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Glucocorticoids Differentially Inhibit Expression of the *RET* Proto-Oncogene

AMANDA CAPES-DAVIS, SCOTT D. ANDREW, VALENTINE J. HYLAND, STEPHEN TWIGG, DIANA L. LEAROYD, TRISHA DWIGHT, DEBBIE J. MARSH, AND BRUCE G. ROBINSON[1]

Kolling Institute of Medical Research and Department of Endocrinology, Royal North Shore Hospital, University of Sydney, Sydney, N.S.W., Australia

The *RET* proto-oncogene encodes a receptor tyrosine kinase activated by the binding of factors from the glial cell line-derived neurotrophic factor (GDNF) family to receptor-α components such as GDNF family receptor α-1 (GFRα-1). Mutations within the sequence of the *RET* proto-oncogene are associated with multiple endocrine neoplasia type 2 (MEN 2), an inherited tumor syndrome characterized by the development of medullary thyroid carcinoma (MTC) and other neuroendocrine tumors. Despite Northern analysis showing that *RET* is expressed in the majority of MTCs, the factors regulating this expression are poorly understood. To address this issue we examined *RET* expression in response to glucocorticoids in the TT cell line, derived from a metastatic MTC. The synthetic glucocorticoid dexamethasone was found to reduce *RET* expression at both mRNA and protein levels. This effect was dose responsive and maximal at 24 h. The reduction in *RET* mRNA was shown to be specific to glucocorticoids and was also seen in a primary MTC culture. Nuclear run-on studies revealed the reduction in steady-state RNA to be due to a decrease in *RET* mRNA transcription and the effect was shown to be independent of new protein synthesis or RNA stability. Dexamethasone was also found to exert an inhibitory effect upon cell growth, suggesting a potential use for glucocorticoids in the treatment of medullary carcinoma and MEN 2.

RET proto-oncogene Multiple endocrine neoplasia type 2 Gene expression Alternative splicing Dexamethasone

THE *RET* proto-oncogene encodes a membrane-bound receptor tyrosine kinase (7). Recent work has shown that the RET tyrosine kinase is activated by factors from the glial cell line-derived neurotrophic factor (GDNF) family, consisting of related neurotrophic factors with homology to transforming growth factor-β (27,31). Factors binding to RET from this family include GDNF, neurturin, persephin, and artemin (3,4,21,46). Physiological activation of RET only occurs in the presence of additional extracellular proteins from the GFRα family such as GFRα-1 (7). Expression of *RET* and its activation by GDNF family members are thought to be especially important in embryogenesis. Embryological studies and *RET* and GDNF knockout mouse models show a role for RET in renal organogenesis and in the development of central and peripheral nervous systems (33,35,41,43). More specifically, RET is believed to be active in proliferation, differentiation, and migration of cells from the neural crest (19,38). Abnormal expression and activation of *RET* can also occur within neural crest-derived tissue as a result of mutations in the *RET* coding sequence. These abnormal events are associated with the inherited disorder multiple endocrine neoplasia type 2 (MEN 2).

MEN 2 is divided into three subgroups, all associated with mutations in the *RET* proto-oncogene. Mutations altering a cysteine-rich extracellular region within the *RET* protein, and less commonly in other areas, are linked to familial medullary thyroid carcinoma (FMTC) (20). Mutations within the cysteine-rich region are also associated with MEN 2A, predisposing

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[1]Address correspondence to Professor B. G. Robinson, Kolling Institute of Medical Research, Royal North Shore Hospital, St Leonards, 2065 N.S.W., Australia. Tel: +61 2 9926 7267; Fax: +61 2 9926 8523; E-mail: bgr@blackburn.med.su.oz.au

affected individuals to MTC, pheochromocytoma, and parathyroid hyperplasia. These mutations generally produce a gain of function effect through dimerization and consequent activation of the RET tyrosine kinase in a manner independent of its ligands (1,42). In contrast, a single mutation affecting the methionine at codon 918 within the intracellular tyrosine kinase domain is linked to MEN 2B (20). This is a more aggressive variant of MEN 2 and is associated with a Marfanoid habitus, mucosal neuroma formation, and ganglioneuromatosis in addition to pheochromocytoma and earlier onset MTC. The codon 918 mutation also occurs as a somatic event in up to 80% of sporadic MTCs and has been found to alter the substrate specificity of the RET tyrosine kinase (42,44).

While the mutations described above provide a clear mechanism for tumorigenesis in MEN 2, many events in tumor formation remain unexplained. Importantly, the factors controlling *RET* expression in both normal and malignant cells remain poorly understood. A single GC-rich promoter region has been identified near the *RET* exon 1 transcription start site containing putative binding sequences for AP-2, SP-1, epidermal growth factor receptor-specific transcription factor, and GC factor, the latter generally acting to suppress transcription (25). No mutations within this sequence or elsewhere have been published to explain the variation in *RET* expression seen in different tumors and cell lines. Similarly, current data do not explain how *RET* mRNA splicing and polyadenylation are regulated. Two protein isoforms can be detected on Western blot arising from this alternative splicing and appear to vary in their transforming ability, especially in the presence of *RET* mutations (2).

In the TT cell line, derived from a metastatic MTC (30) with a codon 634 mutation characteristic of MEN 2A (8), expression of the *RET* proto-oncogene can be silenced by activation of the signal transduction factor Raf-1 (9). This decrease in *RET* mRNA levels occurs in association with cell differentiation and a concomitant decrease in tumor cell growth. A similar decrease in growth has been seen when this cell line was treated with the synthetic glucocorticoid dexamethasone (14). We examined the hypothesis that glucocorticoids could similarly inhibit expression of the *RET* proto-oncogene in MTC cells and here present data to confirm this hypothesis and to show an association between *RET* expression and cell growth.

MATERIALS AND METHODS

Materials

All cell culture media and antibiotics were obtained from Gibco BRL (Mulgrave, Australia) and fetal calf serum (FCS) from Trace Biosciences (Castle Hill, Australia). Dexamethasone, hydrocortisone, and β_2 estradiol were from Sigma Chemical Company (St. Louis, MO), ORG 2058 from Amersham Life Science (Little Chalfont, UK), and dihydrotestosterone from E. Merck (Darmstadt, Germany). Cycloheximide, actinomycin D, and all other reagents, unless otherwise stated, were from Sigma and were of molecular biology grade.

RNA was analyzed with the aid of TRI Reagent (Molecular Research Center, Cincinnati, OH), Genescreen membrane (Dupont NEN Research Products, Boston, MA), and Elutip-D columns (Schleicher and Schuell, Dassel, Germany). Plasmids used in riboprobe synthesis and run-on analysis included pCR-Script (Stratagene, La Jolla, CA) and pGEM-4Z (Promega Corporation, Madison, WI); T3 and T7 polymerases and reagents for riboprobe synthesis were also from Promega. Hybridizations were performed using salmon sperm DNA from Calbiochem (La Jolla, CA) and yeast tRNA from Boehringer Mannheim GmbH (Mannheim, Germany). [α-^{32}P]UTP (800 Ci/mmol, 1480 MBq/ml) was from Dupont NEN Research Products and [methyl-^{3}H]thymidine (60–90 Ci/mmol) from ICN Biochemicals (Costa Mesa, CA).

