

## Stimulation of Rat Placental Cell DNA Synthesis by Transferrin<sup>1</sup>

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### ABSTRACT

*The purpose of the present investigation was to evaluate the in vitro requirements for rat placental cell DNA synthesis. A cell line established from the labyrinth region of midgestation rat chorioallantoic placenta was used to examine the actions of various agents. Transferrin was found to stimulate rat placental cell DNA synthesis and cell proliferation. The effects of transferrin on rat placental cell growth paralleled those observed with fetal bovine serum. Rat placental cells were responsive to both rat and human transferrin. Iron-saturated (holo-) transferrin was a more potent stimulator of rat placental cell DNA synthesis than was iron-free (apo-) transferrin. Addition of insulin, epidermal growth factor, or insulin-like growth factor-II to serum-free medium supplemented with rat transferrin did not significantly enhance rat placental cell DNA synthesis beyond that observed with only transferrin. The results demonstrate that a population of cells exists within the rat chorioallantoic placenta that are highly responsive to transferrin.*

### INTRODUCTION

The regulation of placental morphogenesis is poorly understood. Morphogenesis in the rat chorioallantoic placenta involves extensive cellular proliferation, differentiation (including the formation of glycogen cells, syncytial cells, and giant cells), and the organization of two structurally and functionally distinct regions, the labyrinth and junctional zones (Davies and Glasser, 1968; Soares, 1987; Soares and Glasser, 1987). A cell line has recently been established from the normal rat chorioallantoic placenta that appears to provide a workable in vitro model system for identifying factors controlling placental cell growth and differentiation (Soares et al., 1987). The placental cell line consists exclusively of epitheloid type cells as determined by ultrastructural analysis (Hunt et al., 1988) and cytokeratin expression (Hunt and Soares, 1988). These cells also express alkaline phosphatase (Soares et al., 1987; Hunt and Soares, 1988) and transferrin receptors (Hunt and Soares, 1988), display low levels of class I (RT1-A) histocom-

patibility antigens (Hunt and Soares, 1988), and have the potential to differentiate into trophoblast giant cells (Soares et al., 1987), all characteristics consistent with their placental origin.

The purpose of the present study was to use the placental cell line to examine the in vitro requirements for rat placental cell DNA synthesis.

### MATERIALS AND METHODS

#### Cells

The cell line used in this study was derived from chorioallantoic placentas of the Holtzman rat (Soares et al., 1987) and is designated HRP. The cells were routinely maintained in RPMI-1640 culture medium (Hazelton/KC, Lenexa, KS) supplemented with 5% heat-inactivated fetal bovine serum (FBS, Hazelton/KC), 50  $\mu$ M  $\beta$ -mercaptoethanol (BIORAD, Richmond, CA), 1 mM sodium pyruvate (Sigma Chemical Company, St. Louis, MO), 100 units/ml of penicillin, and 100  $\mu$ g/ml of streptomycin (Hazelton/KC) (complete medium). Experiments determining the effects of transferrin and other test agents on cell growth were conducted in the above medium in the absence of FBS (serum-free medium). The Balb/c mouse embryo fibroblasts (ATCC CCL 163 Balb/3T3 clone A31) used in some experiments were obtained from the American Type Culture Collection (Rockville, MD).

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### *Cell Proliferation Assay*

HRP cells ( $1 \times 10^6$ ) were plated in 60-mm-diameter culture dishes in complete medium. The cells were allowed to attach to the dishes overnight. Complete medium was replaced with serum-free medium, the same medium supplemented with 5  $\mu\text{g/ml}$  of rat transferrin (Pel-Freez, Rogers, AR), or 5% FBS. The rat transferrin used in these experiments was homogeneous by immunoelectrophoresis (Pel-Freez) and by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (5  $\mu\text{g}$  of transferrin separated in 7.5% SDS-polyacrylamide gels and stained with Coomassie Brilliant Blue). Medium was replaced with fresh medium of the same composition after 2 days of culture. The experiments were terminated after 4 days. Cells were detached from culture dishes by exposure to 0.25% trypsin-0.02% ethylenediamine tetraacetate and were disaggregated by repeated passage through a 23-gauge needle. The cells were counted with the aid of a hemacytometer and a light microscope.

