Plant Physiol. (1989) 90, 921-927
0032-0889/89/90/0921/07/$01.00/0

Received for publication September 27, 1988
and in revised form January 25, 1989

Interruption of Somatic Embryogenesis in *Daucus carota* L. by 5-Bromodeoxyuridine

John C. Thomas*, Craig Nessler, and Frank Katterman

Rhône Poulenc Agrochimie, 14-20 Rue Pierre Baizet, 69263 Lyon Cedex 09, France (J.C.T.); *Department of Biology, Texas A&M University, College Station, Texas 77843* (C.N.); *and Genetics Committee, Building 32, University of Arizona, Tucson, Arizona 85743* (F.K.)

ABSTRACT

Embryogenic *Daucus carota* L. cells grown in 9 micromolar 2,4-dichlorophenoxyacetic acid are resistant to greater than 5 micromolar 5-bromodeoxyuridine (BrdU). In contrast, 5 micromolar BrdU strongly inhibits somatic embryogenesis within 24 hours after transfer of cells to an auxin-free medium. DNA synthesis rates in control and BrdU-treated cultures are rapid and similar; however, the DNA content does not reach levels as great in the presence of BrdU as in control cultures. BrdU substitutes for thymidine in the DNA in 28% of the available sites 48 hours after auxin removal. Following DNA repair, somatic embryogenesis resumes. BrdU DNA incorporation leads to somatic embryogenesis inhibition and provides an alternative to auxin treatment for the interruption of carrot cell culture differentiation.

Carrot somatic embryogenesis displays many developmental alternatives, including patterns reminiscent of *in vivo* development (1). Auxin removal is the principal means of beginning this process (14). Rather than utilize the presence of auxin, alternative embryogenesis inhibitors could further help define pathways characteristic of differentiation.

The thymidine (dT[1]) analog BrdU inhibits carrot and animal cell differentiation (5, 22). The mechanism of BrdU action is unclear. Some proposed hypotheses to explain the activity of BrdU include: replication inhibition due to dC depletion (22), gene amplification (3), DNA repair (28), and interference with DNA replication resulting from substitution of BrdU for dT in DNA (2, 16).

This study was conducted to determine the nature of BrdU carrot embryogenesis inhibition reported by Dudits *et al.* (5). Our data suggest that the primary means of developmental arrest is BrdU incorporation into DNA for dT early after auxin removal. The developmentally competent cells are unable to express their totipotency when BrdU is present in the DNA. Following BrdU repair and degradation, totipotential expression again is observed.

MATERIALS AND METHODS

Tissue Culture

Daucus carota L. var. Danvers ½ long (Northrup King) cultures were initiated from 3 d old seedling hypocotyls. Callus was induced and subcultured on 2B5 (11) containing 0.8% w/v Difco Bactoagar and 9 μM 2,4-D. Cultures were grown at 25°C at a light intensity of 100 μE $m^{-1}s^{-1}$ for 16 h/d. After 3 to 5 weeks, approximately 1 g (fresh weight) of callus was transferred to 40 mL of liquid 2B5 medium in a 125 mL Erlenmeyer flask and grown in 10 μE $m^{-1}s^{-1}$ light for 16 h/d at 20 to 22°C with shaking (100 rpm).

Packed cell volume and cell number were correlated using protoplasts. One mL volume of cells (packed at 200*g* for 10 min) was digested 16 h at 22°C in the dark in 20 mL of 0.4 M mannitol, 0.75% (w/v) Cellulysin (Cal Biochem), 0.5% (w/v) Macerozyme R-10 (Yakult Honsha Co, Tokyo, Japan), and 0.5% (w/v) hemicellulase (Sigma) without shaking. The volume was measured and cell-protoplast counts made. One mL of packed carrot suspension cells corresponded to 3×10^7 cells.

Suspension cells were transferred bimonthly by diluting 1:40 in fresh 2B5 medium, with an initial density of 1×10^5 cells/mL. All media supplements (BrdU, dT, etc.) were freshly prepared in DMSO and added aseptically. DMSO levels never exceeded 0.1% (w/v) of the total volume and were not toxic. All experiments were performed in the dark at 25°C.

Seven days following subculture, the suspension cells were washed four times in 0B5 medium (11) and somatic embryogenesis was induced as described (27). The final cell density was 5×10^4 cells/mL and cultures were incubated statically in 20 mL of liquid 0B5 medium. Embryos were scored 28 d later. Callus was defined as any unorganized cell proliferation or irregular structure. Round multicellular structures of 250 to 350 μm were defined as globular embryos. The heart and torpedo stages reached 1 mm.

