CYTOLOGICAL EVENTS INVOLVED IN GLYCOPROTEIN SYNTHESIS IN CELLULAR AND SYNCYTIAL TROPHOBLAST OF HUMAN PLACENTA

An Electron Microscope Autoradiographic Study of [³H]Galactose Incorporation

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ABSTRACT

Electron microscope autoradiography was used to study glycoprotein synthesis in cellular trophoblast (cytotrophoblast) and syncytial trophoblast of term human placental villi incubated in vitro with D-[1-³H]galactose ([³H]gal). Autoradiographs were analyzed using the hypothetical grain analysis of Blackett and Parry (1973. *J. Cell Biol.* **57**:9-15). The results of this study indicated that [³H]gal incorporation into term placental villi was predominantly localized to cytotrophoblast. Utilization of [³H]gal by term syncytial trophoblast was extremely low and yielded too few grains for a quantitative grain analysis. This result is in striking contrast to that found in the preceding study of [³H]leucine incorporation (Nelson, D. M., A. C. Enders, and B. F. King. 1978). Within cytotrophoblast, the rough endoplasmic reticulum incorporated the most [³H]gal into glycoprotein. The Golgi apparatus was another site of [³H]gal incorporation. The vast majority of the [³H]gal incorporated into cytotrophoblast during the pulse incubation remained intracellular through the duration of the experiment. There was little autoradiographic evidence for secretion of tritiated macromolecules. Cytotrophoblast incubated for the longest time period studied (4 h⁺) showed a substantial concentration of tritiated macromolecules in the Golgi complex and in the ground plasm but not in the rough endoplasmic reticulum.

KEY WORDS placenta · glycoproteins · electron microscopy · trophoblast · autoradiography

Tritiated monosaccharides have been used in a number of studies to label glycoproteins whose synthesis and secretion are to be followed by light and/or electron microscope autoradiography. These studies have demonstrated that some glycoproteins that are synthesized remain within the cell (5, 6, 15, 37, 38, 43) while others are added either to the surface coat (3, 4, 6, 23, 28, 29, 41) or to the basal lamina (12, 13). In other cases, the glycoprotein synthesized is destined for secre-

tion (11, 24, 25, 32, 34, 36–39, 50, 51, 53, 56).

The human placenta synthesizes and secretes at least two glycoprotein hormones, human chorionic gonadotropin (HCG),[1] and human chorionic thyrotropin (HCT) (16). In addition, a glycoprotein basement membrane component has been isolated from placental villi (46). A number of poorly characterized pregnancy proteins have also been isolated from placental homogenates, and some of these are glycoproteins (8, 30). While the placenta therefore appears to synthesize a variety of glycoproteins, little is known of the cytological events involved in their synthesis and secretion by trophoblast.

In the preceding paper, both cellular and syncytial trophoblast were demonstrated to have marked capacities for the incorporation of [3H]leucine into proteins (35). To investigate the capacity of both forms of trophoblast to incorporate monosaccharide into macromolecules, term human placental villi were incubated with [3H]galactose and incorporation of radioactivity was followed by electron microscope autoradiography.

MATERIALS AND METHODS

A total of four term human placentas were used. Tissue preparation and incubation conditions were as described in the preceding paper (35), except that Medium 199 (M199) contianed [1H]leucine ([1H]leu) and the radioactive label was 250 μCi/ml (= 42 μM) D-[1-3H]galactose ([3H]gal) (New England Nuclear (Boston, Mass.); lot no. 477-083; sp act, 5.95 Ci/mmol). Chase medium contained 44 mM [1H]gal. Radiochemical purity of [3H]gal in the stock solution and in medium incubated for 40 min with tissue was determined by descending paper chromatography as described by Deppert et al. (14).

