Intermediate Cells during Cytotrophoblast Differentiation in Vitro

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Abstract
Differentiation of the human placental trophoblast cell involves a multistep process, with the generation of several distinct types of intermediate cytotrophoblast cells. Using a short term in vitro cell culture system and centrifugal elutriation, we studied the isolation and morphological and biochemical differentiation of these separated intermediate cell populations. Freshly isolated cell fractions, incubated for 24 h, are heterogeneous in their differentiation stages as determined by the secretion of the proteins chorionic gonadotropin α and β, human placental lactogen, and pregnancy specific β1-glycoprotein. Maintenance in cell culture allows for the further differentiation of these intermediate cells and for syncytiotum formation. With the use of sequential trypsinizations, our data also suggest the parallel differentiation of cytotrophoblast cells into two distinct subsets: one which, through differentiation, gets committed to syncytiotum formation, and the other, which remains mononuclear despite high degrees of biochemical differentiation. These latter cells retain the capacity for syncytiotum formation when reintroduced into appropriate culture conditions. These findings refine the use of the term “intermediate cell” by previous investigators. We suggest that our in vitro system defines normal intermediate stages of trophoblast differentiation, and also serves as a model to simulate adverse conditions of syncytiotum degeneration or injury.

Introduction
Differentiation of the human placental trophoblast cell involves a multistep process which culminates in the formation of terminally differentiated multinuclear syncytiotrophoblasts. The progenitor cytotrophoblasts are mononuclear undifferentiated cells. Biochemical and morphological changes must occur during this process of differentiation whereby mitotically active cytotrophoblast cells fuse to form the mitotically inactive syncytiotrophoblast in vivo and in vitro. Recent literature suggests that there exist transitional or intermediate cells during this process, although no data are currently available examining these cell types separately (1–3).

The term “intermediate” trophoblast has been used with some confusion to describe a variety of both villous and extravillous cells identified within the placenta (3). For the purposes of this report, we use this term to characterize a distinct form of trophoblast cell, presumably villous in origin, that shares some of the morphological and functional features of both cytotrophoblast and syncytiotrophoblast. Although these cells are mononuclear, their cytoplasm shows evidence of differentiation toward a later syncytial state (1, 2). Work by us (4) and other investigators (5) has described an in vitro model that allows for the study of these cells in short term cell culture. In the present series of investigations, we expand on these earlier studies by examining subpopulations of the intermediate cytotrophoblast from both a morphological and functional perspective. Additionally, using sequential periods of trypsinization and centrifugal elutriation, we describe, for the first time, a stable population of cells which differentiate biochemically but do not concomitantly proceed to syncytiotum formation.

Results
Subfractionation of Isolated Cytotrophoblast. Fresh term placentae were digested with trypsin-DNase, followed by Percoll gradient centrifugation, to isolate trophoblast cells. These trophoblast cell cultures have previously been shown to be free of other cell contaminants, and they demonstrated greater than 95% viability (4, 5). By light microscopy examination, these cultures were composed of a heterogeneous cell population.

Subpopulations of cytotrophoblasts were collected using centrifugal elutriation immediately after cell isolation from the placenta, as previously described (6, 7). The percentage of cells in each of the 11 elutriated fractions and their average diameter are shown in Fig. 1A. The recovery, after elutriation, was 72% (n > 45). Fractions 1–5 contained more than 80% of the whole cell populations. The other fractions contained 1–5% each. An evaluation of syncytiotum formation according to cell fraction grown separately in culture is shown in Table 1.

To investigate the secretion of the differentiation-linked placental proteins CGα2 and CGβ, hPL, and SP1, the following experiments were performed on five placental isolates. Equal number of cells from each of the 11 elutriated trophoblast cell fractions were plated separately. [trans135]Cysteine was added either between 0 and 24 h (P24) or between 72 and 96 h (P96). All of the supernatants were collected and immunoprecipitated with specific antisera for CGα, CGβ, hPL, and SP1.

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2 The abbreviations used are: CG, chorionic gonadotropin; hPL, human placental lactogen; SP1, pregnancy specific β1-glycoprotein; HBSS, Hank's buffered salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.
corresponding precipitates were dissolved and separated by gel electrophoresis. The gels were dried and radioautographed (Fig. 2). The bands corresponding to CGα, CGβ, hPL, and SP1 were excised and counted. The protein content of the cells in each plate was measured, and the specific radioactivities (cpm in band per μg protein) were calculated. There was a good correlation between the intensity of the band seen by radioautography and the calculated specific radioactivity.