Cell Culture

TT cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown in RPMI-1640 containing 2 mM glutamine and supplemented with 16% FCS. For steroid specificity studies, phenol red-free RPMI was used because of the estrogen-like activity of phenol red. Penicillin (50 IU/ml) and streptomycin (50 µg/ml) were used in all experiments and cells were routinely tested for Mycoplasma using the Gen-Probe hybridization detection system (Gen-Probe, San Diego, CA). All expression studies were performed at equivalent passage. Before any treatment, cells were plated freshly at 10^5 cells/cm^2 and allowed to settle for 3 days. Cells were then given fresh medium containing either ethanol vehicle at a concentration of 0.1% or dexamethasone in a concentration range of 10^{-10} to 10^{-6} M. Following treatment, cells were trypsinized and pelleted for storage at −70°C.

For primary culture, MTC samples were obtained at surgery from a 27-year-old woman with a sporadic tumor. The samples treated were taken from a metastatic deposit within a cervical lymph node. The tissue was finely minced before being placed as explants in RPMI-1640 supplemented with penicillin, streptomycin, and 10% FCS. A number of dispersed, single cells were included from the mincing process. Dexamethasone or vehicle was added in fresh me-

dium after 24 h. Sampling and culture of this tumor tissue was approved by the Royal North Shore Hospital Medical Research Ethics Committee.

To act as a negative control for *RET* expression, primary fibroblasts were grown from human skin samples within the laboratory of Dr. R. Mason (Sydney University, Australia) and maintained by us for RNA extraction in DMEM supplemented with 10% FCS.

RNA Isolation and Northern Blot Analysis

Total cellular RNA was isolated using TRI Reagent (11). RNA (5 μg) from each treated flask was resolved on a 1% agarose gel containing 4.3 M formaldehyde and transferred to Genescreen membrane by Northern blotting. Membranes were prehybridized and hybridized at 65°C in 50% deionized formamide, 0.8 M NaCl, 1 mM EDTA, 50 mM phosphate buffer, 2% SDS, and 2.5× Denhardt's solution. Salmon sperm DNA (0.1 mg/ml) and tRNA (0.2 mg/ml) were also added. The *RET* riboprobe used was constructed by amplifying a 1.5-kb *RET* fragment encoding exons 11 to 20 from cDNA sequence. This fragment was cloned into pCR-Script and digested for riboprobe synthesis using an *Nhe* I site introduced in the PCR process. 36B4 was derived from a 220-bp *Pst* I fragment cloned elsewhere into pGEM-4Z (16). Sequence was transcribed using T3 and T7 polymerases, respectively, in a mixture containing 100 μCi [α-^{32}P]UTP and then purified using an Elutip-D column. Following hybridization, filters were washed twice at 65°C in 0.1× SSC and 0.1% SDS and analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Bands were quantitated with the aid of ImageQuant software (Molecular Dynamics) and adjusted for loading using 36B4 expression levels.

Western Blot Analysis

TT cell pellets were lysed using TDS lysis buffer [1% Triton X-100 (v/v), 0.5% sodium deoxycholate (w/v), and 0.1% SDS (w/v) in 1× PBS]. Lysates were subjected to SDS-PAGE using 7.5% acrylamide gels (Bio-Rad Laboratories, Hercules, CA) and were transferred to nitrocellulose membranes by Western blotting. Membranes were then blocked in TBS (47 mM Tris, 39 mM glycine, and 20% methanol) containing 1% bovine serum albumin (w/v), 0.02% sodium azide (w/v), and 0.05% Nonidet P-40 (v/v) overnight. Blocked samples were exposed first to rabbit anti-RET antiserum (Santa Cruz Biotechnology, 1:1000) and second to antiserum raised against rabbit and conjugated to horseradish peroxidase (Bio-Rad Laboratories, 1:10000). Bands were detected using SuperSignal chemiluminescent substrate (Pierce, Rockford, IL).

Cycloheximide and Actinomycin D Treatment

Cycloheximide was dissolved in water and added to medium to give a final concentration of 20 μg/ml, a level sufficient to block protein synthesis within 5 min (6). The agent was used to pretreat cells by adding 10 min before dexamethasone.

Actinomycin D was dissolved in DMSO to give a final concentration of 5 μg/ml, a concentration used to examine *RET* stability in neuroblastoma cell lines (6,45). Actinomycin D and dexamethasone were added concurrently.

Nuclear Run-on Analysis

TT cells subjected to run-on analysis were first treated with 10^{-6} M dexamethasone or equivalent vehicle for 24 h. Nuclei were isolated by lysis in Nonidet P-40 (23) and incubated with 400 μCi [α-^{32}P]UTP as previously described (22), except that the run-on reaction was performed at 30°C for 45 min (12). Run-on transcripts were purified by extraction in TRI Reagent as for total RNA and equivalent counts used in the subsequent hybridization.

To act as DNA probes, the *RET* and 36B4 templates used in riboprobe preparation were linearized and immobilized on Genescreen membrane. α-Tubulin was added as an additional control using a 75-bp fragment cloned elsewhere into pBR322 (15). The plasmid pGEM-4Z was also used to control for nonspecific binding. *RET* (5 μg) and 2.5 μg control DNA were applied via a slot-blot apparatus and cross-linked by baking at 80°C.

Slot-blot membranes were prehybridized at 42°C in 50% deionized formamide, 5× SSPE, 5× Denhardt's solution, 0.8% SDS, and 100 μg/ml tRNA (36). Hybridization was performed under the same conditions for 36–72 h. Slot-blots were washed three times for 15 min at room temperature in 2× SSC, 0.1% SDS, and 1× Denhardt's solution without BSA (36) before exposure and PhosphorImager analysis.

Growth Assays

Two assays were used for the analysis of growth. First, the MTS assay was performed using the CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay kit (Promega Corporation). Cells were plated and allowed to settle and grow for 3 days. Each well was then treated with dexamethasone or with an equivalent volume of ethanol vehicle for the times shown. At the commencement of each treatment, the tetrazolium compound MTS was mixed with phenazine methosulphate and added to each well. Plates were incubated for 4 h and their absorbance measured at 490 nm. The background at each time point was also read and subtracted from the readings shown.

[^{3}H]Thymidine uptake assays were performed on cells plated in 24-well plates and allowed to settle and grow for 3 days. Wells were refed with dexamethasone or with an equivalent volume of vehicle for the times shown. At the conclusion of each treatment, 2 μCi/ml [methyl-^{3}H]thymidine was added to each well and cells incubated in this mixture for 4 h. Tritiated material was precipitated with 10% trichloroacetic acid and solubilized in 0.5% SDS. Again, background readings were subtracted from each result before analysis.

Statistical Analysis

RET expression and cell growth were assessed using the Student's two sample *t*-test. Equal variance was assumed for each assay and values are presented from both tails. Probability values (p) come from this analysis and were considered significant when $p < 0.05$. Differences in the *RET* transcripts were further assessed by two-way analysis of variance using the software package SPSS for Windows, with data tested for significance using multiple group comparisons and with Scheffe adjustment for samples in a normal distribution.

RESULTS

Dexamethasone Suppresses RET *Expression Levels*

TT cells express the *RET* proto-oncogene in a characteristic pattern (7), with specific mRNA species of sizes 7.0, 6.0, 4.5, and 3.9 kb seen on Northern blot (Fig. 1A). An additional band noted above the 4.5-kb *RET* transcript was found to be nonspecific and was detected by our *RET* riboprobe in all cell types, regardless of their *RET* expression status (data not shown). Adding dexamethasone to the TT cell line resulted in a decrease in abundance of 7.0-, 6.0-, 4.5-, and 3.9-kb species when compared to vehicle-treated control lanes. This decrease was seen despite loading similar amounts of RNA, as shown by hybridization to 36B4, a probe derived from the gene for human acidic ribosomal phosphoprotein P0 and used extensively as a control for loading (10,28,32).