To determine whether glutaraldehyde nonspecifically bound [3H]gal to tissue, villi were incubated for 5 min in ice-cold M199 with or without 250 μCi/ml [3H]gal, rinsed 3 times with ice-cold M199 (= 8 min total), and prepared for light microscope autoradiographic analysis. All tissues prepared for electron microscope autoradiographic analysis were fixed for 1 h at room temperature in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) containing 0.05% $CaCl_2$. Subsequent tissue proc-

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[1] *Abbreviations used in this paper:* HCG, human chorionic gonadotropin; HCT, human chorionic thyrotropin; [3H]gal, D-[1-3H]galactose (N); [1H]gal, D-[1-1H]galactose; and M199, medium 199 with Earle's salts gassed with 95% air-5% CO_2 and containing 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml Fungizone.

essing was exactly as described in the preceding paper, except that the phosphate buffer rinse was carried out for only 1–4 h.

RESULTS

Chromatography of [3H]Gal-Containing Solutions

Radiochemical purity of stock [3H]gal was determined to be >97% and >97% of the radioactive species present in medium incubated with tissue chromatographed the same as the stock [3H]gal.

Control Experiment for Fixative Binding of [3H]Gal

Both sets of light microscope autoradiographs prepared from the fixation control experiment exhibited background grain levels, indicating that glutaraldehyde did not nonspecifically bind [3H]gal to tissues.

Light Microscope Autoradiography

Light microscope autoradiographs of tissue incubated with [3H]gal had silver grains located predominantly over the perinuclear cytoplasm of cytotrophoblast, with no apparent concentration in any portion of the cytoplasm. There were also clusters of grains that were not adjacent to a cytotrophoblast nucleus, but were located adjacent to the trophoblast basement membrane and appeared to overlie cytoplasm of syncytial trophoblast. However, electron microscope autoradiography demonstrated these clusters to be overlying processes of cytotrophoblast (see below). The number of grains overlying syncytium was very low in all cases, even in autoradiographs from the longest exposure times (2 wk). Although the endothelium of fetal vessels and other cells in the villous core had some overlying grains, connective tissue in the villi had only background grain levels at all time-points studied.

Electron Microscope Autoradiography: Cytotrophoblast

The compartments analyzed as sources and sites in the hypothetical grain analysis are presented in Table 1 along with the numerical distribution of hypothetical grains actually recorded. Since the distribution of activity was assumed to be random, the total number of grains in each row represents the relative area occupied by the organelle (7).

TABLE I

Distribution of Hypothetical Grains for Uniform Activity throughout the Tissue

Position of Grid Point (Source of Hypothetical Grain)	SITE OF HYPOTHETICAL GRAIN (POSITION OF CIRCLE)											
	GP	RER	RER/ Mit	RER/ Golgi	RER/ N	N	Golgi/ Mit	Golgi	Golgi/ RER/ Mit	BM	PM	Total
Ground Plasm (GP)	20	28	12	12	4	0	2	19	1	17	13	128
Basement Membrane (BM)	3	1	1	1	0	2	0	0	0	18	7	33
Rough Endoplasmic Reticulum (RER)	2	35	20	25	4	2	0	5	9	2	1	105
Mitochondria (Mit)	2	1	21	2	1	0	2	2	3	0	4	38
Golgi	1	3	6	15	0	5	8	52	8	1	3	102
Nucleus (N)	0	4	0	0	11	59	0	2	0	1	0	77
Plasma Membrane (PM)	1	0	0	0	0	0	0	2	0	0	7	10
Total	29	72	60	55	20	68	12	82	21	39	35	493
DISTRIBUTION OF REAL GRAINS												
5 min Pulse	8	72	29	54	8	7	8	81	8	20	26	321
20 min Pulse	9	75	42	65	13	4	3	51	10	23	26	321
40 min Pulse	19	103	63	66	23	14	2	95	18	69	24	496
20 min Chase	17	59	39	59	26	10	7	91	8	52	39	407
40 min Chase	18	53	43	54	18	10	5	74	5	47	31	358
4 h Chase	29	59	30	36	9	5	11	85	4	74	14	356

Table I presenting the sites and sources used in the hypothetical and real grain analyses of cytotrophoblast and the numerical totals for each compartment. The column on the right shows the total number of hypothetical grains analyzed and the total number of real grains recorded from autoradiographs of tissue incubated for the time periods indicated. In the hypothetical grain analysis, the total number of grains in each row provides an estimate for the volume occupied by the organelle assumed to be a source of radioactivity in the cell. The total of each column provides the circle analysis for a grain distribution obtainable from cells assumed to contain radioactivity uniformly distributed. All "chase" incubations followed a 20-min "pulse" incubation in radioactive medium.