Cytotrophoblasts incubated from 0 to 24 h (P24) secreted CGα in all 11 cell fractions, although the intensity of the signals in fractions 1 and 11 was lower than that in the other cell fractions (Fig. 2a). Conversely, hPL was secreted only in fractions 5 onward, and the secretion increased with cell size (Fig. 2b). Of interest, CGβ and SP1 were not secreted in significant quantities by any of the cell fractions at the P24 time interval (data not shown).

The secretion of all placental proteins (CGα, CGβ, hPL, and SP1) increased significantly after P24, as can be seen from the change in intensity of their respective radioactive signals (Fig. 2). Calculating the specific activity of each of these proteins demonstrated an approximately 10-fold increase in their secretion.

At 96 h of incubation, the intensity of the signals for CGβ and hPL increased from fraction 1 to 6 and decreased thereafter toward fraction 11 (Fig. 2, d and e). The secretion of CGα and SP1 remained relatively constant across most cell fractions (Fig. 2, c and f). Two fractions, 1 and 11, showed interesting deviations from the above trends. Although fraction 1 demonstrated biochemical differentiation via the increased production of hPL at 96 h, the amount of this protein was lower than in the other cell fractions. For fraction 11, hPL secretion also increased by 96 h, but to a lesser extent than the remaining fractions. These deviations were consistent for the other placental proteins, CGα, CGβ, and SP1, as well.

The same fractions that did not secrete CGβ, hPL, and SP1 at P24 secreted measurable quantities of these proteins at P96 (Fig. 2, d–f). Therefore, by centrifugal elutriation, we isolated several cell fractions (1–4) which initially (P24) secreted CGα only, and several fractions (5–11) which secreted both CGα and hPL. All of these fractions were shown to continue to differentiate biochemically in cell culture, and they subsequently secreted all of the placental protein markers at P96.

**Trypsinization of Cytotrophoblasts in Culture.** Anecdotal observations by us and others (7, 8) have suggested that an identifiable subpopulation of cytotrophoblasts do not form syncytia in vitro. To further examine these cells, repeat trypsinizations of cell cultures were carried out at designated times after initial plating. Previous investigators have reported that trypsin effectively destroys syncytiotrophoblasts, and therefore this method allowed the specific isolation of the remaining cytotrophoblasts (9). The number of cytotrophoblasts isolated by trypsinization was determined, and the percentage of cells surviving from the preceding cell culture was calculated, after correcting for cell loss by protein estimation.

The number of cells in culture gradually decreased over time. However, the percentage of cells isolated by trypsinization of these cultures in relation to the remaining cells remained almost constant during 120 h of incubation (Fig. 3). This occurred despite an increasing number of syncytial structures evident in these cultures as the incubation period was extended (4, 5). Not all cytotrophoblasts are included in this group, in that some of these cells are also lost during the trypsinization process. On average, 20–30% of the initially cultured cytotrophoblasts “self-selected” and were recoverable with trypsinization. Although different placenta showed some biological variation in the percentage of cells that

**Table 1  Syncytium formation according to elutriated cell fraction**

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Eleven cell fractions were individually cultured, and the degree of syncytium formation after 72 h was determined by phase microscopy. This procedure was repeated in 4 individual experiments. Ten different fields were counted in each plate. Each + represents 20% of the nuclei visualized in syncytium.
Fig. 2. Placental protein synthesis and secretion by elutriated cytotrophoblast fractions. Free CGs (a, c, and g), CG dimer (d and h), hPL (b, e, and i), and SP1 (f and j). Autoradiograms of SDS-polyacrylamide gel electrophoresis of labeled immunoprecipitated proteins from media of elutriated and cultured cytotrophoblasts. Cells were incubated with [trans-35S]cysteine for 24 h at initial plating (P24) or after 72 h in culture (P96). Cultured cytotrophoblasts were incubated for 72 h, retrypsinized, elutriated, and incubated for an additional 24 h with [35S]cysteine (P196).

