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Coordinate gene expression during somatic embryogenesis in carrots

(coordinate regulation/carrot culture/two-dimensional gel electrophoresis/cycloheximide inactivation/cycloheximide-resistant mutant)

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ABSTRACT **There are several biochemical differences between the callus and the embryos of carrot culture. Callus tissue produces callus-specific proteins and a conditioning factor that is necessary for the synthesis of callus-specific proteins. By contrast, embryos produce embryo-specific proteins [Sung, Z. R. & Okimoto, R. (1981) *Proc. Natl. Acad. Sci. USA* 78, 3683–3687] and develop the capability to inactivate cycloheximide [Sung, Z. R., Lazar, G. J. & Dudits, D. (1981) *Plant Physiol.* 68, 261–264]. A mutant, WCH105, that can inactivate cycloheximide in the callus as well as in the embryos produces the embryo-specific proteins instead of the callus-specific proteins and fails to produce the conditioning factor by the callus tissue. Callus tissues also produce a conditioning factor for callus growth. This factor is not the same as the conditioning factor for the synthesis of the callus-specific proteins, as WCH105 can grow as callus. The existence of WCH105 demonstrates that the callus-specific and embryo-specific traits are coordinately regulated, but in an opposite manner. A common mechanism apparently activates one set and inactivates the other set of functions. WCH105 seems to be impaired in this mechanism.**

Development involves temporal and spacial changes of cellular phenotype. Accompanying the morphological changes are the biochemical activities—e.g., specific enzyme activities appear and disappear during morphogenesis (1). These activities are characteristically regulated as sets of gene function that are simultaneously activated and inactivated (2, 3). The decision to activate or inactivate certain functions has been proposed to involve dichotomous choices in *Drosophila* development (4).

Carrot cells grown in culture are faced with two major developmental choices: either to proliferate randomly as callus tissue or to undergo organized growth which leads to embryo development (5). This alternative growth pattern is controlled by 2,4-dichlorophenoxyacetic acid (2,4-D, a growth regulator) and high cell density (6, 7). Together, these conditions suppress embryo development and promote callus growth. There are apparently changes in gene expression that occur during embryogenesis, as evidenced by the synthesis of embryo-specific polypeptides (6), increased arginine decarboxylase activity (8), increased enzyme activities in the pyrimidine pathway (9), elevated ratio of rate of synthesis of poly(A)$^+$ mRNA/rRNA (10), and the development of a capacity to inactivate cycloheximide (CH) (11). The embryo-specific proteins and the CH inactivation (CH^i) are new functions that appear during embryogenesis. We are interested in finding functions that disappear during the embryo state—i.e., callus-specific functions—and ascertaining if there is any relationship between the mechanism that activates the embryo-specific function and that which inactivates the callus-specific function.

In this paper, we report (*i*) the finding of callus-specific functions and (*ii*) the use of a mutant, which expresses the CH^i function in the embryo as well as the callus tissue, to demonstrate that at least two embryo-specific functions and two callus-specific functions are regulated by the same mechanism, but in an opposite manner. A "switch" type of control is proposed as the simplest way to regulate the alternate expression of gene functions in two tissue states. To our knowledge, coordinate control of activation of one set and inactivation of another set of functions has not been reported heretofore.

MATERIALS AND METHODS

Cell Lines and Growth Conditions. Plant materials and culture conditions have been described (11). Briefly, a diploid tissue culture line, WOO1C, of wild carrot, *Daucus carota* L., was cultured in liquid Murashige–Skoog medium (MS medium) (12). To maintain unorganized callus growth, the cells were cultured in MS medium supplemented with 0.1 mg of 2,4-D per liter at high cell density, which means the culture is inoculated at about 8×10^5 cells per ml and transferred before it reaches 10^7 cells per ml. The growth of callus cultures was measured by the sidearm-turbidity method (13). The number of cells at any time point of growth was estimated from the turbidity in a Klett–Summerson spectrocolorimeter expressed in arbitrary units. For example, Klett 100 (K100) corresponds to $\approx 2 \times 10^6$ cells.

