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Constitutive expression of growth-related mRNAs in proliferating and nonproliferating lung epithelial cells in primary culture: Evidence for growth-dependent translational control

(pulmonary type 2 cells/lung development/differentiation/protooncogenes/histones)

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ABSTRACT **We describe the control of proliferation and growth-related gene expression in primary cultures of epithelial cells derived from rat lung. Type 2 epithelial cells line the gas-exchange surface of the alveoli where they produce and secrete surfactant. When isolated from adult animals, type 2 cells do not proliferate in culture, although they have a limited ability to do so *in vivo*. We show that type 2 cells isolated from neonatal rats proliferate in culture and that growth can be reversibly arrested by withdrawing serum from the medium. We studied the expression of five genes whose mRNA levels fluctuate with the state of proliferation in several cell systems: the c-*myc* and c-Ha-*ras* protooncogenes and the genes encoding actin, ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17), and histone 3.2. All five mRNAs were constitutively expressed at identical levels in proliferating and nonproliferating (serum deprived) neonatal cells and in adult cells. Thus, at the level of mRNA abundance, the expression of these five genes was uncoupled from the growth state of the cells. By contrast, synthesis of the replication-dependent histones and the activity of ornithine decarboxylase were detectable only in proliferating neonatal cells and not in serum-deprived neonatal cells or in adult cells. The results suggest that, in type 2 cells, growth factors might regulate the translation, rather than the mRNA abundance, of at least some growth-related genes and that the ability to respond to this translational control may be developmentally regulated.**

The proliferation of most differentiated, higher eukaryotic cells is controlled by positive and negative factors that ultimately regulate the expression of specific genes. In recent years, several dozen genes have been identified whose level of expression is coupled to cell proliferation (1, 2). These genes, collectively known as growth-related genes, encode a diverse spectrum of proteins. Much of our understanding about the regulation of cell proliferation and growth-related gene expression derives from studies of fibroblasts in culture. In these cells, it is well established that specific transcriptional or posttranscriptional mechanisms are activated by mitogenic signals, causing increases in growth-related mRNAs. While fewer studies have followed the translation products of these genes, mRNA abundance appears to be the prime, although not necessarily the sole, determinant of their level of expression (3–6).

The mechanisms that regulate growth-related gene expression in fibroblasts also operate in other types of proliferating cells, including hematopoietic cells and some epithelial cells (1, 7–9). However, it is not clear whether the growth control paradigms derived from fibroblasts pertain to all cells, particularly differentiated epithelial cells having a limited capacity for proliferation.

We describe the regulation of proliferation and growth-related gene expression in primary cultures of rat type 2 cells, differentiated epithelial cells isolated from the alveoli of the lung. The epithelial gas-exchange surface is lined by two cell types: type 1 and type 2 cells. Type 1 cells are terminally differentiated and do not proliferate (10). By contrast, type 2 cells retain the ability to divide and to differentiate into type 1 cells. In normal adult animals, type 2 cells undergo minimal cell turnover. However, limited type 2 cell proliferation occurs during compensatory growth after partial pneumonectomy, lung repair following injury, and neonatal lung development (10–13).

Despite their proliferative potential *in vivo*, type 2 cells isolated from the lungs of adult animals do not divide in the presence of serum in culture (14). We show here that type 2 cells isolated from neonatal rats undergo limited proliferation in culture, which can be reversibly arrested by depriving the cells of serum. However, unlike fibroblasts, five growth-related mRNAs were expressed at nearly identical levels in nonproliferating adult type 2 cells and in neonatal type 2 cells, whether or not proliferation was arrested. Despite constitutive expression at the level of mRNA abundance, the translation products of two of these mRNAs were detectable only in proliferating cells. Our results suggest that growth-related genes may be constitutively expressed at the level of mRNA abundance in primary culture of type 2 cells regardless of the growth state, and that growth factors may regulate proliferation by controlling the translation of these mRNAs. Furthermore, the ability to respond to this translational control may change during development or maturation.