Fig. 3. The percentage of recoverable cytotrophoblasts following retrypsinization at different incubation times. Cells were incubated for the times indicated. After incubation, each of the cultures was trypsinized, and the number of cells was determined using a Coulter Counter. Data are expressed as percentage of cells remaining from initial plating, after correcting for cell loss by measuring protein content.

survived trypsinization, this percentage did not change with incubation time for each individual placenta. The retrypsinized cells, when reincubated, demonstrated the capacity to form syncytia similar to the cells in the preceding culture. Repeated trypsinizations of the initial culture at 24-h intervals (up to six times) yielded a progressively smaller absolute number of cells, but the same percentage of isolatable mononuclear cytotrophoblasts. Exposure of the cells to trypsin immediately after isolation, or at up to 9 h after plating, did not reduce their number (data not shown).

As an aside, to speculate as to whether this phenomenon of cell selection occurs in vivo, we isolated cytotrophoblasts from first, second, and third trimester placentae. It is recognized that the ratio of cytotrophoblasts to syncytiotrophoblasts changes throughout pregnancy (10). Trypsinization of placental tissues (n = 10) consistently yielded 1–2 × 10⁶ cytotrophoblast cells/g wet weight, regardless of the trimester from which the placenta was obtained. These findings indirectly suggest a similar process in vivo which conserves a specific fraction of trophoblast cells in a mononuclear, nonsyncytiotrophoblastic state.

Since an increasing number of cells are converted to multinuclear syncytiotrophoblasts during the incubation time, but the percentage of cytotrophoblasts isolated from such cultures by trypsinization remains constant, we attempted to better characterize this isolatable cell subpopulation.
DNA synthesis was determined by replating these retrypsinized cells in the presence of \(^{3}H\)thymidine at 24-h intervals for 120 h. The incorporation of thymidine into DNA was the same when measured in retrypsinized cells at different time points of incubation, as compared to cells from the primary culture that were not retrypsinized (data not shown). In both retrypsinized and native cells, DNA synthesis after 24 h of incubation was negligible (4). These data confirm the absence of actively dividing cytotrophoblasts in culture after 24 h, and that retrypsinization does not alter this process.

We examined multinuclear syncytium formation by the mononuclear cytotrophoblasts isolated from retrypsinized cultures at various times. Using a technique that simultaneously stains cell membranes (R<sub>18</sub>) and nuclei (DAPI), one can determine whether the nuclei are enclosed within the same cell membrane, and therefore distinguish between mono- and multinuclear cells (7). As can be seen in Fig. 4, these cells form syncytia. As previously stated, this occurred to a similar degree as in the primary cultures.

Although the retrypsinized cells appeared to be “quiescent” in their ability to form syncytia in their original cell cultures, a series of experiments were carried out to examine their biochemical differentiation in vitro. This is of critical importance since biochemical differentiation is not necessarily linked to the ability of the cell to form syncytia (8). The expression of trophoblast differentiation markers (CGα, CGβ, and hPL) was measured in cells incubated for 120 h and retrypsinized. The isolatable cells following trypsinization were collected by centrifugation, and RNA was immediately isolated. RNA was also isolated from nonretrypsinized, plated cells (containing both syncytial and nonsyncytial elements) in cultures incubated for the same period of time. The RNA from the retrypsinized and nonretrypsinized cells was separated on agarose gels and hybridized with the appropriate complementary DNA probes.

From the results shown in Fig. 5, it can be seen that there was no difference in the abundance of CGα, CGβ, and hPL mRNA in RNA isolated from retrypsinized and native cells. The same results were obtained when retrypsinized and control cells were compared at 24 h (data not shown), when the expression of these genes is known to be lower (see above). Therefore, the increase in the expression of these genes with incubation time is not dependent upon the formation of the syncytial elements.

The process of retrypsinization of the cytotrophoblasts, however, was shown to have a profound effect on biochemical differentiation of cells in culture. In experiments in which cytotrophoblasts were grown in culture for 24, 48, and 72 h, retrypsinized, and then recultured for an additional 24 h, CG secretion was markedly elevated compared to control, nonretrypsinized cultures for the same periods of time (Fig. 6).

**Subpopulation of Retrypsinized Cytotrophoblasts.** A Coulter Counter was used to determine the cell size distribution of cytotrophoblasts isolated from term pla-

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**Fig. 4.** Syncytium formation by retrypsinized cytotrophoblasts: fluorescence microscopy of cytotrophoblasts. Cytotrophoblasts were isolated from term placenta and incubated in culture for 96 h. After 96 h, the cells were retrypsinized and replated. The cells were fixed and were simultaneously stained for nuclei (DAPI) and membranes (R<sub>18</sub>), demonstrating mononuclear cells after 1 h (A) and syncytium formation after 24 h (B).
centae. These data were compared to similarly isolated cells grown in culture and retpysinized at 24 and 120 h (Fig. 7). The size distribution of cells isolated from placenta at zero time, and cells grown in culture and retpysinized after 24 and 120 h, did not change significantly. The same results were obtained with cells retpysinized after 48, 72, and 96 h (data not shown). Therefore, the cell size populations of cytotrophoblasts isolated from placenta and those isolated after in vitro incubation and retpysinization were identical.

