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# Induction of MUC2 and MUC5AC Mucins by Factors of the Epidermal Growth Factor (EGF) Family Is Mediated by EGF Receptor/Ras/Raf/Extracellular Signal-regulated Kinase Cascade and Sp1\*

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The 11p15 mucin genes (*MUC2*, *MUC5AC*, *MUC5B* and *MUC6*) possess a cell-specific pattern of expression in normal lung that is altered during carcinogenesis. Growth factors of the epidermal growth factor family are known to target key genes that in turn may affect the homeostasis of lung mucosae. Our aim was to study the regulation of the 11p15 mucin genes both at the promoter and protein levels to assess whether their altered expression may represent a key event during lung carcinogenesis. Studies were performed in the mucoepidermoid NCI-H292 lung cancer cell line. Cell treatment with epidermal growth factor (EGF), transforming growth factor  $\alpha$  (TGF- $\alpha$ ), or tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) resulted in a dramatic increase of *MUC2* and *MUC5AC* mRNAs levels, promoter activity, and apomucin expression, whereas those of *MUC5B* and *MUC6* were unchanged. pGL3 deletion mutants of *MUC2*, *MUC5AC*, and *MUC5B* promoters were constructed and used in transient transfection assays to characterize EGF- and TGF- $\alpha$ -responsive regulatory regions within the promoters. They were located in the -2627/-2097 and -202/-1 regions of *MUC2* and *MUC5AC* promoters, respectively. Finally, we demonstrate that transcription factor Sp1 not only binds and activates *MUC2* and *MUC5AC* promoters but also participates to their EGF- and TGF- $\alpha$ -mediated up-regulation. We also show that Sp3 is a strong inhibitor of 11p15 mucin gene transcription. In conclusion, *MUC2* and *MUC5AC* are two target genes of EGFR ligands in lung cancer cells, and up-regulation of these two genes goes through concomitant activation of the EGFR/Ras/Raf/Extracellular Signal-regulated Kinase-signaling pathway and Sp1 binding to their promoters.

Mucins have been postulated to be important molecules in maintaining epithelium homeostasis in inflammatory diseases and cancer in that they are large O-glycoproteins expressed either at the cell surface or as secreted molecules to form a

protective gel. Mucin genes *MUC2*, *MUC5AC*, *MUC5B*, and *MUC6* are clustered on the p15 arm of chromosome 11 (1) and encode large secreted O-glycoproteins that participate in mucus formation and epithelium protection (2, 3). However, their precise biological role as key genes during sequential steps of lung carcinogenesis has yet to be proven.

In the surface epithelium of the respiratory tract, *MUC5AC* is exclusively expressed in mucus-secreting goblet cells, whereas *MUC1* and *MUC4* are expressed in all epithelial cells. *MUC5B* is prominent in the mucous cells of the submucosal glands, and *MUC2* is weakly expressed in both cell types (4–6). In epithelial lung diseases this pattern of expression is altered and has been correlated to poor prognosis of the tumor (invasiveness, metastasis) (7). For example, aberrant expression of *MUC6* in bronchiopulmonary adenocarcinoma, loss of expression of *MUC5AC* and *MUC5B* in epidermoid carcinoma, and a strong expression of the four 11p15 mucin genes in bronchioalveolar carcinoma was recently described (5).

Growth factors are thought to be involved in goblet cell production because hypersecretory diseases are associated with abnormal epithelial cell growth and differentiation, and epithelium wounding leads to repair and remodeling processes (8). More recently, Nadel and co-workers (9, 10) focused on *MUC5AC* regulation by epidermal growth factor (EGF)<sup>1</sup> in the lung. They showed that *MUC5AC* mRNA expression was increased after ligand binding to the EGFR and activation of the mitogen-activated protein kinase cascade. Positive correlation between EGFR and *MUC5AC* expression and localization in goblet cells of human bronchi is also in favor of the activation of a series of events involving EGFR followed by activation of *MUC5AC* expression in lung cells (11). However, these studies only focused on *MUC5AC*, whereas it has been shown by our laboratory that the expression of the other 11p15 mucin genes is also impaired in lung diseases (5, 6). Moreover, despite these promising studies, no data regarding the molecular mechanisms responsible for mucin gene regulation at the promoter level are available. Their identification remains a big challenge but is mandatory if one wants to demonstrate whether mucins are indeed key molecules in carcinogenesis.

At this time, promoter structures of *MUC2* (12, 13),

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<sup>1</sup> The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular signal-regulated kinase; RT, reverse transcriptase; TGF- $\alpha$ , transforming growth factor- $\alpha$ ; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kb, kilobases.

TABLE I

Sequences of the pairs of oligonucleotides used in PCR to produce deletion mutants covering *MUC2* and *MUC5AC* 5'-flanking regions

*SacI* (GAGCTC) and *MluI* (ACGCGT) sites are added at the end of the primers to direct subcloning and are italicized and underlined. Positions of the DNA fragments relative to the published transcription initiation sites (Refs. 12–14) are indicated. S, sense; AS, antisense.

pGL3 deletion mutant	Position of the DNA fragment	Orientation	Oligonucleotide used for PCR (5' → 3')
1824	-947/-1	S	CGC <u>ACG</u> <u>CGT</u> GCC CAG TGG TCT CGT
<i>MUC2</i>		AS	CGC <u>ACG</u> <u>CGT</u> CGC CCG GGG GCA GGG
2214	-2096/+27	S	CGC <u>GAG</u> <u>CTC</u> GCA ATA CGG CTG CCC ACT
<i>MUC2</i>		AS	CGC <u>GAG</u> <u>CTC</u> GGT GGC TGG CAG GGG CGG
1852	-2627/-1	S	CGC <u>GAG</u> <u>CTC</u> CTG GTG AGT CAG GTT
<i>MUC2</i>		AS	CGC <u>GAG</u> <u>CTC</u> CGC CCG GGG GCA GGG GTC
2113	-202/-1	S	CGC <u>GAG</u> <u>CTC</u> TGG GGG GGC CCT CGG AAA
<i>MUC5AC</i>		AS	CGC <u>GAG</u> <u>CTC</u> GAG GGA CCC AAG GTG GCA
2189	-486/-1	S	CGC <u>GAG</u> <u>CTC</u> CAC CCT CCC AGG CAG CCA
<i>MUC5AC</i>		AS	CGC <u>GAG</u> <u>CTC</u> GAG GGA CCC AAG GTG GCA
2144	-1366/-1	S	CGC <u>GAG</u> <u>CTC</u> CTA GGC ACC TGG GCT AAG
<i>MUC5AC</i>		AS	CGC <u>GAG</u> <u>CTC</u> GAG GGA CCC AAG GTG GCA

*MUC5AC* (14) and *MUC5B* (15, 16) are known. All of them are flanked by TATA boxes, and upstream *cis*-elements that confer cell-specific expression to the genes were described. *MUC2* promoter is GC-rich over a very long stretch (4 kb) and, as expected, was shown to be regulated by ubiquitous transcription factor Sp1 (12, 17). *MUC5B* promoter is characterized by two transcription units (15, 16). The proximal unit is also regulated by Sp1/Sp3 factors, whereas the distal unit is under the regulation of ATF/CREB transcription factors. The promoter of *MUC5AC* is only known over 1.4 kb (14). It is characterized by putative binding sites for Sp1, AP-2, and NF- $\kappa$ B transcription factors and key *cis*-elements, yet to be described, operate within 4 kb upstream of the transcription start site (14).

Our aim in the laboratory is to better understand transcriptional regulation of 11p15 mucin genes to propose new therapeutic targets in epithelial diseases (inflammation and cancer) (18). In this study, regulation of the four 11p15 mucin genes *MUC2*, *MUC5AC*, *MUC5B*, and *MUC6* was studied for the first time simultaneously at the mRNA, promoter, and protein levels. We focused on EGF- and TGF- $\alpha$ -mediated regulation of these genes due to the importance of these growth factors both during lung development (19) and carcinogenesis (8). Moreover, because it was previously shown for other genes, such as gastrin (20) or apolipoprotein A-I (21), that Sp1 may participate to EGF-mediated up-regulation of the target gene, we looked at the Sp1 effect on mucin gene regulation. We demonstrate that among the four 11p15 mucin genes only *MUC2* and *MUC5AC* are regulated at the transcriptional (promoter activity and mRNA levels) and protein levels by EGF and TGF- $\alpha$  and that the activated signaling pathway goes through Ras/Raf/ERK kinases. Finally, we show that Sp1 not only transactivates *MUC2* and *MUC5AC* transcription but also participates in their up-regulation by EGF and TGF- $\alpha$ .

