Loss of Tumorigenic Potential by Human Lung Tumor Cells in the Presence of Antisense RNA Specific to the Ectopically Synthesized Alpha Subunit of Human Chorionic Gonadotropin

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Abstract. A clonal strain of human lung tumor cells in culture (ChaGo), derived from a bronchogenic carcinoma, synthesizes and secretes large amounts of alpha (α) and a comparatively lower level of beta (β) subunit of the glycoprotein hormone, human chorionic gonadotropin (HCG). ChaGo cells lost their characteristic anchorage-independent growth phenotype in the presence of anti-α-HCG antibody. The effect of the antibody was partially reversed by addition of α-HCG to the culture medium.

ChaGo cells were transfected with an expression vector (pRSV-anti-α-HCG), that directs synthesis of RNA complementary to α-HCG mRNA. The transfectants produced α-HCG antisense RNA which was associated with the reduced level of α-HCG. Transfectants also displayed several altered phenotypic properties, including altered morphology, less mitosis, reduced growth rate, loss of anchorage-independent growth, and loss of tumorigenicity in nude mice.

Treatment of transfectants with 8,bromo-cAMP resulted in increased accumulation of α-HCG mRNA, no change in the level of α-HCG antisense RNA, release of the inhibition of [^{3}H]thymidine incorporation, and restoration of anchorage-independent growth phenotype. The overexpression of c-*myc*, observed in ChaGo cells, was unaffected by the reduced level of α-HCG.

These results suggest that ectopic synthesis of the alpha subunit of HCG plays a functional role in the transformation of these human lung cells.

LUNG cells that have undergone transformation frequently acquire the property of ectopically synthesizing and secreting hormones (26). The predominant hormone synthesized by many different types of human lung tumors is the human chorionic gonadotropin (HCG)[1] (5, 11, 12, 28, 34). The association of neoplasia with HCG is so great that the increased levels of circulating HCG and its subunits are used as a biochemical marker for malignancy and as a criterion for the successful surgical removal of such tumors. However, it is not known whether such ectopically synthesized hormones play a functional role in the tumorigenesis process.

Under physiological conditions, HCG is synthesized by syncytiotropoblast cells at the very early stages of pregnancy. Consistent production of this hormone is essential for the growth, development, and maintenance of the fetus during the gestation period. The possibility that HCG may also serve as a growth factor in the neoplastic transformation of cells is explored in this investigation. This is achieved by interfering in the synthesis of the hormone in the HCG-producing human lung tumor cells in culture.

It has been demonstrated that microinjection of antibodies to synthetic antigens affected the morphology and function of cells in culture (3, 38). For example, microinjection of anti-*ras* protein antibody into cultured normal rat kidney cells transiently reversed the transformed phenotype of these cells (8, 9, 21). Another experimental approach that has been used by several investigators is to inhibit the translation of mRNA by formation of RNA duplexes with the complementary antisense RNA (18, 19, 23). This is achieved either by introducing a recombinant plasmid expression vector, which generates antisense RNA, or by microinjection of the specific antisense RNA species itself (15, 39). By these means it has been possible to inhibit the synthesis of a variety of cell-specific proteins (20, 23–25). Inducible production of c-*fos*–antisense RNA inhibits proliferation of 3T3 cells (17) and introduction of protooncogene c-*fos*–antisense RNA inhibited the platelet-derived growth factor–induced growth of mouse 3T3 cells that express c-*fos* (24). In some instances, only a transient effect can be observed because of the induction of an unwinding protein by the RNA duplexes (2, 29, 37).

In this investigation, we have examined the role of α-HCG on the regulation of growth of ChaGo cells by specifically reducing the hormone level as the consequence of (*a*) addition of anti-α-HCG antibody in the culture media; and also by (*b*) intracellular synthesis of α-HCG-specific antisense RNA from an expression vector (Rous sarcoma virus [pRSV]-anti-α-HCG). Both maneuvers led to severe alteration of the transformation phenotypes of ChaGo cells.

1. *Abbreviation used in this paper*: HCG, human chorionic gonadotropin.

Materials and Methods

Tissue Culture

All cells were grown in Ham's F-10 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum at 37°C in 95% air and 5% CO_2.

Growth in Soft Agar

5×10^4 cells were uniformly dispersed over top agar (minimal essential medium supplemented with 10% fetal bovine serum and 0.3% bactoagar) and were overlayered on the bottom agar (minimal essential medium supplemented with 10% fetal bovine serum and 0.6% bactoagar), and incubated at 37°C in 95% air and 5% CO_2. Phase-contrast microscopy was used to monitor colony formation and to obtain photographs.

Colony forming efficiency was quantitated 28 d after seeding 3×10^4 cells. Any growth which attained a diameter ≥0.2 mm was considered to be a countable colony. A total of 500 colonies and cells in several representative fields were scored in each plate. In cases where very few or no colonies were formed (such as in the presence of maximum concentration of α-HCG antibody and when 3T3 cells were used), the total number of colonies in the whole plate was counted. Results were expressed as percent colonies formed per total number of seeded cells.

Construction and Characterization of the Plasmid Expression Vector pRSV-Anti-α-HCG

The construction protocol and the structure of pRSV-anti-α-HCG is schematically presented in Fig. 1. The recombinant plasmid pBR322 with α-HCG cDNA insert (621 bp) was a gift from Dr. J. C. Fiddes of California Biotechnology, Inc. (Mountainview, CA) (10). The expression vector pRSV-β-Globin (13) was obtained from the American Type Culture Collection, Rockville, MD. The α-HCG-cDNA was inserted at the Hind III site of the plasmid pBR322 (10). The recombinant plasmid was initially cleaved with Hha I (−58 bp) followed by treatment with the large fragment of the DNA polymerase I (Klenow; New England Biolabs, Beverly, MA). This α-HCG-cDNA fragment was then released from the plasmid by treatment with Hind III. These enzymatic treatments generate a α-HCG-cDNA fragment with the blunt end at the 5′ end and with a Hind III recognition site at the 3′ end of the DNA molecule. The expression vector pRSV-β-globin DNA was treated with the restriction endonuclease Bgl II, followed by generation of the blunt ends with the Klenow fragment of DNA polymerase I. The β-globin sequence was then released from the plasmid backbone by digestion with Hind III. The 3′ Hind III end of the cDNA-α-HCG sequence was then ligated to the Hind III site downstream of the RSV long terminal repeat sequence of the linear plasmid. The recombinant plasmid was then recircularized by blunt end ligation (22). The orientation of the cDNA-α-HCG in the constructed recombinant plasmid was established by digestion with restriction endonucleases followed by Southern blot analysis (33).

The characterization of the expression vector pRSV-anti-α-HCG was focused to establish that the Hind III end (3′) of the α-HCG-cDNA was ligated to the Hind III site located immediately downstream of the RSV promoter sequence (Fig. 1). This was confirmed by digestion of pRSV-anti-α-HCG with the restriction endonucleases, Eco RI and Xba I, followed by identification of a 485-bp DNA fragment which hybridizes with ^{32}P-labeled α-HCG-cDNA probe. Alternative orientation of the inserted α-HCG-cDNA would not have generated such a fragment.

DNA-mediated Transfer of pRSV-Anti-α-HCG into ChaGo and L Cells

The recombinant plasmid pRSV-anti-α-HCG was transferred to L cells and ChaGo cells by a calcium phosphate precipitation method originally described by Wigler et al. (40) and subsequently modified by us (27). The expression vector pRSV-Neo (13) was cotransferred with pRSV-anti-α-HCG and G418-resistant transfectants were selected in the presence of 300 μg/ml of Geneticin (G418; Sigma Chemical Co., St. Louis, MO) for L cells or in the presence of 200 μg/ml for ChaGo cells. The ChaGo cell transfectants were selected in the presence of 100 ng/ml of α-HCG because we anticipated that the growth of anti-α-HCG-expressing transfectants may be dependent on the hormone. However, subsequent characterization of the transfectants demonstrated that although the α-HCG antisense RNA-producing transfectants were very slow growing, they could not be further stimulated by addition of α-HCG in the culture medium.

Other transfectants of ChaGo cells were also obtained with pRSV-Neo plasmid alone and together with another plasmid, pRSV-globin (13). A comparative analysis was conducted of the transfectants with pRSV-Neo alone, with pRSV-Neo plus pRSV-globin, and with pRSV-Neo plus pRSV-anti-α-HCG. Since the plasmid backbone of all three expression vectors was the same, transfectants with pRSV-Neo and pRSV-globin served as a control for the influence of pRSV-anti-α-HCG. Stable expression of the transferred genes suggested that these externally introduced plasmids were integrated stably into the chromosome of the recipient cells. The observed frequency of transfection of the plasmid pRSV-Neo was 2×10^{-5} for ChaGo cells and 1.5×10^{-4} for L cells. The cotransfection frequency of pRSV-anti-α-HCG or pRSV-globin with pRSV-Neo was in the order of 40% in ChaGo cells as calculated from the identified α-HCG antisense RNA or globin mRNA products in the G418-resistant transfectants.

