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Up-regulation of human mucin MUC2 gene transcription by Pseudomonas aeruginosa LPS in the pathogenesis of cystic fibrosis lung disease

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UP-REGULATION OF HUMAN MUCIN MUC2 GENE TRANSCRIPTION BY PSEUDOMONAS AERUGINOSA LPS IN THE PATHOGENESIS OF CYSTIC FIBROSIS LUNG DISEASE

by

Jian-Dong Li, M.D.

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

This thesis is dedicated to

my wife Haidong Xu

PREFACE

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I would like to thank Dr. Carol B. Basbaum for being an excellent mentor and teacher and most especially for being a friend. Her never ending encouragement, support and guidance got me through the roughest times of uncertainty and indecision.

I thank my thesis committee members, Dr. Jerry Cunha, Dr. Sindy Mellon and Dr. Keith Yamamoto for their invaluable advice, scientific discussion and critical reading of my thesis. Dr. Jerry Cunha, chairman of the dissertation committee and qualifying committee, deserves special mention for his persistent inspiration and encouragement.

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I thank my friends Peilin Zhang, Huai-Hu Chuang and Weijun Feng at UCSF for their stimulating discussion and friendship.

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UPREGULATION OF HUMAN *MUC 2* MUCIN GENE TRANSCRIPTION BY *P. AERUGINOSA* IN THE PATHOGENESIS OF CYSTIC FIBROSIS AIRWAY DISEASE

by

Jian-Dong Li, M.D.

ABSTRACT

An unresolved question in CF research is how mutations of CFTR, a Cl ion channel, cause airway mucus obstruction leading to fatal lung disease. Recent evidence has linked the CFTR mutation to the onset and persistence of *Pseudomonas (P.) aeruginosa* infection in the airways and we now provide evidence directly linking *P. aeruginosa* infection to mucus overproduction. We show, by *in situ* hybridization analysis, that *MUC 2* mucin mRNA levels are highly elevated in CF airways and that controlled *in vitro* exposure to *P. aeruginosa* up-regulates *MUC 2* mRNA in human airways. *P. aeruginosa* LPS plays an important role in *P. aeruginosa*-induced *MUC 2* upregulation. This upregulation is mediated by inducible DNA enhancer elements and blocked by the tyrosine kinase inhibitors genistein, tyrphostin AG 126 and PP1, and also by PD98059. Additional studies indicated that *P. aeruginosa* upregulates *MUC 2* transcription via activation of signal transduction pathway involving $PP60^{c\text{-}Src}$, Ras, Raf-1, MEK1/2 and MAPK (ERK1/2). Inhibition of *P. aeruginosa*-induced *MUC 2* upregulation by the oxygen radical scavenger DMTU and antioxidant NAC indicated the involvement of

oxygen radicals. CF epithelial cells were not hypersusceptible to mucin induction by *P. aeruginosa*. These findings bring new insights to our understanding of CF pathogenesis and suggest that the attenuation of mucin production by LPS antagonists, tyrosine kinase inhibitors and oxygen radical scavengers could reduce morbidity and mortality in this disease.

Gerald R. Cunha, Ph.D.
Dissertation Committee Chair

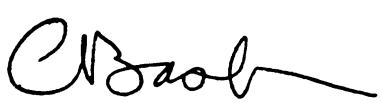
PREVIOUSLY PUBLISHED MATERIALS AND CO-AUTHORSHIP

Portion of this thesis has been previously published in the following paper:

Jian-Dong Li, Austin F. Dohrman, Marianne Gallup, Susumu Miyata, James R. Gum, Young S. Kim' Jay A. Nadel, Alice Prince, and Carol Basbaum: Transcriptional activation of mucin by P. aeruginosa LPS in the pathogenesis of cystic fibrosis lung disease. *Proc. Natl. Acad. Sci. U.S.A.* 94:967-972, 1997. (For commentary, please see S. Sternberg: Cystic fibrosis puzzle coming together. *Science News* 151:85, 1997).

Except for a portion of the experiments described in Figure 1, 2 and 5A, all the experiments and data analysis described in this paper were performed by the first author. Carol Basbaum served as the primary advisor for this research. Austin Dohrman, Marianne Gallup and Susumu Miyata provided some technical assistance in the experiments described in Figure 1, 2 and 5A. James Gum and Young Kim provided the 5'-flanking region of MUC2 and the HM3 cell line. Jay Nadel provided the human bronchial explants and made some suggestions to the experiment described in Figure 1. Alice Prince provided all the P. aeruginosa strains and made some suggestions in experiment described in Figure 4A. This paper was co-written by the first author and Carol Basbaum.

Thesis Research Advisor



Carol Basbaum, Ph.D.