### *DNA Synthesis Assays*

HRP cells ( $2 \times 10^5$ ) were plated in 16-mm-diameter wells in complete medium. The cells were allowed to attach to the dishes overnight, and the medium was replaced with serum-free medium the following day. On the third day of the assay, the medium was replaced with fresh, serum-free medium containing various concentrations of rat transferrin or FBS. After 20 h of incubation, 1  $\mu\text{Ci}$  of  $^3\text{H}$ -thymidine was added to the cultures. After a 4-h incorporation period, medium was removed. The cells were washed twice with phosphate-buffered saline (PBS, 10 mM sodium phosphate, 150 mM sodium chloride pH 7.2), twice with 10% trichloroacetic acid (TCA), and once with ethanol:ether (3:1). The cellular residues were solubilized in 1 ml of 0.2 N sodium hydroxide for 15 min, then 0.75 ml of the solubilized residue was transferred to a scintillation vial together with 100  $\mu\text{l}$  of glacial acetic acid and 5 ml of counting cocktail (Scinti-Verse, Fisher Scientific, St. Louis, MO). The radioactivity present in each sample was then determined with a Packard liquid scintillation counter.

Changes in rat placental cell DNA synthesis following the addition of bovine insulin (Sigma), mouse epidermal growth factor (Sigma), or rat insulin-like growth factor-II (Sigma) to serum-free medium containing rat transferrin (5  $\mu\text{g/ml}$ ) was evaluated with the protocol described above.

The experimental procedure used for the time-course experiments was identical to that presented above, except for the duration of transferrin treatment (see Results section for further information). The protocol used to examine the effects of rat transferrin on mouse embryo fibroblast DNA synthesis was also identical to that presented above for the HRP cells, as were protocols for experiments using human transferrin (Sigma), iron-free (apo-) human transferrin and iron-saturated (holo-) human transferrin (Boehringer-Mannheim Biochemicals Company, Indianapolis, IN).

HRP cells ( $1 \times 10^5$ ) were also plated in Lab-Tek chambers (Miles Laboratories, Naperville, IL). After overnight attachment in complete medium, the medium was replaced with serum-free medium. After a 24-hour incubation, the medium was replaced with fresh serum-free medium, or fresh serum-free medium containing either 5  $\mu\text{g/ml}$  of rat transferrin or 5% FBS. The cells were incubated for 36 h; the incubation was followed by the addition of  $^3\text{H}$ -thymidine at a concentration of 1  $\mu\text{Ci/ml}$  and a subsequent 4-h incubation. The slides were removed from the Lab-Tek chambers, washed with PBS, and dipped in photographic emulsion (Kodak, Rochester, NY). The slides were developed after 3–5 days exposure, and counterstained with Toluidine blue. Coverslips were mounted on the slides, which were then analyzed by light microscopy.

### *Statistical Analysis*

The data were analyzed by analyses of variance. The source of variation from significant F-ratios was determined with Dunn's multiple-comparison test (Keppel, 1973).

## RESULTS

Fetal bovine serum (FBS) was found to be a very potent stimulator of DNA synthesis by HRP cells (Fig. 1). As much as tenfold increases in the incorporation of  $^3\text{H}$ -thymidine were achieved with concentrations of 5–10% of FBS. The minimally effective concentration of FBS ranged from 0.5 to 1.0%.

The addition of purified rat transferrin to serum-free culture medium was nearly as effective as FBS in stimulating DNA synthesis by HRP cells (Fig. 2). Concentrations of 5–10  $\mu\text{g/ml}$  yielded approximately eightfold increases in the incorporation of  $^3\text{H}$ -thymidine into DNA by HRP cells. The minimally effective