#### MATERIALS AND METHODS

**Cell Culture**—Human pulmonary mucoepidermoid carcinoma cell line NCI-H292 was cultured at 37 °C in a humidified 5% CO<sub>2</sub> water-jacketed incubator in RPMI 1640 medium containing 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100  $\mu$ g/ml), Hepes (25 mM), and sodium pyruvate (1 mM). At confluence, cells were incubated for 24 h with EGF (recombinant human EGF, 25 ng/ml), TGF- $\alpha$  (recombinant human TGF- $\alpha$ , 20 ng/ml), TNF- $\alpha$  (recombinant human TNF- $\alpha$ , 40 ng/ml), or EGF plus TNF- $\alpha$  or TGF- $\alpha$  plus TNF- $\alpha$ . In inhibition studies, cells were pretreated for 30 min with tyrphostin AG1478 (10  $\mu$ M, Calbiochem), radicicol (10  $\mu$ M), PD98059 (30  $\mu$ M), or U0126 (10  $\mu$ M, Promega) before adding the growth factors of interest. To inhibit protein synthesis or transcription, cells were pretreated for 30 min with cycloheximide (20  $\mu$ g/ml) or actinomycin D (1  $\mu$ g/ml, Roche Molecular Biochemicals) before adding the growth factors. Inhibition of Sp1 binding was carried out by preincubating cells for 24 h with mithramycin A (100 nM) before adding the growth factors. All reagents were from Sigma unless otherwise indicated.

**Immunohistochemistry**—Confluent cells were trypsinized, centrifuged, washed once with 1 $\times$  phosphate-buffered saline before fixation of the pellet in 4% formaldehyde, and embedding in paraffin. 3- $\mu$ m sections were prepared, and morphological analysis was done after hematoxylin-eosin-safran/Astra blue staining. Pretreatment and immunostaining of the sections were performed as described before (22). Polyclonal anti-MUC6, anti-MUC2, anti-MUC5AC, and anti-MUC5B antibodies were a kind gift from Dr. Carlstedt (University of Lund, Lund, Sweden) (23–26). Anti-CK7 antibody (clone OV-TL 12/30) was purchased from Dako.

**Plasmid Constructions**—The *MUC5B*-pGL3 deletion mutants (2140, 1599, 1596, 1896) covering 2044 nucleotides upstream of the first ATG were previously described in Van Seuning et al. (15) and Perrais et al. (16). *MUC5AC* promoter was obtained by running PCRs on human genomic DNA using the following pair of oligonucleotides: forward primer, 5'-CTA GGC ACC TGG GCT AAG ACA G-3'; and reverse primer, 5'-CTT CCT CCG GCC AAC ACT CAT TGT GTG GAC-3'. PCR conditions with *Taq* polymerase (2 units, Roche Molecular Biochemicals) and 20 ng of DNA were 4 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 64 °C, 2 min at 72 °C and then 2 min at 72 °C. The PCR product obtained in these conditions covers 1.36 kb of the sequence published by Li et al. (14). *MUC2* promoter was obtained by running PCR on the cosmid clone ELO19 isolated from a pWE15 library previously used in the laboratory to isolate the *MUC5AC* gene (27). PCR on ELO19 was performed with 20 ng of DNA and with forward primer, 5'-GGG AGC TCC TGG TGA GTC AGG TTT GTG AT-3', and reverse primer, 5'-GGA CGC GTC AGC CCC ATG GTG GCT GGC AG-3'. PCR was carried out with 2.5 units of *Taq* polymerase (Qiagen) without 5 $\times$  Q buffer in the reaction mixture as follows: 15 min at 95 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 58 °C, and 2 min at 72 °C and then 15 min at 72 °C. The PCR product obtained covers 2.8 kb of the sequence published by Gum et al. (12). Both PCR products were then subcloned into pCR2.1 vector (Invitrogen) before subcloning into *SacI*-*MluI* sites of the promoterless pGL3 Basic vector (Promega). Internal deletion mutants (*MUC2* (1852, 2214, and 1824) and *MUC5AC* (2144, 2189, and 2113)) were generated by PCR using pairs of primers bearing specific restriction sites at their 5' and 3' ends (Table I). PCR products were digested, gel-purified (Qiaquick gel extraction kit, Qiagen), and subcloned into the pGL3 Basic vector (Promega) that had been previously cut with the same restriction enzymes. All clones were sequenced on both strands on an automatic LI-COR sequencer (ScienceTec, Les Ulis, France) using infrared-labeled RV3 and GL2 primers (Promega). Plasmids used for transfection studies were prepared using the Endofree plasmid Mega kit (Qiagen).

**RT-PCR**—Total RNAs from NCI-H292 cells were prepared using the QIAamp RNA blood mini-kit from Qiagen. Total RNA (1.5  $\mu$ g) was used to prepare cDNA (Advantage™ RT-for-PCR kit, CLONTECH). PCR was performed on 5  $\mu$ l of cDNA using specific pairs of primers as previously described (15). Primers are: *MUC2* forward primer (5'-CTGCACCAAGACCGTCCTCATG-3') and *MUC2* reverse primer (5'-GCAAGGACTGAACAAAGACTCAGAC-3') (nucleotide positions 15291–15312 and 15667–15669, respectively accession number L21998) (28); *MUC5AC* forward primer (5'-TGATCATCCAGCAGGCT-3') and *MUC5AC* reverse primer (5'-CCGAGCTCAGAGGACATATGGG-3') (nucleotide positions 2897–2917 and 3284–3305, respectively, accession number AJ001402) (27); *MUC5B* forward primer (5'-CTGCGA-GACCGAGGTCAACATC-3') and *MUC5B* reverse primer (5'-TGGGC-

TABLE II  
Sequences of the sense oligonucleotides used for gel shift assay experiments

Antisense oligonucleotides were also synthesized and annealed to the sense oligonucleotides to produce double-stranded DNA. Positions of the DNA fragments relative to the published transcription start sites (Refs. 12–14) are indicated.

Oligonucleotide name (sense orientation)	Sequence (5' → 3')
T43 (Spl site), -87/-73	CACAGGCCCGCCCT
T44 (CACCC box), -74/-57	CTGCCACCCACGTGAAG
T46 (Spl site), -231/-211	ATGTGGGGAGGACCCTGCAG

AGCAGGAGCACGGAG-3') (nucleotide positions 9057–9078 and 10108–10127, respectively, accession number Y09788) (29); *MUC6* forward primer (5'-GCATGGCGAACGTGACGGTAA-3') and *MUC6* reverse primer (5'-TAGTCTGAGCCCTGCTTGGCA-3') (nucleotide positions 1034–1054 and 1433–1454, respectively, accession number U97698) (30). GAPDH was used as an internal control. *MUC2*, *MUC5AC*, *MUC5B*, *MUC6*, and *GAPDH* PCR product sizes are 401, 409, 415, 421, and 980 base pairs, respectively.

**Transfections**—Transfections and co-transfections were performed using Effectene<sup>®</sup> reagent (Qiagen), and luciferase activities were measured as previously described (15, 16). Total cell extracts were prepared after a 48-h incubation at 37 °C using 1× reagent lysis buffer (Promega) as described in the manufacturer's instruction manual. Total protein content in the extract (4  $\mu$ l) was measured using the bicinchoninic acid method in 96-well plates as described in the manufacturer's instruction manual (Pierce). Relative luciferase activity was expressed per  $\mu$ g of protein. Each plasmid was assayed in triplicate in at least three separate experiments.

**Nuclear Extract Preparation**—Nuclear extracts from NCI-H292 cells were prepared as described by Van Seuning *et al.* (31) and kept at -80 °C until use. Protein content (2  $\mu$ l of cell extracts) was measured using the bicinchoninic acid as described above.

**Oligonucleotides and DNA Probes**—The sequences of the oligonucleotides used for gel shift assays are indicated in Table II. They were synthesized by MWG-Biotech (Germany). Equimolar amounts of single-stranded oligonucleotides were annealed and radiolabeled using T4 polynucleotide kinase (Promega) and [ $\gamma$ -<sup>32</sup>P]dATP. Radiolabeled probes were purified by chromatography on a Bio-Gel P-6 column (Bio-Rad, Ivry-sur-Seine, France).

