Cytological Distribution of Chorionic Gonadotropin Subunit and Placental Lactogen Messenger RNA in Neoplasms Derived from Human Placenta

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ABSTRACT Normal trophoblast of the human placenta elaborates at least two major protein hormones, chorionic gonadotropin (hCG), and placental lactogen (hPL). There are several gestational trophoblastic diseases of the placenta called hydatidiform mole, invasive mole, and choriocarcinoma. Molar and choriocarcinoma tissues characteristically synthesize large amounts of hCG and small quantities of hPL. To examine the role of trophoblast differentiation in the expression of the hCG and hPL genes, we studied the cytological distribution of their messenger RNA (mRNA) in tissue sections of human hydatidiform mole and choriocarcinoma by in situ hybridization. Histologically, these tissues are in different stages of cellular differentiation. In normal placenta, hCGα and -β mRNA can be localized to some cytotrophoblasts and primarily to the syncytium, whereas hPL mRNA appears only in the syncytial layer. In hydatidiform mole, which still retains placental villous morphology, the hPL gene and hCG α and -β genes are expressed but are poorly localized because of the admixture of cyto- and syncytiotrophoblasts. By contrast, choriocarcinoma, which is devoid of placental villous pattern but in which the cyto- and syncytiotrophoblast-like components are distinguishable, expresses hCGα and -β in the syncytial-like areas but little, if any, hPL.

These results suggest that a certain level of trophoblast differentiation, such as villous formation, is associated with hPL expression, while the hCGα gene and the hCGβ gene can be expressed in more disorganized tissues that contain cytotrophoblastic elements.

One of the unique features of the human placenta is its continued differentiation during gestation. Progeny of mitotically active mononucleated cytotrophoblasts fuse to form the mitotically inactive syncytiotrophoblast (1–3). During this differentiation two major peptide hormones are produced, human chorionic gonadotropin (hCG) and human placental lactogen (hPL).[1] Their temporal appearance in maternal serum during pregnancy is different: hCG peaks in the first trimester, while hPL reaches maximal levels at term; thus, the factors controlling their expression are not identical (4–7).

Gestational abnormalities of trophoblast development occur and are called hydatidiform mole, invasive mole, and choriocarcinoma (8–12). Hydatidiform mole is a relatively benign disease of the chorionic epithelium that metastasizes and is invasive in only a small percentage of cases. Invasive mole invades the uterine muscle but retains the same morphological features of hydatidiform mole. Choriocarcinoma is a malignant counterpart that metastasizes early in its course. The risk of choriocarcinoma after molar pregnancy is 2,000–4,000 times higher than after normal pregnancy (8–12). One of the criteria for diagnosing these gestational trophoblastic tumors is the abnormally high serum level of hCG during the course of the disease. In contrast, serum hPL is very low or undetectable (13–17). Therefore, it appears that the aberrant expression of these hormonal genes in these tumors corresponds in some way with alterations in the pathway of placental differentiation. Previously we demonstrated that in normal placenta the cytological distribution of hCGα and

[1] *Abbreviations used in this paper:* hCG, chorionic gonadotropin; hPL, placental lactogen; mRNA, messenger RNA; cRNA, cyclic RNA.

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hPL mRNA are different (21). On the basis of these data we suggested that the expression of these peptides is linked to and/or regulated by the pattern of placental differentiation. The availability of the trophoblastic tumor tissue permits us to test this hypothesis since these neoplasms are in different stages of trophoblast differentiation. Here we report studies of the cytological distribution of the hCGα, hCGβ, and hPL mRNA in choriocarcinoma and hydatidiform mole tissue using the in situ hybridization technique.

In particular, we assessed whether these mRNA are transcribed in tissue components that are similar to their normal placenta counterparts and whether their expression is dependent on particular stages of trophoblast differentiation.

MATERIALS AND METHODS

Preparation of Double-stranded Complementary DNA Probes: pBR322 clones bearing cDNA complementary to hCGα, hCGβ, and hPL mRNA were isolated and purified as previously described (18, 19). The following fragments were used as probes in this study: (*a*) a 440-base-pair PstI restriction fragment that contains the complete 5′ noncoding sequence and all but the last eight amino acids of the coding region of the hCGα subunit; (*b*) a PstI fragment of 540 base pairs that contains the information corresponding to the codons for amino acid 60 to the end of the peptide and the contiguous 3′ noncoding region of hPL; (*c*) an HhaI fragment of 416 base pairs containing 20 base pairs of pBR322 flanking sequences, 90 base pairs of 5′ nontranslated sequence, and the remaining sequences correspond to the translated region of hCGβ (22). Sequence information regarding the 5′ nontranslated region of hCGβ was not known. In our effort to characterize this clone we sequenced the insert by Maxam-Gilbert sequencing gels (20). The sequence of the 90-base-pair nontranslated region is the following:

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−90                     −75
GCG  GCA  ACG  GCA  ACA  ACC  TCC  AGT
          −60                      −45
GCC  CTT  GCC  TGC  CCC  CAC  AAC  CCC
                    −30
GAG  GTA  TAA  AGC  CAA  GTA  CAC  GAG
     −15                 −1
GCA  GGG  GAC  GCA  CCT  TGG
+1
ATG  GAG  ATG  TTC.......
Met  Glu  Met  Phe
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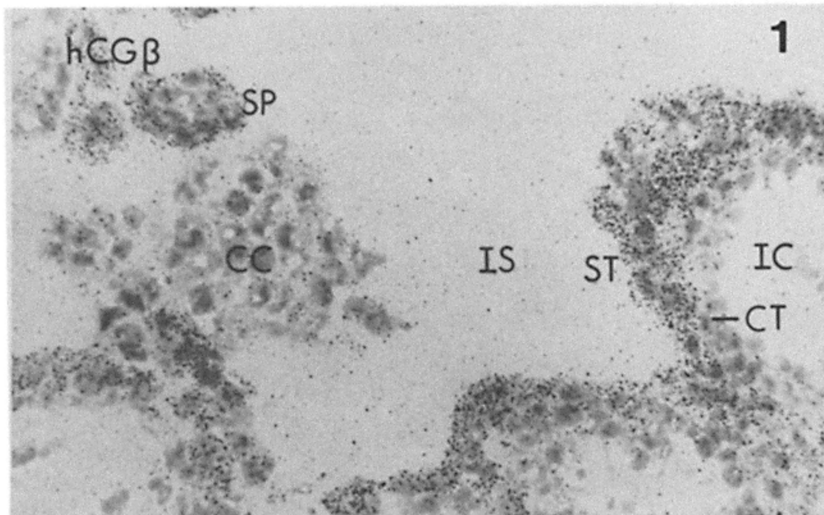


FIGURE 1 Photomicrograph of an autoradiograph of first-trimester (FT, 8 wk) placental tissue (5-μm cryostat sections) hybridized with 3.5×10^5 cpm of hCGβ probe (1-wk exposure, hematoxylin-eosin stain). Silver grains are observed primarily over syncytiotrophoblast (*ST*) and syncytial sprout (*SP*) regions, and some are present over the cytotrophoblasts of villous surface (*CT*). Weak signals are seen over the cell column (*CC*) which consists of undifferentiated cells. *IS* and *IC* denote intervillous space and interstitial cells (villous stroma), respectively. × 200.

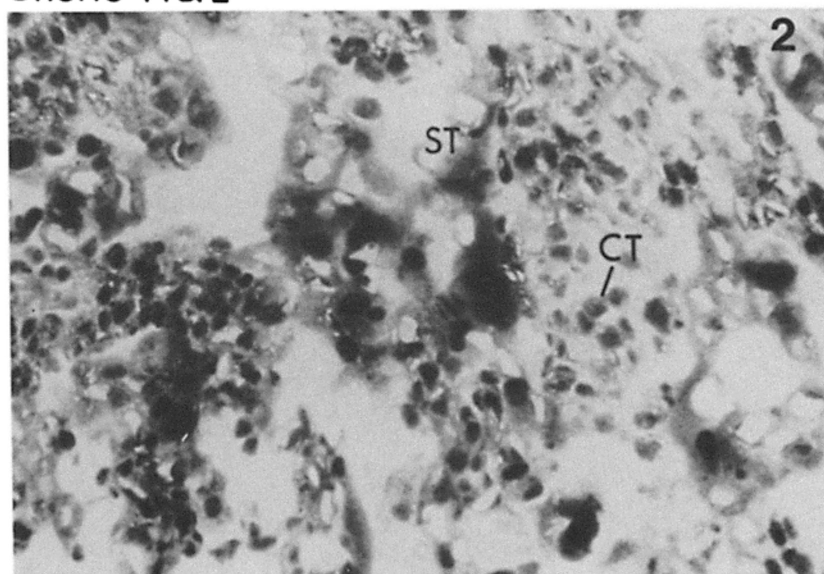


FIGURE 2 Photomicrograph of hematoxylin-eosin stained section (5 μm) of choriocarcinoma obtained after hysterectomy. In contrast to that seen in normal placentae, clusters of pleomorphic mononucleated cytotrophoblastic cells (*CT*) are interlaced by syncytial cells (*ST*). *CT* are apparently mononucleated cells, having discernible cell boundaries, a light cytoplasm, and a clear nucleus with one or two prominent nucleoli. *ST* have no discernible cell boundaries, a hazy darkened (eosinophilic) cytoplasm, and a characteristic multinucleated morphology. *ST* are pleomorphic and have numerous lacunae creating a foamy appearance. No villous pattern can be observed in this tissue. × 200.