To determine whether both *RET* RNA and protein levels are affected by dexamethasone, this experiment was repeated using cells that were lysed for total protein and exposed to a RET polyclonal antibody. Treatment with 10^{-6} M dexamethasone for 24 h resulted in a clear decrease in RET protein levels (Fig. 1B) as well as in *RET* mRNA (Fig. 1A) when compared to vehicle-treated control lanes. Hybridization to α-tubulin antiserum as a control for loading shows that this decrease was not caused by a variation in the total protein present.

We finally explored the applicability of this finding beyond the TT cell line by establishing primary cultures from a 27-year-old female with a sporadic MTC and treating these cultures with dexamethasone (Fig. 1C). The tumor cells expressed all four transcripts, although 7.0- and 6.0-kb species were present at relatively low level compared to TT. Dexamethasone had little impact upon 7.0-kb transcripts but clearly reduced steady-state levels of 6.0-, 4.5-, and 3.9-kb species, as with the TT cell line. Taken together, these results are consistent with dexamethasone exerting an effect on both *RET* mRNA and protein in medullary thyroid carcinoma cells in vitro.

The Effect of Dexamethasone Is Dose and Time Dependent

The effect of dexamethasone on *RET* mRNA expression was further characterized in TT cells by varying the amount given over 24 h (Fig. 2). Increasing the concentration of dexamethasone from 10^{-10} to 10^{-6} M yielded an initial increase in all mRNA species relative to control, followed by a dose-dependent decrease (Fig. 2A). Of these two changes, the decrease seen at higher doses, was more obvious and was found to be significant for all transcripts (Fig. 2B). This decrease was also found to be transcript specific, with levels of 7.0- and 6.0-kb mRNAs declining to a greater degree than 4.5- and 3.9-kb species (Fig. 2B). Analysis of variance showed that these transcript-specific changes were significant for this experiment (data not shown).

To further investigate the changes seen in *RET* expression, TT cells were exposed to 10^{-6} M dexamethasone for up to 72 h (Fig. 3). All *RET* mRNA levels declined significantly in response to treatment, with the greatest reduction at 24 h followed by at least partial recovery for all species. If dexamethasone was removed after 24-h treatment and the effect observed, a rapid increase in *RET* mRNA abundance resulted to greater than control levels over a further 24 h (data not shown). These results would indicate that the effect of dexamethasone on the TT cell line is both dose and time responsive.

The Effect of Dexamethasone Is Steroid Specific

Having characterized the effect of dexamethasone, a panel of steroids was used to treat TT cells and determine the specificity of the effect seen on *RET* expression levels (Fig. 4A). Dexamethasone and hydrocortisone were found to reduce *RET* mRNA levels while β_2 estradiol, dihydrotestosterone, and the bioactive progesterone analogue, ORG 2058, had no net effect (Fig. 4B). Considering the similar effect of hydrocortisone and dexamethasone, we would suggest

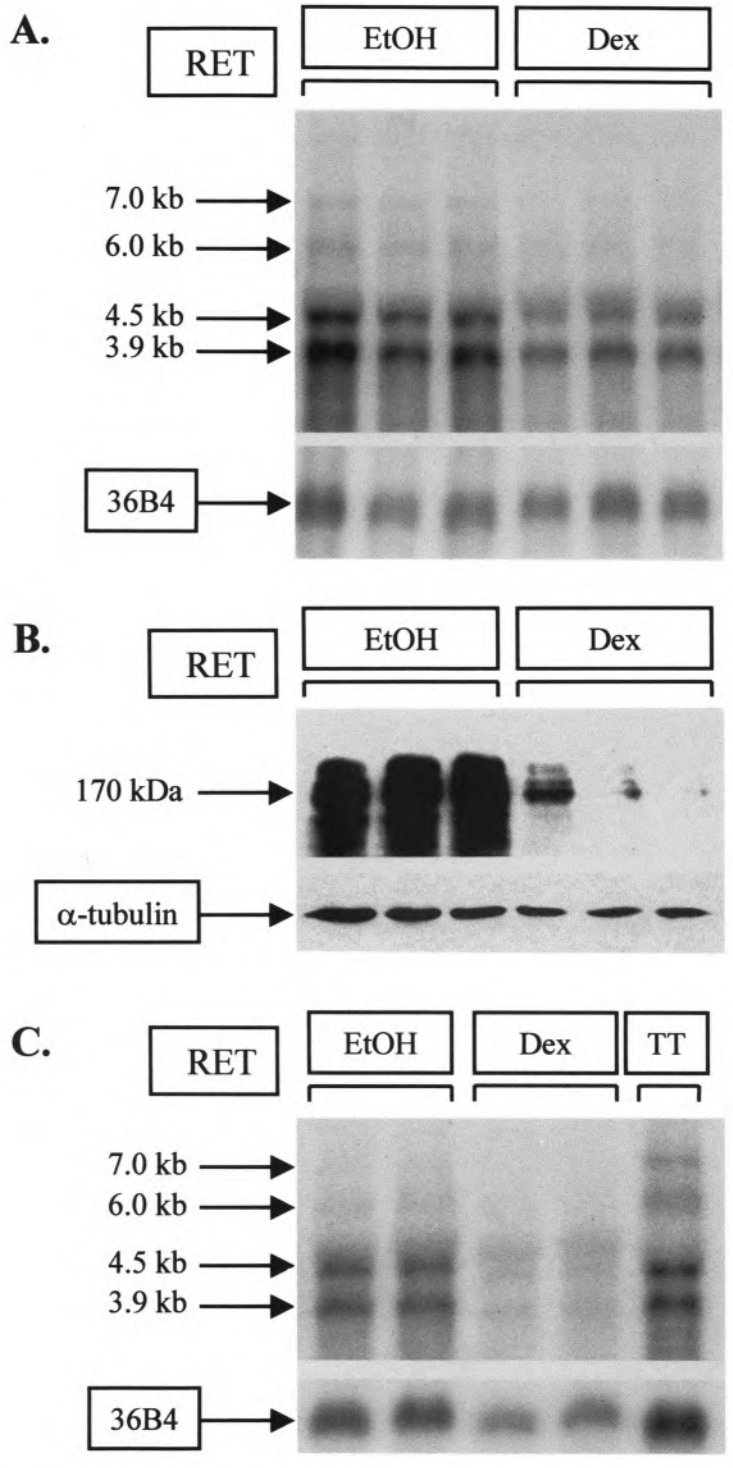

FIG. 1. Effect of dexamethasone on *RET* expression. (A) Assessing RNA expression using the TT cell line. Flasks were treated in triplicate with 10^{-6} M dexamethasone (Dex) or an equivalent volume of ethanol (EtOH) for 24 h. Total RNA (5 μg) was subjected to electrophoresis and Northern blotting. The resulting filter was hybridized to *RET* and 36B4 riboprobes concurrently. (B) Assessing protein expression using the TT cell line. Cells were treated in triplicate with 10^{-6} M dexamethasone or ethanol for 24 h as in (A) and 50 μg protein used for SDS-PAGE and Western blotting. Membranes were hybridized to RET and α-tubulin antibodies sequentially. (C) Assessing RNA expression using primary medullary carcinoma cells. Explant cultures from a 27-year-old woman with metastatic MTC were treated 1 day after establishment with vehicle (EtOH) or 10^{-6} M dexamethasone (Dex) for 24 h. The duplicate lanes shown after Northern blotting and hybridization were derived from single treatment samples; less was loaded in dexamethasone-treated lanes. A single lane of TT RNA was included for comparison with (A).

