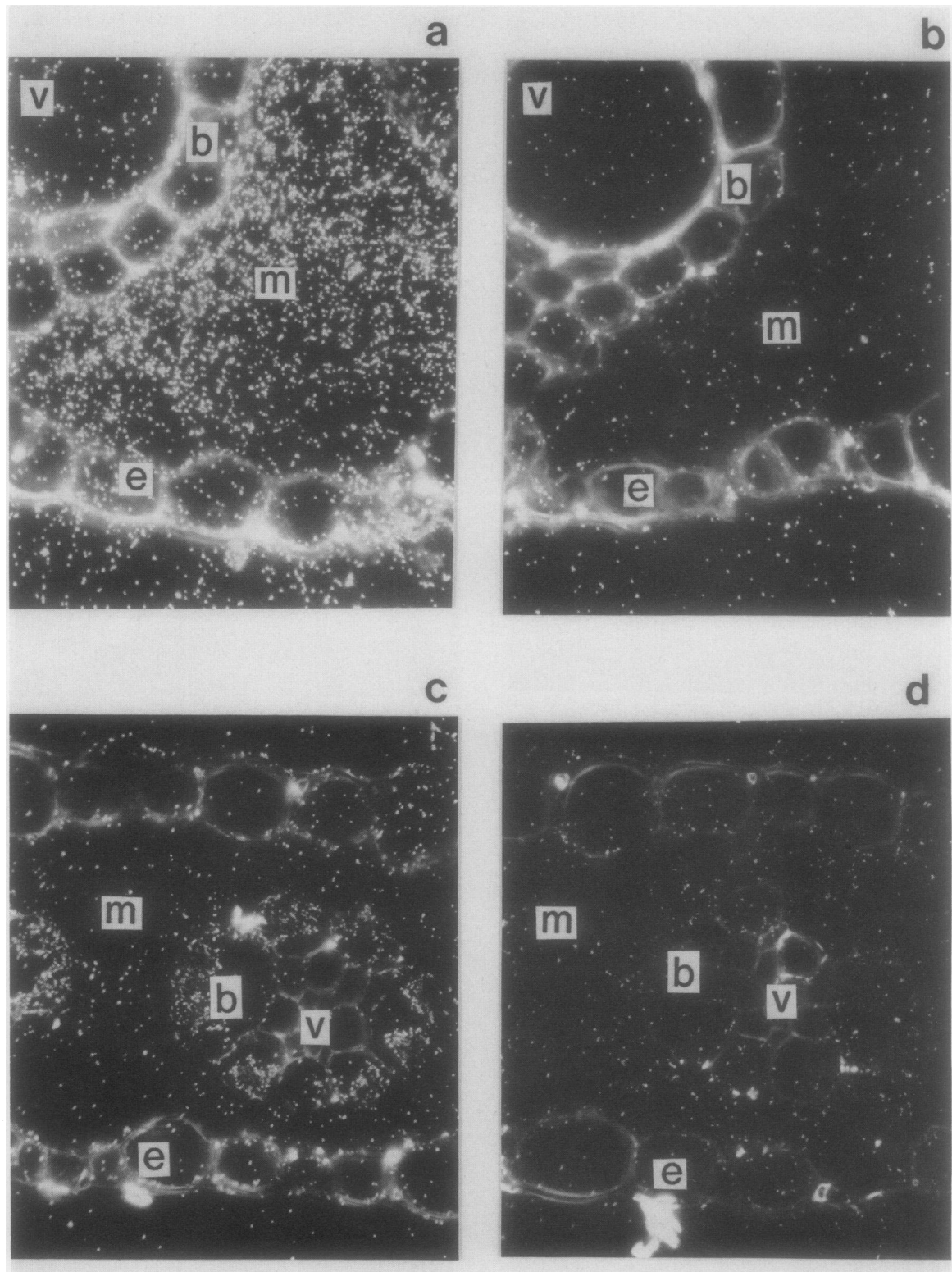


FIG. 3. Cell-specific distribution of PEPCase and RuBPCase mRNAs in maize leaf sections. Higher magnification photomicrographs of leaf sections depicted in Figure 2; sections were incubated with PEPCase antisense (Fig. 3a), PEPCase sense (Fig. 3b), RuBPCase LSu antisense (Fig. 3c), and RuBPCase LSu sense (Fig. 3d) probes, respectively. v = vascular tissue; b = bundle sheath cell; m = mesophyll; e = epidermal cell (magnification of Fig. 3, a and b, approximately $\times 1200$; that for Fig. 3, c and d, $\times 1000$).

RESULTS

Figure 1 shows a representative Northern blot of mesophyll cell-specific RNA, bundle sheath cell-specific RNA and RNA

isolated from unfractionated leaf tissue. The blotted RNAs were hybridized with a nick-translated probes for maize PEPCase and RuBPCase LSu. PEPCase mRNA, 3.4 kilobases, accumulates in maize leaf mesophyll cells but not in bundle sheath cells (Fig. 1).

The opposite is true of RuBPCase LSu mRNA; this mRNA (1.6 kilobases) accumulates in the leaf bundle sheath cells but cannot be detected in the mesophyll cells, in agreement with the previous demonstration by Link *et al.* (17). This Northern blot was also hybridized with a nick-translated cDNA probe for the nuclear-encoded SSu of RuBPCase. RuBPCase SSu mRNA also accumulates exclusively in maize leaf bundle sheath cells (Fig. 1) (6).