Professor in Biomedical Sciences Graduate Program, Department of Anatomy and Cardiovascular Research Institute, UCSF

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

1. Overview

The CF gene mutation causes dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR), a Cl ion channel. Ninety-five percent of the morbidity and mortality associated with this mutation arise from lung disease characterized by chronic infection and airway mucus obstruction (1). The link between the mutation and its lethal sequelae is unknown. Recently some insight has emerged from findings indicating that the CFTR mutation is linked to three abnormalities favoring the onset and persistence of *P. aeruginosa* infection in the lung: (a) undersialylated cell surface glycolipids that act as *P. aeruginosa* binding sites (2), (b) impaired capacity for bronchial epithelial cells to clear *P. aeruginosa* by endocytosis (3) and (c) decreased activity of bronchial bacteriolytic substances due to abnormal airway surface liquid (4). Clinically, the onset of *P. aeruginosa* infection in the CF lung presages airway mucus obstruction and an overall deterioration of lung function. How this occurs is unknown. Here we show that *P. aeruginosa* LPS, a molecule commonly known to stimulate host defense responses in hematopoietic cells, is a potent stimulus of mucin transcription in epithelial cells. Thus, once airway infection has occurred, *P. aeruginosa* LPS is an indwelling stimulus for exaggerated airway mucin synthesis. In the underhydrated CF airway lumen (5), it is not surprising that exaggerated mucin synthesis leads to airway mucus obstruction. We hypothesize that the pathogenesis of CF lung disease proceeds in two stages: first, the increased susceptibility of *P. aeruginosa* infection as a direct consequence of CFTR gene mutation and second, the overproduction of, and airway plugging by, mucin as a consequence of *P. aeruginosa* infection.

2. Hypothesis and specific aims

The hypothesis underlying this work is that the common opportunistic pathogen *P. aeruginosa* upregulates airway *MUC 2* mucin transcription in cystic fibrosis airway disease through specific regulatory mechanisms extending from very upstream intracellular signal transduction molecules to DNA cis- and trans- regulatory elements in the nucleus. To identify these mechanisms, I will:

Specific Aim I. Compare *MUC 2* mucin mRNA levels in non-CF vs CF airway tissue, study the effects of *P. aeruginosa* on mucin *MUC 2* expression at the mRNA level in human CF vs non-CF bronchial explants and epithelial cells, and study the effect of *P. aeruginosa* on *MUC 2* transcription in epithelial cells.

Specific Aim II. Identify the component in the exoproducts of *P. aeruginosa* responsible for *MUC 2* upregulation.

Specific Aim III. Determine whether MUC2 is hyperinducible by *P. aerugionsa* in CF epithelial cells.

Specific Aim IV. Identify *P. aeruginosa*-inducible DNA response elements in the *MUC2* mucin gene regulatory region and cognate transcription factors.

Specific Aim V. Identify the intracellular signal transduction pathways involved in *P. aeruginosa*-induced *MUC 2* upregulation.

3. Cystic Fibrosis

Cystic fibrosis is the most common, fatal, inherited autosomal recessive disorder of the Caucasian population occurring with a frequency of 1 in 2000 live births (38). The gene mutation responsible for CF has been identified on the long arm of chromosome 7, band q31 (39-42). CF gene spans about 230 kb of genomic DNA and encodes a protein of 1480 amino acids named the cystic fibrosis transmembrane conductance regulator (CFTR). Deletion of a phenylalanine residue at position 508 of this protein occurs in 68% of patients (43). The structure of CFTR is characterized by twelve transmembrane spans, two nucleotide binding domains and a cytoplasmic regulatory domain. CFTR acts as a cyclic-cAMP-activated chloride channel (38). CF affects a number of epithelial-lined organs, the most important being the lungs which is responsible for most of the morbidity in the disease. The usual cause of death is respiratory failure caused by chronic infection and mucus overproduction in the lung.

Many epithelial tissues are affected by the disease and manifest abnormal chloride transport (44 - 46). The resulting imbalance of chloride and water flux can explain the relative dehydration of secretions characteristic of cystic fibrosis, but still unexplained is the overproduced mucus that obstructs respiratory tract lumina. The link between defective chloride channel transport and the upregulation of mucus production in CF airway disease is unknown.

4. Airway mucin

Mucin is the major protein in airway mucus. Its extensive glycosylation contributes to its ability to form viscoelasic polymer gels that line the airway surface. The mucus

interacts with epithelial cilia to propel entrapped particles (including bacteria and viruses) to the pharynx. Excessive production of mucus occurs not only in cystic fibrosis, but also in chronic bronchitis, asthma and smoke-related airway diseases, overwhelming the normal ciliary clearance mechanism. Mucus then accumulates in the airway and is the site of recurrent infection. Overproduced mucus and the infection will ultimately lead to mortality in cystic fibrosis patients (12). Inspissated mucus is also a regular finding in those dying in status asthmaticus. The mechanisms underlying mucin overproduction in these diseases have remained unknown.

5. *P. aeruginosa* and LPS

Psedomonas aeruginosa is a gram-negative aerobic rod belonging to the family *Paedomonadacene*. It is an opportunistic pathogen that rarely causes airway disease in normal hosts but is an important cause of airway disease in CF patients, and is found in up to 85% of cultures obtained from CF patients. Increased susceptibility is selective not only for *P. aeruginosa* but also for some other gram-negative bacteria such as *Pseudomonas cepacia*, although it is much less common in CF patients than *P. aeruginosa* . *P. aeruginosa* expresses numerous gene products postulated to contribute to lung pathology (6, 13, 20, 55-57). Such virulence factors include pilin, flagellin, *P. aeruginosa* autoinducer, elastase, alkaline protease, neuraminidase, PLC, exotoxin and endotoxin lipopolysaccharide (LPS). LPS is a conserved component of the gram-negative bacterium's outer membrane, each molecule consisting of a hydrophobic lipid A moiety and a complex array of sugar residues. Upon exposure to LPS, macrophages, endothelial and epithelial cells release a number of immunoregulatory molecules including TNF-a, IL-1, IL-6 (24, 25), IL-8 (26) and mucin (27). The mechanism by which LPS induces these events is only partly understood. The effect of LPS on mucin expression is unknown.