To initiate embryogenesis, the callus tissue was washed with and transferred to MS medium supplemented with or without 2,4-D. Each sample was incubated at 8×10^5 cells per ml for 3 days and then was diluted to low density—i.e., 2×10^4 cells per ml (14). Under this condition, the entire culture converts to somatic embryos and a population of enlarged single cells in 6 days. The enlarged single cells occupy <5% of the fresh weight of the embryo culture. Because such embryo culture is free of any callus tissue, it is defined as 100% embryogenic efficiency (14). Conditioned media were media incubated with cells at an initial density of 8×10^5 cells per ml for 6 or more days. The media were separated from the cells by centrifugation at 2,000 $\times$ *g* for 5 min. All experiments were performed with cultures grown in liquid media; for convenience, the cultures grown at high density in 2,4-D-containing media are referred to as "callus cultures" and those grown at low density as "embryogenic cultures" or "embryo cultures."

Cell lines resistant to CH as callus tissue have been isolated from WOO1C (15). Their callus growth is resistant to growth inhibition by 10 μg of CH per ml, a concentration that inhibits the callus growth of WOO1C. However, embryogenic cultures of both WOO1C and CH-resistant cell lines are resistant to 15

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2D, two-dimensional; CH, cycloheximide; CH^i, CH inactivation; CH^r, CH resistance; MS medium, Murashige–Skoog medium.

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μg of CH per ml (11). One of the CH-resistant cell lines, WCH105, has been characterized genetically by somatic hybridization (16).

Labeling and Extraction of Water-Soluble Proteins. If not specified, the cultures were transferred with minimal disturbance; cells were neither centrifuged nor washed, but were pipetted into 15-ml sterile, conical tubes. The cells or embryos were allowed to settle under 1 × *g* to a pellet of ≈0.05 ml at the bottom of the tube. The rest of the culture was pipetted off (the enlarged, vacuolated cells do not settle under 1 × *g*, thus >90% of them are removed), leaving a volume of 0.5 ml, and 0.125 mCi of [^{35}S]methionine (Amersham, 1 Ci/μmol; 1 Ci = 3.7 × 10^{10} Bq) was added. The tube was placed at an angle of about 10° and shaken on a rotary shaker at 1 revolution/sec at 24°C. After 4 hr of labeling, the samples were washed three times by centrifugation at 1,000 × *g* and were homogenized in 200 μl of extraction buffer [50 mM Tris·HCl, pH 6.8/5% (vol/vol) 2-mercaptoethanol/15% (vol/vol) glycerol] in a mortar and pestle at 4°C. The homogenate was centrifuged at 20,000 × *g* for 20 min at 0°C. Typically, 250,000 cpm (≈10 μl) of the homogenate was loaded onto the native gel.

Gel Electrophoresis. Nondenaturing $NaDodSO_4$ two-dimensional (2D) polyacrylamide gels were prepared as in Sachs *et al.* (17) with the following modifications. The native (nondenaturing) slab gels contained 6.5% (wt/vol) acrylamide and 0.133% (wt/vol) *N,N'*-methylenebisacrylamide. The native gel was run on an apparatus that was cooled to 0°C. Molecular weights of proteins separated on the $NaDodSO_4$ gel were estimated by using phosphorylase, M_r 94,000; bovine serum albumin, M_r 68,000; α-amylase, M_r 48,000; ovalbumin, M_r 43,000; carbonic anhydride, M_r 30,000; and trypsin, M_r 24,000 (Bio-Rad). The autoradiograms (Kodak SB-5 x-ray film) were exposed for ≈6 days.

RESULTS

Synthesis of Callus-Specific Proteins in Response to High Cell Density and 2,4-D. In an earlier investigation, we found that cell density plays a more immediate role in suppressing embryogenesis in carrot culture than does 2,4-D: both embryo-specific proteins and young embryos are formed in 6 days if carrot cells are cultured at low cell density, even if 2,4-D is present (6). However, low-density cultures incubated in 0.1 mg of 2,4-D per liter for 12 days cease to synthesize embryo-specific proteins (Fig. 1*C*) and embryos are converted to callus (7). At this point, the culture reaches a density of ≈4 × 10^5 cells per ml. As the culture grows up to 2–3 × 10^6 cells per ml, at least two new polypeptides are synthesized, which are characterized by two series of radioactive spots on the autoradiogram shown in Fig. 1*A*. Soluble proteins were extracted from a high-density and a low-density culture incubated in media containing 0.1 mg of 2,4-D per liter. The high-density culture produced two polypeptides, C1 and C2, in the size range of M_r 24,000 and M_r 20,000. They are called callus-specific proteins. Apparently, the synthesis of these callus-specific proteins is dependent on cell density. To determine whether the presence of 2,4-D also is essential for their synthesis, cultures grown at high cell density in the absence of 2,4-D were examined. Cultures initiated at high cell density—e.g., 8 × 10^5 cells per ml—in 2,4-D free medium cannot develop into mature embryos: they grow as a mixture of abnormal young embryos and callus (7). In these cultures, the synthesis of callus-specific proteins is not detected (Fig. 2*A*). We conclude that both 2,4-D and high cell density are necessary for the synthesis of callus-specific proteins. The string of spots in each of the two series of callus-specific proteins may be unrelated proteins or may represent products of post-translational modification (18) or degradation products (19).