Measurement of Levels of α-HCG-specific Sense and Antisense RNA in the Transfectants

RNA from recipient cells and transfectants grown in complete medium was isolated by the guanidinium isothiocyanate method (22). The α-HCG-specific sense and antisense RNA species in the total RNA was identified by Northern blot analysis followed by hybridization with strand-specific riboprobes. Human fetal lung tissue was obtained through the courtesy of Dr. Frederick Bieber of Brigham and Women's Hospital (Boston, MA).

The strand-specific riboprobes were synthesized from recombinant phagemid (Bluescript; Stratagene, La Jolla, CA) in which the α-HCG-cDNA was inserted at the Hind III site between the two bacterial RNA polymerases (T7 and T3). The orientation of the inserted α-HCG-cDNA in the Bluescript was established by restriction endonuclease digestion analysis and from the hybridization characteristics of the in vitro-synthesized [^{32}P]RNA products of T3 and T7 polymerase with cellular α-HCG-mRNA. The [^{32}P]RNA probe synthesized from α-HCG-Bluescript DNA in the presence of T3 polymerase (designated as probe T3) recognized the cellular α-HCG-specific mRNA sequences (sense RNA); and the probe synthesized in the presence of polymerase T7 (designated as probe T7) did not recognize α-HCG-mRNA sequence but did recognize the pRSV-anti-α-HCG-specific RNA sequences.

The mouse H3.2 cDNA (32) was provided by Arthur B. Pardee of the Dana Farber Cancer Institute (Boston, MA) and the recombinant plasmid with human c-*myc*-cDNA (35) was made available to us by Dr. Philip Leder of the Department of Human Genetics, Harvard Medical School (Boston, MA).

Measurement of α-HCG Level in ChaGo Cells and Transfectants

The clonal strain of ChaGo cells which synthesizes predominantly α-HCG and very low levels of β-HCG was used in this investigation (34). The α-HCG level in the culture medium was determined by radioimmunoassay as originally described by Vaitukaitis et al. (36) and subsequently modified by Tashjian et al. (34). The polyclonal α-HCG-specific antibody was obtained as a gift from the Hormone Distribution Agency (National Institutes of Health (NIH), Bethesda, MD). The specificity of the antibody was verified by immunoprecipitation of the hormone in the culture medium of [^{35}S]methionine-labeled ChaGo cells. Immunoprecipitated radioactive material was then analyzed by SDS-PAGE and radioactive α-HCG was identified by comparison with the electrophoretic mobility of authentic nonradioactive α-HCG (obtained from Hormone Distribution Agency, NIH) analyzed under identical conditions. 90% of the immunoprecipitated radioactivity was eliminated by performing the immunoprecipitation reaction in the presence of excess (200 ng) nonradioactive α-HCG. Excess amounts of β-HCG did not compete with this immunoprecipitated radioactive material.

[^{3}H]Thymidine Incorporation and Autoradiography

5×10^4 cells were plated in 35-mm tissue culture dishes in F10 medium (supplemented with 10% fetal bovine serum). 48 h later the medium was removed and replaced with 2 ml of fresh medium. [^{3}H]Thymidine (2 μCi/ml) was added to each of these plates and incubated at 37°C in a CO_2 (5%) incubator for the specified time. After the incubation period, the medium was removed and cells were washed free of [^{3}H]thymidine with Hank's buffered saline, fixed with methanol/acetic acid (3:1), and covered with a thin layer of Kodak emulsion (Eastman Kodak Co., Rochester, NY) (1:1, nuclear track emulsion). Autoradiography was carried out at room temperature for 72–96 h. The dishes were developed with Kodak D19 developer and fixed with Kodak fixer. They were then examined under a phase-contrast microscope and photographed.

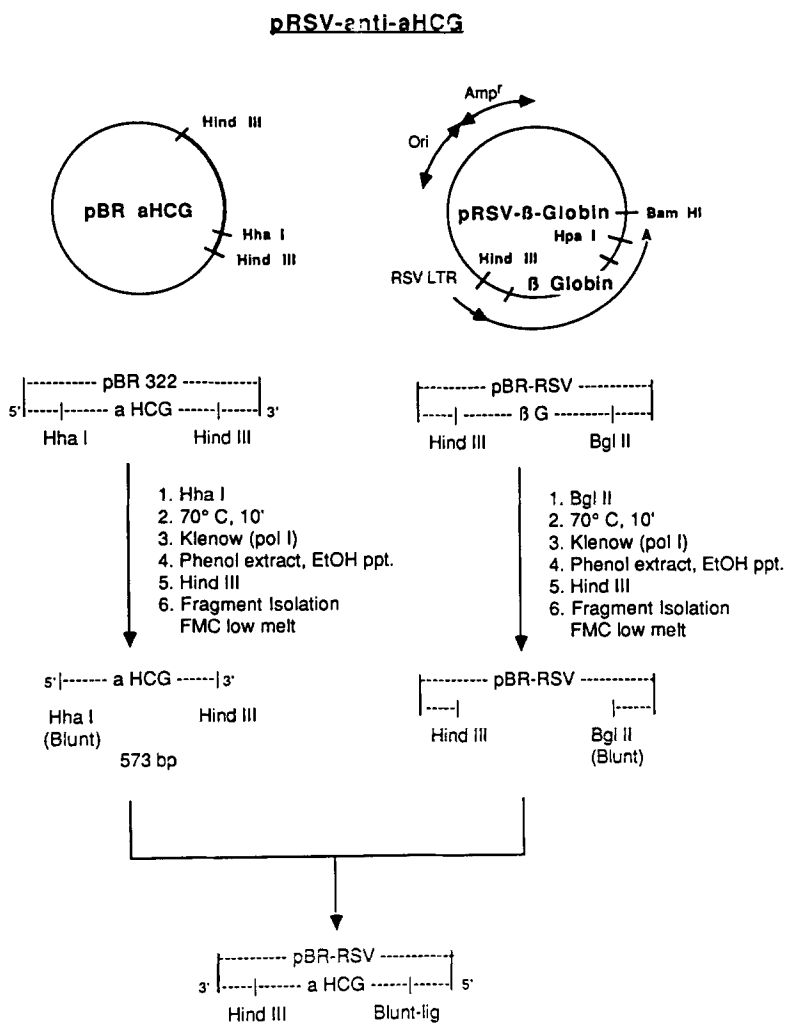

Figure 1. Construction of the pRSV-anti-α-HCG. Construction protocol of the recombinant plasmid pRSV-anti-α-HCG is described schematically. The details are included in Materials and Methods. The left side of the figure shows the isolation of the α-HCG-cDNA insert fragment from the recombinant plasmid pBR-α-HCG (10). The right side shows the structure of the parent plasmid, the expression vector pRSV-globin used for the construction of pRSV-anti-α-HCG. The stepwise treatment of these two plasmids is indicated by the arrows. The restriction endonuclease treatments were carried out under conditions specified by the suppliers. Treatment was with the Klenow fragment of Pol I and blunt-end ligation was performed as described by Maniatis et al. (22). The orientation of inserted α-HCG-cDNA in the pRSV-anti-α-HCG was established by restriction endonuclease mapping and Southern blot analysis (33) of the plasmid DNA.

Results

Inhibition of Anchorage-independent Growth Phenotype of ChaGo Cells by Anti-α-HCG Antibody

Addition of anti-α-HCG antibody to the top agar inhibited the growth of ChaGo cells on soft agar (Fig. 2). The colony forming efficiency of ChaGo cells on soft agar decreased as a function of the α-HCG antibody concentration in the top agar (Fig. 2, *B–D*). This inhibitory effect was partially reversed by addition of α-HCG in the top agar along with the anti-α-HCG antibody (Fig. 2, *E–G*). Neither β-HCG antibody nor the gamma globulin fraction of the rabbit serum caused inhibition of growth of ChaGo cells on soft agar (data not shown). Addition of the β-HCG in the top agar did not reverse the growth inhibiting effect of α-HCG antibody. The growth of α-HCG-nonproducing cell line SSC 15 (30) was not affected by α-HCG antibody. All these results suggested that the loss of the anchorage-independent growth phenotype of ChaGo cells was due to the cell deprivation of one of its own products, the α-HCG. Addition of α-HCG antibody to the tissue culture medium affected the growth of ChaGo cells but this effect was not as dramatic as that observed in the soft agar assay.

