# Conditional BRAF$^{V600E}$ Expression Induces DNA Synthesis, Apoptosis, Dedifferentiation, and Chromosomal Instability in Thyroid PCCL3 Cells

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## Abstract

**The activating mutation *BRAF*$^{T1796A}$ is the most prevalent genetic alteration in papillary thyroid carcinomas (PTC). It is associated with advanced PTCs, suggesting that this oncoprotein confers thyroid cancers with more aggressive properties. *BRAF*$^{T1796A}$ is also observed in thyroid micropapillary carcinomas and may thus be an early event in tumor development. To explore its biological consequences, we established doxycycline-inducible BRAF$^{V600E}$-expressing clonal lines derived from well-differentiated rat thyroid PCCL3 cells. Expression of BRAF$^{V600E}$ did not induce growth in the absence of thyrotropin despite increasing DNA synthesis, which is likely explained because of a concomitant increase in apoptosis. Thyrotropin-dependent cell growth and DNA synthesis were reduced by BRAF$^{V600E}$ because of decreased thyrotropin responsiveness associated with inhibition of thyrotropin receptor gene expression. These results are similar to those obtained following conditional expression of RET/PTC. However, in contrast to RET/PTC, BRAF activation did not impair key activation steps distal to the thyrotropin receptor, such as forskolin-induced adenylyl cyclase activity or cyclic AMP–induced DNA synthesis. We reported previously that acute RET/PTC expression in PCCL3 cells did not induce genomic instability. By contrast, induction of BRAF$^{V600E}$ expression increased the frequency of micronuclei by both clastogenic and aneugenic events. These data indicate that BRAF$^{V600E}$ expression confers thyroid cells with little growth advantage because of concomitant activation of DNA synthesis and apoptosis. However, in contrast to RET/PTC, BRAF$^{V600E}$ may facilitate the acquisition of secondary genetic events through induction of genomic instability, which may account for its aggressive properties.** (Cancer Res 2005; 65(6): 2465-73)

## Introduction

The *BRAF* gene encodes a serine/threonine kinase that serves as an immediate downstream effector of RAS. BRAF transmits signals from RAS to the mitogen-activated protein kinase (MAPK) pathway through mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK) and ERK (RAS-BRAF-MEK-ERK). *BRAF* somatic activating mutations have been identified in various types of cancers, including melanomas (prevalence ~70%) and colorectal and ovarian cancers (~15%; ref. 1). *BRAF* mutations are the most common genetic alteration (36-69%) in papillary thyroid carcinomas (PTC; refs. 2–8). The mutation in PTC is almost exclusively a thymine-to-adenine transversion at nucleotide 1,796 (T1796A), resulting in a valine-to-glutamic acid substitution at amino acid 600 (V600E), designated previously as V599E (9). This mutation is believed to produce a constitutively active kinase by disrupting hydrophobic interactions between residues in the activation loop and residues in the ATP binding site that maintain the inactive conformation and allowing development of new interactions that fold the kinase into a catalytically competent structure (10, 11). Transfection of BRAF$^{V600E}$ leads to constitutive ERK phosphorylation and high transforming activity in NIH3T3 cells (1). However, in melanocytes, oncogenic BRAF is probably not sufficient to induce malignant melanoma because *BRAF* mutations have also been found in ~80% of benign nevi (12).

*RET/PTC* rearrangements are the other genetic hallmarks of PTCs and are particularly prevalent in pediatric cases and in patients with a history of exposure to ionizing radiation (13–16). There is practically no overlap between PTC with *RET/PTC*, *NTRK1*, *BRAF*, or *RAS* mutations, which altogether are found in ~70% of cases (2, 4, 17). The lack of concordance for these mutations provides strong genetic evidence for the requirement of this signaling system for transformation to PTC.

Most studies concur that tumors with *RET/PTC* rearrangements rarely progress to aggressive or undifferentiated carcinomas (18). On the other hand, PTCs with BRAF$^{V600E}$ are more commonly invasive (7) and have a higher likelihood of presenting at a more advanced stage (5, 7). BRAF$^{V600E}$ has been also found in poorly differentiated/anaplastic carcinomas arising most likely from PTCs (5, 7). These findings suggest that this oncoprotein confers thyroid cells with more aggressive properties. Because some thyroid micropapillary carcinomas also have BRAF$^{V600E}$ (7, 19), we have postulated that this oncogenic hit may be an initiating or very early step in tumor development.

To explore early biological consequences following BRAF mutational activation in thyroid cells, we established doxycycline-inducible BRAF$^{V600E}$-expressing clonal lines derived from rat thyroid PCCL3 cells. PCCL3 cells are well-differentiated thyroid cells that require thyrotropin for growth as well as for expression of thyroid-specific genes. Here, we report that expression of BRAF$^{V600E}$ results in impairment of thyrotropin-induced expression of thyroid-specific gene products, including the thyrotropin receptor (TSH-R), consistent with prior studies pointing to dedifferentiating effects through constitutive activation of effectors along the MAPK pathway (20–22). However, BRAF$^{V600E}$ is not sufficient to allow cells to grow in the absence of thyrotropin, although the oncoprotein stimulates DNA synthesis, likely because of concomitant induction of apoptosis, resulting in no net growth in

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the cell population. However, $BRAF^{V600E}$, unlike RET/PTC1 and RET/PTC3, induces genomic instability, suggesting that this oncoprotein may facilitate the acquisition of secondary genetic events necessary for unregulated growth and clonal expansion.

## Materials and Methods

**Cell Lines and Transfections.** PCCL3 cells, a clonal rat thyroid cell line requiring thyrotropin for growth, were maintained in H4 medium consisting of Coon's medium/F-12 high zinc supplemented with 5% fetal bovine serum, 0.3 mg/mL L-glutamine, 1 mIU/mL thyrotropin, 10 μg/mL insulin, 5 μg/mL apo-transferrin, 10 nmol/L hydrocortisone, and penicillin/streptomycin. H3 medium was identical to H4 medium but without thyrotropin. We used a doxycycline-inducible expression system to obtain conditional expression of the oncoprotein in PCCL3 cells as described previously (23). Briefly, we subcloned a *myc*-tagged $BRAF^{V600E}$ cDNA (a gift from Richard Marais, Institute of Cancer Research, University of London, London, United Kingdom) into pUHG10-3, downstream of seven repeats of a tet operator sequence and a minimal cytomegalovirus promoter. This construct was cotransfected into PCCL3 cells stably expressing the reverse tetracycline transactivator rtTA (PC-rtTA cells) with pTK-hygro using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) and clones selected based on absence of expression under basal conditions and strong induction by doxycycline.

**Northern Blotting.** Total RNA was isolated from cells using TRIzol reagent (Invitrogen). Northern blotting was done as described previously (24). The following cDNAs were used to prepare [$^{32}$P]dCTP-labeled probes: *myc* epitope-tagged full-length $BRAF^{V600E}$, full-length rat sodium iodide symporter (NIS) cDNA (a gift from Nancy Carrasco, Albert Einstein College of Medicine, Bronx, NY), 2.9-kb fragment of the 5′ end of mouse thyroglobulin cDNA (a gift from Paul Kim, University of Cincinnati, Cincinnati, OH), and full-length Pax-8 cDNA (purchased from American Type Culture Collection, Manassas, VA).

