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Introduction of v-Ha-*ras* oncogene induces differentiation of cultured human medullary thyroid carcinoma cells

(calcitonin/calcitonin-gene-related peptide/neuroendocrine/secretory granules)

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ABSTRACT **Medullary thyroid carcinoma (MTC) is an endocrine tumor of the thyroid C cells that expresses high levels of the neuroendocrine peptide hormone calcitonin. During tumor progression in the host, there is an apparent loss of differentiation in MTC cells that involves a consistent decrease in calcitonin content of the tumor cells associated with decreased expression of the calcitonin gene and/or changes in a mRNA alternative-processing pattern away from that characteristic of the parent thyroid C cell. We now report that introduction of the viral Harvey *ras* (v-Ha-*ras*) oncogene into cultured human MTC cells can reverse such changes in gene expression and can induce endocrine differentiation of the tumor cells. The expression of v-Ha-*ras* is associated with decreased cellular proliferation and DNA synthesis. There is a marked increase in the number of cytoplasmic secretory granules that are a classic feature of differentiated thyroid C cells. v-Ha-*ras* expression induces increased expression of the calcitonin gene and the processing of the primary gene transcript is shifted to favor calcitonin mRNA rather than calcitonin-gene-related peptide (CGRP) mRNA production. These studies with cultured human MTC cells provide a model system to study the role of Ha-*ras* and related genes in neuroendocrine differentiation. The findings suggest an important approach for identifying genes in solid tumors whose altered expression may play a role in the impaired maturational capacity characteristic of cancer cells during tumor progression.**

Medullary thyroid carcinoma (MTC) is an endocrine tumor that maintains many of the differentiation features of the parent thyroid C cells including the presence of endocrine cell secretory granules, high transcription of the calcitonin gene, and a route of alternative mRNA processing to favor generation of calcitonin-specific mRNA (1–4). Unlike normal C cells, MTC cells often process the primary calcitonin gene transcript to generate not only CT-specific mRNA, but also high amounts of calcitonin-gene-related peptide (CGRP) mRNA (1–4). In patients, there is a distinct relationship between the degree of cellular differentiation and clinical behavior of MTC. Diminished calcitonin production is a constant feature of tumor tissues in patients dying of metastatic MTC (5–9).

The genes responsible for maintenance of proper endocrine cell differentiation and regulation of calcitonin production in thyroid C cells and whose expression might be impaired in tumor cells to prevent proper maturation are not known. In previous studies (10, 11), we demonstrated that chemical agents known to activate the protein kinase A and protein kinase C pathways can induce the appearance of differentiation properties in cultured human MTC cells. In attempting to understand what cellular programs maintain the differentiation status of normal C cells and what alterations might impede normal maturation during tumor progression in MTC, it would be important to define the type of genes that might exert differentiation effects by activating signal-transduction pathways identical or similar to those mentioned above. We now report that increased endocrine cell differentiation properties are acquired by cultured MTC cells with introduction of the viral Harvey *ras* (v-Ha-*ras*) oncogene. This gene is a candidate to interact with protein kinase A and protein kinase C pathways, because its protein product p21 may activate protein kinase A by its GTP-binding capacity and may activate protein kinase C by increasing cellular diacylglycerol (12–16).

MATERIALS AND METHODS

Cell Culture and Virus Infection. Cells from a well-characterized culture line of human MTC, the TT line (17), were grown at 37°C in RPMI 1640 medium (B&B/Scott, Fiskeville, RI) supplemented with 16% (vol/vol) heat-inactivated fetal bovine serum (GIBCO), 2 mM glutamine, 100 units of penicillin per ml, and 100 μg of streptomycin sulfate per ml in a humidified CO_2 incubator. NIH 3T3 cells, infected with Harvey murine sarcoma virus (Ha-MSV) and 1504A amphotropic murine leukemia helper virus (refs. 18 and 19; kindly provided by A. Rein), were grown in Dulbecco's modified Eagle's medium (DMEM, from GIBCO) supplemented with 9% (vol/vol) fetal bovine serum. For viral infection, TT cells were exposed for 3 days to conditioned medium from the infected NIH 3T3 cells, which produced high titers of Ha-MSV and 1504A helper virus. Prior to infection of TT cells, the conditioned medium was mixed with an equal volume of RPMI 1640 medium containing Polybrene (Sigma) at 10 μg/ml.