Protoplast Electroporation

Electroporation of protoplasts from 2,4-D grown cells and cells from dT or BrdU treatment followed the methods of Fromm *et al.* (9). Protoplasts were isolated as described above, filtered through a 95 μm filter, washed three times in CPW salts (8) with 0.4 M mannitol and resuspended in electroporation buffer (0.2 M mannitol, 70 mM NaCl, 1 mM KH_2PO_4, and 4 mM $CaCl_2$). Plasmid CNC17 was kindly provided by Dr. Prem Das (unpublished data). It consists of the 35S promoter from cauliflower mosaic virus fused to the chloramphenicol acetyltransferase gene and the nopaline synthase polyadenylation site in pUC 19. CsCl purified plasmid DNA was prepared as described (20).

[1] Abbreviations: dT, thymidine; BrdU, 5-bromodeoxyuridine; dC, deoxycytidine.

Electroporation was preformed using a "homemade" capacitance discharge machine. The optimum conditions were 350 V (750 μF) across a 0.4 cm gap. Protoplasts (1×10^6 in 1 mL) were incubated on ice with 25 μg of covalently closed circular plasmid DNA for 5 min. After one 75 ms pulse, the protoplasts were chilled for 10 min and thereafter 2 mL/5 min of medium added. Protoplasts were cultured in the dark overnight at a final density of 2×10^5 protoplasts/mL (9). CAT assays were according to Gorman *et al.* (12).

Nucleic Acid Measurements

DNA and RNA synthesis rates and total amounts were determined with cells grown in dT or BrdU according to Smille and Krotov (25). Suspension cells were prepared for somatic embryogenesis as described above with either 5 μM BrdU or dT, and incubated overnight in the dark. [^{32}P]-Orthophosphate (ICN) (1–3 μCi/ml) was added at different times and each culture incubated for 24 h. Cells were harvested (300*g* for 5 min) and ground in cold, distilled water. Cold perchloric acid was then added to 0.5 N. After an overnight 4°C precipitation, the samples were centrifuged (2000*g* for 10 min) and washed with the organic solvents described (25). Synthetic rates and total RNA were estimated from the KOH soluble fraction with scintillation counting and absorbance at 260 nM. The KOH insoluble pellet was washed in cold 0.5 N perchloric acid and then hydrolyzed in 0.5 N perchloric acid at 70°C for 20 min. Aliquots were counted and total DNA estimated (30).

For [^{3}H]uridine labeling, three 20 × 100 mm Petri dishes of cells were pooled for each time point. [^{3}H]Uridine (25 mCi/mM) was added to the cultures (2.5 μCi/mL) with 100 μM of nonradioactive uridine and the cultures incubated for 2 h. RNA was isolated and poly(A$^+$) fractionation were as described elsewhere (20).

Nuclear DNA was isolated according to R. Goldberg (personal communication). Cells were powdered in liquid N_2, extracted in 50× volumes of cold H buffer (4 mM spermidine, 1 mM spermine, 10 mM EDTA, 10 mM Tris [pH 9.5], 80 mM KCl, 0.5 M sucrose, 0.5% (v/v) Triton X-100, and 1 mM PMSF for 10 min. The slurry was filtered (95 μm nylon filter) and the nuclei pelleted at 2000*g* for 10 min. The pellet was resuspended in 10 mL of H buffer, then 10 mL of lysis buffer (2.5% (w/v) Sarkosyl, 0.1 M Tris pH 9.5, and 40 mM EDTA) was added dropwise with mixing. CsCl (19.4 g) was added and the extract then centrifuged at 10,000*g* for 30 min. The protein pellicle was removed and the final volume was measured. Following addition of ethidium bromide (500 μg/ml final), the mixture was centrifuged at 175,000*g* (18°C). DNA bands were recovered and further purified (20).

CsCl gradients were prepared in 10 mM Tris (pH 8), 1 mM EDTA. The refractive index was adjusted to 1.400 g/mL. Analytical ultracentrifugation was performed in a Beckman model E centrifuge with *Micrococcus lysodeikticus* DNA for a standard (1.731 g/cm^3). The preparative CsCl gradients were prepared in a similar way, centrifuged at 175,000*g* for 16 h at 18°C, the tubes punctured, fractionated (3 drops/fraction) and the refractive index and OD 260 nm measured. BrdU incorporation was based on increased DNA density (19).

A modified nick-translation reaction was used to estimate the degree of DNA damage after BrdU incorporation. The conditions were identical to those described (20) with the ommission of DNase. α-[^{32}P]dATP was the labeled dNTP (1 mCi/ml and 500 Ci/mM). As a control, 150 ng of pBR322 plasmid DNA was nick translated in the presence and absence of 8 ng of DNase I. After 1 h at 14°C, 1 mL of cold 10% (w/v) TCA was added, the samples precipitated on ice for 2 h, filtered, washed and counted (20). In the presence of DNase I, ^{32}P-nucleotide incorporation was 10 times greater than the untreated samples. DNA from dT and BrdU incubated cells were then analyzed for damage with nick translation without DNase.

