

# Regulation of Rat Myosin Light-Chain Synthesis in Heterokaryons between 5-Bromodeoxyuridine-blocked Rat Myoblasts and Differentiated Chick Myocytes

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**ABSTRACT** Terminal cell differentiation in a variety of model systems is inhibited by the thymidine analogue 5-bromodeoxyuridine (BUdR). We investigated the mode of action of BUdR by forming heterokaryons between undifferentiated BUdR-blocked rat myoblasts and differentiated chick skeletal myocytes. We analyzed newly synthesized proteins on two-dimensional polyacrylamide gels. The induction of rat skeletal myosin light-chain synthesis was reduced fivefold, as compared with controls, when chick myocytes were fused to BUdR-blocked rat myoblasts. This indicates that plasma membrane effects cannot be the proximate cause for the inhibition of myogenesis by BUdR, since BUdR is able to block the effect of chick inducing factors even when a differentiated chick myocyte is in direct cytoplasmic continuity with the BUdR-blocked rat nucleus.

The observation that chick cells required an 80% substitution of BUdR for thymidine to block myogenesis, whereas L6 rat myoblasts required only a 20% substitution led to a hypothesis involving a DNA-mediated action of BUdR. This model yielded three testable predictions: (a) putative chick inducing molecules should be present in limiting quantities, (b) exploiting gene-dosage effects to increase the quantity of putative chick inducing factors might overcome the inhibition produced in the rat myoblasts by a 35% BUdR for thymidine substitution, and (c) these gene-dosage effects should be abolished by increasing the level of BUdR substitution in the rat myoblast to 60–80%. All three of these predictions have been verified, providing strong indirect evidence that the inhibition of myogenesis produced by BUdR is a direct result of its incorporation into cellular DNA.

The thymidine analogue 5-bromodeoxyuridine (BUdR) has many effects on cultured vertebrate cells. In addition to being toxic (1, 2), mutagenic (3), and an inducer of endogenous viruses (4), it has been found to inhibit the terminal differentiation of cultured melanoma cells (5), chondroblasts (6), erythrocytes (7), and myoblasts (8). Since the expression of specialized functions in postmitotic differentiated cells is not substantially affected by BUdR and since the inhibition of differentiation in dividing cells can occur even if exposure to the drug is limited to a short span during DNA synthesis (9–11), it is thought that the block may be a direct result of the incorporation of BUdR into cellular DNA (12). Recent evidence has suggested that some BUdR effects may result from other metabolic alterations such as deoxycytidine starvation (13) or membrane modifications (14–17). Our technique for isolating

pure populations of heterokaryons (18) suggested a novel method for determining whether the inhibition of differentiation by BUdR occurred by DNA-dependent mechanisms.

If cells are pretreated with lethal doses of different irreversible biochemical inhibitors and then mixed and fused with polyethylene glycol, complementation and specific rescue of the heterokaryons occurs. Parental cells and homokaryons die from the treatments, but each cell in a heterokaryon contributes active molecules to replace those inactivated in its fusion partner and the heterokaryons survive (18). We have used this approach to examine heterokaryons formed by fusing differentiated chick muscle cells to undifferentiated rat myoblasts (19). The induction of rat myosin light-chain synthesis in these heterokaryons suggested that differentiated chick cells contained inducing factor(s). The ability to provide such factors to

a BUdR-blocked rat myoblast by fusing it to a differentiated chick cell should permit discrimination between several possible mechanisms of action for BUdR. For example, if BUdR acts by altering the plasma membrane (14–16) so that the cells no longer recognize environmental signals to differentiate, then rat myosin light-chain synthesis should be inducible in the heterokaryons since the inducing factor(s) provided by the chick cell would be in direct cytoplasmic continuity with the rat nucleus. On the other hand, if BUdR acts by blocking the activating sites for differentiated structural genes, then differentiated rat muscle products should not be inducible. The results of these experiments, in which differentiated chick muscle cells are fused to BUdR-blocked rat myoblasts, are most consistent with the idea that BUdR inhibits myogenesis as a result of its incorporation into DNA.

## MATERIALS AND METHODS

**Cells and Culture Conditions:** All cells were grown in a mixture of four parts Dulbecco's Modified Eagle's Medium to one part Medium 199 (20). A subclone isolated in our laboratory from the rat myoblast cell line L6 derived by Yaffe (20) served as the undifferentiated parent. This subclone has a modal chromosome number of 41 and remains undifferentiated under conditions of exponential cell growth. These cells were subcultured three times per week in the above medium supplemented with 20% newborn bovine serum (NBS). The cells could be stimulated to differentiate at subconfluent densities if they were downshifted to a medium containing 1% NBS and 5  $\mu\text{g}/\text{ml}$  insulin (21). Under these conditions, L6 cells differentiate and form multinucleated myotubes within 2–3 d and initiate the synthesis of muscle-specific proteins. In contrast with differentiated primary cultures of rat myoblasts, differentiated cultures of L6 cells synthesize only the embryonic and not the adult form of skeletal myosin light-chain one (22).

We obtained primary cultures of chick myoblasts by mechanically dissociating thigh muscle from 12-d embryos. The cells were cultivated on gelatin-coated petri dishes supplemented with 5% horse serum and 2% chick embryo extract. To avoid massive gene-dosage effects (23), mononucleated differentiated chick cells were obtained by blocking myotube formation by reducing the calcium concentration with 1.75 mM EGTA 20 h after the initiation of the primary cultures (24). Because many cells detached after long periods in EGTA, after the myoblasts had become postmitotic the medium was changed to normocalcemic medium containing 2  $\mu\text{g}/\text{ml}$  cytochalasin B to maintain the cultures as mononucleated (25). We shall call mononucleated differentiated muscle cells "skeletal myocytes" to distinguish them from both multinucleated myotubes and undifferentiated myoblasts. In some experiments, EGTA was not used and cytochalasin B was added directly to the medium 20 h after the initiation of the culture. Under these circumstances, most of the myoblasts divided once in the presence of cytochalasin B before terminally differentiating, thus yielding a population composed primarily of binucleated myocytes.