MATERIALS AND METHODS

Cell Isolation and Culture. Type 2 cells were isolated from pathogen-free, Sprague–Dawley rats, as described in detail (15) according to the method of Dobbs *et al.* (16). Adult male animals were 2 months old or older; neonatal animals were 10–12 days old. For enzymatic dispersal of lung cells, we used 180 units per adult lung and 36 units per neonatal lung of intratracheal elastase. After panning the cells on IgG-coated plates, unadhered cells were collected by centrifugation and replated onto tissue culture dishes in Earle's minimal essential medium (MEM) supplemented with 4 mM glutamine, 50 units of penicillin per ml, 50 μg of streptomycin per ml, and 10% fetal bovine serum (FBS). Twenty-four hours later, >90% of the adherent cells were type 2 cells, as identified by electron microscopy (data not shown). Cells

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Abbreviations: FBS, fetal bovine serum; ODC, ornithine decarboxylase.

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were harvested with 0.025% trypsin and 0.2 mM EDTA, washed with MEM containing 10% FBS, and plated at 2–3 × 10^4 cells per cm^2. For serum deprivation, type 2 cells were plated as described above but, 4 hr after trypsin treatment and replating, they were shifted into MEM without serum.

Lung fibroblasts were isolated from 10-day-old rats as described (17). After collagenase digestion of lung minces, the cells were collected by centrifugation, resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM glutamine, penicillin G (50 units/ml), streptomycin (50 μg/ml), and 10% FBS and plated at 2–3 × 10^4 cells per cm^2. More than 90% of the cells were fibroblasts judged by positive immunofluorescence staining for vimentin and negative staining for cytokeratins (data not shown). For serum deprivation, the cells were plated identically but, 24 hr after plating, they were shifted into serum-free DMEM.

Determination of Cell Number. Cells were rinsed with phosphate-buffered saline (PBS) and harvested by trypsin-EDTA and centrifugation. The cell pellet was washed twice and resuspended in PBS, and the number of cells was determined with a hemacytometer.

Immunofluorescence. Cells were plated on 12-mm coverslips cultured for 72 hr in 10% FBS, washed with PBS, and fixed in methanol (−20°C, 20 min) and acetone (−20°C, 1 min). Cells were rehydrated in PBS and incubated with monoclonal antibody to either vimentin (1:3 in 0.1% bovine serum albumin in PBS) or cytokeratins (PKK1, 1:10 in PBS) for 1 hr at 37°C. After washing with PBS, the cells were incubated with rhodamine- or fluorescein isothiocyanate-conjugated anti-mouse IgG for 1 hr at 37°C. Coverslips were washed in PBS, dipped in distilled water, and mounted onto glass slides with glycerol/gelatin. Cells were viewed by epifluorescence and photographed.

Disaturated Phosphatidylcholine Analysis. Disaturated phosphatidylcholine was measured by labeling the cells overnight with [*methyl*-^{3}H]choline chloride and separating extracted lipids by one-dimensional thin-layer chromatography as described by Torday *et al.* (18). Disaturated phosphatidylcholine was expressed as a percent of phosphatidylcholine.

RNA Isolation and Analysis. Total cellular RNA was isolated by lysis in guanidine isothiocyanate and centrifugation through CsCl as described by Chirgwin *et al.* (19). Fifteen micrograms of RNA was fractionated on agarose-formaldehyde gels and transferred by blotting onto nitrocellulose or nylon membranes. The blots were prehybridized and hybridized to ^{32}P-labeled probe, washed, and exposed to preflashed XAR-5 film as described (20).

Probes. The viral Ha-*ras* gene [450 base pairs (bp)] (originally from E. Scolnick, Merck Sharp & Dohme) was subcloned into pGEM-1; the probe was an antisense RNA generated by T7 polymerase and [^{32}P]UTP. c-myc, actin, ornithine decarboxylase (ODC; L-ornithine carboxy-lyase, EC 4.1.1.17) and histone probes were generated by random oligonucleotide primer extension using [^{32}P]dCTP. The myc probe was a 5.5-kilobase (kb) murine genomic fragment containing exons 2 and 3 (provided by P. Leder, Harvard Medical School) or a full-length murine cDNA (provided by K. Marcu, State University of New York at Stony Brook). The actin probe was a 1.5-kb cDNA fragment of the rat β-actin gene. Histone sequences were a 207-bp cDNA fragment of a murine histone 3.2 gene (provided by W. Marzluff, University of Florida, Tallahassee). The ODC probe was a 700-bp fragment of a murine cDNA (provided by O. Janne, The Rockefeller University, New York).