Growth Study. Seven days after viral infection, the cells were harvested by trypsinization and seeded in 6-well plates at a density of 10^6 cells per well. As controls, uninfected TT cells or cells infected with 1504A helper virus alone were also identically harvested and plated. Cell number was determined with an automated cell counter (Coulter Electronics). Rates of DNA synthesis were evaluated by measuring the incorporation of [^{3}H]thymidine into acid-insoluble material as described (11).

Secretion of Calcitonin. Medium from Ha-MSV-infected or uninfected TT cells was centrifuged (1500 × *g*, 3 min), and the concentration of calcitonin in the culture medium was determined by a radioimmunoassay (10) and normalized to cell number. Control medium, which was not exposed to the cells, had no detectable calcitonin.

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Abbreviations: CGRP, calcitonin-gene-related peptide; MTC, medullary thyroid carcinoma; Ha-MSV, Harvey murine sarcoma virus.

cDNA Probes. The calcitonin (pTT1062) and CGRP (pTT42) cDNA probes have been described (4). pTT1062 contains only calcitonin-specific sequences (exon 4), no CGRP-specific sequences (4), and no sequences common to both calcitonin and CGRP (exons 1–3). pTT83 has a *Pvu* II–*Pst* I restriction fragment from pTT42 containing only CGRP-specific sequences, including 17 base pairs (bp) of exon 5 and all of exon 6 of the calcitonin gene (4).

Recombinant plasmids containing human β-actin sequences were provided by D. Cleveland (Johns Hopkins University School of Medicine). An *Sst* I–*Pst* I fragment (730 bp) containing only the v-Ha-*ras* gene p21 coding region was obtained from Oncor (Gaithersburg, MD). These probes were labeled with [α-^{32}P]dCTP by nick-translation.

DNA Hybridization. DNA was extracted by a published technique (20), and 10 μg was digested with either *Hin*dIII or *Pst* I for 12–16 hr. Restriction endonuclease-digested genomic DNA for Southern blotting (21) was electrophoresed in a 1% agarose gel and transferred to GeneScreen*Plus* nylon filters (New England Nuclear). After hybridization, filters were washed at 68°C in 15 mM NaCl/1.5 mM sodium citrate, pH 7/0.1% $NaDodSO_4$ for 60 min and then autoradiographed for 3 days at −70°C.

RNA Hybridization. RNA was extracted as previously described (11, 20). RNA (6 μg) for blot hybridization analysis was electrophoresed in a 6% formaldehyde/1.2% agarose gel and transferred to nitrocellulose (20). After hybridization with the probe, filters received a final wash at 68°C in 15 mM NaCl/1.5 mM sodium citrate, pH 7/0.1% $NaDodSO_4$ for 30 min. For autoradiography, filters were exposed for 16 hr at −70°C. The hybridizations and quantitation of the hybridization signal for calcitonin, CGRP, and β-actin mRNAs by densitometry were performed as described (10, 11).

Electron Microscopy. While still attached to the flask, cells were fixed in 2% glutaraldehyde/0.1 M cacodylate buffer (pH 7.4), postfixed in 1% OsO_4/0.1 M cacodylate buffer, sectioned, stained with uranyl acetate/lead citrate, and viewed with a Philips 410 transmission electron microscope at 100 kV (22).

RESULTS

Presence and Expression of Ha-MSV Sequences. The presence of Ha-MSV proviral DNA in infected TT cells was confirmed by Southern blotting of genomic DNA. *Hin*dIII- and *Pst* I-digested DNA from infected cells showed a number of bands when hybridized to a *Pst* I–*Sst* I restriction fragment containing only the v-Ha-*ras* p21 coding region (Fig. 1*A*). *Hin*dIII-digested DNA from infected cells showed a predicted 880-bp fragment consistent with the presence of proviral DNA (23). Also, *Pst* I digests showed, in addition to a 0.8-kilobase (kb) endogenous c-Ha-*ras* fragment in both control and infected cells, a series of bands, including a predicted 4.4-kb band from the Ha-MSV proviral DNA and a series of lower molecular weight bands, especially at 1.8 kb. These smaller fragments suggest rearrangement of some of the integrated sequences.

