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Regulation of Airway Mucin Gene Expression

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Abstract

Mucins are important components that exert a variety of functions in cell-cell interaction, epidermal growth factor receptor signaling, and airways protection. In the conducting airways of the lungs, mucins are the major contributor to the viscoelastic property of mucous secretion, which is the major barrier to trapping inhaled microbial organism, particulates, and oxidative pollutants. The homeostasis of mucin production is an important feature in conducting airways for the maintenance of mucociliary function. Aberrant mucin secretion and accumulation in airway lumen are clinical hallmarks associated with various lung diseases, such as asthma, chronic obstructive pulmonary disease, cystic fibrosis, emphysema, and lung cancer. Among 20 known mucin genes identified, 11 of them have been verified at either the mRNA and/or protein level in airways. The regulation of mucin genes is complicated, as are the mediators and signaling pathways. This review summarizes the current view on the mediators, the signaling pathways, and the transcriptional units that are involved in the regulation of airway mucin gene expression. In addition, we also point out essential features of epigenetic mechanisms for the regulation of these genes.

Keywords

mucus; transcription; lung; epigenetics; differentiation

INTRODUCTION

The conducting airways of the lung are not just passive conduits for the passage of air between the lungs and environment. Cells lining the airway epithelium help to humidify the air as well as to trap environmental pollutants, particles, and pathogens to prevent their entry into the sterile areas of the lower airspace (1, pp. 443–538). To accomplish this function, secretory cells in the airways secrete a viscous mucus layer that helps to trap incoming particles, whereas coordinated beating of cilia from ciliated cells moves them cephalad, where the particles are eventually either expectorated or swallowed into the gastrointestinal (GI) tract (2).

The mechanism of airway mucus production and secretion has gained greater attention in recent years because of its function in mechanical host defense and its pathological ability to plug the airways and impair gas exchange. Asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF) are three of the more prevalent and well-known lung

diseases associated with mucus hypersecretion and accumulation in airways. Previously, research in airway obstruction focused on bronchoconstriction and dynamic airway collapse as the primary causes of airway narrowing. More recently, postmortem studies and biopsies of airways in patients with more severe forms of asthma and COPD have revealed that mucus plugging may be a more significant cause of airway narrowing than previously recognized (2, 3, 3a). Some investigators have questioned whether mucus hypersecretion is of clinical significance in diseases such as COPD when other variables such as age, smoking, and FEV1 (as a rough measurement of lung function) are controlled for (suggesting that excess secretions of mucus are merely a marker for a more severe underlying disease rather than a true cause of physiological impediment) (4). Other studies show that mucus secretion is predictive of mortality, but more so in patients with lower baseline lung function (5). This would suggest that mucus secretions have more physiological effects on gas exchange when it occurs on airways already narrowed from other causes such as bronchoconstriction, dynamic airway collapse, mucosal edema, and subepithelial fibrosis.

Although mucus is composed of various components, including water, glucose, various ionic solutes, and small antimicrobial protein peptides, it is the large, heavily glycosylated proteins called mucins that are primarily responsible for giving mucus their viscoelastic properties. Twenty mucin genes have been identified. Among these, 11 are expressed, either at the message and/or the protein level, in the lungs (6). The rest are found secreted in other body fluids such as saliva, GI secretions, and uterine secretions. To understand the cellular mechanism of mucus secretion, one can study the various steps of their synthesis: transcription of the mucin genes (MUC) into mRNAs, translation into polypeptides and intracellular packaging, glycosylation of the polypeptide backbones in the organelles, and, finally, secretion into the airways. Because of their large size and their complex carbohydrate backbones, mucins are difficult to purify. The protein backbones of various mucins (termed apomucins) have been used to generate antibodies, which have been used successfully in some studies, particularly those involving immunostaining of goblet cells (7). However, more detailed protein studies of mucins are somewhat limited by the facts that mucins exhibit poor migration in various gel matrices and that antibodies sometimes cannot bind to the native protein when their epitopes are hidden by the complex glycosylated backbones. Thus, relatively more data have accumulated on the regulation of these genes at the mRNA level in terms of both their transcriptional and posttranscriptional mechanisms.

This review focuses on the more common mucin genes found in the lungs, particularly those of the secreted gel-forming mucins. We focus on the intracellular signal transduction and transcriptional and posttranscriptional mechanisms that govern their expression. We touch briefly on what upstream signals from cytokines and/or ligands affect these mucins as relating to the intracellular mechanisms, but an in-depth examination of the multitude of mediators reported that can induce or repress mucins is beyond the scope of this review. Readers are referred to another excellent review on these upstream events (6).

MUCINS EXPRESSED IN THE LUNGS

Mucin proteins are synthesized by two types of secretory cells in the lungs: submucosal glandular cells and the surface mucous/goblet cells that line the epithelium. These cells can usually be seen histologically by Alcian blue–periodic acid–Schiff (PAS) staining because of the storage of these heavily glycosylated proteins in cytoplasmic secretory granules (8). Normal airway epithelium contains a paucity of surface goblet cells and a moderate number of submucosal glands. But in inflammatory airway diseases such as asthma and COPD, the surface goblet cells expand considerably (mucous cell hyperplasia/metaplasia), and submucosal gland frequency and size increase (9). The increase in surface goblet cells and sub-mucosal glands in these diseases may be what causes the excessive mucus secretion seen in these disorders (2, 3, 5).

The 11 mucins that have been described to express in the lungs are *MUC1*, *-2*, *-3*, *-4*, *-5AC*, *-5B*, *-6*, *-7*, *-8*, *-13*, and *-19* (6). Among these, *MUC5AC* and *MUC5B* are felt to be the predominant mucins in airway mucus because they are the only ones consistently found at the protein level in mucus and in sputum (10–12). Thus, most of the data on mucins in the respiratory tracts have focused on these two mucins. However, the other mucins are expressed at the mRNA level, both in vitro and in vivo, on airway epithelial cells (13, 14). The significance of these other mucins in the contribution to airway mucus rheology and goblet/mucous cell metaplasia is not as clear as that of these two gel-forming mucins.

The stimuli that upregulate mucin gene expression can be broadly categorized into (*a*) inflammatory cytokines, (*b*) bacterial products, (*c*) growth factors, (*d*) environmental chemicals or pollutants, and (*e*) miscellaneous chemical agents. Among these, inflammatory cytokines can be subdivided further into proinflammatory [tumor necrosis factor-α (TNF-α), interleukin (IL)-1β], TH1 [interferon-γ (IFN-γ)], TH17 (IL-17A), and TH2 (IL-4, IL-9, IL-13) cytokines. Cytokines and bacterial products have been the most extensively studied because of their secretion into airways affected by lung diseases such as asthma, COPD (inflammatory cytokines), and CF (bacterial products) (6). Another focus of research has been growth factors, especially retinoic acids (RA) and ligands that activate epidermal growth factor receptor (EGFR) and cell proliferation, repair, and differentiation (15, 16). Likewise, environmental toxic agents such as cigarette smoke and ozone are important pathophysiological causes of diseases of COPD and emphysema, so their effects on airway epithelial cells and mucins have been explored. And finally, certain chemical agents such as phorbol esters that activate specific intracellular signaling cascades have been used to delineate specific signal transduction mechanisms that affect mucin gene expression (17–19). Despite the large number of heterogeneous mediators involved, patterns have emerged owing to the identification of a few specific signal transduction pathways and transcription mechanisms that are critical to mucin gene expression (Figure 1).

