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Bovine Blastocyst-Derived Trophectoderm and Endoderm Cell Cultures: Interferon Tau and Transferrin Expression as Respective In Vitro Markers

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ABSTRACT

Continuous cultures of bovine trophectoderm (CT-1 and CT-5) and bovine endoderm (CE-1 and CE-2) were initiated and maintained on STO feeder cells. CT-1 and CT-5 were derived from the culture of intact, 10- to 11-day in vitro-produced blastocysts. CE-1 and CE-2 were derived from the culture of immunodissected inner cell masses of 7- to 8-day in vitro-produced blastocysts. The cultures were routinely passaged by physical dissociation. Although morphologically distinct, the trophectoderm and endoderm both grew as cell sheets of polarized epithelium (dome formations) composed of approximately cuboidal cells. Both cell types, particularly the endoderm, grew on top of the feeder cells for the most part. Trophectoderm cultures grew faster, relative to endoderm, in large, rapidly extending colonies of initially flat cells with little or no visible lipid. The endoderm, in contrast, grew more slowly as tightly knit colonies with numerous lipid vacuoles in the cells at the colony centers. Ultrastructure analysis revealed that both cell types were connected by desmosomes and tight junctional areas, although these were more extensive in the trophectoderm. Endoderm was particularly rich in rough endoplasmic reticulum and Golgi apparatus indicative of cells engaged in high protein production and secretion. Interferon tau expression was specific to trophectoderm cultures, as demonstrated by reverse transcription-polymerase chain reaction, Western blot, and antiviral activity; and this property may act as a marker for this cell type. Serum protein production specific to endoderm cultures was demonstrated by Western blot; this attribute may be a useful marker for this cell type. This simple coculture method for the in vitro propagation of bovine trophectoderm and endoderm provides a system for assessing their biology in vitro.

INTRODUCTION

Although several trophectoderm cell cultures or cell lines have been reported for the pig [1,2], rat [3–5], and human [6–8], there are few reports concerning the continuous culture of trophectoderm from ruminants [9]. Indeed, some reports have indicated that primary cultures of ruminant blastocyst cells quickly lost replicative capacity [10,11]. Similarly, although mouse endoderm-like cultures have been produced, usually differentiating from embryonic or embryonal carcinoma stem cell cultures, no published information is available on the continuous culture of extraembryonic endoderm from ruminants [10,12–14].

In vitro models of bovine trophectoderm and endoderm are needed to define better the biological characteristics of these extraembryonic cell types in ruminants. For example, a specific interferon, interferon tau, is produced specifically by the trophectoderm cells of the ruminant conceptus during the establishment of pregnancy, and it was demonstrated to be of importance in maintenance of the corpus luteum [15–18]. Some of the biology of bovine interferon tau (bIFN-τ) is difficult to assess without a culture system for bovine trophectoderm maintenance and propagation [19,20]. Recently, a human choriocarcinoma cell line (JAR) was used to investigate the regulatory domains of bIFN-τ [21,22]. Such studies could be more relevant physiologically if a bovine trophectoderm cell line was available that was known to be competent for the in vitro expression of bIFN-τ. Also, studies related to the induction of bIFN-τ by cytokines such as granulocyte macrophage colony-stimulating factor, or cellular interaction studies (e.g., macrophage interactions), might be more effectively pursued within the context of a bovine trophectoderm in vitro model system [23,24]. For similar reasons, an in vitro model system would also be useful in studying the extraembryonic endoderm of ruminants.

Mouse parietal and visceral endoderm cell lines have been established and used to investigate embryonic differentiation and production of endoderm-specific proteins [12,13,25]. Mummery et al. [13] showed that visceral endoderm-like cells could induce differentiation of mouse embryonal carcinoma cells. This phenomenon was also indicated by the recent study of Brook and Gardner [26] in which the presence of endoderm appeared to be a key factor in preventing the derivation of mouse embryonic stem (ES) cell lines. An in vitro model of ruminant extraembryonic endoderm could therefore be useful in studying similar phenomena in attempts to establish cultures of bovine ES cells. Also, since the development of the blastocyst and yolk sac is profoundly different in ruminants as compared with other species, such as the mouse and human, bovine endoderm and trophectoderm cell cultures could offer more relevant cellular substrates with which to test the biological processes associated with the establishment of pregnancy in ruminants.

Primary outgrowths of bovine trophectoderm and endoderm were observed during attempts to establish bovine epiblast cells in culture [27]. In order to evaluate the potential of these outgrowths as in vitro biological models, we undertook an assessment of their propagation in culture and of some of their functionally relevant gene or protein expression.

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MATERIALS AND METHODS

Cell Culture

All cells were grown on tissue culture plastic ware (Nunc, Roskilde, Denmark; Falcon, Becton-Dickinson, Lincoln Park, NJ). Cryovials (2 ml) were purchased from Nunc. Fetal bovine serum (FBS) was obtained from Gibco (Gaithersburg, MD) or from Hyclone (Logan, UT). Cell culture reagents including Dulbecco's PBS without Ca^{2+} and Mg^{2+}, media, trypsin-EDTA (0.05% trypsin, 0.43 mM EDTA), antibiotics, nonessential amino acids, and L-glutamine were purchased from Gibco. STO cells (CRL 1503; American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Feeder layers were prepared with mitomycin C as previously described [28] or by exposing a suspension of STO cells to 8 krad gamma radiation and plating the cells at 6×10^4 cells/cm^2. Feeder layers were maintained by refeeding with 10% DMEM every 6–7 days.

Bovine blastocysts used as starting material were produced by in vitro maturation, fertilization, and culture (IVMFC) as described elsewhere [29]. Primary cultures of bovine trophectoderm were initiated by plating intact hatched 10- to 11-day bovine blastocysts produced by IVMFC into 4-well tissue culture dishes (Nunc) containing feeder layers of STO mouse fibroblast cells. STO feeder cells were prepared at least 3 days in advance. In a similar manner, primary bovine endoderm cultures were initiated from inner cell masses (ICMs) isolated from 7- to 8-day IVMFC bovine blastocyst. ICMs were isolated by immunosurgery as previously described [27]. Blastocysts and ICMs were plated in 1 ml of 10% DMEM-199 medium (1:1 mixture of DMEM supplemented with 10% FBS, 2-mercaptoethanol [2-ME; 0.1 mM; Sigma Chemical Co., St. Louis, MO], nucleosides [0.03 mM guanosine, 0.03 mM adenosine, 0.03 mM cytidine, 0.03 mM uridine, and 0.01 mM thymidine] from Sigma, nonessential amino acids [single-strength], glutamine [2 mM], and antibiotics [28], and Medium 199 supplemented with 10% FBS, 2-ME, and antibiotics). Fresh 10% DMEM-199 was added to the primary cultures every 3–4 days.

Secondary passage of the trophectoderm and endoderm cell cultures was done by trypsin-EDTA treatment combined with physical dissociation (shearing by repeated pipette aspirations) for the first 2–3 passages. Trypsin-EDTA treatment or EDTA alone was found to be deleterious to the cells, and subsequent passaging of the cultures was performed by physical dissociation only. Physical passage consisted of removing the monolayer of cells from the tissue culture flask surface by shooting jets of medium onto the monolayer surface with a serological pipette. The cell sheets were broken up into relatively large pieces by repeated aspirations through the pipette. The cells were pelleted by centrifugation in a round-bottom centrifuge tube. After the medium was aspirated off, the cell pellet was ground, mortar-and-pestle style, using a 2-ml plastic serological pipette as a pestle. The resulting relatively small clumps of cells were resuspended in 10% DMEM-199 and plated onto STO feeder cells, typically at a 1:10 split ratio.

An alternative method for the passage of the cultures was a modification of a method described by Bednarz et al. [30] for endothelial cells. The medium was withdrawn from confluent monolayers of trophectoderm or endoderm, and the flask was flooded with 2 M urea in culture-grade water. The cells were incubated in 2 M urea at approximately 35°C for 5–6 min, at which time cells were beginning to detach from one another. The cell monolayer was gently washed one time with PBS, and 0.5 ml of trypsin-EDTA was added to the flask (T25) to finish dissociation of the cells by incubating at about 35°C for 5 min. The cells were resuspended in 10% DMEM-199 and plated onto STO feeder cells, typically at a 1:10 dilution. A large percentage of trophectoderm and endoderm cells survived the urea/trypsin-EDTA treatment, whereas most or all of the STO feeder cells exposed to urea appeared to be lysed. This method was effective in producing a single-cell suspension of the trophectoderm or endoderm cells that was particularly useful for cell enumeration and metaphase spread preparation.

