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Developmental modulation of tubulin protein and mRNA levels during somatic embryogenesis in cultured carrot cells

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Abstract. The number of cortical microtubules (MTs) increases considerably as cultured carrot (*Daucus carota* L.) cells initiate and progress through somatic embryogenesis. The basis for this increase in MT number was investigated. A radioimmune assay was used to show that tubulin-protein per cell first decreased as the undifferentiated cells initiated embryonic development, but subsequently increased approximately fivefold between the globular and torpedo/plantlet stages. The increase during the torpedo/plantlet stage was correlated with the increase in cell size that occurred during the latter stages of embryogenesis. The cellular levels of tubulin mRNA were determined by Northern blot analysis, using labeled probes derived from soybean α- and β-tubulin genomic sequences, cloned in the vectors pSP64 and pSP65. This analysis demonstrated that the levels of tubulin-gene transcripts varied with the tubulin-protein levels. Cell-free translation of polyadenylated RNA, followed by immunoprecipitation with an anti-tubulin antiserum, established that these transcripts represented functional tubulin mRNA. These results indicate that MT formation in early embryogenesis is controlled by factors other than the availability of tubulin, but that MT formation later in embryogenesis is coordinated with concomitant changes in tubulin-gene transcription and in the size of the total tubulin-heterodimer pool.

Key words: *Daucus* – Microtubule – Somatic embryogenesis – Tubulin mRNA.

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Abbreviations: kb = kilobase; kDa = kilodalton; Mr = relative molecular mass; MT = microtubule; poly(A)$^+$RNA = polyadenylated RNA; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Introduction

Cell morphology in higher plants is determined by the orientation of cellulose microfibrils within the cell wall (for review, see Palevitz and Hepler 1976). Cortical microtubules (MTs), in turn, may play a role in determining the orientation of the cellulose microfibrils (see Heath and Seagull 1982 for review). In elongating cells, for example, the cortical MTs tend to be oriented at right angles to the axis of elongation, thereby coinciding with the orientation of the more recently deposited cellulose microfibrils (Hardham et al. 1980; Wick et al. 1981; Simmonds et al. 1983). Since treatment of elongating cells with agents that disrupt or destroy the cortical MTs also disrupts the orientation of the cellulose microfibrils and results in a loss of the polarity of cellular expansion, these cortical MTs may determine the vector of cellular expansion (Pickett-Heaps 1967; Palevitz and Hepler 1976; Hogetsu and Shibaoka 1978; Steen and Chadwick 1981). Indeed, the MT/microfibril paradigm has become a central theme in plant development (see discussions in Green and Poethig 1982; Robinson and Quader 1982).

Somatic embryogenesis offers an opportunity to study the role of MTs in plant development at the molecular level. Carrot cells can be grown in vitro either as a mass of single cells and disorganized cell clumps, or as developing somatic embryos (Steward et al. 1964; Halperin and Wetherell 1965). Embryogenesis is induced by transferring the unorganized cells at low cell densities to medium lacking auxin. The development of carrot somatic embryos is similar to that of the zygotic embryos. In the initial globular stage, embryos are composed of approximately isodiametric cells without central vacuoles. Differential cellular elongation in both the procambial files and the epidermis polarizes the embryo by the end of the heart

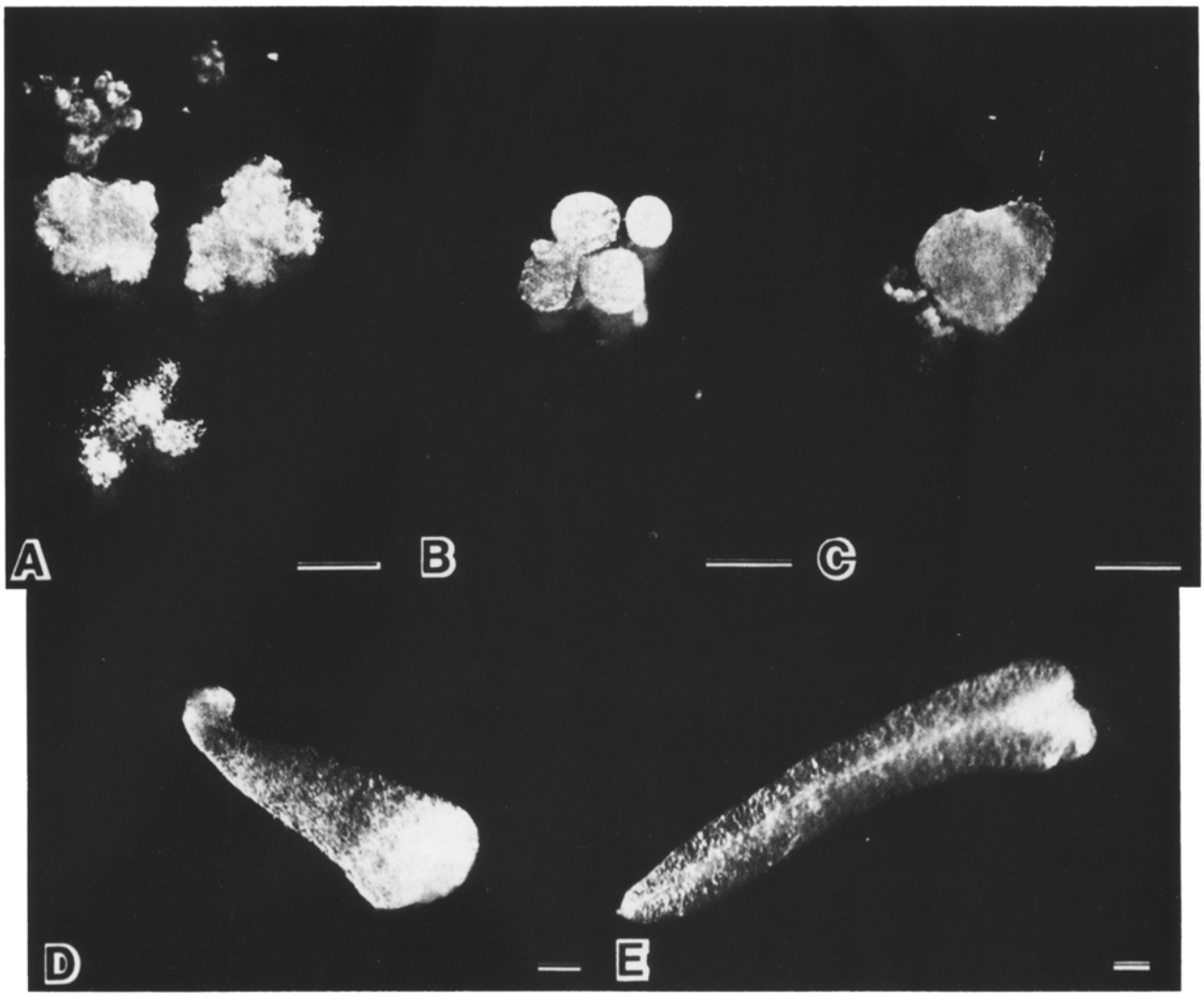

Fig. 1 A–E. Stages of carrot somatic embryogenesis. Cultured carrot cells were induced to undergo somatic embryogenesis by diluting a stationary-phase culture with fresh medium lacking auxin. Shown here are non-embryonic cell clumps and the characteristic stages of embryo development. **A** Non-embryonic, unorganized cell clumps which lack an epidermis, from a cell culture before embryogenic induction. **B** Globular-stage embryos, characterized by an epidermal layer which gives them a smooth surface. **C** Heart-stage embryo, in which the cotyledonary primordia first begin to appear. **D** Torpedo-stage embryo. **E** Plantlet-stage embryo in which vascular tissue has begun to form. Bars = 100 μm

stage. Polarization and cell differentiation continue during the torpedo stage, while the plantlet stage is marked by additional cell elongation and the appearance of vascular elements (Fig. 1).