Preparation of [^{3}H]cDNA: The fragments were nick-translated in a 10-μl reaction mixture containing 200 ng of fragment, 700 pmol each of ([^{3}H]-deoxy-CTP), [^{3}H]dTTP, [^{3}H]dGTP, and [^{3}H]dATP (New England Nuclear, Boston, MA), 25 pg of DNaseI, and 5 units of DNA polymerase I. The reaction mixtures were incubated overnight at 15°C. The specific activity of the radioactive cDNA probe was 7.0–8.7 × 10^7 cpm/μg and the size of the fragments was 60–80 base pairs under nondenaturing conditions. Salmon sperm DNA (denatured in NaOH and neutralized with HCl) was added to the labeled fragments and they were separated from unincorporated nucleotides by gel filtration on Sephadex G50-80, and the excluded material was ethanol precipitated. The precipitates were pelleted in an Eppendorf centrifuge and dissolved in water to a final concentration of 4 ng/μl.

Tissue Preparation and Slide Glass Treatment: The tissues were obtained either from hysterectomies or vacuum curettage, and immediately frozen in liquid nitrogen. In situ hybridization was performed using techniques described previously (21) except for the following modifications. After overnight hybridization, the slides were rinsed for 2 h in one times standard saline-citrate buffer containing 0.1% SDS and for 1 h in 0.1 times standard saline-citrate buffer. Microscope slides were pretreated with autoclaved gelatin instead of bovine serum albumin to avoid possible RNase activity in the albumin.

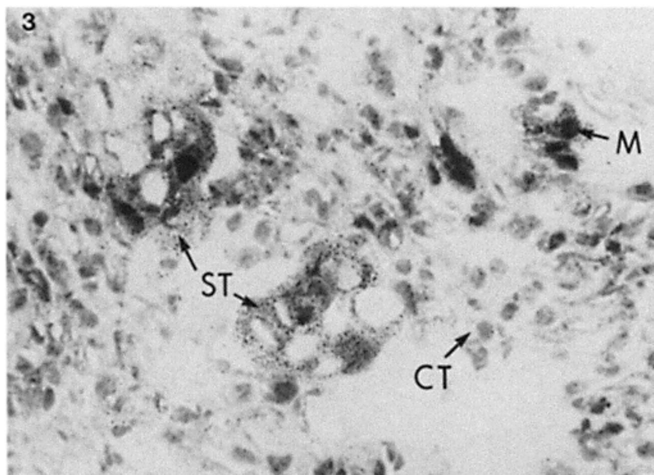
Autoradiography: The slides were dipped in nuclear track emulsion (Kodak NTB-2) diluted with water (1:1) and prewarmed to 45°C (21). They were dried in an upright position and exposed in a light-proof box for 1 wk at 4°C. The slides were developed with Kodak D-19 developer for 3 min at 20°C, rinsed in cold water, fixed with Kodafix for 5 min at room temperature, and stained with hematoxylin-eosin.

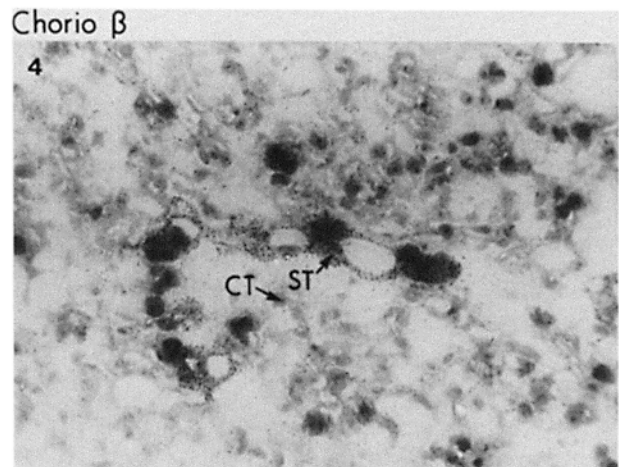
RESULTS

Normal villi of first trimester placenta are covered by syncytiotrophoblast (syncytium; Fig. 1, ST) that overlies a layer of cytotrophoblasts (Langhan cells; Fig. 1, CT) and villous core tissue (Fig. 1, IC). Also present in many villi are collections of cytotrophoblasts referred to as the cytotrophoblastic column (Fig. 1, CC). In this tissue, we previously showed hPL mRNA is localized exclusively to the syncytium and that hCGα mRNA is localized to syncytium and some cytotrophoblasts (21).