The summation of grains in each column provides the circle analysis for a distribution of radioactivity assumed to be uniform (7; see also reference 54). By use of sequential computer analysis of the data obtained from the hypothetical grain distribution,[2] it was possible to make estimates of the relative areas of the organelles (Table II). This is an estimate of the relative volumes occupied by each organelle in the cytotrophoblast represented by the micrographs used in the analysis. As can be seen in Table II, ground plasm,[3] RER and

Golgi complex occupy approximately equal volumes in the cytotrophoblast, and the nucleus somewhat less than these. Table I presents the total number of grains recorded and the distribution of real grains within each organelle compartment at each time-point. The number of hypothetical grains associated with each compartment when the tissue was assumed to contain a uniform distribution of radioactivity was adjusted to fit the real grain data of each time-point. This alteration allowed for the assignment of a "relative activity" for organelles assumed to be a source. Table II presents the relative activity for each organelle at

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[2] Our sincere thanks go to Dr. Nick Blackett from the Institute of Cancer Research, Sutton, Surrey, Great Britain, for completing the analysis of the data by computer.

[3] "Ground plasm" is a compartment used in the Blackett

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and Parry analysis (7), and can be considered synonymous with the phrase "cytoplasmic matrix" or "ground substance."

TABLE II

	Relative Area	RELATIVE ACTIVITY					
		5 min Pulse	20 min Pulse	40 min Pulse	20 min Chase	40 min Chase	4 h Chase
GP	26±1	14±16	10±12	27±10	37±17	32±17	48±14
PM	3±1	10±6	11±5	2±5	9±5	8±4	0±2
BM	5±1	1±2	2±2	11±4	5±4	7±4	18±6
RER	21±2	43±9	61±8	40±8	24±13	27±12	8±7
Mit	8±1	0±1	0±4	0±4	0±5	3±4	0±1
Golgi	21±1	32±7	16±5	19±5	21±5	21±7	26±8
N	16±2	0±1	0±1	1±2	4±3	2±2	0±1

Table II presenting the values of the relative area of each organelle assumed to be a source of radioactivity in cytotrophoblast and the relative activity of each organelle for each incubation period. The relative activity of each organelle as a function of time can be studied by reading across each row. All "chase" incubations followed a 20-min "pulse" incubation in radioactive medium.

each time point.[4] The relative activity estimates in each organelle as a function of time, i.e., reading across each row of the table, indicate that the labeling of ground plasm increased through the entire incubation period, becoming the highest labeled compartment in cytotrophoblast (Fig. 1). Golgi complex labeling was highest in tissue incubated for 5 min, then decreased slightly to a plateau which was maintained throughout the incubation period in chase medium (Figs. 2 and 3). At any one time-point, not all Golgi complexes were labeled (Figs. 2 and 4). Interfaces between RER and Golgi complex were frequently observed to have overlying grains (Figs. 2 and 3). RER had its peak labeling at 20 min (Figs. 2 and 3). The relative activity of RER decreased in cytotrophoblast incubated in chase medium, being lowest after the longest incubation. The basement membrane[5] underlying cytotrophoblast exhibited

[4] The data are presented in tabular (rather than graphic) form so that the relative area of each organelle is presented for convenient comparison with the relative activity at each time-point and so that all time points analyzed can be conveniently presented. (All "chase" incubations were preceded by a 20-min pulse in radioactive medium.)

[5] In the circle analysis, the 50% probability circle centered on grains overlying basal lamina generally included the basal plasma membrane and/or the collagen fibers of the reticular lamina. The phrase "basement membrane" is used in this study to be inclusive of the above-mentioned structures in designating the interface between the epithelium and stroma.

virtually no labeling after short incubations, increased its relative activity with longer periods of incubation, and had its highest activity in tissue incubated for 4 h in chase medium (Fig. 5). Compared to the relative activity after a 20-min pulse, the relative activity of the basement membrane increased ninefold during the 4-h chase incubation. The connective tissue underlying the basement membrane exhibited background grain levels at all times (Fig. 5). Mitochondria, plasma membrane, and nuclei showed very low levels of labeling at all time-points (Fig. 5).