**Western Blotting.** Cells were lysed in a buffer containing 20 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 150 mmol/L NaCl, 0.5% Triton X-100, 50 mmol/L sodium fluoride, 10 mmol/L sodium pyrophosphate, 2 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, and protease inhibitor cocktail (Sigma, St. Louis, MO). After measurement of protein concentration using Micro BCA Protein Assay Reagent (Pierce, Rockford, IL), 25 μg of each sample were separated by 8% SDS-PAGE and blotted onto a polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ). The following primary antibodies were used: anti-*myc* 9E10 (Oncogene, Boston, MA), anti-phospho-ERK E10 (Cell Signaling, Beverly, MA), anti-ERK K-23 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-MEK1/2 (Cell Signaling), anti-BRAF C-19 (Santa Cruz Biotechnology), and anti-BRAF F-7 (Santa Cruz Biotechnology). The antigen-antibody complexes were visualized using horseradish peroxidase–conjugated anti-mouse or rabbit IgG antibody (Santa Cruz Biotechnology) and enhanced chemiluminescence system (Amersham Biosciences).

**Growth Curves.** Cells ($5 \times 10^4$) were plated in each well of six-well plates. The following day, medium was changed to H4 or H3 with or without doxycycline. At the indicated times, the cells were washed with PBS and detached by trypsinization. The cell number was counted using a Z1 Coulter counter (Beckman Coulter, Fullerton, CA).

**Real-time Reverse Transcription-PCR.** Total RNA (2 μg) was reverse transcribed to generate cDNA with SuperScript III (Invitrogen) in the presence of random hexamers. Real-time PCR was done using QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) in a LightCycler instrument (Cepheid, Sunnyvale, CA). The following primer pairs were used: β-actin (5′-CTGAACCCTAAGGCCAACCGTG-3′ and 5′-GGCATACAGGGACAGCACAGCC-3′) and TSH-R (5′-CAAAGATGCCTTTGGAGGAG-3′ and 5′-AGCTCTTTGAGGTGCTCCAG-3′). The cycle threshold value, which was determined using the second derivative, was used to calculate the normalized expression of the TSH-R mRNA using Q-Gene software (25).

### DNA Synthesis

**Thymidine Incorporation Assay.** Cells ($10^4$) were plated in each well of Cytostar-T 96-well scintillating microplate (Amersham Biosciences). After incubation with or without 1 μg/mL doxycycline for the indicated times, medium was replaced with medium containing 0.5 μCi/mL [$^{14}$C]thymidine with or without doxycycline. After the indicated incubation times, the incorporated [$^{14}$C]thymidine was measured using a Packard Top Counter (Perkin-Elmer, Boston, MA).

**Bromodeoxyuridine Uptake Assay.** Cells were incubated with bromodeoxyuridine (BrdUrd) for 1 hour, collected by trypsinization, and fixed with 70% ethanol. Cells were then incubated with 2 N HCl to denature DNA and neutralized with 0.1 mol/L sodium borate (pH 8.5). After labeling with FITC-conjugated anti-BrdUrd antibody (BD PharMingen, San Diego, CA), the percentage of cells positive for BrdUrd incorporation was determined by fluorescence-activated cell sorting (Coulter EPICS, Beckman Coulter).

**Cell Detachment.** Cells ($10^5$) were plated in each well of six-well plates. After the indicated times, the medium was collected to obtain floating cells and the cells remaining on the plate were removed by trypsinization. Detached and attached cells were counted with a Z1 Coulter counter.

**Apo-BrdUrd Assay.** Apo-BrdUrd assays were done according to the manufacturer's protocol (BD PharMingen). Briefly, both floating and attached cells were collected and fixed with 1% paraformaldehyde followed by 70% ethanol. The fixed cells were stored at −20°C until assayed. For DNA labeling, cells were incubated with a DNA labeling solution containing terminal deoxynucleotide transferase and BrdUrd. After labeling, cells were stained with fluorescein-labeled anti-BrdUrd antibody and then incubated with propidium iodide/RNase A solution. The percentage of cells positive for BrdUrd staining (apoptosis) was determined by fluorescence-activated cell sorting.

**Assay for Micronuclei.** Cells were fixed with 10% formaldehyde and stained with 4′,6-diamidino-2-phenylindole (DAPI). The number of micronuclei was obtained by counting cells visually under a fluorescence microscope. For centromere staining, cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 in PBS, and blocked with 10% goat serum in PBS. Anti-kinetochore antibody CREST (Antibodies, Inc., Davies, CA) was used as a primary antibody at a 1:10 dilution in blocking solution. After washing, cells were incubated with anti-human Alexa 488–coupled antibody (Molecular Probes, Eugene, OR) at a 1:500 dilution in blocking solution and stained with DAPI. The number of centromere-positive or centromere-negative micronuclei was counted visually under a fluorescence microscope.

**Cyclic AMP Assay.** Cyclic AMP (cAMP) assays were done using the Adenylyl Cyclase Activation FlashPlate Assay Kit (Perkin-Elmer) as directed by the manufacturer. Briefly, 25,000 cells were suspended in the Stimulation Buffer containing the phosphodiesterase inhibitor isobutylmethylxanthine and added to each well of the 96-well FlashPlate. Cells were then stimulated with 25 μmol/L forskolin at 37°C for 60 minutes. Cells were lysed with Detection Buffer containing cAMP $^{125}$I Tracer and incubated at room temperature for 2 hours. Cellular cAMP levels were measured using a Packard Top Counter.

## Results

**B1 Is the Predominant BRAF Isoform Expressed in Mouse Thyroid and Rat Thyroid PCCL3 Cells.** The *BRAF* gene encodes at least eight different isoforms whose expression varies between tissues (26). Four of the isoforms differ according to the presence or absence of exons 8b to 10 (Fig. 1*A*). In addition, B1 to B4 have short and long forms that result from the deletion of exons 1 to 2 due to an alternative start site within exon 3. The presence of exon 10 (isoforms B3 and B4) enhances the affinity of BRAF for MEK, basal kinase activity, and the mitogenic and transformation properties of BRAF (27). By contrast, the presence of exon 8b has the opposite effect on BRAF activity and function. Mouse thyroid tissue and

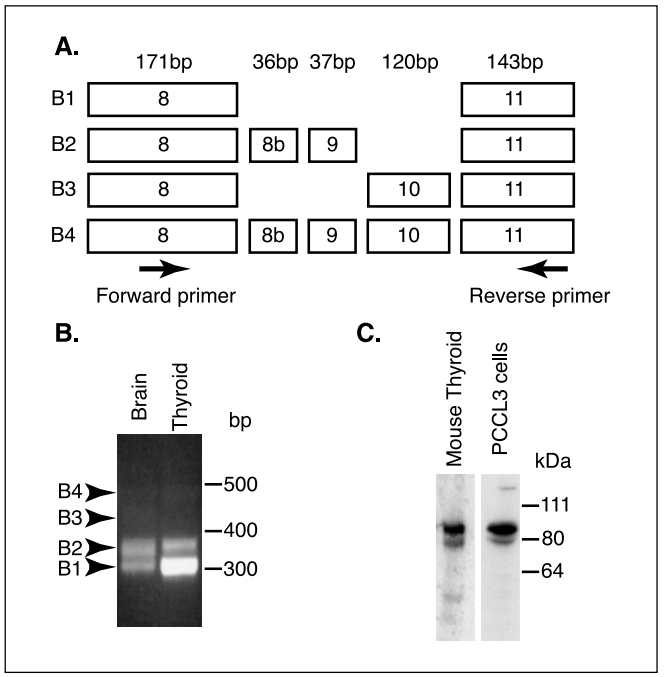



**Figure 1.** B1 isoform of BRAF is expressed in mouse thyroid and rat PCCL3 cells. *A,* alternative splicing of the mouse *BRAF* gene leads to expression of distinct BRAF isoforms. *B,* reverse transcription-PCR of mouse brain/thyroid RNA using primers that amplify the region between exons 8 and 11. *C,* Western blots of protein extracts of mouse thyroid tissue or PCCL3 cells were probed with a rabbit antibody to the COOH terminus of BRAF.

