DIFFERENTIAL EFFECT OF HORMONES ON MACROMOLECULAR SYNTHESIS AND MITOSIS IN CHICK EMBRYO CELLS

JOEL B. BASEMAN and NANCY S. HAYES. From the Department of Bacteriology and Immunology, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27514

The progression of animal cells through the different phases of their cell cycle appears to be dependent upon external regulatory signals as well as the synthesis of specific macromolecules in a coordinate manner (5, 9, 11, 16, 17). Although an extensive literature exists which describes the role of hormones and other factors as governors of macromolecular synthesis and division in eucaryotic cells (1, 4, 6–8, 12, 14, 19, 20) evidence essential to defining the sequence of steps that enables cells to coordinate their metabolism, initiate DNA synthesis, and successfully complete
mitosis is lacking. This information is essential before the role of specific growth factors in different cell types can be defined.

In this study, we report the differential stimulation of macromolecular synthesis and mitosis in chick embryo fibroblasts and indicate that heterogeneous populations of cells exist in the monolayer as determined by their metabolic responsiveness to selected hormones.

MATERIALS AND METHODS

Primary Cell Cultures

Primary cultures of chick embryo cells, prepared from 11-day old embryos, were plated at a cell density of $4 \times 10^5$ cells/cm$^2$ in 1- and 8-ounce bottles and allowed to reach confluence in Eagle's minimum essential medium (MEM) supplemented with 3% calf serum (2, 22). Monolayers were then washed free of serum with MEM and incubated in serum-free MEM for 24 h before the experiment. Serum deprivation reduced the metabolic activity of cells to a basal level, aided in the general synchronization of the cell population, and resulted in a state under which addition of fresh serum produced maximal stimulation. Furthermore, the resultant cell population was virtually all fibroblast as determined by characteristic morphology and the presence of collagen fibers (23).

Incorporation of Radioactive Precursor into Macromolecules

After radiolabeling cells in bottles with uridine, thymidine, or mixtures of amino acids, cell monolayers were washed with cold saline, protein concentrations were determined by the Lowry method (13), and TCA-insoluble material was collected on filters (Millipore Corp., Bedford, Mass.) for counting (scintillation spectrometer with Omnifluor-toluene fluid). In addition, radiolabeled RNA was isolated from intact cells by the cold phenol method (2), and 10 µg of RNA was analyzed by gel electrophoresis according to the method of Peacock and Dingman (15). Gels were then sliced into 1-mm sections that were hydrolyzed in scintillation vials overnight with 0.5 ml of 3 N NH$_4$OH before the addition of scintillation fluid (Omnifluor-Triton-toluene).

Mitotic Indices and Nuclear Autoradiography

For determining mitotic indices and percent radiolabeled nuclei, chick fibroblasts ($5 \times 10^5$ cells/cm$^2$) were added in a volume of 1 ml to Leighton tubes containing single cover slips. After confluent monolayers were incubated in MEM lacking serum for 24 h, test substances were added for a total of 24 h. [H]thymidine (0.3 µCi) was introduced during the last 18 h. Duplicate cover slips were then washed gently in phosphate-buffered saline and fixed in Carnoy's fluid for 30 min. After air-drying, the cover slips were mounted on clean glass slides, and half of the cover slip was dipped in melted Kodak NTB-2 emulsion (Eastman Kodak Co., Rochester, N. Y.). Slides were air-dried, placed in a black box containing Drierite until they were developed 6 days later in Kodak D-19, and then stained for 45 min in a modified Giemsa's solution. The numbers of radiolabeled nuclei were determined by examining with the light microscope 2,000 cells previously exposed to emulsion. Mitotic figures were recorded by examining 2,000 cells contained on the nonemulsion portion of the cover slip. Identical procedures were followed for cell monolayers receiving fresh serum 8 h after the initial exposure of cells to the test substances.