Since overall cell size distribution was similar for native and retpysinized cells, we examined the retpysinized cells according to their sedimentation coefficient with high resolution centrifugal elutriation, after 72 h of incubation. We used this method to compare, from both a morphological and biochemical perspective, the cell fractions isolated from either native placental cells or retpysinized cultures of cytotrophoblasts. Although the elutriation profile of retpysinized cells (Fig. 1B) resembled the elutriation profile of native retpysinized placental cells (Fig. 1A), there is a slight shift in cell size from smaller to larger cells after incubation of cytotrophoblasts for 72 h (compare fractions 1–4 in Fig. 1A to 1–4 in Fig. 1B).

As previously described, differentiation linked placental proteins were investigated in individual fractions of isolated cytotrophoblasts after incubation for 72 h and retpysinization (Fig. 2, Pt96). All four proteins were synthesized and secreted at quantitatively similar levels across fractions. It can be seen that there was no change in pattern between the synthesis and secretion of CGa and SP1 in the different fractions whether cells were elutriated at zero time and incubated for 96 h (Fig. 2, c and f), or first incubated for 72 h and then retpysinized, elutriated, and incubated for an additional 24 h (Fig. 2, g and j). The only difference seen is in fraction 11 in Pt96, where the secretion of these proteins was relatively higher than in the corresponding fractions in Pt96. For CGa and hPL, the previously observed pattern of more hormone production in the middle fractions was not seen in Pt96. Of note, although P24 and Pt96 are similar in that both experiments represent conditions of retpysinization and subsequent short term incubation, the latter condition allowed for differentiation in vitro, even though these cells did not form syncytia.

Discussion

During human intravillous placental differentiation, two distinct trophoblast cell types are usually recognized. These are the mononuclear cytotrophoblast and the multinuclear syncytiotrophoblast. However, cellular differentiation involves a series of continuous stages whereby the undifferentiated cytotrophoblast eventually becomes the syncytiotrophoblast. Indeed, Tighe et al. (11) introduced the term "intermediate cells" to describe a group of cells that appear to be intermediate forms between the cytotrophoblast and the syncytiotrophoblast. This latter refinement in the understanding of trophoblast differentiation remains incomplete, in that Okudaira and colleagues (12) have proposed the existence of "intermediate" cytotrophoblasts which are highly differentiated cells that remain mononuclear, without syncytiitum formation. The data presented in this report confirm the existence of both types of intermediate cells in the dif-
further differentiation process of placenta. The first group of cytotrophoblasts described by our data represent true intermediate cell types which proceed from an undifferentiated stage to syncytium formation. The second group of “intermediate” cells deviate from the terminal process of syncytium formation and remain mononuclear despite biochemical differentiation.

As previously described (5), cytotrophoblast cells isolated by trypsinization and Percoll gradient centrifugation are not homogeneous in their appearance. On a cursory level, this alone suggests that the isolation procedure results in a “snapshot” representation of cells in various stages of differentiation. Using centrifugal elutriation, we further separated and characterized these cells by morphological and biochemical patterns. Centrifugal elutriation is an excellent means by which to examine the issue of cellular differentiation in these cells, for two reasons. First, a more uniform population of cells can be obtained by selecting according to cell size, in addition to density properties. Second, evidence exists supporting a relationship between cell size and stages of the cell cycle (13). We hypothesized that this physical property would also correlate with degrees of differentiation, since these cells are nondividing cells, primarily in stage Go-G1. Previous data from our laboratory with a choriocarcinoma cell line also supported this line of reasoning (6). Our relatively arbitrary use of 11 fractions was based of the suspected range of cell sizes found in the native cytotrophoblast cell culture.