6. LPS-activated intracellular signal transduction pathways and transcription factors

Like numerous other extracellular stimuli, LPS activates gene transcription via activation of a cascade of intracellular signaling molecules involving tyrosine kinases, which in turn activate transcription factors. The activated transcription factors bind to DNA response elements in the regulatory regions of genes. Following binding, the bound transcription factors increase transcription either by directly activating RNA polymerase itself or by facilitating the binding of other transcription factors and the assembly of a stable transcription complex. Initiation of transcription requires general transcription factors to be assembled at the promoter before transcription can begin. This assembly process provides multiple steps at which the rate of transcription initiation can be speeded up or slowed down in response to regulatory signals, and many gene regulatory proteins operate by influencing these steps. Many gene regulatory proteins can activate transcription even when they are bound to DNA enhancer regions thousands of nucleotide pairs away from the promoter that they influence, which means that a single promoter can be controlled by an almost unlimited number of regulatory sequences scattered along the DNA.

Among the several commonly known intracellular signal transduction pathways, the classical mitogen-activated protein kinase (MAP kinase) pathway is thought to be most important in transmitting extracellular LPS signals to the cell interior, including the nucleus. Three subgroups of MAPKs have been shown to mediate LPS-induced responses. These include extracellular signal-regulated kinase 1 and 2 (ERK1/2, also called p44/42 MAPK), c-Jun N-terminal kinase (JNK, also called stress-activated protein kinase, SAPK) (28, 29) and p38 MAPK. The classical ERK1/2 MAPK pathway consists of a protein kinase cascade linking growth and differentiation signals

with transcription in the nucleus. Growth factor receptors and tyrosine kinases activate Ras which in turn activates c-Raf, MEK, and ERK1/2 MAPKs (30). Activated ERK1/2 translocates to the nucleus and activates transcription by phosphorylation of transcription factors such as ELK1 and STAT. LPS has been shown to activate ERK1/2 in macrophages (18, 21). The JNK/SAPK pathway is homologous to the classical ERK1/2 MAPK pathway in overall form but appears to be activated by various stimuli (28). Those extracellular stimuli activating JNK pathway include TNF-a, IL-1, UV light and certain growth factors (31). These stimuli first activate MEKK1. Activated MEKK1 phosphorylates SEK (also known as MKK4) which in turn activates JNK/SAPK. JNK binds tightly to the N-terminal region of c-Jun and phosphorylates c-Jun (32). Activation of JNK by LPS has been recently demonstrated in macrophages (29). The p38 MAP kinase (HOG 1 kinase in yeast), together with its upstream and downstream targets, comprise a third MAP kinase pathway. Like the JNK pathway, p38 MAPK is activated by a variety of cellular stresses including: osmotic shock, inflammatory cytokines, UV light and growth factors (33). Activated p38MAPK can activate transcription factors TCF, ATF2 and Max. Activation of p38 MAPK has been shown in mammalian pre-B cell lines (34). In addition to MAP kinase pathways, protein kinase C (PKC)- and protein kinase A (PKA)-dependent pathways have also been shown to play an important role in cellular responses. However, I obtained no evidence suggesting an important role for these pathways in LPS-induced cellular responses.

7. Significance

(1). Cystic fibrosis lung disease is responsible for 95% of the morbidity and mortality in CF patients. It is characterized by chronic airway infection and mucus overproduction. How a defective Cl^- channel causes mucus overproduction is

unknown. Understanding the mechanisms underlying mucin overproduction in CF airways will provide important insight into the pathogenesis of CF airway disease.

(2). Understanding the signal transduction pathways mediating the P. aeruginosa-induced up-regulation of mucin may represent the first step towards developing therapeutics to inhibit mucin overproduction in the CF lung.

(3). LPS is not only involved in CF pathogenesis but also in other chronic obstructive pulmonary disease (COPD) such as chronic bronchitis. Understanding how mucin is regulated by LPS in CF may shed light on the pathogenesis of other COPD diseases.

MATERIALS AND METHODS

Reagents. *P. aeruginosa* lipopolysachharide (LPS) from serotype 10 was purchased from Sigma Chemical Co. (St. Louis, MO). LPS from PAO1 wild-type and PAO-pmm (algC) mutant were purified as described (6). Lipid A and Genistein were also purchased from Sigma Chemical Co. (St. Louis, MO). Tyrphostin AG126, PP1 and PD98059 were purchased from CALBIOCHEM (La Jolla, CA).

Bacterial Strains and Culture Conditions. The *P. aeruginosa* strains used in these studies were grown in M9 medium with aeration at 37°C to late log phase. The broth cultures were then centrifuged at 10,000 rpm for 50 minutes. The supernatants containing bacterial exoproducts were sterilized by passage through a 0.22-μ polymer filter (Corning Glass Works, Corning, NY 14831) and were then kept at -80°C until use. Bacterial culture supernatants were added to epithelial cell culture medium at a 1:4 dilution ratio (13).

