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Synergistic Stimulation of MUC1 Expression in Normal Breast Epithelia and Breast Cancer Cells by Interferon-γ and Tumor Necrosis Factor-α

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Abstract The MUC1 gene encodes a transmembrane mucin glycoprotein that is overexpressed in human breast cancers. Persistent stimulation by proinflammatory cytokines may contribute to increased MUC1 transcription by tumor cells. We demonstrate that MUC1 expression in T47D breast cancer cells and normal human mammary epithelial cells (HMEC) is enhanced by tumor necrosis factor-α (TNF-α) in the presence of interferon-γ (IFN-γ). MUC1 responsiveness to these cytokines was modest in T47D cells and robustly induced in HMEC. Transient transfection of T47D cells with mutant MUC1 promoter constructs revealed that a κB site at −589/−580 and the STAT-binding element at −503/−495 and were required for cooperative stimulation by TNF-α and IFN-γ. Binding of NFκB p65 to the MUC1 κB site was induced by TNF-α treatment, as demonstrated by electrophoretic mobility shift assay. Specific mutation of the κB site prevented binding of NFκB p65 and blocked TNF-α stimulation of MUC1 promoter activity. Collectively, these studies demonstrate synergistic stimulation of MUC1 expression by TNF-α and IFN-γ that is mediated by independent actions of NFκB p65 and STAT1α upon κB and STAT sites, respectively, in the MUC1 promoter. Strong induction of MUC1 expression by these pro-inflammatory cytokines is clearly evident in normal mammary epithelium. In contrast, breast tumor cells appear to override normal regulatory responses via as yet undefined *cis*-elements. J. Cell. Biochem. 86: 759–772, 2002.

Key words: MUC1; transcriptional regulation; tumor necrosis factor-α; interferon-γ; nuclear factor kappa B; signal transducer and activator of transcription

The full-length product of the MUC1 gene is a large type I transmembrane mucin glycoprotein primarily expressed on the apical surfaces of many mammalian simple epithelial cells that line ducts or glands [as reviewed in Gendler and Spicer, 1995; Lagow et al., 1999; Hanisch and Muller, 2000], although MUC1 expression by nonepithelial cells including hematopoietic cells [Dent et al., 1999], T cells [Agrawal et al., 1998a], and male germ cells [Franke et al., 2001] also has been reported. Proposed functions for MUC1 include modulation of cell adhesion, signal transduction, lubrication and hydration of epithelial surfaces, and protection of epithelial surfaces from infection [Gendler and Spicer, 1995; Lagow et al., 1999; Hanisch and Muller, 2000]. In human breast cancers, regulation of MUC1 expression differs between breast tumor cells and adjacent normal epithelial cells via aberrant glycosylation, cellular distribution, and mRNA splicing; however, the molecular control of any of these events is not well understood [Girling et al., 1989; Hilkens et al., 1992; Zrihan-Licht et al., 1994; Brockhausen et al., 1995]. MUC1 is greatly overexpressed in many epithelial-derived cancers including those of breast, ovary, and pancreas [Gendler and Spicer, 1995]. Overexpression of MUC1 in human breast cancers is frequently due to increased transcription [Hareuveni et al., 1990], possibly as a result of chromosomal rearrangement or gene duplication [Gendler et al., 1990; Bieche and Lidereau, 1997; Waltz et al., 1998].

Normal expression of MUC1 is under the control of steroid hormones in the mammary

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gland, although these responses appear to be indirect [Parry et al., 1992; Zhou et al., 1998]. Expression of MUC1, a constituent of milk, in normal human and mouse mammary epithelial cells is low in virgin glands but increases from mid-pregnancy to lactation [Burchell et al., 1987; Parry et al., 1992]. The development of mice transgenic for human MUC1, antigenically distinct from the endogenous mouse homologue, has brought to light the genomic requirements for expression and regulation of MUC1 in both normal breast and mammary tumors. The smallest transgene utilized to date that displayed expected epithelial-specific expression and regulation of MUC1, including increased expression in mammary tumors, utilized 1.4 kb of 5′-flanking sequence upstream of MUC1 cDNA, implying that introns and 3′-flanking sequence do not participate in transcriptional control [Graham et al., 2001].

A consistent observation among several MUC1-expressing cancer cell lines is the requirement of sequences between −600 and −400 for maximal expression [Abe and Kufe, 1993; Kovarik et al., 1993]. The importance of this region is supported by the finding that the internal sequence −598/−485 enhanced activity of a heterologous promoter more than tenfold in MCF7 breast cancer cells [Abe and Kufe, 1993; Kovarik et al., 1993]. Located within this transcriptionally important region of the MUC1 promoter are a potential binding site for the transcription factor NFκB (nuclear factor kappa B) at −589/−580, overlapping with a putative AP-3 (activator protein 3) element [Abe and Kufe, 1993], and a STAT (signal transducer and activator of transcription) binding element at −503/−495. MUC1 regulation by cytokines and peptide hormones that signal through NFκB and STAT transcription factors is well-documented [Tran et al., 1988; Parry et al., 1992; Clark et al., 1994; Lagow and Carson, 1999; Grunberg et al., 2000; Lagow and Carson, 2000; Gaemers et al., 2001], and evidence suggests that persistent cytokine stimulation may contribute to the increased transcription of MUC1 observed very frequently in breast cancers. The STAT-binding element at −503/−495 was shown to mediate interferon-γ (IFN-γ) responsiveness of the MUC1 gene in a breast cancer cell line, T47D, and specific mutation of the site reduced MUC1 promoter activity, suggesting its involvement in constitutive and stimulated MUC1 expression in these cells [Lagow and Carson, 1999; Gaemers et al., 2001]. However, nuclear extracts from unstimulated T47D cells did not react with the STAT-binding element, indicating that the high level of MUC1 transcription observed in these cells is not due to constitutive activation of this single *cis*-acting promoter element. Furthermore, IFN-γ stimulation of MUC1 promoter activity did not translate into increased MUC1 expression in T47D cells. Therefore, we examined normal mammary epithelial cells to determine whether the mechanism for IFN-γ stimulation of MUC1 expression is conserved or is specific to tumor cells. Importantly, several proinflammatory cytokines including IFN-γ and tumor necrosis factor-α (TNF-α) are produced by lymphocytes associated with mucin-expressing breast tumors [Vitolo et al., 1992, 1993]. Therefore, overexpression of MUC1 may be at least partially the result of a combination of stimulatory factors acting upon multiple regulatory elements. For this reason, we investigated the role of the potential κB site at −589/−580, in the context of the STAT-binding element, in transcriptional regulation of MUC1 by TNF-α and IFN-γ.

METHODS

Cell Culture

T47D human breast ductal carcinoma cells (ATCC, Manassas, VA) were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Gaithersburg, MD). Human mammary epithelial cells, or HMEC (Clonetics, San Diego, CA), were maintained in the recommended mammary epithelial growth medium (MEGM) containing 10 ng/ml recombinant human EGF, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 50 μg/ml gentamicin, 50 ng/ml amphotericin B, and 13 mg/ml bovine pituitary extract (Clonetics).