PCCL3 cells were examined by reverse transcription-PCR and Western blotting to determine the BRAF isoforms expressed in thyroid follicular cells. Reverse transcription-PCR amplification of mRNA from mouse thyroid tissue shows that B1 is the predominant isoform in this tissue (Fig. 1*B*). Western blotting identified an 86-kDa band as the most abundant product, likely corresponding to phosphorylated B1 BRAF, whereas a 79-kDa band is consistent with the unphosphorylated B1 isoform of BRAF (refs. 26, 27; Fig. 1*C*). A similar pattern of expression was also observed in Western blots of extracts from PCCL3 cells (Fig. 1*C*), suggesting that the long form of B1 is also the predominant isotype expressed in rat thyroid. These data informed our choice of BRAF expression vector for these studies, which corresponded to the human B1 isoform.

**Establishment of Doxycycline-Inducible BRAF$^{V600E}$ Thyroid Cells.** Clonal cell lines with doxycycline-inducible expression of BRAF$^{V600E}$ were derived from well-differentiated rat thyroid PCCL3 cells. We selected two lines with low basal BRAF$^{V600E}$ expression and robust induction after addition of doxycycline. As shown in Fig. 2*A*, PC-BRAF$^{V600E}$-6 and PC-BRAF$^{V600E}$-12 cells showed doxycycline-dependent induction of BRAF$^{V600E}$ mRNA. In the absence of doxycycline, the mRNA was barely detected. These lines showed doxycycline dose-dependent BRAF$^{V600E}$ protein expression and ERK phosphorylation (Fig. 2*B*). The two BRAF bands correspond to B1 isoforms of ~86 and 79 kDa. Basal and maximum phosphorylation levels of ERK in PC-BRAF$^{V600E}$-12 cells were higher than in PC-BRAF$^{V600E}$-6 cells. BRAF$^{V600E}$ expression and induction of MEK/ERK phosphorylation were apparent ~12 hours after addition of doxycycline (Fig. 2*C*). Total BRAF expression (endogenous wild-type + induced V600E mutant) at 48 hours was ~2-fold greater than control (Fig. 2*C*).

**Morphologic Change of PCCL3 Cells after Expression of BRAF$^{V600E}$.** When examined by phase-contrast light microscopy, PC-BRAF$^{V600E}$-6 cells in the absence of doxycycline tended to cluster and showed a cobblestone appearance similar to that seen in parental PCCL3 cells (Fig. 3*A*). After addition of doxycycline, a proportion of the cells became rounded and detached, whereas others developed a spindle-like shape (Fig. 3*A*). These changes were first noted at ~48 to 72 hours and persisted through at least 6 days. Similar changes were observed in PC-BRAF$^{V600E}$-12 cells (data not shown).

**Effect of Acute Activation of BRAF$^{V600E}$ on Cell Growth of PCCL3 Cells.** We next investigated the effect of acute BRAF$^{V600E}$ expression on growth of thyroid PCCL3 cells. As shown in Fig. 3*B*, BRAF$^{V600E}$ expression did not confer PCCL3 cells with the ability to grow in the absence of thyrotropin. Moreover, thyrotropin-dependent cell growth was impaired by ~50% to 60% by BRAF$^{V600E}$ expression. PC-BRAF$^{V600E}$-6 cells grew faster than PC-BRAF$^{V600E}$-12 cells, which had higher basal and stimulated activity of ERK. A previous study showed that low-level induction of RAF was growth promoting, but high induction inhibited mitosis and DNA synthesis in NIH3T3 cells (28). Because of this, we examined BRAF$^{V600E}$ effects on cell growth with four different concentrations of doxycycline. As shown in Fig. 3*C*, a dose-dependent inhibition was seen in the presence of thyrotropin and no significant change was found in the absence of thyrotropin.

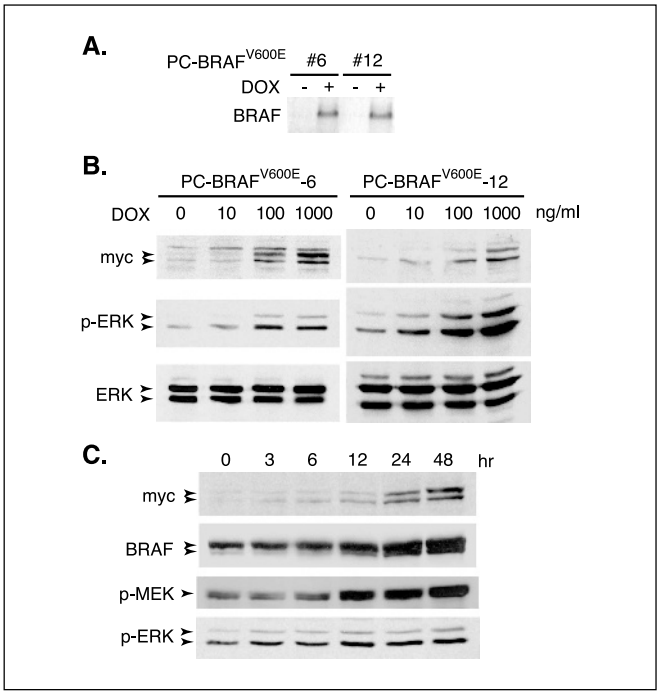



**Figure 2.** Development of PCCL3 clonal lines with doxycycline-inducible expression of BRAF$^{V600E}$. *A,* Northern blot of RNA from PC-BRAF$^{V600E}$-6 and PC-BRAF$^{V600E}$-12 cells treated with or without 1 μg/mL doxycycline for 24 hours. Blots were hybridized with a full-length *myc*-BRAF$^{V600E}$ cDNA probe. *B,* dose-dependent induction of *myc*-BRAF$^{V600E}$ and ERK phosphorylation by doxycycline. Indicated lines were treated with the indicated concentration of doxycycline for 48 hours. Western blotting was done using the indicated primary antibody. *C,* time course of BRAF$^{V600E}$ induction. PC-BRAF$^{V600E}$-6 cells were incubated with 1 μg/mL doxycycline for the indicated times. Total cell lysates (25 μg) were subjected to Western blotting using the indicated primary antibodies. This BRAF antibody recognizes the $NH_2$ terminus of BRAF. Representative of two independent experiments.