ODC Enzyme Assays. Harvested cells were washed with cold PBS, centrifuged, suspended in cold lysis buffer (50 mM Tris·HCl, pH 7.2/0.1 mM EDTA/0.05 mM pyridoxal phosphate/10 mM dithiothreitol) at 6 × 10^6 cells per 0.4 ml, and disrupted by freezing and thawing. The lysates were clarified by centrifugation and assayed for ODC activity as described by Chen *et al.* (21). Triplicate 200-μl reaction mixtures containing 50 μl of lysate and 10 μl of [^{14}C]ornithine (0.05 mmol; 7–8 mCi/mmol; 1 Ci = 37 GBq) were incubated for 60 min at 37°C in microtubes containing a penicillin disc impregnated with NCS toluene tissue solubilizer. The reaction was terminated by injecting 200 μl of 10% trichloroacetic acid and incubation was continued for an additional 90 min at 37°C. Radioactivity absorbed to the discs was measured by liquid scintillation counting. Little or no radioactivity was released from boiled cell lysates.

Histone Protein Synthesis. Cells were washed and preincubated in lysine-free MEM for 20 min at 37°C and labeled for 1 hr at 37°C with [^{3}H]lysine (100 μCi/ml; 100 Ci/mmol) in fresh lysine-free MEM with or without dialyzed FBS. Labeled cells were washed with cold PBS and lysed in 10 mM Tris, pH 7.6/3 mM $MgCl_2$/0.5% Nonidet P-40/1 mM phenylmethylsulfonyl fluoride, and the nuclei were recovered by centrifugation. Detergent-soluble (cytoplasmic) proteins were precipitated from the supernatant with 10% trichloroacetic acid and the precipitates were washed with cold acetone. Acid-soluble proteins were extracted from the nuclei by two 30-min incubations with 0.25 ml of 0.5 M HCl/1% 2-mercaptoethanol/1 mM phenylmethylsulfonyl fluoride and were concentrated by lyophilization (22). Proteins were solubilized in 2× Laemmli sample buffer (23) and were analyzed on 18% SDS/polyacrylamide gels (24). Labeled proteins were visualized by fluorography.

Materials. Rats were obtained from Charles River Breeding Laboratories. Elastase, DNase, and fluorochrome-conjugated antibodies were from Cooper Biomedical. Serum and medium were from GIBCO and nylon filters were from Tetko (Elmsford, NY). Rat IgG and glycerol/gelatin were from Sigma. Radioisotopes were from ICN or New England Nuclear and enzymes used to generate probes were from New England Biolabs. Photographic emulsion, film, and developer were from Kodak. Anti-vimentin antibody was from Boehringer Mannheim and anti-cytokeratin antibody was from Labsystems (Chicago). NCS tissue solubilizer was from Amersham.

RESULTS

Proliferative Characteristics of Adult and Neonatal Type 2 Cells. Type 2 cells isolated from adult rat lungs and cultured in medium containing 10% serum failed to increase in cell number, but type 2 cells from neonatal lungs proliferated under these conditions (Fig. 1). Autoradiography of [^{3}H]thymidine-labeled cells showed that 30–40% of neonatal cells were labeled after a 24-hr pulse (data not shown). When neonatal cells were placed in serum-free medium for 48–72 hr, cell proliferation ceased; when provided with fresh medium containing 10% serum, they resumed proliferation and the cell number doubled within 48 hr (Fig. 1).

After 24 hr in culture, >90% of the neonatal cells were type 2 cells, as judged by the presence of lamellar bodies, surfactant-containing organelles, detectable by electron microscopy (data not shown). After 72 hr, 80–90% of the neonatal and adult cultures consisted of epithelial cells, as judged by the presence of cytokeratins and absence of vimentin (Fig. 2). Phospholipid analysis showed that neonatal cells synthesized similar levels of disaturated phosphatidylcholine, the major surfactant lipid (25), after 24 and 72 hr in culture (49.67% ± 3.18% at 24 hr vs. 43.64% ± 4.49% at 72 hr). These data indicate that the fraction of contaminating fibroblasts remains small throughout the 72-hr culture period in both adult and neonatal cell preparations. We conclude that it is the type 2 epithelial cells that are primarily responsible for the increase in cell number observed in the neonatal cultures.