Generation of Mutant Promoter Constructs

A genomic fragment consisting of the 5′ flanking sequence of the MUC1 gene from −1,406 to +33 was a generous gift from Dr. Sandra Gendler (Mayo Clinic, Scottsdale, AZ). The 1.4MUC construct used in these studies previously was made by cloning this fragment into pGL3basic luciferase reporter vector (Promega, Madison, WI). PCR was used to generate 5′

deletion mutants of 1.4MUC, and inserts consisting of MUC1 promoter fragments −604/+33 and −487/+33 were cloned into pGL3basic. The pGL3-TK plasmid was constructed by cloning the *Bgl*II/*Hind*III fragment of pRL-TK (Promega), containing the HSV-TK promoter, into pGL3basic. Single-stranded oligos (Sigma-Genosys, The Woodlands, TX) encompassing −518/−480 of MUC1 gene sequence were annealed and cloned into pGL3-TK for analysis of the STAT-binding site. Sequence of the STAT-binding site in the native insert: 5′-TTCCGG-GAA-3′; in the mutated insert: 5′-ccCCGGG-AA-3′ (mutated nucleotides in lowercase). The −604/−468 MUC1 promoter segment, containing the STAT-binding site and a potential κB site, was amplified by PCR and cloned into pGL3-TK. All positive clones were confirmed by sequencing.

Site-Directed Mutagenesis

Recombinant PCR, as described by Kovarik et al. [1993], was used to generate specific mutations in the potential κB site at −589/−580 and in the STAT-binding site at −503/−495 in the MUC1 promoter using the 1.4MUC construct as a template. The native sequence of the potential κB site in the sense strand is 5′-GGAAAGTCCG-3′, and the mutated sequence is 5′-GGcccGTCCG-3′. The native sequence of the STAT-binding site is 5′-TTCCGGGAA-3′, and the mutated sequence is 5′-ccCCGGGAA-3′. Recombinant products containing one of the above mutations were used to replace the native sequence in the 1.4MUC construct.

Transient Transfections and Reporter Assays

Transient transfections were performed using LipofectAMINE reagent (Life Technologies) in a 6-well plate format according to the manufacturer's instructions. Two micrograms of pGL3basic-based plasmid and 0.25 μg of pRL-TK plasmid were used per well. For co-transfection of 1.4MUC with STAT1 plasmids, a total of 3.25 μg DNA were used per well: 2 μg of 1.4MUC, 0.25 μg pRL-TK, and 1 μg of expression plasmids for human STAT1α, STAT1β, or vector. STAT1α and STAT1β expression plasmids were gifts from Dr. Ulrike Schindler (Tularik, Inc., South San Francisco, CA). Following transfection, cells were given fresh medium with 5% (v/v) fetal bovine serum in the presence or absence of the following: 200 U/ml recombinant human IFN-γ (Roche, Indianapolis, IN), 25 ng/ml recombinant human TNF-α (Roche), or 10 ng/ml recombinant human IL-1β (Roche). Normal rabbit serum (NRS) was purchased from Calbiochem-Behring (LaJolla, CA), and polyclonal rabbit-anti-human IFN-γ was purchased from BioSource (Camarillo, CA). Luciferase assays were performed using the Dual-Luciferase Assay Kit (Promega) according to the manufacturer's instructions and a Dynex MLX Microplate Luminometer (Dynex Technologies, Chantilly, VA). Reporter activity was expressed as the ratio of firefly luciferase activity (pGL3-based plasmids) to *Renilla* luciferase activity (pRL-TK). Statistical analyses were performed using GraphPad InStat software (GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego California USA, www.graphpad.com), employing one-way ANOVA and the Tukey–Kramer multiple comparisons test.

Northern Blot Analysis

T47D cells were plated in 6-well plates and maintained as described until cells reached confluence. Confluent cells were serum-starved for 24 h prior to treatment. For treatment, T47D cells were cultured in medium containing 5% (v/v) fetal bovine serum ± 200 U/ml IFN-γ and/or 25 ng/ml TNF-α. The same treatments were administered to HMEC in MEGM, but using 50 ng/ml TNF-α. Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA), and Northern blot analysis was performed on total RNA (6 μg/lane for T47D RNA and 10 μg/lane for HMEC RNA) using the NorthernMax-Gly kit (Ambion, Austin, TX) according to the manufacturer's instructions. The cDNA probe used for detection of MUC1 mRNA was a 436-bp RT-PCR product that encompasses parts of exons 6 and 7, encoding the cytoplasmic tail. A cDNA probe for human 18S ribosomal RNA (ATCC) was used as a load control. Probes were non-isotopically labeled using the BrightStar Psoralen-Biotin kit (Ambion), and hybridization signals were detected using the BrightStar BioDetect kit (Ambion). Blots were exposed to X-ray film, and signal intensities were quantified using the Alpha Imager 1D-Multi function (Alpha Innotech, San Leandro, CA). Blots were stripped between probings with boiling 0.1% (w/v) SDS in water, then stored at 4°C.

Western Blot Analysis

Cell treatments and sample preparation. T47D cells were seeded into 24-well plates and maintained as described until cells reached confluence. Confluent cells were serum-starved for 24 h, then cultured in serum-free medium ± 200 U/ml IFN-γ and/or 25 ng/ml TNF-α. The same treatments were administered to HMEC in MEGM. Cells were solubilized with sample extraction buffer [SEB; 0.05 M Tris pH 7, 8 M urea, 1% (w/v) SDS, 0.01% (v/v) phenylmethylsulfonylfluoride (PMSF), 1% (v/v) β-mercaptoethanol], and protein concentration was determined by the method of Lowry et al., [1951]. Culture supernatants were centrifuged briefly at 4°C, 10,000*g*, to pellet any cell debris. Supernatants were precipitated overnight at 4°C using 50 μg of fetal bovine serum protein as carrier. Resulting pellets were rinsed with acetone and resuspended in 25 μl SEB and 25 μl Laemmli sample buffer (LSB, [Laemmli, 1970]). Forty percent of each sample of precipitated culture supernatant protein and 10 μg (T47D) or 50 μg (HMEC) of each sample of cell-associated protein were analyzed. Each sample of cell-associated protein was brought up to a final volume of 25 μl with SEB and LSB at 1:1 (v/v). Before loading, samples were heated at 95°C for 2 min, and 1 μl of bromophenol blue (1% v/v in water) was added to each for visual tracking.