Cytogenetic Analysis

CT-1 cells were analyzed for chromosome content at passages 30 and 54. CT-1 cells were harvested to single cells by treatment with 2 M urea and trypsin-EDTA as described above. The cells were collected in 5 ml of 10% DMEM to inactivate the trypsin, transferred to a 15-ml conical centrifuge tube, and spun in a Sorvall (Newtown, CT) RT6000 centrifuge for 10 min at $180 \times g$ to form a cell pellet. The supernatant was gently aspirated from the cell pellet and discarded. The pellet was gently broken to a slurry with finger flicking, and 5 ml of 75 mM hypotonic KCl was added with a Pasteur pipette while the cells were gently agitated. The cells were allowed to swell for 15 min at room temperature, and 1 ml ice-cold methanol acetic acid fixative (3:1) was added dropwise with constant mixing. An additional 5 ml of fixative was added 1 ml at a time; the tube was mixed by inversion after each addition of fixative. The cells were spun gently for 10 min at $180 \times g$; the supernatant was aspirated, and an additional 6 ml of fixative was added. The cells were allowed to fix for 10 min on ice; then the tubes were spun again (10 min, $180 \times g$) to form a pellet. The supernatant was again aspirated, and the pellet was resuspended in a sufficient amount of fixative (0.2–1 ml) to give a slightly milky suspension of cells.

Metaphase spreads were prepared by dispensing 1 drop of cell suspension from a height of 3–5 cm onto prechilled slides. The slides were flooded with fresh fixative from a Pasteur pipette and allowed to dry in a humidified box at room temperature. The slides were checked at 20× magnification under phase-contrast to ensure proper spreading and proper dilution of the cell suspension. The air-dried slides were stained for 13 min in 5% Gurr's Geimsa stain in PBS, washed briefly with water, and air dried. Chromosomes in metaphase spreads were counted at 400× magnification.

In addition, metaphase spreads on replicate slides were stained with 1 μg/ml propidium iodide for fluorescent observation. Propidium iodide-stained metaphase spreads were imaged at 1000× magnification using a Zeiss (Carl Zeiss, Thornwood, NY) LSM 410 Confocal Microscope equipped with a 100× plan-neofluor oil immersion lens. The 568-nm line of an argon/krypton laser was used for excitation, and emitted light was filtered through a long-pass 590-nm emission filter.

Antiviral Activity Assay

Antiviral assays were completed as described by Roberts et al. [31] at various passages for CT-1 and CE-1 (see Table 1) and for CE-2B at passage 3. Briefly, 50 μl of each sample of cell culture-conditioned medium (48–96 h) was add-

TABLE 1. Antiviral activity of conditioned culture media.[a]

Sample[b]	bIFN-τ[c] (pM)	bIFN-τ[c] (ng/ml medium)
Polyclonal trophectoderm, P5	1,296	30
CT-1 (P12, 13, 14)	31,098 ± 13,613	715 ± 313
CT-1 (P15, 17, 19)	1,793 ± 643	40 ± 14
CT-1 (P21, 22, 26, 32)	5,097 ± 2,021	110 ± 42
CT-1 (P53, 55)	1,574 ± 223	35 ± 5
CT-5 (P4)	3,888	89
Polyclonal endoderm (P4)	ND	ND
CE-1 (P9, 12, 14)	ND	ND
CE-2B (P3)	ND	ND
STO Feeders	ND	ND
DMEM/199 medium	ND	ND

[a] Assay sensitivity was ≤ 209 pM (4.7 ng/ml).
[b] P, Number of passages in culture.
[c] ND, Not detectable.

ed to 100 μl of medium, and 3-fold serial dilutions were made. After coincubation with Madin-Darby kidney cells for 24 h, cells were challenged with vesicular stomatitis virus; and after 18 h, cell viability was determined. The ability of samples to prevent virus-induced cell lysis by 50% was compared to a laboratory standard of recombinant interferon tau, rbIFN-τ1 (5.4×10^7 IU/mg), which provided 50% inhibition from virus-induced cytolysis at 5.3 pM. Assays were completed in duplicate, and results were reported in pM or ng/ml of IFN-τ in culture medium.

Immunoblot Analysis of Conditioned Medium

Four milliliters of serum-free (SF) medium (DMEM-199 containing 10 μg/ml bovine insulin [Collaborative Biomedical Products, Bedford, MA]) was conditioned for 48 h by confluent monolayers of CT-1 trophectoderm (T25 flasks) that had been washed 4 times with SF medium, the last wash including an incubation of several hours at 37°C and 5% CO_2 atmosphere. The washes were performed to remove traces of serum proteins left behind by the FBS-containing growth medium routinely used in the propagation and maintenance of the cell culture. T25 cultures of bovine endoderm, CE-1, CE-2 and CE-2B, and STO feeder cells alone were similarly treated and allowed to condition the medium for 48 h. CT-1 was assayed at passages 15, 16, and 21. CE-2 and CE-2B were assayed at passage 2. CE-1 was assayed at passages 10, 11, and 14. Conditioned-medium samples were centrifuged at $500 \times g$ for 15 min to pellet cell debris, and supernatants were frozen at −20°C. Conditioned-medium samples (single-strength), preconditioned medium, and diluted adult cow serum were mixed 3:1 with 4-strength loading buffer containing SDS and β-mecaptoethanol. Samples were loaded onto either 8% or 13% polyacrylamide gels, and electrophoresis (PAGE) was performed as described by Laemmli [32]. Proteins were transferred to nitrocellulose membrane (0.2 μm), blocked with gelatin, and probed with polyclonal rabbit antisera to transferrin (Sigma) or anti-bIFN-τ (1:2500 dilution) provided by Dr. Michael Roberts (University of Missouri, Columbia) [33]. Specific immunoreactive antigens were visualized with alkaline phosphatase-conjugated secondary antibody reagents (Sigma).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