**Biochemical Selection of Heterokaryons:** The isolation of heterokaryons by using biochemical inhibitors has been described in detail elsewhere (18, 26, 27). The basis for the selective system is the irreversible inactivation of different sets of molecules in two cell populations treated with either iodoacetamide or diethylpyrocarbonate (DEPC). Iodoacetamide inactivates sulfhydryl groups, while DEPC is directed primarily against histidine residues. After a lethal treatment with one of the two inhibitors, the cells are washed free of unreacted agent and then mixed and fused to cells treated with the other inhibitor. Parental cells and homokaryons die from the lethal treatments. However, because different sets of molecules have been inactivated by the two inhibitors, DEPC-treated cells and iodoacetamide-treated cells can complement their molecular lesions and some of the heterokaryons survive (18). 2 d after heterokaryon formation, most of the dead cells are removed by centrifuging the cells onto a Ficoll-sodium diatrizoate cushion (26). The cells floating at the interface are then harvested, washed, and plated in gelatin-coated 0.3-cm<sup>2</sup> microwells. The following day, the cells are fed fresh medium containing 15% fetal calf serum and appropriate concentrations of BUdR to prevent any spontaneous differentiation from occurring. Populations of cells in which >90% of the nuclei were in heterokaryons were labeled overnight in 300  $\mu\text{Ci}/\text{ml}$  of [<sup>35</sup>S]methionine and harvested the sixth day after heterokaryon formation.

A variety of control experiments indicate that the biochemical treatments do not fundamentally affect the ability of the surviving heterokaryons to express differentiated functions. These controls have been discussed in detail elsewhere (18, 19).

**Two-dimensional Gels:** Skeletal myosin is a dimer composed of two

200,000-dalton heavy chains associated with several 16,000–25,000-dalton light chains (28). The skeletal myosin light-chain one was chosen as a marker for muscle differentiation since chick and rat forms have different mobilities on two-dimensional polyacrylamide gels. The spots corresponding to chick fast, rat adult fast, and rat embryonic myosin light chains were identified by their molecular weights and isoelectric points, their co-migration with the light chains of actomyosin purified from chick and rat tissue, their absence in undifferentiated cells and synthesis in differentiated myotubes, their partial purification upon repeated cycles of solubilization at high ionic strength extraction and precipitation at low ionic strength, and their co-purification with the myosin heavy chain using an anti-heavy-chain antibody and inactivated *Staphylococcus aureus* (19).

The actomyosin in heterokaryon cell extracts was enriched by first lysing the cells in 0.05 ml of 0.5% Nonidet P-40, then diluting the extract with 1 ml of buffer containing 15 mM KCl, 10 mM Tris, pH 7.5, 0.1% 2-mercaptoethanol, and 15  $\mu\text{g}$  of cold carrier rat cardiac actomyosin. The extract was centrifuged at 4°C for 10 min in a Beckman microfuge 1–4 d later. The soluble proteins in the supernatant were discarded and the actomyosin-enriched pellet was dissolved in 0.05 ml of O'Farrell's lysis buffer (29). Two-dimensional gels were run as described by O'Farrell (29), with the following modifications. Isoelectric focusing was performed in 19-cm long gels formed in 0.2-ml pipets designed for cotton-plugging. The flared upper end of these pipets provides a large reservoir for samples of 50–100  $\mu\text{l}$ . After isoelectric focusing in the presence of pH 4–6 ampholines, the gels were equilibrated for 1 h in 55% methanol containing O'Farrell's equilibration salts (29). The use of methanol increases resolution, presumably by decreasing diffusion during the equilibration step (30). It also shrinks the first-dimensional gel and permits it to be squeezed directly between the plates of the second dimension. The second dimension consisted of a 5% acrylamide stacking gel and a 12 1/2% acrylamide separating gel. 0.75-mm thick gels were dried under a vacuum and exposed with Kodak AR5 x-ray film for 12 million cpm  $\times$  d.

In some experiments, radioactive spots were quantitated by dissolving small pieces of the gel in an NCS Tissue Solubilizer (Amersham Corp., Arlington Heights, IL) for 48 h before scintillation counting (31). Spots were localized by marking the perimeter of the gel with radioactive ink (15  $\mu\text{Ci}/\text{ml}$  of [<sup>35</sup>S]-methionine) before exposing the autoradiogram. This permitted a precise superposition of the dried gel directly on top of the developed autoradiogram. After the desired spot was circled with ink and the gel and autoradiogram were taped together, regions of the gel corresponding to spots on the autoradiogram could be cut out with a scalpel under direct visualization with strong backlighting through the autoradiogram-gel sandwich. Blank regions of the gel adjacent to the radioactive spots were cut out to determine the gel background. Variable penetration of loaded counts into the gels was compensated for by normalizing the counts with respect to the total counts contained in the region of the gel between the tubulins and the tropomyosins. This region of the gel contained the highest density of spots and thus should give the best estimate of the average relative radioactivity per gel.

The actual amount of induction of rat myosin light chain in control combinations of chick myocytes that were fused to normal undifferentiated L6 rat myoblasts varied widely between experiments (see Tables I and III). This probably reflects variations in the purity and "quality" of the chick myocytes that were produced as primary cultures on a weekly basis. To compensate for this variation, each experiment involving BUdR-blocked rat myoblasts contained an internal control using normal undifferentiated rat myoblasts. Results were then expressed as a proportion of the value obtained with this internal positive control,  $\pm$  SE.

**Creatine Phosphokinase and  $\alpha$ -Bungarotoxin-binding Assays:** Total creatine phosphokinase activity was determined spectrophotometrically by using Kit 45-UV (Sigma Chemical Co., St. Louis, MO) supplemented with 300  $\mu\text{M}$  P<sub>1</sub>P<sub>5</sub> diadenosine pentaphosphate to inhibit myokinase activity (32). Acetylcholine receptor content was determined from the binding of [<sup>125</sup>I]- $\alpha$ -bungarotoxin in the presence vs. absence of the specific inhibitor decamethonium (10<sup>-5</sup> M). Heterokaryons were incubated overnight in 10<sup>-8</sup> M cold  $\alpha$ -bungarotoxin 3 d before being assayed. This treatment exhausts cytoplasmic pools, so the appearance of new binding sites then results from the ongoing synthesis of new receptors (33). Protein was determined by the method of Böhlen et al. (34) by using fluorescamine (Fluoram, Roche Diagnostics, Nutley, NJ). Details of these procedures are presented elsewhere (35).