Electron-microscopic studies of somatic embryogenesis in carrot have shown that cortical MTs become considerably more abundant as carrot cells initiate somatic embryogenesis (Halperin and Jensen 1967; Wochok 1973). How are these changes in MT number brought about? Control of MT formation may be mediated at the level of assembly from a pool of tubulin dimers, without additional tubulin synthesis. For example, in cultured neuroblastoma cells, neurite outgrowth is accompanied by a dramatic increase in MTs, but these are assembled from the pool of soluble tubulin dimers with no change in rate of tubulin synthesis or in the total amount of tubulin per cell (Olmsted 1981). Alternatively, the formation of these additional MTs may be dependent upon, or accompanied by, the synthesis of additional MT protein. Deflagellation initiates flagellar regrowth in *Chlamydomonas*. The MTs of the new flagella are in part assembled from the existing tubulin-dimer pool, but the depletion of this pool is accompanied by a transient increase in tubulin mRNA and in an elevated rate of tubulin-protein synthesis (Weeks and Collis 1976; Lefebvre et al. 1978,

1980). The results of our work, reported in this paper, indicate that the relationship between MT formation and tubulin synthesis in carrot embryogenesis may be similar to that observed in *Chlamydomonas*. Total tubulin-protein levels, as well as tubulin-mRNA levels, are not constant during the course of carrot embryonic development. Except in the initial stages, these parameters tend to vary concomitantly with the number of cortical MTs per cell.

Material and methods

Cell cultures. Cultures of *Daucus carota* L., W001C were kindly provided by Dr. Z.R. Sung, University of California, Berkeley, USA. The cells were grown as suspension cultures in the standard medium of Murashige and Skoog (1962), supplemented with 0.1 $mg \cdot l^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D). To induce embryogenesis, the cells were diluted to a turbidity reading (measured at 600 nm) of 1 with medium lacking 2,4-D (Sung and Okimoto 1981). Globular embryos appeared by day 5. The method of Giuliano et al. (1983) was used for partial synchronization of the embryonic development.

Extraction of tubulin protein. Tubulin was extracted by a modification of the ion-exchange chromatographic method of Morejohn and Fosket (1982). The extraction buffer (PM) contained 50 mM 1,4-piperazinediethanesulfonic acid (Pipes; Sigma Chemical Co., St. Louis, Mo., USA), 0.5 mM magnesium chloride, 2 mM calcium chloride and 1 mM ethylene glycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA; Sigma), pH 6.9. It was supplemented with the protease inhibitors alpha-2-macroglobulin, aprotinin, antipain, chymostatin, leupeptin, and pepstatin (all from Sigma), each at a final concentration of 10 $\mu g \cdot ml^{-1}$, and phenylmethylsulfonyl fluoride (Sigma) at a final concentration of 1 mM. The cells were broken in the extraction buffer as described in Morejohn and Fosket (1982), the homogenate was filtered through Miracloth (Calbiochem, San Diego, Cal., USA) and then centrifuged at $40000 \cdot g$ for 20 min at 4° C. The supernatant was passed through a desalting column, 25 mm diameter, 660 mm long, of P6DG (Bio-Rad, Richmond, Cal., USA) to separate the proteins from secondary metabolites. The protein-containing fraction was brought to 0.4 M KCl, loaded onto a A-50 diethylaminoethyl (DEAE) Sephadex (Pharmacia, Uppsala, Sweden) column and fractionated as previously described (Morejohn and Fosket 1982). The proteins eluted with 0.8 M KCl, consisting mostly of tubulins, were precipitated by bringing the solution to 50% saturation with ammonium sulfate. Rat-brain microtubular protein was isolated by the procedure described by Shelanski et al. (1973). However, the original homogenization buffer was supplemented with the protease inhibitors utilized for plant-tubulin isolation as described above.

Electrophoresis and immunoblot analysis. Proteins were separated on 7.5% acrylamide gels in a modified Studier gel system (Studier 1973), with 150 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris; Sigma), pH 8.8, in the separating gel. Each sample was loaded in duplicate on the slab gels, along with pre-stained protein molecular-weight markers. After electrophoresis at 90 V, constant voltage, one half of the gel was silver-stained (Morrissey 1981), while the proteins in the other half were transferred to nitrocellulose (Towbin et al. 1979). The nitrocellulose blots were blocked overnight with 3% bovine serum albumin in PBS (10 mM sodium phosphate containing 0.9% sodium chloride, pH 7.4) at 37° C. α-Tubulin was detected by incubating the blots with an antiserum, raised in rabbits against taxol-assembled soybean MTs. The antiserum was diluted 1:100 with PBS containing 3% bovine serum albumin. β-Tubulin was detected by reacting the blots with a commercially available monoclonal antibody to chick-brain β-tubulin (Amersham, Arlington Heights, Ill., USA), diluted 1:1000 in the same buffer. In either case the blots were incubated with the antibodies at 37° C, and washed with PBS containing 0.1% Triton X-100. Iodinated protein-A was used to detect polyclonal antibody binding, while the monoclonal antibody was detected with an anti-mouse, alkaline-phosphatase-conjugated antibody (Blake et al. 1984).

Two-dimensional electrophoretic separation of proteins. Proteins were dissolved in an isoelectric focusing sample buffer (described in Bio-Rad Publication No. 1144), which contained pH 4–6.5 ampholines (Pharmacia), and centrifuged at $48000 \cdot g$ for 20 min in an Airfuge (Beckman, Palo Alto, Cal., USA). Between 100 and 600 ng of protein were loaded per gel and the samples were focused for 20 h at 400 V, and then for 4 h at 800 V. Gels were extruded into a modified Studier (1972) sample buffer containing 3% sodium dodecyl sulfate (SDS; Sigma), and loaded onto SDS-acrylamide slab gels, prepared and run as described above, after a 20-min permeation with sample buffer.

Radioimmune assay and cellular protein determination. Tissues at various stages of development were pre-treated with 1 mM Ca^{2+} at 4° C for 2 h to depolymerize MTs; then the tissues were homogenized in PM buffer as described above for tubulin isolation. The homogenates were centrifuged at $12000 \cdot g$ and the supernatants were frozen and stored at −80° C. The protein concentrations were determined using the Bradford (1976) procedure with bovine serum albumin (Sigma) as a standard. To calculate the amount of total extractable protein per cell, cell number also was determined in representative aliquots of each sample, taken before homogenization. For this, the tissue was treated with 5% chromic acid at 37° C to dissociate the tissue and the cells were counted using a Fuchs-Rosenthal chamber. The total protein per cell was calculated by dividing the protein content by the estimate of the total number of cells homogenized.

