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The Irreversible Inhibition of Differentiation of Limb-Bud Mesenchyme by Bromodeoxyuridine

(cartilage/metachromasia/agar/acid mucopolysaccharides)

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ABSTRACT **Growing freshly dissociated chick-limb bud cells (stage 24) over agar for 48 hr permits differentiation into cartilage upon monolayer culture even when initial plating and subculture densities are well below confluency. Addition of 5-bromodeoxyuridine (BrdU) during the initial (48-hr) period over agar irreversibly inhibits chondrogenic differentiation, as characterized by morphology, metachromasia, and sulfate incorporation into acid mucopolysaccharide. Simultaneous, but not subsequent, addition of excess thymidine will prevent the effect of the analogue. Collagen synthesis is not depressed in BrdU-treated cells. Radioautographic studies demonstrate the specific localization of BrdU in the nucleus. Treatment of trichloroacetic acid-precipitable material containing tritiated bromodeoxyuridine with deoxyribonuclease solubilizes 90% of the radioactivity. The loss of the analogue from this precipitable material upon prolonged culture of limb-bud cells is more rapid than can be expected from cell division alone. 5-Bromodeoxyuridine may affect a fraction of DNA involved in stabilization of the differentiated cell phenotype.**

The thymidine analogue 5-bromodeoxyuridine (BrdU) interferes with expression of differentiated function in several cell types (1–6). In all but one of the cases cited (6), the effects of BrdU were reversed by removal of the drug. The reported studies have been concerned primarily with the effect of BrdU on the expression of specialized characteristics of differentiated cells, rather than on the process of differentiation.

Previous studies (Zwilling, E., personal communication) demonstrated that chick limb-bud mesoderm (stage 24) (7) differentiates into cartilage when grown in cell culture at high density (above confluency). Culture at lower cell densities results only in cells resembling fibroblasts. Searls (8) has shown that up to stage 25, the chondrogenic properties of mesodermal limb-bud cells of the chick are influenced by environmental factors, while after this stage, commitment to chondrogenesis is stabilized. Study of limb-bud mesodermal cells in tissue culture before stage 25 might permit examination of factors involved in irreversible commitment to the differentiated state.

This report is concerned with an attempt to study mesodermal cells in tissue culture during this crucial period of chondrogenic determination. A new method is described that permits differentiation of mesodermal cells into cartilage at lower density than previously reported (9). Chondrogenic expression is measured by morphology and by synthesis of chondroitin sulfate proteoglycan. When limb-bud cells grown in culture are subjected briefly to BrdU at this stage (stage 24), differentiation into cartilage cells is irreversibly impaired. Preliminary evidence pertinent to the possible mechanism of action of BrdU is presented.

MATERIALS AND METHODS

Cell Culture. Fore- and hind-limb buds, dissected from stage 23–24 (7) of White Leghorn chick embryos, were trypsinized for 45–60 min in 0.25% trypsin in ethylene diamine tetraacetate (EDTA). Single-cell suspensions containing 1×10^7 cells in 10 ml of medium were placed over 0.5% agar bases containing F-12 medium enriched with 10% fetal-calf serum in 100-mm Falcon tissue-culture dishes. After incubation in 10% CO_2–90% air for 48 hr at 37°, cells were removed by gently scraping the agar with a rubber policeman. Cells were treated for 15 min with 0.25% trypsin in EDTA, washed twice with F-12 medium containing fetal-calf serum, and plated as monolayer cultures at a density of 0.5×10^6 cells per 60-mm Falcon tissue-culture dish. All cultures were grown in F-12 medium supplemented with 10% fetal-calf serum and were fed twice daily with the same medium.

For the culture of cartilage cells, the long bones of stage-32 hind limbs were trypsinized to obtain single-cell suspensions according to Cahn *et al.* (10). The suspension was then treated like the earlier limb-bud tissue, except that 3×10^6 cells were cultured over agar, for 48 hr, then plated at a density of $0.5–1.0 \times 10^5$ cells per 60-mm tissue-culture plate.

Assays. For measurement of synthesis of sulfated glycosaminoglycans, 3.3 μCi of $H_2{}^{35}SO_4$ (carrier free) per ml of medium or 16.7 μCi of [^{3}H]acetate per ml of medium was added to the cultured cells for 6 hr on the day indicated for each experiment. The media and cells were collected, and 1.5–2.0 mg of carrier chondroitin-4-sulfate was added to each sample. The chondroitin sulfate was isolated and radioactivity was determined as described (11). In order to monitor the efficiency of the isolation method, uronic acid was determined in all samples. Recovery of carrier chondroitin-4-sulfate was between 80 and 110%.