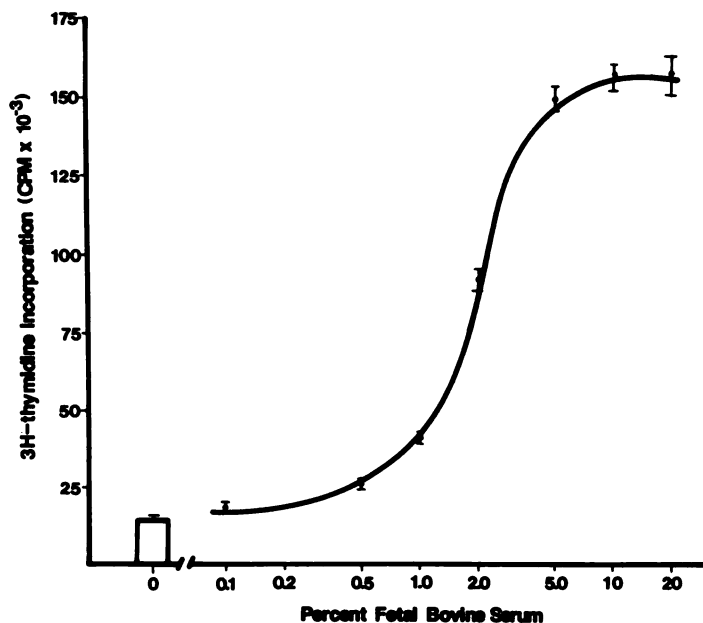


FIG. 1. Effects of fetal bovine serum (FBS) on the incorporation of  $^3\text{H}$ -thymidine into DNA by Holtzman rat placental cells (HRP cells). HRP cells were plated in culture medium containing 5% FBS, transferred to serum-free medium after 24 h, and into the respective treatment after an additional 24 h. After 20 h exposure to the treatments,  $1\mu\text{Ci}$  of  $^3\text{H}$ -thymidine was added. The cells were harvested 4 h later, and the amount of  $^3\text{H}$ -thymidine incorporated into DNA was determined by liquid scintillation counting. Each point represents the mean of five to six replicates, and the vertical bars represent the standard error of the mean. The histogram on the figure represents the serum-free control treatment (0). Values for FBS concentrations greater than or equal to 1.0% were significantly different from the serum-free control,  $p < 0.01$ .

concentration of rat transferrin was approximately  $0.25\ \mu\text{g/ml}$ . Human transferrin was found to stimulate DNA synthesis by HRP cells as effectively as rat transferrin (Table 1). Human holotransferrin was found to be more potent than human apotransferrin (Table 2), suggesting a role for iron transport in the induction of DNA synthesis by transferrin.

Rat transferrin and FBS also stimulated increases in HRP cell numbers after four days of exposure (Table 3), and autoradiographic analysis of  $^3\text{H}$ -thymidine incorporation by HRP cells indicated that both treatments were mitogenic for the rat placental cells (Fig. 3). HRP cells cultured in serum-free conditions showed minimal deposition of silver grains (Fig. 3A), whereas HRP cells exposed to either rat transferrin (Fig. 3B) or 5% FBS (Fig. 3C) showed abundant labeling.

The addition of insulin (0.01, 0.1, 1, or  $10\ \mu\text{g/ml}$ ), epidermal growth factor (0.1, 1, 10, or  $100\ \text{ng/ml}$ ) or insulin-like growth factor-II (0.1, 1, 10, or  $100\ \text{ng/ml}$ ) did not significantly affect the magnitude of trans-

ferrin's effect on placental cell DNA synthesis (data not shown).

The results from experiments to determine the kinetics of transferrin effects on HRP cell DNA synthesis are depicted in Figure 4. An interval of greater than 24 h in serum-free medium was necessary to slow DNA synthesis by the HRP cells. There were no significant differences in cells exposed to  $5\ \mu\text{g/ml}$  of rat transferrin, 5% FBS, or serum-free medium at 4 h. After 8 h of treatment, a significant difference was apparent between serum-free-treated cells and cells exposed to serum or transferrin ( $p < 0.01$ ). This difference was increased through the remainder of the experiment. Growth curves for transferrin and serum-treated cells were similar. The magnitude of the increase in  $^3\text{H}$ -thymidine incorporation by HRP cells to FBS or transferrin was diminished in cells preincubated for 48 h in serum-free medium in comparison to cells preincubated for only 24 h (data not shown).

Transferrin dependency was not characteristic of all cell types. Rat transferrin had no significant effects on the incorporation of  $^3\text{H}$ -thymidine by Balb/c mouse embryo fibroblasts (Table 4). The lack of stimulation of embryo fibroblasts by transferrin