## RESULTS

### *Heterokaryons with Low Dose BUdR-blocked Myoblasts*

The lowest dose of BUdR capable of inhibiting myogenesis was determined to minimize any secondary effects that might occur at high concentrations of BUdR. Fig. 1 shows both the

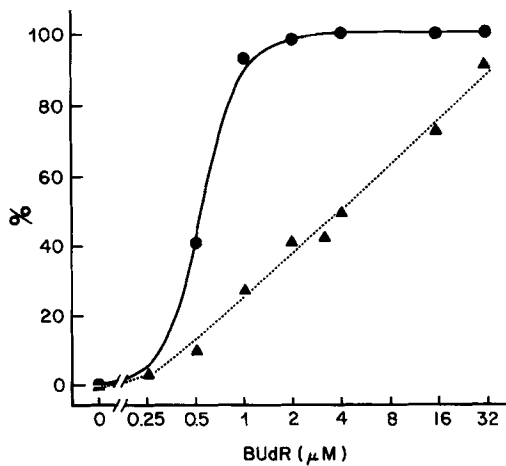


FIGURE 1 The inhibition of differentiation by BUdR. L6 rat myoblasts were plated at low density in increasing concentrations of BUdR and allowed to divide at least three times over a period of 10 d. The medium was then changed to a differentiation-promoting medium containing 1% NBS, 5  $\mu\text{g/ml}$  insulin, and appropriate concentrations of BUdR. 4 d later, aliquots were fixed in 95% ethanol and stained with Giemsa stain. The number of nuclei in myotubes containing more than three nuclei was determined by counting a diameter of each dish. The percentage of BUdR for thymidine substitution ( $\blacktriangle$ ) at each concentration was determined by analyzing the buoyant density of the DNA from sister dishes on cesium chloride gradients as described elsewhere (37). Percentage inhibition,  $\bullet$ .

dosage curve for the inhibition of myotube formation by BUdR in our clone of L6 myoblasts and the percent BUdR for thymidine substitution in cellular DNA that occurred at each BUdR concentration. These data are presented for reference purposes for interpreting the experiments described below.

Fig. 2 compares the proteins synthesized in heterokaryons formed by fusing differentiated chick myocytes to either L6 myoblasts or L6 cells that had been grown in 2  $\mu\text{M}$  BUdR. Both embryonic and adult fast skeletal myosin light chains (22) were induced in control heterokaryons (Fig. 2A). This induction occurred even though 2  $\mu\text{M}$  BUdR was present in the medium continuously after polyethylene glycol-induced cell fusion. Little or no rat light chains were present in heterokaryons involving BUdR-blocked L6 cells (Fig. 2B). A trace of embryonic rat light chain could be identified in the original autoradiogram of the gel in Fig. 2B. To quantitate the relative amounts of light chains synthesized in these heterokaryons, the spots were cut out, dissolved in NCS, and the radioactivity was determined by scintillation counting. Table I presents the data from one experiment. The values for the adult rat fast light-chain one are the least reliable, since a small contamination by the adjacent chick fast light-chain one spot would make a large difference in the values obtained. The following analysis is thus limited to the synthesis of the chick and embryonic rat light chains. The induction of rat embryonic light-chain one in this experiment was reduced >70% in the heterokaryons involving BUdR-blocked as compared with normal undifferentiated myoblasts. A similar quantitation of six additional experiments gave an average induction of the embryonic rat light-chain one in heterokaryons between chick myocytes and rat myoblasts blocked with 2  $\mu\text{M}$  BUdR of  $21 \pm 3\%$  of the induction in control heterokaryons. The synthesis of chick light chains was also reduced to  $53 \pm 5\%$  in these heterokaryons.

The activity of creatine phosphokinase and the acetylcholine

receptor was determined to measure the expression of other myogenic proteins in the heterokaryons. These assays did not distinguish between chick and rat forms. Table II demonstrates

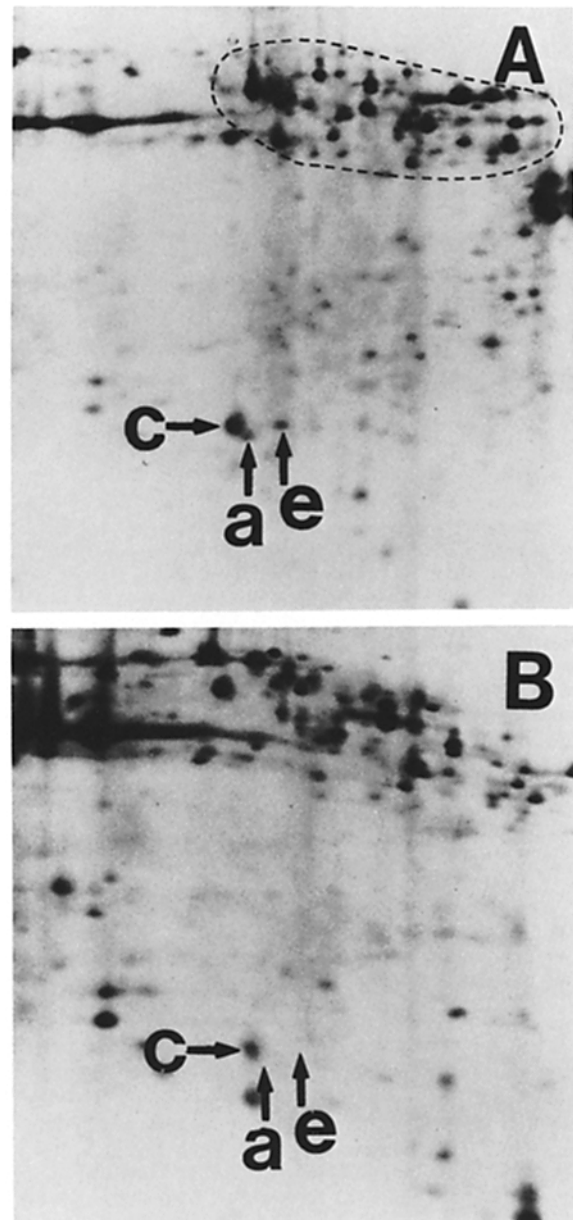


FIGURE 2 Two-dimensional gels of heterokaryon cell extracts. Undifferentiated L6 rat myoblasts or L6 myoblasts that had been grown for three cell generations in 2  $\mu\text{M}$  BUdR were treated with DEPC and fused to differentiated chick myocytes treated with iodoacetamide. The heterokaryons were maintained in BUdR until they were labeled overnight with [ $^{35}\text{S}$ ]methionine and harvested on the sixth day after heterokaryon formation. We analyzed whole cell extracts on two-dimensional gels. The acidic end of the gels are to the right. Only the region of the gels containing the myosin light-chain one is shown. (A) Extract of heterokaryons formed between differentiated chick myocytes and control L6 rat myoblasts. (B) Extract of heterokaryons formed by fusing differentiated chick myocytes and rat myoblasts blocked with 2  $\mu\text{M}$  BUdR. The skeletal myosin light-chain one spots are labeled to indicate chick fast (c), embryonic rat (e), and adult rat fast (a) muscle forms. The region outlined in the top panel is the area used to normalize the radioactivity when spots were excised and quantitated. The induction of rat light chains is inhibited in heterokaryons containing BUdR-blocked rat nuclei.