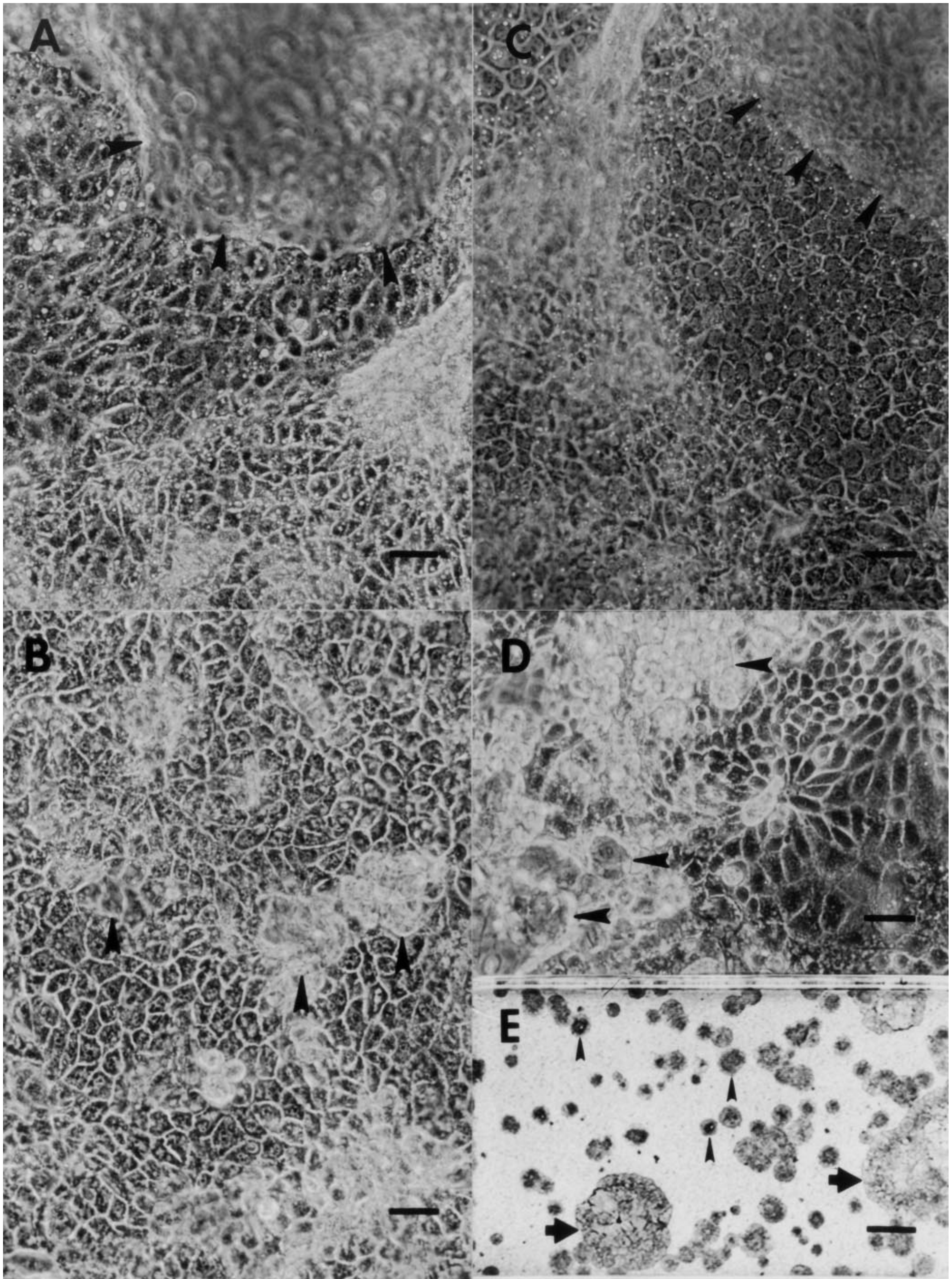
Total RNA was isolated from primary cell culture samples, 500–1000 cells, using RNeasy Mini kit (Qiagen, Chatsworth, CA) or Purescript RNA isolation kit for 100–10 000 cells (Gentra Systems, Minneapolis, MN) according to the manufacturer's protocols. Total RNA was also isolated from an in vivo 17-day bovine conceptus as a positive control for bIFN-τ expression [18] by the method of Chomczynski and Sacchi [34]. RNA transcripts were reverse transcribed to first-strand cDNA using the GeneAmp RNA PCR kit (Perkin Elmer, Foster City, CA) essentially as specified by the manufacturer's instructions. PCR analysis of the RT product was performed with bIFN-τ specific primers, kindly provided by Dr. Michael Roberts (forward primer, 5′-GCCCTGGTGCTGGTCAGCTA-3′ and reverse primer, 5′-CATCTTAGTCAGCGAGAGTC-3′), and with β-actin primers (forward primer, 5′-TACAATGAGCTGC GTGTGG-3′ and reverse primer, 5′-TAGCTCTTCTCCA-GGGAGGA-3′). PCR reactions were prepared as described by the Taq polymerase manufacturer (Perkin Elmer). A 4.5-μl aliquot of the RT product was amplified in a total volume of 25 μl containing single-strength PCR Buffer II (Perkin Elmer), 50 pmol of each primer, 2 mM $MgCl_2$, and 0.625 U Amplitaq (Perkin Elmer). The PCR reactions were carried out in capped PCR microtubes in a Perkin Elmer GeneAmp 9600 PCR System thermocycler. After an initial 2-min heating at 95°C, 50 cycles of 1 min at 95°C for denaturation, 1 min at 56°C for annealing, and 1 min at 72°C for synthesis were carried out. The PCR reaction was completed by 7 min at 72°C followed by a holding temperature of 4°C. PCR products were analyzed on a 1% agarose gel with comparison to a 100-base pair (bp) DNA ladder (Gibco) and had expected product sizes of 584 bp for bIFN-τ and 450 bp for β-actin. The absence of genomic DNA in the RT-PCR reactions was assessed by treating selected RNA isolates with DNase-free ribonuclease A (Boehringer-Mannheim Biochemicals, Indianapolis, IN) prior to RT-PCR. Also, the β-actin primers spanned intron 2 of the β-actin gene, and the presence of genomic DNA would be expected to generate a larger amplicon. All the samples were assayed for β-actin and yielded only the 450-bp amplicon.

Transmission Electron Microscopy

Transmission electron microscopy sample preparation and photomicroscopy were accomplished with the assistance of JFE Enterprises, Brookeville, MD. Trophectoderm culture CT-1 at passage 11 and endoderm cultures CE-2 and its subclone CE-2B (both at passage 2) were fixed in situ with 2.5% glutaraldehyde for 4 h followed by Millonig's phosphate buffer washes [35]. The culture was post-fixed in 1% osmium tetroxide for 1 h. After dehydration in an ethyl alcohol series (70% to 100%, and propylene oxide transitional fluid), the cultures were infiltrated with plastic resin (Spurr's) and polymerized in a 70°C oven. Ultrathin sections (60–80 nm) were stained with 2% uranyl acetate for 1 h prior to examination with a Zeiss EM10 CA transmission electron microscope operating at 60 kV.

Calcium/Phosphate-Mediated DNA Transfection

CT-1 cells were transfected using a calcium/phosphate-mediated DNA transfection protocol [36]. Briefly, 40 μg of DNA in 0.5 ml of 0.25 M $CaCl_2$ was added dropwise to 0.5 ml of double-stength Hepes-buffered saline (HBS 2×) while vortexing. For preliminary experiments, HBS 2× was prepared at pH 6.8 through 7.2 in 0.1 increments. After approximately 10 min of incubation of the DNA precipitate at 22°C, each well of cells of a 6-well plate (35 mm) were


A
B
C
D
E

washed with single-strength HBS of the appropriate pH. The DNA precipitate (0.5–1.0 ml) was then used to replace the single-strength HBS. After an additional incubation of 10 min, fresh medium was added to each well (2.5 ml). After 12–18 h of culture with the DNA precipitate, cells were washed, and fresh medium was replaced. Washing treatments included calcium/magnesium-free PBS, PBS with 1 mM EGTA, or PBS with 10% glycerol for 1 min followed by 3 washes with PBS.

The CT-1 cells were transfected with green fluorescent protein (GFP) reporter plasmids derived from pEGFP-N1 (cat. no. 6085–1; Clontech, Palo Alto, CA) and modified with SV-40 nuclear localization signal sequence. The expression of GFP was driven by either of 4 different promoters as follows: human elongation factor (hEF) [37], phosphoglycerate kinase (PGK) [38], cytomegalovirus (CMV; cat. no. 6085–1, Clontech), and Oct 3/4 [39]. Plasmid DNA was precipitated at pH 6.8, and 40 μg of each plasmid was transfected per 35-mm-well cell culture. The CT-1 cocultures were prepared 3–4 days prior to transfection by plating CT-1 cells, prepared by scrapping and shearing the cells into small clumps, at a 1:5 split ratio.

Cells were assayed for expression of green fluorescence at 48 h after initiation of transfection by blue light excitation (490 nm) epifluorescence using an Olympus (Tokyo, Japan) IMT-2 inverted microscope equipped with an IMT2-RFL reflected light fluorescence attachment.

RESULTS

Cell Culture

Primary outgrowths of bovine trophectoderm were initiated from IVMFC 10- to 11-day hatched blastocysts that were plated on STO feeder cells. The culture system produced hatched blastocysts at 7–8 days with prominent ICMs. Since tissues of the ICM were unwanted, the additional days of culture allowed time for the ICMs to degenerate before the blastocysts were plated (unpublished results). Endoderm outgrowths, recognized by their distinct cell and colony morphology (Fig. 1), were seldom observed to grow out from cultures of 10- to 11-day hatched blastocysts. If endoderm contamination of the primary trophectoderm culture occurred, a micropipette was used to dissect and aspirate the endoderm colony from the culture. This could be accomplished with great efficiency because the endoderm cells were strongly connected to one another so as to form a continuous unit of cells and because the endoderm was not adherent to trophectoderm.

Primary trophectoderm cultures were started by pressing the 10- to 11-day-old IVMFC blastocysts down onto the feeder cells using 25-gauge hypodermic needles. This punctured the blastocysts and helped the trophectoderm cells adhere to the culture substrate. In the first 24 h, many blastocyst cells appeared to undergo some necrosis. However, after several days, outgrowths of healthy trophectoderm cells appeared from the necrotic mass and grew out in a radial fashion. About 75% of the blastocysts gave rise to primary trophectoderm colonies (n ≅ 30). These primary colonies were composed of monolayers of approximately cuboidal cells growing on top of the STO feeder cells with the central-most cells tightly packed and, therefore, smaller and more regular in appearance (Fig. 1A). The primary trophectoderm cells had a granular cytoplasm, and numerous lipid droplets were prominent in these cells, particularly in the central area of the colony.

