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Production of choriogonadotropin-like factor by a microorganism

(ectopic hormone/human choriogonadotropin/bacteria)

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ABSTRACT **Extracts from an acetone powder preparation of a culture of a microorganism tentatively named *Progenitor cryptocides* contain choriogonadotropin (CG)-like factor as determined by radioimmunoassay with antiserum to human (h)CG β subunit COOH-terminal peptide and radioreceptor assay with bovine corpus luteum membranes. Possible interference by proteases in the extracts was excluded. Immunoreactive materials reacting with antisera to hCG β subunit and hCG β subunit COOH-terminal peptide were also found in the extracts. No free α subunit was detected. The CG-like factor was purified by chromatography on Sephadex G-100, concanavalin A-Sepharose, and DEAE-Sephadex A-50. The factor was adsorbed by concanavalin A-Sepharose, suggesting that it contains mannose and glucose moieties. The factor was eluted at the same position as standard hCG on Sephadex G-100. It dissociated into two bands when subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis; the bands corresponded to the respective standard hCG subunits. The biological activity of the purified factor as determined by the rat uterine weight assay and the ovarian weight assay was equivalent to 380 (95% confidence limits: 320–490) and 880 (780–1020) international units/mg, respectively. It can be concluded from the present results that a microorganism produces a glycoprotein that is biologically active and has physicochemical properties similar to those of hCG.**

Human (h-) choriogonadotropin (CG) is a glycoprotein hormone normally produced by the placenta and considered to be a specific hormone of pregnancy and trophoblastic neoplasms. However, it has been reported that nontrophoblastic malignant tumors (1, 2) and normal human tissues (3) produce CG or a CG-like substance. During the course of determining CG in tissues of marine organisms, we have demonstrated that a trypsin-like protease in the tissues mimics hCG in the radioimmunoassay (RIA) and radioreceptor assay (RRA) systems and is capable of stimulating ovarian adenylate cyclase activity *in vitro* (4). Thus, to avoid interference by proteases, measurement of hCG in tissue extracts should be performed with inhibitors in the assay systems.

Related to the problem of extraplacental production of CG, there are reports suggesting that CG is produced by a microorganism (5, 6). However, in the measurements in these studies, possible interference by proteases in the bacterial extracts was not excluded. In the present study, we will show that a CG-like factor is produced by a microorganism and that the observed results are not due to proteases. The factor has been purified and found to be biologically active, and it has immunological and physicochemical properties similar to those of hCG.

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MATERIALS AND METHODS

Bacterial Extracts. A microorganism tentatively named *Progenitor cryptocides* was obtained from V. W.-C. Livingston (5). Cultures of the microorganism were maintained on Muller–Hinton slants. Inoculation of 250 ml of trypticase/yeast extract medium in a 500 ml Erlenmeyer-flask was performed with a single colony from the Muller-Hinton slant. To a 28-liter fermenter, 20 liters of trypticase/yeast extract medium was added and the fermenter was inoculated with an overnight growth of the organism in the 250 ml of medium described above. After incubation for 120 hr at 37°C, the entire culture content was harvested and an acetone-dried powder was prepared according to a prescribed method (5), by J. Majnarich, Biomedical Research Laboratory. Twenty grams of the acetone-dried powder was suspended in 50 ml of 0.01 M Tris·HCl, pH 7.4, for 6 hr at 4°C. The undissolved material was removed by centrifugation at 10,000 × *g* for 20 min and the supernatant was dialyzed against distilled water and lyophilized. The weight of the lyophilized extract was 2.5 g. This lyophilized extract was used as the parent material for this study. A measured amount of the lyophilized extract was reconstituted in 0.01 M sodium/potassium phosphate-buffered saline (pH 7.4), and the existence of CG was determined by RIA and RRA with the appropriate protease inhibitors in the assay medium. Protein was measured by the method described by Lowry *et al.* (7).

Streptococcus faecalis (ATCC 12818) and *Pseudomonas maltophilia* (ATCC 13637) were purchased from the American Type Culture Collection (ATCC) and grown in trypticase soy broth at 37°C. Acetone powders of each of the culture contents were prepared; and the lyophilized extracts were prepared from the powders as described above.