TABLE I  
Relative Light-Chain Synthesis in Heterokaryons

Cell combination	Light-chain 1			Vimentin area*	Gel blanks‡
	Chick	Adult rat	Embryonic rat		
Chick myocyte × rat myoblast					
cpm§	1,101	148	178	52,300	20; 38; 51
cpm as % of vimentin area	2.0	0.21	0.27	—	—
Chick myocyte × BUdR-blocked myoblast					
cpm§	301	38	43	30,000	23; 11; 25
cpm as % of vimentin area	1.0	0.060	0.076	—	—

\* The region of vimentin and its degradation products is outlined in Fig. 2A.

‡ Gel blanks represent regions of the gel the same size as the light-chain spots that contain no apparent proteins. They were dissolved in NCS and counted to yield an average gel background. A machine background of 9 cpm has been subtracted from these values.

§ Counts per minute of [<sup>35</sup>S]methionine of spots from two-dimensional gels dissolved in NCS, based on 20-min counts. A machine background of 9 cpm has been subtracted from these values.

|| The counts per minute in each spot was reduced by the average background of the gel blanks, then divided by the counts per minute in the vimentin area and multiplied by 100.

TABLE II  
Relative Myogenic Expression in Parental Cells and Heterokaryons\*

	(n)‡	Creatine phosphokinase	<sup>125</sup> I- $\alpha$ -Bungarotoxin binding
Parental cells			
Chick myocytes	(3)	250 ± 150	150 ± 50
L6 rat myoblasts	(5)	3 ± 1	0.2 ± 0.1
35% BUdR-L6§	(4)	5 ± 2	0.4 ± 0.3
70% BUdR-L6	(3)	6 ± 4	0.02 ± 0.02
Heterokaryons			
Myocyte × 0% BUdR-L6	(5)	100	100
Myocyte × 35% BUdR-L6	(4)	80 ± 15	95 ± 15
Myocyte × 70% BUdR-L6	(3)	40 ± 5	85 ± 20

\* Values are expressed as a percentage of the myocyte × myoblast result, ±SEM.

‡ n = number of independent experiments. Within each experiment, each cell type was analyzed in triplicate.

§ L6 cells were cultivated in 2  $\mu$ M BUdR, thus producing a 35% substitution of BUdR for thymidine.

|| L6 were grown in 8 or 16  $\mu$ M BUdR, thus producing about a 70% substitution of BUdR for thymidine.

that these two markers of myogenic differentiation were expressed at roughly similar levels in the heterokaryons regardless of whether or not the undifferentiated parental cell type had incorporated BUdR into its DNA. Some reduction in the creatine phosphokinase activity may have occurred, but only when high levels of BUdR were used.

It has been claimed that deoxycytidine can reverse the inhibition of myogenesis produced by BUdR (36). In our hands, 6 mM deoxycytidine does reverse the effects of BUdR, but it also decreases the incorporation of BUdR into cellular DNA (37). We explored the effects of deoxycytidine on the induction of rat myosin light chains by plating heterokaryons in 6 mM deoxycytidine for 3 d before labeling with [<sup>35</sup>S]-methionine. Deoxycytidine did not inhibit the synthesis of rat light chains in control heterokaryons, nor did it reverse the inhibition of light-chain induction produced by prior BUdR incorporation (data not shown). We have shown that only 1–2% of these heterokaryons incorporated [<sup>3</sup>H]thymidine during the first 6 d after heterokaryon formation (19, 35). Deoxycytidine was thus unable to reverse the effects of BUdR in the absence of DNA synthesis under conditions where control L6 rat myoblasts could be induced to synthesize differentiated products.

### Levels of BUdR Required to Inhibit Chick Vs. Rat Myogenesis

During the course of these studies, the amount of BUdR for thymidine substitution associated with the inhibition of differentiation was determined by using the fluorodeoxyuridine, BUdR, and thymidine (FBT) medium developed by Kaufman and Davidson (38). In this medium, we used fluorodeoxyuridine to inhibit endogenous pyrimidine synthesis. The cells were then dependent on exogenous deoxycytidine, BUdR and thymidine, and thus incorporate BUdR and thymidine according to their relative proportions in the medium. Fig. 3 shows the inhibition of differentiation that occurred when primary chick myoblasts and L6 rat myoblasts were grown in FBT medium containing 32  $\mu$ M BUdR and decreasing amounts of thymidine, so that the proportion of BUdR varied from 5 to 100%. The response of the two cell types was very different: L6 was inhibited at ~20% substitution, whereas chick myoblasts required ~80% substitution of BUdR for thymidine before myogenesis was completely blocked. The reliability of the FBT system was verified by analyzing the density of substituted DNA on cesium chloride gradients as described elsewhere (37). L6 cells grown in FBT medium containing 20% BUdR had a density shift on cesium chloride gradients of 0.024 g/ml, corresponding to a 25% substitution of BUdR for thymidine, while chick cells grown in FBT medium containing 80% BUdR had a density shift of 0.0782 g/ml, corresponding to an 84%

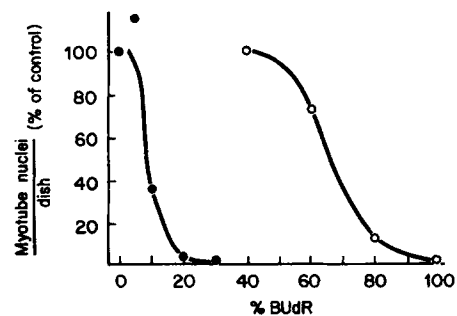


FIGURE 3 Percentage BUdR substitution required to inhibit L6 rat vs. chick myoblasts. L6 myoblasts (●) and primary cultures of chick myoblasts (○) were cultivated in FBT medium (38) containing the indicated percentage of BUdR as a proportion of the total thymidine plus analogue concentration. Stained aliquots were scored for the number of nuclei present in myotubes. Chick cells show a very different sensitivity to BUdR than the L6 rat myoblasts.

substitution. The ratio of BUdR to thymidine in the FBT medium thus provided an accurate measure of the actual level of substitution of BUdR for thymidine in these cells.