Cell Culture. HM3 cells were maintained in Dulbecco's modified Eagles medium. NCIH292 cells were maintained in RPMI 1640 medium. CFTE29O cells were obtained from Dr. D. Gruenert and was maintained in MEM Eagle's with Earle's BSS medium. 16LU cells were maintained in D-MEM/F12 medium. 10% fetal bovine serum was added in all the media.

***In situ* Hybridization Analysis.** The experiments were carried out as described previously (7) and is here described briefly. Tissue preparation-- Human CF bronchial tissue was obtained at lung transplantation from the recipients and non-CF bronchial tissue was obtained from donors. For all experiments reported here, segmental and subsegmental bronchi were used. Slices of bronchial rings (approximately 0.5 mm

long) were prepared within 1 h after transplantation. These human bronchial tissues were rinsed in sterile phosphate-buffered saline (PBS) to remove secretions and incubated in serum-free medium, 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 (DME/F12), supplemented with penicilin (105 U/liter), streptomycin (100 mg/ml), gentamicin (50 mg/ml) and amphotericin B (2.5 mg/ml). The bronchial explants from CF and non-CF individuals were treated with *P. aeruginosa* culture supernatant or vehicle for 6 hours and were then fixed in 4% paraformaldehyde/ 0.1M phosphate buffer for 4 hours and cryoprotected in 30% sucrose/ 0.1M phosphate buffer overnight at 4° C. The next day, samples were embedded in OCT compound and quickly frozen in liquid nitrogen-cooled Freon-22. The frozen tissue was sectioned (6 mm), placed on Superfrost Plus slides (Fisher Scientific, Pittsburgh, Pa.) and air dried quickly. The sections were stored at -80° C until use. RNA probes-- The HAM1 cDNA contained a tandem repeat unit of the *MUC 2* mucin gene. We confirmed by Northern blot that the HAM1 cDNA recognized mRNA transcripts in the human bronchus before using it in in situ hybridization. [^{35}S] UTP-labeled RNA transcripts were synthesized from the cDNAs in linearized pBluescript plasmid using T7 and T3 polymerases to generate antisense and sense probes at concentrations of 2-5 x 10^5 cpm/ml. Frozen sections of human bronchus were air dried quickly, heated at 55° C for 10 min, fixed with 4% paraformaldehyde in PBS for 10 min, washed with 2 x SSC (0.3 M NaCl/0.03 M sodium citrate, pH 7.0), immersed in 0.1 M triethanolamine HCl (pH 7.5) containing 0.25% acetic anhydride for 10 min, rinsed with 2 x SSC, dehydrated with ethanol and air dried. RNA probe was applied in a hybridization mixture containing deionized formamide (50%), dextran sulfate (10%), tRNA (0.5 mg/ml), Ficoll 400 (0.02 % (W/V)), salmon sperm DNA (1 mg/ml), polyvinylpyrrolidone (0.02% (W/V)), 10mM DTT, 0.3 M NaCl, 0.5 mM EDTA, 10 mM Tris-HCL, and 10 mM NaPO4 (pH 6.8). The mixture was heated at 70° C for 15 min and chilled on ice. Fresh DTT was added to achieve a concentration of 20 mM. Then 100 µl of the mixture

was applied to each section, and parafilm coverslips applied. Hybridization was carried out in humid chambers overnight at 55° C. Coverslips were removed in 5 x SSC, 10 mM DTT, at 55° C. Sections were washed three times in wash buffer (2 x SSC, 1 mM EDTA, 10 mM ß-mercaptoethanol (ßME)) for 5 min at room temperature. Subsequently, they were treated with 20 mg/ml of RNase A in 500 mM NaCl, 10 mM Tris (pH 8.0) for 30 min at room temperature. This was followed by 2 x 5 min changes of wash buffer and a high stringency wash in 4 L of a wash solution containing 0.1 x SSC, 1 mM EDTA and 10 mM ßME (2 h at 55° C). Slides were then washed 5 min at room temperature in 0.5 x SSC without ßME and EDTA. Finally, sections were dehydrated with ethanol and air-dried. The slides were exposed to Ilford K5D emulsion, and stored in the dark at 4° C until developed after 3-10 days exposure.

RNase Protection assay (RPA). The experiments were carried out using an RNA probe containing *MUC 2* specific sequence as described previously (8).

Cloning and Sequencing of the 5'-flanking Region of Human MUC 2 Gene, Plasmid Construction, Transfection and Luciferase Assay. The 5'-flanking region of the human *MUC 2* gene was cloned by screening a human placental lFIXII genomic library using the 5'-region of human *MUC 2* cDNA as the probe (9). The 5'-flanking region was sequenced by dideoxy sequencing. Deletional mutants of the 5'-flanking region DNA were obtained by combining restriction digestion of the upstream region of the gene and PCR amplification. The restriction DNA fragments or PCR-amplified fragments were ligated into a luciferase reporter gene. All junctions and identifications of the DNA sequences in the chimeric constructs were confirmed by DNA sequencing. The expression plasmid pREP4.7kbCFTR was obtained from Dr. D. Gruenert. Transfection was performed by a standard electroporation method as described (10). *P. aeruginosa* culture supernatant, LPS and Lipid A was added to the

transfected cells 42 hours after transfection. After 6 hours, the cells were harvested for luciferase assay. All transfections were carried out in triplicate. Luciferase activity was normalized with respect to ß-galactosidase activity.