SDS–PAGE and detection of MUC1 protein. Proteins were separated by SDS–PAGE using a 4.5% (w/v) Laemmli stacking gel [Laemmli, 1970] and a 10% (w/v) Porzio and Pearson resolving gel [Porzio and Pearson, 1977] under constant current. Separated proteins were transferred to Schleicher & Schuell Protran nitrocellulose (Intermountain Scientific, Kaysville, UT) at 4°C. Nitrocellulose blots were blocked at 4°C in phosphate-buffered saline plus 0.1% (v/v) Tween-20 (Sigma) containing 3% (w/v) bovine serum albumin (Sigma). Primary antibody 214D4 (kindly provided by Dr. John Hilkens of The Netherlands Cancer Institute, Amsterdam, The Netherlands), a mouse monoclonal specific for a tandem repeat epitope in the extracellular domain of MUC1 [Wesseling et al., 1995], was added directly to the blocking solution to a final dilution of 1:10,000. In a comparison of 214D4 with several other antibodies directed toward the MUC1 tandem repeats (BC3 [Xing et al., 1989], DF3 [Perey et al., 1992], HMFG1, HMFG2 [Taylor-Papadimitriou et al., 1981; Burchell et al., 1983], and SM3 [Burchell et al., 1987]), 214D4 reacted more strongly and consistently in Western blots than any other antibody in a variety of samples (J. Julian, unpublished observations). Therefore, the 214D4 antibody was used in routine analyses. Following overnight incubation at 4°C, blots were rinsed at room temperature three times, 5 min each, in phosphate-buffered saline plus 0.1% (v/v) Tween-20. For detection, blots were incubated for 2 h at 4°C with horseradish peroxidase-conjugated sheep-anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) at a final dilution of 1:200,000 in blocking solution. After three 5 min rinses at room temperature in phosphate-buffered saline plus 0.1% (v/v) Tween-20, ECL detection was carried out using the SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL) according to the manufacturer's instructions. Blots were exposed to X-ray film, and signal intensities were quantitated using the Alpha Imager 1D-Multi function (Alpha Innotech).

Nuclear Extract Preparation

Three to four confluent T150 flasks were used for each treatment and nuclear extract preparation. Prior to treatment, T47D cells were serum-starved for 24 h. T47D and HMEC cells were untreated or treated for 15 min with 200 U/ml IFN-γ ± 25 ng/ml TNF-α. Nuclear extracts were prepared as described by Dignam et al., [1983] with minor modifications. The following protease and phosphatase inhibitors, along with 1 mM DTT, were added to the PBS/5 mM EDTA (used for detaching cells), hypotonic buffer, and nuclear extraction buffer just before use: 1 mM Na_3VO_4, 1 mM NaF, 1 mM Na_2MoO_4, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, and 1 mM PMSF. Cellular lysis was achieved using final concentrations of 0.1% or 0.5% (v/v) NP-40 for T47D cells and HMEC, respectively. Nuclear extracts were collected and dialyzed in Slide-A-Lyzer MINI dialysis units (Pierce) for 4 h against 250 ml dialysis buffer, changing buffer once. Dialyzed nuclear extracts were flash-frozen in dry ice/ethanol and stored in usable aliquots at −80°C. Protein concentration was determined by the method of Lowry et al., [1951]. Nuclear extracts of at least 5 mg protein/ml concentration were used for EMSA.

Electrophoretic Mobility Shift Assay (EMSA) and Supershift Assay

EMSA and supershift assays for analysis of the MUC1 promoter STAT-binding element at −503/−495 were carried out using the STAT1α NuShift kit (Geneka Biotechnology, Montreal, Canada) according to the manufacturer's instructions. Oligonucleotide probes for STAT1α EMSA included the MUC1 promoter potential STAT-binding site flanked by additional native sequence (−513/−485; 5′GGTGGGGCTA[TTCCGGGAA]GTGGTGGGGG3′; Sigma-Genosys) and a positive control STAT-inducible element (SIE) (5′GTCGACAT-[TTCCCGTAA]ATCGTCGA3′; provided with kit). Corresponding mutant oligos used in competition assays included a mutant MUC1 potential STAT-binding site (5′GGTGGGGCTA[ccCCGGGAA]GTGGTGGGGG3′) and a provided mutant SIE (5′GTCGACAT-[aTagCGTAA]ATCGTCGA3′) (mutated nucleotides in lowercase). Double-stranded oligos were prepared by combining equal parts sense and antisense oligos, boiling for 5 min, and cooling to room temperature overnight to anneal. Wild-type oligos were end-labeled using γ^{32}P-ATP (NEN Life Science Products, Boston, MA) and T4 polynucleotide kinase (Roche). Unlabeled wild-type and mutant competitor oligos were used in a 100-fold molar excess over labeled probe. Five micrograms of T47D or HMEC nuclear extract protein were used in each reaction. Nuclear extracts from IFN-α-treated U-937 cells were provided as a positive control. For supershift assays, nuclear extracts were preincubated with NRS (Calbiochem-Behring) or rabbit polyclonal STAT1α antibody (Geneka Biotechnology). EMSA analysis of the MUC1 promoter potential κB site at −589/−580 was carried out using the NFκB/Rel family NuShift kit (Geneka Biotechnology) according to the manufacturer's instructions. Oligonucleotide probes and competitors for NFκB/Rel EMSA included the MUC1 promoter potential κB site flanked by additional native sequence (MUCκB; −599/−570; 5′-CCAGGCTGCT[GGAAAGTCCG]GCTGGGGCGG-3′); the corresponding mutant, mMUCκB (5′-CCAGGCTGCT[GGcccGTCCG]GCTGGGGCGG-3′; Sigma-Genosys); a provided control binding site for NFκB p65 and c-Rel, Rel (5′-AGCTT[GGGGTATTTCC]AGCCG-3′); the corresponding mutant, mRel (5′-AGCTT[GGcaTAggTCC]AGCCG-3′); a provided control binding site for NFκB p50, NFκB (5′-GCCATGG[GGGGATCCC]CGAAGTCC-3′); and the corresponding mutant, mNFκB (5′-GCCATGG[GccGATCCC]-CGAAGTCC-3′). For supershift assays, nuclear extracts were preincubated with NRS or a rabbit polyclonal antibody to c-Rel, NFκB p65, or NFκB p50 (Geneka Biotechnology).

RESULTS

TNF-α Greatly Enhances MUC1 Transcriptional Activity in the Presence of IFN-γ

A segment of 5′ flanking sequence of the human MUC1 gene from −1,406 to +33 was used in transient transfection assays to study responsiveness to IFN-γ, IL-1β, or TNF-α in T47D cells. A modest but significant stimulation of reporter activity (2.5- to 5-fold) was observed in cells treated independently with IL-1β, TNF-α, or IFN-γ compared with untreated cells (Fig. 1). Reporter activity of cells treated with IL-1β and TNF-α in combination was not significantly different from that in cells treated with each cytokine singly, although combined treatment with IL-1β and IFN-γ resulted in additive stimulation of MUC1 promoter activity. Most notably, a robust stimulation of MUC1 promoter activity (>15-fold) was observed in cells treated with TNF-α in the presence of IFN-γ. The apparent synergy of TNF-α and IFN-γ upon MUC1 promoter activity was not significantly enhanced by IL-1β. Similar treatments had no effect on reporter activity of an unrelated promoter (for the thymidine kinase (TK) gene) in the same vector (data not shown). In transient transfections of T47D cells with the 1.4MUC promoter treated with various concentrations of either TNF-α or IFN-γ, maximal stimulation of MUC1 transcriptional activity was observed in the presence of 25 ng/ml TNF-α and 200 U/ml IFN-γ (data not shown). MUC1 promoter responsiveness to TNF-α and IFN-γ, singly and in combination, was observed within 3 h of treatment, reaching a peak at 24 h (data not shown). For these reasons, 25 ng/ml TNF-α and 200 U/ml IFN-γ were used to treat T47D cells for 24 h in all subsequent transient transfections for promoter construct evaluation. Northern blot analysis was performed on RNA from T47D cells to determine whether the changes in promoter activity translated into increases in steady-state levels of MUC1 mRNA. Two differently sized transcripts were observed as a result of allelic polymorphism in the VNTR