### Hypothesis for the Action of BUdR

The observation of the differential sensitivity to BUdR substitution of chick and rat myoblasts led to the following hypothesis. Let us suppose that this difference is not a result of differential effects of BUdR on chicken and rat DNA, but rather on differential activities of chick and rat factors towards BUdR-containing DNA. Under this hypothesis, although a 20% BUdR for thymidine substitution blocks the activity of rat factors, an 80% BUdR for thymidine substitution is required to block the activity of chick factors. It is likely that lower levels of BUdR substitution that do not entirely block chick inducing factor activity under normal circumstances nonetheless reduce their effectiveness. This might have the following effect on chick myocyte  $\times$  BUdR-blocked rat myoblast heterokaryons. If chick factors are present in limiting quantities, the presence of 35% BUdR in the undifferentiated rat nucleus might be sufficient to cause their activity to fall below a threshold value, and thus inhibit the induction of rat myosin light chains seen in control heterokaryons. This threshold model predicts three testable hypotheses: (a) chick inducing factors should be present in limiting quantities; (b) increasing the amount of chick factors might overcome the effect of a 35% BUdR substitution in the rat myoblast and might increase the amount of induction; and (c) increasing the level of BUdR substitution in the rat nucleus to 60–80% should block the induction even in the presence of increased amounts of chick factors.

### Evidence for a Limited Quantity of Inducing Factor of the Chick Cell

If chick factors were limiting, then two rat genomes should be less inducible than one rat genome by the factors provided by a single chick nucleus. Gene-dosage experiments were conducted to test this possibility. A tetraploid line of L6 rat myoblasts was constructed by first isolating fusion products between iodoacetamide-treated and DEPC-treated L6 cells, then plating the cells at low density and picking clones of dividing hybrid cells. Karyologic analysis confirmed the tetraploid or near tetraploid chromosomal content for most of the isolated clones. More than 90% of the tetraploid clones retained the capacity to differentiate when confluent. Fig. 4 compares the proteins synthesized in heterokaryons formed by fusing differentiated chick myocytes to undifferentiated normal vs. tetraploid L6 rat myoblasts. Lower amounts of rat myosin light chains were induced in the cross with the tetraploid rat cells. This reduction was quantitated by cutting out the spots and determining the radioactivity by scintillation counting. Table III presents the results from three different experiments. These experiments indicate that the induction of rat myosin light chains has been reduced to  $42 \pm 12\%$  of control values as a result of increasing the amount of undifferentiated rat chromosomes per heterokaryon. This provides indirect evidence that the putative chick inducing factors were present in limiting quantities.

### Increasing the Chick Inducing Factors

The relative proportion of chick inducing factors was increased by creating a gene-dosage situation in favor of the chick parent. Binucleated chick myocytes were obtained by

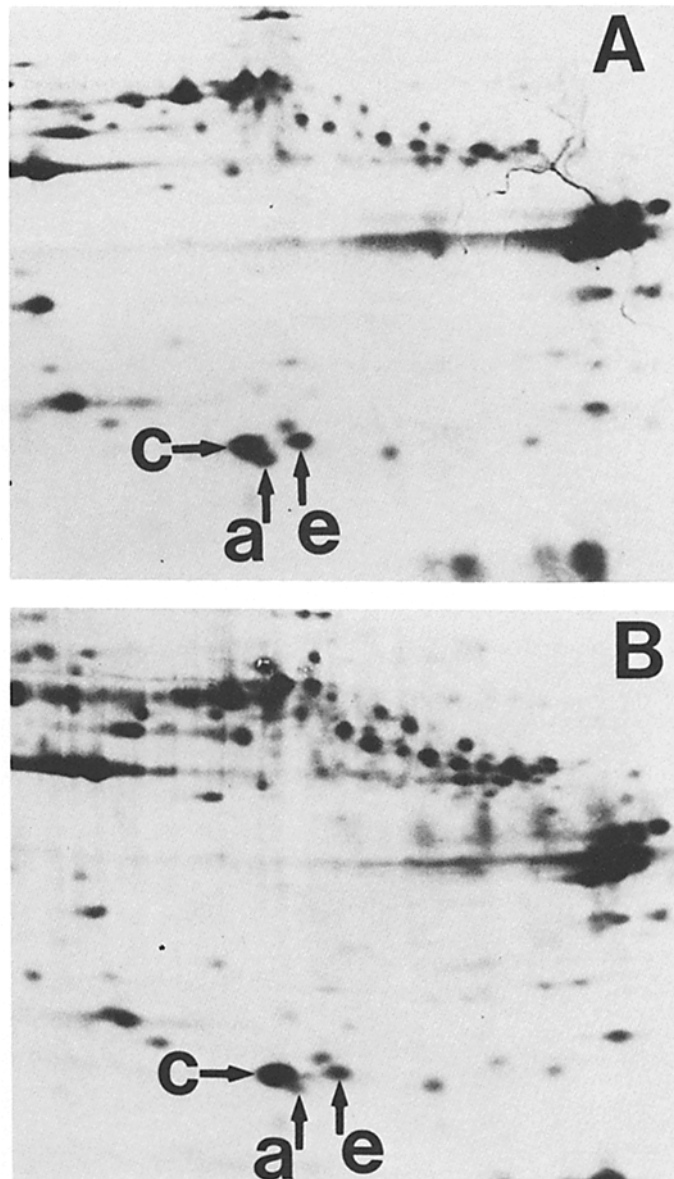


FIGURE 4 Effect of increasing the number of rat chromosomes. The relative quantity of inducing factors was estimated by comparing myosin light-chain synthesis in heterokaryons formed by fusing differentiated chick myocytes to near-diploid (A) or near-tetraploid (B) undifferentiated L6 rat myoblasts. Reduced levels of induced rat myosin light-chain synthesis are present in the tetraploid concentration. Myosin light-chains are labeled as in Fig. 2. This experiment is experiment 2 of Table III.