RESULTS

Our studies indicate that embryogenesis in domestic carrot was more sensitive to BrdU treatment than the growth of the same cells in the presence of 9 μM 2,4-D (Fig. 1). BrdU levels of 5 μM were chosen for future experiments.

Ten days after cell transfer to an auxin-free medium, many globular and heart-shaped embryos were present in untreated cultures (data not shown) and in the dT-treated cells, while the BrdU-treated cultures remain unorganized (Figs. 2 and 3).

The timing of BrdU developmental inhibition was then determined. Fresh BrdU (5 μM) was added at different days following embryo initiation and the resulting embryos scored 28 d later (Fig. 4). Complete embryogenic arrest occurred only when BrdU was added within the first 24 h after the cells were transferred to hormone-free medium (Fig. 4). Also dT rescue was used to confirm the 24 h BrdU sensitive period. BrdU was added at the beginning of embryogenesis and a 10-fold excess of dT added at different times afterward. Complete

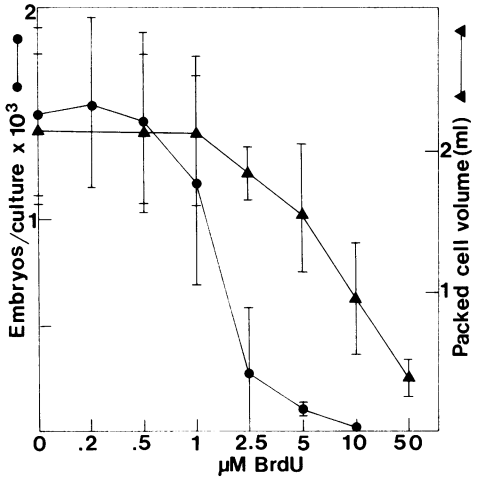

Figure 1. BrdU sensitivity of proliferating cells (▲) and developing somatic embryos (●). Each point represents the mean and standard deviation of four experiments in triplicate. Auxin grown cells were washed as described in 0B5 medium and then subcultured in 40 mL of 2B5 in the dark at 100 rpm at 5×10^4 cells/mL. For embryogenesis the cells were plated at the same density in 0B5 liquid medium. Embryogenesis and suspension culture growth was determined after 3 weeks.

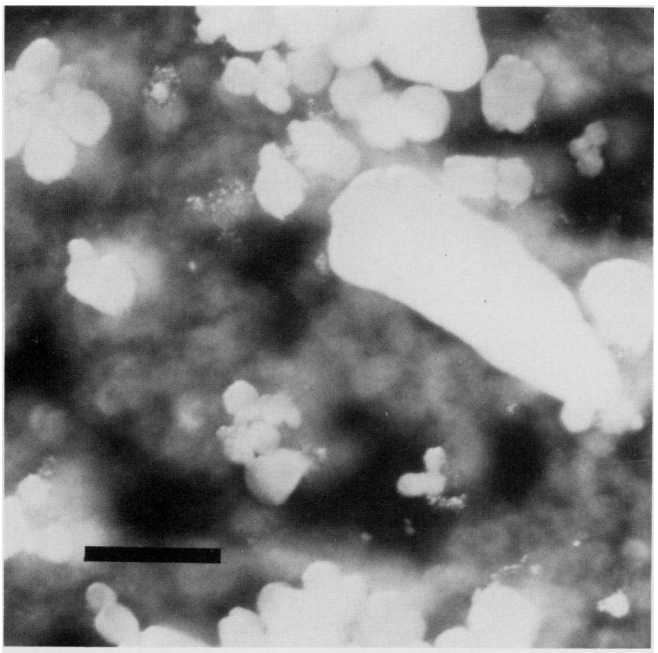

Figure 2. Embryogenesis after 3 weeks in 0B5 with 5 μM thymidine. The bar is 400 μm.