Conditioned Medium Is Necessary for Callus-Specific Protein Synthesis. In searching for the differences between the high- and the low-density cultures that lead to the production of callus-specific proteins, we found that the low-density cultures synthesize the callus-specific proteins if they are incubated in medium previously conditioned with a high-density culture grown for 12 days in a mixture of 40% fresh medium and 60% conditioned medium. Together with 2,4-D, the conditioned medium caused C1 and C2 synthesis in low-density culture. Apparently, the high-density culture has conditioned the medium—e.g., by modifying the culture medium. This conditioned medium in turn causes the cells to produce the callus-specific proteins. The difference between high- and low-

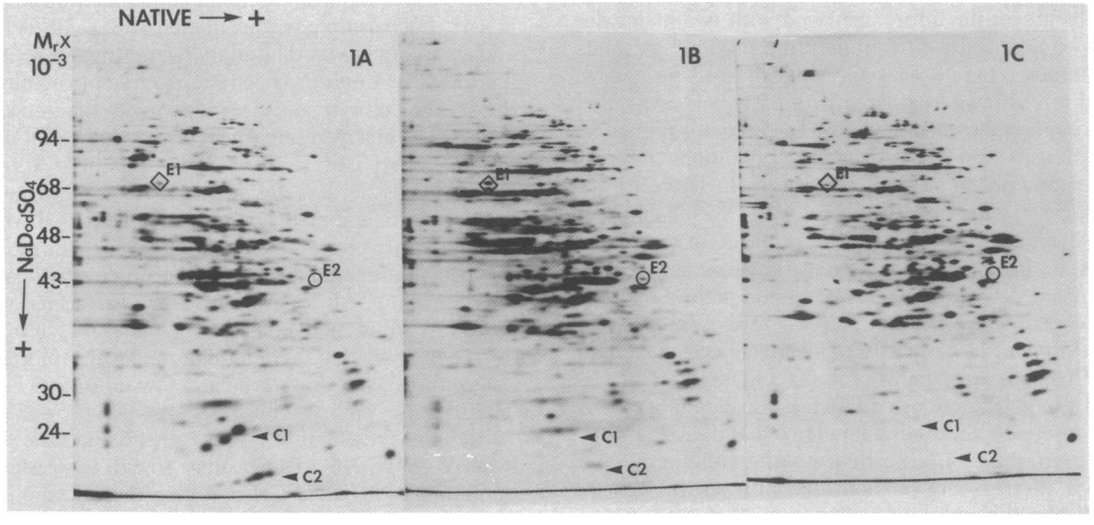

FIG. 1. Translational profiles of callus cultures of WOO1C and WCH105. (*A*) High-density culture of WOO1C. The culture was inoculated at 8 × 10^5 cells per ml in medium with 2,4-D. After 6 days of incubation (2 generations), cells were labeled with [^{35}S]methionine and soluble proteins were extracted and separated by 2D polyacrylamide gel electrophoresis. E1 and E2 are the embryo-specific proteins (6). C1 and C2 point at the two series of callus-specific proteins. (*B*) High-density culture of WCH105. The culture was inoculated at 8 × 10^5 cells per ml, and 2D polyacrylamide gel electrophoresis was performed 6 days (2 generations) after subculture. (*C*) Low-density culture of WOO1C. The culture was initiated at 2 × 10^4 cells per ml in medium with 2,4-D. 2D polyacrylamide gel electrophoresis of the soluble proteins was performed after 12 days of incubation at 25°C.