Electrophoretic mobility shift assays. Nuclear extracts from HM3 and NCIH292 cells were prepared according to (47). Prior to extraction cells were treated with P. aeruginosa culture supernatant for 6 hours. The protein concentration of the cell extract was determined using a BCA protein assay kit (Pharmacia LKB Biotechnology Inc.) using bovine albumin as standard. Different double-stranded oligonucleotide probes as indicated in each experiment were synthesized based on the results from luciferase assay experiment described in RESULTS. The oligonucleotide was labled by [gamma ^{32}P]ATP and T4 polynucleotide kinase and purified on 6% native polyacrylamide gel. The probe was incubated at 37°C for 20 min with nuclear extract (5-10 µg protein) in a solution containing poly dIdC (3 µg) in Hepes-KOH (10 mM, pH 7.9), NaCl (210 mM), $MgCl_2$ (0.75 mM), EDTA (0.1 mM), DTT (0.5 mM), PMSF (0.5 mM) and glycerol (12.5%). For antibody interaction, antibodies specific for CCATT-enhancer binding protein (C/EBP), nuclear factor-kappa B (NF-kappa B), activation protein 2 (AP2) and specificty protein (SP1) were added to the reaction during a 30 min preincubation on ice. Samples were applied to polyacrylamide gels under native conditions in high ionic strength buffer. The dried gel was exposed to X-ray film at -70°C fro 2 -12 h with double intensifying screens.

Purification of LPS. LPS was isolated by a modification of the method of Hitchcock and Brown (48). Strains were harvested in sterile phosphate-buffered saline (PBS) after overnight growth on appropriately supplemented L agar plates. The suspensions were autoclaved and vigorously vortexed, and the cells were pelleted by centrifugation. The supernatants were combined with four volumes of 95% ethanol and

precipitated at -20°C overnight. The precipates were collected by centrifugation and redissolved in sterile PBS. Aliquotes of these preparations were digested with RNAse and DNAse (each at 100 μg/ml) at 37°C overnight and then with pronase (100 μg/ml) for 2 h at 56°C (enzymes were purchased from Sigma) and stored at -20°C until analysis.

SDS-PAGE, Electroblotting and Immunological Detection. Gels for sodium dodecyl sulfate-polyacylamide gel electrophoresis (SDS-PAGE) were run essentially as described by Laemmli (49), using a BIORAD apparatus. SDS-PAGE analyses were performed with precast SDS-12% polyacrylamide gels. To analyze LPS core samples a two-buffer Trycine-based SDS-PAGE system was employed (50, 51). Tricine gels were cast as 18% separating gels. Before analysis by SDS-PAGE, an aliquot of the LPS sample was combined with an equal volume of 2X sample buffer (125 mM Tris, 4.1% SDS, 0.001% bromophenol blue) heated to 65°C for 15 to 30 min. LPS samples separated by SDS-PAGE were transferred to Duralon-UV uncharged nylon membrane. The membranes were air dried and blocked by overnight incubation in blocking solution (5% milk, 10% PBS, 0.005% thimerosal). The blocked membrane were rinsed three times with PBS containing 0.055% Tween 20. The membranes were incubated in polyclonal rabbit antisera (raised against purified P. aeruginosa LPS of the appropriate serotype) diluted 1:400 in PBS containing 10% blocking solution. After being rinsed as described above, the membrane were further incubated for 2 h in alkaline phosphate-conjugated goat anti-rabbit IgG heavy- and light-chain antibodies diluted 1:2,000. Secondary antibody dilution was made in the blocking solution supplemented with 0.005% Tween 20. The membrane was rinsed three times and developed using ECL kit.

RESULTS

SPECIFIC AIM I. COMPARE MUCIN *MUC 2* mRNA LEVEL IN NON-CF VS CF AIRWAY TISSUE, STUDY THE EFFECTS OF *P. AERUGINOSA* ON MUCIN *MUC 2* EXPRESSION AT THE mRNA LEVEL IN HUMAN CF AND NON-CF BRONCHIAL EXPLANTS AND EPITHELIAL CELLS, AND STUDY THE EFFECT OF *P. AERUGINOSA* ON *MUC 2* TRANSCRIPTION IN EPITHELIAL CELLS.

Mucin *MUC 2* mRNA level is highly elevated in CF airways.

As a first step in evaluating the possibility that *P. aeruginosa* upregulates mucin synthesis in CF airways, we monitored airway mucin mRNA levels in CF patients vs controls. Using a probe for the human *MUC 2* mucin gene (9, 11), we showed that a greater proportion of cells express *MUC 2* in CF than in non-CF airways and the level of expression per cell was higher (Fig. 1 A, C, E and G). The results shown in Fig. 1 are typical of 4/4 CF and 4/4 non-CF individual cases and suggest that factor(s) present in the CF airway upregulate *MUC 2* mRNA. As all CF tissues we examined were infected with *P. aeruginosa*, this Gram-negative bacterium that chronically colonizes the airways of approximately 80% of CF patients (12) is a potential source of such factors.