MUC2 EXPRESSION AND REGULATION

MUC2 was one of the first gel-forming mucins to be characterized in detail. It is located next to the *MUC5AC* gene on chromosome 11p15.5. *MUC2* is approximately 17 kb in size, with 48 exons, and the protein product is more than 5100 amino acids long (20). Its

promoter region was the first of the mucins to be elucidated (21). *MUC2* is expressed primarily in the small intestine and colon of the GI tract (22), but its mRNA has also been detected in the airways of the lungs (23). However, there have been questions raised about how much MUC2 contributes to airway mucus secretion in normal and hypersecretory states. In the few biochemical studies of expectorated mucus done to date, the amount of MUC2 present is very small compared with MUC5AC and MUC5B (24–26). Also, although *MUC2* expression is increased in the airways of patients with asthma, the localization of MUC2 to surface goblet cells stained by Alcian blue–PAS staining is not as clear cut as the localization of MUC5AC in the goblet cells of the lung (27–29). Some investigators have postulated that MUC2 is not detected in expectorated mucus because its high insolubility makes it harder to assay (30). Others have also noted that *MUC2* expression occurs early during goblet cell hyperplasia/metaplasia and precedes that of *MUC5AC* expression, suggesting *MUC2* to be an early marker for mucous cell differentiation (13). Numerous inflammatory mediators can stimulate *MUC2* expression in airway epithelial cells in culture. Many of these same mediators, interestingly, can also upregulate *MUC5AC* (31). So far, two main classes of mediators that upregulate *MUC2*—bacterial products and cytokines—have been described in detail. Bacterial gene products include microbial membrane components [lipopolysaccharide (LPS), lipoteichoic acid (LTA), and flagellin]. Cytokines include mainly the proinflammatory cytokines IL-1β and TNF-α.

MUC2 Expression Regulated by Mitogen-Activated Protein Kinase/Nuclear Factor-κB Pathways

The lungs of CF patients are chronically colonized and infected with bacteria, particularly gram-negative bacteria such as *Pseudomonas aeruginosa* and gram-positive bacteria such as *Staphylococcus aureus*. Because goblet cell hyperplasia is abundant in CF, investigators hypothesized that the bacterial products from the chronic infection could induce epithelial cells to express mucin genes and differentiate into goblet cells. Studies to test this hypothesis first started with *MUC2* (Figure 1). Examination of the promoter region of *MUC2* revealed a putative nuclear factor-κB (NF-κB) binding region at −1452/−1441 from the transcriptional start site (32). NF-κB activation in airway epithelial cells is a common event during inflammatory conditions. Utilizing bacterial extracts from *P. aeruginosa* and *Staphylococcus aureus*, investigators found that that LPS and LTA can induce *MUC2* expression in the colon epithelial cell line HM3 (32, 33). Further studies showed that both signaled through the activation of Ras-MEK1/2-ERK1/2 pathways. In the case of LPS, ERK phosphorylation leads to the activation of the 90-kDa ribosomal S6 kinase (pp90sk), which subsequently activates NF-κB and promotes its translocation into the nucleus. There, NF-κB binds to a *cis* element at −1458/−1430 of the *MUC2* gene to promote transcription (32). LTA does not act directly on Ras but instead first activates ADAM10, a matrix metalloprotease (MMP), to cleave membrane-bound pro-EGF (epidermal growth factor) to release free EGF ligands. These EGF ligands then bind to EGFR, which then activates RAS-MEK1/2-ERK1/2, as does LPS, to cause NF-κB activation of *MUC2* transcription (33). *P. aeruginosa* also has a protein called flagellin that can activate airway epithelial cells and *MUC2* expression (34). Although flagellin normally binds to TLR5 and activates NF-κB in airway cells (35), the induction of MUC2 appears to work through the binding of flagella by ASGM1, a membrane ganglioside. After binding ASGM1, ATP is released and binds to an extracellular

nucleotide receptor that signals through phospholipase C activation and the generation of inositol triphosphate and Ca^{2+}. As do LPS and LTA, the signal then converges on the mitogen-activated protein kinase (MAP kinase) ERK and leads to its activation and subsequent downstream transcriptional activation of *MUC2* (34) (Figure 1).

MUC2 Expression Regulated by the MyD88/Nuclear Factor-κB Pathway

Bacterial products usually act on airway epithelial cells by binding to Toll-like receptors (TLRs) on their surface membranes. There are twelve known TLRs that recognize different components of bacteria, fungi, and/or viruses. Signal transduction events after the binding of microbial products to these receptors frequently converge downstream on the activation of NF-κB (36). However, TLR signaling can activate NF-κB through two parallel routes: the MyD88-dependent and -independent pathways (36). In the above studies (32–34), *MUC2* activation by LPS, LTA, and flagellin was suspected not to involve the TLRs at all. LPS, for example, is usually bound to a serum protein called LPS binding protein (LBP) that is then brought to the TLR4 by either a membrane-bound or a soluble receptor called CD14 (37). In the studies above (32–34), however, neutralizing anti-CD14 antibodies could not inhibit *Pseudomonas*'s LPS stimulation of *MUC2*, suggesting that another receptor besides TLR4 is used (32). Likewise, LTA was believed to bind to surface platelet-activating factor receptors (PAFRs), and the flagellin was thought to involve the ASGM1 instead of TLRs (33, 34). Nevertheless, the involvement of TLRs through the MyD88-dependent pathway has been reported for *MUC2* activation. Lysates from nontypeable hemophilus influenza (NTHI) (a pathogen common in COPD patients) were able to upregulate *MUC2* expression through NF-κB activation. Its mechanism involves binding to TLR2, which leads to the activation of the MyD88-TAK-NIK pathway and subsequent IKK phosphorylation (38). Interestingly, this same study also showed that part of the upregulation of *MUC2* depended on a non-TLR/MyD88 pathway that involved transforming growth factor (TGF)-β/Smad signaling.