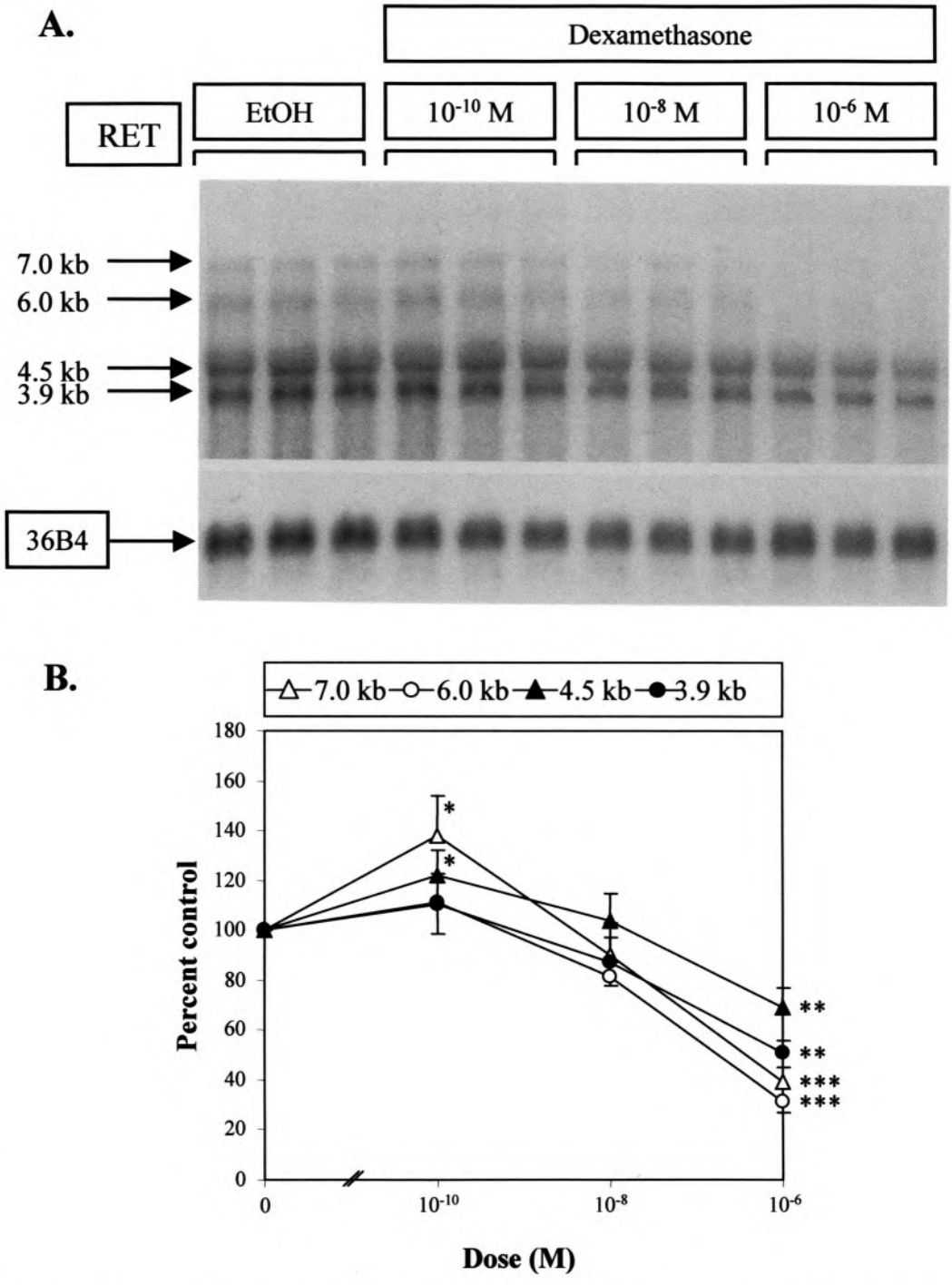

FIG. 2. Dose-dependent effect of dexamethasone on *RET* mRNA. (A) Northern analysis. TT flasks were treated in triplicate with vehicle (EtOH) or with dexamethasone at the doses shown for 24 h. Lanes were hybridized to *RET* and 36B4 riboprobes sequentially. (B) Quantification of the results obtained by Northern analysis. *RET* mRNA species were quantitated and adjusted using hybridization to 36B4 sequence as a control for loading. The mean and SD of each triplicate were then expressed as a percentage of vehicle-treated control samples. Significant differences when compared to control results are shown: $*p < 0.05$, $**p < 0.01$, $***p < 0.005$.

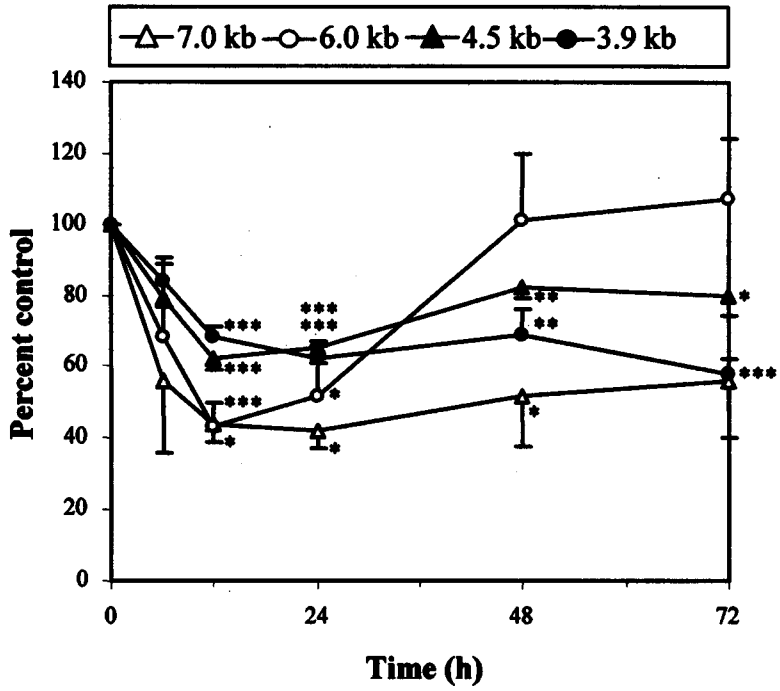

FIG. 3. Time-dependent effect of dexamethasone on *RET* mRNA. Quantification of results obtained by Northern blot analysis (not shown). TT cells were treated in triplicate with 10^{-6} M dexamethasone for 6, 12, 24, 48, and 72 h. *RET* mRNAs were again quantitated, adjusted against 36B4 species for loading, and expressed as a percentage of control lanes. Cells at time zero were used as a control, because *RET* expression had not been found to alter significantly over 72 h in previous experiments (data not shown). Significant differences when compared to control results are shown: $*p < 0.05$, $**p < 0.01$, $***p < 0.005$.

that the inhibition of *RET* expression seen occurs via the glucocorticoid receptor. This receptor was found to be expressed in the TT cell line by hybridizing TT RNA to a glucocorticoid receptor riboprobe, supporting this hypothesis (data not shown).