**Electrophoretic Mobility Shift Assays**—Nuclear proteins (5  $\mu$ g) were preincubated for 20 min on ice in 20  $\mu$ l of binding buffer with 2  $\mu$ g of poly(dI-dC) (Sigma) and 1  $\mu$ g of sonicated salmon sperm DNA. Radiolabeled DNA probe was added (120,000 cpm), and the reaction was left for another 20 min on ice. For supershift analyses, 1  $\mu$ l of the antibody of interest (anti-Sp1, anti-Sp3, Santa-Cruz Laboratories, Tebu, France) was added to the proteins and left for 1 h on ice before adding the radiolabeled probe. Reactions were stopped by adding 2  $\mu$ l of loading buffer. Samples were loaded onto a 4% non-denaturing polyacrylamide gel, and electrophoresis conditions were as described in Van Seuning *et al.* (15). Gels were vacuum-dried and autoradiographed overnight at -80 °C.

## RESULTS

**Expression of 11p15 Mucins in NCI-H292 Cells**—Expression of 11p15 mucin genes was studied by RT-PCR. As shown in Fig. 1, NCI-H292 cells express *MUC2*, *MUC5AC*, and *MUC5B* mRNAs, whereas *MUC6* mRNA is not detected (lane 1). Because NCI-H292 cells are described as a mucoepidermoid cell line, we looked at CK7 and mucin expression by immunohistochemistry using specific antibodies (Fig. 2). CK7, a marker characteristic of a glandular phenotype, is expressed in 100% of the cells (Fig. 2B). *MUC2* (Fig. 2C) and *MUC5AC* (Fig. 2D) apomucins are expressed in 20 and 2–5% of the cells, respectively. *MUC5B* and *MUC6* apomucins were not detected in these cells (not shown). In Alcian blue-stained cells, a few grains of secretion could be detected (Fig. 2A). In conclusion, mucoepidermoid NCI-H292 cells express *MUC2*, *MUC5AC*, and *MUC5B* at the mRNA level, but only *MUC2* and *MUC5AC* apomucins are translated and expressed in the cells.

**EGFR Ligands Stimulate *MUC2* and *MUC5AC* Expression in NCI-H292 Cells**—We then undertook to look at the effect of

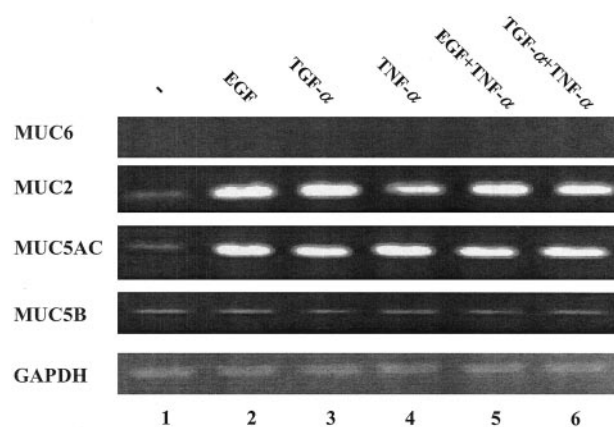


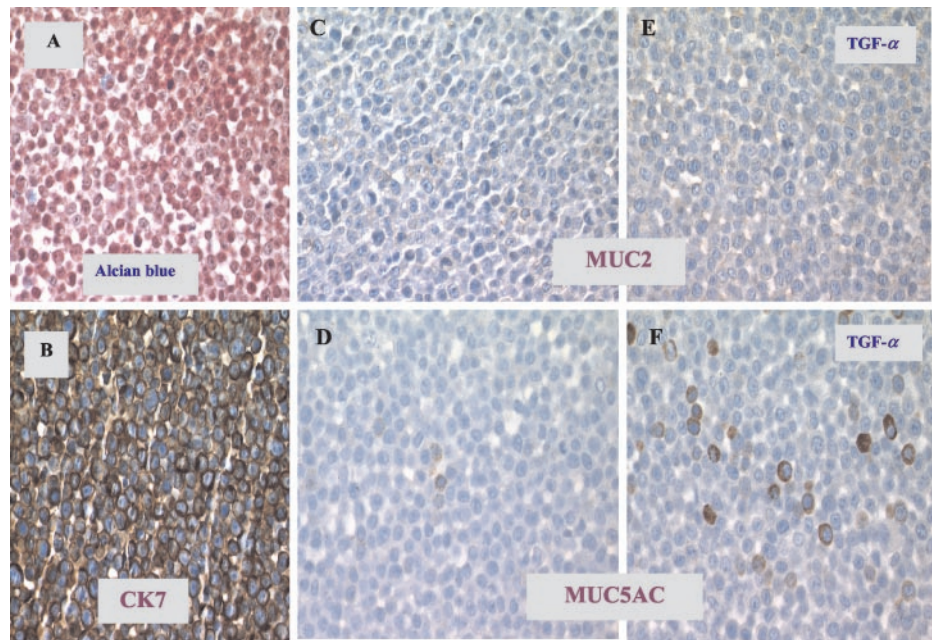
FIG. 1. Study of the expression pattern of 11p15 mucin genes in NCI-H292 cells and effects of EGF, TGF- $\alpha$ , and TNF- $\alpha$  on their expression by RT-PCR. Lane 1, untreated cells. Cells were treated for 24 h with EGF (25 ng/ml (lane 2)), TGF- $\alpha$  (20 ng/ml (lane 3)), TNF- $\alpha$  (40 ng/ml (lane 4)), EGF plus TNF- $\alpha$  (lane 5), and TGF- $\alpha$  plus TNF- $\alpha$  (lane 6). GAPDH was used as an internal control. PCR products were separated on a 2% agarose gel run in 1× Tris-buffered EDTA buffer in the presence of ethidium bromide.

EGF and TGF- $\alpha$  on mucin expression both at the mRNA and protein levels. By RT-PCR (Fig. 1), we show that treatment of the cells with EGF (lane 2) or TGF- $\alpha$  (lane 3) results in a dramatic increase of *MUC2* and *MUC5AC* mRNA levels. Because EGFR is known to be up-regulated by the pro-inflammatory cytokine TNF- $\alpha$ , which is increased in hypersecretory diseases (8), the synergistic effect between TNF- $\alpha$  and EGF or TGF- $\alpha$  was tested. TNF- $\alpha$  up-regulates *MUC2* and *MUC5AC* expression level (lane 4), but no synergistic effect was observed between TNF- $\alpha$  and EGF (lane 5) or TGF- $\alpha$  (lane 6). The different treatments had no effect on *MUC5B* mRNA levels in these cells, and *MUC6*, which is not expressed in this cell line, was not induced either.

The up-regulation of *MUC2* and *MUC5AC* mRNA levels was confirmed at the protein level by immunohistochemistry. As shown in Fig. 2E, a dramatic increase in *MUC2* apomucin expression was observed as 90–100% of the TGF- $\alpha$ -treated cells express *MUC2* compared with 20% in untreated cells (Fig. 2C). *MUC5AC* expression increased to 30% (Fig. 2F) when only a few cells were stained in untreated cells (Fig. 2D). No staining was observed with anti-*MUC5B* and anti-*MUC6* antibodies (data not shown). The same results were obtained when cells were treated with EGF. We can, thus, conclude that *MUC2* and *MUC5AC* up-regulation by EGF and TGF- $\alpha$  in NCI-H292 cells most likely involve regulatory mechanisms at the transcriptional level.