The role of the ectopic synthesis of α-HCG on the growth characteristics of the transformed ChaGo cells was also verified by inhibiting the synthesis of α-HCG and by the α-HCG antisense RNA technique. This is accomplished by transfection of ChaGo cells with a recombinant plasmid in which the α-HCG-cDNA was inserted with a reverse orientation with respect to the reading direction of the RSV promoter.

Expression of pRSV-Anti-α-HCG in ChaGo Cells

The functional state of plasmid expression vector pRSV-anti-α-HCG was verified in both L and ChaGo cells by DNA-mediated transfection studies. The [^{32}P]RNA probe designated by T7 was complementary to the α-HCG antisense RNA and the one designated by T3 was complementary to the α-HCG-specific sense RNA species. Among the 12 ChaGo cell transfectants analyzed, nine in experiment 1 (Fig. 3, *A* and *C*, lanes *1–9*) and three in experiment 2 (Fig. 3, *B* and *D*, lanes *12–14*), the expression of pRSV-anti-α-HCG could be detected in five of the transfectants (Fig. 3, *C* and *D*, lanes *5*, *6*, *8*, *12*, and *13*). The endogenous α-HCG-specific sense RNA can be detected in all the ChaGo cell transfectants (Fig. 3, *A* and *B*, lanes *1–9* and *12–15*). The basis for the different levels of expression of the endogenous α-HCG gene in different transfectants is not clear. The stable integration of externally introduced recombinant plasmid DNA species may have influenced α-HCG gene expression in some transfectants. The size of the [^{32}P]T3 hybridizable sequences

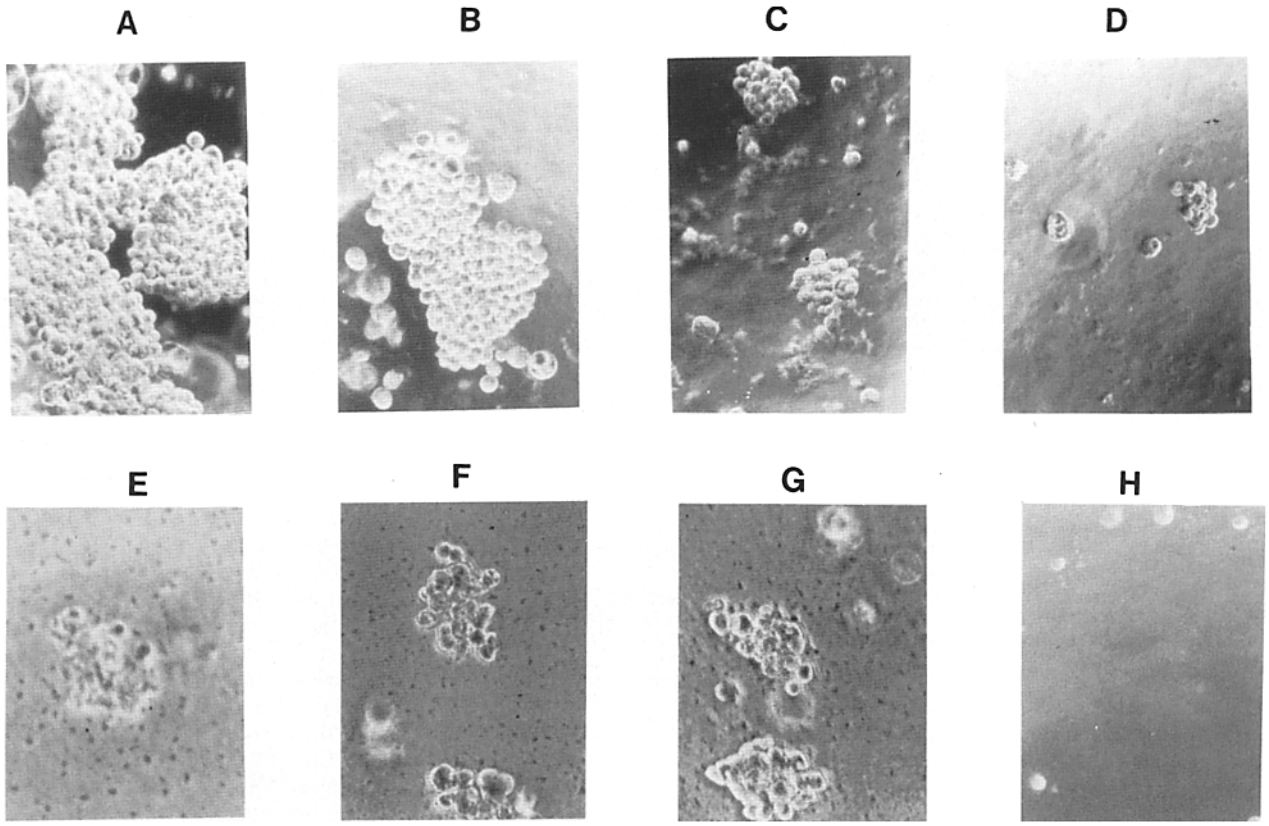

Figure 2. Growth of ChaGo cells on soft agar plates embedded with anti-α-HCG antibody. 5×10^4 cells in top agar were overlayered on bottom agar and were incubated at 37°C in an atmosphere of 95% air and 5% CO_2. The plates were examined by phase-contrast microscopy and photographed. A typical field is presented in each panel. *A* shows colony formation by ChaGo cells on soft agar. 60% of the seeded ChaGo cells (average from duplicate plates) formed colonies on soft agar. *B*, *C*, and *D* show that 12, 0.5, and 0.008%, respectively, of the seeded ChaGo cells formed colonies on soft agar in the presence of anti-α-HCG antibody of dilutions 1:25,000, 1:10,000, and 1:5,000, respectively. This growth inhibition by α-HCG-specific antibody (1:5,000) could be reversed by 50 ng/ml (*E*, 2%), 100 ng/ml (*F*, 11%), and 200 ng/ml (*G*, 25%) of α-HCG in the top agar. (*H*) Same number of NIH 3T3 cells were plated. The alpha subunit of HCG (α-HCG) and the α-HCG-specific antibody (RIA grade) are gifts from the Hormone Distribution Agency (NIH). The colony forming efficiency of ChaGo cells and ChaGo cell transfectants were quantitated by counting the number of colonies (of a diameter ≥0.2 mm) per plate (5×10^4 cells) 28 d after seeding. Results are expressed as the percent of colonies formed per total number of seeded cells per plate.

was the same as the one identified by [^{32}P]cDNA–α-HCG-hybridizable sequences in ChaGo cells; i.e., the same as that of α-HCG-specific mRNA (0.96 kb; Fig. 3 *A*, lane *10*). In L cells, α-HCG sense RNA (T3 hybridization) sequences were not detected (Fig. 3 *A*, lane *11*). The size difference between the sense (0.96 kb) and the antisense RNA (1.40 kb) is believed to be due to the structural organization of the externally introduced plasmid vector pRSV-anti-α-HCG and incomplete processing of its RNA transcripts.

Expression of pRSV-Anti-α-HCG in L Cells

As a control, the same maneuvers were carried out in L cells, which do not synthesize α-HCG. No α-HCG-specific mRNA sequences (T3 hybridizable) were detected in L cells (Fig. 3, compare *A*, lane *11*, with *E*, lanes *3–8*) suggesting that this gene was not active in L cells. However, α-HCG antisense RNA sequences were detected in seven out of ten L cell transfectants derived by cotransfection of pRSV-anti-α-HCG and pRSV-Neo (Fig. 3 *E*, lanes *9–18*). These sequences appeared to be pRSV-anti-α-HCG transcripts, since they hybridized with [^{32}P]cDNA–α-HCG and with [^{32}P]T7. These RNA products do not seem to be the products of the endogenous gene activated by the integration of foreign DNA into the mouse L cell chromosome. The size of the antisense RNA species in L cells was the same as that observed in ChaGo cell transfectants (Fig. 3 *E*, lane *2*).

The results above demonstrate that the constructed expression vector, pRSV-anti-α-HCG, is active in cells that ectopically synthesize α-HCG, as well as in cells that do not produce the hormone.