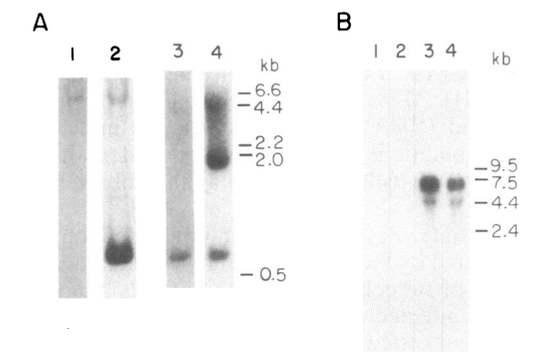

FIG. 1. (*A*) Southern blot of genomic DNA (10 μg) isolated from control (uninfected) TT cells (lanes 1 and 3) or Ha-MSV-infected TT cells (lanes 2 and 4). DNA was digested with *Hin*dIII (lanes 1 and 2) or *Pst* I (lanes 3 and 4), and blots were probed with a ^{32}P-labeled (nick-translated) *Sst* I–*Pst* I fragment (730 bp) containing only the v-Ha-*ras* p21 coding region (Oncor). Positions and lengths (kb) of molecular size markers (*Hin*dIII fragments of phage λ DNA) are given. (*B*) Blot hybridization of total cytoplasmic RNA (6 μg) isolated from control TT cells (lane 1), 1504A helper virus-infected TT cells (lane 2), or two separate Ha-MSV infections of TT cells (lanes 3 and 4). Hybridization was with the same nick-translated probe as in *A*. Note the predominant 6.8- and 4.4-kb transcripts seen only in Ha-MSV infected cells (lanes 3 and 4). RNA "ladder" (Bethesda Research Laboratories) was used as size markers.

Ha-MSV sequences were highly expressed in the infected TT cells (Fig. 1*B*). No transcript homologous to the v-Ha-*ras* p21 coding-region probe could be detected in RNA from uninfected host TT cells or from cells infected by the 1504A helper virus alone. The latter cells did have high expression of the 1504A viral sequences (data not shown).

Morphological Changes. The Ha-MSV-infected TT cells had marked morphological changes in >80% of cells within 5 days after exposure to virus (Fig. 2). Infected cells became smaller and rounded, similar in appearance to TT cells treated with phorbol esters (11). No morphological differ-