MUC2 Expression Regulated by Inflammatory Cytokines

Like bacterial products, inflammatory cytokines induce *MUC2* gene expression in airway epithelial cells. IL-1β, TNF-α, and IL-4 thus far have been identified as having this activity. IL-1β and TNF-α are common proinflammatory cytokines that are elevated in a variety of both airway- and non-airway-related lung disorders and do not necessarily have any specific association with mucous hypersecretory diseases. Nevertheless, airway diseases with mucous cell hyperplasia, particularly COPD, have elevated levels of these cytokines in the blood as well as in the lungs (39, 40). In addition, both IL-1β and TNF-α induce mucous cell hyperplasia in vivo in mouse models (41, 42). Both cytokines can also activate NF-κB signaling in airway epithelial cells (43, 44), which, as shown above, can lead to the activation of *MUC2* expression. Thus, these cytokines may play some role in affecting the expression of mucin genes and mucous cell differentiation.

TNF-α induces the expression of *MUC2* in NCI-H292 cells (31, 45). The detailed mechanism on how this induction occurs is not clear, although TNF-α induction did not require new protein synthesis (45), and it did not increase the activity of a 2.8-kb promoter fragment of *MUC2* (31). This suggests that TNF-α induces *MUC2* by a transcriptional mechanism but that the TNF-responsive element may be beyond the 2.8 kb of the

transcriptional start site. IL-1β also stimulates *MUC2* transcription in NCI-H292 cells (47). In one report, a retinoic acid receptor (RAR)-α antagonist could inhibit the induction of *MUC2* by IL-1β and TNF-α, suggesting that IL-1β and TNF-α act partly through RAR pathways (48). Another report revealed that IL-1β's stimulation of *MUC2* occurs through the protein kinase C (PKC) and ERK pathways (49). In addition, this report also revealed that phosphoinositol 3-kinase (PI3K) inhibitors could partly block the induction of *MUC2* without affecting ERK phosphorylation (49). This finding suggests that IL-1β's effect occurs through a parallel PI3K/AKT pathway (49). IL-4 can induce *MUC2* expression in NCI-H292 cells as well as in colon cell lines (50, 51). IL-4 can induce goblet cell hyperplasia in mouse in vivo. However, this in vivo effect of IL-4 showed that only Muc5ac and not Muc2 was induced (50, 52).

MUC2 Expression Regulated by Epidermal Growth Factor Ligands and Retinoic Acid

Growth factors are important for stimulating the growth and differentiation of airway epithelial cells. Among them, EGF family ligands and vitamin A (all-*trans* RA) are of primary importance in stimulating mucociliary differentiation of airway epithelial cells in culture (53, 54). Not surprisingly, these ligands regulate mucin gene expression as well. EGF family ligands include EGF, TGF-α, amphiregulin- and heparin-bound EGF, betacellulin, epiregulin, epigen, and the neuregulins. They bind to a class of receptors called ErbB receptors, ErbB1–4; the ErbB1 receptor is also known by its more common name, EGFR (55). EGFR signaling regulates *MUC2* expression. Treatment of NCI-H292 cells with either EGF or TGF-α can stimulate *MUC2* transcription. This transcriptional up-regulation was mediated through the activation of RAS/RAF/ERK pathways. Promoter analysis revealed that the EGFR-responsive element was localized to −2627/−2097 of the *MUC2* promoter and that the putative transcription factor responsible for activation was Sp1 (31) (Figure 1). As discussed below, this EGFR/RAS/RAF/ERK/Sp1 pathway is also important for *MUC5AC* regulation.

All-*trans* RA (ATRA or RA) is another growth factor that is important in regulating mucin gene expression. There are reports of *MUC2* induction by RA in human cultured airway epithelial cells, and RAR-α is apparently the primary mediator of this effect (13, 56). Interestingly, RA treatment of primate airway epithelial cells appears to inhibit *MUC2* expression, suggesting that some species differences may exist as to RA's effect on mucin (56, 57).

MUC5AC EXPRESSION AND REGULATION

MUC5AC is located on chromosome 11p15.5, in between *MUC2* and *MUC5B*. Its cDNA is approximately 17.5 kb in length, with a central large exon, similar to *MUC5B*, that is approximately 10.5 kb long. Motifs flanking this central exon are cysteine-rich, von Willabrand–like domains (58–60). *MUC5AC* is expressed primarily on surface mucous cells in the stomach, lungs, endocervix, and conjuntiva (61). In the lungs, goblet cell hyperplasia/metaplasia of surface epithelial cells in inflammatory diseases correlate closely with increases in the expression of *MUC5AC* messages (62, 63). Although surface goblet cells express other mucin genes (14, 64–66), MUC5AC is probably this cell type's most specific marker. Numerous reports have shown that inflammatory cytokines (e.g., TNF-α, IL-1β,

IL-4, IL-6, IL-9, IL-13, IL-17), bacterial products (e.g., LPS, LTA, peptidoglycans, flagellin), growth factors (e.g., EGF, TGF-α, RA, thyroid hormones), proteases [e.g., neutrophil elastase (NE)], environmental pollutants (e.g., cigarette smoke extracts, acrolein, ozone), and viral mediators (e.g., respiratory syncytial virus, rhinovirus) can prominently stimulate *MUC5AC* expression either in vitro or in vivo on airway epithelial cells (Figure 1, middle).

MUC5AC Expression Regulated by MAP Kinases

Like *MUC2*, *MUC5AC* is upregulated by many cytokine mediators and bacterial products. TNF-α and IL-1β induce *MUC5AC* in various airway epithelial cell lines (31, 48, 67–69). One mechanism for TNF-α's induction of *MUC5AC* involves signaling through MAP kinases ERK and p38 downstream to produce cyclic AMP–responsive element binding protein (CREB) activation. A CREB-responsive *cis* element on the *MUC5AC* promoter is present and is localized to the −776 to −929 region (68). Phorbol esters such as phorbol 12-myristate 13 acetate (PMA) can also activate *MUC5AC* expression through ERK pathways, but the downstream effects of PMA involve EGFR and Sp1 rather than CREB (18). A bacterial cytoplasmic protein from NTHI called P6 can activate *MUC5AC* expression. This mechanism appears to involve the binding of P6 to TLR2, which subsequently passes on the signal through the MyD88/TRAF6 pathway to p38 and NF-κB activation (70).

Regulation of *MUC5AC* Expression by Nuclear Factor-κB

Examination of the promoter region of *MUC5AC* reveals several putative NF-κB binding sites (58). Studies have confirmed that NF-κB is a major transcription factor that regulates *MUC5AC* gene expression. Some of the signal transduction events from MAP kinases lead downstream to NF-κB activation. The MyD88 pathway induced by NTHI leads to NF-κB-mediated transcription of *MUC5AC* (48). Pseudomonas extracts can induce *MUC2* and *MUC5AC*, likely by NF-κB mechanisms (58). Although the investigators (58) did not definitively link the pseudomonas induction of *MUC5AC* to NF-κB, the presence of NF-κB sites on *MUC5AC* promoter strongly suggests that they are the putative pseudomonas-responsive elements. The TNF-α induction of *MUC5AC* noted above leads to CREB-mediated transcriptional activation of *MUC5AC* (68). However, another group has shown that TNF-α can also induce *MUC5AC* expression in NCI-H292 cells through NF-κB. Adenoviral delivery of an IKKB mutant could block MUC5AC's stimulation by TNF-α, both in vitro in human cells as well as in vivo in mice (41).