P. aeruginosa upregulates *MUC 2* mRNA in airways.

We directly tested the hypothesis that *P. aeruginosa*-associated factors upregulate mucin mRNA by exposing human bronchial explants to *P. aeruginosa* (strains PAO1 and PA103) culture supernatant or vehicle control (6 hours) and then performing *MUC 2 in situ* hybridization. We found that *P. aeruginosa* culture supernatant greatly increased *MUC 2* expression in both the surface epithelium and submucosal glands of the non-CF explants (Fig. 1, A to H) confirming that *P. aeruginosa*-associated factors

do upregulate *MUC 2* mRNA. The constitutive expression of *MUC 2* mRNA in CF bronchial explants was high relative to that in non-CF bronchial explants and the CF explants showed relatively small increments in *MUC 2* expression in response to exogenous *P. aeruginosa*. This may indicate that *MUC 2* expression had been stimulated near maximally by endogenous factors in the CF explants.

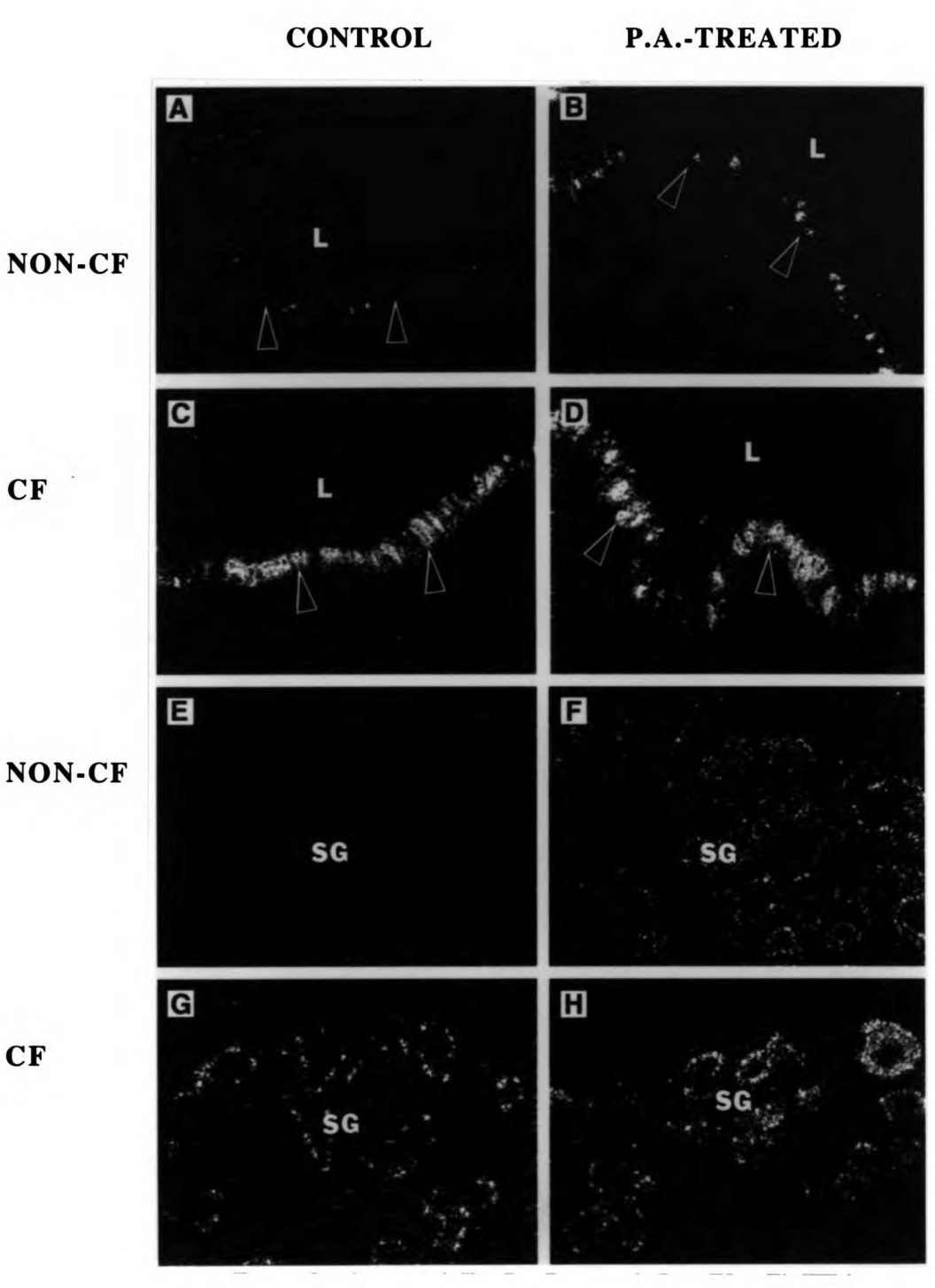
P. aeruginosa directly upregulates *MUC 2* mRNA in epithelial cells.

In explant tissue, *MUC 2* upregulation could have resulted from either a direct effect of bacterial products on epithelial cells or an indirect effect mediated by resident inflammatory cells. To test the hypothesis that the effect was direct, we applied *P. aeruginosa* culture supernatant to cultured epithelial cells and measured *MUC 2* mRNA by RNase protection assay. Because we were interested in the potential generality of the effect, we assayed a variety of epithelial mucin-expressing cell lines. Results from NCIH 292 (human airway epithelial) and HM3 (human colon epithelial) cells are shown in Fig. 2. The *P. aeruginosa* culture supernatant increased *MUC 2* mRNA levels in mucin-expressing epithelial cell lines but not in the non-mucin expressing human adult lung fibroblast cell line 16 LU. These findings indicate that *P. aeruginosa* products act directly and selectively on mucin-expressing cells to upregulate *MUC 2* mRNA.

