Regulation of mucin gene expression in human tracheobronchial epithelial cells by thyroid hormone

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We reported previously that the expression of the gene encoding MUC5AC mucin in human airway epithelial cells is controlled by retinoic acid via the retinoic acid receptor (RAR)-α and that 3,3′,5-tri-iodothyronine (T_3) inhibits the expression of *MUC5AC*. The purpose of the present study was to identify mechanisms mediating the effect of T_3. T_3 has been shown to inhibit gene expression via several mechanisms, either by enhancing or repressing the transcription of target genes or by the regulation of post-transcriptional events. Results showed that T_3 strongly inhibited *MUC5AC*-driven luciferase activity in normal human tracheobronchial epithelial cells that had been transiently transfected with a *MUC5AC*–luciferase reporter construct; however, it did not affect *MUC5AC* mRNA stability. These results indicate that T_3 suppresses *MUC5AC* expression at the transcriptional level. An analysis of deletion constructs showed that deletion of the region downstream of 3 kb resulted in markedly decreased levels of *MUC5AC* transcription in the absence of T_3 (i.e. under control conditions) as well as a loss of responsiveness to the inhibitory effects of T_3. This suggests that this region might contain elements important for the activation as well as the repression of *MUC5AC* transcription. To determine whether T_3 modulates retinoic-acid-dependent *MUC5AC* transcription via an alteration in the abundance of retinoid receptor proteins, we examined the type and abundance of these receptors in nuclear extracts of airway epithelial cells grown in the presence or absence of T_3. Western blots showed that T_3 markedly decreased several types of retinoid receptor while not affecting T_3 receptor proteins. Consistent with this finding were gel-shift assays revealing a decrease in RAR–retinoic acid response element complexes obtained from T_3-treated cells. We propose that T_3 might inhibit retinoid-dependent *MUC5AC* expression by decreasing retinoid receptor levels and thereby decreasing the transcriptional activation of this gene for mucins.

Key words: retinoic acid, retinoic acid receptor, retinoic acid response element, thyroid receptor, tri-iodothyronine.

INTRODUCTION

Mucins are highly glycosylated, high-molecular-mass glycoproteins that are the major components of mucus produced by the epithelia of the respiratory, gastro-intestinal and reproductive tracts. They are responsible for the visco-elastic properties and hydrophilicity of secreted mucus and provide lubrication and protection for the mucous membranes that produce them (reviewed in [1]). In the airways virtually all forms of airway inflammation are associated with the overproduction of mucus, which can lead to airway obstruction. How mucin synthesis is regulated under normal and pathological conditions and how it can be controlled pharmacologically are important questions in respiratory medicine (reviewed in [2]).

Twelve mucin genes, designated *MUCs1–4*, *5AC*, *5B*, *7–8* [2], *MUC9* [3] and *MUCs11–12* [4] have been reported. Three of these mucin genes, *MUC2*, *MUC5AC* and *MUC5B* encode polymeric mucins that are expressed by airway epithelium; however, so far only two major components of airway mucus have been identified, namely MUC5AC and MUC5B [5–7].

In recent years many studies have been conducted aimed at elucidating factors and mechanisms involved in the regulation of mucin genes expressed in the airways (reviewed in [2]). Our efforts have been focused on the role of retinoic acid (RA), a prototypical retinoid, in the differentiation of airway epithelium and in the control of the expression of genes encoding mucin. We and others showed that this expression is dependent on retinoid [8–13] and that the retinoid effect is mediated via retinoic acid receptor (RAR)-α [8]. In separate studies we found that in normal human tracheobronchial epithelial (NHTBE) cultures, the thyroid hormone 3,3′,5-tri-iodothyronine (T_3) represses *MUC5AC* expression and decreases the amount of secreted mucus [9].

The effects of RA and T_3 are mediated via the nuclear receptors RARs, retinoid X receptors (RXRs) and thyroid receptors (TRs) respectively and both RARs and TRs require heterodimerization with RXRs for optimal transcriptional activity [14]. TR–RXR and RAR–RXR recognize the same core sequence of direct repeats (i.e. AGGTCA) in the promoter regions of target genes; the binding specificity is determined by the number of intervening nucleotides between the direct repeats [four and five for thyroid response element and retinoic acid response element (RARE) respectively] [15,16]. Binding of the cognate ligands to the receptors enhances or represses the transcription of their target genes [17] (for a review of T_3 effects see [18]), but without its ligand the TR, but not the RAR, can suppress transcription [19–21]. Furthermore it has been shown that T_3 can regulate RAR activity [19] and that the interaction of RA and T_3 signalling occurs at the level of nuclear receptors [21].

The purpose of the studies described here was to elucidate mechanisms involved in the down-regulation by T_3 of mucin gene expression, which we reported previously [9]. In addition we wished to determine whether *MUC5B*, similarly to *MUC5AC*, is also negatively regulated by T_3.

Our studies showed that the expression of *MUC5AC*, and to a much smaller extent *MUC5B*, is regulated by T_3. We found

that T_3 does not affect *MUC5AC* mRNA stability but rather affects transcription by suppressing promoter activity. We also showed that T_3 significantly decreases the levels of several retinoid receptor proteins and decreases the formation of the RAR–RARE complex. The results suggest that the suppression of *MUC5AC* expression by T_3 might be the result of the decrease in retinoid receptors and lower levels of RAR–RARE complexes, which in turn might be the cause of decreased *MUC5AC* promoter activity and decreased *MUC5AC* transcription.