adding cytochalasin B during the last cell division before terminal differentiation occurred. Since cytochalasin B prevents cytokinesis (39), the cells divided once, became binucleated, then terminally differentiated into postmitotic myocytes. Table IV shows the distribution of chick and rat nuclei obtained in one experiment comparing heterokaryons formed by fusing rat myoblasts to either mononucleated or binucleated chick myocytes. This distribution has been analyzed as follows. If rat myosin light-chain synthesis is induced only in those heterokaryons containing at least a 2:1 ratio of chick to rat nuclei, then the counts per minute in the rat light-chain one spots on two-dimensional gels as a fraction of total counts per minute should be proportional to the number of rat nuclei in chick-dominated heterokaryons as a fraction of total nuclei. In

TABLE III  
Gene-Dosage Effects on Rat Myosin Light-Chain Induction\*

Experiment		cpm in myosin-light chains as % of control area†			cpm in gel background	
		Chick	Adult rat	Fetal rat	As % of control area ± SD	Actual cpm§
1	C × L	9	2	2	0.4 ± 0.2	19
	C × L <sup>2</sup>	11	0.4	0.6	0.3 ± 0.2	19
2	C × L	17	4	10	2.0 ± 0.2	48
	C × L <sup>2</sup>	7	0	2.7	1.0 ± 0.3	29
3	C × L	6	0.3	0.9	0.4 ± 0.1	25
	C × L <sup>2</sup>	6	0.15	0.6	0.4 ± 0.1	27

\* C × L, chick myocyte × L6 rat myoblast heterokaryons; C × L<sup>2</sup>, chick myocyte × tetraploid L6 rat myoblast heterokaryons.

† Calculated as described in Table I with the following modification. In the C × L<sup>2</sup> combinations, 33% of the total counts should be chick and 67% should be rat. The values for C × L<sup>2</sup> have thus been adjusted by multiplying the chick counts per minute by 1.5 (= 0.5 + 0.33) and the rat counts per minute by 0.75 (= 0.5 + 0.67) in order to permit a direct comparison between C × L and C × L<sup>2</sup>.

§ The actual counts per minute are included to permit the percentage values to be reconverted to counts per minute. These values have been reduced by the machine background as in Table I.

TABLE IV  
Gene-Dosage Distribution in Heterokaryons\*

Mononucleated myocyte × rat myoblast				Binucleated myocyte × rat myoblast			
Balanced or rat dominated		≥2:1 Chick dominated		Balanced or rat dominated		≥2:1 Chick dominated	
Nuclei	Number of cells	Nuclei	Number of cells	Nuclei	Number of cells	Nuclei	Number of cells
Chick/rat		Chick/rat		Chick/rat		Chick/rat	
1:1	119	2:1	26	1:1	58	2:1	99
2:2	11	3:1	4	2:2	9	3:1	22
3:3	3	4:1	6	1:2	4	4:1	10
1:2	7	5:1	1	2:3	1	5:1	1
1:3	1	4:2	3	3:2	5	6:1	1
2:3	4	5:2	1	—	—	5:2	1
4:3	1	—	—	—	—	4:2	3
5:3	1	—	—	—	—	—	—
3:2	4	—	—	—	—	—	—

Rat nuclei in chick-dominated heterokaryons = 45. Total nuclei = 535.

Rat nuclei in chick-dominated heterokaryons as percentage of total. Nuclei‡ = 8%.

Rat nuclei in chick-dominated heterokaryons = 141. Total nuclei = 644.

Rat nuclei in chick-dominated heterokaryons as percentage of total. Nuclei‡ = 22%.

\* The number of chick and rat nuclei in heterokaryons was determined after staining with Giemsa stain. Rat nuclei stain more darkly than chick nuclei.

‡ This value was calculated to give the best estimate of the proportion of total radioactivity loaded onto two-dimensional gels that was contributed by induced rat nuclei if more than one chick nucleus were required to induce BUdR-blocked rat nuclei.

the ideal situation where all of the rat nuclei were present in 2:1 chick/rat heterokaryons, 33% of the radioactivity loaded onto the gel would represent rat proteins synthesized in gene-dosed heterokaryons. The data in Table IV show that the use of binucleated chick myocytes did increase the proportion of rat nuclei in gene-dosed heterokaryons from 8 to 22% of total nuclei. Since only two-thirds of the original population of binucleated chick cells actually contained more than one nucleus, this value of 22% is what would be expected, given the maximum value of 33%. A few experiments were eliminated because of the failure to increase this proportion >12%. The analysis of seven remaining experiments yielded average values for the proportion of rat nuclei in gene-dosed heterokaryons as a percentage of total nuclei of  $9 \pm 1$  for heterokaryons involving mononucleated myocytes and  $20 \pm 1$  for heterokaryons involving binucleated myocytes. If rat myosin light-chain synthesis in BUdR-blocked rat myoblasts is induced only in those heterokaryons containing at least twice as many chick as rat nuclei, one could thus expect a 2.2-fold increase in the relative amounts of rat light chain induced by using the binucleated

myocytes. Fig. 5 shows the proteins synthesized when binucleated chick myocytes were fused to either undifferentiated L6 myoblasts or L6 cells that had been blocked with  $2 \mu\text{M}$  BUdR. Radioactive spots from three experiments were excised and quantitated. An average of  $65 \pm 28\%$  of the induction seen in heterokaryons between binucleated myocytes and control myoblasts was seen in the heterokaryons formed by fusing binucleated myocytes to myoblasts blocked with  $2 \mu\text{M}$  BUdR (actual values: 119, 51, and 25%). This is to be compared with the  $21 \pm 3\%$  induction seen in heterokaryons with mononucleated chick myocytes. These results are significantly different at the 0.05-level with a two-sample *t* test (40). The roughly threefold increase in induction observed is close to the 2.2-fold increase predicted under the hypothesis that the induction was occurring only in those BUdR-blocked myoblasts present in chick-dominated heterokaryons.