**Identification of EGF- and TGF- $\alpha$ -responsive Regions within *MUC2* and *MUC5AC* Promoters**—To do that, we first undertook to identify the regulatory regions within the promoters that are responsive to EGF and TGF- $\alpha$ . For these studies, a panel of deletion mutants covering promoter regions of *MUC2*, *MUC5AC*, and *MUC5B* were constructed in promoterless pGL3 basic vector. Cells were then transfected with the different deletion mutants and treated with the growth factor of interest in the same conditions as in Fig. 1. Regarding *MUC2* promoter (Fig. 3A), EGF and TGF- $\alpha$  selectively increased luciferase activity of the longest fragment, which covers the -2627/-1 region of *MUC2* promoter (1852; 3- and 2.5-fold activation, respectively). No effect was seen on fragments covering the -2096/+27 (2214) and -947/-1 (1824) regions, indicating that the -2627/-2097 region of *MUC2* promoter is necessary to observe a response to EGF or TGF- $\alpha$ . Regarding *MUC5AC* promoter (Fig. 3B), EGF and TGF- $\alpha$  increased luciferase activity of the three fragments tested (2–3-fold activation). The

**FIG. 2. Study of the expression of MUC2 and MUC5AC apomucins in NCI-H292 cells and effect of TGF- $\alpha$  on their expression by immunohistochemistry.** Untreated cells (A–D) and TGF- $\alpha$ -treated cells (20 ng/ml, 24 h) (E and F) are shown. A, Alcian blue staining; B, CK7 expression; C and E, MUC2 expression; D and F, MUC5AC expression. Immunostaining protocol is described under “Materials and Methods.” Magnification  $\times 200$ .



region  $-202/-1$  is thus sufficient to get a response, but cooperative elements are present further upstream ( $-1366/-487$ ) since a significant increase is observed when using the longest deletion mutant (compare *black bars* with *gray bars*).

Having shown that treatment of NCI-H292 cells with TNF- $\alpha$  resulted in an increase of *MUC2* and *MUC5AC* mRNA levels (Fig. 1, *lane 4*), we also looked at its effect on their promoters. As shown in Figs. 3, A and B, TNF- $\alpha$  did not have any significant effect of *MUC2* and *MUC5AC* promoter activity. As expected, no synergistic effect between TNF- $\alpha$  and EGF or TGF- $\alpha$  was observed on either promoter, thus confirming RT-PCR results (see Fig. 1, *lanes 5* and *6*). Finally, the different treatments had no effect on the two promoters of *MUC5B* (proximal and distal, Fig. 3C), those results being in agreement with RT-PCR data (see Fig. 1). From these studies it can be concluded that EGF and TGF- $\alpha$  activate *MUC2* and *MUC5AC* promoter activity and that responsive elements are present in the  $-2627/-2097$  and  $-1366/-1$  regions of *MUC2* and *MUC5AC* promoters, respectively. In the experiments that follow we, thus, focused our work on the regulation of these two genes.

**EGF, TGF- $\alpha$ , and TNF- $\alpha$  Stimulate *MUC2* and *MUC5AC* Gene Expression at the Transcriptional Level**—To further identify the intracellular mechanisms used by EGFR ligands and TNF- $\alpha$  that lead to a higher expression of *MUC2* and *MUC5AC* in NCI-H292 cells, a 30-min pretreatment with actinomycin D, a transcriptional inhibitor, or cycloheximide, an inhibitor of protein synthesis, before exposure to EGF, TGF- $\alpha$ , or TNF- $\alpha$ , was applied. As shown in Fig. 4, no effect of actinomycin D and cycloheximide is seen on the basal level of *MUC2* or *MUC5AC* mRNAs in these cells (*lanes 1–3*). On the other hand, actinomycin D treatment completely blocked the EGF-, TGF- $\alpha$ -, and TNF- $\alpha$ -mediated up-regulation of *MUC2* and *MUC5AC* (*lanes 6, 9, and 12*). In the same conditions, cycloheximide had no effect (*lanes 5, 8, and 11*). Thus, these data indicate that EGF, TGF- $\alpha$ , and TNF- $\alpha$  increase *MUC2* and *MUC5AC* expression at the transcriptional level and that activation does not require *de novo* protein synthesis.

**EGFR Ligands Stimulate *MUC2* and *MUC5AC* via EGFR/Ras/Raf/ERK-signaling Cascade**—To determine which signaling pathway is induced by EGF or TGF- $\alpha$ , three pharmacological agents were used in inhibition studies. Tyrphostin AG1478 is a selective inhibitor of EGFR tyrosine kinase activity, radi-

cicol selectively depletes cells in Raf kinase (33), PD98059 is a mitogen-activating protein/ERK kinase 1 (MEK-1) inhibitor, and U0126 is an inhibitor of MEK-1 and MEK-2. As a control, cells were treated with these inhibitors with no further treatment. As shown in Fig. 5A, these inhibitors had no effect on the basal expression of *MUC2* and *MUC5AC* mRNAs (compare *lanes 2–4* to *lane 1*). On the contrary, pretreatment with tyrphostin AG1478 led to a complete inhibition of *MUC2* and *MUC5AC* gene up-regulation by EGF or TGF- $\alpha$  (compare *lanes 6* and *10* to *lanes 5* and *9*, respectively). Pretreatment with radicicol (*lanes 7* and *11*), PD98059 (*lanes 8* and *12*), or U0126 (data not shown) partially inhibited the response to EGFR ligands. Thus, these results indicate that EGF- and TGF- $\alpha$ -mediated up-regulation of *MUC2* and *MUC5AC* in NCI-H292 cells goes through a signaling pathway involving sequential activation of EGFR kinase, Ras/Raf, and ERK but that most likely another signaling pathway is involved since inhibition by radicicol, PD98059, and U0126 is only partial.

In functional studies, in which transfected cells were pretreated with tyrphostin AG1478 or radicicol in the same conditions, a total inhibition of the EGF- and TGF- $\alpha$ -mediated up-regulation of *MUC2* and *MUC5AC* deletion mutants was observed (Fig. 5B). These results indicate that the responsive elements to the activated EGFR/Ras/Raf/ERK cascade are present in the  $-2627/-1$  and  $-1366/-1$  regions of *MUC2* and *MUC5AC* promoters, respectively.

**Binding of Transcription Factors Sp1 and Sp3 to *MUC5AC* Promoter**—Transcription factors of the Sp family are important regulators of *MUC2* and *MUC5B* mucin gene expression (12, 15–18) and are known to interfere with EGF-mediated regulation of numerous genes (34). The high density of GC-rich boxes upstream of the transcriptional start site of *MUC5AC* is also in favor of such a regulatory role for Sp1 on that gene. However no data regarding regulation of *MUC5AC* by Sp1/Sp3 has been published so far. Electrophoretic mobility shift assays studies were thus carried out to identify new Sp1/Sp3 binding sites within *MUC5AC* promoter. Two shifted bands were visualized with probe T44 (Fig. 6, *lane 2*). This probe contains a CACCC box binding site located at  $-74/-57$  in *MUC5AC* promoter. Upon the addition of anti-Sp1 antibody (*lane 3*), no effect is seen, whereas the addition of anti-Sp3 antibody resulted in a decrease of the band intensities (*lane 4*). Thus, complexes 1 and 2 correspond to the two forms of Sp3 (70 and 105 kDa). T43

FIG. 3. Effect of EGF, TGF- $\alpha$ , and TNF- $\alpha$  on *MUC2*, *MUC5AC*, and *MUC5B*. A, pGL3 deletion mutant transcriptional activity in NCI-H292 cells is shown. Schematic representations and localization of the different pGL3-mutants covering 2627 nucleotides of *MUC2*, 1366 nucleotides of *MUC5AC* (B), and 2044 nucleotides of *MUC5B* (C) promoters with Sp1/Sp3 putative binding sites are shown on the left part of the figure. Luciferase diagrams are on the right-hand side. REF. refers to the normalized luciferase activity of the fragment without any treatment.