MATERIALS AND METHODS

Air/liquid interface cultures

NHTBE cells (passage 2) (Clonetics Corp., La Jolla, CA, U.S.A.) were seeded on uncoated, semipermeable Transwell clear membranes (Corning Costar, Cambridge, MA, U.S.A.) at 2×10^4 cells/cm². Cells were cultured in hormone-supplemented, serum-free medium containing all-*trans*-RA (Sigma, St Louis, MO, U.S.A.) either with or without T_3 (6.5 ng/ml unless indicated otherwise). For the complete medium formulation see [12]. Cultures were grown submerged for the first 7 days, at which time the air/liquid interface was created. Cultures were maintained at 37 °C in a humidified air/CO_2 (19:1) atmosphere.

Immunodetection and quantification of secreted MUC5AC and MUC5B mucin

To monitor the production of MUC5AC and MUC5B mucin, apical secretions accumulating over a 24 h period (triplicate samples per group) were collected and assayed by dot-blotting [12]. In brief, diluted apical secretions were applied to nitrocellulose membranes, which were incubated with either the mouse monoclonal anti-(human MUC5AC) antibody (NeoMarkers, Freemont, CA, U.S.A.) or the rabbit polyclonal anti-MUC5B antibody [6] MAN5BIII, which was a gift from Dr John Sheehan (The Wellcome Trust Centre for Cell Matrix Research, University of Manchester, Manchester, U.K.) followed by reaction with horseradish-peroxidase-conjugated goat anti-mouse or anti-rabbit IgG. The signal was detected by chemiluminescence (ECL® kit; Amersham, Little Chalfont, Bucks., U.K.). The levels of MUC5AC and MUC5B reactivity were measured densitometrically. Statistical comparisons were made by Student's t test.

Competitive reverse-transcriptase-mediated PCR (RT–PCR)

Methods to detect and quantify *MUC5AC* and *MUC5B* have been reported previously in detail [8,11]. In brief, total RNA was collected from triplicate separate cultures and was reverse-transcribed into cDNA. Oligonucleotide primers were designed on the basis of published sequences for *MUC5AC* [22] and *MUC5B* [23], and β_2-*microglobulin* (*β2M*, RNA control) and interleukin-8 (*IL-8*) which were purchased from Clontech (Palo Alto, CA, U.S.A.). PCRs for *MUC5AC* and *MUC5B* were performed in the presence of internal standards (so-called MIMICs) (PCR MIMIC Construction Kit; Clontech) with 1.0 and 0.1 attomoles per reaction for *MUC5AC* and *MUC5B* respectively. Specific amplification for *MUC5AC* and *MUC5B* was confirmed by sequencing. The amplification efficiency for *MUC5AC* and *MUC5B* cDNA and MIMIC was verified by determining the amount of cDNA and MIMIC produced after various numbers of PCR cycles; the quantifiability of the assays was determined by titrating MIMIC against a constant amount of cDNA. *IL-8* PCRs were performed in accordance with the manufacturer's directions. PCR products were separated on 2 %

(w/v) agarose gels (containing EtBr) and the resulting bands were analysed with a digital imaging system (Alpha Innotech, San Eleandro, CA, U.S.A.). The ratio of the signal intensity of the target cDNA to the MIMIC was determined for each sample and the mean ratio was calculated for treated and control groups. Statistical comparisons were made by Student's t test and linear-regression analysis.

Transient transfections

Methods to measure *MUC5AC* promoter activity with luciferase as a reporter have been reported previously [24]. Fragments ranging in size from 1.3 to 1.02 kb, which were located immediately adjacent to the 5′ transcriptional start site of human *MUC5AC*, were generated by digestion with exonuclease (Erase a Base System; Promega Corp., Madison, WI, U.S.A.) of the 1.3 kb fragment of the *MUC5AC* promoter and cloned into the pGL3-Basic luciferase vector (Promega Corp.).

NHTBE cells were seeded in six-well tissue-culture plates (2×10^4 cells per well) in bronchial epithelial cell growth medium (BEGM; Clonetics Corp.) containing growth factors and supplements as recommended by the supplier, except that RA was omitted from the medium. At 2 days before transfection (i.e. when the cultures had reached approx. 60–70 % confluence), T_3 was removed from the medium. Transfection with LIPO-FECTAMINE Plus (Gibco BRL, Gaithersburg, MD, U.S.A.) was performed in accordance with the manufacturer's directions. After 3 h the transfection medium was replaced with BEGM with or without T_3 and indicated concentrations of RA. The cultures were maintained for an additional 48 h. *MUC5AC* promoter activity was determined by measuring luciferase activity in accordance with manufacturer's directions (Promega Corp.) and normalized by co-transfection with the *β-galactosidase* expression plasmid pβgal-control vector (Clontech). *β-Galactosidase* activity was measured in accordance with the manufacturer's instructions (Clontech). All transfections were performed in triplicate wells; results are reported as emitted light per well (means ± S.D.).

Preparation of nuclear extracts

NHTBE cells were cultured for 14 days in culture medium without or with T_3. The cultures were washed twice with PBS, the cells were dissociated after trypsin/EDTA treatment and cell extracts were prepared in accordance with published procedures [25]. The protein concentrations were determined by bicinchoninic acid protein assay (Pierce, Rockford, IL, U.S.A.) and extracts were aliquoted and stored at -70 °C before use.