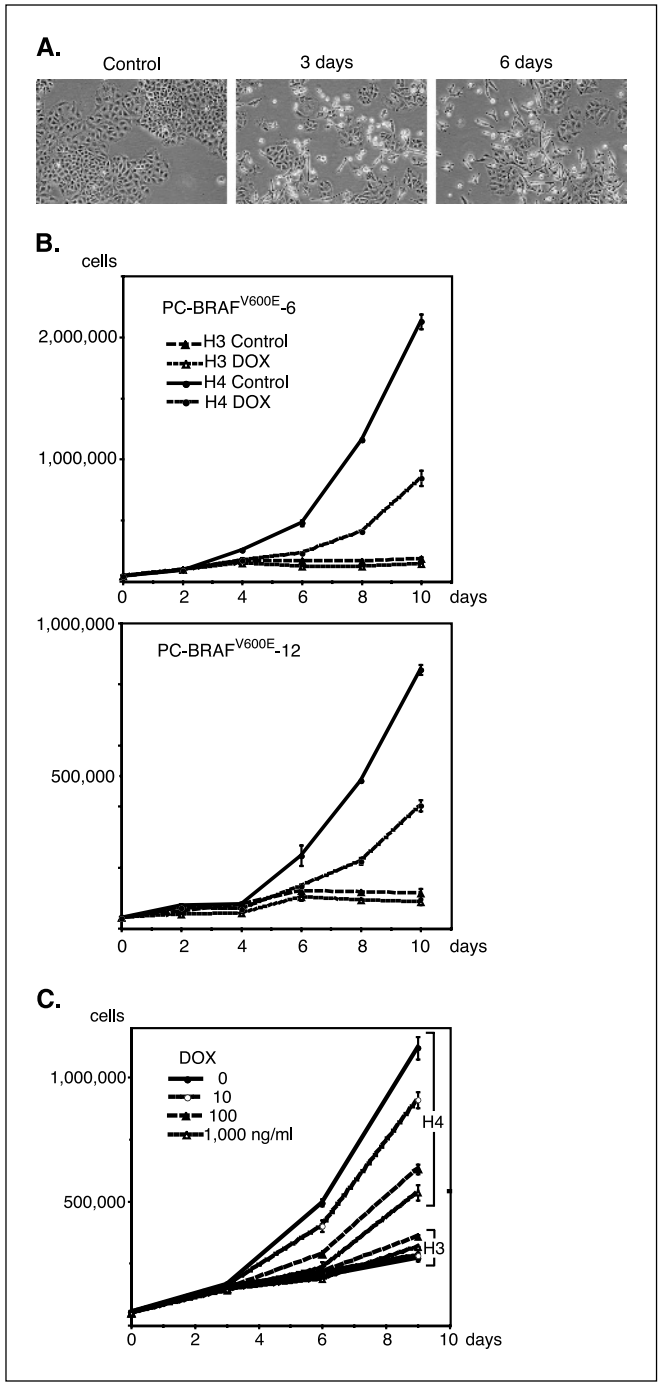



**Figure 3.** *A,* morphologic change of PCCL3 cells after expression of BRAF$^{V600E}$. PC-BRAF$^{V600E}$-6 cells were cultured in H4 medium with or without 1 μg/mL doxycycline for the indicated times before taking images with an inverted microscope with phase contrast. *B,* effect of acute activation of BRAF$^{V600E}$ on cell growth of PC-BRAF$^{V600E}$-6 or PC-BRAF$^{V600E}$-12 cells. Cells were treated with or without 1 μg/mL doxycycline in the presence (H4) or absence (H3) of thyrotropin. Medium was changed every 2 days. *C,* dose-dependent effect of doxycycline-induced BRAF$^{V600E}$ expression on cell growth. PC-BRAF$^{V600E}$-6 cells were treated with the indicated concentration of doxycycline in H4 or H3 medium. Medium was changed every 3 days. *Points,* mean of three wells of a six-well plate; *bars,* SD. Representative of two independent experiments.

**Effect of BRAF$^{V600E}$ on Thyroid-Specific Gene Expression.** The impairment of thyrotropin-induced cell growth by BRAF$^{V600E}$ prompted us to explore whether the oncoprotein might interfere with TSH-R mRNA abundance. Indeed, previous studies from several groups have shown that constitutive activation of effectors along the RET/PTC-RAS-MAPK pathway impair thyroid-specific gene expression (20–22). As shown in Fig. 4*A*, real-time reverse transcription-PCR confirmed that BRAF$^{V600E}$ expression induced time-dependent reduction of TSH-R mRNA expression. Expression of NIS, thyroglobulin, and Pax-8 mRNA as determined by Northern blotting showed similar reduction patterns (Fig. 4*B*). The effects of BRAF$^{V600E}$ on expression of NIS mRNA cannot be simply attributed to impairment in thyrotropin action through decreased abundance of TSH-R, because BRAF$^{V600E}$ also inhibited cAMP-induced NIS mRNA levels (Fig. 4*C*).

**Effect of BRAF$^{V600E}$ Expression on DNA Synthesis.** We next examined the effect of BRAF$^{V600E}$ oncoprotein on DNA synthesis. We first investigated this by [$^{14}$C]thymidine incorporation into control and doxycycline-treated PC-BRAF$^{V600E}$-6 cells. In the absence of thyrotropin, BRAF$^{V600E}$ induced DNA synthesis by

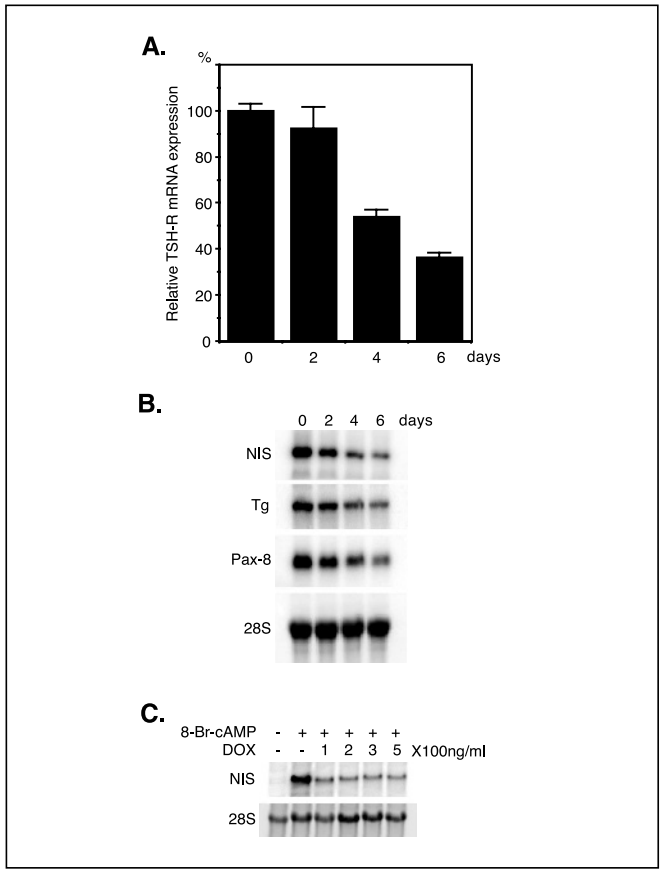



**Figure 4.** Effect of BRAF$^{V600E}$ expression on thyroid cell differentiation. PC-BRAF$^{V600E}$-6 cells were cultured in H4 medium with or without 1 μg/mL doxycycline for the indicated times. Cells were then harvested and total RNA was extracted. *A,* time course of TSH-R mRNA abundance following BRAF$^{V600E}$ expression. TSH-R mRNA was determined by real-time reverse transcription-PCR and expression was normalized for β-actin mRNA. Relative to expression of TSH-R mRNA in the absence of doxycycline. *Columns,* mean of a single experiment done in triplicate; *bars,* SE. Similar results were obtained in two additional independent experiments. *B,* effects of BRAF$^{V600E}$ on NIS, thyroglobulin (*Tg*), and Pax-8 mRNA levels. Northern blots were hybridized with the indicated probes. Ethidium bromide staining of 28S rRNA was used as a loading control. Similar results were obtained in two separate experiments. *C,* effects of BRAF$^{V600E}$ on 8-Br-cAMP-induced NIS mRNA. Cells were pretreated with H3 medium for 3 days. Doxycycline (*DOX*) was added 12 hours before addition of 500 μmol/L 8-Br-cAMP and cells were harvested after a further incubation for 48 hours. Northern blot was hybridized with NIS cDNA, and ethidium bromide–stained 28S rRNA is shown for verification of loading.