Hormones and Reagents. Highly purified hCG (CR-119), hCG α subunit (hCGα) (CR-119), and hCG β subunit (hCGβ) (CR-119-2) used as standard preparations in each assay were gifts from the Center for Population Research of the National Institute of Child Health and Human Development and R. Canfield, Columbia University. Specific antisera to hCG, hCGα, and hCGβ were generated by C. C. Chang, using the multiple intradermal injection technique as described by Vaitukaitis *et al.* (8). Highly purified hCG (per mg, 12,000 international units equivalent to Second International Standard hCG by the mouse uterine weight method), hCHα, and hCGβ used as antigens to produce the antisera were prepared by Y.

Abbreviations: CG, chorionic gonadotropin; hCG, human CG; hCGα and hCGβ, α and β chains of hCG; RIA, radioimmunoassay; RRA, radioreceptor assay; Con A, concanavalin A; $NaDodSO_4$, sodium dodecyl sulfate.

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Y. Tsong from commercial hCG (2700–3000 international units/mg, Organon), by a modification of the method described by Canfield and Morgan (9). Specific antiserum to hCGβ COOH-terminal peptide (H93) was a generous gift of H. C. Chen and G. D. Hodgen of the National Institute of Child Health and Human Development.

N-α-*p*-Tosyl-L-lysine chloromethyl ketone, L-1-tosylamide-2-phenylethyl chloromethyl ketone, soybean trypsin inhibitor (type 1-S), and methyl α-D-glucopyranoside were purchased from Sigma. Sephadex G-100, concanavalin A (Con A)-Sepharose, and DEAE-Sephadex were obtained from Pharmacia, and Na^{125}I from New England Nuclear. Leupeptin and antipain were donated by the U.S.–Japan Cooperative Medical Science Program.

RIA and RRA. hCG (CR-119), hCGα (CR-119), and hCGβ (CR-119-2) were radioiodinated with Na^{125}I by using the lactoperoxidase method as described (10). All RIAs were carried out by the double-antibody technique as described (4). hCG (CR-119) showed 1.4% and 9.0% crossreactivity in the homologous RIA for hCGα and hCGβ, respectively. RRA was performed by the method described by Saxena *et al.* (11), using plasma membranes of bovine corpus luteum. The membranes were generously supplied by Wampole Laboratories.

Sephadex Column Chromatography. A sample of 100 mg of the lyophilized extracts of the bacterial culture content was dissolved in 2.0 ml of 0.01 M Tris·HCl buffer (pH 7.4)/0.15 M NaCl, and the mixture was applied to a column (1.5 × 85 cm) of Sephadex G-100 equilibrated at 4°C with 0.01 M Tris·HCl/0.15 M NaCl. The eluate was collected in 2.0-ml fractions. The void volume was determined with blue dextran. The column was standardized on separate days with ^{125}I-hCG, ^{125}I-hCGα, and ^{125}I-hCGβ.

Con A-Sepharose Column Chromatography. Columns (9.0 × 130 mm) of Con A-Sepharose were prepared and extensively washed at 4°C with 0.01 M phosphate-buffered saline (pH 7.4). Fractions containing hCG activity obtained from Sephadex G-100 were pooled, diaylzed, lyophilized, and applied to the columns. The unadsorbed substances were eluted with 0.01 M phosphate-buffered saline (pH 7.4). Con A-adsorbed glycoproteins were eluted with phosphate-buffered saline containing 0.2 M methyl α-D-glucopyranoside; 1.0-ml fractions were collected.

Ion-Exchange Chromatography. Fractions containing hCG activity obtained from Con A-Sepharose column were pooled, dialyzed, lyophilized, and applied to a column (1.5 × 40 cm) of DEAE-Sephadex A-50, equilibrated at 4°C with 0.01 M Tris·HCl (pH 7.4). The adsorbed protein on the column was eluted by a linear gradient to 0.5 M NaCl in 0.01 M Tris·HCl buffer (pH 7.4); 2.0-ml fractions were collected.

Polyacrylamide Gel Electrophoresis. Electrophoresis was performed in a 7.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (NaDodSO$_4$), as described by Weber and Osborn (12). The gels were stained with 0.2% Coomassie blue.