Fig. 1 shows the distribution of hCGβ in normal first trimester placenta. Most of the signal was seen on the syncytial regions including the syncytial sprouts and little, if any, was observed on the cell column region. Some cytotrophoblasts beneath the syncytium may contain signal suggesting that the hCGβ subunit mRNA is not exclusively localized to syncytial trophoblast. The signal corresponding to hCGβ mRNA in sections of term placenta was at background level (data not shown). This is consistent with previous observations that the translatable level of hCGβ mRNA from term placenta was undetectable (7).

On the basis of this result and our earlier studies, we proposed that the expression of hCG and hPL is linked to the differentiation of trophoblasts (21). Specifically, we suggested that the transcription of hCG subunit genes is activated during cytotrophoblast/syncytium conversion but before fusion into syncytium. In contrast, hPL mRNA synthesis starts later in trophoblast differentiation, very likely concurrent with or after the stage of syncytiotrophoblast formation and persists to the





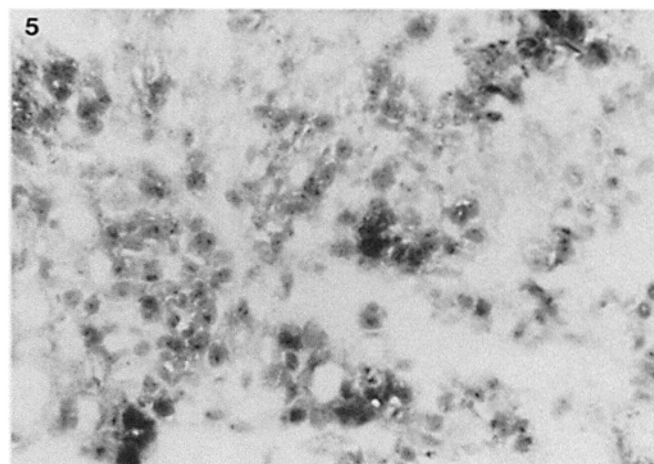


FIGURE 3, 4, and 5 In situ hybridization of sections of choriocarcinoma with hCGα (Fig. 3), hCGβ (Fig. 4), and hPL (Fig. 5) cDNA probes (hematoxylin-eosin). 4 ng of cDNA probe was applied to each slide. This corresponds to 3.2 × 10^5 cpm for α, 3.5 × 10^5 cpm for β, and 2.8 × 10^5 cpm for hPL. The slides were exposed for 1 wk. Multinucleated syncytial cells (*ST*) with prominent vacuolization show well localized signals for hCGα and hCGβ but few, if any, signals for hPL. Cytotrophoblastic cells (*CT*) show no signal except for a mononucleated large cell in Fig. 3 (*M*, see text). All micrographs, × 200.

third trimester.

Because choriocarcinoma contains trophoblasts that are not fully differentiated, we wanted to examine the distribution of hCGα, hCGβ, and hPL mRNA in sections derived from choriocarcinoma tissue. Our hematoxylin-eosin stained sections of this tissue show no villi, which is the typical morphology of this neoplasm (Fig. 2). There are clusters of mononucleated cytotrophoblastic cells interspersed with foci of multinucleated cells reminiscent of syncytiotrophoblasts. Occasionally, some enlarged mononucleated cells are present. After hybridization of choriocarcinoma sections with hCGα and hCGβ probes, abundant grains are seen in syncytial cells (ST, Figs. 3 and 4). Few grains are seen in the cytotrophoblastic cells. The large mononucleated cells, which have syncytial-like cytoplasm but still maintain a recognizable cell boundry, however, also show hybridization of this probe (Fig. 3, M). In contrast to these findings, no signal above the background was seen when sections were hybridized to hPL cDNA probe with comparable specific activity to the hCG probes (Fig. 5).