Chemicals

[5-H]Uridine and [methyl-3H]thymidine were purchased from Nuclear Dynamics Inc. (El Monte, Calif.), [H]amino acid mixtures from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y., and [methyl-3H]aminoisobutyric acid from ICN Pharmaceuticals, Inc., Cleveland, Ohio. Bovine insulin and hydrocortisone were obtained from Sigma Chemical Co., St. Louis, Mo., and calf serum from Grand Island Biological Co., Grand Island, N. Y.

RESULTS

Stimulation of DNA Synthesis by Hormones

Earlier studies indicated that insulin (0.4 µg/ml) stimulated RNA synthesis in serum-starved confluent monolayers of primary chick embryo cells to at least 70% of the level obtained with 3% calf serum, a concentration sufficient for optimal growth of the cells (2). Under these conditions, RNA species as measured by acrylamide gel electrophoresis appeared similar after exposure of cells to either agent. In addition, autoradiographic data obtained using [H]uridine indicated that the majority of cells were heavily radiolabeled after exposure to insulin (0.4 µg/ml) when compared to untreated controls (unpublished data). Nevertheless, insulin only slightly stimulated DNA synthesis in the cell population and had no significant effect on the mitotic index, in contrast to serum.

Therefore, attempts were made in this study to develop a means by which insulin's incomplete stimulation of DNA synthesis could be supplemented by other regulatory factors. Previous reports implicated hydrocortisone as such a potential synergistic signal (3, 7). Hydrocortisone at a
concentration of 0.5 μg/ml plus insulin (0.4 μg/ml) significantly increased the rate of incorporation of [3H]thymidine into TCA-precipitable material (Fig. 1A), although the maximal effect was still considerably less than that obtained with 3% calf serum. Hydrocortisone-treated cells incorporated thymidine at a low and constant rate not significantly different from that of untreated control cultures.

Since the maximal stimulation of DNA synthesis by insulin or insulin plus hydrocortisone ranged from 10 to 25% of that observed with 3% calf serum and since the effect of somatomedin C on DNA synthesis in primary chick monolayers was

50% of that of serum (21), it seemed possible that heterogeneous cells existed within the fibroblast population that were uniquely responsive to specific hormones. Therefore, nuclear autoradiography of [3H]thymidine-labeled cells was performed and correlated with determinations of mitosis (Table 1, data presented under "— serum added at 8 h" [see footnote to Table]). Only 8% of the fibroblasts actively engaged in DNA synthesis after insulin stimulation, while 14% and 27% of cells synthesized DNA after exposure to insulin plus hydrocortisone and somatomedin, respectively. 75% of the nuclei were radiolabeled after the addition of 3% calf serum to serum-starved mono-

**Figure 1** Kinetics of macromolecular synthesis in chick cells exposed to various regulatory signals. Chick embryo cells (4 × 10⁶ cells/cm² in MEM with 3% calf serum) were added in a volume of 3 ml to 1-ounce glass bottles. Confluent monolayers were established and serum-starved for 24 h as described in Materials and Methods. After exposure of cells to insulin (0.4 μg/ml), hydrocortisone (0.5 μg/ml), insulin (0.4 μg/ml) plus hydrocortisone (0.5 μg/ml), serum (3%), or MEM alone, monolayers were radiolabeled with [3H]thymidine, [3H]uridine, or [3H]amino acids (0.6 μCi/ml of medium) for 2 h before harvesting. The reported values are the average of duplicate samples from three separate experiments. Except in the case of panel A, the differences between means at each point for insulin or serum determinations varied by less than 10% of the highest value. (A) thymidine to DNA; (B) uridine to RNA; (C) amino acids to protein. (A) A, serum; O, insulin + hydrocortisone; ■, insulin; O, hydrocortisone; □, control. (B and C) □, serum or insulin; O, insulin + hydrocortisone; O, hydrocortisone; □, control.
TABLE 1