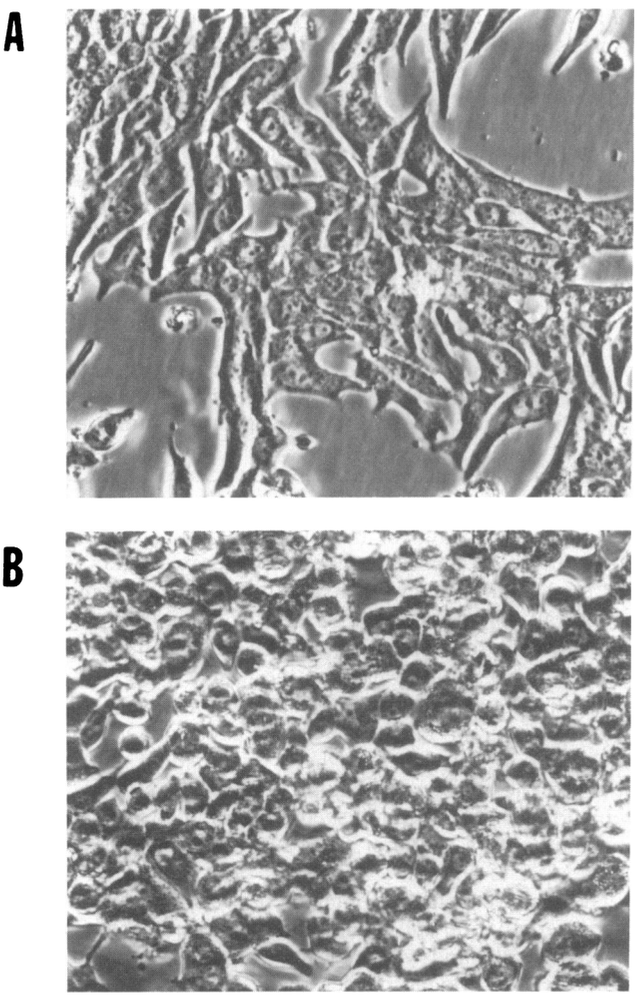

FIG. 2. Micrographs of uninfected TT cells (*A*) and Ha-MSV-infected TT cells 10 days after infection (*B*). Note the flat, large uninfected TT cells (*A*), and the large populations (often 90% or more) of small, rounded cells in Ha-MSV-infected TT cells (*B*). These morphological changes were maintained for at least three passages in culture. TT cells infected with 1504A helper virus alone showed no morphological changes. (×200.)

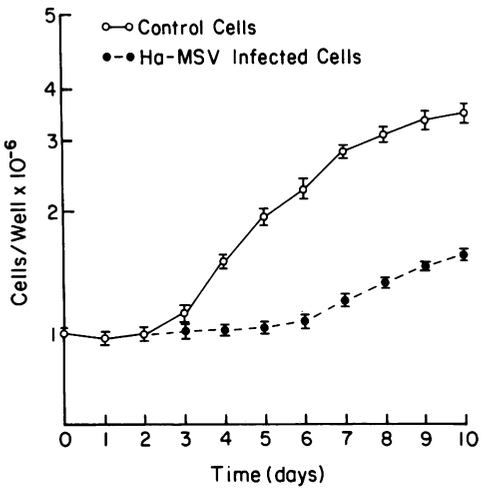

FIG. 3. Growth curves of Ha-MSV-infected and uninfected TT cells. Seven days after viral infection, the cells were trypsinized and seeded in 6-well plates at a density of 10^6 cells per well. Cell number was determined at each time point, using an automated cell counter (Coulter Electronics). Each point represents the mean (± SD) of triplicate wells in three independent Ha-MSV infections. Growth of cells infected with 1540A helper virus alone was identical to those of uninfected control cells.

ences were observed in cultures infected only with the 1504A helper virus.

Changes in Cell Growth. The integration and expression of Ha-MSV had marked effects on the growth of TT cells, producing a decrease in proliferation similar to that observed when these cells were treated with phorbol esters or cyclic AMP (10, 11). Doubling time of the Ha-MSV-infected cells was 216 hr, whereas that of uninfected cells was 84 hr (Fig. 3). This decrease in growth was also evident in a comparable decrease in the incorporation of [^{3}H]thymidine (Table 1). No effect on growth was seen in cells infected with 1504A helper virus alone.

Table 1. Ha-MSV infection decreases [^{3}H]thymidine incorporation in TT cells

Days after subculture	Incorporation, cpm per 10^5 cells		
	Uninfected	Helper virus alone	Ha-MSV plus helper virus
3	3442 ± 517	ND	3305 ± 421
6	4452 ± 189	4400 ± 430	1990 ± 87*
9	2981 ± 353	2844 ± 78	1579 ± 93*

Values are means ± SD of triplicate wells. ND, not done.
*$P < 0.001$, compared to uninfected or helper virus-infected cells.

Changes in Neuroendocrine Differentiation. The v-Ha-*ras*-infected cells showed an ultrastructural change suggestive of a dramatic increase in endocrine cell differentiation. Mature endocrine cells and well-differentiated endocrine tumor cells, including MTC cells, contain well-formed cytoplasmic secretory granules, which serve to package and store mature forms of small polypeptide hormones such as calcitonin (24, 25). Despite the relatively high calcitonin content of the TT cells, such granules are sparse in uninfected cells. Introduction of v-Ha-*ras* leads to a dramatic increase in secretory granules (Fig. 4), identical in morphological appearance to those seen in normal human thyroid C cells and in differentiated MTC cells (25).