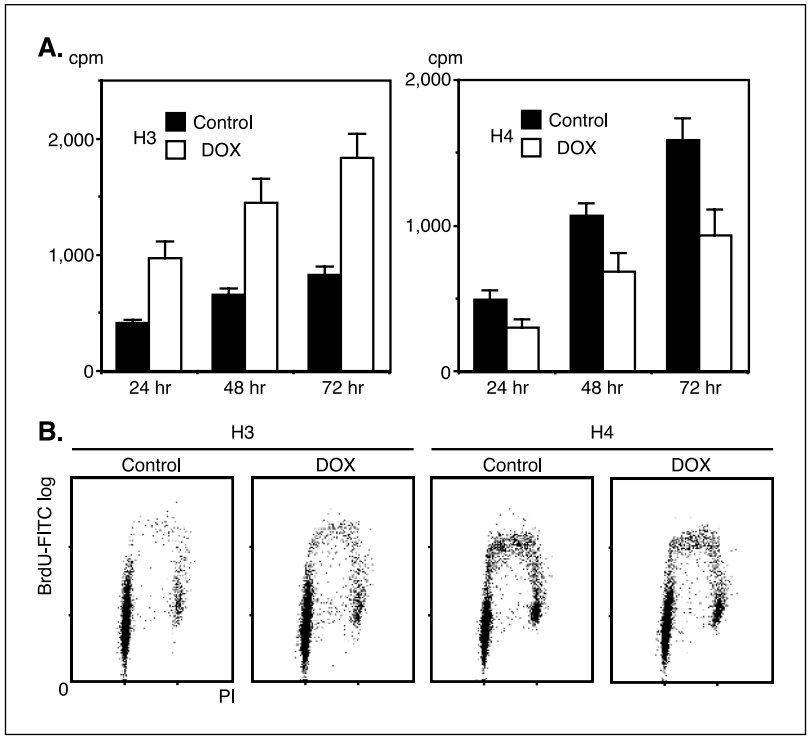



**Figure 5.** Effect of expression of BRAF$^{V600E}$ on DNA synthesis. *A,* PC-BRAF$^{V600E}$-6 cells were cultured with or without 1 μg/mL doxycycline for 24 hours in H3 or H4 medium. In the H3 experiment, the cells were preincubated with H3 medium for 3 days before addition of doxycycline. The medium was then replaced with medium containing [$^{14}$C]thymidine with or without doxycycline and incubated for the indicated times. Incorporated thymidine was measured as described in Materials and Methods. *Columns,* mean of eight wells; *bars,* SD. Similar results were obtained in two separate experiments. *B,* PC-BRAF$^{V600E}$-6 cells were cultured with or without 1 μg/mL doxycycline in H3 or H4 medium for 6 days. Cells were incubated with 10 μg/mL BrdUrd for 1 hour before harvesting. Incorporated BrdUrd was analyzed by fluorescence-activated cell sorting as described in Materials and Methods.

~2-fold at various time points over a 72-hour incubation. By contrast, BRAF$^{V600E}$ expression inhibited thyrotropin-induced DNA synthesis, consistent with the impairment of other thyrotropin-mediated responses described above (Fig. 5*A*). Because sustained activation of BRAF$^{V600E}$ in these cells is associated with changes in cell number and induction of apoptosis (see below), both of which could confound results derived from prolonged incubations with [$^{14}$C]thymidine, we reexamined effects of BRAF$^{V600E}$ on DNA synthesis at a later time point using a BrdUrd incorporation assay following a short (1-hour) pulse of the compound. As shown in Fig. 5*B*, BrdUrd incorporation was induced in the absence of thyrotropin and very slightly reduced in the presence of thyrotropin after treatment with doxycycline for 6 days, indicating that the effects on DNA synthesis persisted despite concomitant activation of cell death. Note that despite the induction of DNA synthesis in the absence of thyrotropin, BRAF$^{V600E}$ did not stimulate cell growth.

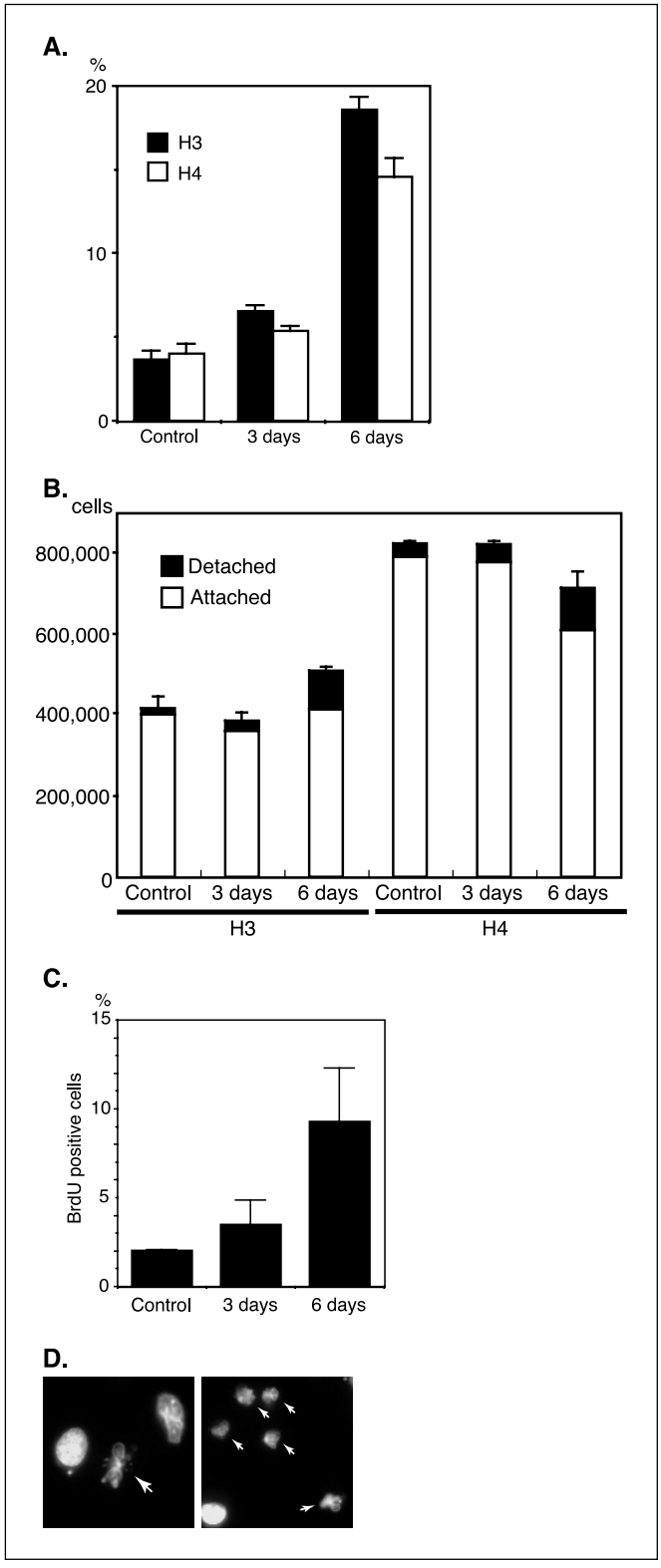
**Cell Detachment and Apoptosis after BRAF$^{V600E}$ Expression.** Because of the discrepancy between the effects of BRAF$^{V600E}$ on cell growth and DNA synthesis in the absence of thyrotropin, we explored the possibility that the oncoprotein may be inducing cell detachment and/or apoptosis, which would dampen putative changes in cell number. As shown in Fig. 6*A*, ~15% to 20% of cells treated with doxycycline for 6 days were detached in both H3 and H4 conditions. Figure 6*B* shows the total cell counts and the fraction of detached and attached cells. In the absence of thyrotropin, the number of attached cells remained fairly constant between control and doxycycline-treated cells. However, the total count was increased by BRAF$^{V600E}$ if detached cells are also considered. On the other hand, both total and attached cell counts are reduced by BRAF$^{V600E}$ in the presence of thyrotropin.