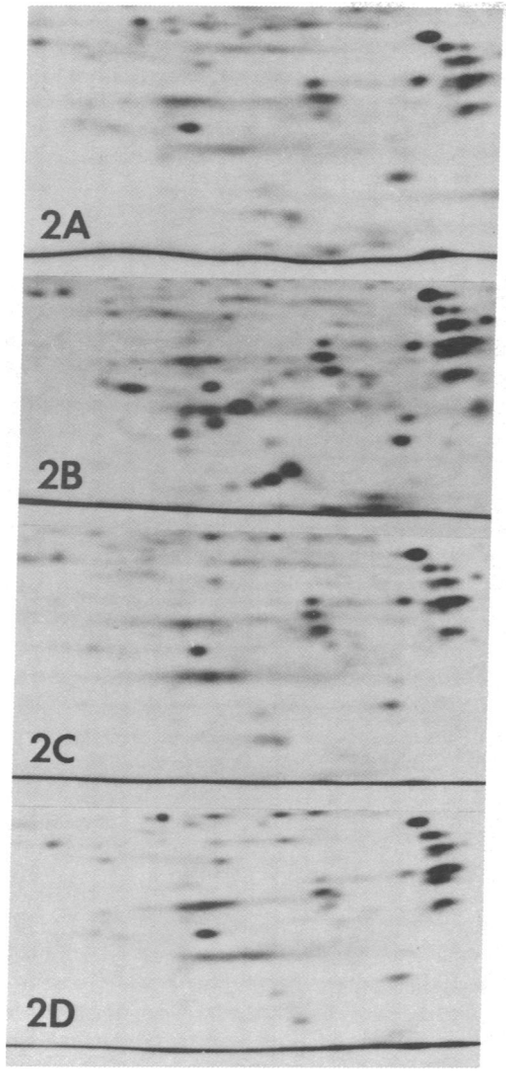

FIG. 2. Synthesis of callus-specific proteins in WOO1C and WCH105 cultures. (*A*) High-density WOO1C culture grown in medium without 2,4-D. A logarithmic culture of WOO1C grown in medium with 2,4-D was washed, resuspended in 2,4-D-free medium at a density of 10^6 cells per ml, and subcultured at $\approx 5 \times 10^6$ cells. 2D polyacrylamide gel electrophoresis was performed 6 days later when the culture reached $\approx 3 \times 10^6$ cells per ml. (*B*) Low-density WOO1C culture in medium conditioned by WOO1C. Conditioned medium was the medium of a high-density culture grown in medium with 2,4-D from 8×10^5 to 3×10^6 cells per ml. A low-density culture was incubated in a medium containing 60% conditioned medium and 40% fresh medium with 2,4-D at an initial cell density of 2×10^4 cells per ml. The 2D polyacrylamide gel electrophoresis was performed in 12 days. (*C*) Low-density WOO1C culture grown in medium conditioned by WCH105 callus. The conditioned medium of WCH105 was prepared as described above. WOO1C cells were inoculated in 60% conditioned WCH105 callus medium and 40% fresh medium with 2,4-D at a cell density of 2×10^4 cells per ml. 2D polyacrylamide gel electrophoresis was performed 12 days later. (*D*) Low-density WCH105 culture grown in medium conditioned by WOO1C callus. WCH105 were inoculated in 60% medium conditioned by WOO1C and 40% fresh medium at a cell density of 5×10^4 cells per ml. 2D polyacrylamide gel electrophoresis was performed 12 days later.

density cultures could be qualitative or quantitative—i.e., either the low-density culture cannot condition the medium at all or it conditions to a degree that is insufficient to elicit the production of callus-specific proteins. In any case, at least two functions are involved in the appearance of the callus proteins: the conditioning factor and the synthesis of the callus-specific proteins.

WCH105 Expresses Neither Function That Results in the Production of Callus-Specific Proteins. CH resistance (CH^r) is considered as a differentiated function in carrots because normal cell lines—e.g., WOO1C—are resistant to inhibition by CH when grown under embryogenic conditions but are sensitive to CH when grown as callus. However, both embryonic and callus cultures of WCH105 are resistant to CH. The mechanism of CH^r, either as an embryonic function or as a CH^r callus function, was found to be CH^i (11). The CH^i in WCH105 callus was shown to be a recessive trait by somatic hybridization (16). It appears that WCH105 is altered in the timing of expression of the CH^i function—expressing an embryonic function while growing as callus tissue.