We show here that freshly isolated cytotrophoblasts, separated by size groups, are in various stages of biochemical differentiation as evidenced by the variable production and secretion of hPL. This, in fact, correlated closely with cell size, as hypothesized above: the larger the cell size, the greater the secretion of hPL. The fact that all of the cell fractions secreted CGα is consistent with the model of cytotrophoblast maturation proposed by Boime (14), in which this placental protein appears early in the differentiation process. Synthesis and secretion can be used interchangeably here, since we have previously shown that these proteins are released immediately after production (4). Similar results have been obtained in studies of hPL mRNA abundance across these fractions (7).

Maintenance of these cell fractions in culture allowed for further biochemical differentiation of all groups and was accompanied by syncytium formation in most fractions. Biochemical differentiation was demonstrated by the appearance of CGβ and SP1 and the augmented secretion of CGα and hPL. Of interest, fractions 1 and 11 were distinct from the other fractions in their relatively reduced secretion of the investigated placental proteins. These latter data support our belief that these two fractions represent “aging” stages of differentiation, leading to apoptosis. Fractions 1 and 11 did not demonstrate the capacity to form syncytiotrophoblasts, further supporting this possibility. Preliminary results in our laboratory using electron microscopy show evidence of heterochromatinization and necrosis in both fractions. These are well recognized physical signs of cellular aging and degeneration. Syncytiotrophoblasts in vivo have similar degenerative properties, including a reduction in RNA synthesis and decreased hormonal production (15). It is therefore likely that fractions 1 and 11 represent perisyncytial events in our native cell cultures. All of the cell fractions discussed above fit the first definition of intermediate cells proposed by Tighe et al. (11), representing the continuum from the undifferentiated cytotrophoblast to the formation of the highly differentiated syncytiotrophoblast.

We also isolated a stable self-selecting subpopulation from the cytotrophoblasts which deviated from the path of the above intermediate cells. These cells were at the same stage of differentiation as the entire culture at each time point, as measured by the expression of placental protein mRNAs. However, they remained mononuclear, retaining the capacity for cellular fusion only when recultured. Previous studies (8) have also shown cytoplasmic and biochemical differentiation in the absence of syncytium formation. We propose that these cells are the “intermediate cells” suggested by Okudaira et al. (12). In all likelihood, our initial digestion and trypsinization of the native placenta yielded this same group of cytotrophoblasts from the remainder of the villous trophoblast population. In support of this speculation, we demonstrated a consistent percentage of isolatable cells, regardless of the gestation age, or in culture, regardless of the number of sequential trypsinizations and times of incubation. Additionally, by elutriation, we found a similar profile of cell fractions between cytotrophoblasts derived from native placenta and those obtained by trypsinization of cell cultures. Lastly, others (3) have described the existence of a stable population of cytotrophoblast cells, which comprise between 15 and 30% of the total villous trophoblast volume throughout pregnancy. The mechanism by which these cells escape syncytium formation in native tissue (or preceding cultures) remains unclear. However, based on preliminary

experiments in our laboratory, this event cannot be overridden by the addition of conditioned media or by altering the degree of cell-cell contact and fusion. The absolute number of mononuclear cytotrophoblasts that remain after trypsinization cannot be determined, because some cytotrophoblasts are lost during the trypsinization process as well.

The biological role of these two types of “intermediate” cells remains speculative. Benirschke and Kaufmann (15) have reported that under normal in vivo conditions, survival of the syncytiotrophoblast layer depends on cytotrophoblast fusion to regenerate and maintain its functional and structural integrity. Cytotrophoblasts shortly before syncytial fusion are highly differentiated and have ultrastructural similarities with the syncytiot. It is likely that our in vitro system mimics this normal process by which cytotrophoblasts differentiate prior to cellular fusion with the syncytiot. Additionally, under pathological clinical conditions, increased numbers of villous cytotrophoblasts are seen in vivo, and these cytotrophoblasts can reach high degrees of differentiation (15). Use of trypsin to isolate cells in native placenta, or in established trophoblast cultures, may be a means to simulate in vivo conditions of syncytial degeneration or injury and therefore model the triggering mechanism by which mononuclear cytotrophoblasts, previously held in reserve, are now recruited for differentiation and syncytial reparation.