Several groups have reported that NE can induce *MUC5AC* in airway epithelial cells (71–73). One group found that the mechanism involved activation of the ERK and NF-κB (71), although other groups reported different mechanisms such as mRNA stability and EGFR activation (72, 73). The differences in signaling and mechanistic steps are difficult to sort out because different cell systems and treatments were used. However, there are compelling data that reactive oxygen species (ROS) formation is required upstream for NE-mediated *MUC5AC* expression (74–76). The generation of ROS by NE appears to be mediated by different types of NADPH oxidases (77, 78) and may also require PKC activation (74). This may mean that ROS act upstream of NF-κB in *MUC5AC* expression because ROS activation of NF-κB in airway epithelial and smooth muscle cells has been observed (79–81).

Transcriptional Activation of *MUC5AC* Through Epidermal Growth Factor Receptor Signaling Pathways

As noted above, the ErbB family receptors play important roles in growth, repair, and tumorigenesis in epithelial cells. Signaling through EGFR (the ErbB1 receptor) has long been known to play an important role in modulating the expression of mucin genes (82, 83). Many stimuli, including cigarette smoke, NE, phorbol esters, eosinophil products, bacterial products, and human airway trypsin-like protease, can upregulate *MUC5AC* through EGFR (18, 73, 74, 84–86). EGFR is expressed on airway epithelial cells, and in many cases its ligands, such as TGF-α and amphiregulin, are also present on the membrane in their inactive forms (55). Stimuli work by cleaving these ligands to their active forms, which then act in an autocrine or a paracrine manner to upregulate *MUC5AC* (18, 73, 74). As noted for NE, ROS are an important mechanism for stimulating *MUC5AC*. Cigarette smoke is also an important environmental stimulus for generating ROS (87). One group has shown that ROS generation from NE and cigarette smoke leads to EGFR activation, through the activation of TNF-α-converting enzyme (TACE), to cleave pro-TGF-α from the membrane into the active soluble form of TGF-α (74, 88). Because dual oxidases are expressed in airway epithelial cell surface, dual oxidase-1 (Duox-1) may be involved in TACE activation (78). Other enzymes besides TACE that can also cleave pro-EGFR ligands to activate *MUC5AC* include MMPs, dis-integrin and metalloproteinase domain proteins (ADAMs), and tissue kallikrein (TK) (89, 90). Downstream of EGFR, the mechanism that leads to *MUC5AC* expression can involve ERK activation with subsequent binding of transcription factors Sp1 (18) and Fra-2 to the *MUC5AC* promoter (91). However, ROS generated by cigarette smoke can also induce *MUC5AC* by a non-EGFR, non-ERK pathway that involves JNK activation and subsequent binding of the JunD transcription factor to the promoter (91).

TH2 Cytokines' Effects on *MUC5AC*: The Importance of Injury and Repair Mechanisms

Among the inflammatory cytokines that have been associated with mucous cell hyperplasia, the TH2 cytokines, such as IL-4, IL-9, and IL-13, have been most frequently noted to induce *MUC5AC* gene expression both in vitro and in vivo (6). This observation is consistent with the close association of mucous cell hyperplasia/metaplasia seen in allergic airway diseases such as asthma. IL-9 induces *MUC5AC/Muc5ac* both in vitro and in vivo (92–96), although few data exist as to the intracellular mechanisms of its action. IL-9's effect on *MUC5AC* may be mediated through IL-13 (95, 96), although other studies dispute the role of IL-13 (92, 94). IL-9's ability to directly induce *MUC5AC* gene expression on airway epithelial cells is controversial (92, 97). Under one scenario, IL-9's ability to directly induce mucous cells in culture may require a coexisting degree of injury and repair (98). IL-9 may have some role in inducing *MUC5AC* expression, although its requirement in mucous cell differentiation in the mouse is not absolute (99).

IL-4 and IL-13 are the TH2 cytokines most frequently associated with *MUC5AC*/*Muc5ac* induction. The IL-4 and IL-13 receptors are heterodimers and share a common alpha receptor (IL-4Rα), which may explain why much of their cellular effector functions are similar. After receptor binding, these cytokines' signals are transduced through the Janus kinase (JAK) pathways that ultimately lead to STAT6 nuclear translocation and the transcriptional regulation of various genes by STAT6. IL-4 and IL-13 stimulate *MUC5AC*

expression both in vitro and in vivo (50, 52, 100). Although the predominant signaling for both these two cytokines may be through IL-4Rα, the association between IL-4's *MUC5AC* induction is less clear than for IL-13. IL-4's effect on mucous cell metaplasia/hyperplasia and *MUC5AC*/*Muc5ac* has been predominantly observed in vivo (50, 52), whereas in vitro experiments with defined IL-4 administration to airway epithelial cells have produced conflicting results (97, 100–103). Hence, IL-4's role in inducing mucous cell differentiation may be due to indirect mechanisms from the inflammatory cells and mediators it recruits to the lung rather than a direct effect on airway epithelial cells.

IL-13 has a greater association with mucous cell differentiation in asthma. Although there are also conflicting data, there is more abundant and stronger evidence for IL-13's role in directly inducing airway epithelial cells to upregulate *MUC5AC*/*Muc5ac* and differentiate into mucous cells (97, 103, 104). However, IL-13's ability to induce mucous cell formation and *MUC5AC* in culture appears to require a much more prolonged exposure (4 days to 2 weeks) than is typical for most cytokine responses (100, 101). Because IL-13's ability to induce STAT6 phosphorylation occurs very rapidly, with nuclear translocation seen within 30 min, direct transcriptional activation of *MUC5AC* by STAT6 seems unlikely (105). Examination of the promoter region of *MUC5AC* in mouse and humans also does not reveal clear-cut putative STAT6 binding sites, and other putative transcription factor binding sites such as SMAD4 and HIF-1α are more likely the direct inducers of *Muc5ac* transcription (106). Despite its importance in inducing *MUC5AC* expression, the EGFR pathway does not appear to be involved in IL-13's effects (107). STAT6 likely is an upstream signal for IL-13 that induces other transcriptional signals and/or the release of other soluble factors that then act downstream on *MUC5AC* expression and mucous cell differentiation.