Transcriptional activation of *MUC 2* by *P. aeruginosa*.

We next performed experiments to determine whether transcriptional control mechanisms are involved in the observed *MUC 2* upregulation. We transfected the epithelial cell lines mentioned above and CFTE29O (a human airway CF epithelial cell line homozygous for ΔF508 CFTR) with an expression vector containing 2.8 kb of the *MUC 2* 5'-flanking region fused to a luciferase reporter gene. When we exposed the transfected cells to *P. aeruginosa* culture supernatant (6 hours), luciferase activity

Fig. 1. *In situ* hybridization analysis of *MUC 2* mRNA expression in non-CF and CF human bronchial explants. *In situ* hybridization was performed with the HAM1 anti-sense probe recognizing human *MUC 2* mRNA as described previously (7). *MUC 2* mRNA expression is shown in vehicle control-treated and *P. aeruginosa*-treated bronchial explant surface epithelia from non-CF (**A** and **B**) and CF (**C** and **D**) individuals; in vehicle control-treated (**E** ang **G**) and *P. aeruginosa* -treated (**F** and **H**) bronchial explant submucosal glands from non-CF (**E** and **F**) and CF (**G** and **H**) individuals. Arrowheads in A, B, C and D indicate the position of the epithelial basement membrane. In these experiments both non-CF and CF bronchial explants were exposed for 6 hours to *P. aeruginosa* culture supernatants. Similar results were observed in bronchial explants from four CF and four non-CF individuals treated with *P. aeruginosa* strain PAO1 or PA103. L: airway lumen. SG: submucosal glands. In *Proc. Natl. Acad. Sci. USA* 94, 967-972, 1997. Reproduced with permission.


CONTROL
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NON-CF
CF
NON-CF
CF
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Figure 1

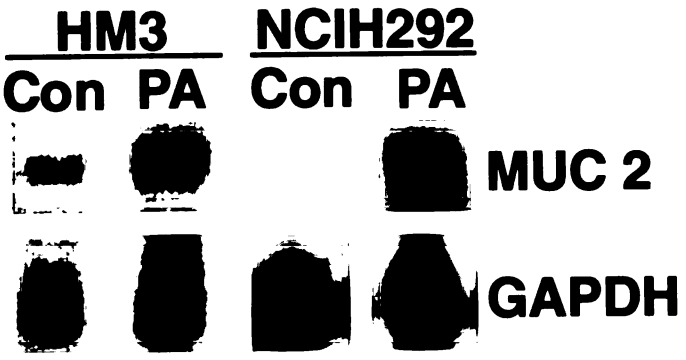


Figure 2

Fig. 2. RPA analysis of *MUC 2* mRNA expression in human *MUC 2*-expressing epithelial cell lines. Upregulation of *MUC 2* mRNA by *P. aeruginosa* culture supernatants occurred in both HM3 (human colon epithelial) and NCIH292 (human airway epithelial) cells. Cells were treated with *P. aeruginosa* culture supernatants (PA) or vehicle (CON) for 6 hours prior to cell lysis and RNA extraction. The results are typical of 4 separate experiments for each cell line. In *Proc. Natl. Acad. Sci. USA* 94, 967-972, 1997. Reproduced with permission.

Fig. 3. Upregulation of *MUC 2* transcriptional activity by *P. aeruginosa*.. A 2.8 kb DNA fragment of the 5'-flanking region of the human *MUC 2* gene cloned into a luciferase reporter gene (p-2864luc) (-2864 to +14 bp) was transfected into NCIH292, HM3 and CFTE29O cells. Luciferase activity was then assessed in *P. aeruginosa* treated- and non-treated cells. Induction by *P. aeruginosa* was detected in all cell lines. Transfected cells were treated with either *P. aeruginosa* culture supernatants or vehicle for 6 hours prior to cell lysis. All transfections were carried out in triplicate. Values are the means ± SD; n = 5. Luciferase activity was normalized with respect to ß-galactosidase activity. In *Proc. Natl. Acad. Sci. USA* 94, 967-972, 1997. Reproduced with permission.