Ha-MSV Increases Calcitonin Secretion. Consistent with the increased numbers of secretory granules described above, the Ha-MSV-infected cells had markedly increased calcitonin secretion. Calcitonin in the culture supernatant, measured over a 6-day period by radioimmunoassay (10),

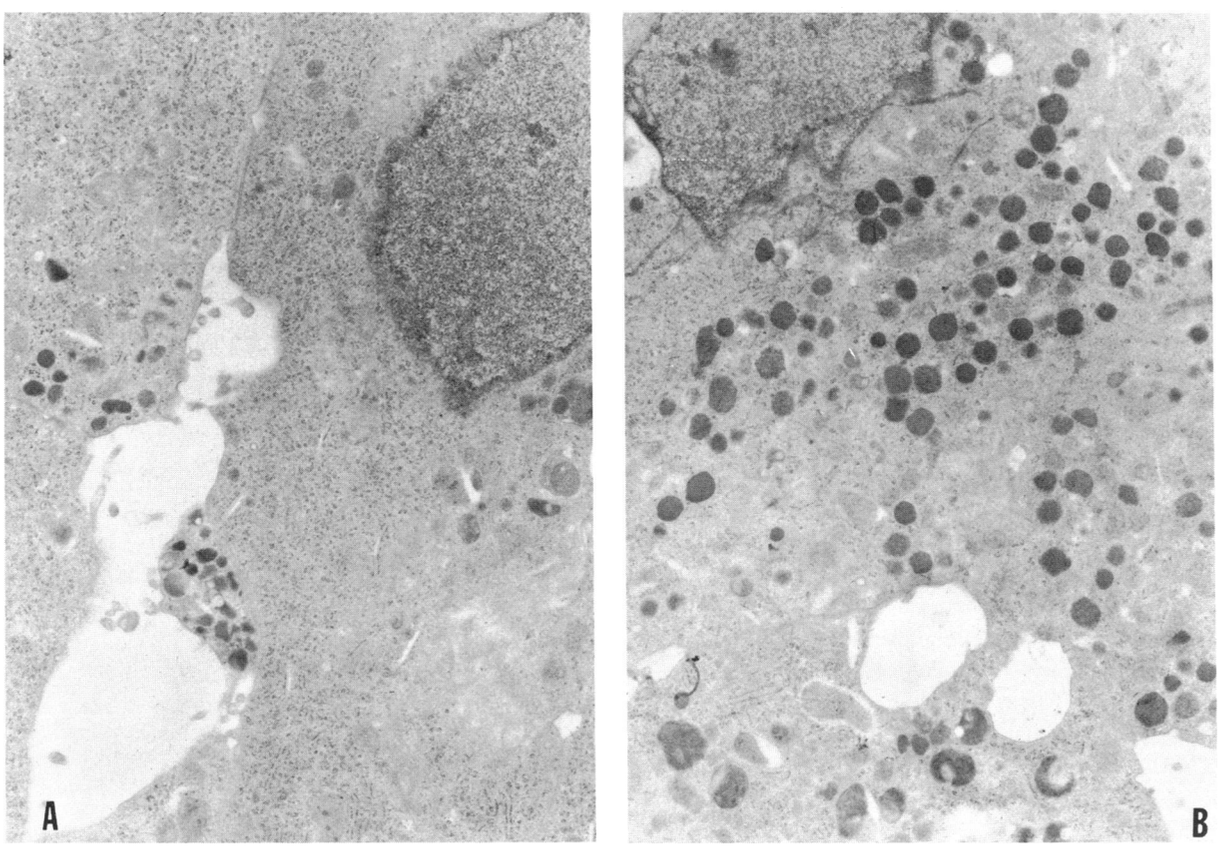

FIG. 4. Electron micrographs of TT cells (*A*) and Ha-MSV-infected TT cells 10 days after infection (*B*). Note the profound increase of dense spherical secretory granules in Ha-MSV-infected TT cells (*B*). (×9000.)

increased up to 3- to 4-fold in Ha-MSV-infected cells compared to control cells (Fig. 5).