Bioassay. Biological activity of the purified bacterial CG factor was determined by measuring the increase in uterine weight and ovarian weight of immature female rats as described by Diczfalusy and Loraine (13). Wistar rats, weighing 36–44 g and 20–22 days old, were subcutaneously injected with 0.5 ml of test solution every 24 hr three times. The rats were sacrificed 96 hr after the initial injection, and the uteri and ovaries were removed, dried on filter paper, and weighed. The Second International Standard hCG was used as reference. Bovine serum albumin was added as a stabilizing agent to all test solutions to a final concentration of 0.1%.

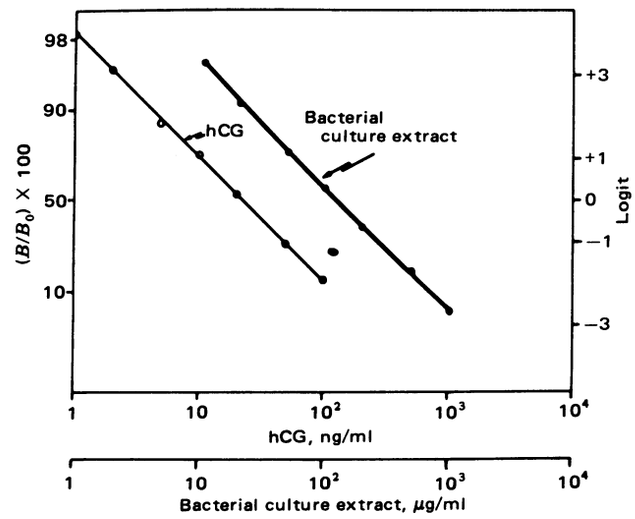

FIG. 1. Dose–response curves for hCG (CR-119) and the lyophilized bacterial culture extract in the RRA. Results are shown as the dose of the hCG reference preparation or of the lyophilized extract plotted on logarithmic scale against the logit transformation of the response. B, cpm bound in the presence of ^{125}I-hCG and unlabeled ligand; B_0, cpm bound in the presence of ^{125}I-hCG alone.

RESULTS

Serial dilutions of the lyophilized extract derived from the culture of *Progenitor cryptocides* demonstrated dose–response curves that paralleled those of hCG (CR-119) in RRA (Fig. 1), in homologous hCG RIA (not shown), and in RIA utilizing antiserum (H93) to hCGβ COOH-terminal peptide (Fig. 2). To exclude the influence of protease in the assays, tosyllysine chloromethyl ketone (1 mM), tosylamide-phenylethyl chloromethyl ketone (1 mM), leupeptin (1 mM), antipain (1 mM), and soybean trypsin inhibitor (0.1%) were added to the RRA and RIA systems. The addition of these protease inhibitors did not affect the displacement of ^{125}I-hCG in RRA and RIAs, suggesting that the results were not due to proteolytic activity. The extracts derived from the cultures of *S. faecalis* and *Pseudomonas maltophilia* showed no displacement of ^{125}I-hCG in the RRA and RIAs.

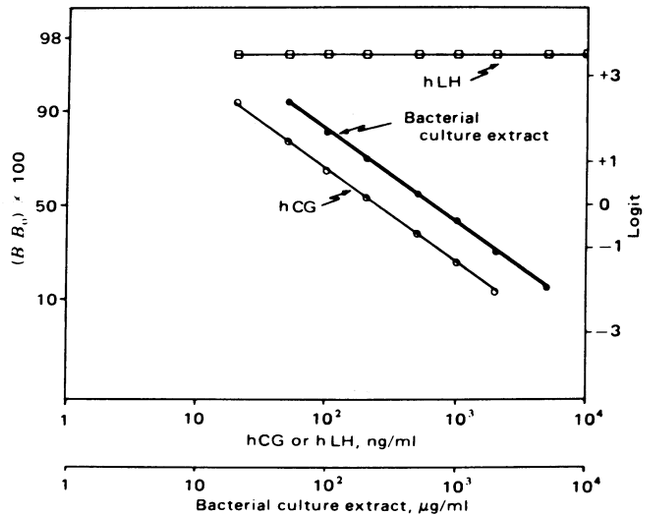

FIG. 2. Dose–response curves for hCG (CR-119) and the lyophilized bacterial culture extract in the RIA using antiserum (H93) to hCGβ COOH-terminal peptide. hLH, human luteinizing hormone.