The  $21 \pm 3\%$  induction of light-chain synthesis seen in the heterokaryons formed by fusing BUdR-blocked myoblasts to mononucleated chick myocytes can also be explained by gene-dosage effects. The data of Table IV indicate that in the

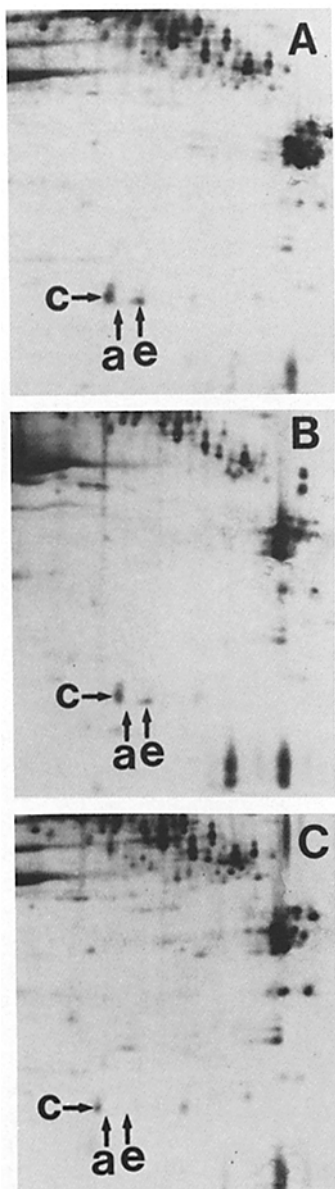


FIGURE 5 Effect of increasing the number of chick chromosomes and the level of BUdR substitution. Binucleated chick myocytes were fused to L6 myoblasts (A) or L6 cells that had been grown in 2  $\mu$ M BUdR (B) or 16  $\mu$ M BUdR (C). Rat myosin light chains are now induced in cells in which 35 but not 70% of the thymidine residues have been replaced by BUdR. Faint adult fast skeletal myosin light-chain one spots could be seen in the original autoradiograms of A and B.

mononucleated myocyte experiment, 44% of the total nuclei were rat, while 8% of the total nuclei were rat nuclei in chick-dominated heterokaryons. If all of the rat nuclei in control heterokaryons formed by fusing chick myocytes to normal rat myoblasts were induced but only the chick-dominated heterokaryons if the combination used 2  $\mu$ M BUdR-blocked myoblasts, then the expected induction in the latter would have been  $8 + 44 = 18\%$  of the control value. The fivefold decrease in the induction observed in myoblasts blocked with 2  $\mu$ M BUdR can thus be entirely explained by the number of chick-dominated heterokaryons.

#### Increasing the Level of BUdR Substitution

Fig. 1 shows that the level of BUdR substitution in the above experiments using 2  $\mu$ M BUdR was  $\sim 35\%$ . L6 cells grown in

16  $\mu$ M BUdR contain  $\sim 70\%$  BUdR. Fig. 5 also shows the proteins synthesized when binucleated chick myocytes were fused to L6 myoblasts blocked with 16  $\mu$ M BUdR (Fig. 5 C). Although sufficient chick factors were present to induce rat myosin light-chain synthesis in cells containing 0 or 35% BUdR, no induction was observed in heterokaryons in which the nucleus contained 70% BUdR. Quantitation of two experiments showed only background levels of radioactivity in the rat light-chain areas of these gels.

The inability of binucleated chick myocytes to induce myosin light-chain synthesis in myoblasts containing high levels of BUdR substitution was confirmed by using quail myoblasts. We have shown that mononucleated chick myocytes can induce normal diploid undifferentiated quail myoblasts to synthesize quail fast skeletal myosin light-chain one (41). Fig. 6 shows that although binucleated chick myocytes could induce light-chain synthesis in control quail myoblasts, no light-chain synthesis was induced in quail myoblasts grown in FBT medium containing 80% BUdR.

#### DISCUSSION

These results indicate that BUdR inhibited the induction of rat myosin light chains when BUdR-blocked L6 rat myoblasts were fused to mononucleated differentiated chick myocytes. The simple presence of BUdR in the medium could not explain the effect, since differentiated chick myoblasts were able to induce normal undifferentiated rat myoblasts even if BUdR was continuously present after the time of heterokaryon formation. Prior exposure of the undifferentiated myoblast to BUdR is thus necessary to block the induction.

The regulation of different myogenic functions does not appear to be completely uniform in these heterokaryons. Chick myosin light-chain synthesis in heterokaryons involving myoblasts blocked with 2  $\mu$ M BUdR was consistently reduced to 50% of that seen in control heterokaryons. In contrast, the synthesis of total acetylcholine receptor as measured by the reappearance of  $^{125}$ I- $\alpha$ -bungarotoxin binding sites remained at control levels. Although the activity of creatine phosphokinase was maintained in heterokaryons between chick myocytes and rat myoblasts blocked with 2  $\mu$ M BUdR, at higher levels of BUdR substitution in the rat nucleus the activity fell to 40% of control values. Although these results are consistent with a noncoordinate regulation of different myogenic functions, the data must be regarded as only suggestive. The different assays are in fact measuring somewhat different processes. The overnight [ $^{35}$ S]methionine label of myosin light chains is measuring a combination of synthesis and degradation and distinguishes between chick and rat isoforms. The other assays do not distinguish the species origin. The creatine phosphokinase assay only measures total activity without demonstrating synthesis. Furthermore, total activity measures both muscle (M) and nonmuscle (B) subunits of the enzyme, and the regulation of these two forms in heterokaryons with BUdR-blocked myoblasts may well be different. Nonetheless, the results do suggest that prior BUdR exposure of the rat myoblast is capable of producing differential effects on the expression of several chick muscle proteins in heterokaryons.

There are multiple potential explanations for this phenomenon. The factors maintaining the synthesis of each specific chick muscle protein may not be present in the same effective concentration. The simple dilution of these factors by a non-inducible BUdR-blocked rat nucleus might then produce the observed effect. There might also be differences in the binding of the different factors to BUdR-containing DNA that result