Western blotting

Nuclear extracts (50 μg per lane) from cultures grown in the absence or presence of T_3 were separated by SDS/PAGE [10 % (w/v) gel] and transferred to nitrocellulose membranes that were blocked in 5 % (v/v) dried milk. Blots were incubated at room temperature for 1 h with nuclear-receptor-specific antibodies [anti-RARs α, β and γ; anti-RXRs α, β and γ; anti-TRs α and β; and anti-actin, all obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.)] at concentrations suggested by the supplier. Blots were washed and the signals detected by chemiluminescence (ECL® kit).

Electrophoretic mobility-shift assay

Double-stranded oligonucleotides for the consensus RARE-binding site (DR-5, 5′-GGTTCANNNNNAGTTCA-3′; Santa Cruz Biotechnology) were end-labelled with [γ-^{32}P]ATP (NEN

Dupont, Wilmington, DE, U.S.A.). Nuclear extracts prepared from cultures grown in the absence or presence of T_3 were incubated with radiolabelled oligonucleotide in binding buffer (Gel Shift Assay Systems; Promega Corp.) for 30 min at room temperature. Excess unlabelled oligonucleotide or anti-$RAR\alpha$/anti-$RAR\beta$ antibodies (5 μg of each) (Santa Cruz Biotechnology) were added to indicated reactions. Products were separated on non-denaturing polyacrylamide gels [6 % (w/v) gel run at 250 V and 4 °C for 50 min] and transferred to nitrocellulose membranes; the signal was detected with a PhosphorImager (Molecular Dynamics, Palo Alto, CA, U.S.A.).
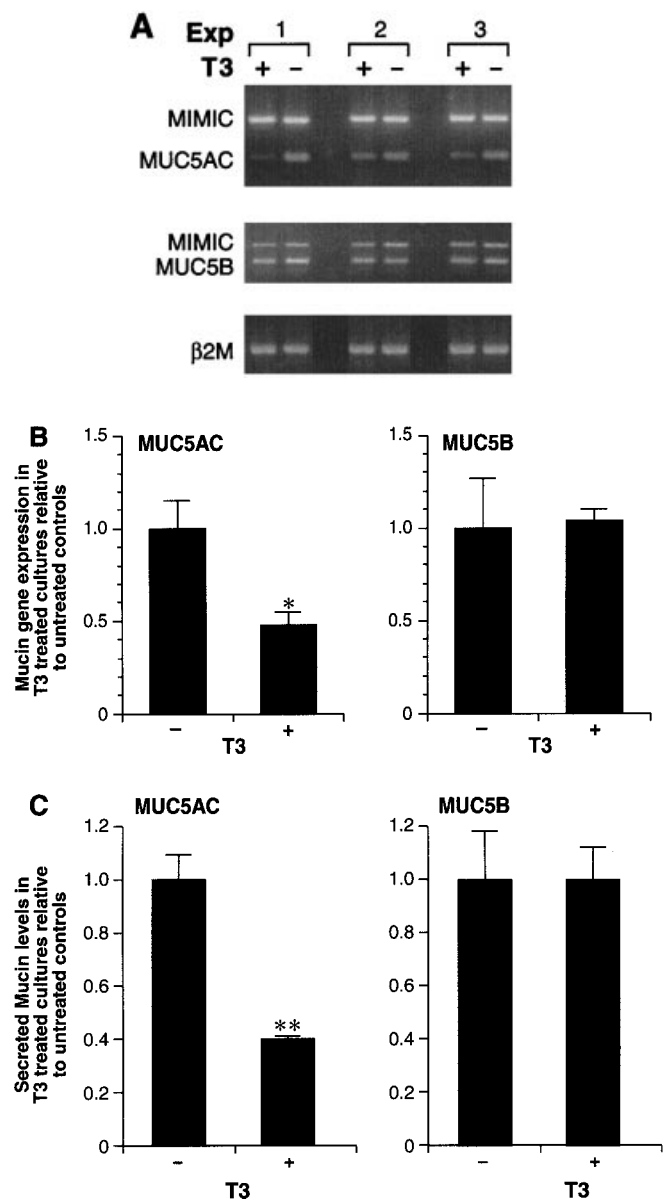
RESULTS

Effects of T_3 on mucin gene expression and MUC5AC and MUC5B mucin production

Previously we showed that T_3, a hormone supplement commonly used in epithelial growth medium, inhibited the production of mucus and the expression of *MUC5AC* in NHTBE cell cultures [9]. To determine whether T_3 inhibits MUC5AC and MUC5B mRNA expression and mucin production, NHTBE cells were grown for 14 days in medium containing 50 nM RA with and without T_3. Total RNA from three independent experiments was collected and the levels of MUC5AC and MUC5B mRNA expression were determined by competitive RT–PCR. As seen in Figures 1(A) and (B), the addition of T_3 caused a 60 % decrease in the levels of steady-state MUC5AC mRNA. However, MUC5B and β2M (PCR control gene) mRNA levels were unaffected by treatment with T_3. In the same experiments we also collected apical secretions produced over a 24 h period; with the use of anti-MUC5AC and anti-MUC5B specific antibodies we found that MUC5AC mucin levels were also decreased by approx. 50–60 % in T_3-treated cultures (Figure 1C, left panel), whereas the levels of MUC5B were not affected (Figure 1C, right panel).