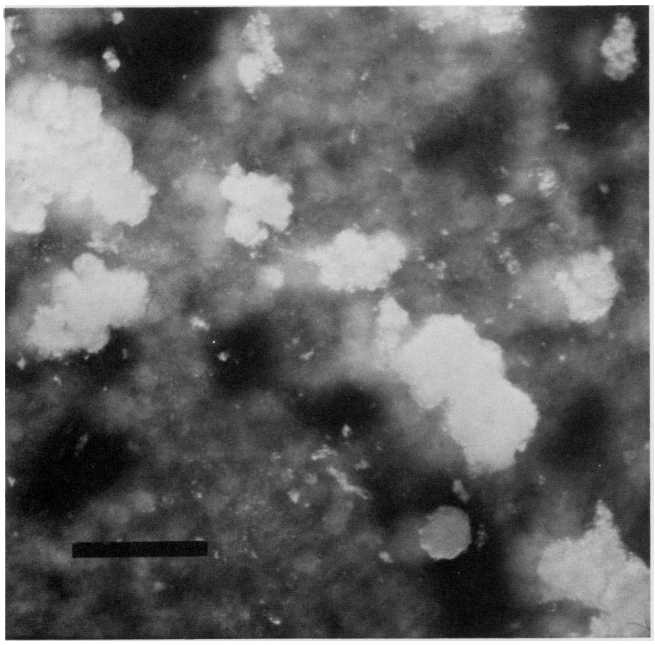

Figure 3. Effect of 5 μM BrdU on embryogenesis in 0B5 medium after 3 weeks. The bar is 400 μm.

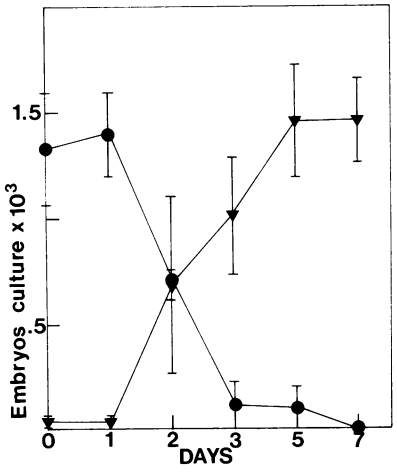

Figure 4. Determination of the BrdU sensitive period. After washing cells in 0B5 medium, 5×10^4 cells/mL were plated as described. At 0, 1, 2, 3, 5, and 7 d after washing, 5 μM BrdU was added and embryogenesis was determined 4 weeks later (▼). In addition, cells were initially plated in 5 μM BrdU and at different days after 50 μM thymidine was added (●). Vertical lines indicate the standard deviations while the points represent the mean of three separate experiments done in triplicate.

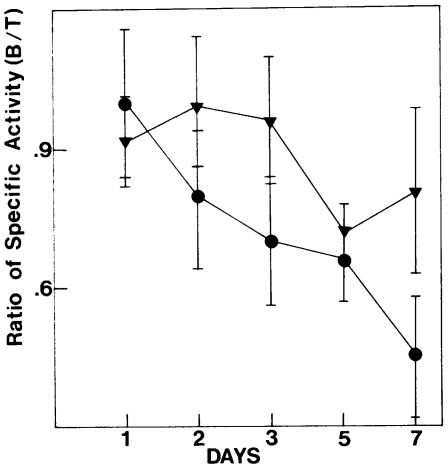

Figure 5. [^{32}P]Orthophosphate incorporation into DNA (▼) and RNA (●) after a 24 h incubation. Data are presented as the specific activity of incorporated polynucleotides per total amount (RNA or DNA). The B/T ordinate represents the ratio of nucleic acid synthesis in BrdU grown cells divided by that obtained in dT grown cells. Vertical lines indicate standard deviations of the points (means of duplicates of three experiments).

rescue only occurred when dT was added 24 h or less after the addition of BrdU (Fig. 4).

The BrdU effect on DNA and RNA synthesis in carrot cells was measured using [^{32}P]orthophosphate incorporation. DNA synthesis (specific activity) remained similar for BrdU and dT incubated cells 3 d after transfer to hormone-free medium (Fig. 5), long past the BrdU-sensitive period as determined above. However, the DNA accumulation in BrdU treated cells was slower. Their DNA doubling time approached ½× the rate of cells differentiating in the presence of 5 μM dT after 1 week (Fig. 6A).

RNA synthesis rates (specific activity) decreased in cells grown in the presence of BrdU (Fig. 5). Total RNA accumulation was also slower in the BrdU-treated cells (Fig. 6B). The poly(A$^+$) RNA synthesis was not selectively BrdU inhibited after auxin withdrawal for up to 3 d (Table I). As for the 2,4-D-grown cells, synthetic rates 24 h after culture transfer lag behind auxin free (dT or BrdU) cells in agreement with an earlier study (24).