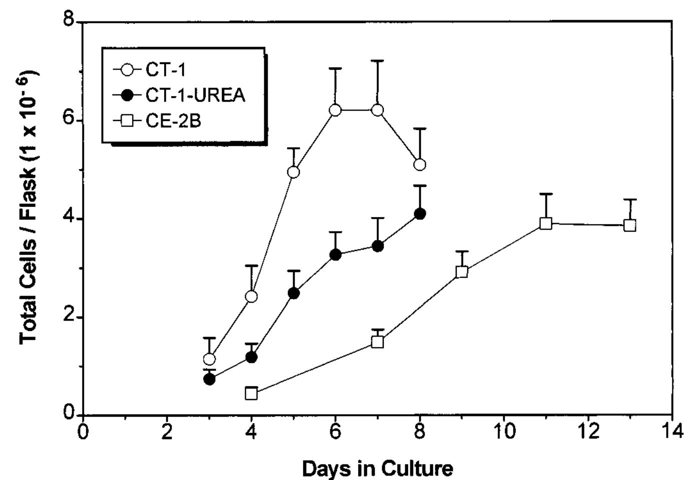


FIG. 2. Cell replication assay over a 10-day culture period. CT-1 cells, CT-1-Urea, and CE-2B cells were grown on STO feeder layers at passage 31, 33 and 10, respectively. CT-1-Urea refers to CT-1 cells that were passaged by the urea/trypsin-EDTA protocol for six consecutive passages prior to assay. Total cells per T25 flask was derived by averaging the counts of eight hemocytometer squares (1 mm^2) for CT-1 cells or 16 hemocytometer squares for CT-1-Urea and CE-2B. Input of CT-1, CT-1-Urea, and CE-2B cells was undefined, but was a 1:10 split ratio for CT-1 and CT-1-Urea and a 1:5 split ratio for CE-2B.

Secondary culture of the trophectoderm was initially done by exposing the colonies to trypsin-EDTA after two washes with Ca^{2+}/Mg^{2+}-free PBS. Trypsin-EDTA had little effect on the cells, and they were poorly separated from one another. The trophectoderm cells could, however, be separated into small clumps of cells with shearing action created by forceful and repeated aspirations with a pipette. To expand the culture, the clumps of surviving trophectoderm were plated onto fresh STO feeder layers in T25 tissue culture flasks. Outgrowths from the dispersed clumps of cells were robust for the first 2–4 passages at 1:2 split ratios. After this time, however, the continued use of trypsin-EDTA was too damaging, and the cultures would not survive or grow well. If, instead, the trophectoderm cultures were dispersed into small clumps of cells by physical shear force alone (see *Materials and Methods*), they did survive and grew well after each passage. By this means a culture derived from a single blastocyst, designated CT-1, was established for characterization.

The CT-1 bovine trophectoderm cells were cultured continuously for over 2 yr and 76 passages with portions frozen every 5–10 passages. CT-1 was passaged at 1:3 or 1:5 split ratios for the first 15 passages and thereafter routinely split at a 1:10 ratio. The CT-1 cells grew without senescent changes being observed over this time. The CT-1 cells grew relatively rapidly with a doubling time estimated at 24 h after a lag period of 2–3 days following each passage (Fig.

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FIG. 1. Phase-contrast photomicrographs of bovine trophectoderm and endoderm in STO coculture. **A.** Primary trophectoderm colony outgrowth; note dome formation (arrowheads). Bar = 44 μm. **B.** CE-2B cell monolayer, passage 6; note vesicle formations and piled cells (arrowheads). Bar = 46 μm. **C.** CT-1 cell monolayer, passage 31; note dome formation (arrowheads). Bar = 46 μm. **D.** Primary endoderm colony outgrowth; note vesicle formations and piled cells (arrowheads). Bar = 52 μm. **E.** Mixed secondary culture of trophectoderm and endoderm colonies fixed and stained with Giemsa stain; note the smaller size and more tightly organized endoderm colony morphology (arrowheads) compared with the larger, more widely spreading trophectoderm colony morphology (arrows). Bar = 6.5 mm.

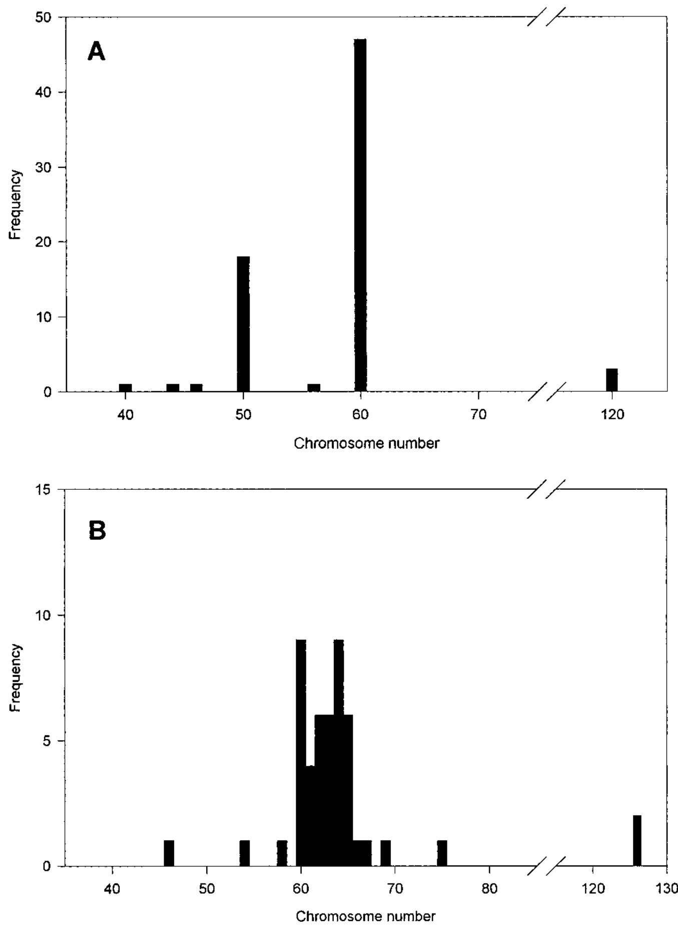

FIG. 3. Karyotype analysis of CT-1 cells. Metaphase spreads of CT-1 cells were prepared and counted at passage 30 (**A**) and passage 54 (**B**).

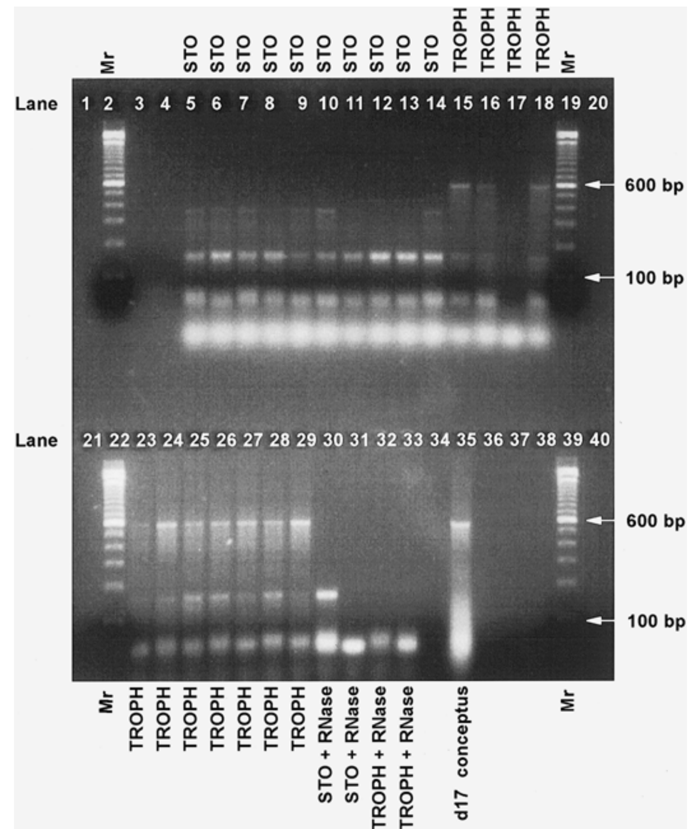

FIG. 4. RT-PCR analysis of bIFN-τ expression in primary trophectoderm colonies grown on STO feeder cells. The cDNA from individual independent trophectoderm primary colonies (TROPH) consisting of approximately 1000 cells or patches of approximately 1000 STO feeder cells (STO) was assayed by RT-PCR using primers specific for bIFN-τ that produced a full-length amplicon of 584 bp from 17-day conceptus cDNA. Mr: relative migration of 100-bp DNA ladder.

2). After passage at a 1:10 split ratio, the cells reached confluency in about 7 days. The secondary cultures were morphologically similar to the primary outgrowths after reaching confluency (Fig. 1B). Prior to reaching confluency, the cells were less distinct and somewhat larger in appearance, being flat, spread out, and thin. The individual colonial outgrowths after each passage enlarged rapidly to produce a distinct macroscopic colony morphology. During this outgrowth the cells often pushed down to the plastic substrate underneath the STO feeder cells. After reaching confluency, the cells became progressively more crowded, and they formed numerous domes by transporting fluid basolaterally (Fig. 1C). The cultures could be maintained at confluency for many weeks by refeeding every 3–4 days with fresh medium. Confluent cultures released noticeable amounts of flocculent material in the medium between feedings. The flocculent material appeared to be composed of detached cells or cellular debris, presumably of trophectoderm origin and not of feeder cell origin because of its constant production. After 1–2 wk at confluency the culture was nearly opaque when viewed macroscopically from underneath.