Table III presents the results of the chi-squared test. Chi-squared values <19 are reasonable data fits at P values of 0.05 or greater. The high value at 20-min chase (45 ± 20) is probably not significant because the variation in x^2, i.e., SE is very high, indicating that lower values were obtained in the calculation of the standard error.

Electron Microscope Autoradiography: Syncytial Trophoblast

Electron microscope autoradiographs of tissue incubated with [3H]gal exhibited too few grains overlying syncytium to allow a grain analysis to be performed. Even after exposure periods that yielded high grain densities over cytotrophoblast cytoplasm, very few grains were located overlying syncytium (Figs. 1 and 6). The clusters of grains observed in the light microscope autoradiographs apparently overlying syncytium adjacent to base-
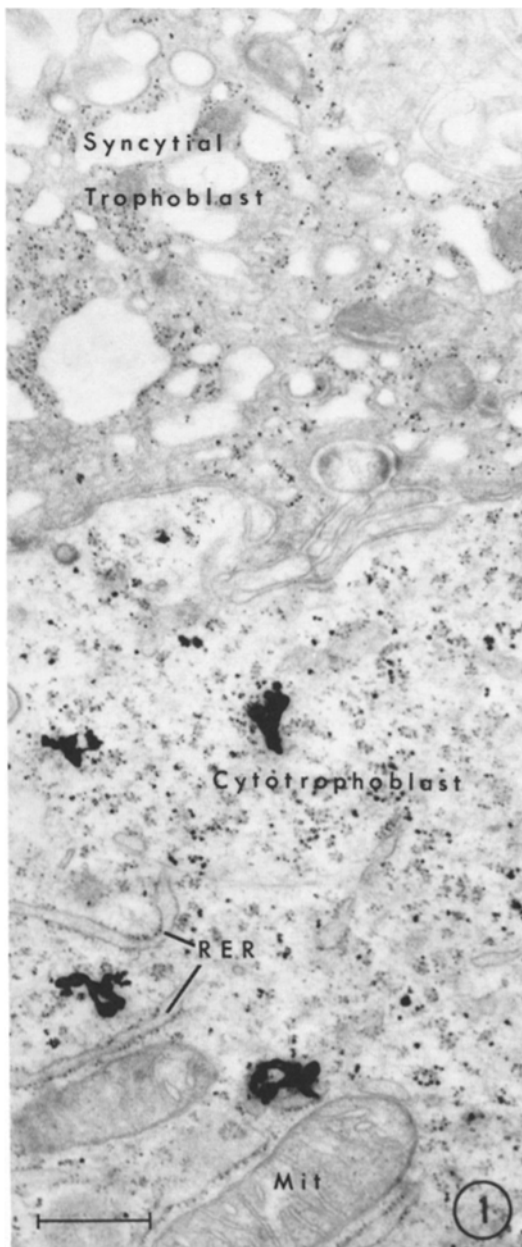
DISCUSSION

This study has demonstrated that [³H]gal incorporation into term placental villi was predominantly localized to cytotrophoblast while utilization of [³H]gal by syncytial trophoblast was extremely low. This result is in striking contrast to that found in the preceding study of [³H]leucine incorporation by trophoblast where both cellular and syncytial trophoblast exhibited marked capacities for incorporation of the radioactive amino acid (35). Within cytotrophoblast, the RER incorporated the most [³H]gal into glycoprotein. The Golgi apparatus was another active site of [³H]gal incorporation. Some of the newly synthesized molecules labeled during the pulse incubation eventually appeared in the cytotrophoblast cytoplasm. A substantial concentration of glycoproteins remained associated with elements of the Golgi apparatus, but not with the RER, in cytotrophoblast incubated for the longest time period studied (4 h⁺). There was little autoradiographic evidence for glycoprotein secretion by cytotrophoblast and no evidence on which to propose a pathway for processing secretory product.