To measure collagen synthesis, 0.67 μCi of [^{14}C]proline per ml of medium was added to cultured cells for 16 hr. The medium was removed and the cells were washed with 2 ml of phosphate-buffered saline solution (pH 7.4). The wash was

TABLE 1. *Effect of growth on agar on differentiation of limb-bud mesenchyme*

Experiment no.	*A* cpm/10^6 cells	*B* cpm/10^6 cells	*C* cpm/10^6 cells
1	16,500	348	—
2	6,200	590	—
3	4,630	595	428
4	53,800	5,760	7,050
5	245,000	4,950	—
6	26,300	—	1,610

3.3 μCi $H_2{}^{35}SO_4$ per ml of medium was added to each culture 6 hr before harvest. On the ninth day of culture, chondroitin sulfate was isolated as indicated in *Methods*. The descriptions of cells used in *A*, *B*, and *C* are as follows: (*A*) Dissociated cells plated at 10^7 cells in 10 ml media for 48 hr in 100-mm dishes containing 0.5% agar bases, then redissociated and placed on 60-mm plates at a density of 0.5×10^6 cells per dish for 7 days; (*B*) Dissociated cells plated for 48 hr at a density of 10^7 cells in 10-ml of medium on 100-mm dishes without agar, then redissociated and placed on 60-mm dishes at a density of 0.5×10^6 cells per dish for 7 days; and (*C*) Dissociated cells plated at a density of 3.6×10^6 cells in 3-ml of medium on 60-mm tissue-culture dishes and grown for 9 days. All three cell densities are below confluency.

combined with the media and the mixture was dialyzed for 24 hr against six changes of distilled water. The washed cells were scraped from the plates, and cells and media were hydrolyzed separately with 6.0 N HCl for 24 hr. Each sample was analyzed for hydroxyproline and proline incorporation into protein by the method of Lukens (12). Cell counts were also determined for each sample.

Protein synthesis was measured by addition of 1.7 μCi of [^{14}C]leucine per ml of medium 6 hr before harvest of cells. After the cells were washed with balanced salt solution, protein was precipitated with 5% Cl_3CCOOH. The precipitate was collected on a Millipore filter, washed with cold 5% Cl_3CCOOH, dried, and counted. Total protein content was determined by the method of Lowry *et al.* (13).

RNA synthesis was measured by addition of 1 μCi of [^{14}C]uridine per ml 6 hr before harvest. The cells were then treated as described above. For study of DNA synthesis, 1 μCi per ml of [^{3}H]thymidine was added to the medium and samples were prepared as indicated above.

For radioautographic studies, 0.75 μCi of [^{3}H]BrdU per ml was added to cells grown over agar for 48 hr. Cells were collected on coverslips that had been placed in the culture dishes, washed with balanced salt solution, and fixed in Bouin's solution. In each experiment, one slide was heated in 5% Cl_3CCOOH at 90° for 15 min to determine whether grains were produced by Cl_3CCOOH-soluble material. Both slides were then washed with cold 5% Cl_3CCOOH to remove unincorporated nucleotides, washed in graded alcohol:water mixtures, and dipped in Ilford K5 emulsion. Radioautographs were stored for 2–4 weeks in the dark at 4° and then developed in Kodak D-19 developer.

The persistence of [^{3}H]BrdU in cells after subculture as monolayers was also determined by radioautography. The cells were fixed with Bouin's solution on the seventh day after subculture. The plastic plates were cut into strips, taped onto slides, and treated like the coverslips.

For the study of the rate of disappearance of BrdU, 0.75 μCi of [^{3}H]BrdU plus 32 μM (10 μg/ml) of nonradioactive BrdU per ml were added to freshly dissociated limb-bud cells (stage 23–24) that were grown over agar for 48 hr. After removal from agar, the cells were washed three times with complete medium. The amount of BrdU incorporated while the cells were over agar and the persistence of label in these cells on subculture in the absence of BrdU was determined as follows: Each sample was divided into three portions. To one portion, 50 μg of DNase per ml of solution was added. This portion, as well as the untreated portion, was sonicated for 5 sec, then incubated for 45 min at 37°. These samples were passed over Millipore filters and washed with an excess of cold 5% Cl_3CCOOH. The filters were dried and counted. The third portion of cells was plated on 60-mm dishes at a density of 0.5×10^6 cells per plate. At various intervals, cell numbers were determined and radioactivity in the cold Cl_3CCOOH-precipitable material was determined as indicated above.