Primary bovine endoderm cultures were initiated on STO feeder cells from ICMs immunodissected from 7- to 8-day hatched blastocysts. Endoderm cell outgrowths were recognized by their characteristic cell and colony morphology (Fig. 1). Contaminating trophectoderm cells, recognized by their morphology, were rarely observed in the primary endoderm colonies, but, if found, were removed by dissection and aspiration with a mouth-controlled micropipette. Any surviving epiblasts were similarly identified and removed. The primary endoderm cell culture was grown for 2–4 wk before the first secondary passage. The primary colony was composed of approximately cuboidal epithelial cells closely packed together in the center of the colony; cells were flatter and more spread out at the periphery of the colony. The primary colony would frequently have domes or areas of cells that formed vesicle-like structures by transporting fluid between two or three adjacent cells (Fig. 1). The cells were not as granular in appearance as trophectoderm cells, had scant amounts of visible lipid, and, particularly at the colonies' periphery or at domes, had distinct, dark, weblike arrangements of cytoskeletal fibers. The cells grew predominantly on top of the STO feeder cells.

Secondary culture of endoderm cells was accomplished by physically shearing the primary colonies into small clumps of cells and passaging them onto new STO feeder cells. The endoderm cells were more adherent to each other than the trophectoderm cells. Therefore, passage by exposure to urea/trypsin-EDTA (see *Materials and Methods*) was particularly effective and was routinely used in later secondary passages. Exposure to trypsin-EDTA alone was deleterious to the cells and was not effective at separating the cells from one another.

Secondary cultures of endoderm cells were collections of individual colonial outgrowths from individual clumps

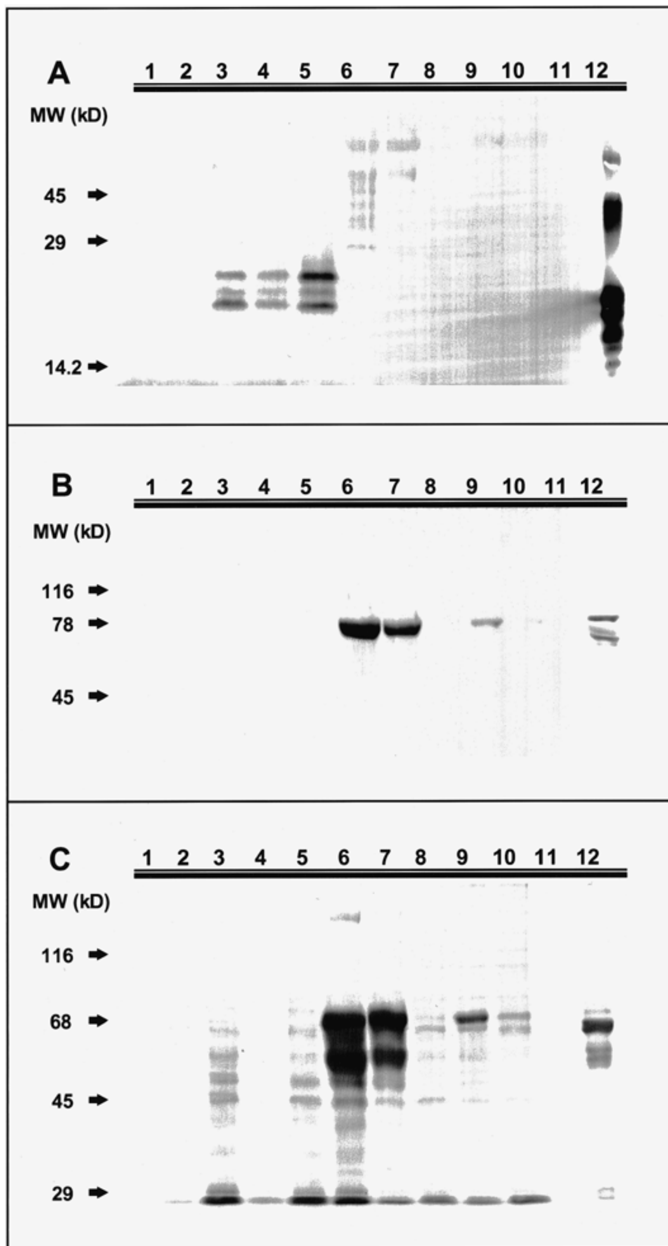

FIG. 5. Immunoblot of CT-1 and CE-2B conditioned medium demonstrating specific expression of bIFN-τ and transferrin, respectively. Western blots of single-strength conditioned medium (48–72 h) were probed with anti-bIFN-τ (**A**) or anti-bovine transferrin (**B**) or were stained for total protein with Coomassie blue (**C**). Lane 1, unconditioned-medium control; lane 2, STO feeder cell-conditioned medium control; lane 3, CT-1 at passage 21 (P21); lane 4, CT-1 at passage 15; lane 5, CT-1 at passage 16; lane 6, CE-2B at passage 2; lane 7, CE-2 at passage 2; lane 8, CE-1 at passage 14; lane 9, CE-1 at passage 11; lane 10, CE-1 at passage 10; lane 11, Blank; lane 12, 75 ng of recombinant bIFN-τ in (**A**), or 0.1 μl of adult bovine serum in (**B**), or 0.05 μl of adult bovine serum in (**C**).

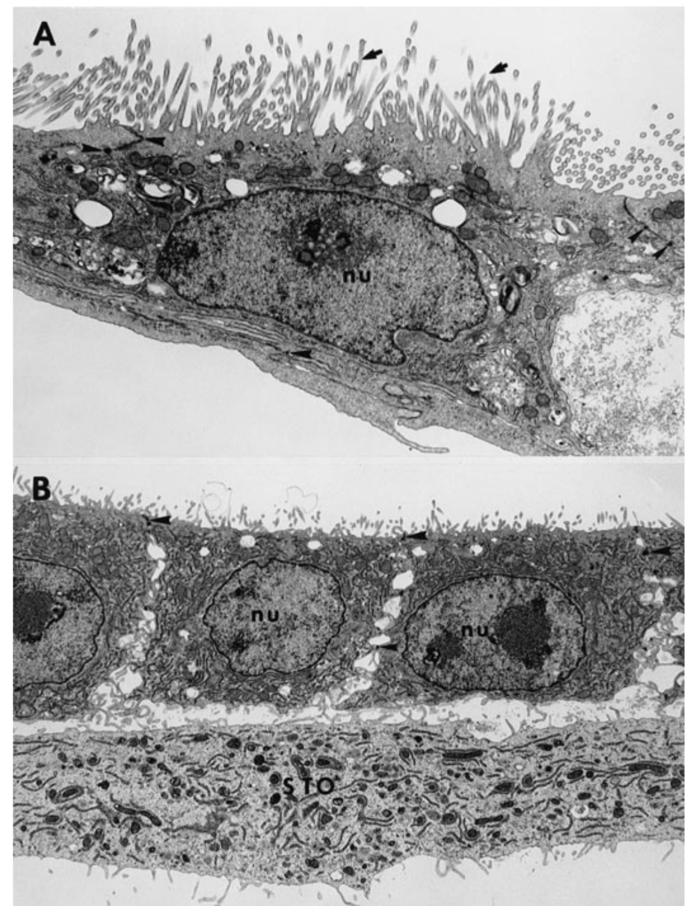

FIG. 6. Transmission electron micrograph of bovine trophectoderm CT-1 cells and bovine visceral endoderm CE-2B cells in culture. **A.** CT-1 cells at passage 11 in coculture with STO feeder cells. Note the numerous and long microvilli at the apical surface of the CT-1 cells (arrows) and desmosomal and tight junction connections between the CT-1 cells (arrowheads). The CT-1 monolayer was elevated above the STO feeder cell substrate as a result of dome formation, i.e., fluid transport under the basal membrane. ×9500. **B.** CE-2B cells at passage 2 in coculture with STO feeder cells. Note large STO feeder cells underneath the three cuboidal CE-2B cells, and tight junctions and the cell-to-cell spot desmosomal attachments at characteristic ladder-like spokes (arrowheads). ×4800. nu, Nucleus. (Published at 49%.)

of cells attached to the feeder cells. Two independent endoderm cell cultures were propagated, designated as CE-1 and CE-2. In both CE-1 and CE-2, an unknown epithelial cell type that grew faster than the typical primary endoderm cell type was discovered. After about 10 passages at 1:2 or 1:3 split ratios, this unidentified epithelial cell took over the CE-1 culture. CE-2 was subcloned at passage 3 by picking individual colonial outgrowths (colony cloning) by mouth-controlled micropipette and transferring each into the well of a 4-well tissue culture plate (Nunc) containing STO feeder cells. By this means, pure cultures of endoderm, as recognized by morphology, were established. The subclones appeared to grow at varying rates but were all similar to the primary endoderm cultures in cell and colony morphology. One of these cultures, designated CE-2B, grew the fastest, with a doubling time of approximately 48 h (Fig. 2); and it was effectively passaged by physical shearing or, subsequently, by the urea exposure method. The colony and cell morphology of CE-2B, and the other subclones, differed from that of primary endoderm outgrowths in that the cells accumulated large amounts of lipid, particularly those located at the center of the colonies. CE-2B was passaged at 1:3 ratios in coculture on STO feeder cells for 12 passages over the 6-mo period of the study. Aliquots of the cells were frozen at several passage levels.