Interaction of RA and T_3 in the regulation of mucin gene expression

We hypothesized that T_3 might interfere with RA signalling. T_3 and RA bind to their cognate nuclear receptors, TR and RAR respectively, both of which form heterodimers with RXRs, thereby modulating the transcriptional activity of target genes. We speculated that the degree of suppression of mucin mRNA levels might depend on the relative concentrations of RA and T_3 in the medium. To test this, NHTBE cells were cultured in the absence of T_3 for 14 days in medium containing 5 nM RA, 50 nM RA (the standard concentration used in all previous experiments) or 500 nM RA. On day 14, cultures were treated for 4 days with various concentrations of T_3 (0.0065–6.5 ng/ml), after which total RNA was isolated and the mRNA levels of *MUC5AC* and *MUC5B* were determined by RT–PCR and quantified by densitometry. As seen in Figure 2(A), in cultures containing only 5 nM RA (known to be a suboptimal RA concentration [9]), T_3 concentrations as low as 0.065 ng/ml suppressed MUC5AC mRNA and MUC5B mRNA levels to approx. 60 % of controls. In cultures grown in 50 nM RA (Figure 2B), a 100-fold higher concentration of T_3 (i.e. 6.5 ng/ml) was required to obtain a similar degree of suppression of MUC5AC. Under these conditions, treatment with T_3 resulted in a dose-dependent decrease in MUC5AC mRNA; however, MUC5B mRNA levels were not significantly affected. At 500 nM RA, T_3 did not significantly suppress either MUC5AC or MUC5B mRNA expression. These results indicate that the

suppression of MUC5AC mRNA levels by T_3 is dependent on both RA and T_3 concentration; however, MUC5B mRNA levels were suppressed only under suboptimal concentrations of RA.

Time course of T_3 suppression of mucin gene mRNA levels

To determine how rapidly T_3 suppressed mucin mRNA levels, cultures grown for 14 days in medium containing 50 nM RA without T_3 were treated with T_3 (6.5 ng/ml) for up to 48 h. Total RNA was collected at indicated times and assayed for MUC5AC
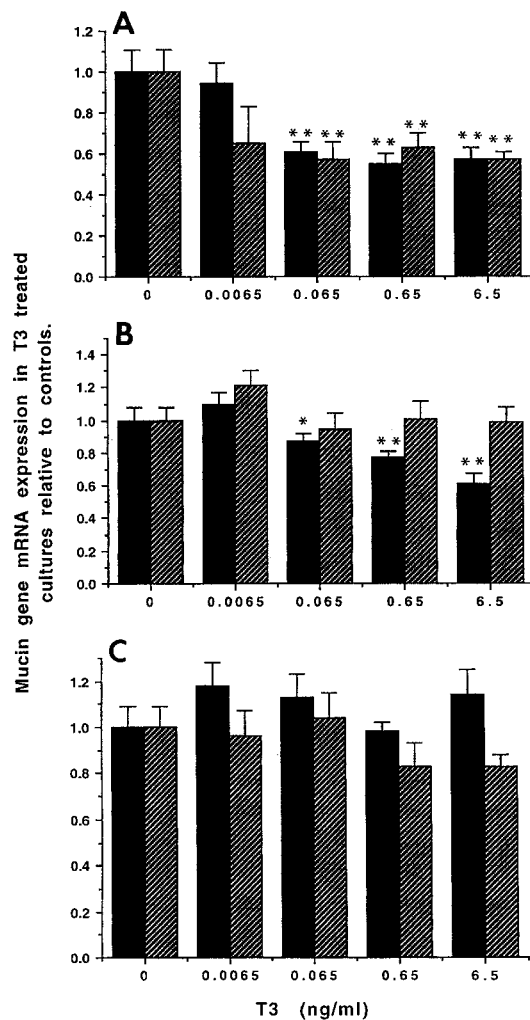

Figure 1   Effect of chronic treatment with T_3 on mucin gene expression and MUC5AC and MUC5B mucin production

In three independent experiments NHTBE cells were grown for 14 days in medium without T_3 or containing T_3 (6.5 ng/ml). Total RNA was isolated from triplicate wells from each experiment; the levels of MUC5AC, MUC5B and β2M mRNA species were analysed by competitive RT–PCRs (**A**) and the relative levels were determined by densitometry (**B**). Apical secretions for 24 h were collected from triplicate cultures and assayed by dot-blot analysis for the levels (means $\pm$ S.D.) of MUC5AC and MUC5B mucins (**C**). *$P < 0.05$, **$P < 0.01$.
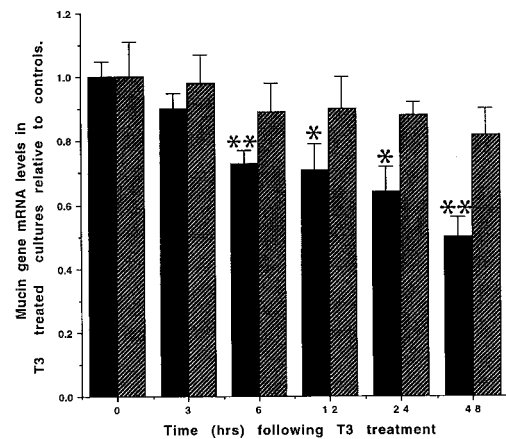
Figure 2 Effect of RA concentration on the inhibition of mucin gene expression by T$_3$

NHTBE cells were cultured for 14 days in medium containing 5 nM RA (**A**), 50 mM RA (**B**) or 500 nM RA (**C**) without T$_3$. Starting on day 14, different concentrations of T$_3$ were added to the cultures. After 4 days of treatment, total RNA was collected from three separate cultures and the levels of MUC5AC (filled bars) and MUC5B (hatched bars) mRNA species were determined. The results are expressed as relative mRNA levels (means ± S.D.) in T$_3$-treated cultures compared with untreated cultures. The results are representative of data obtained from two separate experiments. *P < 0.05, **P < 0.01.