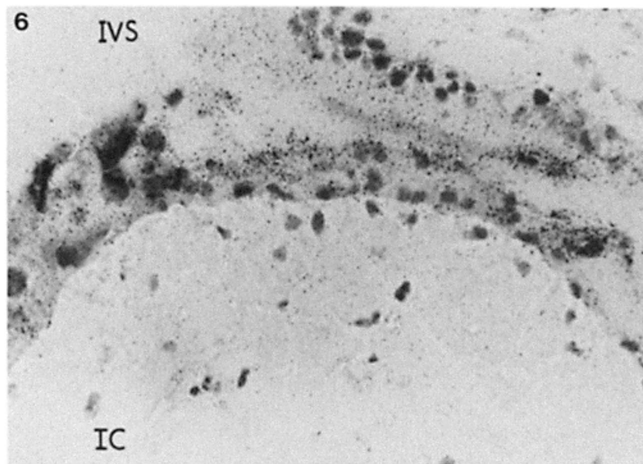
Another form of trophoblast tumor, hydatidiform mole, still maintains the general morphology of a normal placental villus but there is swelling of villous stroma and hyperplasia of the trophoblasts (Figs. 6–8). The latter results in the admixture of cyto- and syncytiotrophoblasts. As expected, sections of molar tissue hybridized significantly with the hCGα (Fig. 6) and hCGβ (Fig. 7) cDNA probes but in contrast to choriocarcinoma sections, a significant hPL signal over the background was seen (Fig. 8).

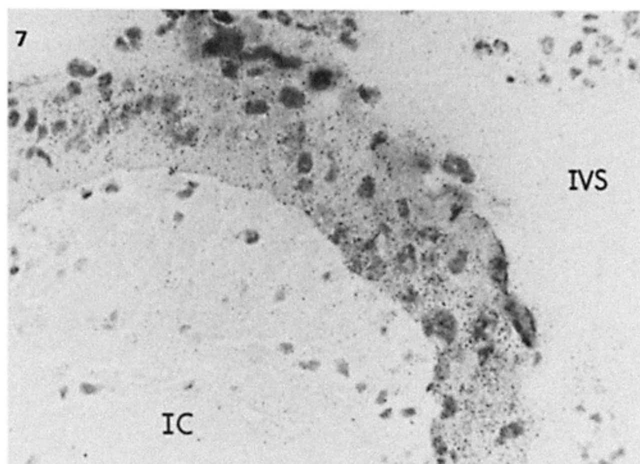
Specificity of Hybridization

Several controls were done to show the specificity of the mRNA:cDNA hybridization. First sections of choriocarcinoma and hydatidiform mole were hybridized to fragments derived from an HhaI digest of pBR322 (Fig. 9). The specific activity of this probe was at least two times higher than the placental cDNA fragments. The amount of DNA probe applied to the slide and the duration of exposure were identical to those used in the hybridization with placental probes. Only a few scattered grains were observed over the sections.

As an additional control, sections of choriocarcinoma were treated with RNase and then hybridized to hCGβ probe (Fig. 10). To ensure that untreated and treated sections were from similar regions of the tissue, we compared nonadjacent sections of each complete series of sections. Before hybridization, sections were incubated with either buffer or RNase for 1 h at 37°C, followed by several washings of twice standard saline-citrate buffer for 6 h at 4°C. In the absence of RNase, strong signals were observed, whereas in the presence of RNase the signal was reduced to background level. The same results were seen with hCGα or hPL cDNA probes (21). Further evidence for the specificity of the hybridization is included in several of the above sections where nontrophoblastic cells such as those of the villous stroma do not contain signal when hybridized to placental probes.

These data together with our results from the specificity of in situ hybridization with sections from normal placenta (21) show that the grains in the trophoblasts were the result of