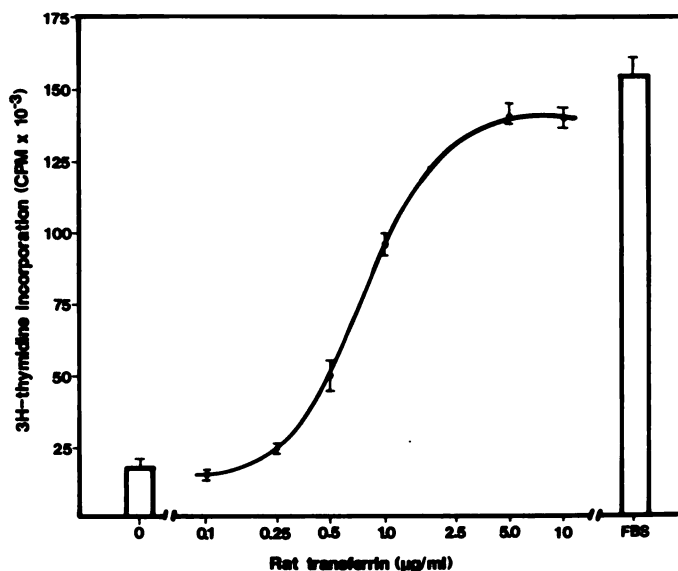


FIG. 2. Effects of rat transferrin on the incorporation of  $^3\text{H}$ -thymidine into DNA by Holtzman rat placental cells (HRP cells). See Figure 1 for further details regarding the experimental protocol. Each point represents the mean of five to six replicates, and the vertical bars represent the standard error of the mean. The histograms on the figure represent the serum-free control treatment (0) and the 5% fetal bovine serum control (FBS). Values for transferrin concentrations greater than or equal to  $1.0\ \mu\text{g/ml}$  were significantly different from the serum-free control,  $p < 0.01$ .

TABLE 1. Effect of human transferrin on DNA synthesis by Holtzman rat placental cells (mean  $\pm$  SEM).

Treatment	N	<sup>3</sup> H-thymidine incorporation (cpm $\times$ 10 <sup>3</sup> )
Serum-free control	6	18.9 $\pm$ 1.6
5% Fetal bovine serum	6	162.2 $\pm$ 4.5*
Rat transferrin (2.5 $\mu$ g/ml)	6	126.8 $\pm$ 3.2*
Human transferrin (2.5 $\mu$ g/ml)	6	127.4 $\pm$ 5.3*

\*Values are significantly different from serum-free control values,  $p < 0.01$ .

also indicates the absence of contamination of the transferrin preparation with a number of different types of growth factors known to stimulate embryo fibroblasts (O'Keefe and Pledger, 1983).

### DISCUSSION

We have demonstrated that transferrin is a potent regulator of rat placental cell growth. Exposure of rat placental cells to transferrin under serum-free conditions resulted in approximately an eightfold stimulation of DNA synthesis. The stimulatory effects of transferrin approached those obtained with FBS. Transferrin also stimulated the proliferation of rat placental cells *in vitro*. Transferrin is routinely

TABLE 2. Effects of human apotransferrin and holotransferrin on DNA synthesis by Holtzman rat placental cells (mean  $\pm$  SEM).\*

Treatment	<sup>3</sup> H-thymidine incorporation (cpm $\times$ 10 <sup>3</sup> )
Serum-free control	24.6 $\pm$ 1.3
5% Fetal bovine serum	91.0 $\pm$ 4.0 <sup>a</sup>
Apotransferrin ( $\mu$ g/ml)	
0.01	25.9 $\pm$ 0.3
0.1	41.6 $\pm$ 3.1
1.0	72.5 $\pm$ 4.6
10.0	75.0 $\pm$ 2.8
Holotransferrin ( $\mu$ g/ml)	
0.01	27.6 $\pm$ 11.1
0.1	83.2 $\pm$ 9.5 <sup>b</sup>
1.0	93.5 $\pm$ 12.7
10.0	111.9 $\pm$ 14.6 <sup>c</sup>

\*Values represent means of nine measurements (serum-free control and 5% fetal bovine serum) and means of a representative experiment performed in triplicate (apotransferrin and holotransferrin).

<sup>a</sup>Significantly different from serum-free control,  $p < 0.01$ .

<sup>b</sup>Significantly different from apotransferrin, 0.1  $\mu$ g/ml,  $p < 0.01$ .

<sup>c</sup>Significantly different from apotransferrin, 10  $\mu$ g/ml,  $p < 0.05$ .

TABLE 3. Rat placental cell proliferation following four days of exposure to rat transferrin (mean  $\pm$  SEM).

Treatment	N	Cells ( $\times$ 10 <sup>6</sup> )
Serum-free control	5	2.6 $\pm$ 0.3
5% Fetal bovine serum	4	10.3 $\pm$ 1.3*
Rat transferrin (5 $\mu$ g/ml)	5	7.6 $\pm$ 0.6*

\*Values are significantly different from serum-free control values,  $p < 0.01$ .

used as a growth supplement for cell culture but generally is relatively ineffective in stimulating cell proliferation without the addition of other growth factors (Barnes and Sato, 1980). In the present study, the addition of insulin, epidermal growth factor, or insulin-like growth factor-II did not significantly alter the magnitude of the effect of transferrin on placental cell DNA synthesis. Although the results obtained with transferrin on rat placental cells differ somewhat from those obtained with adult cell types, they are in agreement with the stimulatory effects of transferrin on embryonic cell growth (Ekblom et al., 1983).