Cell numbers were determined in a Coulter counter after treatment with 0.25% trypsin–EDTA.

Source of Materials. F-12 medium was obtained from North American Biologicals, Inc., and fetal-calf serum was obtained from Grand Island Biological Co. Bacto-agar was manufactured by Difco Laboratories. The following radioactive precursors were obtained from New England Nuclear Corp.: $H_2{}^{35}SO_4$, carrier free, 43 Ci/mg; [^{14}C]uridine, 50 Ci/mmol; [^{14}C]leucine, 52 Ci/mmol; [^{3}H]thymidine, 18.3 Ci/mmol; [^{3}H]BrdU, 12.7 Ci/mmol; [^{14}C]proline, 260 Ci/mmol; [^{3}H]-acetate, 100 Ci/mmol. BrdU was purchased from Calbiochem.; thymidine, proline, and hydroxyproline were purchased from Sigma and DNase from Worthington (Type 1 RNase free, 2500 U/mg). Carrier chondroitin-4-sulfate was a gift from Dr. J. A. Cifonelli, and twice recrystallized papain was a gift from Dr. Lennart Rodén.

RESULTS

Effect of agar on differentiation of limb-bud cells

Previous studies by Zwilling (personal communication) had shown that limb-bud mesenchyme cells obtained from stage-24 chick embryos do not differentiate to cartilage cells in culture unless plated at a density greater than confluency. It had been demonstrated in this laboratory (14) that embryonic chick cartilage cells grown in liquid suspension above agar exhibited enhanced chondrogenic expression. Therefore, an attempt was made to determine whether culture on agar would promote differentiation of early embryonic limb-bud mesenchyme when plated at densities below confluency. For this purpose the following experiment was designed with freshly liberated limb-bud cells (stage 24). (*A*) Cells were cultured at a density of 1×10^7 cells per 100-mm dish over agar for 48 hr and then plated at a density of 0.5×10^6 cells per 60-mm dish, (*B*) Cells were plated at a density of 1×10^7 cells per 100-mm dish without agar, and after 48 hr, subcultured at a density of 0.5×10^6 cells per 60-mm dish, and (*C*) Cells were plated directly at a density of 3.6×10^6 cells per 60-mm dish and were not subcultured. The initial plating density for each of the cultures was less than required for a confluent monolayer. Sulfate uptake was measured on day 7 after subculture for *A* and *B* and on day 9 for *C*.

The development of cartilage was monitored by phase-contrast microscopy and by staining with 1% aqueous tolu-

idine blue. Cultures first grown on agar (*A*) showed a large number of cells with typical epitheloid morphology and the presence of metachromatic matrix. The data presented in Table 1 demonstrate a striking increase in chondroitin sulfate synthesis by cultures derived from cells first grown over agar.

The mechanism by which culture over agar promotes differentiation into cartilage is not clear. Since it seemed possible that cell aggregation might be an important factor, the technique of Moscona (15) was used for aggregation of limb-bud cells in a gyratory shaking flask. 3 ml of a limb-bud cell (stage 24) suspension (10^6 cells per ml) were incubated for 48 hr in 25-ml Ehrlenmeyer flasks on a New Brunswick gyratory shaker rotating at 70 rpm, at 38°. The cells were then dissociated by treatment with 0.25% trypsin in EDTA and placed on plastic dishes at a density of 0.5×10^6 cells per 60-mm plate. After 7 days of growth on the tissue-culture plates, there was no recognizable cartilage formation based on morphology, metachromasia, or chondroitin sulfate synthesis. These results indicate that aggregation of limb-bud cells is not in itself sufficient to promote chondrogenesis on subsequent monolayer culture.

Prevention of differentiation of cartilage cells by BrdU

Since previous studies (1, 2) had shown that BrdU prevents the expression of cartilage phenotype, experiments were performed to determine the effect of this analogue on the differentiation of dissociated limb-bud cells. The data in Table 2 show the effect of BrdU added during the initial 48-hr period when the mesenchymal cells were cultured over agar. In these experiments *no BrdU was present* during subsequent subculture on plastic dishes, when the cartilage phenotype normally makes its appearance in untreated cells. These data indicate that the presence of BrdU in concentrations of 32 μM during the period of culture over agar inhibits the subsequent appearance of the cartilage phenotype. BrdU did not exert its effect simply as a result of toxicity since cells exposed to the analogue continued to exhibit normal rates of DNA, RNA, and protein synthesis and cell division (Table 3).