Ha-MSV Increases Calcitonin Gene Expression. Insertion of the v-Ha-*ras* gene led to changes in expression of the calcitonin gene in MTC cells consistent with a state of increased differentiation. Ha-MSV-infected cells had an increase in the expression of the gene and an increase of the ratio of calcitonin- to CGRP-specific mRNA. This change in mRNA ratio is in a direction more typical of the calcitonin gene expression pattern in normal thyroid C cells, which predominantly produce calcitonin.

Previous work showed that calcitonin gene expression in cultured MTC cells is tightly coupled to the growth status of the cells (ref. 26; B.D.N., K.Y. Chen, A.D.B., B. A. Roos, and S.B.B., unpublished data). As shown in Fig. 6, in uninfected control cells, calcitonin-sequence-specific mRNA rises during the late growth phase, whereas CGRP mRNA increases earlier in growth and decreases as cells reach confluence. Hence, we studied calcitonin and CGRP mRNA levels at multiple time points during the growth of the Ha-MSV-infected TT cells. Steady-state calcitonin-specific mRNA is markedly increased (range 2- to 4.5-fold in seven separate experiments) in cytoplasm from Ha-MSV-infected TT cells at all time points studied (Fig. 6). However, CGRP-specific mRNA, which is derived from the same gene, increased little in cells infected with Ha-MSV (Fig. 6; range, 0–25% in seven separate experiments). Thus, the ratio of calcitonin mRNA to CGRP mRNA was increased 2-fold or more at each time point in infected TT cells. No changes in calcitonin or CGRP mRNA expression were seen in cells infected by 1504A helper virus alone (data not shown).

DISCUSSION

Ha-MSV infection of cultured human MTC cells alters cell morphology, decreases cell proliferation, increases the numbers of cytoplasmic secretory granules, increases calcitonin gene expression, and increases the ratio of calcitonin mRNA to CGRP mRNA. These changes are all consistent with acquisition of increased endocrine cell differentiation properties by these cells. The formation of secretory granules is consistent with a general increase in endocrine cell maturation capacity, and the increased calcitonin/CGRP mRNA ratio is a specific feature of mature thyroid C cells.

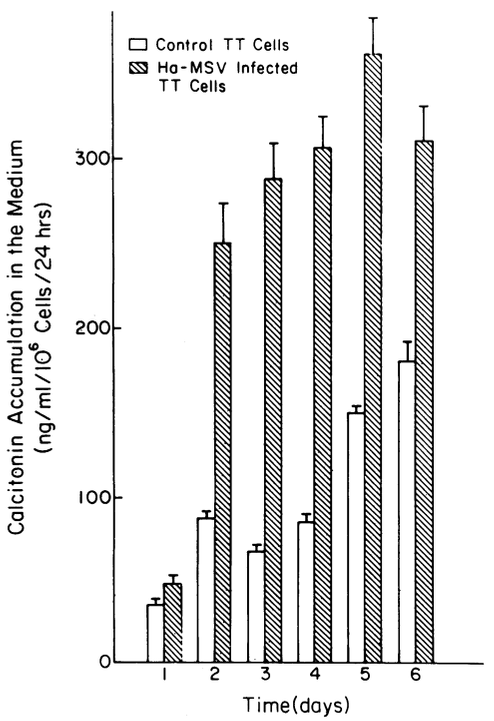

FIG. 5. Calcitonin secretion into the medium. Seven days after viral infection, the cells were trypsinized and seeded in 6-well plates at a density of 10^6 cells per well. Calcitonin content in the medium was measured by radioimmunoassay at each time point (10). Calcitonin content in the medium of TT cells infected with 1504A helper virus alone was identical to that of uninfected control cells. Each bar represents the mean (+ SD) of triplicate wells (ng/ml for 10^6 cells over 24 hr).