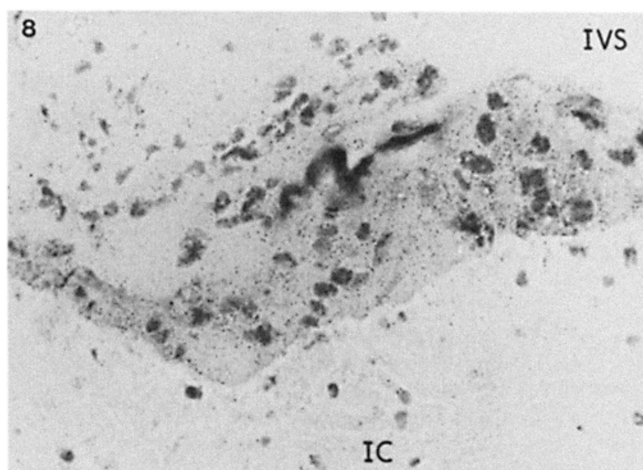
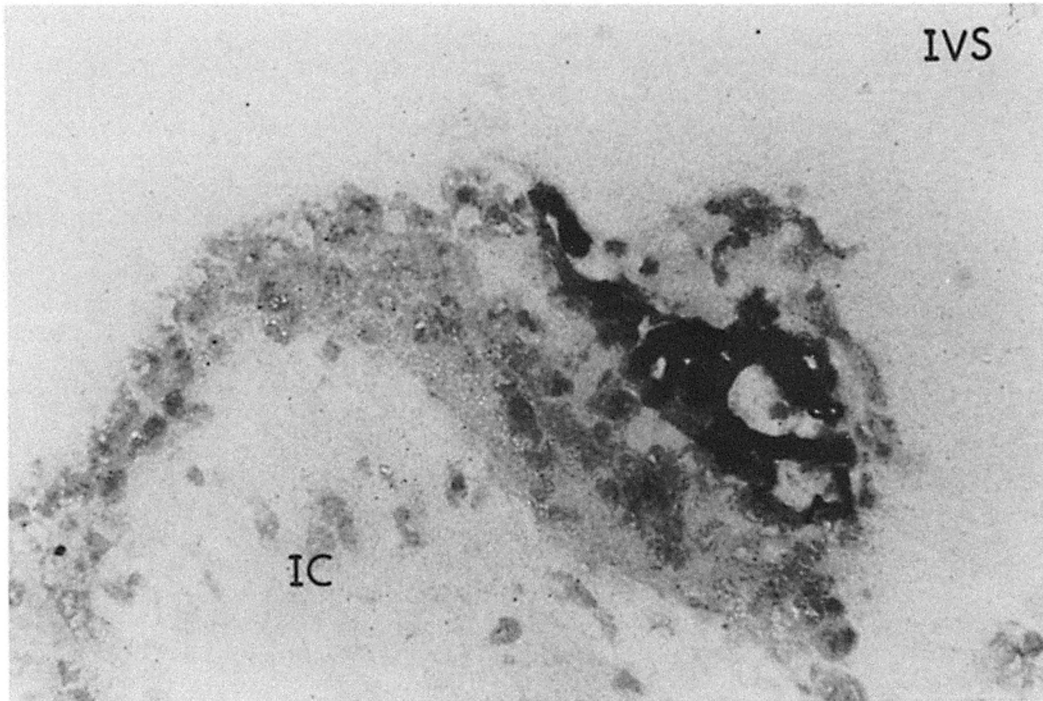


FIGURE 6, 7, and 8 In situ hybridization of sections of hydatidiform mole with hCGα (Fig. 6), hCGβ (Fig. 7), and hPL cDNA probes (Fig. 8). The amount and specific activity of cDNA applied was the same as described for Figs. 3–5. The molar tissue still maintains a significant villous morphology but there is swelling of the interstitial tissue and a large cystic central area. In mole there is hyperplasia of the trophoblast and a loss of the boundary between syncytio- and cytotrophoblast (i.e., layer). The hCGα and -β and hPL mRNA signals are distributed rather homogeneously throughout villi. All micrographs, × 200.