To determine the competence of transcription and trans-

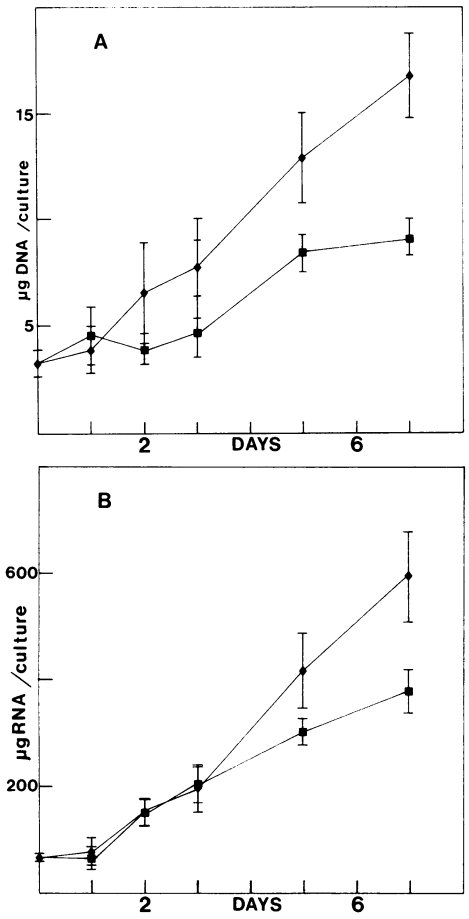

Figure 6. Total DNA (A) and RNA (B) content per culture as extracted following Smille and Krotov (25). Cells treated with BrdU from the onset of the incubation (▼) are compared to control cultures in 5 µM thymidine (■). Data represent the mean and standard deviation of three experiments done in triplicate.

Table I. *Poly(A⁺) RNA Synthesis in Differentiating Carrot Cells in the Presence of 5 µM dT ± 9 µM 2,4-D or 5 µM BrdU − 2,4-D*

Carrot cells were labeled for 2 h in 100 µM uridine + 2.5 µCi/mL of [³]uridine, extracted using the hot phenol method and fractionated on oligo-dT cellulose. The mean and standard deviation (in parentheses) of three experiments are presented below. The data represent the percentage of the total counts in the RNA fraction.

Time after Transfer	5 µM dT + 2,4-D	5 µM dT − 2,4-D	5 µM BrdU − 2,4-D
h			
24	1.4 (0.2)	2.3 (0.3)	2.1 (0.2)
48	ND[a]	0.9 (0.1)	1.5 (0.3)
72	ND	1.9 (0.7)	1.7 (0.6)

[a] Not done.

lation of BrdU-treated cells, protoplast electroporation was employed. Neither RNA nor protein would be observed following electroporation if BrdU selectively inhibits these processes. The results (Fig. 7) indicate that protoplasts isolated from cells treated with BrdU during the first 24 h after auxin

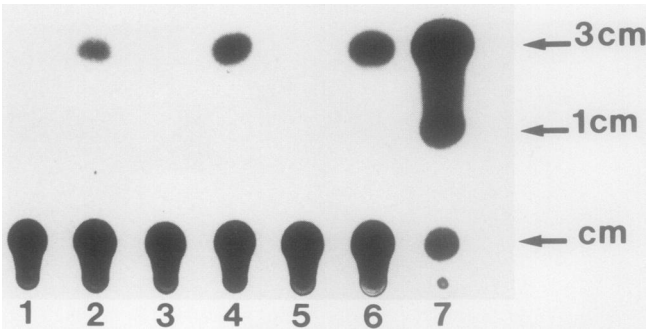

Figure 7. Cat assays of CNC17 electroporated carrot protoplasts grown in 5 µM dT (*1*, *2*), 5 µM BrdU (*3*, *4*), or 9 µM 2,4-D and 5 µM dT (*5*, *6*). Cells had been previously washed and resuspended in either 0B5 with dT, BrdU, or 9 µM 2,4-D + dT and incubated for 24 h. After an overnight isolation (including either pyrimidine and with or without 2,4-D), 1×10^6 protoplasts were suspended in 25 µg plasmid DNA. Either protoplasts remained on ice (*1*, *3*, and *5*) or were electroporated (*2*, *4*, and *6*). An extract of *E. coli* HB101 containing plasmid pBR325 encoding the CAT gene was used as a positive control (*7*). After medium addition, either pyrimidine and/or 2,4-D was added. Cells were recovered 16 h later and CAT assays performed. Lines indicate the substrate (cm) chloramphenicol, the major reaction product (3-cm) 3-acetylchloramphenicol and a minor reaction product (1-cm) 1-acetylchloramphenicol.

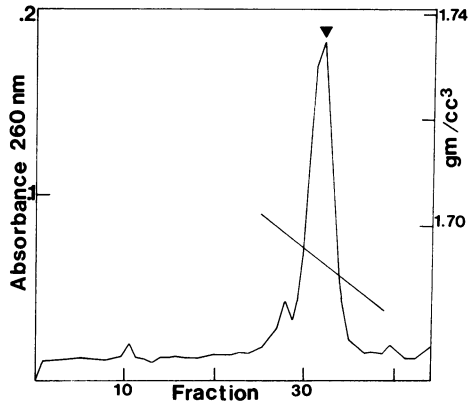

Figure 8. CsCl density gradient of DNA isolated from nuclei of carrots grown in 0B5 with 5 µM dT for 3 d. The ▼ indicates the average density of 1.695 g/cm³.

removal express CAT activity following electroporation as do the thymidine and 2,4-D-treated cells.