α-HCG Production by pRSV-Anti-α-HCG-expressing Transfectants

The influence of α-HCG antisense RNA on the hormone production by ChaGo cells was examined by measurement of α-HCG in the culture medium of transfectants. The α-HCG level in the culture medium of the five pRSV-anti-α-HCG-expressing transfectants (NH17, NH18, NH22, NH23, and NH24) was significantly lower than the level in the parental strain (Table I). Though these five transfectants synthesize significant amounts of α-HCG-specific mRNA, the α-HCG antisense RNA species in the same transfectants has effec-

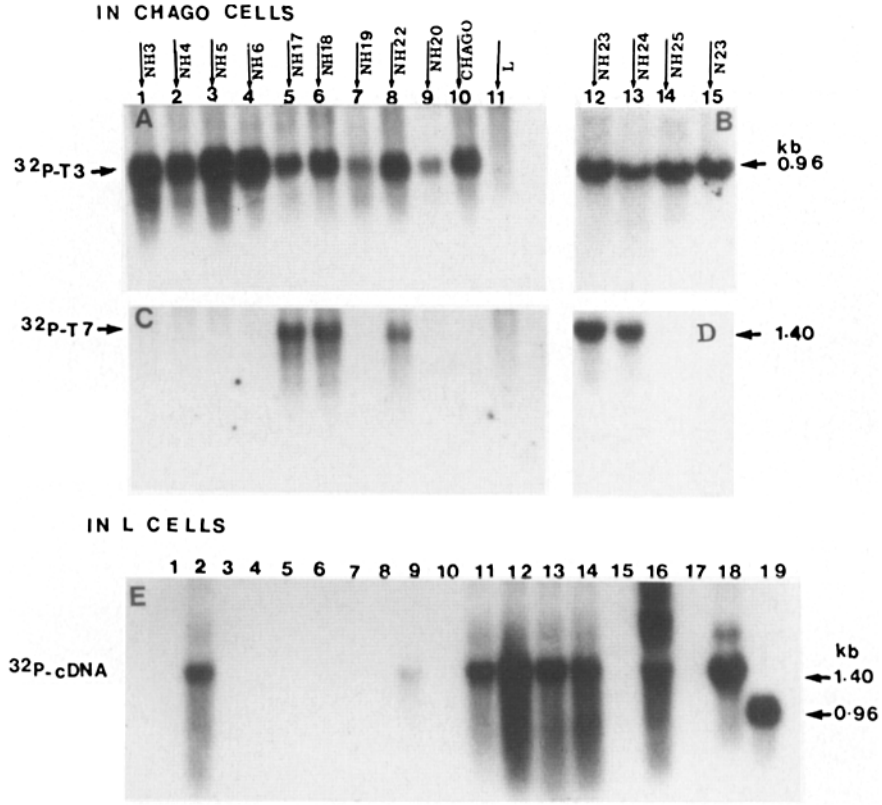

Figure 3. Identification of sense and antisense mRNA-α-HCG in ChaGo cell and L cell transfectants. Cellular total RNA was isolated and analyzed by the Northern blotting technique (33). 50 μg of RNA was loaded in each lane. *A* and *B* show the [^{32}P]T3 hybridizable sequences (α-HCG sense RNA) in ChaGo cell transfectants with pRSV-Neo and pRSV-anti-α-HCG (*A*, lanes *1–9*, and *B*, lanes *12–14*). In *B*, lane *15*, RNA from a transfectant derived with pRSV-Neo alone was applied. In *A*, lanes *10* and *11*, RNA from ChaGo cells and L cells were applied, respectively. *C* and *D* show the [^{32}P]T7 hybridizable sequences (α-HCG antisense RNA) of the same amounts of RNA samples analyzed under identical conditions. *E* shows the [^{32}P]α-HCG-cDNA hybridizable sequences in L cell transfectants. In *E*, lanes *3–8* contain RNA from transfectants with pRSV-Neo alone and lanes *9–18* contain RNA from transfectants with pRSV-Neo and pRSV-anti-α-HCG. *E*, lanes *1* and *19*, contain RNA from L cells and ChaGo cells, respectively. RNA from one of the ChaGo cell transfectants, NH17, was applied in *E*, lane *2*.

Table I. HCG Production by ChaGo Cells and Transfectants

Strains	Donor DNA	α-HCG antisense RNA	α-HCG (ng/μg cell DNA/day)
ChaGo	None	–	10.02
NH17	pRSV-Neo and pRSV-anti-α-HCG	+	0.56
NH18	" "	+	1.05
NH22	" "	+	0.86
NH23	" "	+	0.67
NH24	" "	+	0.78
NH3	" "	–	9.89
NH4	" "	–	10.90
NH5	" "	–	9.72
N23	pRSV-Neo	–	10.56
NG3	pRSV-Neo and pRSV-G1b	–	9.53
NG5	" "	–	11.02

1×10^5 cells were plated in 35-mm tissue culture dishes in 2 ml of complete medium. The next morning the medium was removed and 2 ml of fresh medium was added. The medium was changed every 2 d thereafter till the date of withdrawal for analysis on day 10 of growth. The HCG production by the transfectants in the culture media was quantitated by radioimmunoassay (34, 36). A polyclonal antibody and the RIA-grade authentic α-HCG was obtained as a gift from the National Hormone Distribution Agency). The ^{125}I-α-HCG and all the other reagents were obtained from Cambridge Medical Diagnostics (Billerica, MA). Cellular DNA content in each plate (on the day of withdrawal of the media) was measured as described previously (27). (–) Absence of α-HCG antisense RNA; (+) presence of α-HCG antisense RNA. NH17, NH18, NH22, NH23, and NH24 were pRSV-anti-α-HCG-expressing ChaGo cell transfectants. In NH3, NH4, and NH5 were G418-resistant ChaGo cell transfectants but α-HCG antisense RNA was not detected in these transfectants. N23 was a G418-resistant transfectant which was derived by transfection of only the pRSV-Neo plasmid and NG3 and NG5 were G418-transfectants of ChaGo cells in which globin mRNA was detected.

tively blocked the synthesis of α-HCG. The level of α-HCG synthesis was not affected in the globin gene expressing G418-resistant transfectants (Table I).

Influence of Expression of pRSV-Anti-α-HCG on the Morphology and Growth Characteristics of ChaGo Cells

Transfection of pRSV-anti-α-HCG drastically affected the morphology and growth pattern of ChaGo cells. The transfectants, expressing pRSV-anti-α-HCG were very much stretched, flattened, and multinucleated in comparison to the parental strain (Fig. 4). The postmitotic round cells which could be seen among the ChaGo cell population (Fig. 4 *A*) were practically absent in the pRSV-anti-α-HCG-expressing transfectants NH17, NH18, and NH23 (Fig. 4, *B*, *C*, and *D*, respectively). The morphology of the transfectants with pRSV-Neo and pRSV-globin genes were comparable to those of ChaGo cells. The morphology of the pRSV-anti-α-HCG-expressing transfectants were characteristic of resting cells. In fact, despite similar plating efficiencies, the growth rate of transfectants was significantly lower than that of ChaGo cells, especially when the plating density was very low (Fig. 5 *A*). The transfectants NH17, NH18, and NH22 remained quiescent during 10 d of growth in tissue culture conditions, but not because of nutritional deficiency (fresh medium was supplied every 3 d). Under these conditions, ChaGo cells doubled at their usual rate of 80–96 h. The growth could be accelerated by increasing the number of seeding cells (Fig. 5, *B* and *C*). Supplementation with α-HCG or with HCG (200 ng/ml) did not significantly affect the growth rate of these transfectants. Since serum can shift cells