The effect of BrdU as measured by sulfate incorporation into chondroitin sulfate proteoglycan was dose dependent up to a concentration of 10 μg/ml (32 μM). Doses larger than 10 μg/ml did not further depress radioactive sulfate incorporation. Inhibition was observed with as little as 1 μg of BrdU per ml.

TABLE 2. *Effect of BrdU on differentiation of cartilage*

Experiment no.	Control cpm/10^6 cells	BrdU cpm/10^6 cells
1	13,400	2,380
2	53,800*	8,920
3	76,000	6,250
4	245,000*	65,200
5	26,300*	3,040
6	15,400	2,540

Cells were grown over agar for 48 hr and then plated at a density of 0.5×10^6 cells per 60-mm dish. BrdU was added at a concentration of 10 μg (32 μM) per ml of medium during culture over agar. No BrdU was present on subculture. Cells were labeled with 3.3 μCi $H_2{}^{35}SO_4$ per ml of medium, 6 hr before harvest, on the seventh day of subculture.

* Compare with *A*, Table 1.

TABLE 3. *Effect of BrdU on cell multiplication and on protein, DNA, and RNA synthesis*

	Control	BrdU
Cell no. per dish	3.2×10^6	3.3×10^6
mg Protein per dish	0.42	0.37
	cpm/10^6 cells	cpm/10^6 cells
[^{3}H]thymidine incorporation	1,420	990
[^{14}C]uracil incorporation	139	126
[^{14}C]leucine incorporation	13,600	14,500

All experiments were performed on cells exposed to 50 μg of BrdU per ml of medium during growth over agar. Isotopes were added 6 hr before harvest of cultures on the seventh day after subculture.

In order to be certain that the effects observed were not simply on sulfation, another precursor of chondroitin sulfate, [^{3}H]acetate, was used. The results were identical with experiments with $H_2{}^{35}SO_4$. In other experiments, it was shown that exposure of cells over agar to BrdU for as little as 18 hr was sufficient to prevent subsequent chondrogenesis.

Thymidine, at a concentration of 160 μM, prevented the effect of 32 μM BrdU only if it was added simultaneously with the analogue. Subsequent addition of the same concentration of thymidine when cells previously exposed to BrdU were subcultured on plates, did not restore the cartilage phenotype. The inhibition of differentiation during this special period of determination is reversed neither by removal of BrdU nor by the subsequent addition of excess thymidine.

That the effect was irreversible was demonstrated by the decrease in the rate of chondroitin sulfate synthesis by BrdU-treated cells that were maintained through serial subculture in the absence of the drug. At higher doses of BrdU (32–160 μM), the diminution of the capacity to synthesize chondroitin sulfate was observed through four subcultures in 31 days. Even at a dose of 1 μg/ml of BrdU (3.2 μM), the difference between BrdU-treated and control cells was evident through five subcultures, as illustrated by the data summarized in Table 4.

If limb-bud cells at stage 24 that had undergone differentiation (during the period of culture over agar) were exposed to BrdU during subsequent subculture, a decrease in sulfate uptake occurred. However, further subculture for 6 days in the absence of BrdU resulted in restoration of high incorporation of sulfate.

TABLE 4. *Effect of BrdU after serial subculture*

	cpm/10^6 control cells	cpm/10^6 BrdU-treated cells
First subculture (6 days)	15,400	8,250
Second subculture (10 days)	9,300	5,260
Third subculture (21 days)	13,200	6,975
Fourth subculture (27 days)	5,020	2,740
Fifth subculture (41 days)	20,900	11,400

$H_2{}^{35}SO_4$ was added 6 hr before harvest of cells on the day indicated. BrdU at a concentration 3.2×10^{-6} M (1 μg/ml) was used only during initial growth over agar.