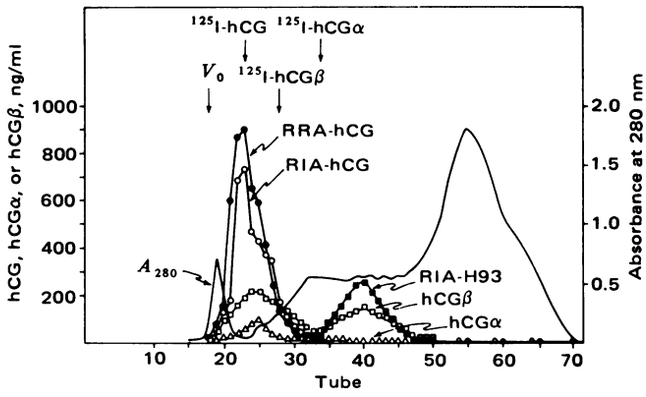

FIG. 3. Sephadex G-100 elution profile of the bacterial culture extract. Each 2.0-ml fraction was assayed by the RRA and by the RIA systems for hCG, hCGα, hCGβ, and hCGβ COOH-terminal peptide. Protein content of the eluted fractions was measured by absorbance at 280 nm. V_0 represents void volume determined by elution of blue dextran.

To verify that the putative CG factor present in the extracts derived from the culture of *Progenitor cryptocides* is CG, the factor was purified by a combination of gel filtration on Sephadex G-100 and chromatography on Con A-Sepharose and DEAE-Sephadex A-50. When the extract was filtered through a column of Sephadex G-100, CG-like factor determined by both RRA and RIA was found to be eluted at the position corresponding to that of ^{125}I-hCG used as a marker, suggesting that its apparent M_r is similar to that of hCG. A noteworthy finding was that immunoreactive material that reacted only with antisera to hCGβ or to hCGβ COOH-terminal peptide was eluted (Fig. 3). This elution peak was considerably retarded compared to ^{125}I-hCGβ, suggesting that the apparent M_r of the immunoreactive β fragment is much lower than that of hCGβ (CR-119-2). However, no free α subunit was detected. After gel filtration of a total of 2.4 g of the lyophilized extract, the fractions containing CG (tubes 21–27) were pooled, dialyzed against distilled water, and lyophilized. The yield of the CG fraction obtained at this step was 31.2 mg of protein.

To determine whether or not the CG fraction obtained from Sephadex G-100 contained proteolytic activity, ^{125}I-hCG (500,000 cpm) used as a tracer in the RRA and RIA was incubated with the CG fraction (1 mg) at 37°C for 60 min. The reaction mixture was analyzed by gel filtration on Sephadex G-100. The radiolabeled hCG was recovered intact, indicating

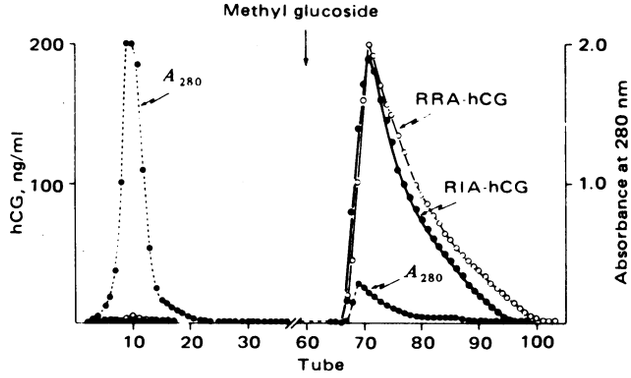

FIG. 4. Elution profile on Con A-Sepharose column chromatography of the CG-containing fraction obtained from the Sephadex G-100 column. Fractions 1–59 were eluted with phosphate-buffered saline; the following fractions were eluted with the same buffer containing 0.2 M methyl α-D-glucopyranoside. Each 1-ml fraction was assayed for hCG by the RRA and the RIA systems. Protein content was measured by absorbance at 280 nm.