One reason why IL-13 requires a prolonged exposure time to affect *MUC5AC* expression is because of coexisting injury and repair of the airway. Indeed, epithelial denudation and sloughing have long been observed in the respiratory tracts of asthmatic patients (108). IL-13 induces proliferative response in various airway epithelial-type cells, although it does not directly damage epithelial cells, and it is not clear whether this proliferation is related to mucous cell formation (103, 104). However, recent evidence suggests that other airway epithelial cells can transdifferentiate (i.e., exhibit metaplasia) into mucous cells (109, 110). Allergen-mediated inflammation appears to recruit Clara cells to differentiate into mucous cells, although it is not clear whether IL-13 is the agent involved (109). It is also unclear if such a finding in mouse airways can be translated to humans.

Ciliated cells, long thought to be terminally differentiated, can also transdifferentiate to repopulate the airway epithelium during injury (111). One recent report, using a Sendai virus infection model in mouse, suggested how IL-13, EGFR, and injury and repair could work in concert. The Sendai virus in this model causes significant injury to the airways of mice so that a repair response is triggered. In the C57B6 strain of mice, this injury leads to persistent goblet cell metaplasia/hyperplasia along with IL-13 upregulation. The investigators noted that this injury leads to hyperplasia of the ciliated cells that was dependent on EGFR and PI3K signaling. However, this ciliated hyperplasia could not lead to goblet cells directly; rather, IL-13 was needed to induce these ciliated cells to trans-differentiate into goblet cells (110).

Negative Regulators of *MUC5AC* Expression and of Mucous Cell Differentiation

Fewer data have accumulated on the mechanisms whereby *MUC5AC* and mucous cell differentiation can be downregulated. Some anti-inflammatory pharmaceutical agents have been shown to inhibit *MUC5AC* expression. Macrolide antibiotics, felt to have some intrinsic anti-inflammatory effects independent of its bactericidal actions, can inhibit *MUC5AC* expression (112). Glucocorticoids, a mainstay pharmacological agent to treat asthma and COPD, can inhibit *MUC5AC* expression by transcriptional repression (113). A transcription factor named Foxa2 (HNF-3β) appears to repress the formation of mucous cells and *MUC5AC* expression. Conditional knockout of the Foxa2 gene in mice leads to impaired alveolarization and goblet cell hyperplasia. Mucous cell differentiation and upregulation of *Muc5ac* lead to the downregulation of Foxa2 (114). IL-13 and EGFR signaling can also downregulate Foxa2. Some evidence indicates that Foxa2 can promote Clara cell formation (115). These data are consistent with the above data regarding the ability of Clara cells to transdifferentiate into mucous cells, and Foxa2 may be the key transcription factor that modulates this process.

MUC5B EXPRESSION AND REGULATION

MUC5B is located next to *MUC5AC* on the chromosome 11. The gene structure reveals that *MUC5B* is approximately 39 kb long, with 49 exons, and produces a polypeptide estimated to be approximately 5701 amino acids. The central large exon region is 10,713 bp long and includes the entire tandem repeat region. In intron 36, a variable number of tandem repeats (VNTR) polymorphism is noted among different individuals, but so far no linkage to specific genetic disorders has been identified (116). In the 5′ region upstream of the transcriptional start site, putative binding sites for AP-1, Sp1, NF-κB, and other transcription factors exist (116). Less is known about the mechanisms of *MUC5B* regulation because fewer studies of *MUC5B* have been performed than for *MUC5AC*.

In adult tissues, *MUC5B* is localized almost exclusively to submucosal glands in the airways. In fetal development, however, *MUC5B* is coexpressed with *MUC5AC* on surface epithelial cells from week 13 onward. At approximately week 23, its expression on the surface is reduced, and its expression in the glands is more predominant (14). When primary airway epithelial cells are plated in cultures and mucociliary differentiation is initiated in air-liquid interface and RA treatment, upregulation and coexpression of both *MUC5AC* and *MUC5B* occur, suggesting that cultured cells show some similarity to surface airway cells during fetal development (13). In addition, pathological examination of certain human lung diseases such as emphysema and usual interstitial pneumonia has shown that *MUC5B* messages can be upregulated in surface airway epithelial cells in association with mucous cell hyperplasia/metaplasia (116). In mouse models of asthma, airway epithelial cells have also shown upregulation of *Muc5b* in association with mucous cell hyperplasia (64). Cumulatively, these data suggest that, although *MUC5AC*/*Muc5ac* is the predominant marker for surface goblet cells, *MUC5B*/*Muc5b* may also be upregulated and be a disease marker in certain circumstances. This coexpression of *MUC5B* and *MUC5AC* in surface goblet cells is similar to that seen in the fetal lung (14). This suggests that injury may induce

some type of progenitor cell proliferation or transdifferention of differentiated cells that mimics the behavior of airway epithelial cells in fetal lung development.

Upregulation of *MUC5B* Expression in Airway Epithelial Cells

Because *MUC5B* is upregulated in airway epithelial cells in inflammatory lung diseases, it is natural to suspect that inflammatory cytokines stimulate *MUC5B* expression as they do for *MUC5AC*. In a study on primary human airway epithelial cultures, a panel of 20 cytokines (IL-1α, IL-1β, IL-2 to -18, and TNF-α) revealed that IL-6 and IL-17 were the only cytokines able to upregulate *MUC5B* and *MUC5AC* (97). Other agents that can upregulate *MUC5B* expression in airway epithelial cultures include RA, rhinovirus 14 infection, alpha defensins (human neutrophil peptides 1–3), and PMA (19, 118, 119). One report showed that IL-13 could upregulate *MUC5B*, although other studies have shown no effect (97, 120).

In vivo, upregulation of *MUC5B*/*Muc5b* could be seen on surface airway goblet cells in a mouse model of asthma as well as in human airway sections from patients with inflammatory lung disease (64, 116). *MUC5B* is a major component of secreted mucus and, in some reports, was found in concentrations approximately ten times that of *MUC5AC* (11, 12). In one report, patients with a rare pulmonary disorder known as diffuse panbronchiolitis —a progressive airway disorder found mostly in Asian countries (particularly Japan) and characterized by airway narrowing, mucous hyperplasia, and distal airspace enlargement— showed high levels of *MUC5B* expression on the surface goblet cells (121). Examination of the genomic DNA of patients with this disease revealed a high correlation between specific polymorphisms in the promoter region of *MUC5B* and *MUC5B* expression and severity of the disease (121). The etiology of this disease is not clear, but associations with specific bacterias such as *P. aeruginosa* have been noted clinically, and the disease is amenable when treated with macrolide antibiotics such as erythromycin. CF is also associated with significant goblet cell hyperplasia, and *P. aeruginosa* chronically colonizes the airways and causes clinical exacerbations. However, the relationship between *Pseudomonas* and the mucous cell hyperplasia/metaplasia between these very different pulmonary disorders is not clear.