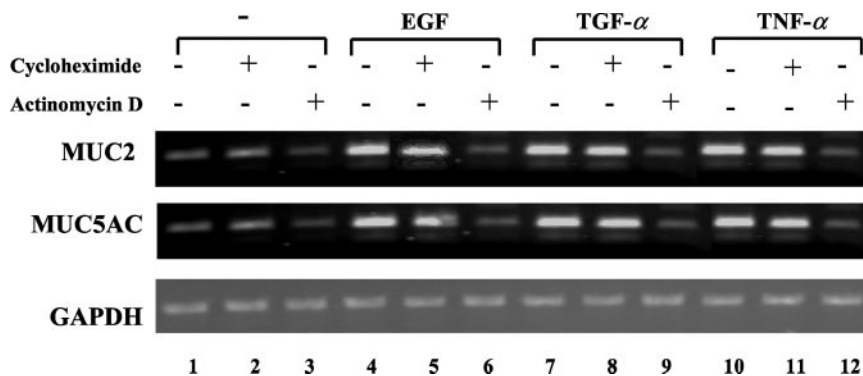
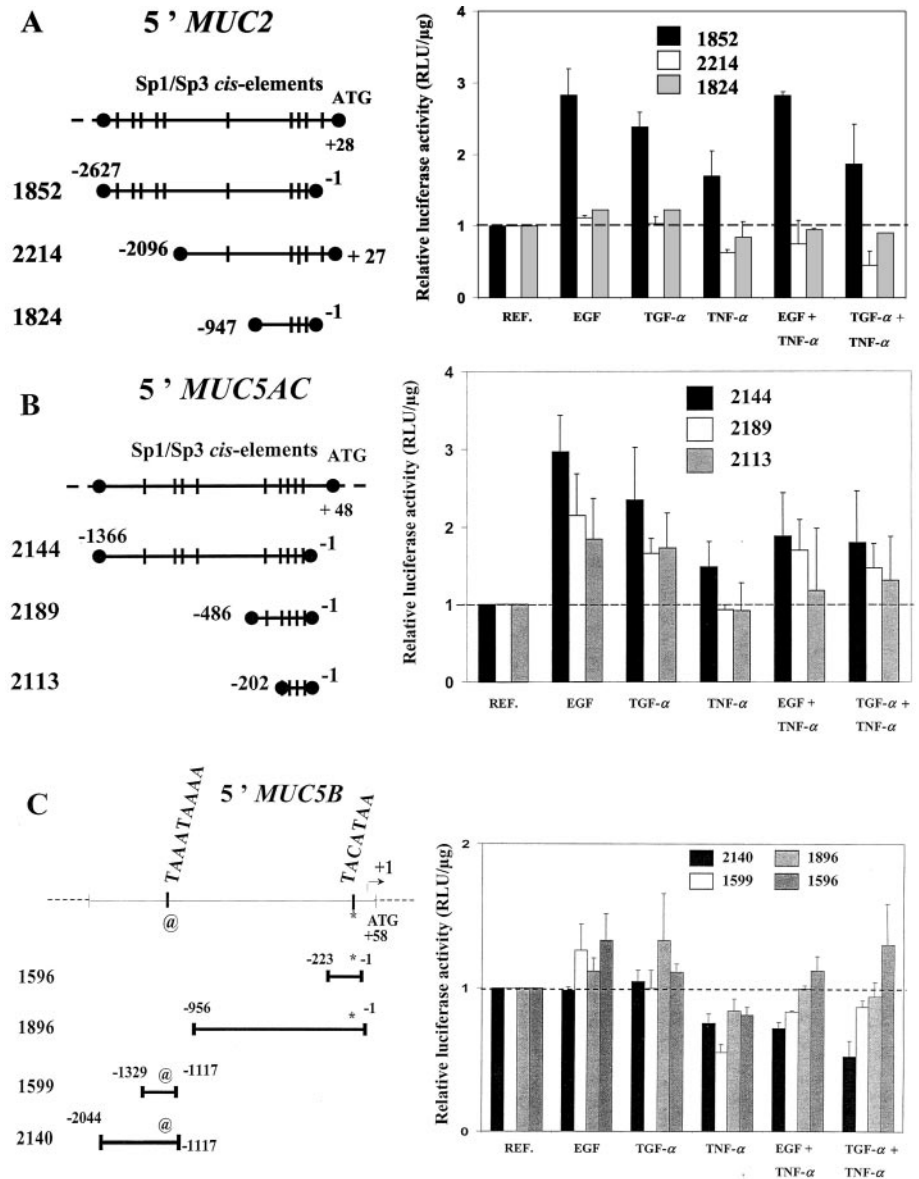
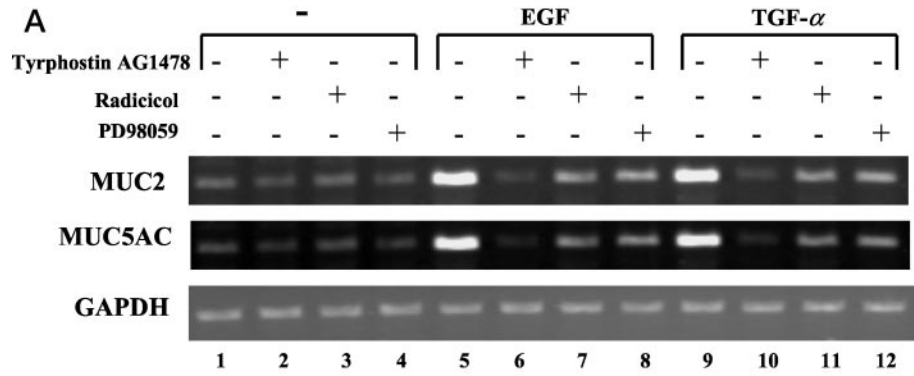


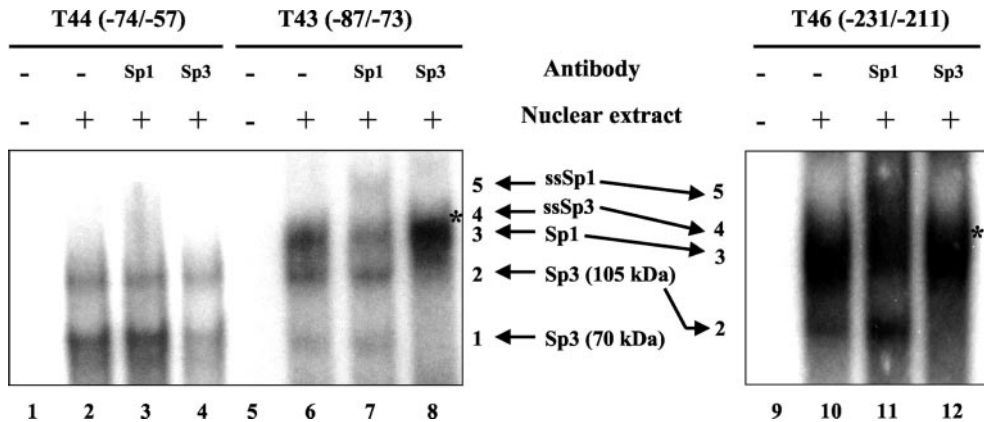
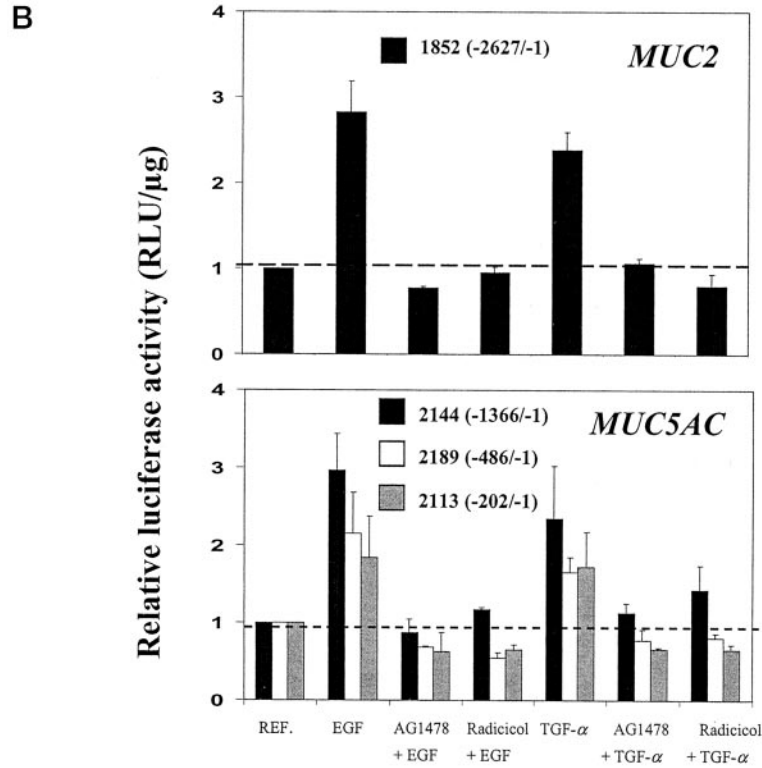
FIG. 4. Study of the effect of cycloheximide and actinomycin D on EGF-, TGF- $\alpha$ -, and TNF- $\alpha$ -mediated up-regulation of *MUC2* and *MUC5AC* in NCI-H292 cells by RT-PCR. Cells were pretreated with cycloheximide (20  $\mu$ g/ml) or actinomycin D (1  $\mu$ g/ml) for 30 min (lanes 1-3) before EGF (lanes 4-6), TGF- $\alpha$  (lanes 7-9), or TNF- $\alpha$  (lanes 10-12) treatment. GAPDH was used as an internal control. PCR products were separated on a 2% agarose gel run in 1 $\times$  Tris-buffered EDTA buffer in the presence of ethidium bromide.