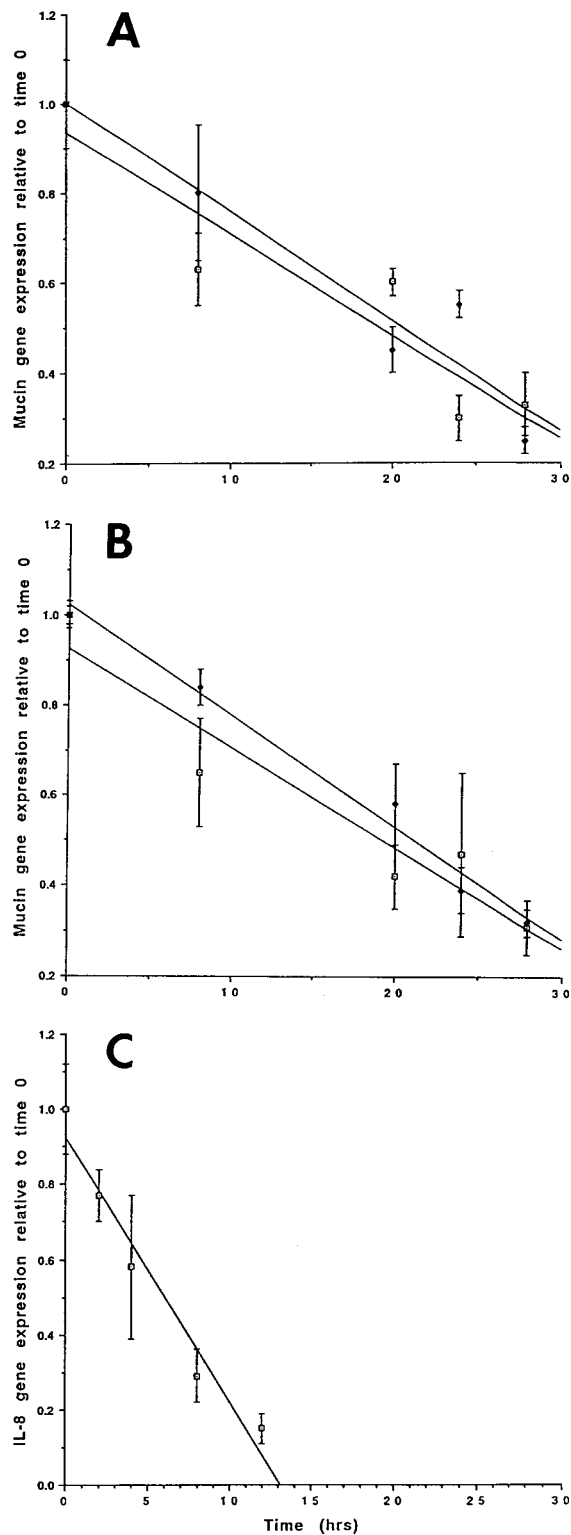**Figure 3 Time course of the effect of T$_3$ on mucin gene expression**

NHTBE cells were grown for 14 days without T$_3$. Starting on day 14, T$_3$ (6.5 ng/ml) was added to half of the cultures. Total RNA was isolated at indicated times from three separate cultures and the levels of MUC5AC (filled bars) and MUC5B (hatched bars) mRNA species were determined by competitive RT–PCR and quantified by densitometry. Results are expressed as the relative levels in T$_3$ treated group compared with untreated controls (means ± S.D.). The results are representative of data obtained from three separate experiments. *P < 0.05, **P < 0.01.

(10 μg/ml) to the cultures. Total RNA was collected over a period of 28 h and mRNA levels were determined and plotted. Longer treatment times with actinomycin D resulted in significant cellular destruction. The results are summarized in Figure 4. The lines connecting the different time points have slopes of 0.022 compared with 0.024 for *MUC5AC* (Figure 4A) and of 0.022 compared with 0.025 for *MUC5B* (Figure 4B) from cultures grown in the absence and in the presence of T$_3$, respectively. The half-lives of MUC5AC and MUC5B mRNA species were estimated to be 21–23 h regardless of the absence or presence of T$_3$. Linear regression analysis, performed to fit the two data sets (i.e. mRNA levels from cultures grown in the absence or presence of T$_3$), indicated that the slopes were the same under both conditions for both *MUC5AC* and *MUC5B*. These results indicate that T$_3$ did not alter MUC5AC or MUC5B mRNA stability, which suggests that T$_3$ was either directly or indirectly affecting mucin gene expression via transcriptional regulation. IL-8 mRNA, which served as a control, was found to have a rapid rate of decay (Figure 4C): the line drawn by linear regression had a slope of 0.07. The half-life of IL-8 mRNA was calculated to be approx. 5 h.