Karyotype Analysis of CT-1 Cells

Cytogenetic analysis of the CT-1 cells was undertaken using urea/trypsin dissociation to create single-cell suspen-

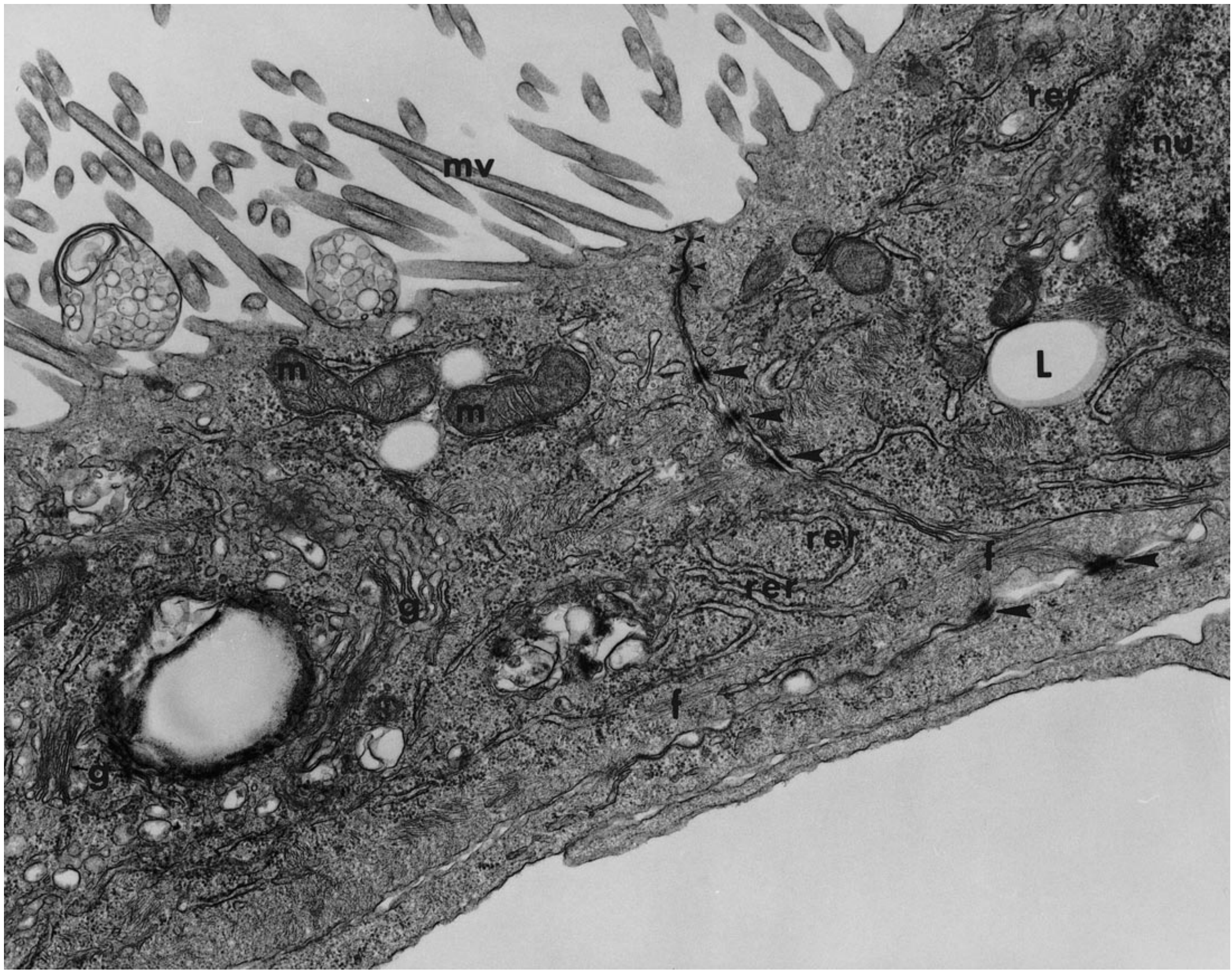

FIG. 7. CT-1 cells in a dome area of the monolayer culture on STO feeder cells at passage 11. Tight junctional area (small arrowheads) and desmosomes (large arrowheads) connected the CT-1 cells together. Note the interdigitated connection between the two cells at their basal membrane and numerous long microvilli (mv) at their apical membrane. ×30 000. m, Mitochondria; rer, RER; g, Golgi complexes; f, microfilaments; L, lipid vacuole; nu, nucleus. (Published at 74%.)

sions (see *Materials and Methods*). Figure 3A shows the distribution of chromosome counts obtained from the enumeration of 73 CT-1 metaphase spreads at passage 30. A bimodal distribution was found in which 64% of the spreads had a normal diploid number of 60 chromosomes. Hypodiploid counts were found in 30% of the metaphase spreads, and, notably, the majority of these (82%) had 50 chromosomes. A few cells were found to have a tetraploid number of 120 (Fig. 3A).

Analysis of 49 metaphase spreads prepared at passage 54 showed a shift to a unimodal distribution with 82% of the cells having between 60 and 65 chromosomes (Fig. 3B). A majority of these were distributed at and around a content of 64 chromosomes (Fig. 3B). The remaining 18% of metaphase spreads consisted of cells with various hypo- and hyperdiploid contents or near tetraploid cells (Fig. 3B). All the chromosomes at both passage levels were acrocentrics or telocentrics with the exception of the X chromosome, which was submetacentric and of which there appeared to be two.

Trophectoderm and Visceral Endoderm mRNA and Protein Expression Analysis

Primary cultures of bovine trophectoderm were assayed for expression of the bIFN-τ gene by RT-PCR and Northern blotting. The RT-PCR analysis detected the predicted 584-bp bIFN-τ amplicon in the Day 17 cow conceptus positive control and in most, 9 of 11, of the independent primary trophectoderm cultures assayed (Fig. 4). This specific amplicon was not detected in primary and secondary endoderm cultures (not shown) or in STO feeder cells, although smaller amplification products of approximately 380 bp or 150 bp were often detected in all tissue culture samples. Pretreatment of the primary trophectoderm cultures with RNase abolished all amplicons from the primary trophectoderm cultures (Fig. 4). Northern blot analysis using a full-length bIFN-τ probe detected a single transcript of approximately 0.8 kb in the 17-day cow conceptus and primary trophectoderm culture total RNAs (not shown), thus confirming the RT-PCR results.

Conditioned media (single-strength) from the CT-1, CE-2B, and STO feeder cells were analyzed for expression of bIFN-τ and transferrin by immunoblotting. Western blots that were reacted with anti-bIFN-τ antibody detected proteins of 20–23 kDa, which would be expected for the differentially glycosylated isoforms of bIFN-τ (Fig. 5A, lanes 3, 4, and 5). Conditioned medium from endoderm cultures CE-1, CE-2, and CE-2B, STO, and unconditioned medium were all negative for reactivity with the anti-bIFN-τ antibody (Fig. 5A). The recombinant bIFN-τ control showed a single protein band by Coomassie staining at the expected size of 19 kDa (not shown). However, the bIFN-τ preparation was apparently contaminated with small amounts of various bacterial proteins that were detected by the antibody preparation (Fig. 5A, lane 12).

Western blots of single-strength conditioned medium showed that CE-2B cells produced numerous proteins in large amounts that corresponded in profile to cow serum proteins (Fig. 5C, lanes 6 and 7). In contrast, CT-1 trophectoderm cell and STO feeder cell-conditioned media did not present a protein profile resembling that of serum (Fig. 5C, lanes 2–5). The endoderm culture taken over by the unidentified epithelial cell type, CE-1, had much less of the proteins produced by the CE-2B clonal culture. Western blots probed with antiserum to bovine transferrin showed strong reaction with a 72-kDa protein as expected in the CE-2 and CE-2B cultures (Fig. 5B, lanes 6 and 7), but little or no reaction in conditioned medium from CE-1 endoderm cultures overgrown by the variant epithelial cell type (Fig. 5B, lanes 8–10). Unconditioned medium and conditioned medium from CT-1 cultures and STO feeder cells showed no reaction with the anti-transferrin antibody (Fig. 5B, lanes 2–5).