BrdU incorporation into DNA was examined using CsCl density gradients. Main band DNA from carrot cells grown for 2 d in hormone-free medium has a mean density of 1.695 gm/cm³ (Fig. 8). This value is in agreement with published measurements (6). Following 2 d of BrdU treatment, a very different density profile is observed (Fig. 9). The Br ion is more dense than the CH_3 group of thymidine. When BrdU substitutes for dT the DNA density increases. The main band DNA density after 2 d of BrdU treatment is 1.728 g/cm³ and represents 28% BrdU substitution. It is important to note the extensive variation of DNA densities. By 3 d of BrdU exposure the main band DNA density increases to 1.760 g/cm³, again displaying great variation in density (Fig. 10). The degree of substitution for dT at 3 d is 68%.

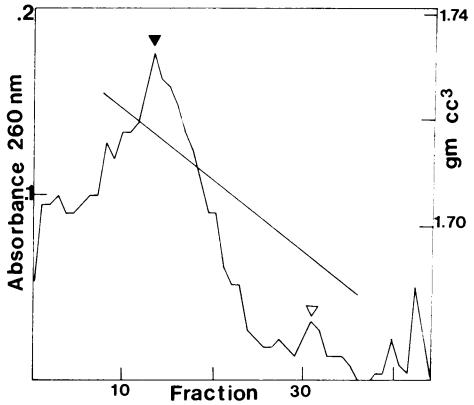

Figure 9. Nuclear DNA of carrot cells grown on 0B5 with 5 μM BrdU. The ▼ indicates an average density of 1.716 g/cm^3 and ▽ indicates a smaller underreplicated fraction DNA (1.695 g/cm^3).

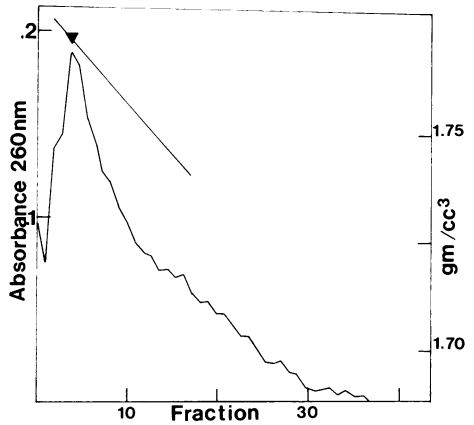

Figure 10. Density of DNA from carrot cells grown for 3 d in 5 μM BrdU. The ▼ indicates an average density of 1.756 g/cm^3.

Table II. *DNA Density Summary of Carrot Cells Grown for 2 and 28 d in 5 μM dT or BrdU*

DNA was isolated from nuclei and the density determined with CsCl analytical ultracentrifugation. In the case of cells treated with BrdU for 2 d after subculture to hormone-free medium, two peak densities (a, b) were observed as in Figure 9.

Time in Culture	Density	Percent Substitution
	g/cm^3	
2 Days:		
5 μM dT	1.695	0
5 μM BrdU	1.699[a]	1.7
	1.729[b]	28.5
28 Days:		
5 μM dT	1.695	0
5 μM BrdU	1.697	0.5

Analysis of DNA isolated from cultures treated with BrdU for 28 d revealed that the BrdU is eventually removed, perhaps by DNA repair (Table II). Only 0.5% of the previously occupied sites contain BrdU 28 d after treatment. Furthermore, BrdU was found labile in the culture medium, where degradation was estimated at 45% every week using the change in absorption at 278 nm/ 260 nm (data not shown). After the cells had recovered (*i.e.* repaired their DNA in 28 d) somatic embryogenesis began. There then appears to be a correlation between DNA substitution and the inability to complete embryogenesis.

Table III. *Estimation of DNA Damage in Carrot Cells Grown in Either 5 μM BrdU or dT Using a Nick-Translation Assay Without Added DNase*

DNA Origin	^{32}P	Percent of BrdU Grown
	cpm	
Extraction 1:		
dT	2.1×10^5	74
BrdU	2.8×10^5	100
Extraction 2:		
dT	3.9×10^5	78
BrdU	5.0×10^5	100

In *Escherichia coli* BrdU incorporation into DNA results in an increase in DNA repair (28). For this reason a modified nick translation of DNA isolated from BrdU- and dT-treated cells was used. In two separate preparations of DNA, BrdU-treated cells contain 25% more nicks than the dT controls (Table III).