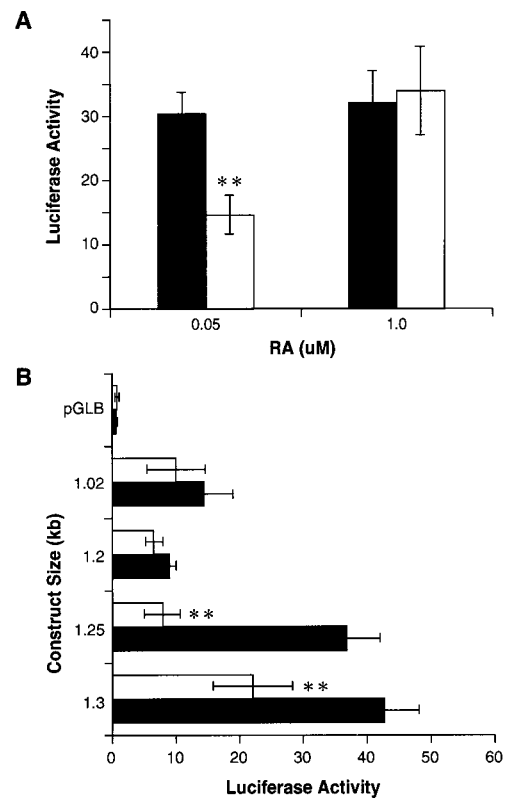## Effect of T$_3$ and RA on *MUC5AC* promoter activity

To determine whether T$_3$ was regulating *MUC5AC* transcription, we transfected NHTBE cells with a luciferase reporter vector containing the 1.3 kb 5′ flanking region from the transcription start site of the human *MUC5AC* promoter and three deletion constructs of 1.25, 1.2 and 1.02 kb. As shown in Figure 5(A), T$_3$ suppressed luciferase activity in NHTBE cells transfected with the 1.3 kb construct by approx. 50 % in cultures containing 50 nM RA. The addition of higher concentrations of RA abolished the T$_3$ suppressive effect.

Analysis of the deletion constructs (see Figure 5B) in the absence of T$_3$ (control conditions) showed that luciferase activity was 3–4-fold higher in cells transfected with 1.3 and 1.25 kb constructs than in cells transfected with constructs of 1.2 and 1.02 kb, indicating that a critical element regulating *MUC5AC*

and MUC5B mRNA levels. As seen in Figure 3, MUC5AC mRNA levels decreased between 3 and 6 h after the start of treatment and continued to decrease over the next 45 h. MUC5B mRNA levels were not significantly different from controls even after 48 h of treatment.

Effect of T$_3$ on mucin mRNA stability

Reports in the literature indicate that T$_3$ can destabilize the mRNA transcribed from certain genes [26]. Therefore we considered whether the observed effects were due to changes in mucin mRNA stability. We examined the effect of T$_3$ on the half-lives of MUC5AC and MUC5B mRNA species. NHTBE cells were cultured for 14 days in the absence or presence of T$_3$ (6.5 ng/ml) in medium containing 50 nM RA; at that point transcription was blocked by the addition of actinomycin D

Figure 4 Effect of chronic treatment with T_3 on MUC5AC and MUC5B mRNA stability

Cultures were grown in the absence (□) or presence (◆) of T_3 (6.5 ng/ml). On day 14, actinomycin D (10 μg/ml) was added to the culture medium and at indicated times total RNA was isolated from three independent cultures and the levels of MUC5AC (**A**), MUC5B (**B**) and IL-8 [actinomycin D control, (**C**)] mRNA species were determined by RT–PCR and quantified by densitometry. The results are expressed as mucin gene or *IL-8* expression (means ± S.E.M.) relative to zero time; the curves were determined by linear regression analysis. The results are representative of data obtained from three separate experiments.


Figure 5 Effect of T_3 and RA on the suppression of *MUC5AC* promoter activity

(**A**) NHTBE cells were transfected with a luciferase reporter vector containing the 1.3 kb 5′ flanking region of the human *MUC5AC* promoter and a *β-galactosidase* control construct. Luciferase activity was measured after 48 h of treatment (triplicate cultures per condition) in medium containing either 0.05 or 1 μM RA without T_3 (filled bars) or with T_3 (open bars). (**B**) NHTBE cells were transfected with different deletion constructs containing 1.3–1.02 kb of the 5′ flanking region of the *MUC5AC* promoter or vector alone and co-transfected with *β-galactosidase* construct. Luciferase activity was measured after 48 h of treatment (triplicate cultures per condition) in medium containing 0.05 μM RA without (filled bars) or with T_3 (open bars). Results are plotted as luciferase activity (light units) (means ± S.D.). The results represent data obtained from two independent experiments. **$P < 0.05$.

transcription lay within the region between 1.25 and 1.2 kb. *MUC5AC* transcriptional activity was suppressed by T_3 in cells transfected with the 1.3 and 1.25 kb constructs; however, no significant suppression was measured with the 1.2 and 1.02 kb constructs.