For the radioimmunoassays, the samples were diluted with PM buffer containing 1 $mg \cdot ml^{-1}$ ovalbumin added as a carrier. Tubulins were quantitated by a direct-binding dot-blot assay similar to the method of Jahn et al. (1984). Known amounts of total calcium-solubilized proteins, at various dilutions, were dotted in 10-μl aliquots onto nitrocellulose strips. All samples were applied in triplicate. The blots were treated with the polyclonal antiserum, and antibody binding to tubulin was detected with iodinated protein-A. A 1.5-cm cork-borer was used to remove equal areas of nitrocellulose, and the filter pieces were counted in a γ-counter. Tubulin levels were computed by linear regression analysis, using known amounts of purified tubulin as standards. The linear binding region was defined as that portion of the standard curve represented by values which could be fitted to a straight line showing a correlation coefficient of at least 0.99.

Immunolocalization of MTs. Protoplasts were isolated according to the method of Hahne et al. (1983). Cortical MT arrays were visualized immunochemically according to the method of Lloyd et al. (1980), except that 0.1% Triton X-100 was used in the extraction buffer, along with the protease inhibitors used for tubulin isolation. The lysed protoplasts were reacted with the

antiserum to taxol-assembled soybean MTs. A fluorescein-conjugated goat anti-rabbit secondary antibody (Sigma) was used for detection. Random photographs were taken with Tri-X film (Eastman-Kodak, Rochester, N.Y., USA), using a Zeiss Photomicroscope (Carl Zeiss, Oberkochen, FRG) and the standard epifluorescence filters for fluorescein.

Isolation of genomic clones encoding tubulin. Sequences encoding both α- and β-tubulin were isolated from a soybean (*Glycine max* (L.) Merr.) genomic library cloned in bacteriophage Lambda (provided by Dr. R. Goldberg, University of California, Los Angeles, USA), which had been constructed from randomly sheared DNA. The isolation and characterization of the β-tubulin genomic fragment, cloned in the plasmids pSP64 and pSP65, and used to generate labeled probes, has been described by Guiltinan et al. (1987). All procedures, unless noted, were performed according to Maniatis et al. (1982). The α-tubulin genomic fragment was isolated by screening 1.5 genomic equivalents of the Lambda library with a full-length nick-translated copy DNA encoding a *Chlamydomonas* α-tubulin (provided by C. Silflow, University of Minnesota, Minneapolis, USA; Silflow and Rosenbaum 1981) using plaque hybridization (Benton and Davis 1977). Four clones were isolated which contained DNA that hybridized to the *Chlamydomonas* α-tubulin cDNA at high stringency (hybridization at 50° C in 3 × SSC [SSC = 0.15 M NaCl + 0.015 M Na citrate, pH 7.0], 10 × Denhart's solution [Maniatis et al. 1982] and 0.1% SDS, followed by washing at 60° C in 1 × SSC, 10 × Denhart's and 0.1% SDS). One of these Lambda clones was selected for subcloning. A 2.2-kilobase (kb) Xho-I fragment was inserted into the plasmid pSP64 (Promega-Biotec, Madison, Wis., USA). A portion of this plasmid was sequenced (Sanger et al. 1977) using a primer to the SP6 promotor region (Promega Biotec) and the sequence data was analyzed with the Bionet Resource Computer (Intelligenetics, Palo Alto, Cal., USA).

Isolation and Northern blot analysis of polyadenylated RNA (poly(A)$^+$RNA). RNA was extracted by the method of Cashmore (1982) and fractionated on an oligo-dT cellulose column (Type III; Collaborative Research, Waltham, Mass., USA). The poly(A)$^+$RNA was further fractionated by formaldehyde-agarose gel electrophoresis (Derman et al. 1981) and the RNA was blotted onto nitrocellulose (Thomas 1980). The filters were hybridized to UT^{32}P-labeled RNA transcripts of the soybean-tubulin genomic fragments cloned either in pSP64 or pSP65 (Melton et al. 1984). The hybridizations were conducted in 50% formamide containing 4 × Denhardt's solution (Maniatis et al. 1982), 4 × SSC, 17 μg·ml^{-1} salmon-sperm DNA (Sigma), 0.1 mg·ml^{-1} tRNA, 0.1% SDS at 60° C. The blots were washed at 60° C in 0.1 × SSC with 0.1% SDS and exposed at −60° C to Kodak XAR (Eastman-Kodak) film using Lightning Plus intensifier screens (Du Pont, Wilmington, Del., USA).

In-vitro translation and immunoprecipitation. A rabbit reticulocyte lysate (Promega-Biotec) was used to translate the poly(A)$^+$ RNA in vitro. Typically, 6–7 μg of poly(A)$^+$RNA were used in the reaction in the presence of $1.85 \cdot 10^5$ Bq of [^{35}S]methionine (Amersham). After translation, aliquots were precipitated with 10% trichloroacetic acid to determine translation efficiency. The α-tubulin protein was immunoprecipitated using the polyclonal antiserum. The translation mixture was brought to 500 μl with PBS containing 0.05% SDS. Protease inhibitors (as used in tubulin extraction) were added to 1 μg·ml^{-1}. Eight microliters of antiserum were added and allowed to react for 2 h at 37° C. Then 20 μl of protein-A Sepharose (Sigma) were added and allowed to incubate for 1 h at 37° C. The Sepharose beads, with adsorbed proteins, were washed four times with PBS and were then added to 20 μl of 8 M urea with 4 μl isoelectric focusing sample buffer. Purified tubulins were added as marker proteins. The samples were analyzed using two-dimensional gel electrophoresis. After separation in the second dimension, the gels were silver-stained to locate the marker tubulins, impregnated with Enhance (New England Nuclear, Boston, Mass., USA), and exposed to Kodak XAR film using intensifying screens at −60° C. After exposure, one major reactive spot was identified on each autoradiogram which comigrated with the α-tubulin marker. This was scanned with a densitometer in the two directions corresponding to the isoelectric focusing and molecular-weight separatory axes.

Results

Changes in cell size during embryogenesis. Previous authors have described qualitatively the change in cell size accompanying carrot somatic embryogenesis (Halperin and Wetherell 1965; Schiavone and Cooke 1985). To quantify these changes, cell size was measured in a randomly selected sample of cells before the initiation of embryogenesis, and in embryos at subsequent stages of development. Cell-surface area was calculated since most cortical MTs are not distributed throughout the cell volume, but are displayed in a narrow layer of cytoplasm near the cell surface, particularly in the more elongate cells. Average cell size decreased as the globular-stage embryos were formed. This decrease in cell size was followed by a progressive increase as the embryos went through subsequent developmental stages (Fig. 2). The average cells of the torpedo/plantlet stages were larger than those of the globular stage by a factor of approx. 5.5.

Tubulin isolation and electrophoresis. The isolation of tubulins from carrot embryos initially was hampered by the presence of secondary metabolites which caused extensive protein denaturation. The manipulation of three factors during tubulin isolation alleviated this problem: elevation of the concentration of dithiothreitol in the extraction buffer to 25 mM, removal of secondary metabolites by passing the homogenate through a desalting column, and the removal, or avoidance, of copper ions during homogenization (the bronze bushings in the Polytron apparatus were replaced with teflon material). When these three steps were taken, the tubulin isolated from carrot callus and somatic embryos exhibited an electrophoretic mobility similar to rat-brain tubulin in the Studier (1973) SDS polyacrylamide gel system (Fig. 3).