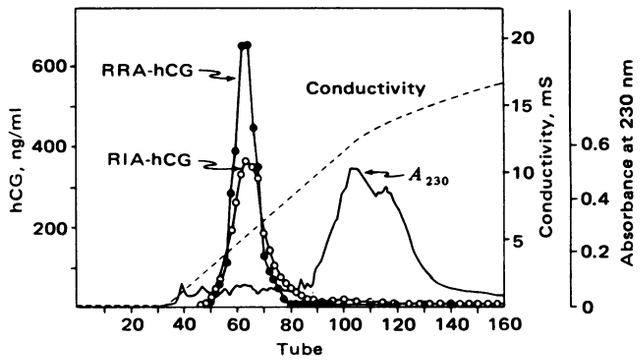

FIG. 5. Elution profile of the Con A-adsorbed CG fraction on DEAE-Sephadex A-50 column chromatography. The adsorbed protein on the column was eluted with a linear 300-ml gradient from 0 to 0.5 M NaCl in 0.01 M Tris·HCl buffer (pH 7.4). Each 2.0-ml fraction was assayed for hCG by RRA and RIA. Protein content was measured by absorbance at 230 nm.

that the CG fraction does not contain any proteases that hydrolyze ^{125}I-hCG. It can be concluded from the present results that the bacterial factor is CG-like and that interference from proteases in the RIA and RRA is unlikely.

To ascertain whether or not the bacterial CG factor contains sugar moieties, each 6 mg of the CG fraction obtained from Sephadex G-100 was reconstituted in 2.0 ml of 0.01 M phosphate-buffered saline (pH 7.4) and applied to a column of Con A-Sepharose. The CG factor was adsorbed to a Con A-Sepharose column and eluted with 0.2 M methyl α-D-glucopyranoside (Fig. 4), indicating that the CG factor contains glucose or mannose moieties. After a total of 30 mg of the CG fraction was chromatographed on Con A-Sepharose, the immunoreactive CG fractions were pooled, dialyzed against distilled water, and lyophilized. The yield of the CG fraction at this step of purification was 6.4 mg of protein. The lyophilized Con A-adsorbed material was reconstituted in 0.01 M Tris·HCl buffer (pH 7.4) and applied to a column of DEAE-Sephadex A-50. When the column was eluted with a linear gradient, the CG factor was eluted in a single peak at a conductivity of 4.0–6.0 mS (Fig. 5). This elution position corresponded to that found for placental hCG (14). The fractions containing CG activity were combined, dialyzed against distilled water, and lyophilized. The final yield was 570 μg of protein. Table 1 shows a summary of the yield and hCG potency of the fractions containing CG-like activity obtained at each step of the purification procedure. The hCG potency determined by RRA and RIA increased with each purification step.

To establish that the purified bacterial CG is composed of subunits, the factor was analyzed by electrophoresis on Na-DodSO$_4$/polyacrylamide gels (Fig. 6). The bacterial CG factor separated into two major bands with mobilities corresponding

Table 1. Yield and relative hCG potency at each purification step

Fraction	Yield, mg	hCG potency, international units/mg RRA	RIA
Lyophilized extract	2500*	3.4 ± 0.2	2.1 ± 0.1
Sephadex G-100	31.2	231 ± 11	150 ± 7
Con A-Sepharose	6.4	880 ± 45	542 ± 27
DEAE-Sephadex A-50	0.57	3580 ± 160	2260 ± 102

Relative hCG potency (mean ± SD) was determined by the RRA and homologous hCG RIA, using the Second International Standard hCG as a reference preparation. The yield is expressed as amounts of protein, except that of the starting extract.
* Weight of dry material.

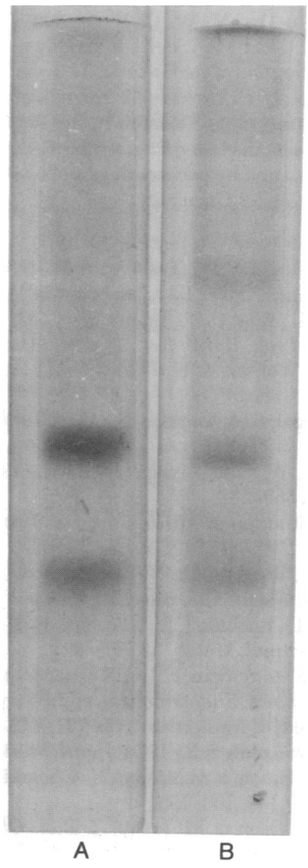

FIG. 6. $NaDodSO_4$/polyacrylamide gel electrophoresis. Gel A shows the electrophoretic pattern of hCG (CR-119) (20 μg), which dissociated in $NaDodSO_4$ gel into α and β subunits. Gel B represents the electrophoretic pattern of the purified bacterial CG-factor (20 μg) obtained from DEAE-Sephadex A-50 chromatography. The migration is toward the anode at the bottom of the figure.