The rodent placenta has previously been shown to be a target tissue for transferrin (McArdle et al., 1984a,b; 1985). Receptors for transferrin have been preferentially localized to the labyrinth region of the chorioallantoic placenta of the mouse (Müller et al., 1983; Adamson, 1986) and rat (Hunt and Soares, 1988). Transferrin has been shown to be synthesized by a variety of embryonic and extraembryonic cells, including those forming the visceral yolk sac, a major source of transferrin during midgestation, and by cells in adult tissues (Adamson, 1982; Meek and Adamson, 1985). Although transferrin is not synthesized by the rodent placenta (Adamson, 1986), our results suggest that placental cells may be dependent on transferrin for growth.

The growth and differentiation of the chorioallantoic placenta are essential for the maintenance of normal fetal development. Increased placental size results in increased surface area for nutrient and waste exchange and increased number of cells capable of secreting hormones and growth factors that influence maternal and fetal environments. Some of the hormones and growth factors produced by the placenta have also been proposed as regulators of placental cell growth (see Adamson, 1986, for a

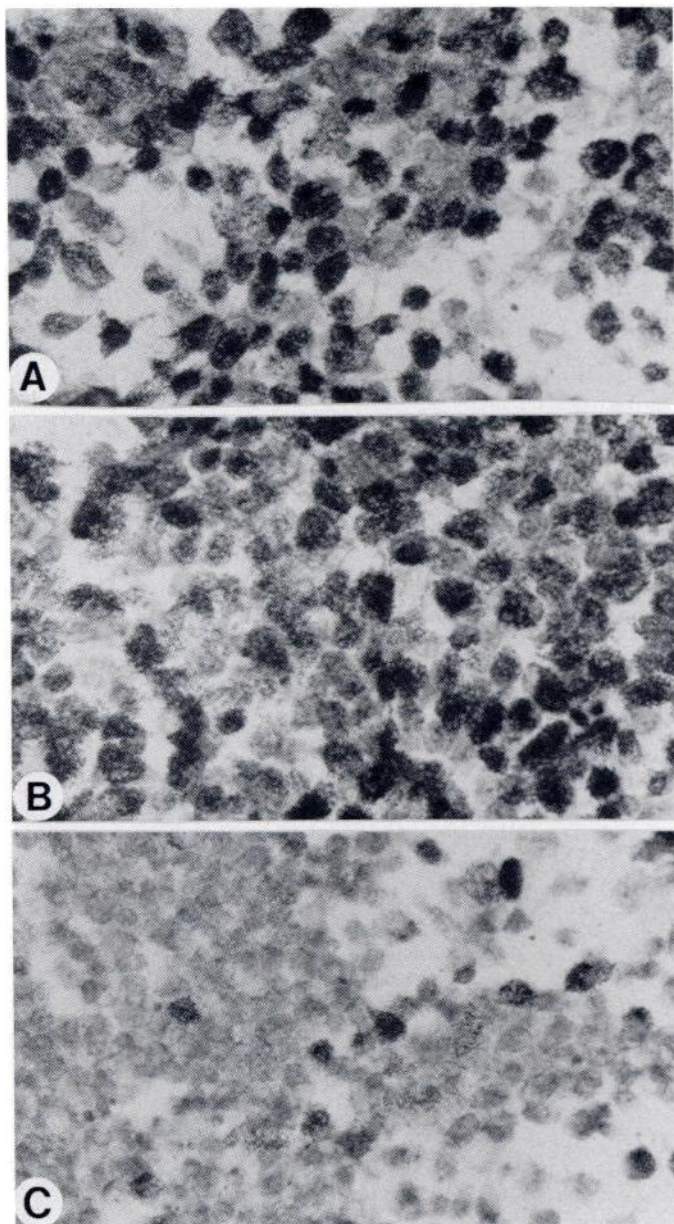


FIG. 3. Autoradiograms of incorporation of <sup>3</sup>H-thymidine into DNA by Holtzman rat placental cells (HRP cells). HRP cells were plated in the presence of 5% fetal bovine serum (FBS), transferred to serum-free culture medium for 24 h, and then incubated with A) 5% FBS containing medium, B) serum-free medium containing rat transferrin (5 μg/ml), or C) serum-free culture medium without any supplements. The cells were incubated with the respective treatments for 36 h, then exposed for 4 h to <sup>3</sup>H-thymidine. The cells were dipped in photographic emulsion, exposed for 3 days, developed, and counterstained. This figure depicts representative autoradiograms from three experiments.