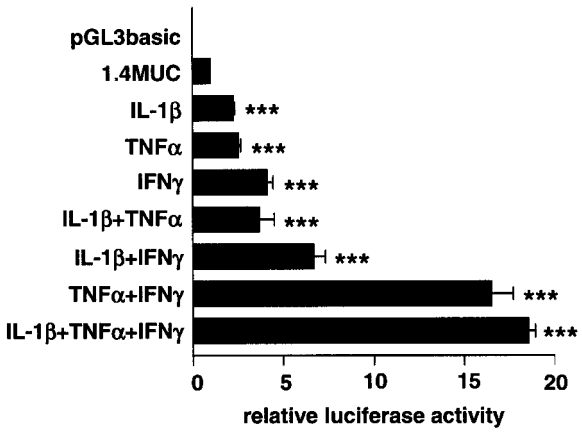

Fig. 1. MUC1 promoter response to proinflammatory cytokines. T47D cells were transiently transfected with control plasmid (pGL3basic) or 1.4 kb MUC1-luciferase (1.4MUC). Cells transfected with 1.4MUC were treated with the indicated cytokines for 24 h (IL-1β, 10 ng/ml; TNF-α, 25 ng/ml; IFN-γ, 200 U/ml). Promoter activity was determined as described in Methods and is expressed as the ratio of firefly luciferase activity (pGL3 plasmids) to the activity of the co-transfected internal control, *Renilla* luciferase. Error bars reflect results of at least two experiments performed in duplicate ±SEM. ***$P<0.001$ versus 1.4MUC in untreated cells by ANOVA and Tukey–Kramer multiple comparisons test.

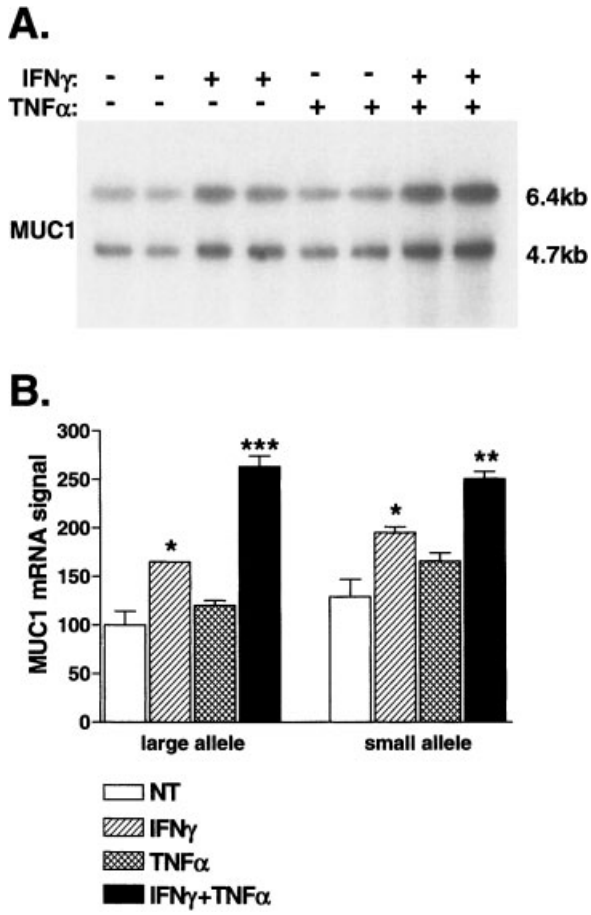

Fig. 2. Northern blot analysis of MUC1 mRNA in T47D cells cultured in the presence or absence of IFN-γ and/or TNF-α. Following a 24 h serum starvation, T47D cells were treated with 200 U/ml IFN-γ and/or 25 ng/ml TNF-α in the presence of 5 % (v/v) serum for 24 h. (**A**) Total RNA was extracted and analyzed (6 μg/lane) for MUC1 mRNA as described in Methods. (**B**) Signal intensities were quantified using the 1D-Multi function of an Alpha Imager and normalized to that of the larger MUC1 transcript in untreated cells. *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus signal of transcript in non-treated (NT) cells.

domain (Fig. 2A). Treatment of the cells with TNF-α alone did not noticeably affect the steady-state level of MUC1 mRNA from either allele, and IFN-γ alone resulted in a mild (approximately 50%) increase in the steady-state level of MUC1 mRNA (Fig. 2B). Combined treatment with TNF-α and IFN-γ increased the level of MUC1 mRNA by 2- to 2.5-fold, a significantly greater increase than with IFN-γ alone but less than predicted by reporter activity in transfected cells. Despite these apparent changes in MUC1 mRNA in response to TNF-α and IFN-γ, similar changes in the level of MUC1 protein were not evident (data not shown).

The high basal level of MUC1 expressed in T47D cells, along with variability in MUC1 promoter responsiveness to IFN-γ in other epithelial cancer cell lines (data not shown), prompted us to examine cytokine responsiveness in normal breast epithelial cells. HMEC, isolated from normal breast tissue and having a finite life span in culture, were treated with TNF-α and IFN-γ, independently and in combination for up to 48 h. Northern blot analysis of HMEC total RNA indicated that MUC1 mRNA was not detectable in either untreated cells or cells treated with TNF-α alone (Fig. 3A). However, transcription of MUC1 was induced by IFN-γ alone, and this induction of expression was further enhanced by TNF-α. The effects of these treatments on MUC1 expression were observed within 12 h (data not shown). Comparison of the combined signals of both transcripts indicates that the apparent TNF-α-induced enhancement of the steady-state level of MUC1 mRNA in IFN-γ-treated cells is twofold, although when compared to untreated cells, the overall increase in MUC1 mRNA is essentially infinite. Separate analyses revealed that the level of the larger, less abundant transcript in IFN-γ-treated cells was increased fourfold by TNF-α, as compared to a 50% increase in the smaller transcript (Fig. 3B). Western blot analysis of HMEC total