As shown in Fig. 6*C*, ~10% of cells treated with doxycycline for 6 days showed evidence of apoptosis as determined in an Apo-BrdUrd assay. Nuclear apoptotic changes, such as fragmentation or chromatin condensation, were also observed in both attached and detached cell fractions by DAPI staining (Fig. 6*D*). These data suggest that the cell detachment is due at least in part to apoptosis. Moreover, the fact that nuclear fragmentation was also seen in attached cells suggests that cell death was not due to anoikis.

**Effect of BRAF$^{V600E}$ Expression on Adenylyl Cyclase Activity.** We showed that the impairment of thyrotropin-dependent gene expression by BRAF$^{V600E}$ could be attributed at least in part to decreased abundance of TSH-R. We next explored whether BRAF activation may also interfere with thyrotropin-mediated responses at a more distal step. We therefore explored the effect of BRAF$^{V600E}$ expression on adenylyl cyclase activity. As shown in Fig. 7*A*, forskolin increased cAMP level by ~50-fold and this effect was not impaired by BRAF$^{V600E}$. Moreover, BRAF$^{V600E}$ had no discernible effect on cAMP-induced DNA synthesis. As shown in Fig. 7*B*, doxycycline treatment increased DNA synthesis by ~2-fold, consistent with the results shown in Fig. 5. Predictably, 8-Br-cAMP stimulation for 24 hours induced DNA synthesis by ~5-fold in the absence of doxycycline. Interestingly, BRAF$^{V600E}$ expression did not interfere with cAMP-induced DNA synthesis in thyroid PCCL3 cells. Thus, the oncoprotein interferes with thyrotropin-mediated responses primarily at the receptor level and does not impair adenylyl cyclase activity or cAMP-induced DNA synthesis.

**Micronuclei Formation after BRAF$^{V600E}$ Expression.** We showed previously that acute activation of a RAS oncoprotein leads to development of chromosomal instability in PCCL3 cells manifesting as loss of chromosomal material, mitotic bridge formation, and misaligned chromosomes (29). As shown in Fig. 8*A*, BRAF$^{V600E}$ expression induced micronuclei and mitotic bridge formation. In untreated cells, when micronuclei were present, as a rule only a single micronucleus was observed in the affected cell. By contrast, after BRAF activation, two or more micronuclei were frequently found. Figure 8*B* shows quantitative

data in the two independently derived BRAF$^{V600E}$-expressing lines. Micronuclei formation was increased by ~2-fold in both PC-BRAF$^{V600E}$-6 and PC-BRAF$^{V600E}$-12 cells. Basal and doxycycline-induced micronuclei formation in PC-BRAF$^{V600E}$-12 cells was ~2 times higher than in PC-BRAF$^{V600E}$-6 cells. Micronuclei are generally believed to form either by disruption of the mitotic spindle, leading to the loss of a whole chromosome (an aneugenic event), or by induction of double-strand DNA breaks with loss of a portion of a chromosome (a clastogenic event). Micronuclei resulting from aneugenic events can be identified by staining with anti-kinetochore antibody. By contrast, micronuclei resulting from clastogenic events would primarily consist of chromosome fragments and would be negative for centromere staining (although fragments that include a centromere may be positive). Examples of centromere-negative or centromere-positive micronuclei are presented in Fig. 8*C*. The ratio of centromere-positive to centromere-negative micronuclei was not changed after treatment with doxycycline, indicating that both aneugenic and clastogenic events were induced by BRAF$^{V600E}$ expression in thyroid PCCL3 cells.




## Discussion

Activating mutations of either *RET/PTC*, *NTRK1*, *RAS*, or *BRAF* are found in 70% of PTCs and are mutually exclusive (2, 4, 17). This is consistent with requirement for constitutive activation of the MAPK pathway that can be achieved by mutation of any of these effectors, which are thought to develop early in tumor evolution. However, there is good evidence that PTCs with *RET/PTC*, *RAS*, or *BRAF* mutations differ in morphologic characteristics and biological behavior (7, 18). PCCL3 cells are well-differentiated rat thyroid clonal cells and have been used extensively to explore the effects of oncogene activation on thyroid cells *in vitro* (30). They require both thyrotropin and insulin (or insulin-like growth factor I) for proliferation and cannot be transformed by the single expression of any of the above-mentioned oncoproteins. RET/PTC promotes thyrotropin-independent growth and loss of thyroid differentiated gene expression following stable transfection (20–22, 31). However, the ability of RET/PTC-expressing cells to grow in the absence of thyrotropin may require secondary events developing during clonal selection and adaptation, because RET/PTC does not induce thyrotropin-independent growth after short-term expression (32). The primary purpose of this study was to examine the mechanisms of thyroid tumor initiation by oncogenic BRAF. To accomplish this, we established doxycycline-inducible BRAF$^{V600E}$-expressing cell lines derived from PCCL3 cells. This system allowed us to minimize secondary changes taking place during selection, to understand early effects of the oncoprotein, and to compare these with those observed after acute expression of other constitutively active components of the MAPK pathway. We selected the two best clones, PC-BRAF$^{V600E}$-6 and PC-BRAF$^{V600E}$-12 cells, which showed low or

**Figure 6.** *A* and *B,* expression of BRAF$^{V600E}$ increases cell detachment. PC-BRAF$^{V600E}$-6 cells were cultured with or without 1 μg/mL doxycycline in H3 or H4 medium. Doxycycline was added at the indicated times before harvest. In the H3 experiment, cells were preincubated with H3 for 3 days before addition of doxycycline. *A,* effects of BRAF$^{V600E}$ on cell detachment. *Columns,* mean of detached cells expressed as a percentage of total cells in three wells of a six-well plate; *bars,* SD. *B,* effects of BRAF$^{V600E}$ on total cell counts. *White columns,* attached cells; *black columns,* detached cells. *Columns,* mean of three wells of a six-well plate; *bars,* SD of the total cell count. Similar results were obtained in two independent experiments. *C* and *D,* expression of BRAF$^{V600E}$ induces apoptosis. *C,* PC-BRAF$^{V600E}$-6 cells were cultured with or without 1 μg/mL doxycycline in H4 medium. Doxycycline was added at the indicated times before harvest. Apoptotic cells were counted using an Apo-BrdUrd assay. *Columns,* mean of three independent experiments; *bars,* SD. *D,* PC-BRAF$^{V600E}$-6 cells were cultured with 1 μg/mL doxycycline in H4 medium for 6 days. Attached cells (*left*) were fixed and stained with DAPI. Floating cells (*right*) were collected and attached to silane-coated slide glass using cytospin before fixation and staining. *Arrows,* apoptotic cells. Similar results were obtained in cells grown in H3 medium (data not shown).