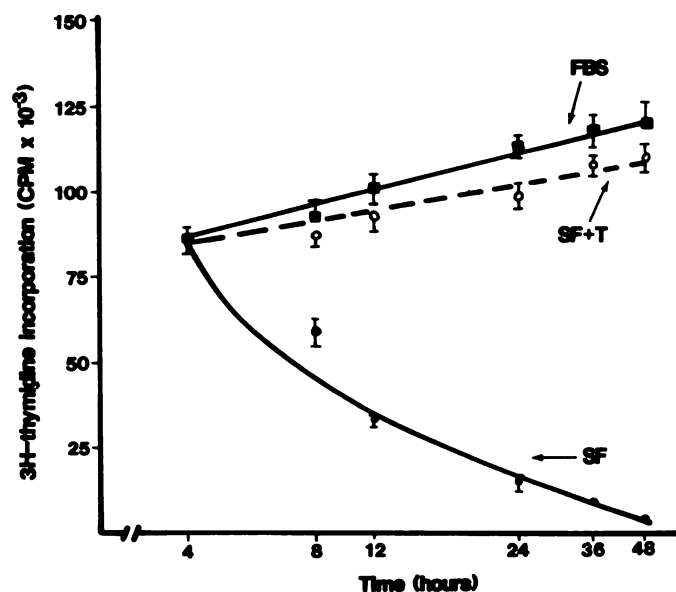


FIG. 4. Time-course effects of rat transferrin on the incorporation of <sup>3</sup>H-thymidine into DNA by Holtzman rat placental cells (HRP cells). HRP cells were plated in culture medium containing 5% fetal bovine serum (FBS), transferred to serum-free medium for 24 h, and then into 5% FBS containing medium (FBS), serum-free medium (SF), or serum-free medium containing 5 μg/ml of rat transferrin (SF + T). The cells were harvested at various time points after initiation of the treatments. A 4-h incubation with <sup>3</sup>H-thymidine preceded the cellular harvests. The amount of <sup>3</sup>H-thymidine incorporated into DNA was determined by liquid scintillation counting. Each point represents the mean of five to six replicates, and the vertical bars represent the standard error of the mean. Significant differences were not observed between the FBS and SF + T treatments at any time points; however, values for SF treatment were significantly different from the SF + T and FBS treatments at time points from 8 h to 48 h, *p* < 0.01.

the visceral yolk sac is a derivative of the inner cell mass and is a major source of transferrin. Whether the visceral yolk sac is the major source of transferrin responsible for placental cell growth remains to be determined. Uterine tissue has been shown to produce other iron-transporting proteins, including lactotransferrin (Pentecost and Teng, 1987) and

review). The inner cell mass and its derivatives have also been shown to be important modulators of placental cell growth (see Gardner, 1983, and Ilgren, 1983, for reviews). In keeping with this latter notion,

TABLE 4. Effect of rat transferrin on DNA synthesis by mouse embryo fibroblasts (mean ± SEM).

Treatment	N	<sup>3</sup> H-thymidine incorporation (cpm × 10 <sup>3</sup> )
Serum-free control	5	1.0 ± 0.1
Rat transferrin (5 μg/ml)	5	1.5 ± 0.1
5% Fetal bovine serum	5	27.7 ± 4.4*

\*Values are significantly different from serum-free control values, *p* < 0.01.

uteroferrin (Roberts and Bazer, 1985). The role of these proteins in regulating placental cell growth is unknown.

The importance of iron delivery in transferrin-mediated cell growth has been previously demonstrated (Thesleff et al., 1985). Comparison of the actions of apotransferrin versus holotransferrin is consistent with the transfer of iron being an integral regulatory step in the control of rat placental cell DNA synthesis.

Our observations on transferrin-stimulated placental cell growth may not be generalized to all placental cell types. In this report, we have examined the effects of transferrin on a population of cells isolated from the midgestation chorioallantoic placenta (Soares et al., 1987). This population of placental cells is relatively autonomous; they produce their own extracellular matrix (Soares et al., 1987), are transplantable (Soares et al., 1987), and have minimal external requirements for growth (present study). These characteristics are not unlike those expected for a stem cell population (Adamson, 1986). A more complex array of growth factors may be required for placental cells with differentiated phenotypes (Goustin et al., 1985; Fant et al., 1986; Athanassakis et al., 1987).

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