There is evidence to indicate that the carbohydrate chains of glycoproteins are synthesized by the sequential addition of monosaccharides either directly to a polypeptide backbone or to a lipid intermediate that subsequently transfers a growing oligosaccharide chain to the polypeptide (10, 31, 40, 49). Cell fractionation studies have shown that glycosyltransferases are found in high concentration in the Golgi complex especially (21, 33, 44, 45). Electron microscope autoradiographic studies have localized the Golgi complex as a principal site for the incorporation of tritiated monosaccharides into the oligosaccharide chains of glycoproteins (3-6, 18, 22-25, 32, 34, 36-39, 43, 50, 53, 56). The role of the Golgi complex in this aspect of glycoprotein synthesis has been reviewed in detail (52). The addition of monosaccharides to the oligosaccharide chains of glycoproteins occurs in a specific order determined by the sequence of sugars in the oligosaccharide chain itself and is not exclusively the result of Golgi glycosyltransferases. Electron microscope autoradiographic studies of thyroglobulin (25, 53) and immunoglobulin G (56) synthesis have indicated that monosaccharides located proximal to the polypeptide backbone, e.g., mannose, N-acetylglucosamine, are added to the oligosaccharide chains of the glycoprotein molecule while it is still in the RER, while sugars located distally in the
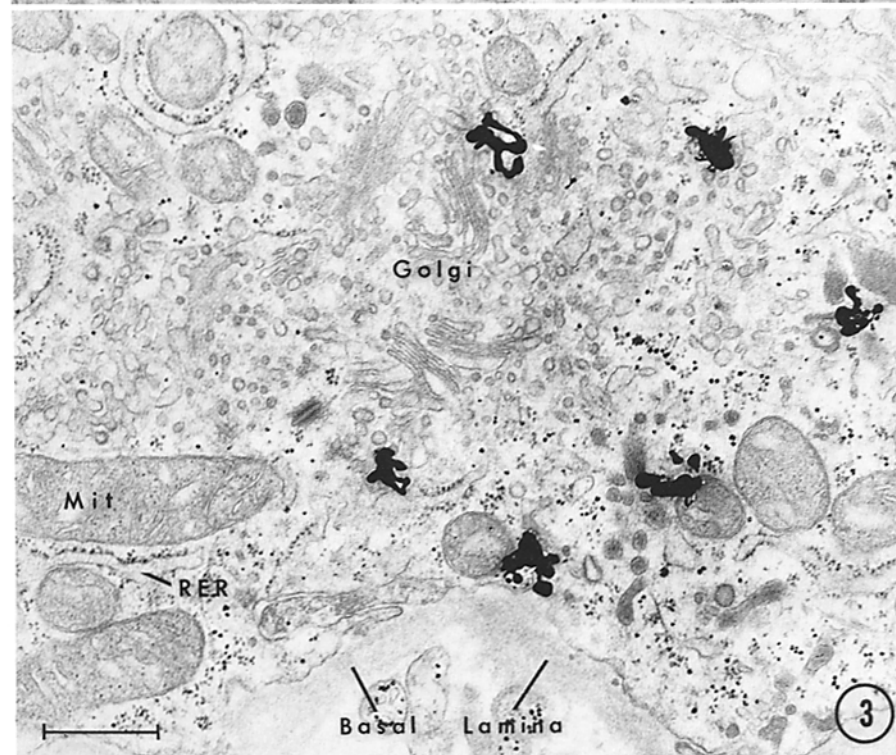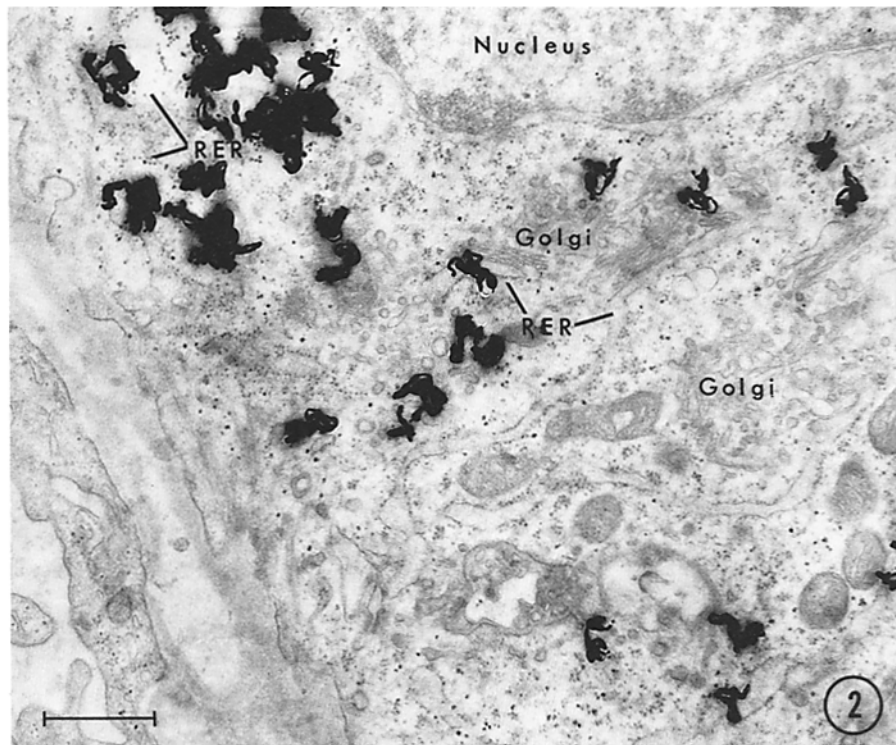