covers the nucleotides -87/-73 of *MUC5AC* promoter and contains a Sp1/Sp3 putative binding site. When incubated with nuclear proteins, three shifted bands were visualized (lane 6; complexes 1, 2, and 3). Complex 3 corresponds to Sp1 binding since a supershift occurred upon the addition of the anti-Sp1 antibody in the mixture to produce complex 5 (lane 7). Com-

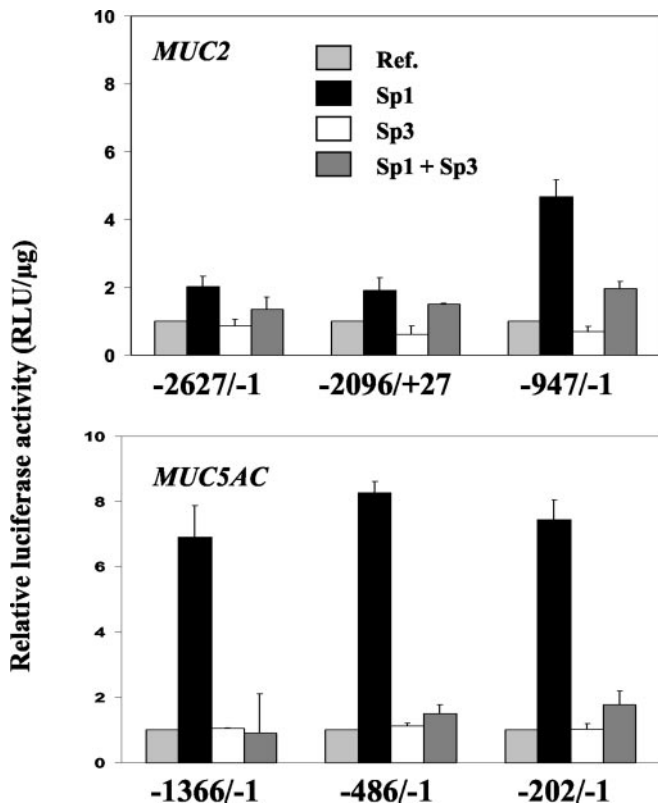
plexes 1 and 2 correspond to the two forms of Sp3 (lane 6), since these two bands were supershifted upon the addition of the anti-Sp3 antibody to produce complex 4 (lane 8). T46, which is representative of another Sp1/Sp3 binding site at -231/-211 in *MUC5AC* promoter, also binds the two transcription factors (lanes 9-12). Altogether, it can be stressed from these experi-



**FIG. 5. Effect of tyrphostin AG1478, radicalcol, and PD98059 on MUC2 and MUC5AC expression in NCI-H292 cells.** A, effects on mRNA levels were measured by RT-PCR. Cells were pre-treated with tyrphostin AG1478 (10  $\mu$ M), radicalcol (10  $\mu$ M), or PD98059 (30  $\mu$ M) for 30 min (lanes 1-4) before EGF (lanes 5-8) or TGF- $\alpha$  (lanes 9-12) treatment. GAPDH was used as an internal control. PCR products were separated on a 2% agarose gel run in 1 $\times$  Tris-buffered EDTA buffer in the presence of ethidium bromide. B, effects on promoter activity was studied by transient transfection assays in the same conditions. REF. refers to the normalized luciferase activity of the fragment without any treatment.



**FIG. 6. Identification of Sp1 and Sp3 cis-elements in MUC5AC promoter by electrophoretic mobility shift assays.** 5  $\mu$ g of nuclear extracts prepared from NCI-H292 cells were incubated with the radiolabeled DNA probes as indicated. Lanes 1-4, T44, Sp1-binding site at -74/-57; lanes 5-8, T43, CACCC box at -87/-73; lanes 9-12, T46, Sp1 binding site at -231/-211. Supershift experiments (ss-) were carried out by adding 1  $\mu$ l of the antibody of interest (lanes 3, 7, and 11, anti-Sp1; lanes 4, 8, and 12, anti-Sp3). Radiolabeled probe alone was loaded in the first lane of each series (lanes 1, 5, and 9). DNA-protein complexes with Sp1 (complex 3) and Sp3 (complexes 1 and 2) and supershifted complexes with anti-Sp1 (complex 5) and anti-Sp3 (complex 4) are indicated by arrows. An asterisk indicates the position of the supershift obtained with anti-Sp3 antibody (lanes 8 and 12), which is located just above the DNA-protein complex obtained with Sp1.



**FIG. 7. Regulation of the promoters of *MUC2* and *MUC5AC* by Sp1 and Sp3 transcription factors.** Co-transfection experiments were performed in the presence of 1  $\mu$ g of *MUC2* and *MUC5AC* pGL3 deletion mutants and 0.25  $\mu$ g of pCMV-Sp1 (black bars), pCMV-Sp3 (white bars), or 0.25  $\mu$ g of pCMV-Sp1 + 0.25  $\mu$ g of pCMV-Sp3 (dark gray bars) expression vectors. Ref. refers to the normalized luciferase activity of the pGL3 deletion mutants of interest co-transfected with the empty expression vector pCMV4 (gray bars).

ments that Sp1 and Sp3 engage with two and three cognate *cis*-elements within the first 300 bp of *MUC5AC* promoter, respectively.

**Role of Transcription Factors Sp1 and Sp3 on *MUC2* and *MUC5AC* Promoter Activity**—Knowing that Sp1 and Sp3 interact with their cognate *cis*-elements within *MUC2* (17) and *MUC5AC* (this report) promoters, co-transfection studies were then performed to study their biological effect on *MUC2* and *MUC5AC* transcription activity. pGL3-*MUC2* and pGL3-*MUC5AC* deletion mutants were transfected in the presence of expression vectors encoding either Sp1 (pCMV-Sp1) or Sp3 (pCMV-Sp3) (Fig. 7). Empty pCMV4 vector was used as the reference (gray bar). Co-transfection studies performed on *MUC2* indicate that Sp1 strongly transactivates the -947/-1 region of the promoter (black bars, 5-fold), whereas a weak effect is observed on the two other fragments tested (2-fold). Sp3 inhibits *MUC2* promoter activity (white bars, 25–50% inhibitory effect) and competes away Sp1 transactivating effect (dark gray bars). *MUC5AC* is strongly transactivated by Sp1 throughout the 1.4 kb of the promoter region (black bars, 6–8-fold induction), whereas Sp3 has no effect (white bars). As for *MUC2*, Sp3 competed away Sp1 positive regulatory effect on *MUC5AC* promoter activity (dark gray bars). From these studies, it can be concluded that ubiquitous transcription factors Sp1 and Sp3 are important regulators of *MUC2* and *MUC5AC* transcription with positive and negative regulatory effects, respectively.

**Sp1 Acts as a Mediator in EGF- and TGF- $\alpha$ -mediated Up-regulation of *MUC2* and *MUC5AC***—Having shown that Sp1 binds and transactivates *MUC2* and *MUC5AC* promoters, we

then undertook to study whether it mediates EGF/TGF- $\alpha$  activation of these two genes. To this end, we used mithramycin A, a drug that modifies GC-rich regions of the DNA and blocks Sp1 binding. As shown in Fig. 8A, inhibition of Sp1 binding by mithramycin A dramatically reduced *MUC2* mRNA levels in the cells, whereas the effect was not as pronounced on *MUC5AC* (compare lane 2 with lane 1). More interestingly, pretreatment of the cells with mithramycin A before EGF or TGF- $\alpha$  treatment significantly decreased the EGF (compare lane 4 with lane 3)- and TGF- $\alpha$  (compare lane 6 to lane 5)-mediated up-regulation of *MUC2* and *MUC5AC* mRNA levels. Again, the inhibitory effect was more pronounced on *MUC2*.