Antiviral Activity Assay of Conditioned Media

Conditioned media from CT-1, CT-5, CE-1, CE-2B, and polyclonal secondary trophectoderm cultures and STO feeder cells were analyzed for the presence of antiviral activity in comparison to an unconditioned-medium negative control and a recombinant bIFN-τ positive control (Table 1). Only the conditioned media from the CT-1, CT-5, and polyclonal secondary trophectoderm cultures contained antiviral activity. The amount of antiviral activity in the CT-1 culture varied from sample to sample but did not appear to be related to passage level.

Transmission Electron Microscopy

The CT-1 bovine trophectoderm culture and CE-2B bovine endoderm culture were prepared for ultrastructural analyses at approximately 3 wk postpassage at passage 11 and passage 2, respectively.

CT-1 cells were arranged in a single layer of elongated cuboidal cells growing on top of or between the STO feeder cells (Figs. 6A and 7). The CT-1 cells were usually in close proximity to the STO feeder cells, except where dome formation occurred, but were never intimately joined to the STO cells. The CT-1 cells displayed a polarized morphology with prominent and numerous microvilli at their apical surface facing the medium. The CT-1 cells were joined by numerous desmosomal elements and tight junctional complexes at their lateral surfaces (Fig. 7). Golgi complex, smooth and rough endoplasmic reticulum (RER), mitochondria, and microfilaments were all numerous and well represented in the CT-1 cells (Fig. 7). Large lipid vacuoles were occasionally found, although the lipid was often completely or partially removed during preparation. Overall the CT-1 cells were similar to in vivo bovine trophectoderm examined from expanded preimplantation blastocysts (Fig. 8).

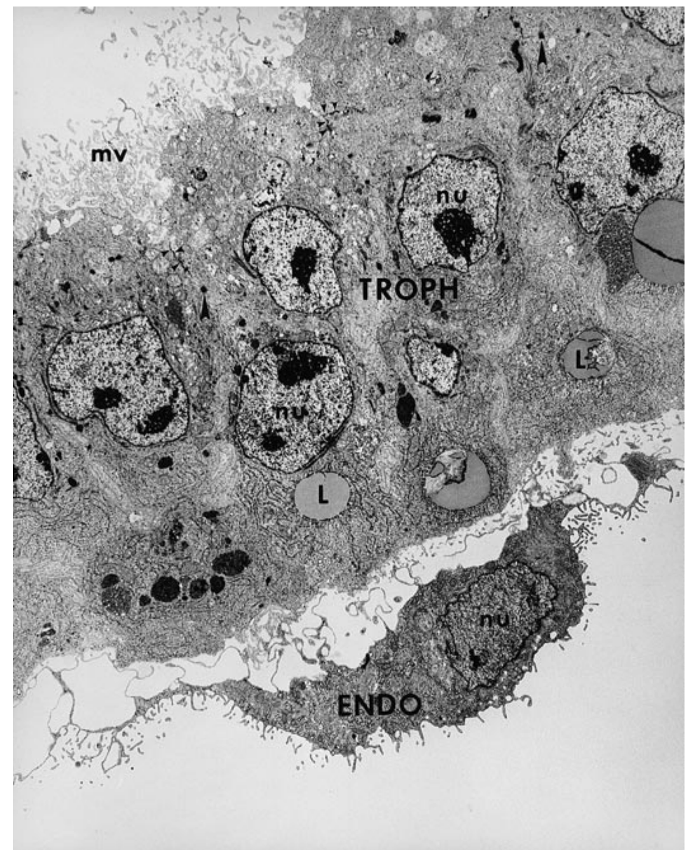

FIG. 8. In vivo cow trophectoderm and endoderm from a 19-day bovine blastocyst. This portion of the expanded blastocyst had closely organized trophectoderm cells (TROPH) with abundant apical microvilli (mv) facing the uterine environment. Note the tight junctional (small arrowheads) and desmosomal (large arrowheads) areas of trophectoderm cell union. The endoderm cells (ENDO) were positioned just underneath the basal membrane of the trophectoderm cells. ×4800. nu, Nucleus; L, lipid vacuole. (Published at 45%.)

The CE-2B bovine endoderm cell culture was composed of markedly cuboidal cells primarily arranged in a single layer growing on top of the STO feeder cells (Figs. 6B and 9). The CE-2B cells were not intimately joined to the STO feeder cells, and they had a polarized morphology with numerous microvilli confined to the apical cell surface. Tight junctional complexes connected the cells to one another, and intercellular lateral connections were also mediated by numerous spot desmosomal connections. Outstanding among the cytoplasmic features of the CE-2B cells were their large amounts of RER and Golgi complex (Figs. 6B and 9). Extensive stacks of RER were observed frequently in the cells. Mitochondria were also abundant, and large lipid vacuoles were observed often (Figs. 6B and 9). Overall the cells were similar in appearance to in vivo bovine endoderm in expanded preimplantation blastocysts except that Golgi complexes were not apparent in the in vivo endoderm (Fig. 8).

Transfection of CT-1 Cells

Transient expression of GFP occurred in the CT-1 cells 48–72 h posttransfection. Of the four promoters tested,

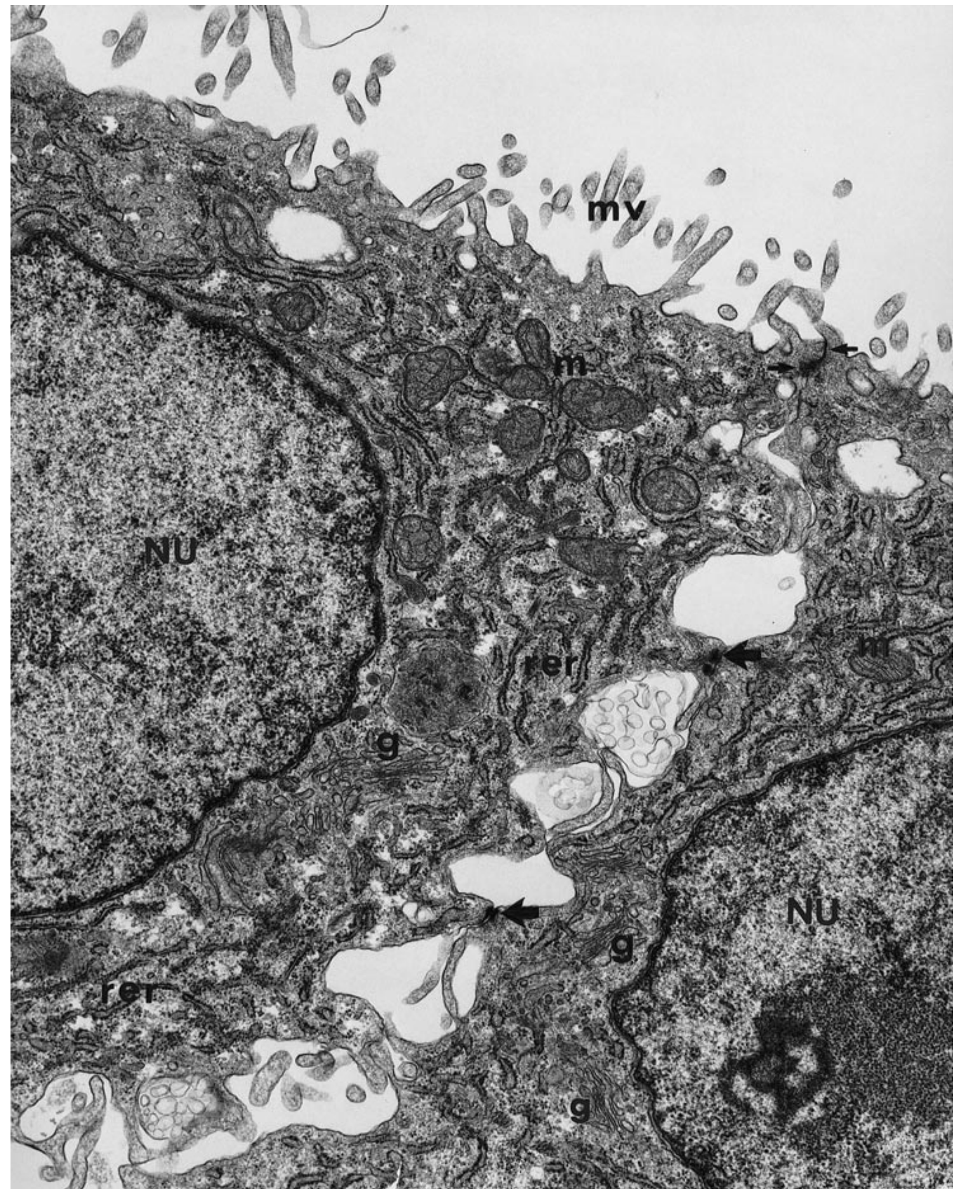

FIG. 9. CE-2B cell culture on STO feeder cells at passage 2. Tight junctional area (small arrows) and desmosomes (large arrows) connected the CE-2B cells together. Note the prominence of RER (rer) and Golgi complexes (g) in the cytoplasm and the smaller, less numerous microvilli (mv) at the apical surface of the cells. ×24 000. m, Mitochondria; NU, nucleus. (Published at 45%.)