FIGURE 1 Electron micrograph of trophoblast in an autoradiograph of tissue incubated for 20 min in [³H]gal-containing medium and then an additional 40 min in nonradioactive medium. Grains in the cytotrophoblast overlie the ribosome-rich ground plasm in the RER/Mit. No grains are present overlying the syncytial trophoblast. Exposure, 2.5 mo. Bar, 0.5 μm. × 30,000.

ment membrane were, on examination of electron microscope autoradiographs, seen to be located overlying cytotrophoblast processes (Fig. 6).

FIGURE 2 Electron micrograph of a portion of a cytotrophoblast cell in an autoradiograph of tissue incubated for 5 min in [³H]gal-containing medium. A high grain concentration overlies the tangentially sectioned RER in the upper left of the figure. Some Golgi areas have a few overlying grains. Several profiles of RER apparently fusing with Golgi membranes are present. Exposure, 2 mo. Bar, 0.5 μm. × 30,000.

FIGURE 3 Electron micrograph of cytotrophoblast in an autoradiograph of tissue incubated for 20 min in [³H]gal-containing medium and then an additional 20 min in nonradioactive medium. The numerous Golgi-associated vesicles have overlying grains, but the RER/Mit of this cells is unlabeled. Exposure, 4 mo. Bar, 0.5 μm. × 30,000.
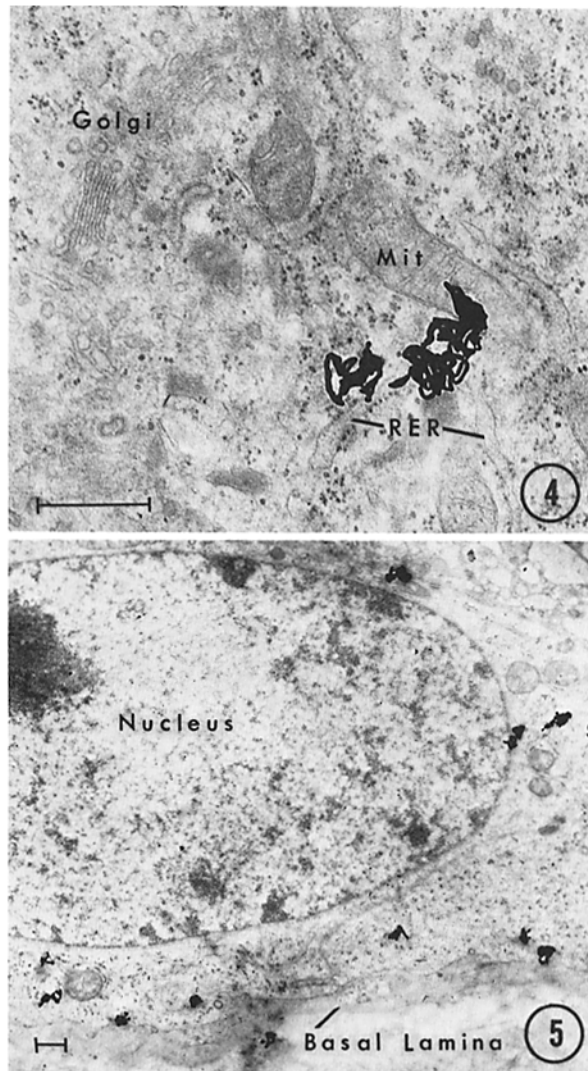
FIGURE 4 Electron micrograph of cytotrophoblast in an autoradiograph of tissue incubated for 5 min in [³H]gal-containing medium. The RER adjacent to mitochondria (Mit) in this cell evidenced overlying grains, but no grains were associated with any of the Golgi stacks present. Exposure, 1 mo. Bar, 0.5 μm. × 30,000.