Expression of Growth-Related mRNAs. We measured the abundance of five mRNAs in proliferating and nonprolifer-

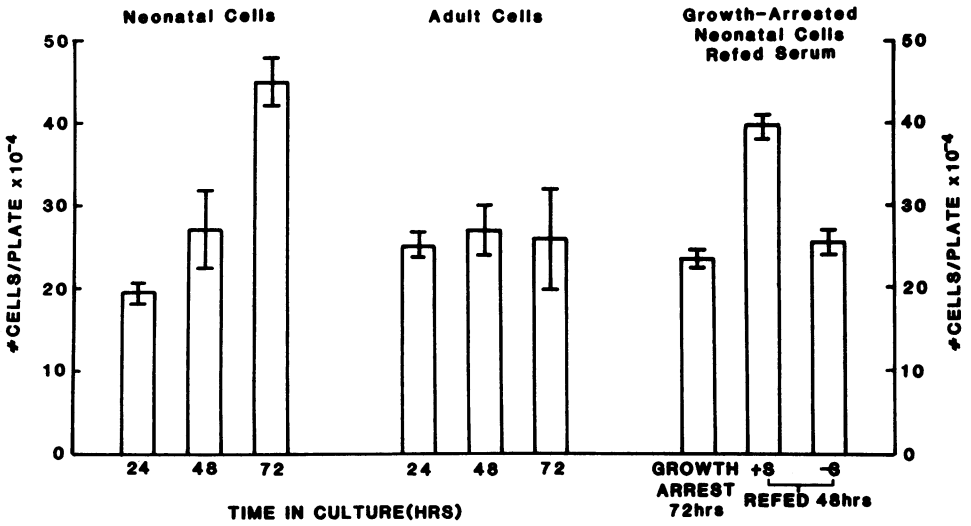

FIG. 1. Neonatal type 2 cells proliferate in culture. Type 2 cells were isolated from neonatal or adult rat lung and plated in 10% serum, and cell numbers were determined at the indicated intervals. In refeeding studies, 4 hr after plating, neonatal type 2 cells were deprived of serum for a period of 72 hr. Cells were then refed either with medium containing 10% serum or with medium containing no serum.

ating type 2 cells. In fibroblasts, four of these increase during progression from G_0 through G_1: the cytoskeletal protein actin and the protooncogene c-*myc*, which are induced early (within 1–2 hr) in this transition, and the c-Ha-*ras* protooncogene and the polyamine biosynthetic enzyme ODC, which are induced somewhat later (2–4 hr); the mRNA for histone 3.2 is expressed only during S phase (1, 2).

Growing neonatal cells fully expressed the mRNAs for all five genes (Figs. 3*A* and 4). However, the prevalence of these mRNAs was nearly identical in growing neonatal cells and in nonproliferating adult cells (Figs. 3*B* and 4). When either neonatal or adult cells were deprived of serum, the level of all five mRNAs remained constant (Figs. 3 and 4) despite the arrest of cell division in the neonatal cells. When serum-deprived neonatal cells were given fresh serum-containing medium, they resumed cell proliferation (Fig. 1); however, the abundance of actin, c-myc, c-Ha-*ras*, and histone 3.2 mRNA remained unchanged after serum stimulation (Fig. 3*A*).

Proliferating type 2 cells and proliferating fibroblasts expressed similar levels of ODC and histone mRNAs (Fig. 4). Therefore, the small contaminating population of fibroblasts in type 2 cell cultures was responsible for only a small proportion of the mRNA detected in the cultures. When type 2 cells were deprived of serum, ODC and histone mRNAs were unchanged. In contrast, ODC mRNA declined to a modest extent and histone 3.2 mRNA fell to undetectable levels in serum-deprived fibroblasts.

ODC Activity and Histone Protein Synthesis. To test the possibility that the expression of growth-related genes in type 2 cells might be controlled at the level of protein, as opposed to mRNA abundance, we measured the translation products of the ODC and histone mRNAs in proliferating and nonproliferating type 2 cells and lung fibroblasts.