We decided to find out if WCH105 callus expressed the embryo- and the callus-specific proteins. Fig. 1*B* shows that WCH105 callus grown at high density in 0.1 mg of 2,4-D per liter does not synthesize the callus-specific proteins, but it synthesizes the two embryo-specific proteins, E1 and E2, in the size range of M_r 77,000 and M_r 43,000, respectively. The translational profile of WCH105 embryo is the same as that of its callus (Fig. 1*B*). Subsequently, we set out to investigate which of the two functions responsible for callus protein synthesis was absent in WCH105 callus. To see if WCH105 callus can condition the medium, conditioned medium obtained from a high-density callus culture of WCH105 was incubated with low-density WOO1C cells for 12 days. We found that conditioned medium of WCH105 cannot elicit callus-specific protein synthesis in WOO1C (Fig. 2*C*). Similarly, to test if WCH105 callus has the ability to synthesize C1 and C2, WCH105 callus was incubated at low density in conditioned WOO1C medium. Again, C1 and C2 were not detected in the autoradiograph (Fig. 2*D*). Therefore, WCH105 can neither produce callus-specific proteins nor condition the medium to elicit the protein synthesis in wild-type cells.

Effect of Conditioned Medium on Morphology. The finding that high density promotes callus growth and low density promotes embryonic growth suggests that the conditioned medium, which is necessary for callus protein synthesis, also affects culture morphology. The following observations were made to investigate whether the conditioned medium will turn an embryonic culture to callus. Table 1 shows that a low-density WOO1C culture grown in its own conditioned medium would not form globular embryos (Fig. 3*C*) as the control cultures do (Table 1, experiments 1 and 2; Fig. 3 *A* and *B*); instead, it grows as callus. The degree of embryo development is progressively inhibited as the concentration of conditioned medium increases from 10% to 90% (Table 1, experiments 3–6 and 8). Like the conditioned medium of WOO1C, medium conditioned by WCH105 cells (experiment 12) also is effective in inhibiting embryogenesis in low-density cultures.

Plant cells may condition the medium by excreting substances, modifying or depleting the nutrients in the medium. It is possible to distinguish the first two possibilities from that of nutrient depletion by mixing 60% conditioned medium with 40% 2× concentrated MS medium (Table 1, experiment 7). Although high salt concentration is known to promote embryogenesis (20), the 2× concentrated medium improved embryogenesis in the conditioned medium by 2.7% only. This result implies that it is the presence, not the absence, of certain factor(s) in the conditioned medium that promotes callus growth. Furthermore, this factor is heat-stable (Table 1, experiment 9).

High-density cultures grown in 2,4-D-free medium produce a mixture of abnormal young embryos and callus but cannot produce callus-specific proteins. We examined the effect of the medium from such cultures on embryogenesis in low-density cultures. Table 1 (experiments 10 and 11) shows that a mixture

Table 1. Effect of conditioned medium on embryo and callus growth

Experiment	Strain	Conditioned medium			Fresh medium		% of embryos (50–200 μm)	% of callus clumps (50–200 μm)
		Culture	%	2,4-D	%	2,4-D		
1	WOO1C				100	−	100 ± 21	0
2	WOO1C				100	+	57 ± 13.9	12 ± 5.6
3	WOO1C	WOO1C	10	+	90	+	7.8 ± 1.2	58.8 ± 20.5
4	WOO1C	WOO1C	20	+	80	+	1.7 ± 1.7	54 ± 17.5
5	WOO1C	WOO1C	40	+	60	+	0	78.5 ± 25
6	WOO1C	WOO1C	60	+	40	+	0	100 ± 19
7	WOO1C	WOO1C	60	+	40*	+	2.7 ± 2.3	80.2 ± 11.4
8	WOO1C	WOO1C	90	+	10	+	0	94.7 ± 18.6
9	WOO1C	WOO1C	90 (boiled)	+	10	+	0	93.2 ± 16.1
10	WOO1C	WOO1C	60	−	40	−	100 ± 12.0	0
11	WOO1C	WOO1C	60	−	40	+	28.7 ± 5.7	39.4 ± 6.2
12	WOO1C	WCH105	60	+	40	+	0	98 ± 10.5
13	WCH105				100	−	100 ± 14.5	30 ± 3.2
14	WCH105				100	+	1.2 ± 0.6	100 ± 12.0
15	WCH105	WCH105		+	40	+	0	100 ± 5.2