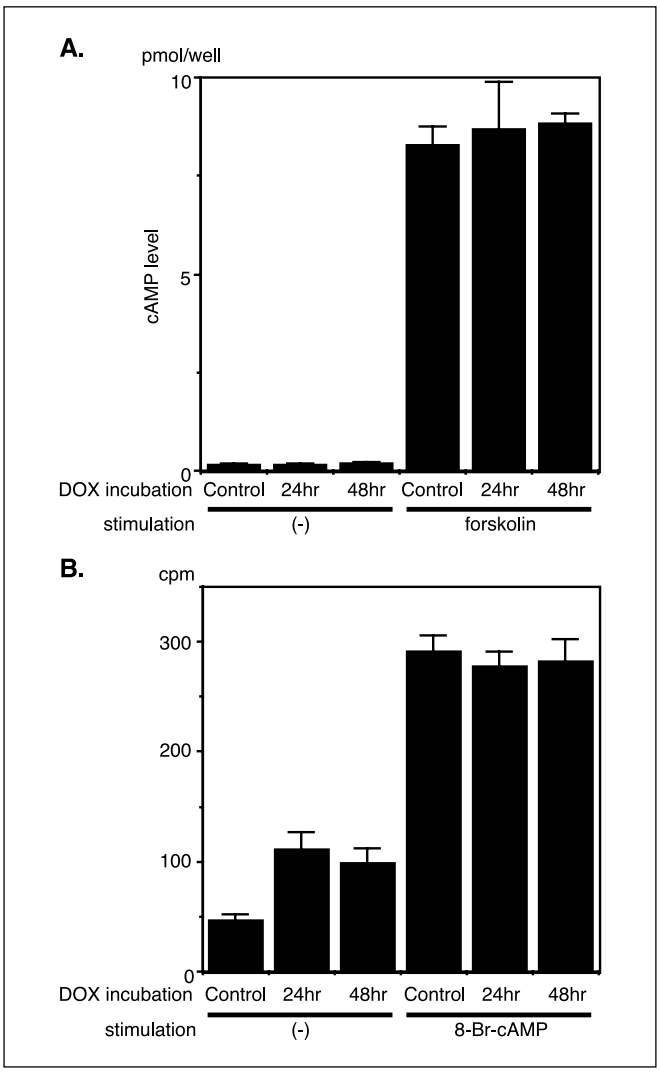


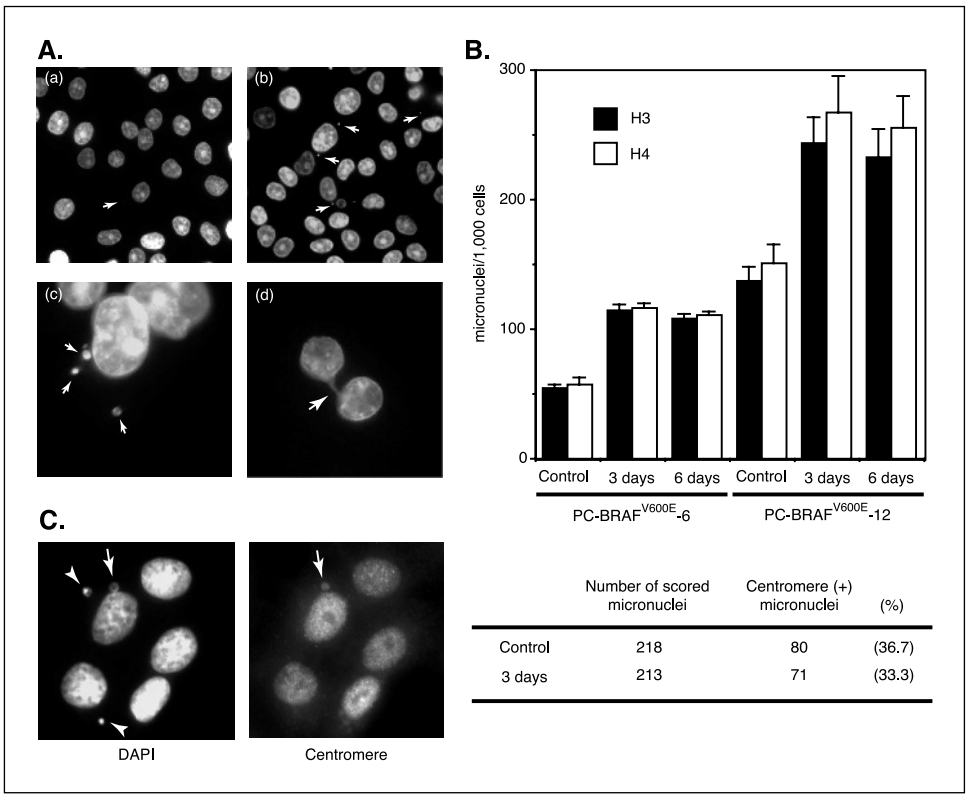

**Figure 7.** Effect of BRAF$^{V600E}$ expression on signaling distal to TSH-R. *A,* effect of BRAF$^{V600E}$ expression on adenylyl cyclase activity. PC-BRAF$^{V600E}$-6 cells were cultured until 70% confluent in H4 medium. The medium was then replaced with H3 medium for 3 days. Doxycycline (1 μg/mL) was added at the indicated times before assay. After incubation with or without 25 μmol/L forskolin, cellular cAMP levels were measured as described in Materials and Methods. *Columns,* mean of a single experiment done in triplicate; *bars,* SD. Similar results were obtained in another two independent experiments. *B,* effect of BRAF$^{V600E}$ expression on cAMP-stimulated DNA synthesis. PC-BRAF$^{V600E}$-6 cells were preincubated with H3 medium for 3 days. Doxycycline (1 μg/mL) was added at the indicated times before starting stimulation. Cells were then stimulated with 1 mmol/L 8-Br-cAMP and incubated with [$^{14}$C]thymidine for 24 hours. Incorporated thymidine was measured as described in Materials and Methods. *Columns,* mean of four wells of the plate; *bars,* SD. Similar results were obtained in two independent experiments.

undetectable BRAF$^{V600E}$ under basal conditions and high induction of the oncoprotein and robust phosphorylation of MEK and ERK in the presence of doxycycline. In addition, the level of BRAF$^{V600E}$ expression after 24 to 48 hours of treatment with 1 μg/mL doxycycline was approximately equal to that of endogenous wild-type BRAF.

As stated, thyrotropin is required for growth of PCCL3 cells *in vitro*, and most human thyroid cancer cell lines lose this requirement. Notably, BRAF$^{V600E}$ expression did not result in thyrotropin-independent growth of PCCL3 cells. On the other hand, BRAF$^{V600E}$ induced DNA synthesis in the absence of thyrotropin. This is explainable by a concomitant increase in cell detachment and apoptosis, which is quite similar to the previously reported effects of conditional expression of RET/PTC1 and RET/PTC3 in these cells (32). The fact that constitutive activation of BRAF recapitulates effects of RET/PTC on growth and apoptosis implicates the MAPK pathway in these effects. The evidence that BRAF$^{V600E}$ expression drives a weak growth stimulus is also consistent with its putative role as a tumor-initiating event. Presumably, additional genetic or epigenetic changes are required for thyroid cells to grow in these conditions, which would likely require disabling the apoptotic program activated by this oncoprotein.

Acute expression of BRAF$^{V600E}$ reduced thyrotropin-dependent cell growth in a doxycycline dose-dependent manner, indicating that constitutive activation of BRAF$^{V600E}$ signaling is growth inhibitory in thyroid PCCL3 cells even at low concentration. Thyrotropin-induced DNA synthesis was also impaired. Again, these findings are quite comparable with our prior observations following acute expression of RET/PTC1 and RET/PTC3. Both RET/PTC and BRAF decrease expression of the TSH-R (32), which could explain in part these findings. However, the mechanisms by which RET/PTC and BRAF interfere with thyrotropin action distal to the receptor differ in important respects. Whereas RET/PTC markedly impairs adenylyl cyclase activity (32), BRAF does not alter forskolin-induced cAMP levels. We reported previously that RET/PTC-induced inhibition of adenylyl cyclase activity required coupling to both phospholipase Cγ and Src homology and collagen (32). Vanvooren et al. showed that dog and human thyroid cells express primarily adenylyl cyclase isoforms AC3, AC6, and AC9 (33). AC6 activity is subject to inhibition by protein kinase Cδ and protein kinase Cε in PC12 cells (34). Indeed, RET/PTC1 and RET/PTC3 induce a fairly selective activation of protein kinase Cε (35), which could explain the RET/PTC requirement for phospholipase Cγ for adenylyl cyclase inhibition. By contrast, other receptor tyrosine kinases, such as insulin-like growth factor I receptor, enhance adenylyl cyclase activity in HEK293 cells, and this is not blocked by inhibitors of protein kinase C, ERK, protein kinase A, or phosphatidylinositol 3-kinase (36). However, this effect is blunted by a dominant-negative RAF-1, which did not seem to act via MEK (36). Moreover, RAF-1 was found to phosphorylate AC6 on specific serine residues, and these were required for enhancement of activity. The lack of effect of BRAF$^{V600E}$ on forskolin-induced adenylyl cyclase activity in PCCL3 cells was therefore unexpected and may indicate a preferential effect of RAF-1 on AC6 or a comparatively modest contribution of this isozyme to total adenylyl cyclase activity in thyroid cells.