ODC enzyme activity fluctuated with the growth state in both type 2 cells and lung fibroblasts (Table 1). After serum deprivation, ODC activity declined to nearly undetectable

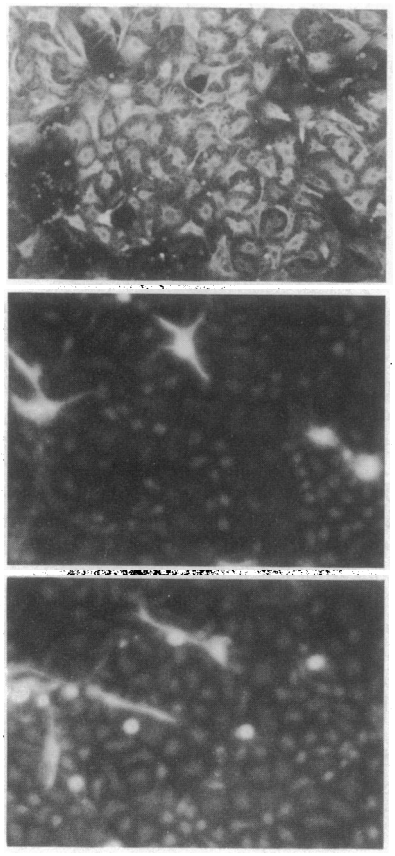

FIG. 2. Intermediate filament staining of type 2 cell cultures. Type 2 cells isolated from adult or neonatal animals were plated onto glass coverslips and cultured for 72 hr in 10% serum. The cells were then fixed and stained for cytokeratin or vimentin. (*Top*) Neonatal type 2 cells stained for cytokeratin. (*Middle*) Neonatal type 2 cells stained for vimentin. (*Bottom*) Adult type 2 cells stained for vimentin.

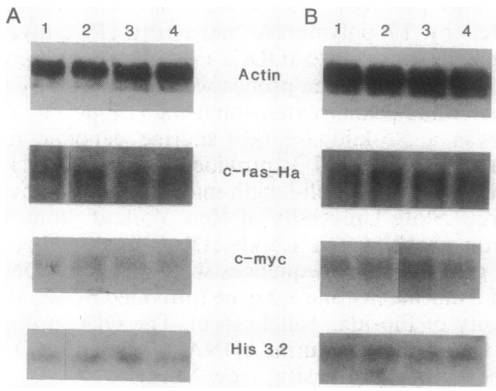

FIG. 3. Abundance of actin, c-Ha-*ras*, c-myc, and histone 3.2 mRNA in neonatal (*A*) and adult (*B*) type 2 cells. Lanes: 1, Cells cultured for 48 hr in 10% serum; 2, cells cultured for 48 hr in serum-free medium; 3, serum-deprived cells stimulated for 2 hr with 10% serum; 4, serum-deprived cells stimulated for 24 hr with 10% serum.

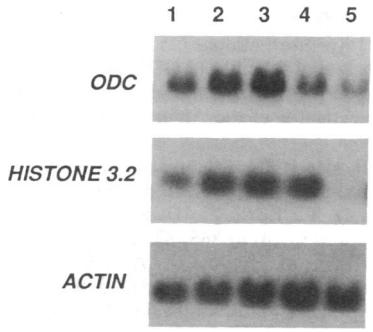

FIG. 4. Abundance of actin, c-Ha-*ras*, ODC, and histone mRNA in adult and neonatal type 2 cells and neonatal lung fibroblasts. Lanes: 1, adult type 2 cells cultured for 48 hr in 10% serum; 2, neonatal type 2 cells cultured for 48 hr in 10% serum; 3, neonatal type 2 cells cultured for 72 hr in serum-free medium; 4, lung fibroblasts cultured for 48 hr in 10% serum; 5, lung fibroblasts cultured for 72 hr in serum-free medium.

levels in both cell types (Table 1). These changes were associated with a modest decline in ODC mRNA in lung fibroblasts but unchanged levels of ODC mRNA in type 2 cells. Moreover, adult type 2 cells in 10% serum expressed a high level of ODC mRNA (Fig. 4), yet enzyme activity was nearly undetectable (Table 1).