Conditioned media were obtained from 6-day-old cultures. 100% embryogenesis in WOO1C is equivalent to 570 embryos per ml and 120 embryos per ml in WCH105. In addition to these clumps, WCH105 cultures grown in 2,4-D often contain a population of loose clumps made of large cells about 10–40 μm in diameter; they occupy about 10% of the total cell mass and are not counted in these experiments. Embryogenesis is scored in 6 days. Presence and absence of 2,4-D are indicated by + and −, respectively. Values are shown as mean ± SEM.
*Two times concentrated medium.

of the conditioned medium with fresh medium containing 2,4-D is effective in inhibiting embryo formation and promoting callus growth, but the mixture with fresh 2,4-D-free medium is not effective. This result suggests that to promote callus growth, the "factor" in the conditioned medium must interact with an auxin, either the endogenous auxin (indole acetic acid) from the high-density culture or 2,4-D supplied in the low-density culture.

In general, WCH105 responds to conditioned medium in the same way as do WOO1C cells (Table 1, experiments 13–15). However, the phenomenon is not as pronounced as in WOO1C, because WCH105 regenerates less efficiently (about 1/5th as slowly and less efficiently than WOO1C) (Fig. 3*D*).

DISCUSSION

How Does High Cell Density Promote Callus Growth? Early studies have suggested that carrot cells become embryogenic as soon as tissue cultures are initiated from the explants, only to be prevented from forming embryos by 2,4-D (21). The disruption of embryonic growth results in callus growth. The action of auxins is believed to promote cell enlargement, which may result in disorganized growth, thus disrupting embryo development. Indeed, cells in a callus clump are larger and more randomly arranged than those in an embryo. However, 2,4-D cannot act alone in suppressing embryo development; such suppression also requires high cell density. Recent studies have shown that embryogenesis is triggered whenever callus is subcultured into fresh medium, even in the presence of 2,4-D (5–7). However, embryo development can proceed only if the cells are cultured at low density (6). When the embryonic cultures grow to a higher density, 2,4-D becomes effective in suppressing further embryonic growth, thus promoting callus growth.

Carrot cells grown in culture are capable of synthesizing indole acetic acid. Embryonic culture contains at least 10 times more indole acetic acid than does callus cultures (22). On the basis of these facts, it was suspected that high-density cultures condition the medium by excreting indole acetic acid. However, boiled conditioned medium is just as effective in inhibiting embryogenesis as unheated medium (indole acetic acid is expected to be destroyed by boiling). A high concentration of nutrients is necessary for somatic embryogenesis (20); conditioned medium may well be depleted of these nutrients. However, when nutrient concentration is adjusted to about the level of fresh medium by mixing 2× concentrated MS medium with the conditioned medium (Table 1), it still promotes callus growth and inhibits embryo development. Thus, although the active factor in conditioned medium is unknown, it is not likely to be either indole acetic acid or the depletion of certain nutrients in the medium. The identification of this factor would contribute to the understanding of its interaction with 2,4-D and how this interaction affects the mode of growth in plant cells.

The Factor That Promotes Callus Growth Is Different from That Which Results in Callus-Specific Protein Synthesis. Both callus growth and callus-specific protein synthesis are dependent on the simultaneous presence of conditioned medium and auxin. However, whatever promotes callus growth must be different from that which leads to the protein synthesis, because WCH105 cannot condition the medium to provoke callus protein synthesis in WOO1C, yet its medium can promote callus growth.

Coordinate Expression of Several Temporal Functions During Embryogenesis. In practice, the distinction between callus and somatic embryos is made on the basis of morphology. During the transition from callus to the first stage of embryo, the globular embryo, there are a number of biochemical functions that change their expression—e.g., the disappearance of the callus-specific proteins, the appearance of the embryo-specific proteins, and the expression of the CH^i. The existence of the phenotype of WCH105 callus—expressing the embryonic functions (embryo-specific proteins and CH^i) but not the callus functions (callus-specific proteins and the "conditioning factor" for the protein synthesis)—suggests that these four temporal functions are regulated by a common mechanism. Under this regulatory mechanism, "activation" of one embryo-specific function is accompanied by activation of another and "inactivation" of two callus-specific functions. We propose a simple model for this type of regulation—a switch type of control, which turns on a set of functions while turning off another set at the same time. In WCH105, this switch is stuck at the embryonic direction and it no longer responds to suppression by 2,4-D and high-density conditions.