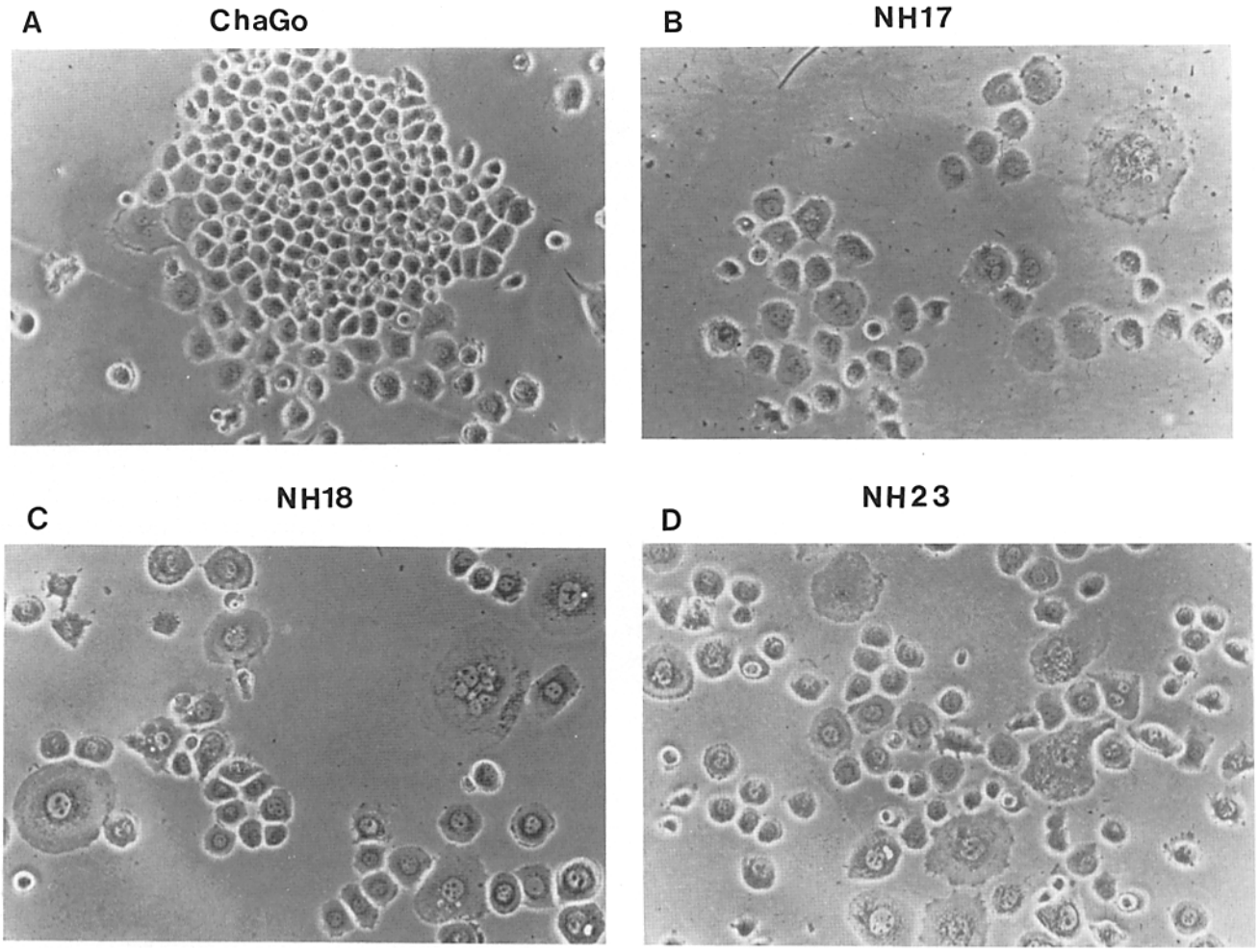

Figure 4. Morphology of ChaGo cells and transfectants. 1×10^5 cells were plated in 60-mm tissue culture dishes in complete medium (Ham's F10 medium supplemented with 10% fetal bovine serum). Cells were incubated in the CO_2 incubator under conditions described above. Cells were fed with fresh medium every fourth day. Photographs were taken under a phase-contrast microscope at 10×. ChaGo cells were photographed after 10 d of growth, whereas the pRSV-anti-α-HCG-expressing transfectants, NH17, NH18, and NH23, were photographed after 18 d of growth.

from a nonproliferating to a proliferating stage, additional serum (up to the concentration of 30%) was added to the culture medium of transfectants. This supplement-stimulated growth, which is in line with the morphologic evidence for the quiescent nature of α-HCG antisense-producing ChaGo cell transfectants (Fig. 5, *D* and *E*). Transfection of pRSV-anti-α-HCG did not affect the morphology or growth rate of L cells.

Stimulation of α-HCG Sense RNA Synthesis by 8,Bromo-cAMP and Its Effect on the Growth Phenotypes of α-HCG Antisense RNA-producing ChaGo Cell Transfectants

In seeking to elevate the level of α-HCG-specific mRNA, ChaGo cells and ChaGo cell transfectants were exposed to 8,bromo-cAMP which stimulates the synthesis of α-HCG-specific mRNA in other α-HCG-producing cells, such as BEWO (human choriocarcinoma) cells (6, 31). This treatment resulted in an elevated level of α-HCG-specific mRNA synthesis (sense RNA) in ChaGo cells and also in transfectants (Fig. 6 *A*, *top*). The synthesis of α-HCG antisense RNA, which is driven by the RSV promoter in the transfectants NH17 and NH18, was not affected by such treatment (Fig. 6 *A*, *middle*), nor was the expression of the α-tubulin gene (Fig. 6 *A*, *bottom*). When transfectants that express the pRSV-anti-α-HCG were exposed to 8,bromo-cAMP, they were shifted from the resting to growing phase (monitored by [^{3}H]thymidine incorporation; Fig. 6 *B*), and recovered their anchorage-independent growth phenotype (as shown by colony formation on soft agar; Table II).

[^{3}H]Thymidine Incorporation

The rate of [^{3}H]thymidine incorporation into DNA has been used as an index of the number of cells in S phase. The proportion of ChaGo cells in S phase was much higher relative to that of α-HCG antisense RNA-producing transfectants (50–55% for ChaGo cells [Fig. 6 *B*, *middle*], and only 15–20% in NH17 and NH18 [Fig. 6 *B*, *top* and *bottom*]). 8,Bromo-cAMP treatment of the transfectants stimulated [^{3}H]thymidine incorporation in these cells (Fig. 6 *B*, +; NH17, 60%, and NH18, 67%). The G1/S block, which was observed in NH17 and NH18, was reversed by increased synthe-