Proliferating neonatal type 2 cells incorporated substantial radiolabel into histones H3, H2A, H2B, and H4. By contrast, the labeling of these histones was barely detectable in adult type 2 cells, yet other nuclear proteins were synthesized at comparable rates by adult and neonatal cells (Fig. 5). The labeling of histones in neonatal cells was markedly reduced when proliferation was arrested by serum deprivation (Fig. 5) despite continued expression of histone 3.2 mRNA. In contrast, histone protein synthesis correlated with the expression of histone 3.2 mRNA in lung fibroblasts.

Table 1. ODC activity in proliferating and nonproliferating type 2 cells and lung fibroblasts

Cell type	% serum	ODC activity
Neonatal type 2	10	639.5 + 98.6
Neonatal type 2	0	9.3 + 4.9
Adult type 2	10	31.2 + 8.7
Fibroblast	10	401.5 + 63.8
Fibroblast	0	12.1 + 5.0

ODC activity is expressed as pmol of $^{14}CO_2$ liberated per 10^6 cells per hr. Values represent mean + SEM for three separate experiments.

DISCUSSION

We describe a system of primary lung epithelial cells in which proliferation in culture is developmentally regulated and can be controlled by serum growth factors. We show that the mRNAs for five growth-related genes are constitutively present in these cells, regardless of their proliferation status, and that the expression of at least two of these genes appears to be regulated at the level of translation.

Standard culture conditions support several rounds of cell division of type 2 cells isolated from neonatal animals in contrast to cultures of adult type 2 cells. *In vivo*, the neonatal lung is actively growing in contrast to the adult lung. This proliferative difference *in vivo* may explain why a portion of neonatal type 2 cells retain their ability to undergo at least limited cell division in primary culture.

From studies of fibroblasts, it is generally accepted that serum-dependent growth is coupled to a time-dependent increase in certain mRNAs that correlates with growth state or position in the cell cycle (1, 2). Our results clearly indicate that for type 2 cells in culture the mRNA levels for five such genes do not correlate with growth state. These results suggest several possibilities. First, the abundance of these mRNAs may be coupled to growth state and nondividing type 2 cells may be blocked in S phase. Second, the levels of these