DISCUSSION

Halperin and Jensen (13) have proposed that the auxin 2,4-D induces carrot cell dedifferentiation, proliferation and the promotion of a totipotent state. In this context, 2,4-D acts as both a totipotent inducer and repressor of the totipotent expression. Auxin grown carrot cells can be considered poised just prior to morphologically recognizable embryogenesis. Perhaps for this reason, many genes optimally expressed during embryogenesis are expressed at lower levels prior to auxin removal (26, 27, 29).

A comparison between auxin and BrdU inhibition may provide insight into somatic embryogenesis initiation. For example, quantitative changes in the level of total transcription may not be important in the transition between suspension cells and early embryogenesis. Using [^{3}H]uridine labeling we have not seen significant differences in poly A$^+$ synthesis between dT- and BrdU-treated cultures grown without auxin. In contrast, cells growing in the presence of 2,4-D produce less poly(A$^+$) RNA 24 h after induction (Table I), in agreement with data obtained by Sengupta and Raghavan (24).

Using bacterial RNA polymerase, Scheit (23) found no difference in *in vitro* RNA transcription rates when the DNA template contained dT or BrdU. In addition, electroporated CNC17 plasmid is transcribed and translated in BrdU-treated cells (protoplasts) as in dT or 2,4-D-grown cells. The idea that BrdU acts only by transcriptional alteration, independent of DNA incorporation is improbable.

Following BrdU DNA incorporation, the transition from an unorganized cell cluster to an embryogenic cluster fails. If this effect is due to a lack of endogenous dC (22), then exogenously supplied dC might be expected to rescue somatic embryogenesis. Exogenous dC does rescue BrdU repressed

embryogenesis, but 10 times more dC is needed as compared to dT (data not shown).

DNA replication interference has been suggested after BrdU incorporation into the DNA of tobacco suspension cultures (2). In *Xenopus* oocytes, BrdU-substituted DNA can serve as a replicative host, but not all substituted strands permit a second round of replication (16). Incorporation into DNA of BrdU is itself is not responsible for *E. coli* replication arrest directly, rather it is the deoxyuracil from BrdU dehalogenation and the consequent repair (28). The enzyme responsible for dU repair is found in many organisms including plants (18).

In this study, BrdU was substituted for dT in up to 68% of all the available residues within 3 d of auxin removal. In addition, the DNA displayed a wide range rather than a single predominant density. This density diversity could be explained by BrdU substitution and repair. Using DNase-free nick translation, BrdU-substituted DNA contains about 25% more single-stranded sites compared to dT-treated cellular DNA, and may be undergoing DNA repair (Table III). After nearly all (0.5%) of the BrdU is replaced somatic embryogenesis resumes (Table II).

Because of BrdU excision and DNA repair, replication completion and transcription also continues at slower rates. BrdU substitution and consequent repair could increase the normal replicon pausing time during the S-G2 interphase and delay carrot DNA synthesis completion (15). This idea is consistent with the thought that the normal replication rate is so rapid that the repair rate of DNA is the rate-limiting step (17). Flickinger *et al.* (7) found that BrdU-substituted DNA initiates a similar number of replication bubbles, but the rate of replicon elongation was reduced. Slower replication completion could explain why *de novo* DNA synthesis of BrdU-treated cells is unchanged while DNA accumulation slows as compared to the dT-treated cultures.

Early replication events in carrot somatic embryogenesis are important. The cell cycle transit time increases from 58 h to 6 h for the first 3 d without auxin (10), and only the rapidly replicating cells develop into somatic embryos. The initial cell replication has been shown to create developmental polarity (13), which is maintained throughout development (21).

This study indicates that there exist at least two separate events prior to the beginning of somatic embryogenesis. First, the release of a developmental block occurs with 2,4-D removal. Next the cells pass through a replication cycle when BrdU is incorporated and thus presents a second developmental block. As genes of different embryonic stages have been isolated (4) developmental inhibitors could be used to more precisely measure when, after 2,4-D removal, these genes become transcriptionally active.

ACKNOWLEDGMENTS

Our thanks to Dr. Bob Goldberg for the method of nuclear DNA extraction, to Dr. Prem Das for furnishing us with the CNC17 plasmid, and to Dr. William Seffins for his encouragement and the building of the electroporation device.