to α and β subunits dissociated from hCG (CR-119). An additional minor band was observed, which was slightly retarded compared to standard hCG. To identify each band, the gel was sliced into 2.2-mm segments. Each segment was homogenized in 500 μl of phosphate-buffered saline (pH 7.4) and centrifuged at 3000 × *g* for 20 min, and the supernatant was assayed for hCG, hCGα, and hCGβ using the respective homologous RIA systems. The peaks designated as segments 28, 22, and 11 showed immunoreactivities with antisera to hCGα, hCGβ, and hCG, respectively (Fig. 7). Hence, the immunoreactivity and protein peaks of the bacterial CG subunits coincided with the migration bands of α and β subunits dissociated from hCG (CR-119). The retarded minor band corresponding to a M_r of 86,000 might represent a large precursor species of the bacterial CG factor.

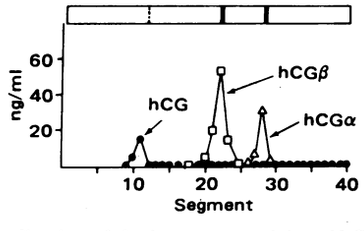

FIG. 7. Localization of the immunoreactivity of hCGα and hCGβ in a gel prepared in the same manner as gel B of Fig. 6. Each 2.2-mm segment of the gel was homogenized in phosphate-buffered saline and the supernatant was assayed for hCG, hCGα, and hCGβ by the respective homologous RIA.

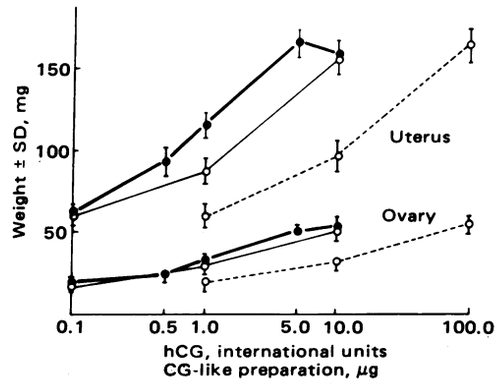

FIG. 8. Dose–response curves of standard hCG and bacterial CG-like preparations determined by measuring the uterine and ovarian weights in immature female rats. ●—●, Second International Standard hCG; O---O, CG-like preparation obtained from Sephadex G-100; O—O, CG-like preparation from DEAE-Sephadex A-50.

The biological potency of the bacterial CG-like preparations was determined by the rat uterine weight assay and ovarian weight assay in immature female rats at three dose levels. The dose–response curves for the uterine weight and ovarian weight with these CG-like preparations appeared to be similar to the curve of standard hCG (Fig. 8). The mean biological potency of the CG-like preparation obtained from Sephadex G-100 and DEAE-Sephadex A-50 was equivalent to 55 (95% confidence limit: 45–70) and 380 (320–490) international units/mg, respectively, by the uterine weight assay and 93 (85–118) and 880 (780–1020) international units/mg, respectively, by the ovarian weight assay.

DISCUSSION

It can be concluded that the microorganism *Progenitor cryptocides* produces a CG-like factor. This conclusion is based on the following observations. First, the bacterial culture extract showed dose–response curves that paralleled those of hCG (CR-119) in the RRA system using bovine corpus luteum membranes as well as in the RIA system using antiserum (H93) generated against hCGβ COOH-terminal peptide, which interacts specifically with the unique COOH-terminal peptide sequence of hCGβ (15). Interference by proteases has been excluded by the findings that inhibitors added to the RRA and RIA systems did not influence the results and that radiolabeled hCG incubated with the partially purified bacterial CG fraction was not hydrolyzed. Second, the elution profiles of the bacterial CG-like factor on Sephadex G-100 and Con A-Sepharose were consistent with those of hCG. These findings indicate that the bacterial CG-like factor is a glycoprotein containing glucose or mannose moieties or both, and that the apparent M_r of the glycoprotein is similar to that of hCG. Third, after extensive purification the bacterial CG-like factor consists of two subunits with electrophoretic mobilities corresponding to the mobilities of the subunits of standard hCG on a $NaDodSO_4$/polyacrylamide gel. Fourth, the bacterial factor manifested biological activity as determined by the rat uterine weight assay and the rat ovarian weight assay. The present results obtained with the bacterial CG-like factor indicate that this protein is quite different from that isolated from the crab stomach, which was shown to be a protease (4).