A polyclonal antiserum raised against soybean MTs reacted with a single band in Western blots of electrophoretically separated whole carrot-cell proteins. This single immunoreactive band had an apparent relative molecular mass (M_r) = 56000 Da

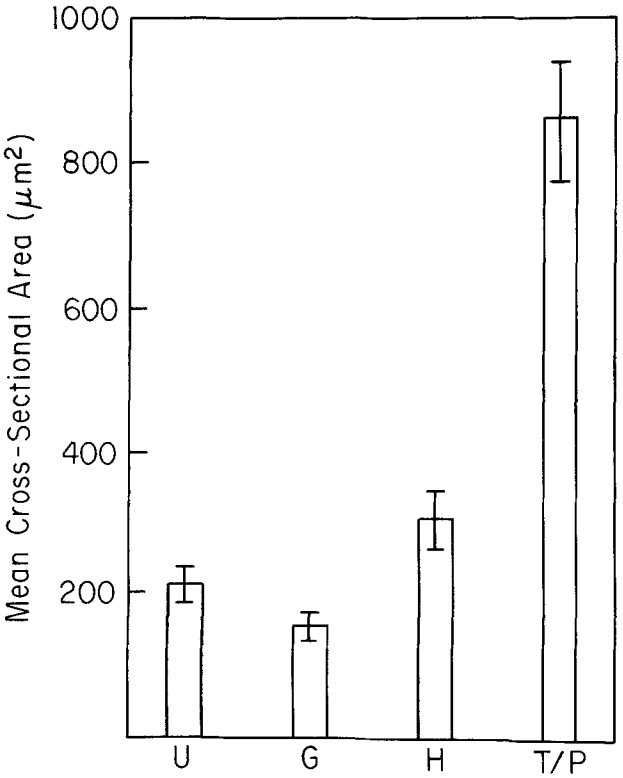

Fig. 2. Cell size is not constant during carrot embryogenesis. Mean cell size (as indicated by the calculated mean cross-sectional area) was determined as described in *Material and methods*. Bars = 1 SE. *U* = uninduced tissue, *G* = globular-stage embryos, *H* = heart-stage embryos, *T/P* = torpedo- and plantlet-stage embryos

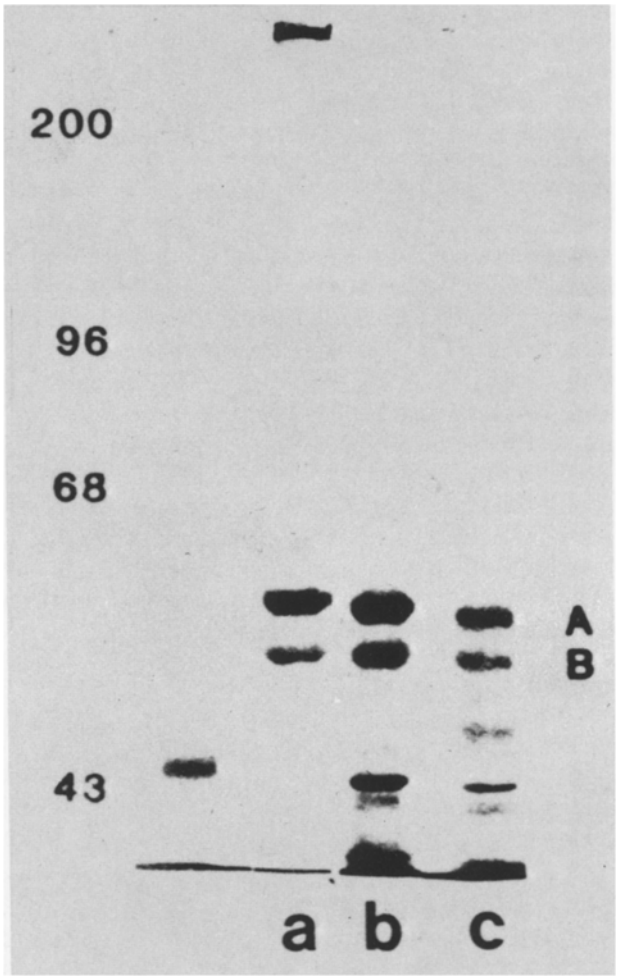

Fig. 3. Tubulin subunits isolated from uninduced carrot cells and from embryos co-migrate with rat-brain tubulin subunits in SDS-PAGE. Tubulin was isolated from both undifferentiated cells (*lane c*) and torpedo/plantlet stage embryos (*lane b*) and subjected to SDS-PAGE, prepared as described by Studier (1973), along with rat-brain tubulin (*lane a*). The proteins were silver stained after electrophoresis. The positions of the MW markers are indicated on the *left*

(Fig. 4A, lane 2). Similarly, the antiserum reacted with the slower-migrating band of the purified carrot tubulin, after its separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 4B, lane 1). In contrast, a β-tubulin-specific monoclonal antibody, prepared against chick-brain tubulin, reacted with the faster-migrating band, which exhibited an apparent M_r = 54000 Da (Fig. 4B, lane 2). The data in Fig. 4B were obtained using the same nitrocellulose filter which was sequentially reacted with a β-tubulin-specific monoclonal antibody and then with the polyclonal antiserum. The monoclonal antibody was detected with an anti-mouse alkaline-phosphatase secondary antibody, while the polyclonal was detected with iodinated protein-A. These data demonstrate that the polyclonal antiserum contains antibodies specific for α-tubulin in carrot cell homogenates.

Quantitation of tubulin. Tubulin levels were determined as a function of the embryonic stage. The technique of Giuliano et al. (1983) was used to synchronize embryogenesis at the pre-globular stage. The cultures were scored for embryonic stage at daily intervals. Complete synchrony was not achieved, although one stage usually predominated. Asynchrony tended to become more pronounced during the advanced stages. Therefore, before protein extraction, each culture was scored for the predominant embryonic stage and, because of asynchrony, data from both torpedo- and plantlet-stage embryos are reported together. Cell counts were performed to ascertain the number of cells from which protein was extracted. Seventy-two percent of the cell clumps in cultures called "globular" were clearly globular-stage embryos, while the remainder of the clumps were not embry-