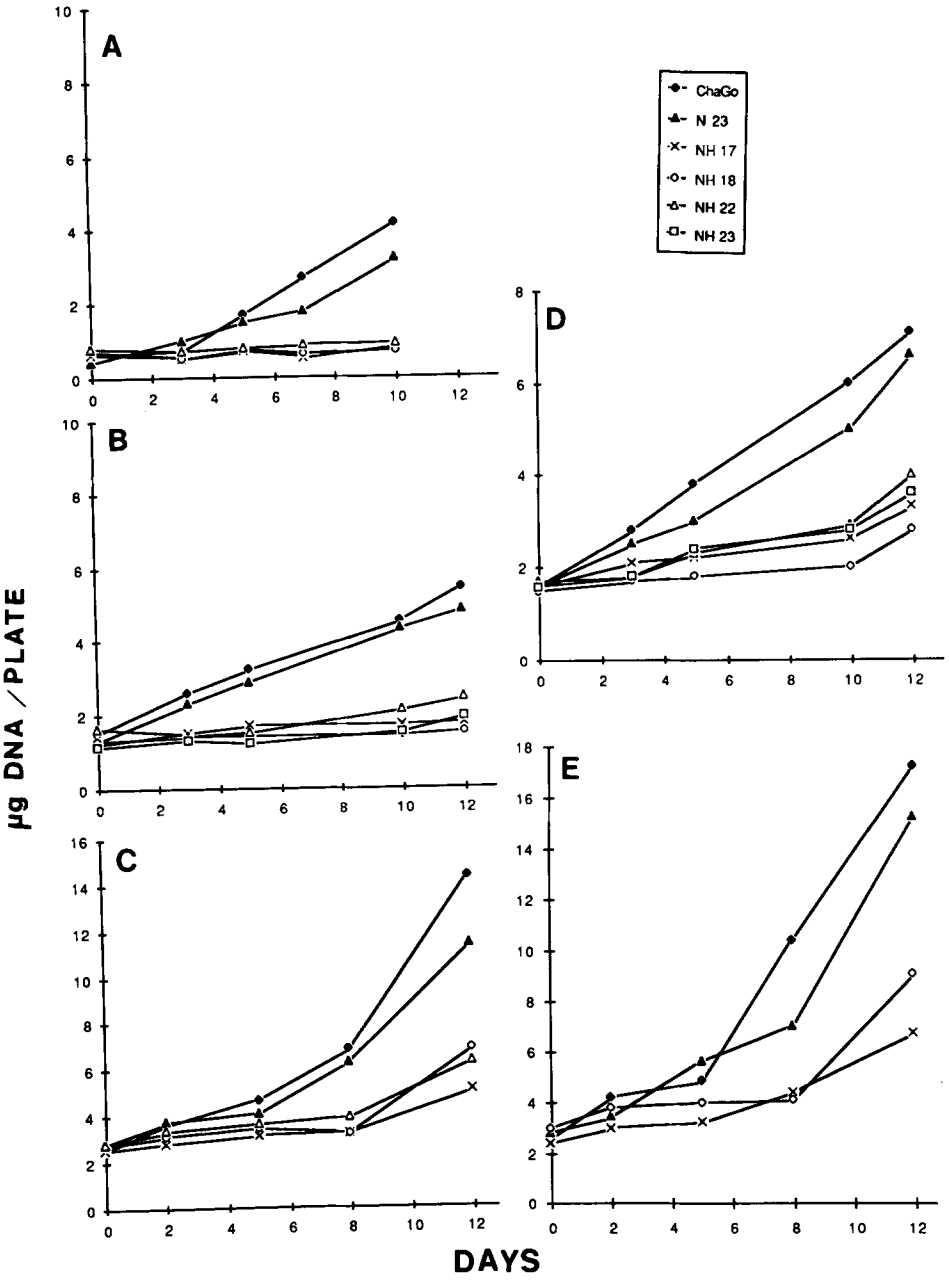

Figure 5. Growth rate of ChaGo cells and transfectants. ChaGo cells, the pRSV-anti-α-HCG-expressing transfectants NH17, NH18, NH22, and NH23, and the pRSV-Neo-expressing (only) transfectant N23 were plated in 35-mm tissue culture dishes in 2 ml of complete medium and were incubated under CO_2. The medium was replaced by serum minus medium after 24 h and by complete medium after another 48 h. Plates were examined thereafter as indicated. The α-HCG level in the medium was determined as described in Materials and Methods. Cells were washed with Hank's buffered saline, and scraped and resuspended in 1 ml of water. Cell extracts were prepared by sonication followed by centrifugation at 1,000 *g* for 5 min in a refrigerated centrifuge. DNA content in an aliquot was determined by fluorometry. Plating density: (*A*) 1×10^4; (*B*) 5×10^4; (*C*) 1×10^5 cells/plate in 10% serum-containing medium; (*D*) 5×10^4 cells/plate in 30% serum-containing medium; and (*E*) 1×10^5 cells/plate in 30% serum-containing medium.

sis of α-HCG-mRNA, which presumably increased production of the hormone. Treatment with 8,bromo-cAMP increased the intracellular ratio of sense to antisense RNA species. The presumed resultant increase in the intracellular level of α-HCG apparently drove the G1/S-blocked cells into the S phase.

Growth on Soft Agar

That the α-HCG level influenced growth was further supported by experiments on the anchorage-independent growth phenotype of both control and 8,bromo-cAMP-treated transfectants. The transfectants NH17 and NH18 which did not form colonies on soft agar did so in the presence of 8,bromo-cAMP (Table II). Addition of anti-α-HCG antibody inhibited the 8,bromo-cAMP-induced stimulation of growth of NH17 and NH18 on soft agar which suggests that the stimulated growth resulted from the increased intracellular synthesis of the hormone. In light of the similar behavior of ChaGo cells, it is concluded that the anchorage-independent growth phenotype of ChaGo cells and NH17 and NH18 is linked, either directly or indirectly, to the net level of α-HCG synthesis by these cells.

The Level of Expression of Cell Proliferation-dependent Histone H3.2 Gene and c-myc Gene in ChaGo Cells and in pRSV-Anti-α-HCG-expressing Transfectants

The level of histone (H3.2) gene expression increases in parallel with increased cell proliferation (4, 7, 14, 16). The influence of the synthesis of α-HCG antisense RNA on the proliferation of ChaGo cells was verified by monitoring the expression of the histone H3.2 gene. Results presented in Fig. 7 *A* demonstrate that the level of expression of H3.2 gene

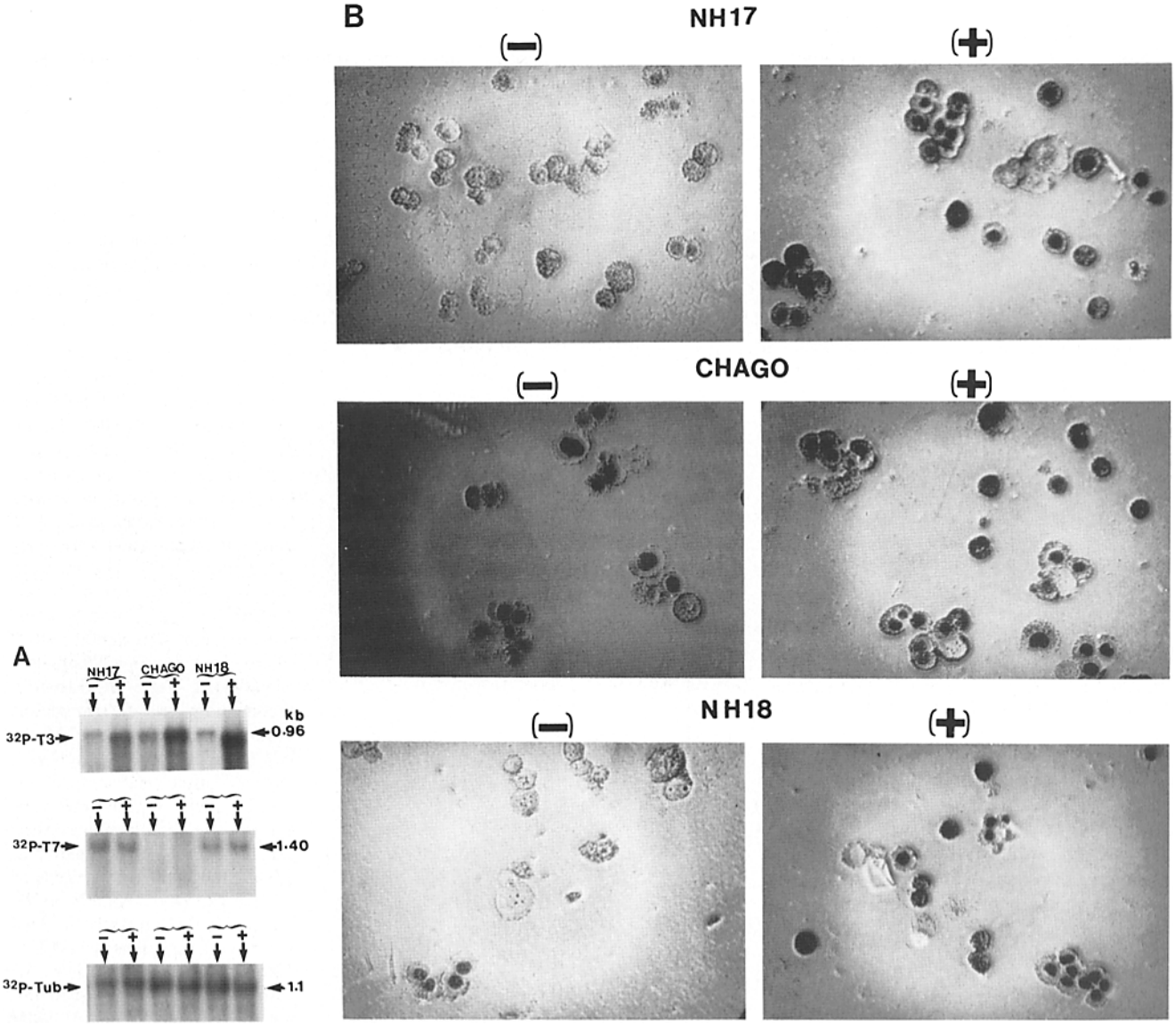

Figure 6. Influence of 8,bromo-cAMP on the synthesis of α-HCG-specific sense and antisense RNA and [^{3}H]thymidine incorporation in control and treated ChaGo cells and transfectants. ChaGo cells and the pRSV-anti-α-HCG-expressing transfectants, NH17 and NH18, were grown as described in Materials and Methods. (*A*) Northern blot analysis of RNA isolated from control (−) and 8,bromo-cAMP-treated (1 mM for 48 h; +) cells. 25 μg of total RNA was applied to each lane. Top autoradiograph shows the levels of α-HCG-specific sense RNA (hybridizable by [^{32}P]T3 riboprobe). Middle autoradiograph shows the levels of α-HCG-specific antisense RNA (hybridizable by [^{32}P]T7 riboprobe). The bottom autoradiograph shows the level of alpha 2-tubulin-specific mRNA sequences (hybridizable by [^{32}P]alpha 2-tubulin cDNA probe; see reference 1) in control (−) and treated (+) cells. The numbers on the right indicate the size in kilobase pairs (*kb*) of the specific RNA sequences. (*B*) Autoradiographs of [^{3}H]thymidine incorporation in control (−) and 8,bromo-cAMP-treated (+) (1 mM) ChaGo, NH17, and NH18 cells. Cells were incubated with 2 μCi/ml of [^{3}H]thymidine for 6 h, fixed, and autoradiographed under conditions described in Materials and Methods.

in the transfectants NH17 (Fig. 7 *A*, lane *2*), NH18 (Fig. 7 *A*, lane *3*), and NH22 (Fig. 7 *A*, lane *4*) was reduced almost to that of the normal fetal lung tissue (Fig. 7 *A*, lane *1*) and was significantly lower than that observed in ChaGo cells (Fig. 7 *A*, lane *5*). Rehybridization of the same filter with [^{32}P]c-*myc* probe demonstrated that the level of expression of this gene in all the three transfectants (Fig. 7 *B*, lanes *2*, *3*, and *4*) and in ChaGo cells (Fig. 7 *B*, lane *5*) were similar. The overexpression of c-*myc* gene in ChaGo cells that did not change with the transition of cells from growing to resting state suggested a stable deregulation of the gene.