Signal Transduction Pathways in the Regulation of *MUC5B* Expression

The upregulation of *MUC5B* by IL-17A on primary airway epithelial cultures appeared to be partly indirect and due to the release of IL-6 and autocrine stimulation. IL-6's upregulation of *MUC5B* appears to depend on the ERK MAP kinase pathway because the inhibitor U0126 could partly block its stimulation (97). PMA is capable of inducing *MUC5B* expression. This chemical ligand is an activator of PKC. A study showed that activation of *MUC5B* by PMA required the p38 and JNK pathways, which led to transcriptional activation of the *MUC5B* promoter by Sp1 (19) (Figure 1, bottom). This mechanism is somewhat different from the PMA induction of *MUC5AC*, which involved the EGFR-mediated pathway, which also led to Sp1 activation of gene transcription (18). The nucleotide UTP can induce both *MUC5B* secretion as well as *MUC5B* expression. UTP stimulation of *MUC5B* expression depends on MAP kinase pathways because inhibitors to ERK could block UTP-dependent induction. Transduction of signals from the surface P2Y

receptors through a G protein–coupled intermediate receptor through ERK pathways is thought to lead to *MUC5B* upregulation by UTP (123).

REGULATION OF OTHER MUCIN GENES IN THE LUNGS

MUC1, a membrane-bound mucin with a cytoplasmic tail, was the first mucin molecule to be cloned. It is expressed in secretory epithelial cells of the airways, GI tract, and female reproductive tract and is associated with goblet cells (124). Its function is not well defined yet, but it appears to play a role in antiadhesion of tumor cells or bacteria (124, 125). It can mediate signal transduction responses through Ras, β-catenin, and p53. Signaling through these pathways may affect the apoptotic state of the cell, which may explain the greater expression of *MUC1* in neoplastic cells (126). *MUC1*'s expression in airway epithelial cells can be induced by NE and CpG DNA from bacteria (127, 128). Otherwise, it is regulated by inflammatory mediators in noncancerous airway cells.

MUC3 is primarily an intestinal mucin, and it is not expressed in the fetal or normal adult lung (28, 29). However, some studies have shown that *MUC3* is expressed at detectable levels in lung adenocarcinomas and bronchoalveolar carcinomas (129). Airway cultures of primary nasal and airway epithelial cells appear to upregulate *MUC3* during differentiation (130).

MUC4 is another membrane-bound mucin. Its expression in airway epithelial cells appears to be nonspecifically localized to basal, ciliated, and goblet cells (29). Mucociliary differentiation of cultured primary airway epithelial cells increases *MUC4* expression (130). IL-4, IL-9, and NE have also been reported to increase expression of *MUC4* in airway epithelial cells. In the case of the IL-4 and IL-9, transcriptional activation was believed to be the mechanism (131, 132), whereas for NE increase in mRNA stability occurred (133).

MUC6, a secreted mucin, is similar to MUC3 in that it is expressed primarily in intestinal cells, except in some cases in which it is upregulated in lung carcinoma cells (129). *MUC7* is expressed in the lungs primarily in the serous cells of the submucosal glands (134). Its expression increases in response to RA in culture, and it can be upregulated by inflammatory cytokines (IL-1β, TNF-α, IL-4, and IL-13), EGF, and bacterial products such as LPS (135). Analysis of the promoter region of *MUC7* revealed that a region −138/+30 containing AP-1 and NF-κB sites is critical to basal expression as well as the responsiveness of MUC7 to TNF-α. Mutagenesis experiments further elucidated that the AP-1 sites are important for *MUC7*'s basal expression whereas NF-κB is important for the responsiveness of MUC7 to TNF-α (136). MUC8 is a secreted mucin that is also primarily localized to the submucosal glands of the trachea (137). Its expression, however, does not appear to be related to differentiation (130). Although MUC11 and MUC13 have been localized to the lung, few data exist regarding their regulation. MUC19 is a recently identified secreted gel-forming mucin. Its expression is localized mainly to the mucous cells of the salivary glands and submucosal glands of the trachea (138). The extent to which MUC19 secretion is significant to airway mucus rheology remains to be defined.

EPIGENETIC MECHANISMS: A RELATIVELY UNEXPLORED AREA OF GENE REGULATION IN MUCIN BIOLOGY

Beyond the typical transcriptional and post-transcriptional processes reviewed here, another mode whereby mucin genes may be regulated is through epigenetic mechanisms (139). Epigenetics is defined as heritable changes in gene function that are not associated with actual changes in genomic DNA sequences. In practical terms, it is usually used to describe the turning on and off of genes due to DNA methylation, histone modifications, and microRNA silencing. DNA methylation involves the addition of a methyl group to the cytosine nucleotide of cytosine-guanine (CG) dinucleotide sequences in the genomic DNA (Figure 2). This methylation tends to inhibit the binding of transcription factors and to turn off gene expression. In addition, methylation can also repress transcription indirectly by promoting the binding of a family of proteins called methyl binding proteins (MBDs), which act as repressors of transcription (140). A clustering of CG dinucleotide sequences in a region of DNA is referred to as a CpG island. These CpG islands tend to play important regulatory roles when they occur in the promoter regions of genes. The chemical addition of a methyl group to the DNA backbone is usually catalyzed by enzymes called DNA methyltransferases or methylases. One example of this type of methylase is the enzyme called maintenance methylase enzyme or DNA methyltransferase 1 (DNMT1). This enzyme acts during DNA replication in cell division to reproduce the methylation imprinting of the old DNA strand on the newly synthesized DNA strand. Thus, this methylase only replicates the existing methylation and does not add de novo methylation to previously unmethylated DNA elements.

Other DNA methylases include the DNMT2, -3a, and -3b. These DNA methylases, unlike maintenance methylase, can de novo methylate previously unmethylated DNA regions. How these methyltransferases bind with specificity to methylate some regions of DNA and not others is not entirely clear. Also, although methylation and demethylation of genes do occur, specific DNA demethylating enzymes have not been conclusively identified. Biochemically, there is evidence that DNA demethylating enzymes exist in nuclear extracts. Some reports suggest that the methyl binding protein MBD2 has demethylating enzymatic activities, but other studies have disputed this contention (140a, 140b). In either case, methylation and demethylation in DNA promoter regions around specific genes can turn on and off gene expression, especially affecting the overall level of expression. Changes in the total levels of DNA methyltransferases are believed to alter the methylation levels of specific genes (Figure 2). For example, knockdown experiments of DNA methyltransferases with siRNA and inhibition with the inhibitor 5′-azacytidine can decrease the methylation of certain genes, leading to their increased transcriptional activity.