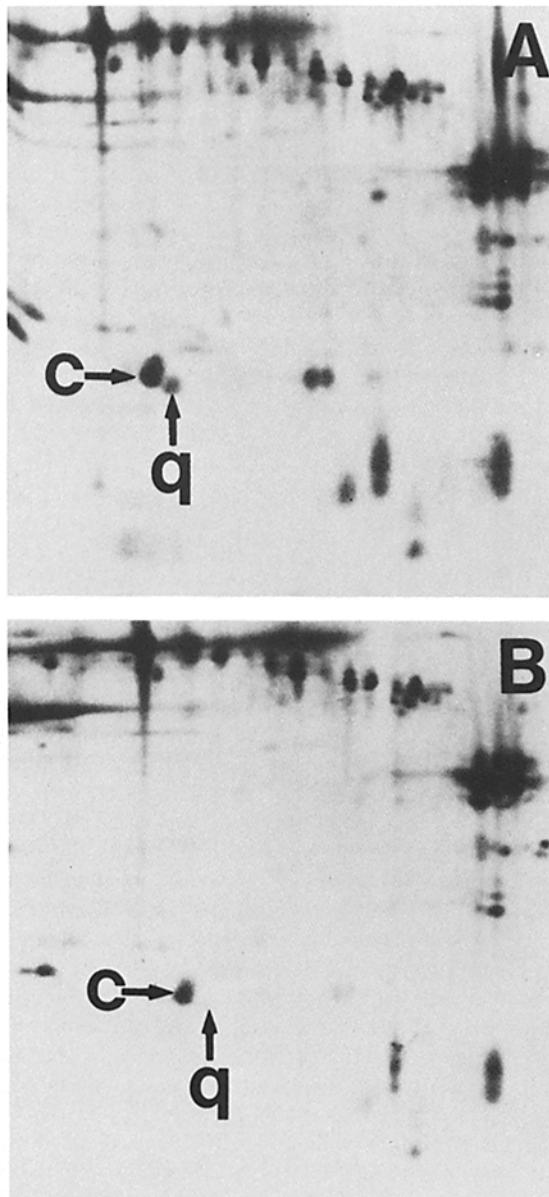


FIGURE 6 80% BUdR for thymidine substitution blocks light-chain synthesis in avian-avian crosses. Binucleated chick myocytes were fused to undifferentiated quail myoblasts that had been grown in FBT medium producing an 80% BUdR for thymidine substitution. If the BUdR-block is removed by cultivating the quail myoblasts in BUdR-free medium for 2 d before heterokaryon formation, quail myosin light-chain synthesis (*q*) is induced (*A*). However, light-chain synthesis is not present if the quail myoblasts are maintained in FBT medium before fusion (*B*). Both sets of heterokaryons were cultivated in FBT medium after heterokaryon formation.

in different dilutional effects for each factor. Although the phenomenon could be a consequence of differing amounts of suppressive factors produced by the BUdR-containing nucleus, BUdR is not acting by converting the myoblast into a fibroblast-like pattern of suppression. We have found that chick myosin light-chain synthesis, creatine phosphokinase activity, and the level of  $\alpha$ -bungarotoxin binding were all inhibited >90% in heterokaryons formed by fusing chick myocytes to rat fibroblasts (36). The small reduction in some chick muscle functions in heterokaryons produced by BUdR-blocked rat myoblasts is thus qualitatively and quantitatively different from that produced by fibroblasts.

These results are not consistent with a mechanism of action for the inhibition of myogenesis by BUdR that depends exclusively upon an alteration in plasma membranes (14–17). Chick factor(s) fail to induce rat myosin light-chain synthesis even when both chick and rat nuclei coexist within a common cytoplasm, thus bypassing the plasma membrane. However, we cannot exclude a mechanism of action that involves alterations in intracellular membranes.

The levels of BUdR substitution required to inhibit myogenesis were very different for chick and rat cells. Although Rogers et al. (36) reported that 3.2  $\mu$ M BUdR produced a 1% substitution of BUdR for thymidine in L6 rat myoblasts, a recalculation of their data (37) indicates that an actual equilibrium incorporation of ~32–40% would have been obtained in their study. This value is comparable to our result of 40% substitution in 3  $\mu$ M BUdR (Fig. 1). The 80% substitution of BUdR for thymidine that we found was required to inhibit myogenesis in chick cells is similar to the 60% level reported by O'Neill and Stockdale (10). The relative substitution we found in chick and rat cells is thus approximately the same as that found by other laboratories.

The differential sensitivity of chick and rat myoblasts to BUdR suggested the hypothesis that BUdR was inhibiting differentiation by altering the effective activity of myogenic inducing factors. This hypothesis explained the fivefold decrease in the induction of rat myosin synthesis in heterokaryons involving myoblasts blocked with 2  $\mu$ M BUdR as a result of the reduction in activity of limiting quantities of chick inducing factors below a threshold level due to the presence of a 35% BUdR for thymidine substitution in the target undifferentiated rat nucleus. All three predictions of this model have been confirmed. The reduced induction seen in heterokaryons between mononucleated chick myocytes and tetraploid rat myoblasts indicates that the putative chick inducing factors are present in limiting quantities. Increasing the relative amount of chick inducing factors by forming heterokaryons between binucleated chick myocytes and 35% BUdR-blocked rat myoblasts is successful in increasing the induction of rat myosin light-chain synthesis. Finally, increasing the level of BUdR substitution in the rat myoblast to ~70% is effective in preventing this induction even after fusion to binucleated chick myocytes. Although indirect, the verification of all three predictions provides strong suggestive evidence for the validity of the hypothesis.

It is tempting to describe the reduced activity of the putative chick inducing factors as due to a reduced affinity for BUdR-blocked DNA binding sites. However, the evidence is not sufficient for this conclusion. Studies on the effect of BUdR on the binding of known bacterial inducers and repressors (42, 43) have shown that BUdR increases the affinity of the regulatory proteins for their binding sites. The prediction from these studies has been that processes under positive regulation should be induced by BUdR while those under negative control should be inhibited (43). Under this interpretation, BUdR would inhibit myogenesis by increasing the affinity of negative elements for the regulatory sites. Our data could thus be explained either as a reduced capacity of a chick positive regulatory factor to displace the repressive elements or as a reduced capacity for the chick cytoplasm to dilute the negative elements sufficiently to cause them to dissociate from the BUdR-containing DNA.

The major evidence against BUdR inhibiting myogenesis as a result of its incorporation into DNA is the report by Rogers et al. (36). They claimed that deoxycytidine could reverse the



effects of BUdR on myogenesis without changing its incorporation into DNA. However, we have repeated their experiments, have shown that deoxycytidine does in fact reduce BUdR incorporation, and have provided an explanation why Rogers et al. obtained erroneous results (37). The available evidence thus strongly supports a DNA-mediated role for the action of BUdR. As Ashman and Davidson have indicated (44), BUdR may inhibit different cell types by a variety of different mechanisms. The conclusion from these experiments may thus apply only to the action of BUdR on myogenic cells. Our results are most consistent with a direct effect of BUdR on myoblast DNA that prevents the induction of previously unexpressed differentiated functions.

Received for publication 16 July 1982, and in revised form 22 February 1983.

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