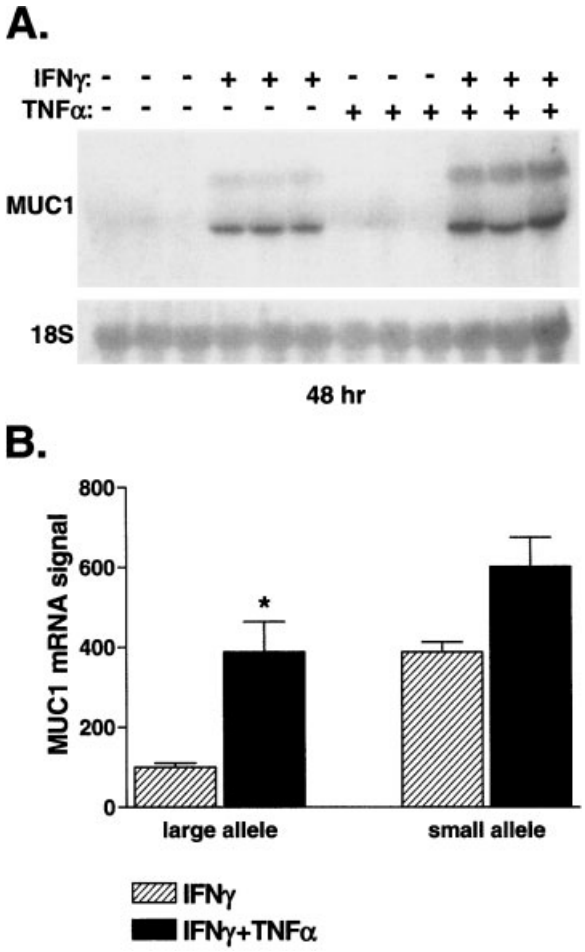

Fig. 3. MUC1 mRNA in HMEC cultured in the presence of IFN-γ and/or TNF-α. HMEC were treated with 200 U/ml IFN-γ and/or 50 ng/ml TNF-α for the indicated times. (**A**) Total RNA was extracted and analyzed (10 μg/lane) for MUC1 mRNA or 18S ribosomal RNA as described in Methods. (**B**) Signal intensities were quantified using the 1D-Multi function of an Alpha Imager and normalized to the larger MUC1 transcript in IFN-γ-treated cells. $*P = 0.0200$ versus signal of transcript in IFN-γ-treated cells by unpaired *t*-test.

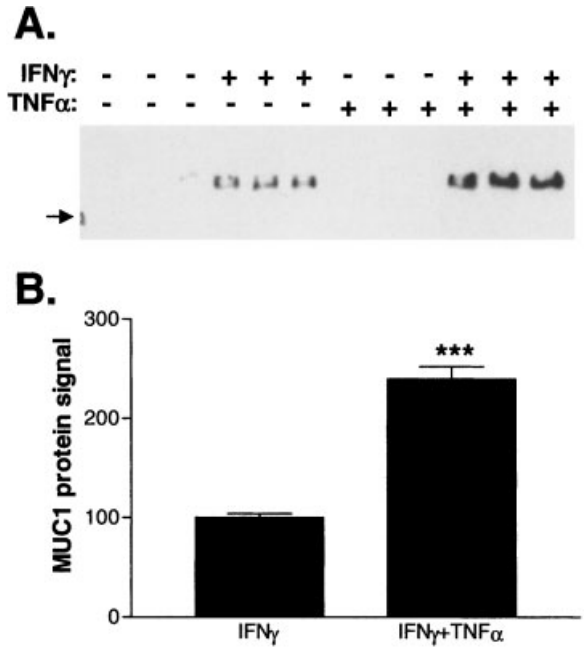

Fig. 4. Western blot analysis of MUC1 in HMEC cultured in the presence of IFN-γ and/or TNF-α. HMEC were treated with 200 U/ml IFN-γ and/or 50 ng/ml TNF-α for 48 h. (**A**) Total cellular protein (50 μg/lane) was analyzed for changes in MUC1 expression using the 214D4 antibody. The migration position of myosin (202 kDa) is indicated by the arrow. (**B**) Signal intensities of cell-associated MUC1 were quantified using the 1D-Multi function of an Alpha Imager and normalized to that in IFN-γ-treated cells. $***P=0.0004$ versus MUC1 signal in IFN-γ-treated cells by unpaired *t*-test.

cellular protein demonstrated induction of MUC1 expression in response to IFN-γ alone that was further enhanced by co-treatment of the cells with TNF-α (Fig. 4A). MUC1 protein levels were increased by TNF-α at least 2.5-fold over that observed in cells treated with IFN-γ alone (Fig. 4B). After prolonged exposure, induction of MUC1 by TNF-α alone was also detectable (data not shown).

The Mechanism of IFN-γ Stimulation of MUC1 Expression is Conserved Between Breast Cancer Cells and Normal Mammary Epithelia

We determined that the *cis*-acting element required for stimulation of the MUC1 promoter by IFN-γ in T47D cells was the STAT-binding element at −503/−495, and this finding was independently confirmed [Lagow and Carson, 1999; Gaemers et al., 2001]. However, IFN-γ responsiveness of MUC1 at the level of transcript differed greatly between T47D cells and HMEC. For this reason, we examined HMEC to determine whether STAT1α also mediated induction of MUC1 expression by IFN-γ in normal breast epithelial cells using electrophoretic mobility shift assays (Fig. 5). A 29-bp double-stranded oligonucleotide encompassing the STAT-binding element from the MUC1 promoter (−513/−485) and a control SIE oligonucleotide were used to probe nuclear extracts prepared from untreated HMEC and T47D cells or cells treated with 200 U/ml IFN-γ for 15 min. No complexes were observed with nuclear extracts from untreated HMEC or T47D cells (lanes 1 and 7); however, incubation of the MUC1 or SIE probe with extracts from IFN-γ-treated cells or control extracts resulted in distinct protein–DNA complexes that migrated

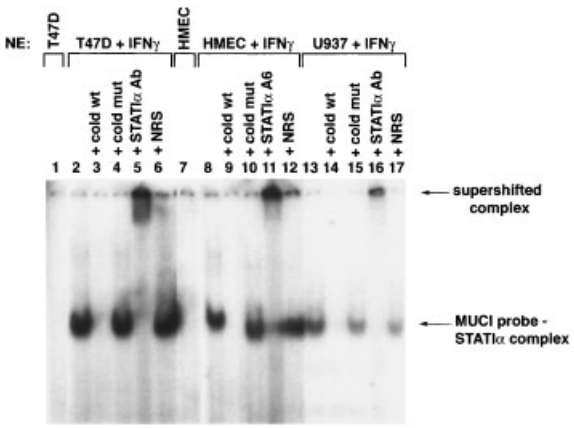

Fig. 5. Electrophoretic mobility shift assay of the MUC1 promoter STAT-binding element and nuclear extracts from T47D and HMEC cells. A 29-bp end-labeled fragment of the MUC1 promoter (−513/−485) or a control STAT-inducible element (SIE) was incubated with 5 μg of T47D or HMEC nuclear protein. The SIE probe was also incubated with positive control nuclear protein from IFN-α-treated U937 cells. Unlabeled wild-type (wt) or mutated (mut) fragments were added at a 100-fold molar excess compared to labeled probes. For supershift analysis, rabbit polyclonal anti-STAT1α or normal rabbit serum (NRS) was preincubated with nuclear proteins before addition of probe (1×10^5 cpm/reaction).