Dexamethasone Suppresses RET *Expression Through Directly Altering RNA Transcription*

The 12-h delay between the addition of dexamethasone and an effect on *RET* expression suggested that this effect could be mediated via a separate, newly synthesized protein. In order to investigate this possibility, TT cells were treated with cycloheximide to eliminate all new protein synthesis (Fig. 5A). Cycloheximide alone (C) produced an increase in *RET* mRNA levels compared to control lanes (Et). Adding dexamethasone in conjunction with cycloheximide (D/C) resulted in a decrease in *RET* mRNA levels relative to cycloheximide alone (C) at 12 h, a time at which suppression of these levels by dexamethasone could be seen (Fig. 5B). The fact that this decrease could still be seen even after adding cycloheximide would indicate that the effect of dexamethasone is not dependent on new protein synthesis.

Another possible mechanism for a decline in *RET* mRNA levels could involve a change in RNA stability. Such a possibility was tested by treating TT cells with actinomycin D to eliminate all new RNA synthesis (Fig. 6A). Treatment with actinomycin D (A) for 24 h resulted in a decline in 7.0- and 6.0-kb species to approximately 50% and in 4.5- and 3.9-kb transcripts to 70% of control (Et) levels. Adding dexamethasone as well as actinomycin D (A/D) did not provoke a more rapid decrease in any of these four transcripts relative to actinomycin D alone (Fig. 6B). This was true even at 24 h, the time at which dexamethasone was found to exert its greatest effect on *RET* expression (Fig. 3). The fact that adding dexamethasone did not induce a more rapid decline in RNA expression than actinomycin D alone would imply that its effect on *RET* mRNAs was not caused by a change in RNA stability.

To finally investigate the effect of dexamethasone on overall *RET* transcription, nuclear run-on transcription studies were performed to compare cells treated with dexamethasone to others treated with vehicle alone. Nuclear RNA transcripts from treated cells were extended and labeled in run-on assays before their hybridization to slot-blots containing *RET* sequence encoding transmembrane and tyrosine kinase domains (Fig. 7). Slot-blots also included 36B4,

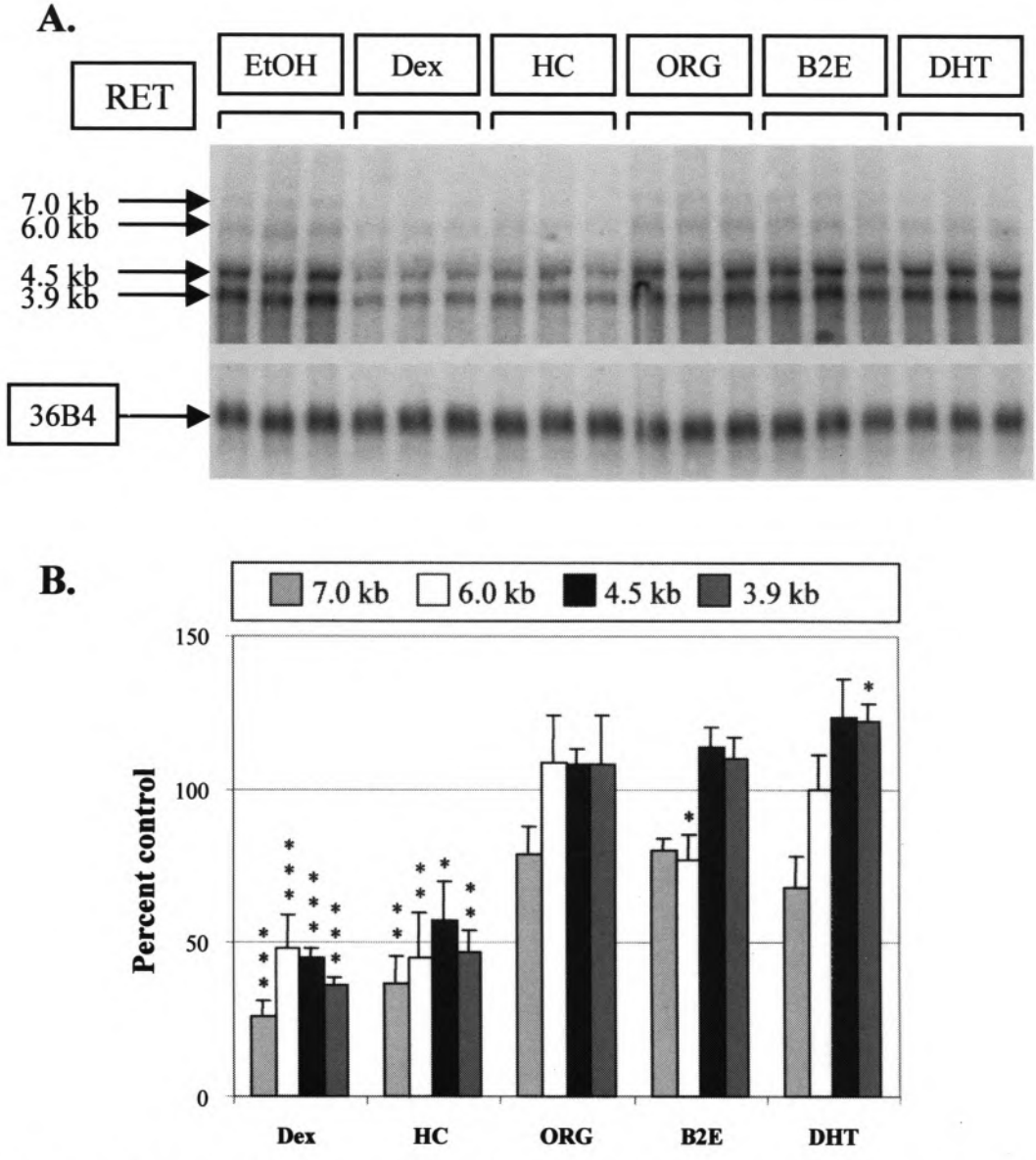

FIG. 4. Steroid specificity and *RET* mRNA expression. (A) Northern analysis. TT cells were treated in triplicate with the steroids shown, all at 10^{-6} M, for 24 h. ORG 2058 was used as an active analogue of progesterone. EtOH, ethanol vehicle; Dex, dexamethasone; HC, hydrocortisone; ORG, ORG 2058; β_2E, β_2 estradiol; DHT, dihydrotestosterone. (B) Quantification of results obtained from Northern analysis. Band intensities were again corrected for loading with 36B4 and expressed as a percentage of the mean of vehicle-treated control lanes. Significant differences when compared to control results are shown: $*p < 0.05$, $**p < 0.01$, $***p < 0.005$.

α-tubulin, and plasmid pGEM-4Z sequence as controls. Treatment with dexamethasone resulted in a specific decrease in *RET* hybridization, indicating that its inhibitory effect on *RET* expression was due to a direct effect on the transcription rate.