FIGURE 5 Electron micrograph of cytotrophoblast in an autoradiograph of tissue incubated for 20 min in [³H]gal-containing medium and an additional 4 h in nonradioactive medium. Grains are distributed along the trophoblast basement membrane and throughout the cytoplasm of the cell but are absent from the nucleus. Exposure, 3 mo. Bar, 0.5 μm. × 8,100.

oligosaccharide side chains, e.g., galactose, fucose, are incorporated at the level of the Golgi complex. Recent biochemical studies by Waechter and Lennarz (see reference 49 for review) suggest that a lipid intermediate is important in receiving some monosaccharides, e.g., mannose, N-acetylglucosamine, from nucleotide-sugar donors. The sequential addition of specific sugars to such lipid carriers serves to build at least a part of the oligosaccharide side chain of some glycoproteins before the time when the chain is transferred to the polypeptide backbone of the glycoprotein molecule. Galactose transfer to lipid intermediates has not been studied in detail (49). Autoradi-

TABLE III

Incubation Time		$X^2 \pm SE$	Degrees of Freedom
5 min	Pulse	16±10	10
20 min	Pulse	13±10	10
40 min	Pulse	28±14	10
20 min	Chase	45±20	10
40 min	Chase	26±11	10
4 h	Chase	17±10	10

Table III giving the results of the chi-squared analysis of the grain data for cytotrophoblast.

ographic studies of a wide variety of cells have demonstrated [³H]galactose to be incorporated into the oligosaccharide chains of glycoproteins predominantly in the Golgi complex (3, 18, 22–24, 34, 36, 43, 50, 56). This study has shown that the cytotrophoblast RER incorporated the most radioactivity into macromolecules when term villi were incubated with [³H]gal. Whether the galactose was incorporated directly into the oligosaccharide chain of a glycoprotein or into a lipid intermediate is unknown but would be an interesting possibility to investigate. The Golgi apparatus was another site of incorporation of radioactivity in these studies. The observations provide suggestive evidence for the presence of galactose-containing macromolecules in both the RER and Golgi complex. Biochemical studies are needed, however, to confirm the presence of macromolecular bound [³H]galactose in these organelles.

Similarly, biochemical studies are needed to identify and characterize the macromolecules containing radioactivity in the ground plasm of cytotrophoblast, although the radioactivity is assumed to be due to [³H]gal-containing macromolecules. The presence of ground plasm labeling in tissue incubated for longer, but not shorter times does suggest that some galactose-containing macromolecules from RER and/or Golgi apparatus enter the cytoplasm. Studies of glycoprotein formation in liver cells indicated that cytoplasmic glycoproteins existed and that some of these were released from the Golgi complex (1, 2, 19). Cytotrophoblast undergoes division, differentiation, and fusion in a process that forms more syncytial trophoblast (9, 20, 42). Ground plasm labeling detected

in this study may represent glycoproteins that contribute to these processes.

Recent immunofluorescence studies of human placenta suggest that cytotrophoblast cells synthesize some of the pregnancy-specific β_1-glycoprotein found in maternal serum (48). Whether cytotrophoblast contributes to the production of HCT, a glycoprotein hormone whose site of production in the placenta has not been localized (26, 27), is unknown. Glycoproteins synthesized by cytotrophoblast presumably do not include HCG since this hormone has been localized to the syncytial trophoblast (17).