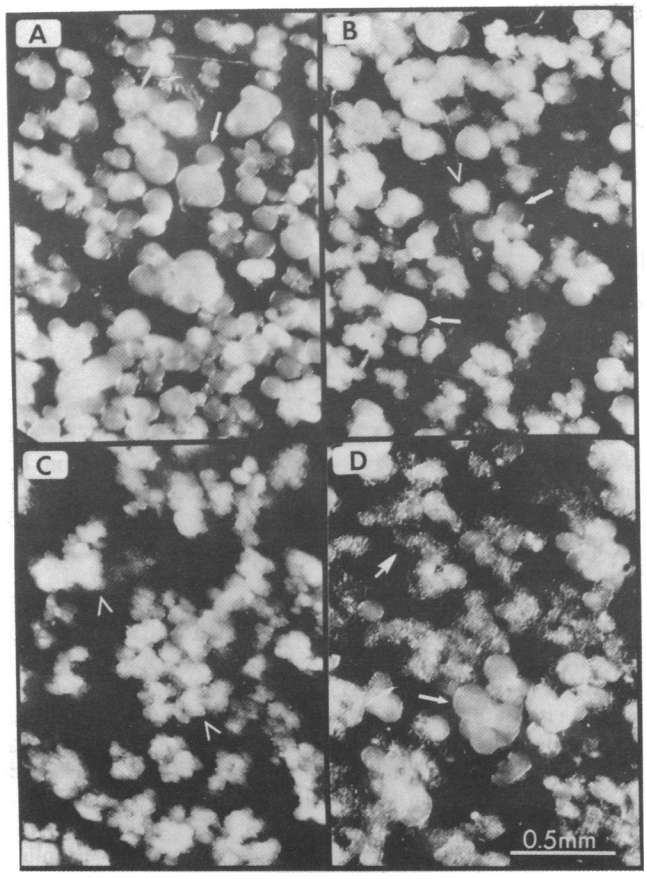

FIG. 3. Morphology of the carrot cells grown in culture. (*A*) Low-density WOO1C cells in 2,4-D-free medium. Cells, prepared according to the *Materials and Methods*, were cultured at high density in 2,4-D-free medium for 3 days. One-half milliliter of the culture was diluted in 20 ml of fresh MS medium without 2,4-D at a density of 2×10^4 cells per ml. The photograph was taken 6 days after the culture had been diluted. This culture consists of embryos only. (*B*) Low-density WOO1C in conditioned medium. Cells were incubated at high density in medium with 2,4-D for 3 days and were diluted to low density in medium with 2,4-D for 6 days. This culture consists of embryos and callus. (*C*) Low-density WOO1C in conditioned medium. One-half milliliter of WOO1C cells grown for 3 days in medium with 2,4-D at high density was diluted to low density in 60% conditioned medium (prepared as described in the legend to Fig. 2) and 40% fresh medium containing 2,4-D. This culture consists of callus clumps only. (*D*) Low-density WCH105 cells in medium with 2,4-D. Cultures were prepared as described in the legend to Fig. 3. This culture consists of embryos and highly vacuolated and elongated cells. ↑ points at embryos (spherical structures with a smooth surface); V points at callus (irregular-shaped clumps of cells); and ↟ points at elongated cells (large, translucent single cells).

Relationship of the Biochemical Functions to Morphology. Previous studies have shown a temporal relationship among several functions during embryogenesis (6, 7). The relationship between the above-described genetic program and the morphology—i.e., embryo formation—can be explained by a linear pathway based on the temporal sequence of events: callus and callus proteins $\xrightarrow{4\text{ hr}}$ embryonic proteins $\xrightarrow{3\text{ days}}$ CH^i $\xrightarrow{5\text{ days}}$ globular embryos. Alternatively, they may be viewed as parallel pathways controlled by the same factors, such as fresh medium, cell density, and 2,4-D. In WCH105, only the genetic program described above is altered such that CH^1 and embryo-specific proteins are constitutively expressed, whereas callus-specific proteins are never produced. But the pathway leading to morphogenesis remains sensitive to control by 2,4-D and high density; thus, WCH105 still grows as callus under these conditions.

In conclusion, these results indicate that although the synthesis of callus-specific proteins correlates with the morphology of callus tissues, such proteins apparently are not required for callus growth. Embryo-specific proteins alone cannot evoke embryogenesis, but mutants impaired in embryogenesis do not produce embryo-specific proteins (6). To resolve whether embryo-specific proteins are necessary for embryo development in wild carrots will require the isolation of a mutant whose embryos do not produce the embryo-specific proteins.

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