Dexamethasone Alters Cell Proliferation in Association With its Effect on RET *Expression*

The functional significance of dexamethasone was last assessed by measuring cell growth in two different assay systems. Treatment with 10^{-6} M dexamethasone for up to 6 days resulted in a decrease in cell number when compared using the MTS assay to vehicle-treated control samples (Fig. 8A). This decrease became apparent after only 1 day but did not become significant until the second day of treatment. Similarly, treatment with dexamethasone for 24 h resulted in a decrease in cell growth when assessed by [^{3}H]thymidine uptake assay (Fig. 8B). This decrease became significant after treating cells with more than 10^{-8} M dexamethasone. The results of both assays

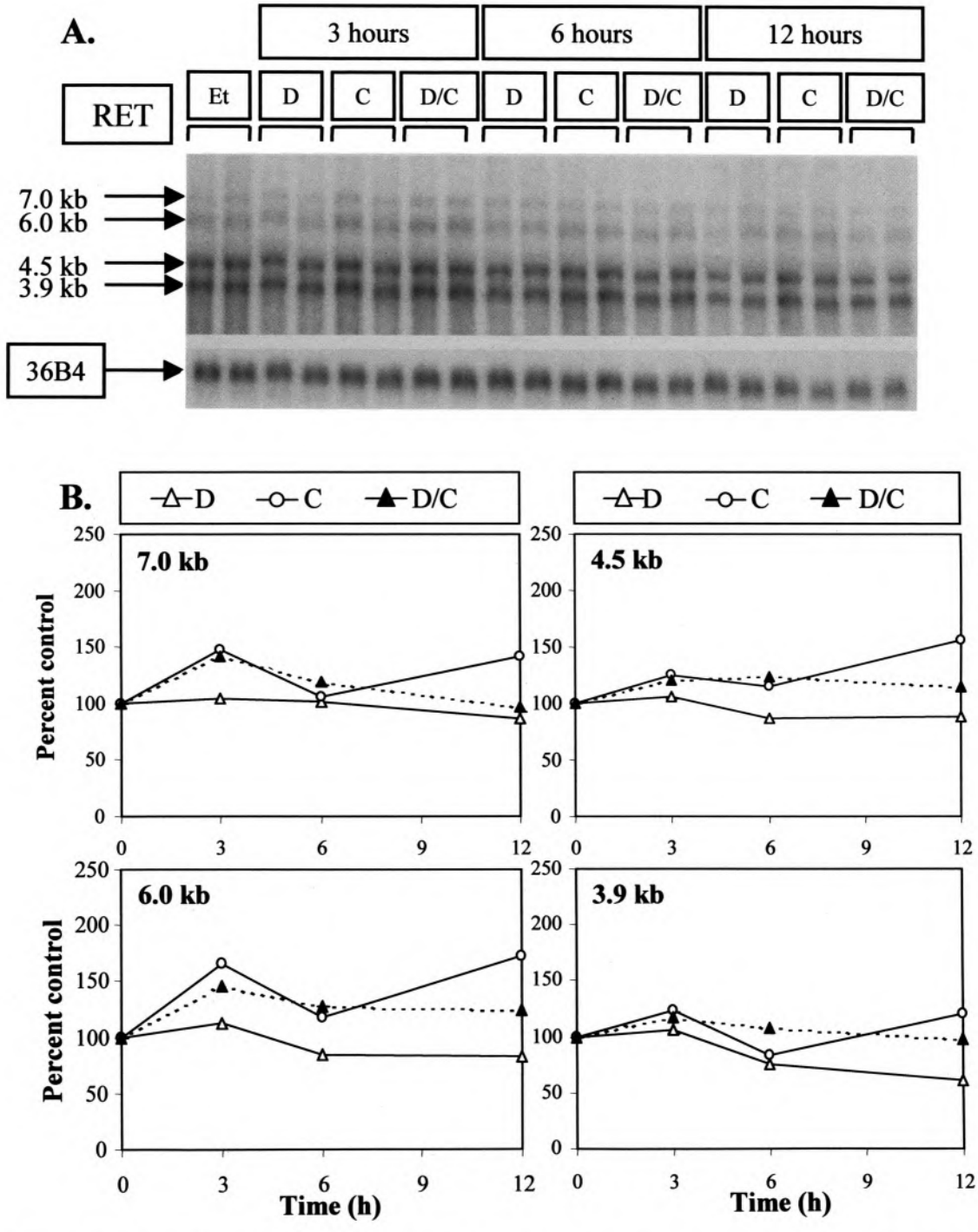

FIG. 5. Effect of cycloheximide on *RET* mRNA levels and their dexamethasone-induced inhibition. (A) Northern analysis. TT cells were treated for the time shown with 10^{-8} M dexamethasone (D), 20 μg/ml cycloheximide (C), or both substances (D/C) as described in Materials and Methods. Representative results from duplicate samples are shown. (B) Quantification of results obtained from Northern analysis. Each of the 7.0-, 6.0-, 4.5-, and 3.9-kb *RET* species are assessed in separate panels here. The effects of dexamethasone alone (D), cycloheximide (C), or both substances (D/C) are expressed as a percentage of the mean vehicle-treated control lanes (Et) after correction for loading with 36B4.

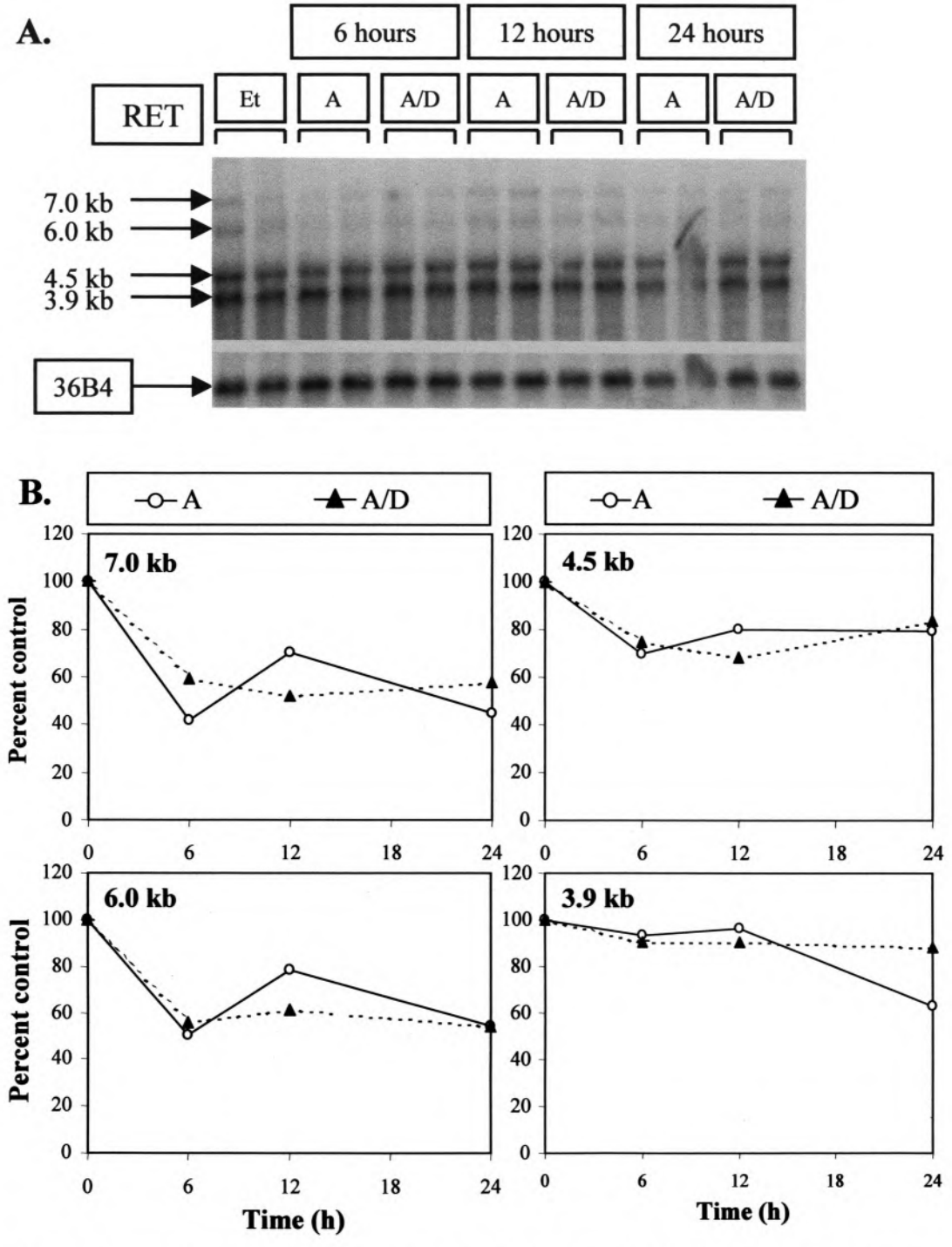

FIG. 6. Effect of actinomycin D on *RET* mRNA levels with and without dexamethasone. (A) Northern analysis. TT cells were treated with 5 μg/ml actinomycin D alone (A) or with the addition of 10^{-8} M dexamethasone (A/D) for the times shown. Again, representative results from duplicate samples are shown. (B) Quantification of results obtained from Northern analysis. Each of the 7.0-, 6.0-, 4.5-, and 3.9-kb *RET* species are again assessed in separate panels. The effects of actinomycin D alone (A) or in combination with dexamethasone (A/D) are expressed as a percentage of the mean vehicle-treated control lanes (Et) after correction for loading with 36B4.