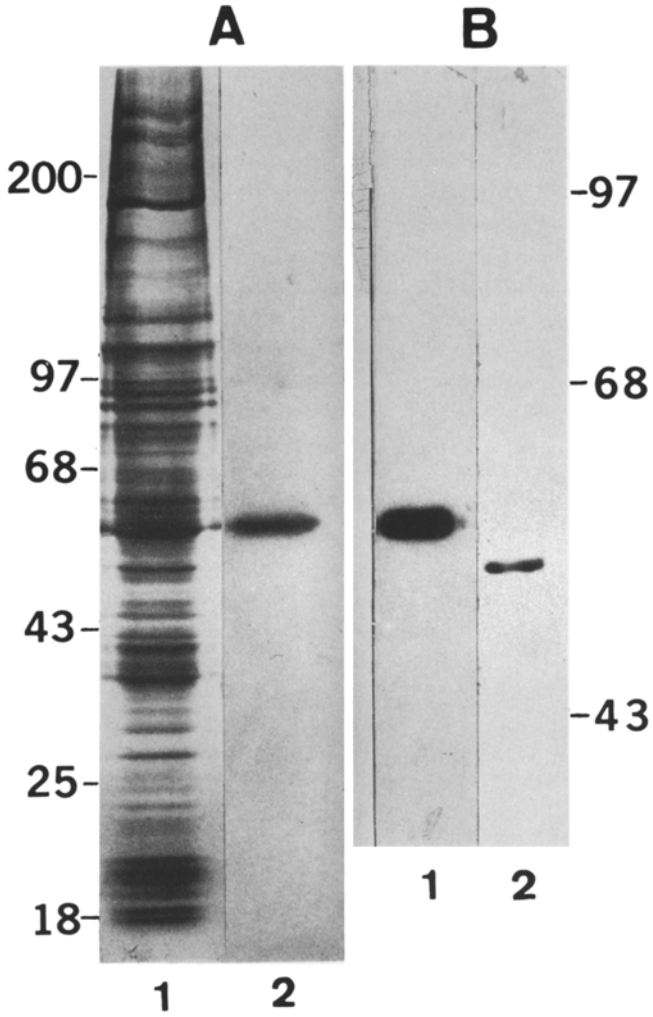

Fig. 4A, B. The antiserum raised against taxol-assembled MTs reacted with carrot α-tubulin. **A** Whole carrot-cell proteins were solubilized by homogenization in extraction (PM) buffer containing 2 mM Ca^{2+}, and separated on a 5–15% gradient acrylamide gel. The silver-stained gel is shown in *lane 1*. The proteins from a comparable gel were blotted onto nitrocellulose and reacted with the anti-soybean-tubulin antiserum. The blot then was treated with iodinated protein-A, washed, and an autoradiogram was prepared. A single band of reactivity was observed which migrated with a $M_r = 56$ kDa (*lane 2*). The position of the marker proteins is noted on the left. **B** Isolated carrot tubulin was subjected to electrophoresis on a 7.5% acrylamide gel prepared according to Studier (1973). The proteins were blotted onto nitrocellulose and reacted with the polyclonal antiserum, raised against taxol-assembled soybean MTs. The antibody reacted to the slower-migrating tubulin monomer (*lane 1*). Conversely, when the same nitrocellulose filter was reacted to a β-tubulin-specific monoclonal antibody, only the faster-migrating monomer was reactive (*lane 2*). The positions of the MW marker proteins are noted on the *right*

onic. In the cultures designated "torpedo/plantlet", an estimated 70% of the total cells were from embryos in one of these two stages of development.

The amount of tubulin extracted from the carrot cells was increased by approx. 50% when the cells were treated with 1 mM Ca^{2+} before homogenization and Ca^{2+} were included in the homogenization buffer. Similarly, a treatment with 1 mM Ca^{2+} markedly reduced the number of MTs detectable in carrot protoplasts by immunofluorescence (Fig. 5), presumably because this treatment disassociated the MTs. As a result, Ca^{2+} was used to disassociate the MTs and solubilize the tubulin for the tubulin determinations.

Tubulin determinations were made from carrot cultures at each stage of embryogenesis by means of a radioimmune assay utilizing the anti-soybean tubulin antiserum characterized above. The data from these assays demonstrated that the amount of extractable tubulin changed during development. Total protein per cell did not change during the initiation of embryogenesis, but it doubled during the transition from the globular to the torpedo/plantlet stages. The amount of tubulin per cell initially decreased as the cultured cells initiated embryogenesis, but it exhibited over a fivefold increase during the later stages of embryogenesis (Table 1).

Partial characterization of genomic clones encoding α-tubulin. Partial restriction maps of the plasmids designated pAST164 and pAST164′ are shown in Fig. 6. The two Bam H1 fragments hybridized under high stringency to an α-tubulin copy DNA (cDNA) from *Chlamydomonas* (but not to a *Chlamydomonas* β-tubulin cDNA). The region extending from the SP6 promoter approximately 200 bp in the 3′ direction of pAST164 was sequenced to verify that it represented a fragment of a soybean α-tubulin gene. A comparison of this putative α-tubulin sequence with both chicken (Valenzuela et al. 1981) and rat (Lemischka and Sharp 1981) α-tubulin sequences demonstrated a homology of 70% between nucleotide positions 425 and 502 of a rat α-tubulin. This homologous region is in the third exon of this rat α-tubulin gene. Similar homology was found to the chicken α-tubulin.

The plasmid pAST164 was used to synthesize probes to determine the cellular levels of α-tubulin transcripts. The SP6 transcript made from the coding region in the orientation shown in Fig. 6 (and predicted to produce an α-tubulin anti-sense RNA sequence), hybridized to blots of electrophoretically separated carrot poly(A)$^+$RNA to produce a diffuse band of approx. 1.7 kb (Fig. 7).

Quantitation of tubulin mRNA levels. Are the increases in tubulin protein levels seen in later stages of embryogenesis paralleled by a comparable increase in tubulin mRNA? Polyadenylated RNA