Tumorigenic Potential of ChaGo Cells and pRSV-anti-α-HCG-expressing Transfectants in Athymic Mice

The tumorigenic potential of ChaGo cells and α-HCG antisense RNA-producing transfectants was verified by implantation of these cells under the dorsal skin of athymic mice (Nu Nu mice, Frederick Cancer Center, Frederick, MD). Implantation of ChaGo cells (1×10^6) once under the back skin of athymic mice (Nu Nu mice) induced palpable tumors within 3 wk which grew vigorously within the next few weeks (Fig.

Table II. Colony Forming Efficiency of ChaGo Cells and ChaGo Cell Transfectants in the Presence of 8,Bromo-cAMP in Soft Agar

Additions	ChaGo	NH17	NH18
	%	%	%
None	52.0	0.004	ND
Anti-α-HCG antibody	0.01	ND	ND
8,Bromo-cAMP	73.0	30.0	26.0
8,Bromo-cAMP and Anti-α-HCG antibody	2.4	ND	ND

The colony forming efficiency was calculated as described in Materials and Methods. 5×10^4 cells were seeded in triplicate plates; the top agar in the presence of 8,bromo-cAMP (1 mM), or anti-α-HCG antibody (1:2,500), or both. Colony formation in several fields were counted and the percent of colony formation was calculated from a total of 500 colonies and single cells in these fields. Plates with no colonies (*ND*) were scanned for the entire area.

8; examined and photographed 6 (*6W*) and 12 wk (*12W*) after implantation). Histological examination of the tumor confirmed these growths as epithelial cell carcinoma. No visible tumor was induced by NH17 and NH18 cells in any animals over the course of 6 wk (Fig. 8, *top*, *6W*). After 12 wk, a small tumorous growth appeared in only one of the animals that had received NH17 cells (Fig. 8, *bottom*, *12W*). The tumorigenic potential of ChaGo cells was practically lost in the presence of α-HCG antisense RNA, presumably due to the reduced intracellular level of hormone synthesis.

Discussion

These findings suggested that the intracellular level of α-HCG in ChaGo cells is critical for certain transformation phenotypes. The depletion of α-HCG by extracellular or intracellular manipulations had consequences. ChaGo cells lost their tumor phenotypes and exhibited those more characteristic of resting normal cells such as altered morphology, reduced growth rate, less mitosis, loss of anchorage-independent growth, and loss of tumorigenicity in athymic mice.

Hormones and growth factors regulate proliferation of eukaryotic cells in a very subtle manner. The appropriate site of synthesis, the site of action, and also the appropriate level of synthesis are the key determinants for the regulated growth of specific cell types under the influence of these physiological substances. Regulatory mechanisms of cell proliferation may thus be perturbed significantly by inappropriate and high levels of expression of these genes. Inappropriate gene expression leading to ectopic hormone production in varieties of human tissues has been correlated with neoplastic transformation of specific cell types (26). In this investigation, we have attempted to establish a causal relationship between α-HCG production and growth regulation of ChaGo, a cell line derived from a bronchogenic carcinoma. Our results demonstrated that deprivation of ChaGo cells of the alpha subunit of the hormone, either by α-HCG-specific antibody or by the expression of the recombinant plasmid pRSV-anti-α-HCG, drastically affected the growth pattern

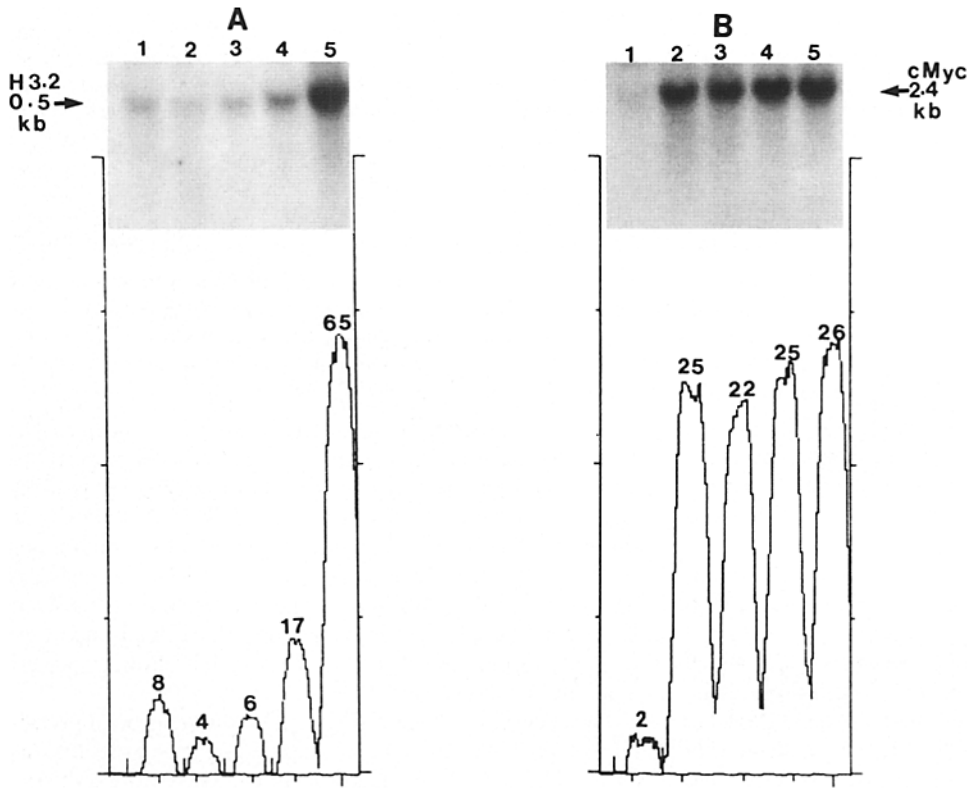

Figure 7. Level of expression of histone H3.2 and c-*Myc* in ChaGo cells and pRSV-anti-α-HCG-expressing transfectants. The level of expression of histone H3.2 gene (*A*) in ChaGo cells (lane *5*) and in transfectants NH17 (lane *2*), NH18 (lane *3*), and NH23 (lane *4*) was determined by Northern blot analysis of cellular total RNA (33). 50 μg total RNA was applied to each lane. The RNA samples were denatured and electrophoresed on 1.4% agarose, followed by blot transfer and subsequent hybridization with [^{32}P]H3.2 probe, and then autoradiographed. The same membrane filter was subsequently stripped of the [^{32}P]H3.2 by boiling three times (15 min each) in distilled water. The stripped membrane was then rehybridized with ^{32}P-labeled c-*myc* (human cDNA) probe (*B*). The arrows show the sizes in kilobase pairs of the specific RNA species. In lane *1*, the same amount of denatured total RNA from human fetal lung tissue was applied. The lower section of each panel demonstrates the densitometric scanning of the autoradiographic signals performed in a transmission densitometer with a 3-mm slit (EC Apparatus Corp., St. Petersburg, FL). The peak height and area was integrated in the Apple IIE computer with the Zenith electrophoresis program. The numbers on top of each peak show the percent of peak area.