The biological potency of the purified bacterial CG factor was extremely low compared to purified urinary hCG preparations (>10,000 international units/mg) (16). The basis for the low biological activity was not apparent in the present study. One possible reason may be that bacterial CG-like factor has

low sialic acid content. It is well known that desialylated hCG possesses extremely low biological activity *in vivo* due to its more rapid removal from the blood (17, 18). In spite of this, desialyted hCG retains full activity in the RRA and RIA systems. In addition, recent studies from this laboratory established that hCG purified from placental tissues has a lower sialic acid content than standard urinary hCG and exhibits biological potency equivalent to 750 international units/mg (unpublished data). Hence, the bacterial CG-like factor appears to resemble placental hCG rather than urinary hCG. In fact, the elution pattern on DEAE-Sephadex A-50 indicated that the electrical charge of the bacterial CG-like factor is similar to that of placental hCG.

An interesting observation is that significant amounts of free hCGβ immunoreactive fragments were eluted during gel filtration on Sephadex G-100 (Fig. 3). On the other hand, no detectable free α subunit was found in the bacterial culture extract. This is in contrast to placental tissue, which contains substantial amounts of free hCGα without accompanying free hCGβ (19, 20). Furthermore, substantial amounts of free α subunit are present in sera of pregnant women but minimal or no free β subunit (21). These observations suggest that the production of hCGβ might be a rate-limiting step in the biosynthesis of hCG in human placenta. Hence, the unbalanced formation of significant quantities of free hCGβ immunoreactive subunits with no detectable free α subunit in the bacterial preparation observed in this study suggests that the regulatory mechanism controlling the biosynthesis of CG-like factor in bacteria may differ from that of placenta.

Recently, Acevedo *et al.* (22), using immunocytochemical techniques, reported that CG-like protein is present in several microorganisms that were isolated from patients bearing malignant neoplasms. However, these findings should be substantiated by other methods in view of the fact that β subunits of glycoprotein hormones and serine proteases possess a homologous peptide sequence (23). Jagiello and Mesa-Tejada (24) recently demonstrated that an antiserum raised against β subunit of human luteinizing hormone contains antibodies that interact with peptides containing the homologous sequence. Hence, to establish that CG-like factor is produced by extraplacental tissues, the crossreactivity of hCGβ and protease should be considered and excluded. Richert and Ryan (25) reported that the culture media of *Pseudomonas maltophilia* may contain a protein molecule that crossreacts with antisera to hCG as well as to hCGβ in addition to the presence of a receptor to hCG. Moreover, they reported that the hCG-like material from *Pseudomonas maltophilia* was due to two artifacts, a protease and a soluble form of the hCG binding site (26). Nevertheless, we have recently isolated a strain of *Streptococcus faecalis* from the urine of a patient (A.K.) with adenoid cystic carcinoma of the ethmoid sinus. This microorganism apparently produces only CGβ-immunoreactive fragment (unpublished data). These findings of production of CG-like factor and CGβ fragments by microorganisms are demonstrations of an expression of a mammalian gene. To explain this observation, two alternative hypotheses are offered. One is that the CG gene has its origin early in evolution and the other is that its expression in bacteria is a consequence of a natural process of DNA recombination.

We are grateful to Drs. V. W. C. Livingston, J. Majnarich, A. Strampp, K. Horiuchi, and Ms. J. Bell for making available to us the acetone powder preparation of the bacteria. This work was performed as part of the Contraceptive Development Program of the International Committee for Contraceptive Research of the Population Council. T.M. is supported by Rockefeller Foundation Fellowship Award in Reproductive Biology and by a Biomedical Fellowship of the Population Council, Rockefeller University.

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