In functional studies, similar effects were observed on *MUC2* and *MUC5AC* basal transcriptional activities, thus confirming RT-PCR data. As shown in Fig. 8B, cells treated with mithramycin A had a much lower basal activity of *MUC2* promoter (fragment 1852, 50% inhibition). The effect on *MUC5AC* was much weaker and restricted to the first 202 nucleotides of the promoter (fragment 2113; 25% inhibition). The role of Sp1 as a mediating agent in TGF- $\alpha$ -mediated up-regulation of *MUC2* and *MUC5AC* promoters was also studied by means of transfections. The results shown in Fig. 8C confirm the data obtained by RT-PCR. Indeed, transfected cells pretreated with mithramycin A before TGF- $\alpha$  incubation resulted in a total loss of *MUC2* and *MUC5AC* promoter activity (black bars) when compared with TGF- $\alpha$  treated cells (white bars). The same results were obtained with EGF (not shown). Altogether these results demonstrate that Sp1 binding is essential for *MUC2* and to a lesser extent for *MUC5AC* basal expression and that it also participates to the EGF- and TGF- $\alpha$ -mediated up-regulation of these two genes.

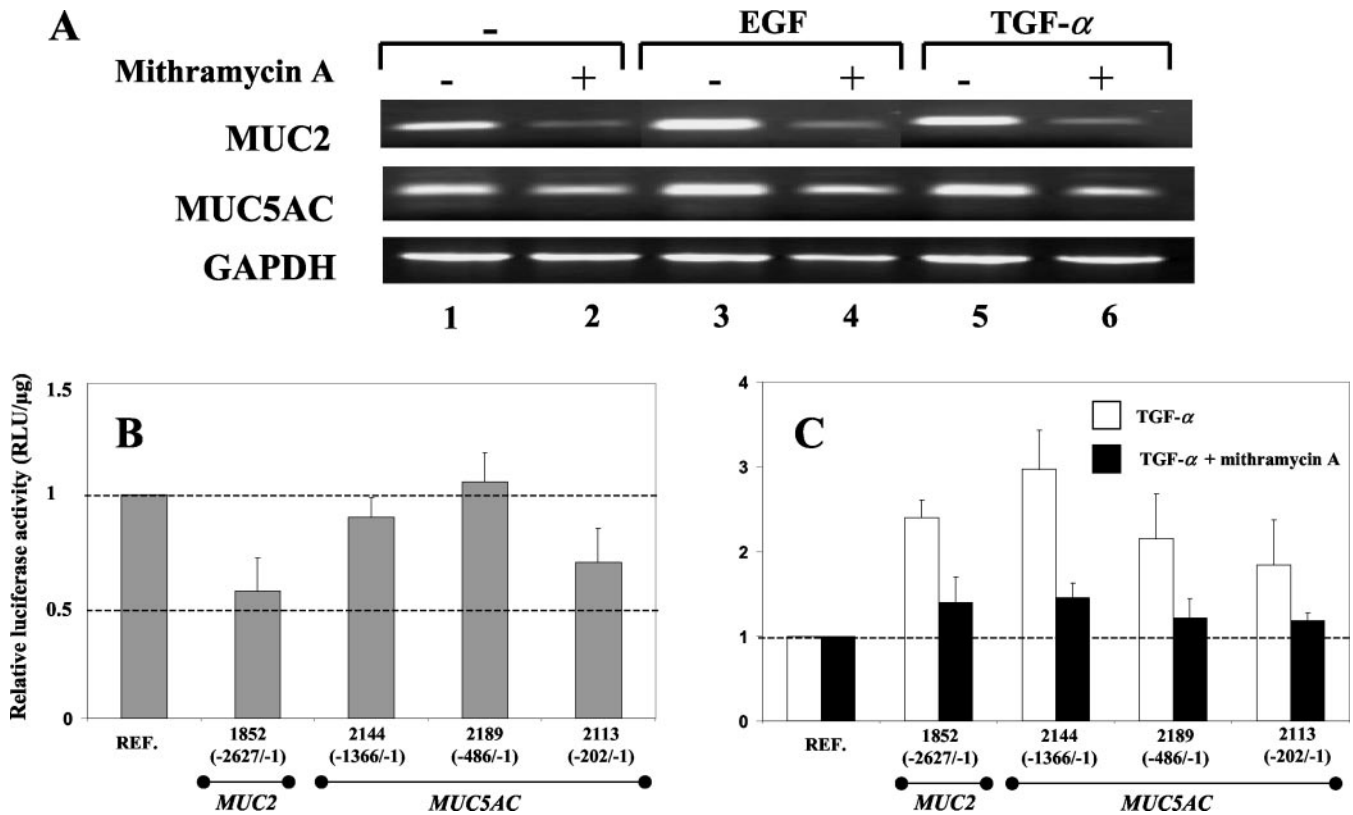
#### DISCUSSION

Organization, evolution, and regulation of the four 11p15 mucin genes, *MUC2*, *MUC5AC*, *MUC5B*, and *MUC6* have been extensively studied in our laboratory (1, 4, 5, 15, 16, 18, 35–37). *In situ* hybridization and promoter functional studies conducted by us and others indicate that they are tightly regulated (2, 17) and have an altered profile of expression in human diseases of the respiratory, gastrointestinal, and urogenital tracts (2, 5, 38–42). Such alterations in expression (overexpression, repression, neoexpression) is usually characteristic of genes playing important roles in carcinogenesis.

Mucin hypersecretion and goblet cell hyperplasia are characteristics of inflammatory diseases and carcinogenesis of the lung (37, 40, 43, 44). Besides inflammatory mediators, growth factors could be involved in goblet cell production, because hypersecretory diseases are associated with abnormal epithelial cell growth and differentiation, and epithelium wounding leads to repair and remodeling processes (8). It has been postulated that EGF and its receptor EGFR could be good candidates. In human airways, EGFR is expressed in the fetus, where it is involved in cell proliferation, branching, morphogenesis and epithelial cell differentiation (19). Its expression is sparse in the adult lung but is expressed in lung tumors and asthma and is up-regulated by the pro-inflammatory cytokine TNF- $\alpha$ . Furthermore, K-Ras mutations are frequent in mucinous bronchioalveolar carcinoma and often associated with mucinous differentiation (45).

In this paper, we thus undertook to study the intracellular molecular mechanisms induced by EGF and TGF- $\alpha$  alone or in synergy with TNF- $\alpha$ , regulating the expression of the four 11p15 mucin genes, *MUC2*, *MUC5AC*, *MUC5B*, and *MUC6*, as well as identify promoter regions involved in that regulation. For that, studies were conducted in the human lung mucoepithelial cell line NCI-H292, which has been extensively used until now to study mucin gene regulation in lung cancer cells.





**FIG. 8. Effect of mithramycin A on EGF- and TGF- $\alpha$ -mediated up-regulation of *MUC2* and *MUC5AC* in NCI-H292 cells.** **A**, study of *MUC2* and *MUC5AC* mRNA levels by RT-PCR. Untreated cells without (lane 1) or with (lane 2) mithramycin A. Cells were treated for 24 h with EGF (25 ng/ml) or TGF- $\alpha$  (20 ng/ml) in the presence (lanes 4 and 6) or absence (lanes 3 and 5) of mithramycin A (100 nM). GAPDH was used as an internal control. PCR products were separated on a 2% agarose gel run in 1 $\times$  Tris-buffered EDTA buffer in the presence of ethidium bromide. **B**, transfected cells with *MUC2* and *MUC5AC* pGL3-deletion mutants were treated for 24 h with mithramycin A (100 nM) before luciferase activity measurement. REF. refers to the activity of the fragment of interest without any treatment. **C**, transfected cells with *MUC2* and *MUC5AC* pGL3 deletion mutants were treated for 24 h with TGF- $\alpha$  (20 ng/ml) in the absence (white bars) or presence (black bars) of mithramycin A (100 nM). REF. refers to the activity of the fragment of interest without any TGF- $\alpha$  treatment.