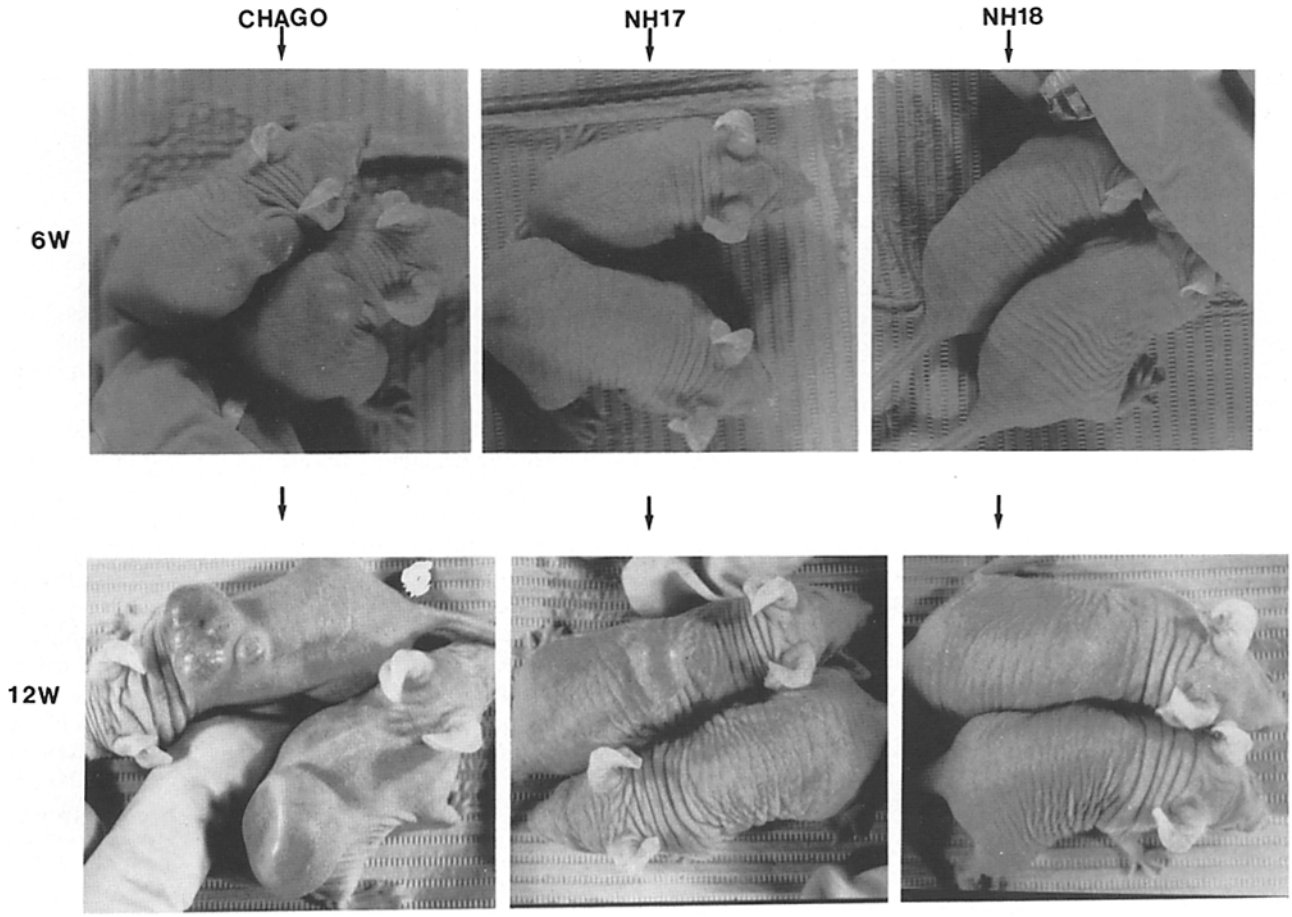

Figure 8. Tumorigenic potential of ChaGo and ChaGo cell transfectants (NH17 and NH18) in athymic mice. Athymic mice (Nu Nu mice from the Frederick Cancer Center) were housed in sterile cages separated from other animals. 1×10^6 cells of each cell strain in 0.5 ml sterile Hank's buffered saline were implanted under the back skin of the mice (in duplicate). The animals were examined routinely for palpable tumor growth. The top and bottom panels show the animals, as examined and photographed, 6 (*6 W*) and 12 wk (*12 W*) after sample implantation of the respective cell strains.

of these cells. Continuous proliferation and the anchorage-independent growth phenotypes were lost by these cells concurrent with the synthesis of α-HCG antisense RNA and reduced level of α-HCG production. The transition of pRSV-anti-α-HCG-expressing cells from a continuously proliferating state to the resting state was correlated with a reduced level of expression of the cell cycle-dependent H3.2 gene and [^{3}H]thymidine incorporation in the pRSV-anti-α-HCG-expressing transfectants. The proportion of the cells entering S phase was directly correlated with the level of α-HCG production.

The critical role of α-HCG on the regulation of growth of ChaGo cells was more evident from the results of modulation of the intracellular ratio of α-HCG-specific sense to antisense RNA in favor of the former by 8,bromo-cAMP, which reestablished the transformation phenotypes in these cells. The 8,bromo-cAMP-induced growth stimulation of transfectants could be reversed by anti-α-HCG antibody, suggesting that the observed growth stimulation was mainly due to the increased α-HCG production.

The level of α-HCG antisense RNA synthesized by the transfectants, though significant, was not higher than the level of endogenous sense RNA product. However, it seems that this level of antisense RNA was sufficient to inhibit significantly the synthesis of the protein product in these cells. It was suggested that a >50-fold higher level of antisense RNA over the endogenous sense RNA is required for the effective inhibition of synthesis of a specific protein in other cell types (2, 17, 20, 24, 29, 37, 41). However, it is evident from these results that different levels of antisense RNA synthesis was necessary in different cell types to achieve the effective inhibition of the synthesis of the target gene product. For example, in certain cell systems RNA duplexes induced the production of an unwinding protein, which might explain why such a high level of antisense RNA is necessary for the effective inhibition of specific protein synthesis in these cells (2, 29, 37). It seems that the recipient cell type is a determining factor for the effective inhibition of protein synthesis by antisense RNA.

The specificity of the α-HCG effect on the growth phenotype of ChaGo cells was demonstrated by the results that (*a*) anti-α-HCG antibody addition to a α-HCG-nonproducing human cell type (30); and (*b*) anti-α-HCG RNA synthesis in L cells in which no α-HCG-specific mRNA could be detected did not affect the growth pattern of these cells. These results, thus, suggest that the influence of α-HCG is re-

stricted to the ChaGo cells which ectopically synthesized the hormone. Though α-HCG partially reversed the antibody-induced inhibition of growth of ChaGo cells, the beta subunit of the hormone was unable to do so demonstrating the specificity of the antibody effect. Similarly, anti-β-HCG antibody did not affect the growth phenotype of ChaGo cells, again suggesting that the observed growth modulation was mainly dependent on the α-HCG concentration and not the β-HCG level in the culture medium. Although external addition of α-HCG was unable to reverse the α-HCG antisense–induced growth inhibition, the increased intracellular level of the subunit by 8,bromo-cAMP stimulated the cell proliferation. These results suggested that it is the intracellular level of the specific hormone which influenced the overall growth pattern of ChaGo cells. As the number of cells in the total population entering S phase was significantly reduced, or stimulated concurrently with a reduced or increased level of α-HCG production by these cells, it may be postulated that the lowered level of α-HCG led to a growth arrest, presumably before S phase. Although α-HCG reversed the antibody-induced growth inhibition of ChaGo cells, the hormone did not significantly improve the growth pattern of pRSV-anti-α-HCG-expressing transfectants. This, however, could be achieved by increasing the intracellular level of α-HCG. These results suggested that intracellular concentration of the hormone was the critical determinant of its proliferative signal, which probably was mediated via a cascade of events. In this case, exogenously administered hormone may reverse the intracellular depletion caused by antibody presentation, but could not access the intracellular compartment to substitute for endogenously produced hormone in antisense-blocked cells. The inability of externally added α-HCG may be also due to the defective receptor mechanism through which the hormone action is normally mediated. The possibility that α-HCG antibody- and α-HCG antisense RNA–induced growth effects were due to the altered level of β-HCG seems to be remote because neither of the effects could be reversed by addition of β-HCG to ChaGo cells or to the α-HCG antisense RNA–producing transfectants. The overexpression of the c-*myc* gene, which is believed to be a G1-specific event, may also play a very important role in the growth phenotypes of transformed ChaGo cells. Though the role of c-*myc* overexpression in ChaGo cells was not defined as yet, it may be postulated that the c-*myc* gene product and α-HCG may work in synchrony. The former may increase the number of cells in G1 and the latter drive the cells to S phase, similar to the one designated as a progression factor. Overexpression of both these genes, caused by the carcinogenic event, may maintain the continuous growth phenotype of ChaGo cells. While the level of deregulated overexpression of c-*myc* remained unaltered, reducing the level of α-HCG alone blocked continuous proliferation and arrested the growth of these lung tumor cells, presumably in the G1/S junction of the cell cycle. Voluminous reports demonstrate extensive correlation of ectopic synthesis of HCG and subunits, predominantly α-HCG, with tumors of different human tissues. Though the mechanism by which its effect is exerted on the continuous growth phenotype of lung tumor cells is not clearly defined in molecular terms, experimental evidence is presented in this report which demonstrates a possible direct or indirect role of the ectopic synthesis of the glycoprotein hormone in the carcinogenic process.

The authors are thankful to Drs. Arthur B. Pardee of the Dana Farber Cancer Institute (Boston, MA), Jane Chisholm of the Harvard School of Public Health (Boston, MA), Mortimer Litt of Harvard Medical School (Boston, MA), and P. V. Cherington of Tufts University School of Medicine (Boston, MA) for their help and suggestions in the preparation of this manuscript.

This work was supported by NIH grant HL3203 awarded to D. K. Biswas.

Received for publication 2 August 1988 and in revised form 27 December 1988.

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