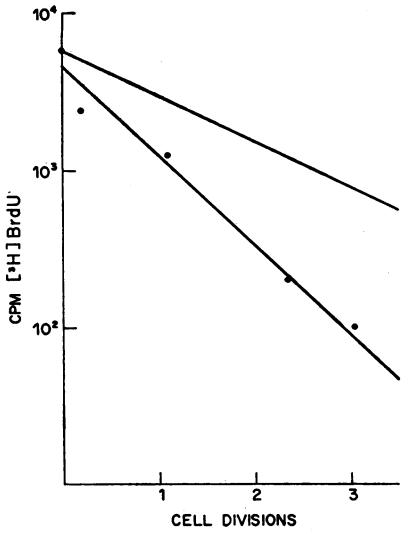

FIG. 1. Decay of [³H]BrdU-labeled, Cl_3CCOOH-precipitable material in stage 24 limb-bud cells upon subculture on tissue-culture dishes. Freshly dissociated cells were labeled with 0.75 μCi [³H]BrdU per ml of medium plus 10 μg of unlabeled BrdU per ml of medium and were grown over agar bases. After 48 hr, cells were removed from agar, redissociated, washed, counted, and replated on 60-mm plastic dishes. Radioactivity in cold Cl_3CCOOH precipitate and cell counts were determined upon removal of cells from agar, twice daily for 8 days. *Ordinate:* cpm [³H]BrdU/10^6 cells. *Abscissa:* cell divisions expressed as doublings of total cell number per 60-mm plate.

The inhibitory effect of BrdU on expression of cartilage phenotype in differentiated chondrocytes is reversible. An additional confirmation of this conclusion was obtained when cartilage from stage-32 hind limbs were first cultured with BrdU and then subcultured without it. No inhibition of sulfate incorporation was observed after subculture for 7 days in the absence of BrdU.

Effect of heterotypic and BrdU-treated cells on chondroitin sulfate synthesis

Chacko and Holtzer (16) have reported that cocultivation of BrdU-treated cartilage cells with untreated cartilage cells suppressed normal chondrocyte morphology. We, accordingly, mixed equal numbers of limb-bud mesenchyme cells that had been exposed to BrdU while cultured over agar with cells cultured over agar but untreated with the analogue. After 7 days of culture, minimal inhibition of expression of the cartilage phenotype was observed.

However, when equal numbers of cells that had been cultured over agar but untreated with BrdU and rat glial tumor cells (clone C6) (17) were mixed and plated on tissue-culture dishes, a striking inhibition of sulfate uptake was observed.

Collagen synthesis

Since chondrocytes characteristically synthesize collagen as well as chondroitin sulfate proteoglycan, an attempt was made to study the effect of BrdU treatment on subsequent collagen production by the differentiating limb-bud cells. The data in Table 5 show that, in contrast to the effect on acid mucopolysaccharide synthesis, BrdU did not depress the appearance of hydroxyproline in macromolecules by the limb-bud cells (stage 24). In fact, the BrdU-treated cells seemed to be producing higher concentrations of both cell- and medium-associated collagen than the untreated (control) cultures.

Mechanism of action of BrdU

In view of the short exposure to BrdU in these experiments, it seemed appropriate to determine whether the BrdU was incorporated into nuclear DNA. Autoradiographs of limb-bud cells treated with [³H]BrdU after being cultured for 48 hr over agar showed a dense accumulation of silver grains over the nucleus of 95% of these cells. However, after 7 days of subculture with BrdU in plastic dishes, during which time the cells had divided about three times, silver grains were no longer detectable. An investigation of the kinetics of this rapid loss of [³H]BrdU was, therefore, undertaken. That the label was incorporated into DNA was demonstrated by the loss of 85–90% of the counts from the cold Cl_3CCOOH-precipitable material as a result of treatment with RNase-free DNase.

Fig. 1 compares the observed loss of labeled BrdU with that which might be anticipated on the basis of the rate of cell division. These data indicate an unexpectedly rapid disappearance of labeled BrdU that cannot be explained on the basis of the observed rate of cell division.

DISCUSSION

The results reported in this study indicate that early embryonic limb tissue is relatively unstable with respect to differentiation into mature cartilage. When freshly dissociated limb-bud mesenchyme cells are grown at low density or are exposed to BrdU, there is permanent inhibition of chondrogenic expression. The effects of culturing the cells at low density can be alleviated by initially placing the cells in liquid suspension over agar, while the suppressive effects of early exposure to BrdU can only be overcome by simultaneous addition of high concentrations of thymidine.