The photomicrograph in Figure 2a illustrates the results attained for PEPCase mRNA localization using the *in situ* hybridization technique. Duplex RNA molecules have been formed between an Sp6 promoter-generated PEPCase 'antisense' mRNA probe and the PEPCase mRNA fixed and retained in the leaf tissue sections. As indicated by the presence of silver grains (white specks in these dark field photomicrographs) in the photographic emulsion, PEPCase mRNA is found localized in the leaf mesophyll cells. When viewed at low magnification (as in Fig. 2a), this mesophyll cell-specific hybridization is seen as a figure eight pattern, contained between the epidermal cell layers and weaving its way around the vascular bundles and the leaf's intercellular regions. The photomicrograph in Figure 2b illustrates the results of an identical experiment except that the radioactivity labeled probe consisted of RNA molecules identical in nucleotide sequence to a stretch of maize PEPCase mRNA. This use of the sense PEPCase RNA probe served as a control experiment; only background levels of hybridization were expected and observed.

Similar experiments were conducted using Sp6 promoter-generated RuBPCase LSu antisense RNA probe experimentally and RuBPCase LSu 'sense' RNA probe as a control. In Figure 2c and d, duplex RNA molecules have been formed between the antisense probe and the RuBPCase LSu RNA fixed and retained in the leaf sections. These hybrids, as evidenced by the localization of silver grains in the photographic emulsion, are localized in the bundle sheath cells which are seen (Fig. 2c) surrounding several intermediate leaf veins. Only background hybridization was seen when identically prepared leaf sections were incubated with labeled sense RNA transcripts (Fig. 2d). The somewhat higher signal to noise ratios seen in Figure 2c (despite longer exposure times) as compared to Figure 2a is due, at least in part, to the fact that the slides depicted in the former figure were developed at 15°C while those in the latter were developed at room temperature.

Figure 3 illustrates more clearly the cell-specificity of PEPCase mRNA and RuBPCase LSu mRNA accumulation in maize leaves. The micrographs in this figure were photographed at a higher magnification than those in Figure 2. Only background levels of hybridization can be seen in the epidermal and vascular cells depicted in these photomicrographs. In Figure 3a, however, strong hybridization is detected in the mesophyll cells of a leaf section hybridized with PEPCase antisense probe. Conversely, in Figure 3c, it is the bundle sheath cells, in a leaf section containing a smaller vascular bundle than that depicted in Figure 3, a and b, in which RuBPCase LSu mRNA is strongly detected. This chloroplast-encoded mRNA also appears to be appropriately localized in the bundle sheath cell plastids. Organelle-specific hybridization is, however, difficult to distinguish from hybridization in the bundle sheath cell cytosol itself; both cellular components appear localized along the periphery of the cell nearest the mesophyll cell layer. In the mesophyll cells, which make up most of the rest of the interior of the leaf (Fig. 3c), background hybridization is all that can be detected.

DISCUSSION

Data presented in Figure 1 demonstrate that the strict localization of PEPCase in maize leaf mesophyll cells is paralleled by accumulation of its mRNA exclusively in the same cell type. It has been previously demonstrated that PEPCase is immunolog-

ically identified in the *in vitro* translation products encoded by maize mesophyll cell mRNA but not in those encoded by bundle sheath cell mRNA (6). The results presented here effectively eliminate the possibility that translationally inactive ('masked') RNAs are significant factors in the cell-specific regulation of this gene family. PEPCase cell-specific gene expression is, rather, a result of either transcriptional control or the rapid degradation of PEPCase mRNA in maize leaf bundle sheath cells. Data presented in Figure 1 corroborate the results attained by Link *et al.* (17); maize mesophyll cells lack RuBPCase LSu mRNA. The cell-specific expression of the gene encoding RuBPCase LSu is also, therefore, most likely a result of cell-specific transcriptional regulation or mRNA degradation.

While studies of cell-specific gene expression in mature leaf tissue can, with perseverance, be conducted on isolated maize leaf cell fractions (Fig. 1) (6, 15, 17, 23), cell-specific studies of relatively undifferentiated tissues cannot. Mechanical and enzymic (*e.g.* Ref. 15) cell separation methods depend upon physical differences between the mature cell types. Also, the cell fractions obtained using these separation methods do not consist exclusively of mesophyll cells or bundle sheath cells. Sheets of epidermal cells and bundle sheath strands, for example, are isolated in the same cellular fraction (15; B Martineau, WC Taylor, unpublished data). Therefore, we adapted protocols used to examine the accumulation of mRNAs in sea urchin (1, 2) and *Drosophila* (11) tissue sections for use in maize leaf tissue sections (see "Materials and Methods"). The results of these *in situ* hybridization experiments (Figs. 2 and 3) corroborate those presented in Figure 1 for PEPCase mRNA and RuBPCase mRNA localization.

This report represents the first demonstration of the technique of *in situ* hybridization to cellular RNA molecules in photosynthetic plant tissues. As such, it provides a tremendously useful method for relating plant anatomy to molecular aspects of plant gene expression. Our previous studies (18) demonstrated that small but significant amounts of PEPCase and RuBPCase mRNAs (but not their respective proteins) (19) accumulate in maize leaf tissues prior to mesophyll and bundle sheath cell maturation. Results of our preliminary experiments examining younger tissue indicate that we are able to detect at least 250 mRNA molecules in a cell of average volume. It may now be possible, therefore, to conduct cell-specific expression studies of relatively undifferentiated plant cells.

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