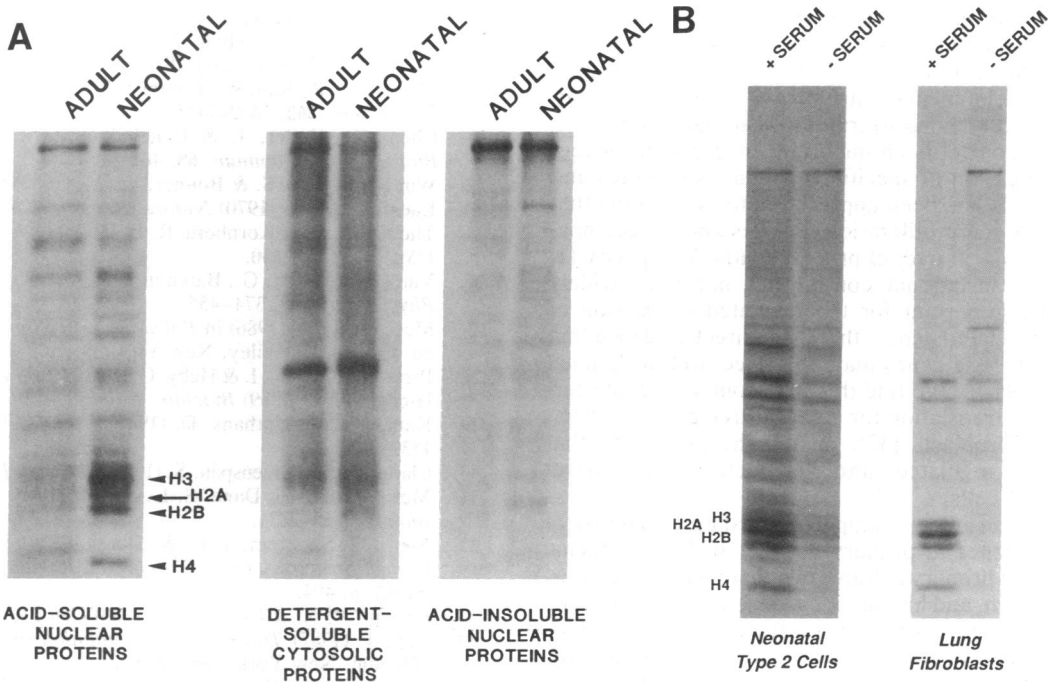

FIG. 5. Histone protein synthesis is coupled to the growth state of type 2 cells and lung fibroblasts in culture. (*A*) Adult and neonatal type 2 cells and neonatal lung fibroblasts labeled after 48 hr in culture with [^{3}H]lysine for 1 hr; 1.5×10^5 cpm of acid-soluble and acid-insoluble nuclear proteins and 4.5×10^5 detergent-soluble cytosolic proteins were loaded onto the gel. (*B*) Neonatal type 2 cells and lung fibroblasts labeled and processed as described above after 48 hr in 10% serum (+ SERUM) or after 72 hr in serum-free (− SERUM) medium; 2×10^5 cpm of acid-soluble nuclear proteins were loaded onto the gel.

mRNAs may be uncoupled from the growth state of type 2 cells, and other factors may regulate type 2 cell proliferation. Finally, growth-related gene expression may be coupled to growth state in type 2 cells, but not at the level of mRNA abundance. Our results on the synthesis of histone proteins and the activity of the ODC enzyme, while not eliminating the first two, support this last possibility.

It is well-established that ODC activity is growth-state dependent in a number of cell systems (26). Although enzyme activity is only an indirect measure of translation, our results clearly show that despite high levels of ODC mRNA, enzyme activity is undetectable in nonproliferating type 2 cells. In quiescent lung fibroblasts, there was also a discrepancy between ODC mRNA and enzyme activity levels; the quiescent cells expressed reduced but nonetheless detectable levels of mRNA, whereas enzyme activity was undetectable. This has been observed in other fibroblast cultures (26). ODC activity has been shown to be regulated primarily at the level of transcription, although translational and posttranslational control has been reported (27–31).

It is also well established that histone protein synthesis begins at the onset of DNA synthesis (32). Exceptions have been noted; in S49 and CHO cells, histones are synthesized at equivalent rates throughout the cell cycle, but the newly synthesized histones remain in the cytoplasm until S phase (33). This is not the case in type 2 cells, since we could not detect newly synthesized histones in either the nuclear or cytoplasmic fractions of nondividing cells. In quiescent lung fibroblasts, histone 3.2 mRNA was expressed at a low level and histone protein was not synthesized. In contrast, the lack of histone synthesis cannot be attributed to a decrease in mRNA in nonproliferating type 2 cells. We conclude that histone 3.2 and possibly the other replication-dependent histone mRNAs are not translated when type 2 cells cease proliferation.

We have previously found that actin and many other proteins are synthesized at the same rate in adult and neonatal type 2 cells (15). This suggests that translational control may be confined to a subset of growth-related genes. Moreover, in neonatal cells, serum factors regulate the translation of the histone and ODC mRNAs. This finding suggests that developmental stage or age of the animal may alter the ability to respond to translational regulation.

Translational control is an important mechanism for regulating the expression of specific genes in prokaryotes and eukaryotes (34). It has been appreciated for some time that the activation of cell proliferation requires and is accompanied by an increase in overall protein synthetic capacity (1, 35). Although translational control has not been widely observed to be important for the regulated expression of specific growth-related genes, there is indirect evidence that at least some of these genes may be subject to translational control in fibroblasts. We find that antiproliferative interferons suppress the translation for at least three growth-related genes in 3T3 fibroblasts (37), raising the possibility that developmentally regulated autocrine factors may control growth in type 2 cells.

Our data provide a new example of translational control of growth-related genes in primary cultures of lung epithelial cells. Although in primary cultures of hepatocytes the expression on c-*fos*, actin, and histone 3.2 is coupled to growth state (9), c-*myc* mRNA levels have been found to be uncoupled from growth state in keratinocytes (36). These findings suggest that epithelial cells from different organs may regulate expression of growth-related genes by different mechanisms.

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