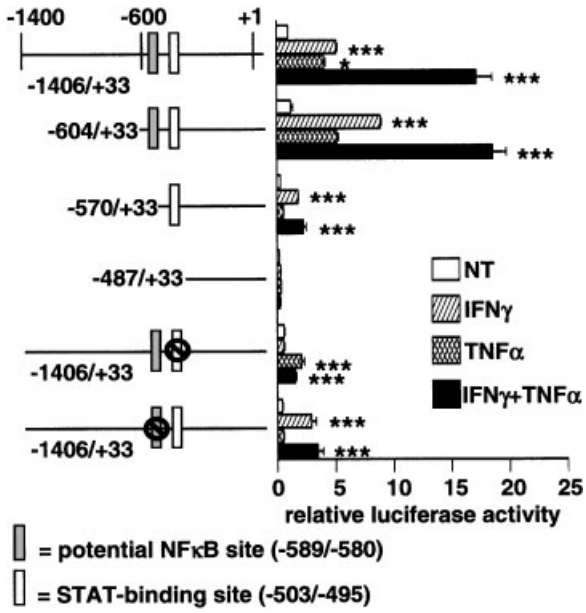

Fig. 6. Synergistic response of the MUC1 promoter to IFN-γ and TNF-α requires a κB site at −589/−580 and the STAT-binding site at −503/−495. T47D cells were transiently transfected with the full-length MUC1 promoter construct, 1.4MUC, or the indicated mutant constructs and subsequently treated with 200 U/ml IFN-γ and/or 25 ng/ml TNF-α for 24 h. All promoter activities were normalized to that of 1.4MUC in untreated cells. Error bars reflect results of at least two experiments performed in triplicate ± SEM. $^*P<0.05$, $^{***}P<0.001$ vs. activity in non-treated (NT) cells by ANOVA and Tukey–Kramer multiple comparisons test.

similarly (lanes 2, 8, and 13). Inclusion of excess unlabeled wild-type oligonucleotide in the binding reaction completely prevented complex formation, demonstrating specificity of the bound factor(s) for the STAT-binding element (lanes 3 and 9). Unlabeled mutant oligonucleotide did not affect complex formation (lanes 4 and 10). Pre-incubation of nuclear extracts with a rabbit polyclonal STAT1α antibody, but not NRS, further retarded the mobility of the complex observed with extracts from IFN-γ-treated cells, demonstrating that STAT1α was a component of the complex in the cancer cell line and in normal mammary epithelia (lanes 5, 6, 11, and 12).

Transcriptional Synergy by IFN-γ and TNF-α Requires the κB Site at −589/−580 and the STAT-Binding Site at −503/−495 in the MUC1 Promoter

We assessed the requirement of *cis*-acting elements in the MUC1 promoter for mediating synergistic responses to IFN-γ and TNF-α, initially employing deletion analyses and transient transfection assays (Fig. 6). The −604/+33 construct retained the full synergistic response to IFN-γ and TNF-α, indicating that the *cis*-acting elements necessary for this response were present. Deletion to −570, excluding a potential κB site at −589/−580, but retaining the STAT-binding site at −503/−495, reduced basal promoter activity by 60%. The response of the −570 construct to IFN-γ was approximately fivefold over that in untreated cells; however, TNF-α responsiveness was eliminated. Additionally, the magnitude of the response to IFN-γ in the presence of TNF-α was not significantly greater than with IFN-γ alone. Further deletion to −487/+33 eliminated both TNF-α and IFN-γ responsiveness as well as 75–80% of the basal promoter activity. Specific mutation of the STAT-binding element at −503/−495 abolished IFN-γ responsiveness and prevented IFN-γ/TNF-α synergy; however, a three to fourfold response to TNF-α alone was retained, similar in degree to that observed with the intact 1.4 kb promoter. Likewise, specific mutation of the κB site at −589/−580 eliminated TNF-α responsiveness, but retained a five to sixfold response to IFN-γ, and promoter activity in the presence of both TNF-α and IFN-γ was not greater than with IFN-γ alone. Furthermore, specific mutation of either the STAT site or the κB site reduced basal MUC1 promoter activity by 45–50%.

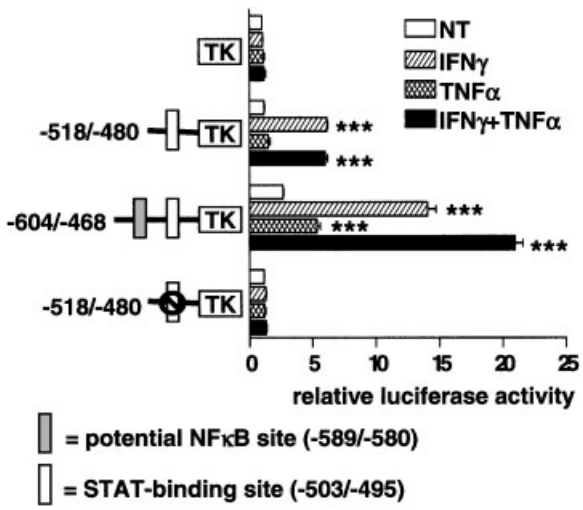

Fig. 7. The MUC1 promoter STAT-binding element is necessary but not sufficient for transcriptional synergy by IFN-γ and TNF-α. Fragments of the MUC1 promoter (−518/−480) containing a wild-type (TTCCGGGAA) or mutated (ccCCGGGAA) STAT-binding element, and a fragment containing both the STAT-binding element and a potential κB site (−604/−468), upstream of the TK promoter in pGL3basic were transfected into T47D cells treated with 200 U/ml IFN-γ and/or 25 ng/ml TNF-α for 24 h. Construct activities were normalized to that of the vector, pGL3-TK, in untreated cells. Error bars reflect results of at least two experiments performed in triplicate ±SEM. ***$P<0.001$ versus activity in non-treated (NT) cells by ANOVA and Tukey–Kramer multiple comparisons test.

Heterologous promoters containing a wild-type or mutant STAT-binding element (−518/−480) upstream of the TK promoter were also used in transient transfections of T47D cells to assess the response to TNF-α and IFN-γ (Fig. 7). The construct containing a wild-type STAT-binding element displayed no responsiveness to TNF-α alone, and in cells treated with both IFN-γ and TNF-α, the promoter activity was not significantly greater than with IFN-γ alone. Inclusion of additional 5′ sequence in the −604/−468 construct, containing both the potential κB site at −589/−580 and the STAT-binding site at −503/−495, not only conferred responsiveness to TNF-α alone upon the TK promoter, but also a synergistic response to TNF-α and IFN-γ. As expected, specific mutation of the STAT-binding site completely abolished responsiveness to IFN-γ, and activity of this construct was not affected by TNF-α.