Materials and Methods

Cell Culture. Cytotrophoblasts were purified from human term placenta according to Gileadi et al. (4). Briefly, 200 g of minced placenta, scraped free of blood vessels and connective tissue, were incubated in 1 liter of sterile HBSS, containing 25 mm HEPES (pH 7.4), 0.12 mm CaCl₂, 0.8 mm MgCl₂, 1 g trypsin (Sigma), 135 mg DNase I (Sigma), and antibiotics. After incubation for 30 min at 37°C, the solution was filtered, and newborn calf serum (15% final concentration) was added. Cytotrophoblasts were obtained by centrifugation through a discontinuous Percoll gradient (15~70% in HBSS), at 3000 rpm for 30 min and collection of the fractions between 35% and 55% (5). Approximately 4 x 10⁵ cells/cm² were plated in polystyrene culture dishes (Nunc). Cells were maintained in Medium 199 containing 10% fetal calf serum, 25 mm HEPES (pH 7.4), penicillin (180 units/ml), streptomycin (100 μg/ml), and amphotericin B (0.2 μg/ml). The medium was changed every 24 h.

Centrifugal Elutriation. Two to 2.5 x 10⁶ cytotrophoblast cells were concentrated into 10 ml of medium containing 25 mm HEPES (pH 7.4) and loaded into a Beckman J2-21M elutriator rotor (Beckman, Palo Alto, CA), using a standard chamber and a Masterflex peristaltic pump (Cole-Parmer Instruments, Chicago, IL). Constant rotor speed was maintained at 2000 rpm. The elutriation buffer was HBSS, containing 2% newborn calf serum at 4°C. The cells were loaded at a flow rate of 10 ml/min. The first 150 ml served as a washing step and were discarded. The flow rate was increased from 15 to 65 ml/min, in steps of 5 ml, and 11 fractions (100 ml each) were collected. At the last flow rate, the centrifuge was stopped. Cells from each fraction were precipitated by centrifugation for 5 min at 2000 rpm, and the cells were suspended in incubation medium.

Cell Size Determination. Cells from each of the fractions were suspended in HBSS, and their number and size distribution in each fraction were determined by using a Coulter Counter (Coulter Electronics, Harpenden, Hertfordshire, United Kingdom).

Trypsinization. Cells grown for 24 and 120 h were washed with HBSS. Trypsin solution (0.25% trypsin-0.3% EDTA) was added to the cells and incubated for 20 min. The cell suspension was transferred to a conical tube with 2 volumes of newborn calf serum, collected by centrifugation, and resuspended in Medium 199.

Determination of Macromolecule Synthesis. Approximately 0.5 x 10⁶ cytotrophoblasts were cultured in 24-well multidishes, in 0.5 ml Medium 199 containing HEPES, fetal calf serum, and antibiotics as described above.

To determine incorporation of [³H]thymidine into DNA, 5 ml of (methyl-¹H)thymidine (1 μCi/ml, ICN Biomedicals, Irvine, CA) were added to the culture medium in each well. The incorporation of Tras[³S]-label (ICN Biomedicals, Cat. No. 51006) into cellular proteins was determined by changing the culture medium to Medium 199 without cysteine and methionine, supplemented with 10% dialyzed fetal calf serum and 10 μl/ml of Trans[³S]-label. The labeling was initiated immediately or 72 h after plating.

After 24 h of labeling, the medium was removed, the cells were washed twice with HBSS, 0.5 ml distilled water was added, and the cells were scraped from the plate and frozen. After thawing, aliquots of the lysate were taken for protein determination by the Bio-Rad protein assay dye reagent (Bio-Rad Laboratories GmbH, Munich, Germany; Cat. No. 500-0006). [³H]Leucine and [³H]thymidine incorporation was determined by trichloroacetic acid precipitation.

Isolation of RNA. Total cellular RNA was isolated from cells by the guanidinium thiocyanate-cesium chloride method (16).

Northern Blotting. Ten mg of each RNA sample were separated by 1% agarose-formaldehyde gel electrophoresis and transferred to Hybond-N nylon filters (Amer sham, Amersham, United Kingdom). The blots were stained with methylene blue (17) and hybridized with 18S ribosomal RNA (data not shown), in order to ascertain that equal amounts of RNA had been loaded in each lane. The blots were prehybridized at 42°C in 50% formamide, 5X saline-sodium phosphate-EDTA, 5X Denhardt's solution, 0.1% SDS, and 0.1 mg/ml herring testes DNA and hybridized with specific complementary DNA probes. The probes used for hybridization were prepared from the appropriate clones of H19, insulin-like growth factor II, CGA, CGB, and hPL. The probes were labeled according to the random primed labeling kit (Boehringer Mannheim) protocol. The blots were washed twice in 0.1X standard saline citrate-0.1% SDS at 65°C and exposed to AGFA Curix film at ~80°C.

References