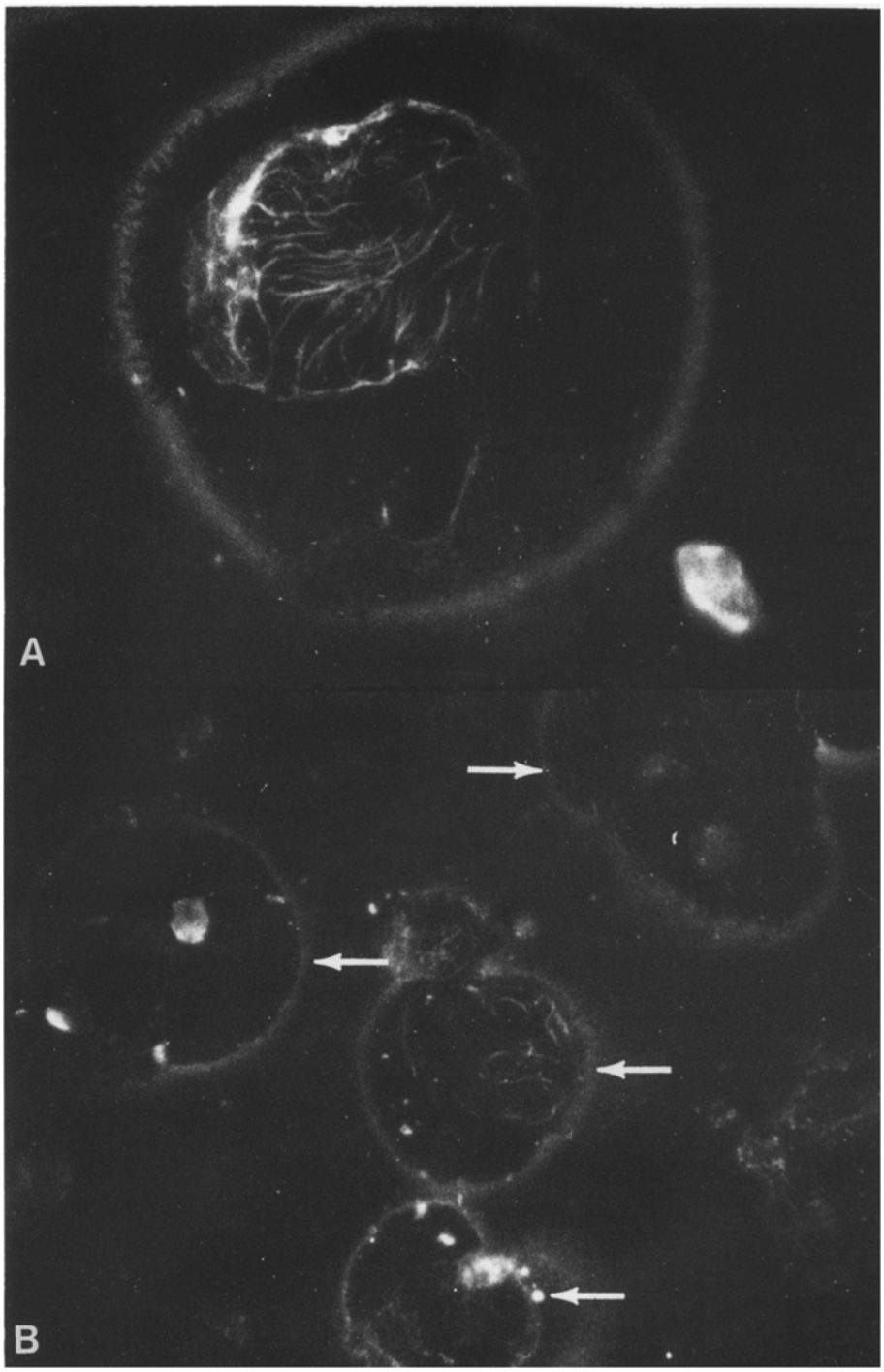

Fig. 5A, B. Ca^{2+} disrupts immunocytochemically demonstrable MTs in carrot protoplasts. Carrot protoplasts were allowed to settle onto poly-L-lysine-coated coverslips and then extracted for 20 min with 50 mM Pipes buffer, pH 6.9, containing 0.1% Triton X-100, with or without 1 mM Ca^{2+}. **A** Protoplasts were extracted with buffer containing no added Ca^{2+}; ×1134. **B** Protoplasts were extracted with buffer containing 1 mM Ca^{2+}, ×882. In either case, the protoplasts were reacted with a polyclonal antiserum raised against soybean MTs, and antibody binding was detected with a second, fluorescein-conjugated goat anti-rabbit antibody, as described in *Material and methods*

was extracted from undifferentiated cells and from plantlet/torpedo-stage embryos and the relative abundance of tubulin-RNA sequences was determined in an effort to answer this question. The soybean-tubulin probes hybridized under high-stringency conditions to complementary sequences in RNA extracted from carrot cells (Fig. 7). Using either the α- or the β-tubulin probes for the detection of tubulin sequences in carrot, only one major band of hybridization was evident with a size of 1.7 kb. This length would be sufficient to encode a 50–56-kDa protein. Densitometric analysis of the autoradiograms indicated that the plantlet/torpedo-stage embryos have an average of 2.8 times more α- and β-tubulin mRNA than undifferentiated cells.

Table 1. Cellular tubulin levels change during carrot embryogenesis. The protein concentration/cell was determined for each sample as described in the text, and the tubulin levels in calcium-solubilized cellular proteins were quantitated by means of a radioimmune assay that employed the antiserum characterized in this study. Each value represents the average of three determinations and is given along with the relative error. Tubulin/cell was computed from these values

Tissue	Protein level (pg/cell)	Tubulin level (ng tubulin/ μg protein)	Tubulin/cell (pg/cell)
Uninduced cells	87±17	120±12	10±3
Globular embryos	85±16	72± 7	6±2
Torpedo/plantlet	190±32	170±15	32±8

In-vitro translation of carrot poly(A)$^+$RNA and immunoprecipitation of the translation products. To see if the rise in the abundance of tubulin sequences represented an increase in functional tubulin mRNA, the isolated carrot poly(A)$^+$RNA was translated in a reticulocyte cell-free system containing [^{35}S]methionine, and the translation products were immunoprecipitated with the antiserum used to quantitate the tubulin-protein levels. Purified carrot tubulin was added to the immunoprecipitate as a marker, and the products were separated by two-dimensional gel electrophoresis. The silver-stained gels are shown in Fig. 8A. The autoradiograms of these gels are shown in Fig. 8B. The major signal in both of these autoradiograms co-migrated with the α-tubulin of the added marker protein. Silver-stained gels of purified tubulins extracted from the torpedo/plantlet-stage embryos exhibited several closely migrating α-tubulin isoforms after two-dimensional gel electrophoretic separation (Fig. 9). The radioactivity co-migrated with the entire α-tubulin region on each of the gels, with no indication that any particular isotype was synthesized preferentially. Densitometric measurements of the areas shown in Fig. 8B showed that the torpedo/plantlet embryos contained 2.7 times the amount of translatable RNA encoding α-tubulin, compared with the uninduced cells.

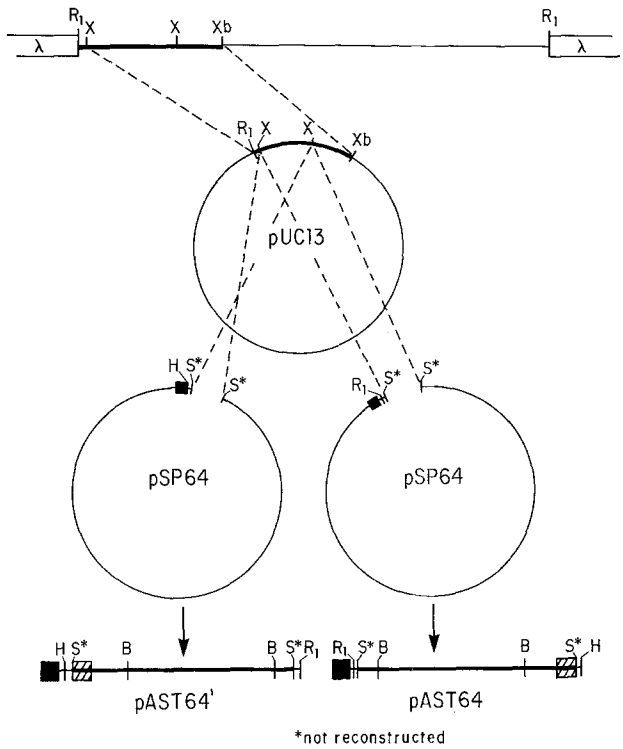

Fig. 6. Cloning strategy and restriction maps of plasmids containing a genomic fragment of a soybean α-tubulin gene. A clone from a soybean genomic library in Lambda was identified as containing a putative α-tubulin sequence by hybridization to a *Chlamydomonas* α-tubulin cDNA. An Eco R1-Xba I fragment of the Lambda clone, which contained the soybean sequences that hybridized to the *Chlamydomonas* α-tubulin cDNA probe, was subcloned into the plasmid pUC13. A 2.2-kb Xho I-Xho I fragment of the pU13 clone, which contained the sequence that hybridized to the *Chlamydomonas* α-tubulin cDNA, was ligated into the Sal I site of the pSP64 poly-linker and used to transform *Escherichia coli*. The Bam H1 restriction pattern of the plasmid isolated from several of the transformed bacterial colonies was determined. Two restriction patterns were observed, representing the plasmid with the putative α-tubulin sequence inserted in the two possible orientations. Representatives colonies containing each of these plasmids were isolated and designated pAST64′ (oriented to give a "sense" transcript from the SP6 promoter) and pAST64 (oriented to give an "antisense" transcript). The vector sequences from pSP64 are denoted by the *thin line*. Regions hybridizing to the *Chlamydomonas* α-tubulin cDNA are denoted with a *thick line*. The region sequenced is shown with *cross-hatching*, and the SP6 promoter is designated by a *solid box*. The position of Hind-III, Eco R1, and Bam H1 restriction sites are shown, designated, in the order given, H, R1, and B. The position of the original Xho I sites which mark the ends of the subcloned soybean sequence were destroyed upon ligation to the Sal I site of the pSP64 or pSP65 polylinker