These cells are known to express EGFR, *MUC2*, and *MUC5AC* (9, 10, 46–50). In this report we also show that these cells express *MUC5B* mRNA but not the protein. Our data quickly showed that *MUC5B* and *MUC6* were not submitted to EGF and TGF- $\alpha$  regulation. On the other hand *MUC2* and *MUC5AC*, the two central genes in the cluster, are up-regulated both at the mRNA and protein levels (Fig. 9). The data regarding *MUC5AC* regulation is in agreement with those described by Nadel and co-workers (9, 10), who had focused their work on the regulation of that gene. However, we clearly show in this report that *MUC2* is also up-regulated by EGF and TGF- $\alpha$ , raising the question whether those two genes contain common regulatory elements in their promoters.

To answer this question, pharmacological agents with specific inhibitory activity were used both in RT-PCR experiments and in functional studies. We have demonstrated that EGF- and TGF- $\alpha$ -mediated up-regulation of *MUC2* and *MUC5AC* goes through binding of EGFR and then activation of the Ras/Raf/ERK-signaling cascade. Responsive elements were localized in the -2627/-2097 and -202/-1 regions of *MUC2* and *MUC5AC* promoters, respectively. However, ERK-signaling cascade is not the exclusive pathway since expression of *MUC2* and *MUC5AC* mRNAs was not totally abolished after inhibition of ERK by PD98059 or U0126. Because EGFR may also transduce signals through JNK (51), p38 (51), phosphatidylinositol 3-kinase (52), or protein kinase C (51), these signaling cascades will also have to be investigated in the future. Preliminary data in the laboratory suggest that protein kinase C-signaling pathway is also involved in mediating *MUC2* and *MUC5AC* up-regulation by EGFR ligands since partial inhibi-

tion of their expression was observed when NCI-H292 cells were pretreated with protein kinase C inhibitor GF109203X.

Regarding synergism between TNF- $\alpha$ - and EGF-mediated activation of mucin gene expression, previous work carried out in NCI-H292 cells showed that activation of EGFR tyrosine kinase by its ligands (EGF, TGF- $\alpha$ ) leads to the synthesis of *MUC5AC* mRNA and protein via mitogen-activated protein kinase-p44/42<sup>MAPK</sup>-signaling pathway and that this phenomenon is potentiated by TNF- $\alpha$  (9). We, however, did not find any synergistic effect between TNF- $\alpha$  and EGF or TGF- $\alpha$ , both, at the mRNA level or on promoter activity. Regarding the direct effect of TNF- $\alpha$  on mucin gene expression, previous studies also carried out in NCI-H292 cells have shown that TNF- $\alpha$  up-regulates *MUC2* (47) but not *MUC5AC* (9). In this paper, we show that both genes are up-regulated at the mRNA level by TNF- $\alpha$ , but we could not show any significant up-regulation of *MUC2* and *MUC5AC* promoters. This means that TNF- $\alpha$ -responsive elements are located in other parts of the 5'-flanking regions of the genes and/or post-transcriptional mechanisms like mRNA stabilization are occurring or that an indirect mechanism via activation of EGF by TNF- $\alpha$  is involved (19).

We then went further into our analysis of *MUC2* and *MUC5AC* regulation at the promoter level by EGF and TGF- $\alpha$ . Recently, it was shown that the up-regulation by EGF of genes that are involved in carcinogenesis often implicates cooperative mechanisms mediated by ubiquitous transcription factor Sp1. The mechanism either involves activation of Sp1 by a specific kinase (53) or uses Sp1 as a docking platform to convey EGF-activated AP-1 transcription factor toward the promoter of the target gene when AP-1 binding sites are absent in the promoter

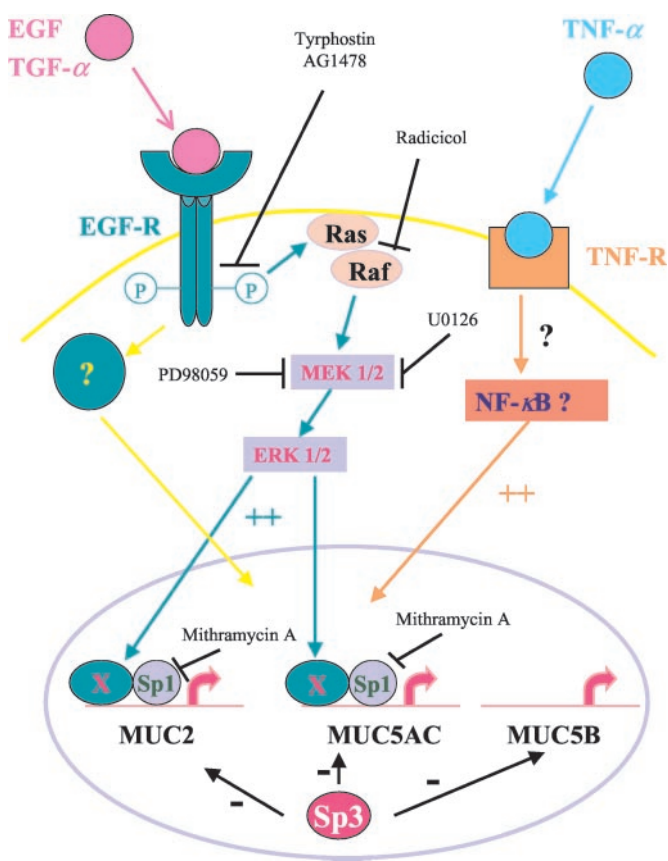


FIG. 9. Schematic representation of the signaling pathways leading to *MUC2* and *MUC5AC* up-regulation in response to EGF, TGF- $\alpha$  and TNF- $\alpha$  in NCI-H292 lung cancer cells. Sp1 and Sp3 biological roles on 11p15 mucin gene expression are also indicated. MEK, mitogen-activating protein/ERK kinase.

(54). Because Sp1 is an important regulator of *MUC2* (12, 17), *MUC5AC* (this report), and *MUC5B* (15, 16) expression, we tested its potential involvement in their EGF-mediated up-regulation. Our data clearly indicate that Sp1 is essential to get efficient up-regulation of *MUC2* and *MUC5AC* after EGF and TGF- $\alpha$  stimulation, since inhibition of Sp1 binding to DNA by mithramycin A efficiently inhibited EGF- and TGF- $\alpha$ -mediated up-regulation of these two genes. So far, electrophoretic mobility shift studies performed in the laboratory failed to identify AP-1 binding sites in *MUC2* and *MUC5AC* promoters. We, thus, hypothesize that Sp1 may act as a docking platform to help convey the transcription factor(s) activated by EGF or TGF- $\alpha$ .

Transcription factors of the Sp family have recently emerged as potent regulators of mucin genes (18). Having previously shown that *MUC5B* is regulated not only by Sp1 but also by Sp3 transcription inhibitor (16), we studied its effect on *MUC2* and *MUC5AC* promoters as well. It is of importance to note that when both transcription factors are present in equimolar amounts, Sp3 inhibitory effect prevails and leads to the absence of transactivation of the genes. Sp3 appears thus as a potent inhibitor of 11p15 mucin gene expression. Our results indicate also that Sp1 binding is implicated in *MUC2* basal transcriptional activity in NCI-H292 cells since mithramycin A blocks its expression at promoter and mRNA levels. This transcription factor appears important for *MUC2* gene expression since it interacts and transactivates murine (17), rat (55), and human (Ref. 17 and this report) *MUC2* promoters. This result is of importance because 11p15 mucin genes are clustered in a GC-rich region of chromosome 11 (1, 18), where gene methylation is often associated with their repression in cancer and

poor prognosis (16, 18, 32, 42). The relationship between Sp1/Sp3 expression and 11p15 mucin gene methylation status in cancer cells appears as a common regulatory mechanism (18) and will have to be evaluated in pathologies in which repression of mucin genes is observed.

In conclusion, our study performed on the four 11p15 mucin genes show that they are tightly and differentially regulated in human NCI-H292 lung cancer cells. The two central genes of the cluster, *MUC2* and *MUC5AC*, are up-regulated by EGF and TGF- $\alpha$ , whereas *MUC6* and *MUC5B* are not. TGF- $\alpha$ -mediated up-regulation of *MUC2* and *MUC5AC* was shown to be mediated by Sp1, a ubiquitous transcription factor that itself is able to induce transcription of all 11p15 mucin genes. Our studies will certainly help define new therapeutic strategies in airway hypersecretory diseases and lung carcinogenesis in which one may want to efficiently and selectively inhibit the EGFR-signaling pathway to reduce/prevent 11p15 mucin hypersecretion and/or 11p15 mucin gene-altered expression.

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