TNF-α Induces Binding of NFκB to the MUC1 Promoter κB Site

We assessed function of the putative κB site in TNF-α regulation of MUC1 transcription using electrophoretic mobility shift assays. MUC1 promoter sequence from −599 to −570 was used to probe nuclear extracts from T47D cells that were treated for 15 min with 25 ng/ml TNF-α in the presence or absence of 200 U/ml IFN-γ (Fig. 8). Several complexes of different mobilities were observed and were numbered in order of slowest to fastest mobility. Complex 1 (C1) was observed with all T47D nuclear extracts tested, regardless of treatment; however, C2 and C3 were specifically formed with nuclear extracts from T47D cells that had been treated with TNF-α (Fig. 8A; lanes 3 and 4). The

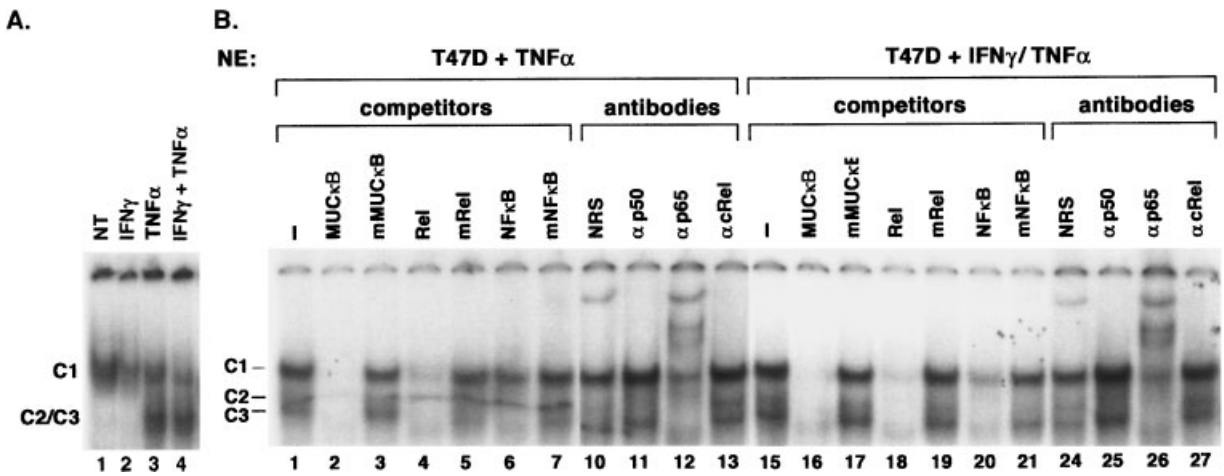

Fig. 8. Electrophoretic mobility shift assay of the MUC1 promoter potential κB site and nuclear extracts from T47D cells. (**A**) A 30-bp end-labeled fragment of the MUC1 promoter (−599/−570) was incubated with 5 μg of nuclear extract protein from T47D cells treated with or without 25 ng/ml TNF-α ± 200 U/ml IFN-γ for 15 min. (**B**) Unlabeled wild-type or mutant (m) oligonucleotides were added at a 100-fold molar excess compared to labeled probe in competition assays as described in Methods. For supershift analysis, nuclear extracts were preincubated with NRS or rabbit polyclonal antibodies to NFκB p50, NFκB p65, or c-Rel for 20 min before addition of probe ($>1\times10^5$ cpm/reaction). Observed complexes were labeled as C1–C3.

intensities of C2 and C3 were similar in reactions containing nuclear extracts from cells treated with TNF-α in the presence or absence of IFN-γ, indicating that the bound factor(s) were not induced to bind differently upon the addition of IFN-γ. In competition assays, excess unlabeled MUCκB, but not a corresponding mutant sequence, mMUCκB, completely abolished C1, C2, and C3, although a very faint band was still observed migrating slightly faster than C3 (Fig. 8B, lanes 2, 3, 16, and 17). Excess unlabeled Rel control oligonucleotide (a binding site for c-Rel and NFκB p65), but not a corresponding mutated sequence, competed similarly (lanes 4, 5, 18, and 19); however, neither excess unlabeled NFκB control (a binding site for NFκB p50) nor its mutated control, competed for binding (lanes 6, 7, 20, and 21).

Supershift assays were performed with a series of antibodies specific for several NFκB/Rel family members to determine the identity of the bound factor(s). Preincubation of nuclear extracts with NRS did not affect the mobility of any of the complexes, but did yield a nonspecific band of slower mobility than C1 (Fig. 8B, lanes 10 and 24). Antibodies for NFκB p50 (NFκB1) and c-Rel did not retard the mobility of any complex (lanes 11, 13, 25, and 27); however, in addition to generating the nonspecific band observed with NRS, antiserum for NFκB p65 (RelA) partially shifted C1 and completely shifted C2 and C3, identifying p65 as a component of these complexes (lanes 12 and 26).

DISCUSSION

Studies of MUC1 transcriptional regulation so far have relied heavily on the use of transformed cell lines. Breast and pancreatic cancer cell lines in which MUC1 promoter analyses have been conducted overexpress MUC1, indicating gene regulation gone awry. While MUC1 promoter studies in cancer cell lines are informative, comparison with normal, untransformed cells are needed to provide insight as to where regulatory aberrations may occur. We have demonstrated that MUC1 expression is amplified by TNF-α in the presence of IFN-γ, modestly in T47D breast cancer cells and robustly in normal HMEC. At the transcriptional level, this regulation requires the independent actions of STAT1α and NFκB on their respective binding sites at −503/−495 and −589/−580 in the MUC1 promoter. Given that the binding of NFκB to the MUC1 κB site was not enhanced by the addition of IFN-γ to the TNF-α treatment, the transcriptional synergy appears not to be mediated by cooperative binding of these transcription factors. In addition, the MUC1 STAT-binding site did not compete out any complexes formed by the MUC1 κB site incubated with nuclear extracts from IFN-γ/TNF-α-treated cells, and an antibody for STAT1α did not affect any of the complexes (data not shown), demonstrating that the synergy is mediated by the independent actions of STAT1α and NFκB on their respective sites in the MUC1 promoter and subsequent cooperative action of these factors upon basal transcription elements.

We found that the steady-state levels of MUC1 mRNA in T47D cells treated with IFN-γ in the presence or absence of TNF-α did not reflect promoter activity as measured in transient transfection assays. Several factors might potentially account for these discrepancies. Despite the fact that overexpression of MUC1 in mammary tumors was observed using the same 1.4 kb MUC1 promoter [Graham et al., 2001], additional regulatory elements present in the endogenous MUC1 gene may account for the high basal level of MUC1 expressed by T47D cells. Therefore, activity of the 1.4 kb MUC1 promoter in T47D cells may be considerably less than that of the endogenous promoter so that the basal level of promoter activity appears to be lower, allowing for greater apparent stimulation by proinflammatory cytokines. Conversely, further stimulation of MUC1 expression may be dependent on the basal level of transcription in the cell line being studied. In this regard, T47D cells have been reported to express very high basal levels of MUC1 relative to other breast cancer cell lines examined [Walsh et al., 1999].