hEF promoter gave the most expression, with an estimated 4–5% of the CT-1 cells expressing. The number of CT-1 cells expressing GFP from the CMV promoter was nearly equivalent to that found with the hEF promoter. In contrast, very few (less than 0.1%) GFP-expressing CT-1 cells were observed after transfection with the PGK and Oct 3/4 promoter constructs. GFP expression was usually associated with the peripheries of CT-1 cell colonial outgrowths. GFP expression in STO feeder cells was not observed.

DISCUSSION

Bovine trophectoderm and endoderm cell cultures were initiated and propagated on STO feeder layers from IVMFC hatched blastocysts. The trophectoderm and endoderm cells appeared to retain in vivo-like morphology and function. A specific function of in vivo bovine trophectoderm, bIFN-τ expression, was maintained by the CT-1 trophectoderm culture over extended secondary passage [15–18]. Similarly, the CE-2B endoderm cell culture was continuously cultured, and it exhibited the specific functional characteristic of visceral endoderm in its expression of the protein transferrin [10,40]. Thus, although anatomical source and morphological criteria can be used for identification of these polarized epithelia, expression of bIFN-τ or transferrin provides specific markers to discriminate between the two cell types.

Ultrastructural morphological features of the CT-1 and CE-2B cell cultures were similar to those of in vivo trophectoderm and endoderm. A characteristic in common was the presence of intercellular junctional complexes. The presence of tight junctions and desmosomes defines the cells as epithelial and provides ultrastructural confirmation of vectorial fluid transport across the cells as exemplified by dome formation in vitro and expansion of the blastocyst in vivo. The bunched-up morphology (Fig. 8) of the in vivo cow trophectoderm cells was variable, and in many areas the cells were flatter like the in vitro CT-1 cells (Fig. 6). Microvilli appeared to be more numerous and shorter on the CT-1 cells in comparison with those on the in vivo trophectoderm cells, although this varied. In vivo trophectoderm in general appeared to

have a more dense cytoplasmic matrix than the CT-1 cells, perhaps resulting from the presence of more free ribosomes and possibly glycogen particles [41]. Also, as previously seen in our fixation protocol, lipid contained within lipid vacuoles was better retained by the in vivo trophectoderm and endoderm than by their in vitro counterparts. This apparently reflects some qualitative difference in the lipid as processed and stored under in vitro conditions [42]. RER was prominent in the in vitro cultures, particularly in the CE-2B cells, where stacked RER was commonly observed. However, abundant RER was also observed in the in vivo cells, particularly in the endoderm, and extensive and swollen RER is characteristic of endoderm cells [43–46]. The most prominent difference between in vivo and in vitro conditions in both cell types, but particularly between in vivo endoderm and CE-2B cells, was the extensive and numerous Golgi complexes present in every section of every CE-2B cell (Figs. 7 and 9). In contrast, in vivo endoderm cells contained few and small Golgi complexes. This difference probably reflects a response to in vitro conditions and not a developmentally relevant change, since in vivo ruminant yolk sac endoderm cells (4.5-mm crown-rump length) have few Golgi complexes [44]. However, in humans [43], cats [45], and dogs [46], Golgi complexes are a prominent feature of yolk sac endoderm cells.

Western blot (Fig. 5) and antiviral (Table 1) assays indicated that the levels of bIFN-τ produced by the CT-1 cell cultures were comparable to those produced by blastocyst-stage embryos and could be estimated to be between 100 and 1000 ng/ml [47]. The posttranslational modifications of the CT-1-produced bIFN-τ might be equivalent to the bIFN-τ produced in vivo. The amounts of bIFN-τ transported into the medium by the CT-1 cells could potentially be optimized by adjusting the culture environment. If so, this might be of some utility in the production of native bIFN-τ, although other eukaryotic or prokaryotic expression systems would probably remain more efficient at producing the unmodified bIFN-τ. For example, a recombinant yeast expression system produces apparently fully functional sheep IFN-τ in milligrams per milliliter amounts [48,49].

Chromosomal content of the CT-1 cells assayed at passage 30 showed that the majority of cells in the culture had a diploid complement (Fig. 3A). A few tetraploid metaphase spreads were found; this was not unexpected for trophectoderm cells, since terminal differentiation can produce binucleated cells in ruminants [50]. However, the relatively large number of hypodiploid cells within the CT-1 culture (30%) may have represented a stable subpopulation of abnormal CT-1 cells with about 50 chromosomes, rather than experimental error in metaphase spread preparation. Examples of hypodiploid bovine cell lines exist [51]. However, by passage 54 the chromosome complement had shifted to the point where most cells had slightly more than 60 chromosomes, indicating that the cell population was in flux over this period and becoming near diploid in character (Fig. 3B).

Stringfellow et al. [9] reported a trophectoderm-like cell culture established from a bovine blastocyst designated BE-13. BE-13 cells were similar to CT-1 cells in their sensitivity to trypsin, but otherwise they differed in several ways. BE-13 was established from an 11-day in vivo blastocyst without feeder layer support in Ham's F10 medium supplemented with FBS, insulin, and epidermal growth factor (EGF). In contrast, the CT-1 cells required feeder cells for establishment and for maintenance of phenotype and growth. However, it may be that CT-1 cells would grow slowly in the absence of the feeder cells as evidenced by our observations of pig trophectoderm culture. The presence of EGF and insulin in the medium might enhance such feeder cell-independent growth. Stringfellow et al. [9] found that putative trophectoderm cells lines could not be established from bovine blastocysts less than 11 days old. This contrasts with the present results showing that bovine trophoblast and endoderm cell cultures can be established from prehatched (6–8 day) blastocysts (unpublished results) and even blastomeres dissociated at the morula stage [52]. The cell morphology of the BE-13 cells differed from that of CT-1 cells. The BE-13 cells were squamous-like cells that exhibited prominent stress fibers, dinucleated cells, swollen RER, few Golgi complexes, and microvilli concentrated at one end of the cell. The BE-13 cells were not shown to possess tight junctions and desmosomes, and dome formation was not reported. These morphological features differ markedly from those of CT-1 cells and from in vivo trophectoderm, and may reflect a trophectoderm cell struggling to survive in vitro. In any case, the BE-13 cells senesced after the 38th passage, and a 1:2 transfer ratio at each passage was necessary to achieve this passage level. In contrast, the CT-1 cell culture has been in continuous culture for over 76 passages, with most passages at a 1:10 transfer ratio; and no apparent change in morphology or expression of bIFN-τ has occurred over this time. The CT-1 cells therefore constitute a cell line.

The continuous culture of preimplantation trophectoderm and endoderm on STO feeder cells may be a good in vitro model for studying these cell types. The expression of bIFN-τ and transferrin by CT-1 and CE-2B cells, respectively, distinguishes these cells from other cells that might be cultured from bovine blastocysts such as putative bovine ES cells. The CT-1 cell line and the CE2B cell culture may also be unique reagents with which to study X-inactivation and nuclear totipotency because endoderm and trophectoderm specifically inactivate the paternal X chromosome [53,54]. Also, the bovine blastocyst-derived cell culture established by Stringfellow et al. [9] was susceptible to infection by several bovine viruses as indicated by cytopathic effects and virus titer. The authors speculated that trophoblast cell lines could serve as models for studying the trophoblast tropism of several bacterial agents associated with abortifacient diseases in ruminants [9]. The CT-1 cell line might also be useful for these purposes, assuming that the endogenous bIFN-τ expression would not inhibit viral replication [55]. Primarily, however, the CT-1 cell line and the CE-2B cell culture should be useful for studying cellular, cytokine, and hormone interactions involved with recognition of pregnancy and placentation in ruminants [56,57].

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