Why the incorporation of [³H]gal by syncytial trophoblast was at such a low level is somewhat of an enigma. On the basis of its cytologic appearance and presumed secretory functions (55), syncytial trophoblast was expected to be the predominant location for the incorporation of a glycoprotein precursor such as [³H]gal. It is unlikely that the low level of incorporation is due to poor viability of syncytium since syncytial trophoblast incorporates [³H]leucine actively when incubated under the same conditions used in this study (see companion to this paper). In addition, the small number of grains overlying syncytium can not be attributed to grain loss during processing of the autoradiographs since the adjacent cellular trophoblast cells had numerous grains present.

Only term placental tissue was used in this study, and, thus, the glycoprotein synthetic activity of syncytial trophoblast at the end of its normal lifespan is reflected by this study. Syncytial trophoblast from placentas obtained earlier in gestation may be more active in glycoprotein synthesis.
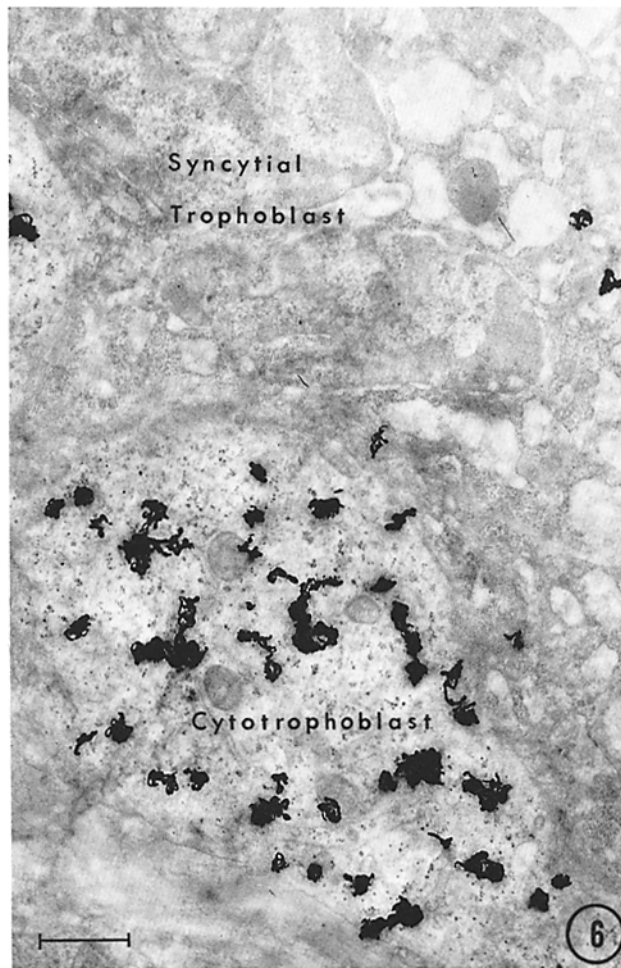
FIGURE 6 Electron micrograph of syncytial trophoblast adjacent to a cytotrophoblast process in an autoradiograph of tissue incubated for 40 min in [³H]gal-containing medium. A high grain density is present overlying the cytotrophoblast process while the syncytium has very few grains. Exposure, 2.5 mo. Bar, 0.5 μm. × 23,600.

In addition, secretion by cellular and/or syncytial trophoblast may be more active earlier in gestation so that an autoradiographic study of villi obtained earlier in gestation might provide the information about intracellular pathways followed by placental secretory products that this study was unable to illustrate. One glycoprotein secretory product, HCG, has been localized to the syncytium and is known to contain galactose (47). However, placental secretion of this glycoprotein hormone is highest in first trimester and lowest in term placentas. Thus, a small amount of synthesis by the large mass of syncytium present in the term placenta could provide the serum concentrations present at the end of gestation and yet

synthesis of HCG molecules might not be detected by an electron microscope autoradiographic study of glycoprotein synthesis.

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