Mole pBR



Chorio pBR

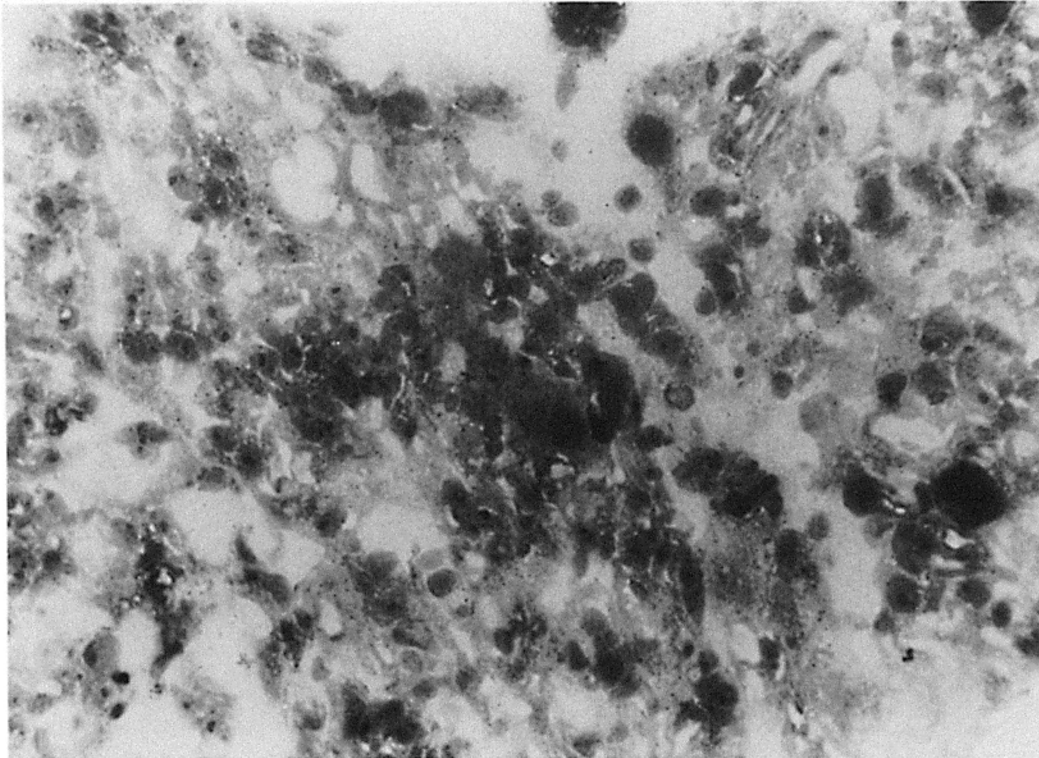
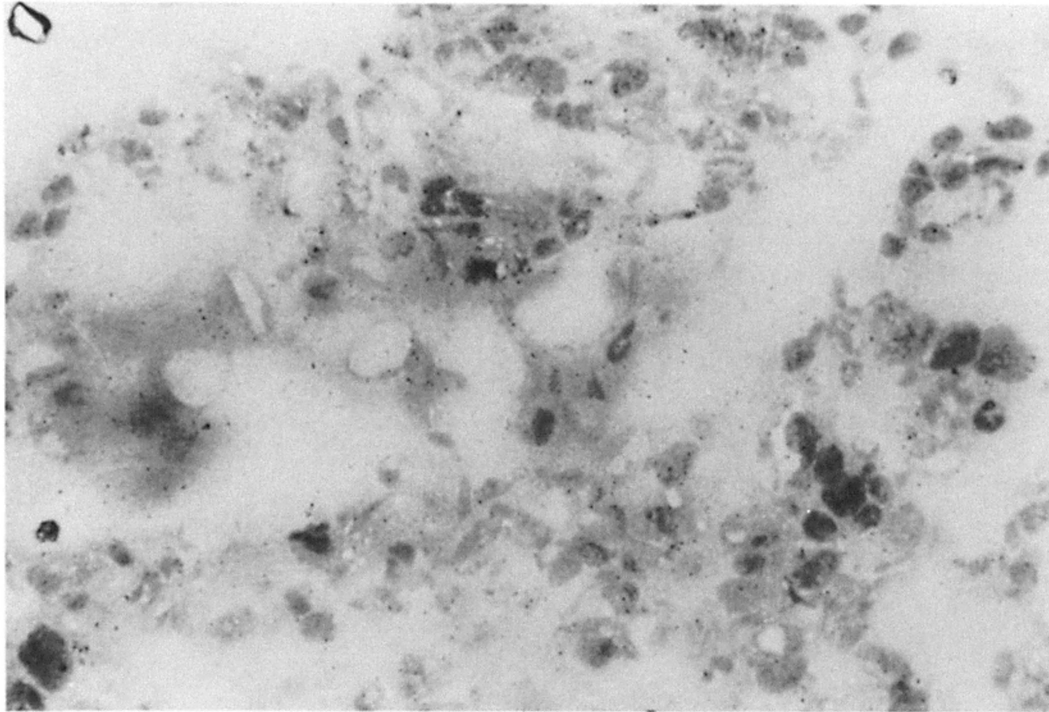


FIGURE 9 Hybridization of molar and choriocarcinoma sections with a nick translated Hha I digest of pBR322 (8.0×10^5 cpm/slide, 1-wk exposure). × 200.

specific DNA-RNA hybrids.

The data presented here are the results of analyses on over 100 sections of two hydatidiform moles and one case of choriocarcinoma. Because of the low incidence of these diseases, especially choriocarcinoma, it is difficult to obtain nonnecrotic tissue. Regarding this point it is crucial that the tissue examined here was the actual tumor; analyses were not performed on cells derived from the tumors obtained after