LITERATURE CITED

1. **Ammirato PV** (1983) Embryogenesis. *In* DA Evans, WR Sharp, PV Ammirato, Y Yamada, eds, Handbook of Plant Cell Culture, Vol I. Macmillan, New York, pp 82–123
2. **Bezdek M, Vyskot B** (1981) DNA synthesis in cytokinin-autotrophic tobacco cells. Planta **152:** 215–224
3. **Biswas DK, Lyons J, Tashjian A** (1977) Induction of prolactin synthesis in rat pituitary tumor cells by 5-bromodeoxyuridine. Cell **11:** 431–439
4. **Choi JH, Liu L-S, Borkird C, Sung ZR** (1987) Cloning of genes developmentally regulated during plant embryogenesis. Proc Natl Acad Sci USA **84:** 1906–1910
5. **Dudits J, Lazar G, Bajszar G** (1979) Reversible inhibition of somatic embryo differentiation by bromodeoxyuridine in cultured cells of *Daucus carota* L. Plant Sci Lett **8:** 315–323
6. **Duhrssen E, Neumann K-H** (1980) Characterization of satellite DNA of *Daucus carota* L. Z Pflanzenphysiol **100:** 447–454
7. **Flickinger RA, Grabar F, Kundal E, Shepherd GW** (1980) Replicon size increase due to 5-bromodeoxyuridine incorporation. Cell Biol Int Res **4:** 129–135
8. **Frearson EM, Power JB, Cocking EC** (1973) The isolation, culture and regeneration of Petunia leaf protoplasts. Dev Biol **33:** 130–137
9. **Fromm M, Taylor LP, Walbot V** (1985) Expression of genes transfered into monocot and dicot plant cells by electroporation. Proc Natl Acad Sci USA **82:** 5824–5828
10. **Fujimura T, Komamine A** (1980) The serial observation of embryogenesis in a carrot cell suspension culture. New Phytol **86:** 213–218
11. **Gamborg OL, Miller RA, Ojima K** (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res **50:** 151–158
12. **Gorman CM, Moffat LF, Howard BH** (1982) Recombinant genomes which express choramphenicol acetyltransferase in mammalian cells. Mol Cell Biol **2:** 1044–1051
13. **Halperin W, Jensen WA** (1967) Ultrastructural change during the growth and embryogenesis in carrot cell cultures. J Ultrastruct Res **18:** 428–443
14. **Halperin W, Wetherall DF** (1964) Adventive embryony in tissue cultures of wild carrot. Nature **205:** 519–520
15. **Hernandez P, Lamm SS, Bjerknes CA, Van't Hof J** (1988) Replication termini in the rDNA of synchronized pea root cells (*Pisum sativum*). EMBO J **7:** 303–308
16. **Hutchinson CJ, Cox R, Drepauln RS, Gomperts M, Ford CC** (1987) Periodic DNA synthesis in cell-free extracts of *Xenopus* eggs. EMBO J **6:** 2003–2010
17. **Kornberg A** (1988) DNA replication (minireview). J Biol Chem **263:** 1–4
18. **Lindahl T** (1979) DNA glycosylases, endonucleases for apurinic/apyrimidinic sites, and base excision-repair. Prog Nucleic Acids Res Mol Biol **22:** 135–192
19. **Luk DC, Bick MD** (1977) Determination of 5-bromodeoxyuridine in DNA by buoyant density. Anal Biochem **77:** 346–349
20. **Maniatis T, Fritsch EF, Sambrook J** (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. **Nomura K, Komamine A** (1986) Polarized DNA synthesis and cell division in cell clusters during somatic embryogenesis from single carrot cells. New Phytol **104:** 25–32
22. **Rutter WJ, Picket RL, Morris PW** (1979) Toward molecular mechanisms of developmental processes. Annu Rev Biochem **42:** 601–646
23. **Scheit KH** (1979) Effects of substrate modification on transcription of DNA by DNA-dependent RNA polymerase. *In* J Skoda, P. Langen, eds, Antimetabolites in Biochemistry, Biology and Medicine, FEBS Vol 57. Pergamon Press, New York, pp 127–138
24. **Sengupta C, Raghavan V** (1980) Somatic embryogenesis in carrot suspensions. II Synthesis of ribosomal RNA and poly(A^+) RNA. J Exp Bot **31:** 259–268
25. **Smille RM, Krotov G** (1960) The estimation of nucleic acids in some algea and higher plants. J Can Bot **38:** 31–49
26. **Sung ZR, Okimoto R** (1981) Embryonic proteins in somatic embryos of carrot. Proc Natl Acad Sci USA **78:** 3683–3687
27. **Sung ZR, Okimoto R** (1983) Coordinate gene expression during somatic embryogenesis in carrots. Proc Natl Acad Sci USA **80:** 2661–2665

28. **Szyszko J, Pietrzykowska I, Tardowski T, Shugar D** (1983) Identification of uracil as the major lesion in *E. coli* DNA following the incorporation of 5-bromodeoxyuracil and some accompanying effects. Mutat Res **108:** 13–27
29. **Thomas TL, Wilde D** (1987) Analysis of carrot somatic embryo gene expression programs. *In* CE Green, DA Somers, WP Hackett, DD Besboer, eds, Plant Tissue and Cell Culture. Alan R Liss, New York, pp 83–93
30. **Vytasek R** (1982) A sensitive flurometric assay for the determination of DNA. Anal Biochem **120:** 243–248