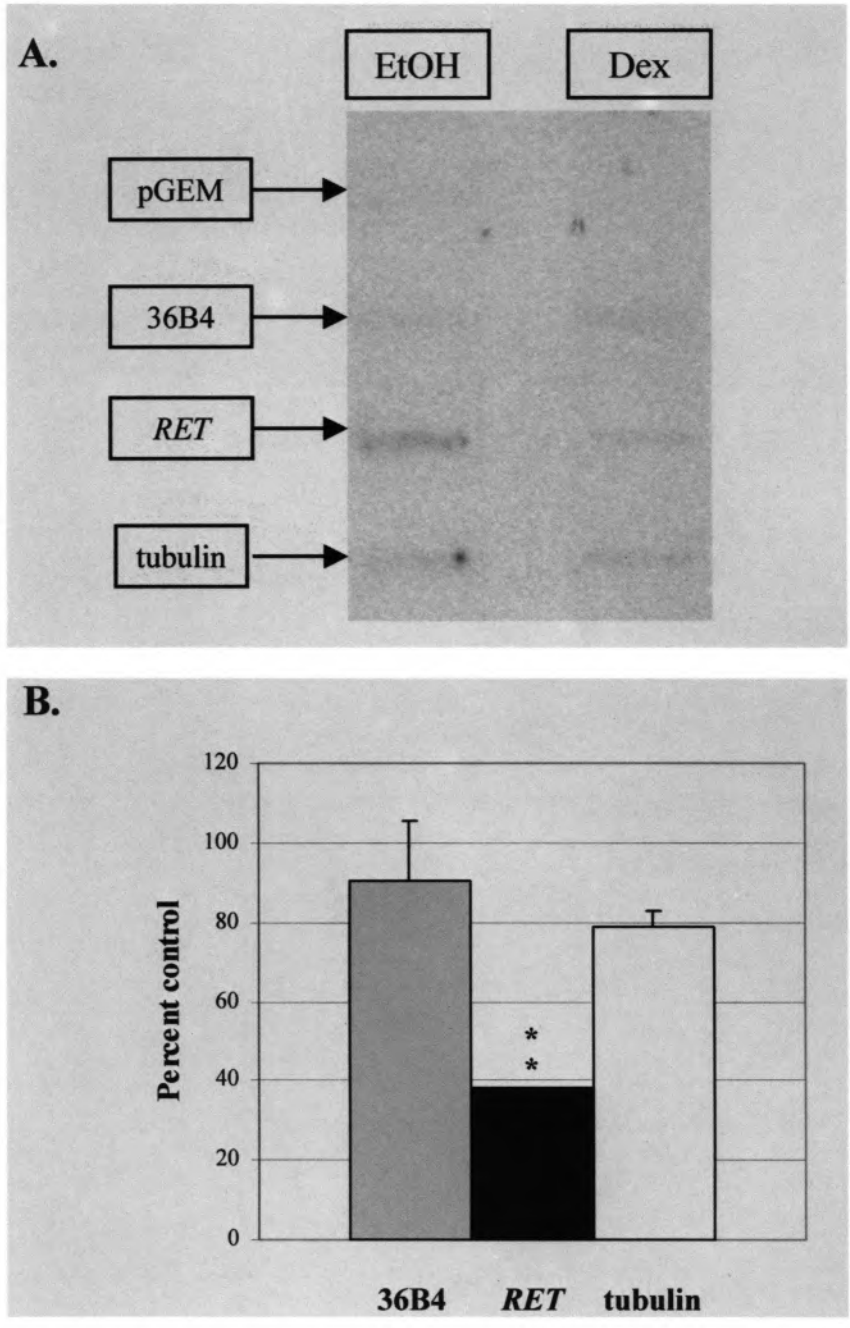

FIG. 7. Effect of dexamethasone on *RET* transcription. TT cells were treated with 10^{-6} M dexamethasone (Dex) or equivalent vehicle (EtOH) for 24 h and cells harvested for nuclear extraction. Relative nuclear transcription rates were determined by run-on assay, allowing the elongation of nascent RNA transcripts in the presence of [α-^{32}P]UTP as described in Materials and Methods. Transcripts were hybridized to identical slot-blots containing *RET* transmembrane and tyrosine kinase sequence. As controls for the action of dexamethasone on other transcripts, 36B4 and α-tubulin constructs were included on these slot-blots. To further control for hybridization specificity, the plasmid pGEM-4Z was also added. Results were reproducible on two occasions. Significant differences when compared to control results are shown: $^{**}p < 0.01$.

would indicate that the decrease in *RET* expression seen with dexamethasone is associated with a similar decrease in cell proliferation.

DISCUSSION

Expression of the *RET* proto-oncogene and its activation by the mutations of MEN 2 are important events for tumorigenesis in certain neuroendocrine tissues. Silencing of *RET* expression has been found to be associated with endocrine differentiation and a decrease in cell growth (9,40), a finding of potential interest for tumor treatment. Because dexamethasone has previously been found to have a similar effect on cell growth, an investigation of its effect on *RET* expression was warranted.