Another epigenetic mechanism involves histone modification (Figure 2). Genomic DNA is wrapped around histones that help to package and compact the extremely long DNA strand into the small volume of the nucleus. Covalent modification of histones can to some degree open or close this packaging around specific regions of DNA and alter the access of transcription factors and RNA polymerase complexes to specific genes or loci. These covalent modifications include acetylation, methylation, and phosphorylation. Acetylation of

histones typically unwinds the packaging and tends to turn on the genes around that area, whereas deacetylation closes the genes and turns it off (Figure 2). Acetylation and deacetylation are catalyzed by histone acetylases (HATs) and histone deacetylases (HDACs), respectively. In addition, HATs and HDACs tend to bind coactivator or corepressor proteins and recruit them to specific regions of DNA where they bind to other transcription factors to promote and repress transcription, respectively.

Although studies of epigenetic mechanisms in mucins have been few and most have been limited to *MUC2* expression, there are compelling reasons why they may be an important means of control for this family of genes. First, mucin genes clearly have cell and tissue specificity, and epigenetic mechanisms are frequently involved in the tissue-specific expression of other genes. Second, the use of epigenetic mechanisms can add an extra layer of control to help specific cells respond to the environment. For example, cytokine mediators that can activate NF-κB or MAP kinase pathways can potentially cause widespread and unwanted activation of mucin genes because these signaling pathways are active and ubiquitous in many different cell types. However, cell specificity of response can be achieved if cells primed to have mucin genes respond, e.g., airway epithelial secretory cell types, have their promoter regions relatively open by unmethylated CpGs and acetylated histones, whereas cells that are not primed to have mucin genes respond (e.g., inflammatory cells, mesenchymal cells, endothelial cells) have them turned off by methylated CpGs and deacetylated histones.

MUC2: An Example of an Epigenetically Controlled Mucin Gene

MUC2 is an example of a mucin with tissue-specific expression in the colon, small intestine, and respiratory tract. Most studies show little or no expression of *MUC2* in the normal gastric mucosa. However, in carcinogenesis, there are alterations in the mucin expression pattern, with a decrease in *MUC2* expression associated with de novo colon carcinogenesis (141). Studies of *MUC2* in the GI tract and its expression during carcinogenesis reveal that promoter DNA methylation is a frequent mechanism that controls *MUC2* expression (142, 143). Previous data indicate that the sequence of the *MUC2* promoter region approximately 343 bp upstream of the transcription start site contains elements regulating *MUC2* expression (21, 22). Recent results demonstrate that the methylation level of this region, comprising nine CpG sites, is related to *MUC2* expression in colon cancer cells in vitro (143) and pancreatic cancer in vivo (142). The mechanisms underlying the absence of *MUC2* expression in normal stomach and de novo *MUC2* expression in intestinal metaplasia and gastric carcinoma have not been studied. For cases of gastric carcinogenesis, comparison was made in the levels of methylation in nine CpG sites in the −289 and +1 region of the *MUC2* promoter. Methylation of this CpG region showed a significant correlation with low expression of *MUC2* in normal gastric mucosa, whereas demethylation of this region was associated with high *MUC2* expression in mucinous gastric carcinomas (144). Several notable CpG sites in the wider area of the *MUC2* promoter region, including those in AP2 and Sp1 binding motifs, showed obvious differences in methylation level between PANC1, BxPC3, and normal colonic crypt cells (145). Similar observations showing that site-specific methylation can downregulate *MUC2* gene expression have been

reported in pancreatic cancer cells (142, 146). These specific sites of methylation changes in the promoters may be a road map to the critically important regulatory regions of *MUC2*.

Few studies have looked at the role of histone modifications in *MUC2* expression. One study examined the histone deacetylase inhibitor trichostatin A (TSA) on *MUC2* expression in pancreatic cancer cell lines. Treatment with TSA resulted in the upregulation of *MUC2* expression, suggesting that histone acetylation can to some degree turn on the *MUC2* gene (146). In the future, further studies in other epigenetic mechanisms, including histone modification and chromatin modification in *MUC2* inactivation, are expected.

Other Mucin Genes and Epigenetic Mechanisms

There have been very few studies of epigenetic mechanisms in other mucin genes. *MUC5B* is suspected to be regulated to some degree by promoter methylation. Treatment with 5′-azacytidine can increase its expression in the KATO III and AGS gastric carcinoma cell lines. Mapping of the 5′ promoter region of *MUC5B* revealed, between −2044 and +3, nine putative CpG sites, some of which were methylated. Methylations of these CpGs were confirmed and were suspected to be responsible for the repression of *MUC5B* in these cell lines (147). For *MUC5AC*, a study in a pancreatic adenocarcinoma cell line showed that methylation of its 5′ promoter region showed correlation of approximately 77% with the degree of *MUC5AC* expression. However, when 5′-azacytidine was given, no increase of *MUC5AC* occurred, making the role of methylation in this cell line unclear (148). *MUC1* has a CpG island in a 60-bp tandem repeat region. Comparison of the methylation of this region between breast cancer cells, noncancerous breast cells, and other non-breast tissues revealed a similar correlation between lower methylation and higher expression of the *MUC1* gene (149).

CONCLUSION

The accumulated data suggest that secreted gel-forming mucins are regulated by specific pathways in surface airway epithelial cells. MAP kinase pathways, EGFR, and RA receptors signal downstream to Sp1, NF-κB, and AP-1 transcription factors that then alter the expression of various mucin genes. As our knowledge of mucins increase over time, it is becoming more and more evident that molecules of this class have an extremely complicated biology. Because of the large size and complex carbohydrate structures of mucins, mucin studies at the protein level will continue to be challenging in the future. As such, studies at the molecular genetic level on transcriptional, posttranscriptional processing, and epigenetic mechanisms should yield a greater understanding of how mucin genes are regulated in a cell- and tissue-specific manner and how their aberrant expression occurs in inflammatory lung diseases and carcinogenesis.