Discussion

Our results do not explain the electron-microscopic observations of a paucity of MTs in undifferentiated cultured carrot cells versus their relative abundance in the cells of carrot somatic embryos (Wochok 1973; Halperin and Jensen 1967). Although the tubulin levels, whether expressed on a per-cell or per-unit of cellular protein basis, increased during the later stages of embryogenesis, they either decreased or remained unchanged in early embryogenesis. Thus, the increased density of cortical MTs in the globular-stage embryos must result either from a change in the stability of the MTs or an increase in the rate of MT assembly from the soluble tubulin pool. Cytoplasmic MTs

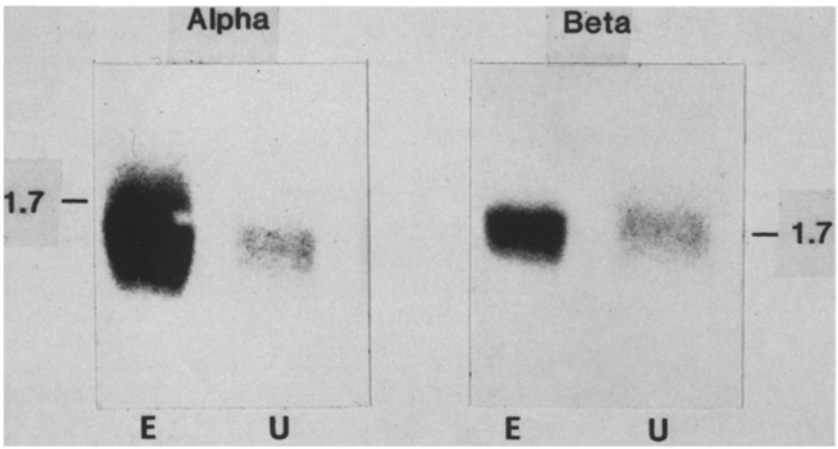

Fig. 7. Tubulin poly(A)$^+$RNA levels are higher in torpedo/plantlet-stage carrot embryos than in undifferentiated cells. Polyadenylated RNA was isolated from uninduced cells and from torpedo/plantlet-stage embryos and fractionated by formaldehyde-agarose gel electrophoresis. The RNA was blotted to nitrocellulose and hybridized to radioactive transcripts of the α-tubulin sequence in plasmid pAST164, or to an RNA transcript from a cloned β-tubulin sequence. Polyadenylated RNA derived from embryos is shown in *lane E*, and poly(A)$^+$RNA derived from uninduced cells is shown in *lane U*. Equal amounts of RNA from embryos and uninduced cells were loaded onto the gels. Blots were probed with the SP6 transcript encoding soybean β-tubulin (*Beta*) and exposed. The probe was eluted and the nitrocellulose filter was probed again with the SP6 transcript encoding soybean α-tubulin (*Alpha*). Only the reactive areas of the gel are shown

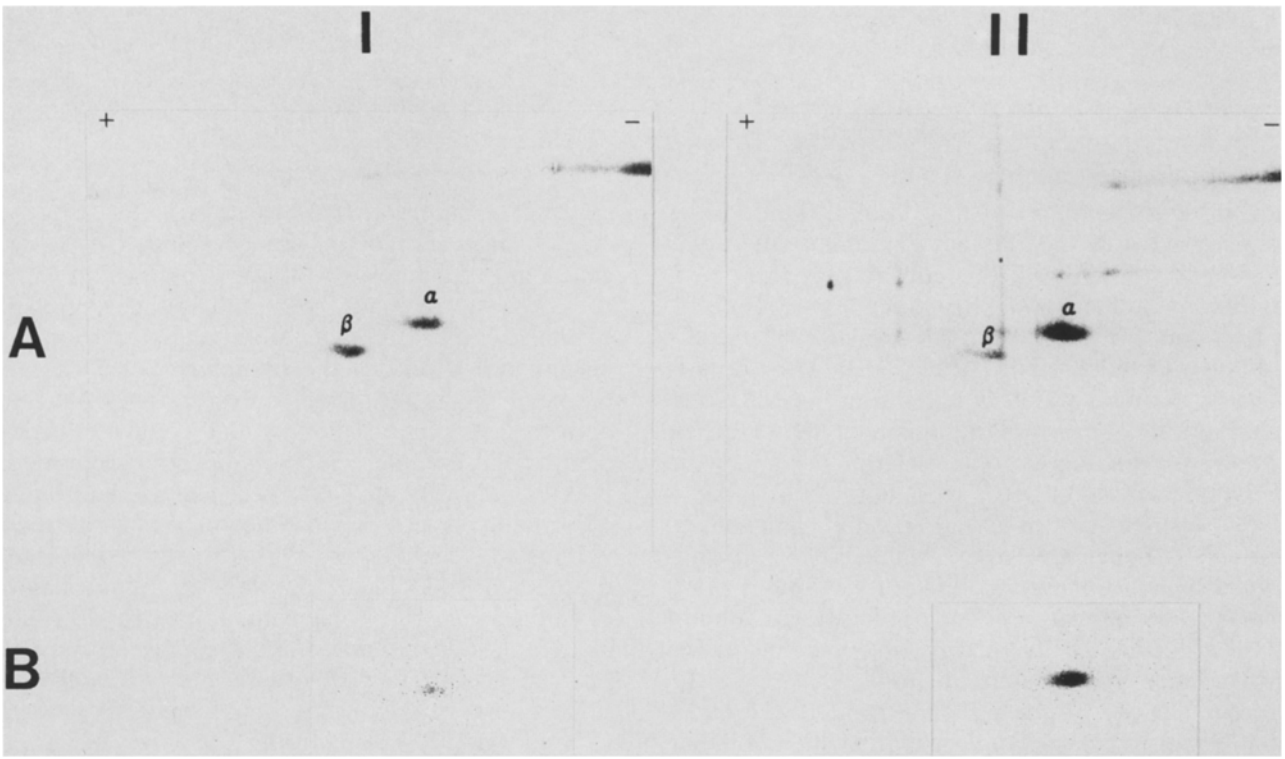

Fig. 8A, B. Separation of in-vitro-synthesized, immunoprecipitated translation products by two-dimensional gel electrophoresis. Polyadenylated RNA isolated from either uninduced cells or torpedo/plantlet-stage embryos was translated in a reticulocyte cell-free system in the presence of [^{35}S]methionine, following which the α-tubulin products were immunoprecipitated as described in *Material and methods*. Purified carrot tubulin was added as a marker and the polypeptides were separated by two-dimensional gel electrophoresis and autoradiographed. **A** Shown here are the silver-stained gels, photographed before the fluorographic procedure. **B** Autoradiographs of the silver-stained gels. *I* = Immunoprecipitated products from the undifferentiated cell poly(A)$^+$RNA translation and marker tubulin; *II* = immunoprecipitated translation products and marker tubulin from the embryonic cell poly(A)$^+$-RNA