The effect of BrdU on these "protodifferentiated" cells (18) contrasts markedly with its influence on differentiated cells. The repression of differentiated expression by BrdU is both permanent and time-specific in the early embryonic cell type; however, it is reversible in mature cells. Wilt (19) has recently demonstrated a suppressive effect of BrdU associated with a specific period of embryonic chick erythropoiesis. However, this inhibition could be reversed by subsequent addition of thymidine at twice the molarity of BrdU. Gontcharoff and Mazia (6) have also shown a critical period during

TABLE 5. *Effect of BrdU on biosynthesis of collagen*

	Control hyp/pro/10^6 cells		BrdU hyp/pro/10^6 cells	
	Cells	Medium	Cells	Medium
I	5.0×10^{-3}	1.4×10^{-2}	7.1×10^{-3}	2.8×10^{-2}
II	9.0×10^{-3}	2.8×10^{-2}	15.8×10^{-3}	5.1×10^{-2}

Cells grown over agar for 48 hr with or without 10 μg BrdU per ml of medium, then placed on tissue-culture dishes, were incubated for 16 hr with 16.7 μCi [¹⁴C]proline. Medium was removed from each plate, and the plates were washed once with phosphate-buffered saline solution (pH 7.4). This wash was combined with the media; cells were scraped from the dishes in phosphate-buffered saline, and label incorporated into hydroxyproline (hyp) and proline (pro) were determined as described in *Methods*. *I* and *II* are replicate experiments.

which BrdU perturbs sea urchin development, the effect being irreversible. The similarity between their finding with sea urchins and the one reported in this paper cannot be readily determined, although superficially the sequence of events appears comparable.*

Recent studies with transplanted fragments of chick-limb mesenchyme indicate that a relatively fixed time period exists during which limb-bud cells are stabilized into a chondrogenic versus soft tissue phenotype (8, 20). Before this crucial stage, cells in certain areas of the limb seem to be directed toward cartilage production (21, 22) but can become redirected by modification of their environment. Intrinsic cellular qualities appear to prevent environmental factors from determining cell expression after this important period. We have shown that the exhibition of the differentiated state by early limb mesenchyme cells (stage 23–24) is exquisitely sensitive to their surroundings, while older embryonic cells (stage 32) are only transiently affected by their mileu and, thus, possess the ability to revert to normal expressivity once their environment becomes favorable.

The lack of inhibition of collagen synthesis in BrdU-treated cells points to a dual mechanism for control of synthesis of this protein and synthesis of chondroitin sulfate glycosaminoglycan during cartilage differentiation. To invoke the interpretation of Holtzer and Abbott (16) that BrdU preferentially represses the synthesis of "luxury molecules," one must be willing to assume collagen production is necessary for cell viability. Since ascorbic acid was not added to the medium, the possibility arises that collagen biosynthesis was occurring at basal concentrations in both BrdU-treated and control cultures (26). Ascorbate might enhance a preferential stimulation of collagen production in untreated cells in comparison with cells in cultures treated with BrdU; experiments are now in progress to evaluate this possibility.

The mode of action of BrdU may allow the elucidation of mechanisms involved in cellular differentiation. On the basis of the specific activity and total incorporation of [^{3}H]BrdU, calculations indicate that BrdU is initially incorporated into DNA at a fairly low concentration (about 2% substitution for thymidine). It is then rapidly lost from the genome. Radioautographs revealed that 95% of the cells exposed to BrdU incorporate the analogue into their DNA during the 48-hr culture period over agar. It could not be determined whether inhibitors of DNA synthesis prevent BrdU suppression of differentiation since previous studies with cytosine arabinoside, nitrogen mustard, and 5-fluorodeoxyuridine revealed that these inhibitors themselves disturbed the development of cartilage from limb-bud mesenchyme (Levitt and Zwilling, unpublished).

The possible importance of specific satellites of DNA or selective gene amplification in differentiation are only beginning to be appreciated. Rutter (23) has recently shown BrdU to be selectively incorporated into a "light" fraction of DNA. Others have postulated the involvement of cytoplasmic DNA molecules in influencing both tissue differentiation and gene expression (24, 25). Since we have observed that even a relatively brief exposure to BrdU (18–24 hr) during the initial period of cultivation of chick limb-bud cells will permanently repress chondrogenic differentiation, it appears plausible that some of the DNA synthesized by the limb-bud mesenchymal cell during this early period possesses characteristics not retained in mature cells. If this fraction of the DNA is modified before differentiation is stabilized, the permanent loss of specialized function in affected cells might be expected. A possible explanation of these events is that BrdU is incorporated into a specific region of DNA, exerts its repressive action at a crucial time of differentiation, and then is rapidly lost from this now functionless region.

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* While this communication was in preparation, an abstract of a paper presented at the 11th Annual Meeting of the American Society of Cell Biology by Joan Abbott came to our attention; irreversible inhibition of chondrogenesis by BrdU in chick-embryo somites was observed.