T_3 suppresses retinoid receptor expression

We next determined whether T_3 affects the expression of either RARs, RXRs or TRs. Nuclear extracts were prepared from NHTBE cells that had been cultured for 14 days in medium containing 50 nM RA without or with T_3 (6.5 ng/ml). The receptors were identified by Western blotting with antibodies directed against specific receptor isotypes. As seen in Figure 6, the levels of RAR α, β and γ and RXR α and γ proteins (51–54 kDa) were lower in cultures grown in the presence of T_3. In contrast, the expression of TRβ and β-actin protein was affected only marginally, if at all, by T_3. No TRα was detected (results not shown). These results indicate that T_3 suppressed retinoid receptor expression in NHTBE cells.

RARα   RXRα
-      +   -   +

RARβ   RXRγ
-      +   -   +

RARγ   TRβ
-      +   -   +

β-actin
-      +
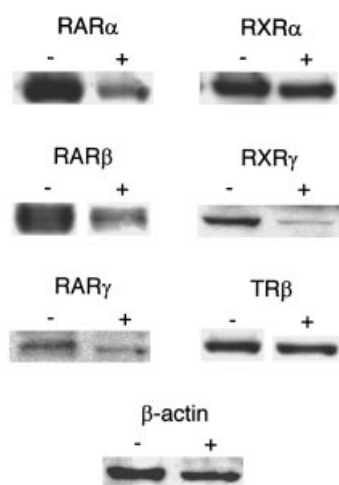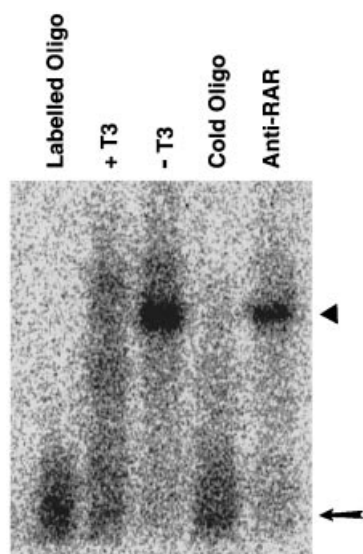
Figure 6   Effect of T₃ on RAR expression

Nuclear extracts were prepared from cultures grown for 14 days in medium containing 0.05 μM RA and either 0 or 6.5 ng/ml T$_3$. Nuclear lysates (50 μg) were separated by SDS/PAGE and the level of each receptor protein was examined with relevant specific antibodies. The results represent data obtained from two independent experiments.

T₃ reduces retinoid receptor binding to RARE

Previous studies showed that retinoid receptors, especially RARs, are involved in the regulation of *MUC5AC* and *MUC5B* expression [8]. To determine whether the decrease in RAR proteins caused by T$_3$ (see Figure 6) also resulted in decreased

Figure 7   Effect of T₃ on RAR–RARE complex formation

Nuclear extracts (50 μg) obtained from the same experiment as that described in the legend to Figure 6 were incubated with ^{32}P-labelled consensus, RARE double-stranded oligonucleotides (5×10^4 c.p.m. per reaction). RXR–RAR/RARE complexes were separated by non-denaturing PAGE and the bands were detected by densitometry on a PhosphorImager. Lane 1, only labelled oligonucleotide; lane 2, nuclear extract obtained from cultures grown in the presence of T$_3$; lane 3, nuclear extract obtained from cultures grown in the absence of T$_3$; lane 4, as lane 3 but also containing excess unlabelled ('cold') oligonucleotide; lane 5, as lane 3 but also containing anti-RARα and anti-RARβ antibodies (5 μg of each antibody). The filled triangle indicates RAR–RARE complexes; the arrow indicates free probe.

retinoid receptor binding to RARE, nuclear extracts obtained from cultures grown for 14 days in the absence or presence of T$_3$ (6.5 ng/ml) were incubated with a consensus ^{32}P-labelled RARE oligonucleotide. As seen in Figure 7, nuclear extracts from cultures grown without T$_3$ in the culture medium (lane 3) had much higher levels of retinoid receptor–RARE complexes than cultures grown in the presence of T$_3$ (lane 2). The specificity of the reaction was confirmed in lanes 4 and 5 in in Figure 7: the addition of excess unlabelled oligonucleotide decreased the level of the signal (compare lane 4 with lane 3) and the addition of anti-RARα/anti-RARβ specific antibodies (the epitope is the DNA-binding domain of the receptor) also resulted in a significant decrease in the level of complex (compare lane 5 with lane 3). These results indicated that the decrease in the levels of retinoid receptor proteins by T$_3$ resulted in decreased formation of receptor–response element complexes.

DISCUSSION

The present studies confirm and extend our previous findings [9], which showed that T$_3$ markedly decreased MUC5AC mRNA expression and mucus secretion by cultured NHTBE cells. We now show that, in contrast with MUC5AC mRNA, MUC5B mRNA levels are affected only marginally by T$_3$, suggesting important differences in the regulation of these two mucin genes. For *MUC5B*, a suppressive effect of T$_3$ was demonstrated only if the cultures were maintained in low RA concentrations known to be suboptimal for mucin gene expression (see below). The sequence for the *MUC5B* 5′ flanking region of the transcription start site was published [27] as this manuscript was being prepared. Additional studies will be required to determine critical features in the transcriptional machinery responsible for differences in the regulation of *MUC5AC* and *MUC5B* expression.

We recently demonstrated that NHTBE cultures secrete both MUC5AC and MUC5B, but little if any MUC2 [28]. For that reason, and because MUC5AC and MUC5B have been shown to be important airway mucins in humans, we limited the present studies to the investigation of these two mucins. We found, by using antibodies specific for MUC5AC and MUC5B, that the level of secreted MUC5AC was decreased by approx. 50–60 % in T$_3$-treated cultures; in contrast, secreted MUC5B levels were not affected by treatment with T$_3$.