Influence of Various Regulatory Factors on DNA Synthesis and Mitosis in Confluent Monolayers of Serum-Starved Chick Cells

| Factor                    | Concentration | Radiolabeled nuclei* 1 | Mitotic figures* 1 |
|---------------------------|---------------|------------------------|-------------------|
|                           | μg/ml         | %                      | per 2,000 cells    |
| MEM                       | —             | 3                      | +                 |
| Hydrocortisone            | 0.5           | 2                      | 38                |
| Insulin                   | 0.4           | 8                      | 45                |
| Insulin and hydrocortisone| 0.4 + 0.5     | 14                     | 62                |
| Somatomedin C (750–1,200 units/mg) | 1         | 27                     | ND                |
| Calf serum                | 3%            | 75                     | 79                |

Test substances were added to quiescent cultures of chick embryo fibroblasts for a total of 24 h. During the last 18 h of incubation, cells were radiolabeled with [3H]thymidine (0.3 μCi) and then processed as described in Materials and Methods.

* Each value is the average of determinations on duplicate cultures from four separate experiments representing composite data from 8,000 cells. In no case did duplicate counts differ by greater than 15% of the highest value.

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protein, and DNA in monolayers of chick cells. However, the simultaneous addition of hydrocortisone and insulin stimulated DNA synthesis considerably above levels reached in cultures exposed to insulin alone, although mitosis was still not apparent. Under these same experimental conditions, the rates of synthesis of RNA and protein were decreased in cells incubated with insulin plus hydrocortisone when compared to insulin-treated monolayers. Therefore the combined effects of insulin and hydrocortisone appeared both synergistic and antagonistic as determined by the specific macromolecular events. It was significant that the addition of serum to insulin plus hydrocortisone-treated cultures at a critical time in the cell cycle allowed certain cells to enter mitosis. Together, insulin and hydrocortisone apparently furnished usable information for initiating mitosis but required regulatory assistance from serum, the complete signal. Although there are quantitative limitations to monitoring similar effects of serum addition on insulin-stimulated cells (only 8% of the cell population was stimulated by insulin to engage in DNA synthesis, Table I), no mitotic figures were observed. These results further indicate that the physiologic role of insulin may not be mitogenic, as we and others have previously suggested (2, 10, 18).

Somatomedin appears to be a growth-promoting hormone for chick cells since its presence at relatively low concentrations allows the progression of certain cells through the growth cycle (Table I, [21]). In unpublished studies performed in collaboration with J. J. Van Wyk and L. E. Underwood, we have demonstrated separate binding sites for somatomedin and insulin in cultures of primary chick cells and have shown that the somatomedin binding site is highly insensitive to competitive binding by insulin. These observations reinforce the critical nature of unique receptor sites in regulating cell growth. Gospodarowicz has proposed the interesting concept that hydrocortisone may regulate receptor sites for fibroblast growth factor in 3T3 cells, increasing the number of available sites and thus the level of stimulation by specific concentrations of the factor (7). Although this possibility may also exist in primary chick cell monolayers, the fact that the syntheses...
of RNA, protein, and DNA are differentially affected in the cell population by the simultaneous addition of insulin and hydrocortisone suggests a more complex relationship. Whatever the explanation, nuclear autoradiography and mitotic indices indicate that heterogeneous populations of cells exist in monolayers of chick embryo fibroblasts and that these cells differ in sensitivity to selected hormones.

**SUMMARY**

Exposure of serum-deprived confluent monolayers of chick embryo cells to fresh serum results in maximal stimulation of synthesis of RNA and protein followed by increased DNA synthesis and mitosis. The addition of insulin to quiescent cultures effects a similar acceleration of synthesis of RNA and protein, but little stimulation of DNA synthesis and mitosis is evident. However, the simultaneous addition of insulin and hydrocortisone to resting cells causes a significant increase in the rate of DNA synthesis although the level reached is considerably lower than that obtained with serum and still no mitosis occurs. Unexpectedly, insulin plus hydrocortisone prevents maximal synthesis of RNA and protein, in contrast to insulin-treated cultures. Nuclear autoradiography and percent mitosis of cells incubated with various regulatory factors indicate that cell heterogeneity exists and is reflected in the metabolic responses of subpopulations to specific regulatory signals.

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