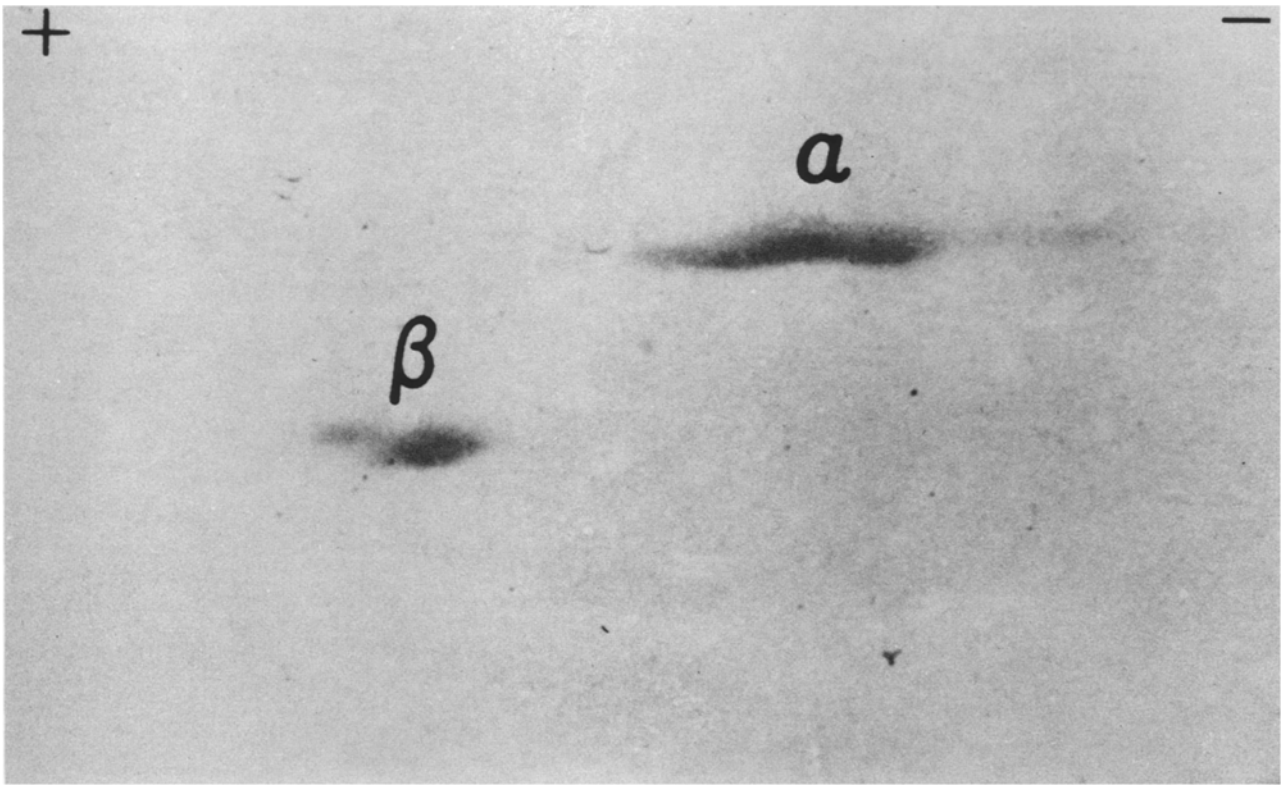

Fig. 9. Two-dimensional gel electrophoretic separation of torpedo/plantelet stage embryonic tubulin. Tubulin, extracted from embryos as described in *Material and methods* was separated by two-dimensional electrophoresis and silver-stained. Closely migrating isoforms of both α- and β-tubulins are evident. The positions of the cathode and anode are indicated on the photograph

are in equilibrium with the soluble tubulin-dimer pool in many of the animal cell systems where this has been studied, and this equilibrium can be affected by numerous factors such as calmodulin, Ca^{2+} concentration, and the presence of specific MT-associated proteins (Raff 1979). Very little is known about the factors regulating the MT/soluble tubulin equilibrium in plant cells. However, an apparently analogous phenomenon occurs in the nerve-growth-factor-promoted outgrowth of neurites from cultured mammalian cells. Neurite outgrowth is dependent upon enhanced MT formation which occurs from the soluble tubulin pool, without an increase in the total cellular tubulin level (Olmsted 1981). In this case, the shift in the MT/soluble tubulin equilibrium seems to come about because of the appearance in the cells of additional MT-associated proteins which enhance the stability of the assembled MTs (Black et al. 1986).

When cultured carrot cells are induced to undergo embryogenesis, both the cell doubling time and the average cell size decrease (Halperin and Wetherell 1965; Warren and Fowler 1978). The decrease in cell size was confirmed in the present study. Furthermore, the reduction in the average cell size was shown to be accompanied by a decrease in tubulin levels. Volume expansion in plant cells occurs principally as a result of the expansion of the vacuole. The expanding vacuole of an enlarging cell displaces the cytoplasm such that it becomes tightly adpressed to the plasmalemma. At maturity, the majority of cellular volume is occupied by the vacuole, which contains mostly water. Previous authors have concluded that the amount of protein per cell is an indicator of cytoplasmic volume (Wyegalla et al. 1985). If this is the case, the decrease in cell size during the initial stages of embryogenesis is not accompanied by a decrease in cytoplasmic volume since the protein content per cell of globular-stage embryos is almost the same as that of the undifferentiated cells.

Cell size increased during the later stages of embryogenesis. The increase in cell size was accompanied by an increase in the cytoplasmic volume, as indicated by cellular protein levels. Electron-microscopic studies have shown that developing carrot embryos tend to maintain a relatively constant density of cortical MTs during cell enlargement (Halperin and Jensen 1967). This is con-

sistant with previous observations in plant cells that the number of cortical MTs increases as cells enlarge (Traas et al. 1984). The present study demonstrates that tubulin-protein levels also increase during the cell enlargement that occurs during the transition of somatic carrot embryos from the heart stage to the plantlet stage, whether expressed on a per-protein or a per-cell basis.

The increase in tubulin-protein levels that occurred with cell enlargement during the later stages of carrot somatic embryogenesis was accompanied by an increase in tubulin poly(A)$^+$RNA levels. There was a very good correlation between the amount of translatable message encoding α-tubulin and the amount of labeled α-tubulin genomic probe hybridizing to total poly(A)$^+$RNA on blots. The change in tubulin message could be achieved by either an increase in tubulin-gene transcription or an increase in mRNA stability. Although our data do not allow us to distinguish between these possibilities, tubulin-protein levels are likely to be controlled at least in part by the level of message availability during carrot embryogenesis. In other organisms, tubulin synthesis appears to be autoregulated by a mechanism in which the tubulin-protein synthetic rate and half-life of the tubulin mRNA are controlled by the amount of non-polymerized tubulin (Ben-Ze'ev et al. 1979; Cleveland et al. 1981; Caron et al. 1985; Pittenger and Cleveland 1985). The relationship between tubulin synthesis and the soluble tubulin pool has not been investigated in plant cells, but such studies, using cultured carrot cells, were initiated recently in this laboratory.

It is difficult to know if the observed increase in tubulin and MTs is causative to, or a consequence of, the cellular enlargement that occurs during carrot somatic embryogenesis. When undifferentiated carrot cells are treated with non-lethal levels of colchicine, cell expansion is not blocked, although the cells lose the polarity of enlargement and become isodiametric (Lloyd et al. 1980). This indicates that cell expansion per se can occur in the absence of assembled cortical MT arrays. However, the establishment of a preferential vector of elongation, e.g., anisotropic expansion, probably requires the presence of properly oriented cortical MTs. Our data as well as previous reports indicate that an increase in tubulin protein is necessary for the formation of the MTs which will control the axis of cellular expansion as the embryos become polarized. The increase in MT number seen during cell expansion indicates that a critical number of MTs are necessary per unit area to regulate this cellular process.

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