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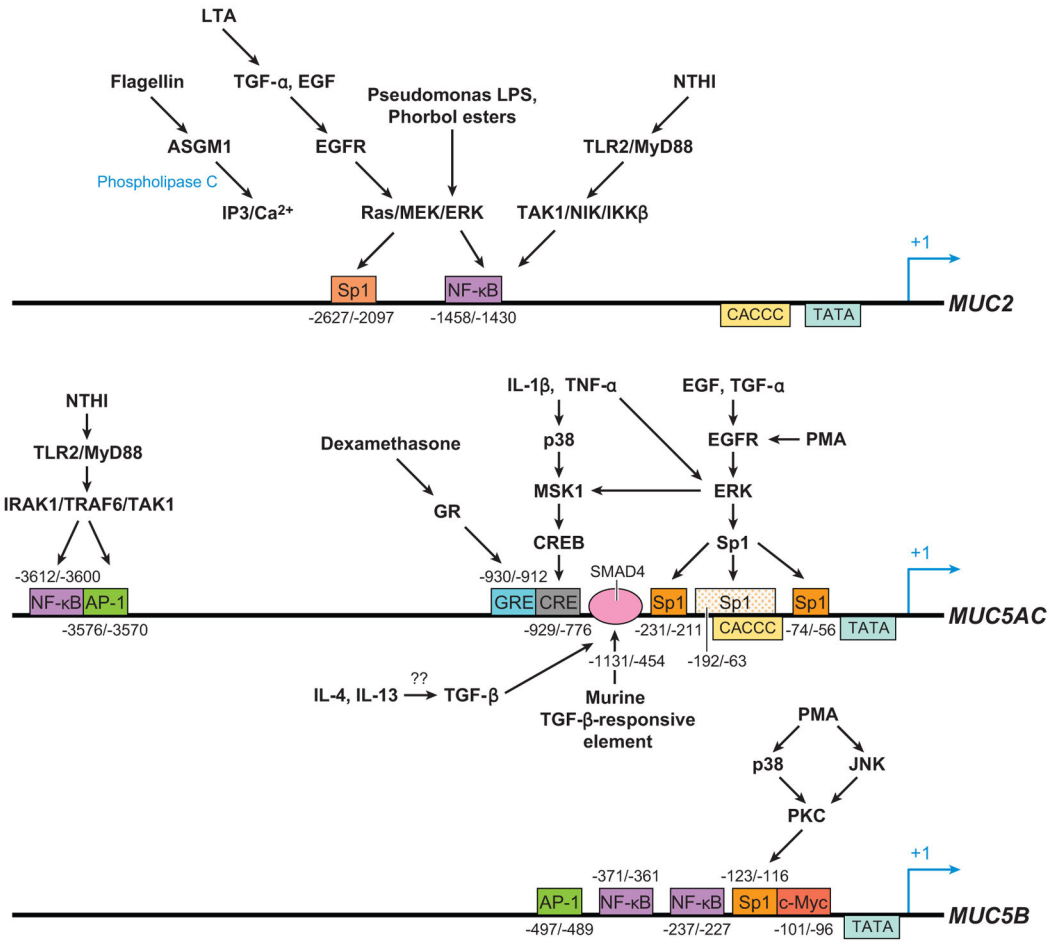

Figure 1.

A rough outline of 5′ promoter regulatory regions for *MUC2*, *MUC5AC*, and *MUC5B*. Although this picture is not comprehensive, some specific pathways are provided to illustrate the stimulus and signal transduction pathways that activate certain transcription factors to their binding sites on the mucin promoters. The mapping of specific regulatory regions for *MUC5B* to their stimulus has not been elucidated in detail, but some putative sites from promoter sequence analysis are provided.

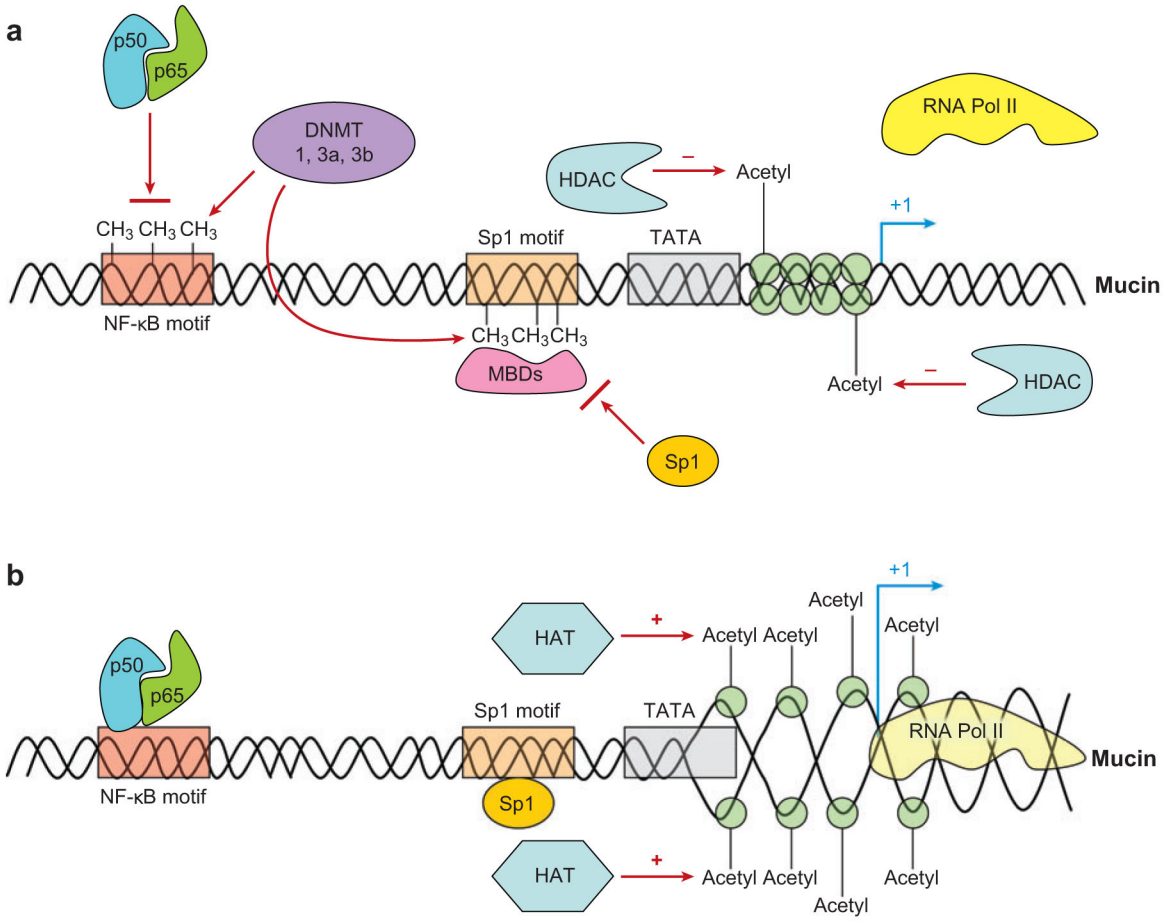

Figure 2.

Theoretical mechanism of how epigenetic mechanisms can regulate mucin gene transcription. (*a*) Mucin gene expression is low. DNA methylation decreases the binding of transcription factors either directly or through methyl binding proteins (MBDs). DNA methyltransferases (DNMT) catalyze the transfer of methyl groups de novo to CpG sites. Nucleosome structure is relatively closed by histones deacetylated by histone deacetylases (HDAC). (*b*) Mucin gene expression is high. Loss of DNA methylation allows transcription factors to bind to their putative binding sites. Acetylation of histones by histone acetylases (HAT) leads to the opening of nucleosomes and increased transcription. NF-κB, nuclear factor-κB; RNA Pol II, RNA polymerase II.