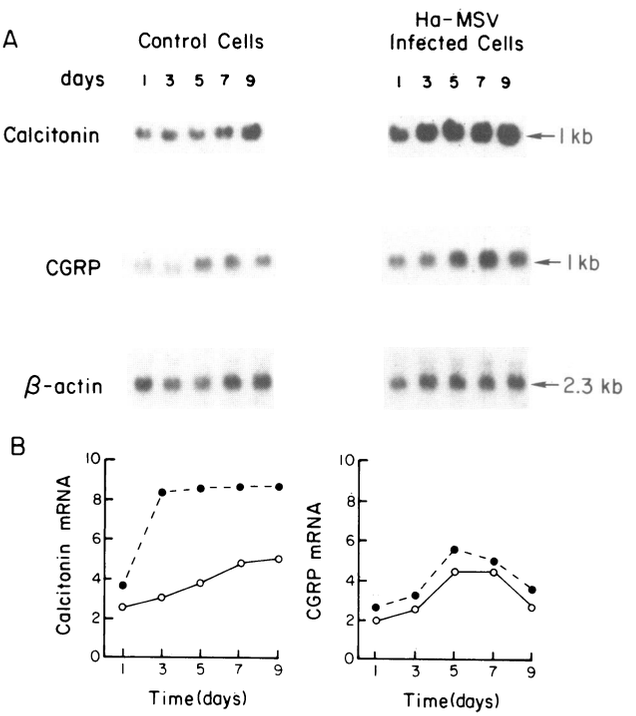

FIG. 6. (*A*) Blot hybridization of calcitonin and CGRP mRNAs during the growth of Ha-MSV-infected and uninfected TT cells. Total cytoplasmic RNAs were extracted, electrophoresed, blotted, and hybridized to ^{32}P-labeled cDNA probes for calcitonin-specific sequences (pTT1062) (4), for CGRP-specific sequences (pTT83) (4), and for human β-actin sequence. (*B*) Calcitonin and CGRP mRNA levels as determined by densitometric analysis and expressed relative to the signal for β-actin mRNA during the growth of the TT cells. ○, Control, uninfected TT cells; ●, Ha-MSV-infected TT cells. The results for the one representative experiment shown are typical of those obtained in seven separate studies.

Two of the differentiation-related changes seen in the present work (decreased growth and increased calcitonin gene expression) were observed previously (10, 11) when MTC cells were treated with phorbol esters or cyclic AMP (activators of protein kinase C and protein kinase A, respectively). It is then possible that the *ras* oncogene induces some of its effects on MTC cells through signal-transduction pathways identical or similar to those activated by phorbol esters and cyclic AMP. However, the v-Ha-*ras*-induced formation of secretory granules and the increase in calcitonin/CGRP mRNA ratio are additional effects to those seen with phorbol esters and cyclic AMP and could represent activation of additional signal-transduction pathways.

The transforming role of the v-Ha-*ras* oncogene and the ability of this gene to immortalize some normal cells has been well established in multiple studies (27–34). Much less well studied is the role that c-Ha-*ras* may play in maintaining proper differentiation in normal or neoplastic cells (35–39). Interestingly, Ha-MSV has been shown to cause changes in differentiation-associated properties of an endocrine tumor that is closely related developmentally to MTC cells, the rat pheochromocytoma PC12 (39). This differentiation process was also induced in PC12 cells by microinjection of activated c-Ha-*ras* p21 proteins (38). The growth-arresting and differ-

entiation-inducing effects of Ha-MSV on MTC and pheochromocytoma cells suggest that c-Ha-*ras* or a similar gene may have a role in normal neuroendocrine differentiation. While defining the exact gene(s) involved will require further study, the approach used in our present investigation should help to define the types of genes that induce and/or maintain neuroendocrine cell differentiation *in vivo*.

A cardinal feature of tumor progression in human cancers is the loss of, or decrease in, the differentiation properties characteristic of the normal parent cells from which the tumor arises. This is a particularly striking feature of MTC, which retains high calcitonin content in patients with more indolent tumors but decreases or even loses content of this hormone in patients who die of virulent metastatic disease (5–9). The loss of expression for a key gene(s), which may activate signal-transduction pathways involved in normal cell differentiation, could play a role in the type of differentiation block that accompanies cell transformation and/or tumor progression in MTC and related tumors. One could envision that we have restored such a lost gene function by introduction of the Ha-*ras* gene into MTC cells in our present study. Our system, which stresses modulation of neuroendocrine differentiation phenotype at the morphologic level and at the level of gene expression, provides an important model for further dissection of the mechanisms through which alterations in genes such as Ha-*ras* may interfere with normal differentiation in cancer cells.

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