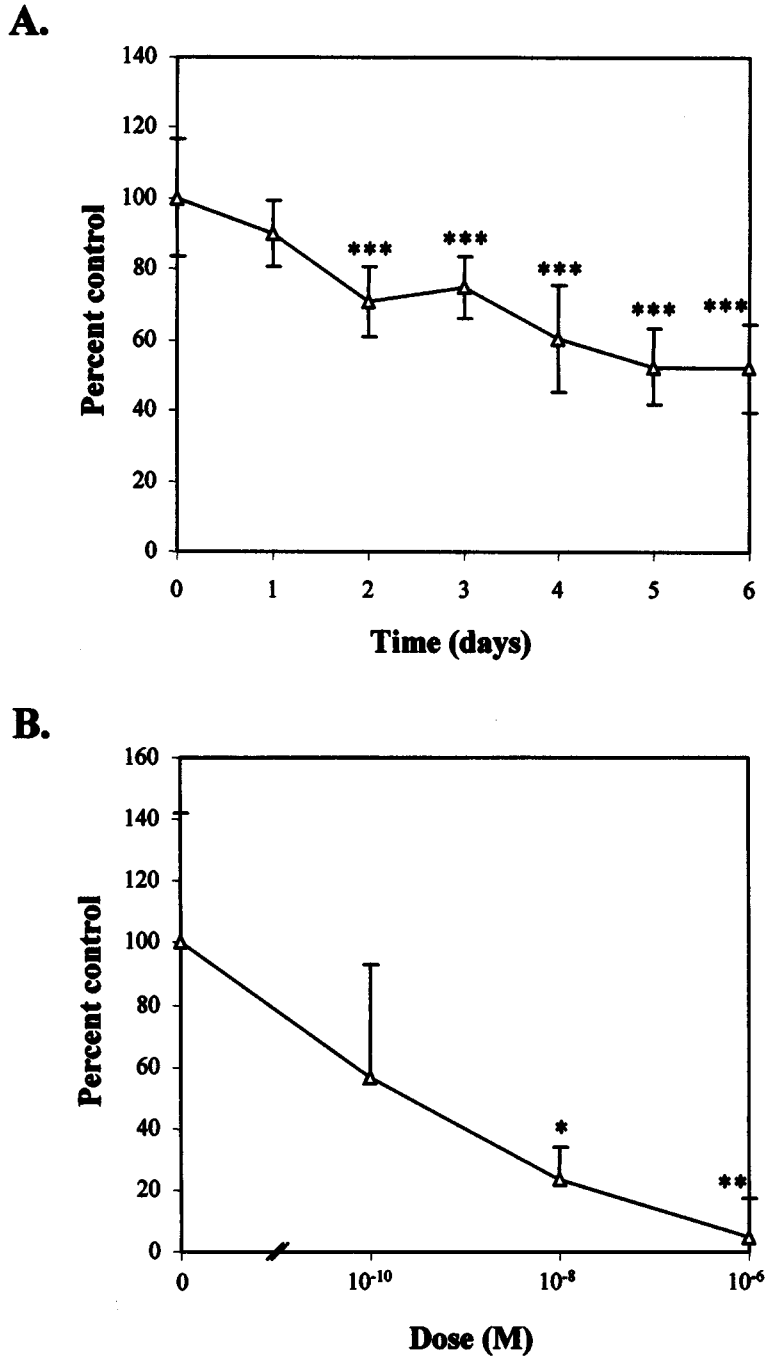

FIG. 8. Growth in response to dexamethasone. (A) Using the MTS assay. TT cells were treated with 10^{-6} M dexamethasone or with an equivalent amount of ethanol for up to 6 days. MTS assays were performed daily and background readings subtracted from each day's result. Treatment results are presented here as a percentage of vehicle-treated control lanes ± 1 SD ($n = 8$). Significant differences when compared to control samples are shown: $*p < 0.05$, $**p < 0.01$, $***p < 0.005$. (B) Using the [^{3}H]thymidine uptake assay. TT cells were treated with either 10^{-6} M dexamethasone or vehicle for 24 h. [^{3}H]Thymidine was then added for the final 4 h of this incubation and the uptake assay performed. Treatment results are presented, minus background readings, as a percentage of vehicle-treated control lanes ± 1 SD ($n = 3$). Significant differences are shown as in (A).

Treatment of the TT cell line, an accepted in vitro model for medullary thyroid carcinoma, with dexamethasone resulted in a clear decrease in both *RET* mRNA and protein levels (Fig. 1). The decrease in RNA expression seen with dexamethasone was evident with concentrations at or above 10^{-8} M (Fig. 2) and appeared at higher concentrations after only 12 h of treatment (Fig. 3). This effect was found to be specific to glucocorticoids (Fig. 4). While neither β_2 estradiol nor dihydrotestosterone reduced *RET* mRNA levels, both hydrocortisone and dexamethasone produced a significant decrease in *RET* expression for all transcripts. Interestingly, the progesterone analogue ORG 2058 did not produce a similar decrease. This lack of activity is surprising, because progesterone is known to bind competitively to the glucocorticoid receptor (24), but may be explained by the existence of a separate progesterone receptor in the TT cell line (13). The fact that dexamethasone reduced *RET* mRNA levels in both TT cells and in primary culture (Fig. 1C) would suggest that this finding is applicable to more than a single cell line.

The various methods used to study the effects of glucocorticoids on *RET* expression suggested a direct effect on *RET* transcription. First, cycloheximide was used to eliminate protein synthesis and assess the importance of new protein (Fig. 5). Cycloheximide alone was found to yield a net increase in *RET* mRNA levels, implying that protein was normally synthesized to suppress *RET* expression. A similar effect has been noted elsewhere in SK-N-BE cells (6). Adding dexamethasone resulted in a decline in *RET* mRNA levels relative to cycloheximide alone, suggesting that the steroid exerted its effect directly without new protein mediators. Second, actinomycin D was employed to abolish new RNA synthesis and assess the importance of changes in RNA stability (Fig. 6). Adding actinomycin D showed that existing *RET* mRNA species degraded slowly, with half-lives of greater than 24 h. Similar half-life observations have previously been made in neuroblastoma cell lines (6,45). It could be seen that 7.0- and 6.0-kb species declined more rapidly with actinomycin D alone, suggesting that these longer species are more susceptible to degradation. Adding dexamethasone, however, did not materially affect the degradation of any of the four *RET* species when compared to treatment with actinomycin D alone. Finally, transcription run-on assays supported this result by showing that treatment with dexamethasone resulted in a decrease in overall *RET* mRNA synthesis (Fig. 7).

The mechanism for such a decrease in *RET* transcription remains unclear after analysis of known regulatory sequence. Binding of a glucocorticoid–receptor complex to specific regulatory elements is likely, but neither a full glucocorticoid regulatory element (GRE) nor the rarer negative GRE (18) have been identified within the *RET* promoter region [(25), L. Mulligan, personal communication]. Additional regulatory sequence may lie further upstream. Further examination of the published promoter and intronic sequence (25), however, has identified two glucocorticoid receptor half-sites. The first lies 382 bp upstream from the transcription start site, while the second is located 947 bp downstream of exon 19 (data not shown). Both half-sites are identical to that implicated in repression of calcitonin/calcitonin gene-related peptide expression by glucocorticoids (47). The importance of either glucocorticoid receptor half-site for *RET* expression is currently unknown. Alternately, it remains possible that glucocorticoids exert their effect through interaction with other transcription factors in the absence of DNA binding. Such a mechanism frequently results in suppression of gene transcription (5) and may involve transcription factors such as AP-1 (26,48) or NF-κB (37). Neither of these factors has yet been implicated in binding to the *RET* promoter and additional promoter sequence is required to investigate these mechanisms.

The functional significance of glucocorticoids is confirmed by studying TT cell growth. As previously documented by other groups, treatment with dexamethasone resulted in a significant growth inhibition using two assay methods. This inhibition was dose responsive and became more pronounced with time, as seen in both TT and CA77 cell lines (14,29,39). It should be noted that a similar effect on cell growth and *RET* expression has also been noted after activation of Raf-1 (9) and after treatment with phorbol esters and sodium butyrate [(17,34), A. Capes-Davis, submitted]. Considering these results, we suggest that the decrease seen in *RET* mRNA levels may be responsible for the changes in cell behavior seen with these agents.

The effects of glucocorticoids on *RET* expression and cell behavior are likely to have clinical significance for the treatment of MTC. Dexamethasone has been shown here to have significant effects on a cell line that is held to be an in vitro model for medullary carcinoma, with a particular impact on the expression of a proto-oncogene known to cause this tumor. Although the adverse effects of glucocorticoids such as dexamethasone can be significant, these steroids are currently available for clinical use and may be helpful for treatment or palliation of patients with end-stage tumors. In the future, with development of synthetic glucocorticoids varying in their metabolic action, further agents may become available minimizing these adverse reactions. The in vitro work described here thus forms a rational basis for animal studies and may

lead to subsequent human trials for the use of glucocorticoids in MTC.

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