In contrast to the modest cytokine responsiveness of MUC1 in T47D cells, robust induction of expression was observed in the normal mammary epithelial cells treated with IFN-γ alone or in combination with TNF-α, resulting from transcriptional stimulation. However, we noted that the degree of MUC1 induction in HMEC by IFN-γ alone appeared to increase with increasing passage number of the cells. In earlier passage HMEC, the difference in MUC1 protein level in cells treated with IFN-γ alone versus IFN-γ and TNF-α was more pronounced relative to the difference in the level of transcript, suggesting the potential involvement of post-transcriptional mechanisms in addition to

transcriptional mechanisms in normal cells. Several mechanisms for IFN-γ/TNF-α synergy have been reported, including cross-regulation of receptors, enhancement of STAT1 activity by TNF-α, cooperation between STAT1α or IRF-1 and NFκB, and IFN-γ augmentation of NFκB activation via rapid IκB degradation [Sanceau et al., 1995; Cheshire and Baldwin, 1997; Ohmori et al., 1997; Lee et al., 2000]. Augmentation of NFκB or STAT binding falls within the realm of transcriptional control and would be most likely be detectable in gel shift assays. MUC1 mRNA was detectable in HMEC after 12 h of treatment with IFN-γ alone or in combination with TNF-α, and although earlier timepoints were not examined in these cells, increased promoter activity was observed within 3 h of treatment in T47D cells. In this respect, synthesis of additional factors that may participate in the regulation is unlikely. The discrepancy between the level of MUC1 protein and mRNA observed in early passage HMEC treated with TNF-α and IFN-γ might be explained by increased protein stability as a result of increased translation or decreased degradation. Factors potentially affecting the rate of degradation may include post-translational modification, such as phosphorylation or glycosylation, or association with other proteins. Treatment of primary cultures of rat mammary epithelial cells or a rat mammary carcinoma cell line, 13,762, with TGFβ1 results in differences in post-transcriptional regulation of another transmembrane mucin, SMC/Muc4 [Price-Schiavi et al., 1998]. Thus, post-transcriptional regulation may modulate expression of multiple mucins.

Glycosylation of MUC1 also is regulated and can vary within a tissue [Aplin et al., 1998; Hanisch and Muller, 2000]. Whether this post-translational modification of MUC1 is affected by the cytokine treatments described has not yet been addressed. The apparent difference in MUC1 protein levels in HMEC treated with IFN-γ alone versus IFN-γ and TNF-α might reflect differential recognition by the 214D4 antibody; however, this would be true only if a particular treatment induced a glycoform switch, i.e., IFN-γ and TNF-α treatment together producing an underglycosylated, more recognizable, form of MUC1 in contrast to treatment with IFN-γ alone. Comparison of 214D4 with other antibodies directed toward the MUC1 tandem repeats [Xing et al., 1989], DF3 [Perey et al., 1992], HMFG1, HMFG2 [Taylor-Papadimitriou et al., 1981; Burchell et al., 1983], and SM3 [Burchell et al., 1987] indicated that unlike most, 214D4 appears to recognize MUC1 protein independently of glycosylation state (J. Julian, unpublished observations).

At first glance, the STAT-binding element at −503/−495 appeared to be important for constitutive as well as stimulated MUC1 expression, as specific mutation of this element reduced promoter activity by 40–50% in T47D cells [Lagow and Carson, 1999; Gaemers et al., 2001]. Nonetheless, the STAT element did not bind components of nuclear extracts from unstimulated T47D cells in gel shift assays. Furthermore, unpublished studies conducted in our lab demonstrated that MUC1 expression was unchanged by overexpression of STAT1α in T47D cells, indicating that the high basal level of MUC1 expression in T47D cells was not accounted for by the action of this factor upon the STAT-binding element. Conversely, the κB element may be involved in constitutive activation of the MUC1 promoter. Regardless of treatment, a prominent complex was observed in gel shift assays of the MUC1 κB site and nuclear extracts from T47D cells. Constitutively active NFκB has been reported in some non-epithelial, normal cell types [May and Ghosh, 1997], although NFκB DNA-binding activity is more often induced. Studies of the MUC1 κB site in normal mammary epithelial cells are currently underway to determine whether constitutive activation of this element is a normal feature of MUC1 transcriptional regulation. When analyzing nuclear extracts from T47D cells, the constitutive complex of slowest mobility (C1) was specifically competed by excess unlabeled wild-type but not the mutated MUC1 κB site, indicating binding specificity. An antibody to NFκB p65 partially shifted complex C1 in addition to the complete shift of induced complexes C2 and C3, indicating that p65 may participate in both basal and TNF-γ-stimulated MUC1 transcription. Besides forming homodimers, NFκB p65 has been shown to form heterodimers with c-Rel and p50 [May and Ghosh, 1997]; however, supershift assays did not indicate involvement of these factors in the constitutive or TNF-α-induced complexes. As C1 was not an induced complex, additional assays with antibodies for other NFκB/Rel family members were not performed.

Overexpression of MUC1 is a common characteristic of many human epithelial-derived cancers including those of the breast, ovary, and pancreas, and malignant transformation is associated with abnormal regulation of MUC1 expression at multiple levels [Abe and Kufe, 1990; Gendler et al., 1990; Bieche and Lidereau, 1997; Waltz et al., 1998]. In contrast to the apically-restricted, highly glycosylated molecule observed in normal luminal epithelial cells, MUC1 in tumor cells is expressed over the entire plasma membrane [Hilkens et al., 1992], accumulates in the cytoplasm [Peterson et al., 1990; Rahn et al., 2001], and is drastically underglycosylated and antigenically distinct in tumor cells [Girling et al., 1989], so that humoral and cell-mediated immune responses are elicited; however, these responses are often insufficient to destroy the tumor cells [Jerome et al., 1991; van de Wiel-van Kemenade et al., 1993; Kotera et al., 1994]. The demonstration that MUC1 expressed by cancer cells inhibits T-cell proliferation or activation may provide a mechanistic explanation for the ineffectiveness of the immune response [Gimmi et al., 1996; Agrawal et al., 1998b; Chan et al., 1999, 2000]. In this context, persistent cytokine stimulation of breast tumor cells may result in a positive feedback loop promoting tumor cell survival through overexpression of MUC1.

In conclusion, we report that TNF-α amplifies MUC1 expression in IFN-γ-treated breast cancer cells and to a much greater degree in normal mammary epithelial cells. At the transcriptional level, we show that this concerted regulation requires the independent actions of NFκB p65 and STAT1α upon their respective binding sites at $-589/-580$ and $-503/-495$ in the MUC1 promoter. Additionally, our findings indicate that post-transcriptional mechanisms also may be involved in TNF-α/IFN-γ regulation of MUC1 expression in the normal cells. The discrepancy between MUC1 promoter activity and expression in T47D cells treated with IFN-γ in the presence or absence of TNF-α suggests that elements outside of the 1.4 kb 5′ flanking region override these normal controls in tumor cells.

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