RA and T$_3$ regulate the transcriptional activity of a multitude of genes through a closely related family of nuclear receptors [29–31]. It is well established that the signalling pathways of the two hormones and their receptors can interact at various levels [20,32–34]. As a first step in examining the interaction of RA and T$_3$ in the regulation of mucin gene expression we conducted RA/T$_3$ concentration–response studies. The results showed that the lower the RA concentration the less T$_3$ was required to suppress MUC5AC mRNA levels. The experiments showed further that at the lowest RA concentration studied (5 nM) as little as 6.5 pg/ml T$_3$ significantly suppressed MUC5B mRNA levels, indicating that MUC5B is also subject to regulation by T$_3$; however, this was demonstrated only at low, non-physiological, retinoid concentrations. These studies clearly indicated the interaction of RA and T$_3$ in regulating MUC5AC mRNA levels and, to a much smaller degree, MUC5B mRNA levels.

The suppression of *MUC5AC* expression was shown to be rapid and clearly time-dependent. The first statistically significant suppression of MUC5AC mRNA occurred after 6 h of treatment with T$_3$ (in cultures maintained in 50 nM RA) and continued to increase until the end of the experiment at 48 h. In contrast, MUC5B mRNA levels were decreased only marginally by treatment with T$_3$, confirming previous observations suggesting

that the two mucin genes are differentially regulated [8]. The rapidity with which T_3 suppressed MUC5AC mRNA levels raised the possibility that T_3 acts by down-regulating mucin gene transcription (see below).

Previous studies indicate that T_3 decreases the mRNA stability of some target genes [26]. We therefore examined the half-lives of MUC5AC and MUC5B mRNA during treatment with T_3. In accordance with a previous paper [35] we found that the half-lives of these mucin mRNA species are long (between 21 and 23 h) and that treatment with T_3 had no measurable effect on the stability of either mRNA, supporting the notion that T_3 might decrease mucin gene transcription.

The thyroid hormone receptor has been shown to activate or repress transcription in either a ligand-dependent or a ligand-independent manner, depending on the target gene in question [20]. Tomic et al. [21] demonstrated that T_3 suppresses the expression of several keratin genes in epidermal keratinocytes by binding to negative response elements ('nTRE') in the promoter region. We showed that the treatment with T_3 of NHTBE cells transfected with a luciferase reporter construct of the *MUC5AC* promoter suppressed *MUC5AC* promoter activity, indicating that T_3 suppresses *MUC5AC* transcription. By using a series of deletion constructs we found that, in the absence of T_3, loss of the region between 1.25 and 1.2 kb led to a 3–4-fold decrease in *MUC5AC* transcriptional activity, indicating that within this region lies a critical element regulating *MUC5AC* transcription. T_3 suppressed *MUC5AC* transcription in cells transfected with the 1.3 and 1.25 kb constructs but not in cells transfected with the 1.2 kb and 1.02 kb constructs. We interpret these findings to indicate that, in the *MUC5AC* promoter, the elements responsible for high levels of transcriptional activity and those responsible for the suppression of *MUC5AC* transcription by T_3 are close to each other. However, we cannot completely rule out the possibility that the transcriptional activity of the 1.2 and 1.02 kb constructs was too low in the absence of T_3 for the reliable detection of any suppressive effect of T_3 on transcription. Interestingly, there is neither a putative TRE nor a RARE located within the 50 bp region between 1.2 and 1.25 kb [36]. Gel-shift assays and site-directed mutagenesis experiments will be required for identifying the regulatory elements involved in the basal regulation and T_3-mediated suppression of *MUC5AC*.

Because the ligand binding of T_3 receptors can either activate or repress the transcription of target genes [18,20,21], we considered the possibility that T_3 might decrease the levels of RARs, which are critical for mucin gene expression in NHTBE cells [8]. It has been shown in rats that thyroid hormone up-regulates RXR-β and down-regulates RXR-γ [37,38]. We found that treatment with T_3 markedly decreased the levels of RAR-α, RAR-β, RXR-α and RXR-γ proteins in NHTBE cells. In addition, gel-shift assays indicated that ligand-activated retinoid receptor–RARE complex formation was markedly decreased by T_3, presumably because of the lowered retinoid receptor protein levels. Because mucin gene expression in NHTBE cells is highly dependent on retinoid receptors, these findings suggest that one of the mechanisms leading to the T_3-induced suppression of mucin gene expression is via the decrease in nuclear retinoid receptors.

Several key findings emerge from our studies. First, *MUC5AC* and *MUC5B* are differentially regulated by T_3. Under normal culture conditions (i.e. 50 nM RA and 6.5 ng/ml T_3), *MUC5AC* is far more responsive to T_3 suppression than *MUC5B*. Secondly, the suppression of *MUC5AC* expression by T_3 is not mediated via a change in mRNA stability; rather, T_3 directly or indirectly regulates *MUC5AC* transcription, as indicated by transfection assays with *MUC5AC* promoter constructs. Thirdly, we showed

that, in NHTBE cells, T_3 suppresses retinoid receptor levels and RXR–RAR/RARE complex formation, which explains, at least in part, the decrease in *MUC5AC* transcription.

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