By contrast to RET/PTC, BRAF does not inhibit cAMP-induced DNA synthesis. In thyroid cells, cAMP promotes the assembly and activation of cyclin D3-cyclin-dependent kinase 4 complexes (37, 38) and increases nuclear expression of p27$^{kip1}$. The protein kinase A–anchoring protein AKAP95 associates with cyclins and has been proposed to participate in regulation of DNA synthesis in thyroid cells (39). The MAPK pathway has been shown to play a permissive role in the modulation of thyroid cell growth by cAMP (30). Our data suggest that constitutive high-intensity activation of MAPK provides no additional stimulus to DNA synthesis induced by cAMP in these experimental conditions. By contrast, BRAF activation markedly impairs cAMP-induced expression of NIS. This is consistent with previous studies implicating effectors along the MAPK pathway in blunting expression of thyroid-specific genes (22, 40, 41) through a mechanism that involves in part decreased transcriptional activity of TTF-1, a homeodomain-containing




| | Number of scored micronuclei | Centromere (+) micronuclei | (%) |
|---|---|---|---|
| Control | 218 | 80 | (36.7) |
| 3 days | 213 | 71 | (33.3) |

**Figure 8.** Expression of BRAF$^{V600E}$ induces formation of micronuclei. *A,* PC-BRAF$^{V600E}$-6 cells were cultured with or without 1 μg/mL doxycycline in H4 medium for 6 days. Cells were fixed and then stained with DAPI. *(a),* control cells; *(b)-(d),* doxycycline-treated cells; *small arrows,* micronuclei [*(a)-(c)*]; *large arrow,* chromosome bridge [*(d)*]. *B,* PC-BRAF$^{V600E}$-6 or PC-BRAF$^{V600E}$-12 cells were cultured with or without 1 μg/mL doxycycline in H3 or H4 medium as described in Fig. 6*A* and *B* legend. Cells were collected and stained with DAPI. The number of micronuclei was visually counted using fluorescence microscopy. *Columns,* mean of three independent experiments; *bars,* SD. *C,* PC-BRAF$^{V600E}$-6 cells were cultured with or without 1 μg/mL doxycycline in H4 medium for 3 days. Cells were then fixed and stained with DAPI and anti-kinetochore antibody. Representative centromere-positive (*arrow*) and centromere-negative (*arrowhead*) micronuclei. Percentage of centromere-positive micronuclei was visually counted using fluorescence microscopy. Representative of two independent experiments.

transcription factor required for normal thyroid development and expression of thyroid-specific proteins (40, 41). The biochemical basis for the clear difference in the way BRAF activation affects cAMP effects on DNA synthesis and differentiated gene expression in PCCL3 cells requires further study.

We reported previously that 2 to 4 days after H-RAS$^{V12}$ activation (first or second cell cycle) there was a significant increase in the percentage of cells with micronuclei, small nuclear-like structures containing chromosomes or chromosome fragments that form during mitosis as a result of chromosome missegregation (29). Quantification of micronuclei has been used to measure the extent of chromosomal loss resulting from DNA-damaging agents, such as ionizing radiation (clastogens), or toxins that interfere with the proper functioning of the mitotic spindle (aneugens; refs. 42, 43). The degree of increase in micronuclei was equivalent to that seen after exposure of PCCL3 cells to 5 Gy γ-irradiation. The effects of H-RAS$^{V12}$ were mediated by activation of MAPK, as treatment with PD98059 at concentrations verified to selectively inhibit MEK1 (and not phosphatidylinositol 3-kinase or p38 MAPK) reduced the frequency of micronuclei formation. In addition, doxycycline-inducible expression of a constitutively active MEK1, but not of a mutant RAC1 (which activated c-Jun $NH_2$-terminal kinase and p38 MAPK in these cells), mimicked the effects of H-RAS$^{V12}$ (29). The effects of H-RAS$^{V12}$ on genome destabilization were apparent although the sequence of p53 in PCCL3 cells was confirmed to be wild type. H-RAS$^{V12}$ and activated MEK1 also induced centrosome amplification and chromosome misalignment (29). The present data showing similar effects of BRAF$^{V600E}$, a naturally occurring oncoprotein that constitutively activates the MAPK pathway, is consistent with these previous findings. Indeed, PC-BRAF$^{V600E}$-12 cells that have higher basal and greater BRAF-induced levels of ERK phosphorylation than PC-BRAF$^{V600E}$-6 cells also showed higher frequency of micronuclei formation. Both clastogenic and aneugenic events are possibly involved in BRAF$^{V600E}$-induced micronuclei formation, suggesting that at least two mechanisms may be operating to induce genomic instability. By contrast, conditional expression of RET/PTC1 or RET/PTC3 did not induce micronuclei formation (29). Conditional RET/PTC expression evokes more transient and less intense ERK phosphorylation than either RAS or BRAF,[1] and in addition to the MAPK pathway, RET/PTC can signal through many other effectors that could alter the predisposition to generate chromosomal abnormalities.

Recent studies suggest that MAPK may play a direct role in mitosis and chromosome segregation. Activated ERK localizes to kinetochores in early and midmitosis, in asters during all stages of mitosis, and in the chromosome midbody in late anaphase (44). It associates with the motor protein CENP-E and phosphorylates it *in vitro*. CENP-E localizes to kinetochores during prometaphase and regulates attachment of chromosomes to microtubules. MAPK also phosphorylates proteins containing the 3F3/2 phosphoantigen, which recognizes an epitope that disappears with kinetochore attachment to the spindles (45). Evidence for a temporal sequence of localization of activated MAPK in different nuclear compartments during mitosis (44, 45) suggests that phosphorylation-dephosphorylation steps are needed for orderly progression, a step that may be disrupted when MAPK activation is constitutive. This could conceivably explain in part the pronounced decrease in chromosome stability following deregulation of activators of MAPK in fibroblasts (46) and thyroid cells (29).

[1] Unpublished data.

Extrapolating from these *in vitro* observations, one can speculate that the greater predisposition to aggressive thyroid cancers in BRAF-positive tumors may be due in part to induction of genomic instability. The fact that neither forskolin (which, like mutant forms of the TSH-R and Gs, activates adenylyl cyclase) nor RET/PTC induced detectable chromosomal changes is consistent with the low frequency of aneuploidy seen in thyroid tumors harboring these latter defects *in vivo*.

In conclusion, BRAF$^{V600E}$ expression confers normal thyroid cells with little growth advantage because of concomitant activation of DNA synthesis and apoptosis. It is not sufficient to transform the cells. However, in contrast to RET/PTC, BRAF$^{V600E}$ may facilitate the acquisition of secondary genetic events through induction of genomic instability, which may in turn account for its aggressive properties.

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