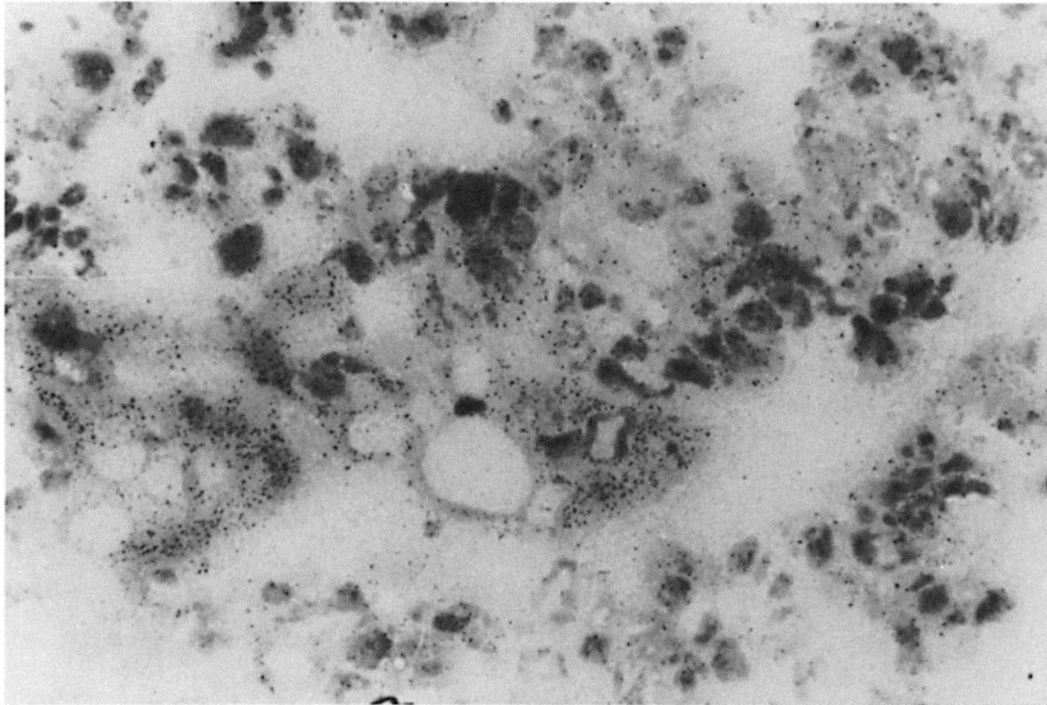


FIGURE 10 Effect of RNase pretreatment on the hybridization of the hCGβ cDNA probe to choriocarcinoma sections. Before hybridization the sections were treated with RNase (100 μg/ml, 1 h at 37°C) or buffer (-RNase). × 200.

several passages in culture. Thus, the conclusions drawn here are not influenced by changes that could occur during cell culture.

DISCUSSION

Previously we showed that the hCGα subunit mRNA is localized predominately in syncytial regions but that it is present in some differentiating cytotrophoblasts (21). Here we show that the signal for hCGβ mRNA in first trimester placenta was primarily localized to syncytiotrophoblast regions although it appears some is found in cytotrophoblasts.

HPL mRNA by contrast was exclusively localized to the

syncytium. Moreover, hCGα mRNA decreased about sixfold in term placenta, in which cytotrophoblasts disappeared, whereas the hPL mRNA level remained constant in first trimester and term sections. During pregnancy there is a gradual decrease of cytotrophoblasts until, at term, the syncytiotrophoblast is the major cellular component of placental villi. Taken together the data suggest that the expression of hCGα and -β subunit genes is dependent on the presence of cytotrophoblasts and that the decreasing levels of the subunits during gestation is associated with the depletion of these cells. Apparently the presence of cytotrophoblasts is not necessary for maintaining the production of hPL in the syncytium. It is also clear that hPL is expressed only in fully differentiated trophoblast whereas, at least in the case for the hCGα subunit, expression is initiated prior to syncytial formation.

Data obtained from sections of neoplastic trophoblast are consistent with this model. Choriocarcinoma is composed of trophoblasts that are not fully differentiated. There are clusters of cytotrophoblastic cells and multinucleated cells that are apparently immature syncytial elements. In these sections, hCGα and -β mRNA signals were seen in syncytial cells while the signal for hPL mRNA was almost at background. Nevertheless, it appears that certain cytotrophoblast elements also contain α and β mRNA, since some large mononucleated trophoblasts having both syncytio- and cytotrophoblastic morphology showed significant amounts of message. These mononucleated trophoblasts may represent the equivalent of an intermediate cell formed during the development of syncytiotrophoblasts from progenitor cytotrophoblasts.

In the case of hydatidiform mole, the hPL gene, as well as the hCG subunit genes, is expressed. It is significant that one of the distinguishing features of molar tissue compared to choriocarcinoma tissue is that the former retains a significant placental villous morphology.

Thus, it seems reasonable that a certain level of differentiation such as that indicated by villus formation is essential for hPL gene expression. By contrast, the hCGα gene, and the hCGβ gene, can be expressed in more poorly differentiated tissue which contain cytotrophoblastic elements.

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