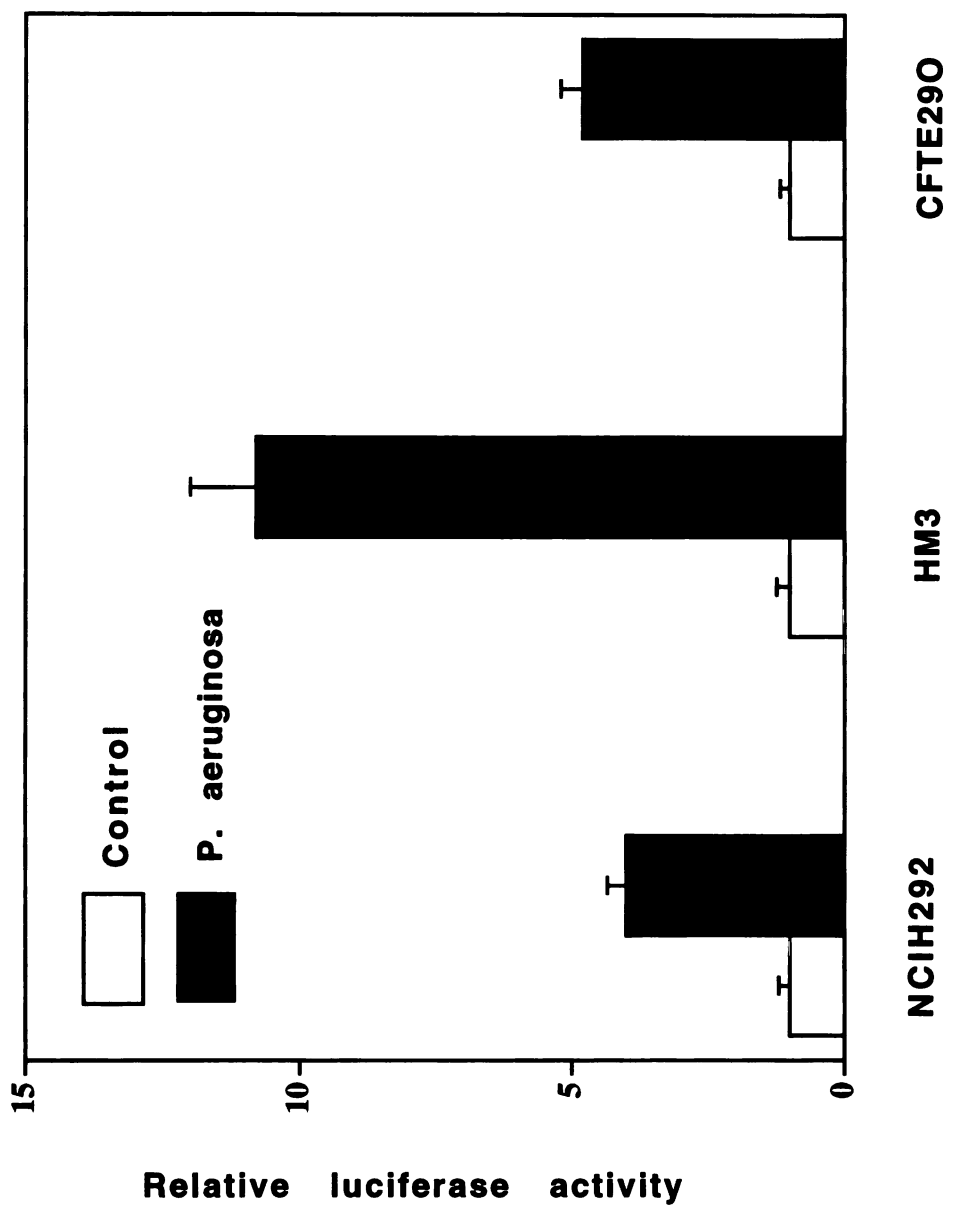

Control
P. aeruginosa
15
10
5
0
Relative luciferase activity
NCIH292
HM3
CFTE29O

Figure 3

SPECIFIC AIM II. IDENTIFY THE COMPONENT IN THE EXOPRODUCTS OF *P. AERUGINOSA* RESPONSIBLE FOR *MUC 2* UPREGULATION.

Characterization of LPS as the major inducer in *P. aeruginosa* exoproducts.

A. Genetic approach

We next addressed the question of which components of *P. aeruginosa* are responsible for *MUC 2* upregulation. Via the screening of *P. aeruginosa* mutants, it has been found that diverse products of this organism can affect host gene expression (13). We assessed the role of some of these in *MUC 2* upregulation by testing the potency of culture supernatant from bacterial mutants PAOR1 (deficient in production of the autoinducer PAI, elastase, alkaline protease and neuraminidase) (14-16), AK1152 (deficient in production of pilin and flagellin) (17) and PAO/NP (deficient in production of pilin) (13). That these media were as potent as that of wild-type essentially excluded a role for the mutant gene products (Fig 4).

B. Biochemical approach and physical characterization based on MW using spin column.

To obtain additional information regarding the molecular source of *MUC 2* stimulatory activity, we analyzed the activity in *P. aeruginosa* culture supernatant with respect to molecular size as well as to heat- and enzyme- sensitivity. Fractionation of wild-type culture supernatant using centricon filters with graded molecular weight cut-offs revealed stimulatory activity across a wide range of molecular sizes (Fig. 5). Further analysis showed that the activity is resistant to heat, proteolysis and DNA digestion (Fig. 6). Although not diagnostic of any specific molecule, these physico-

Fig. 4. Characterization of the virulence factor of *P. aeruginosa* responsible for *MUC 2* upregulation using genetic approach. HM3 cells were transfected with p-2864luc. After 42 hours the cells were exposed to culture supernatants (CS) from bacterial mutants PAOR1 (deficient in production of the autoinducer PAI, elastase, alkaline protease and neuraminidase), PAO/NP (deficient in production of pilin), AK1152 (deficient in production of pilin and flagellin), PAO/SRN (deficient in production of phospholipase C), PAO-pmm (deficient in making the O-side antigen of LPS) and PAO/DB2 (a double pilin mutant). After 6 hours the cells were harvested for luciferase activity measurement. All transfections were carried out in triplicate. Values are the means ± SD; n = 4. Luciferase activity was normalized with respect to ß-galactosidase

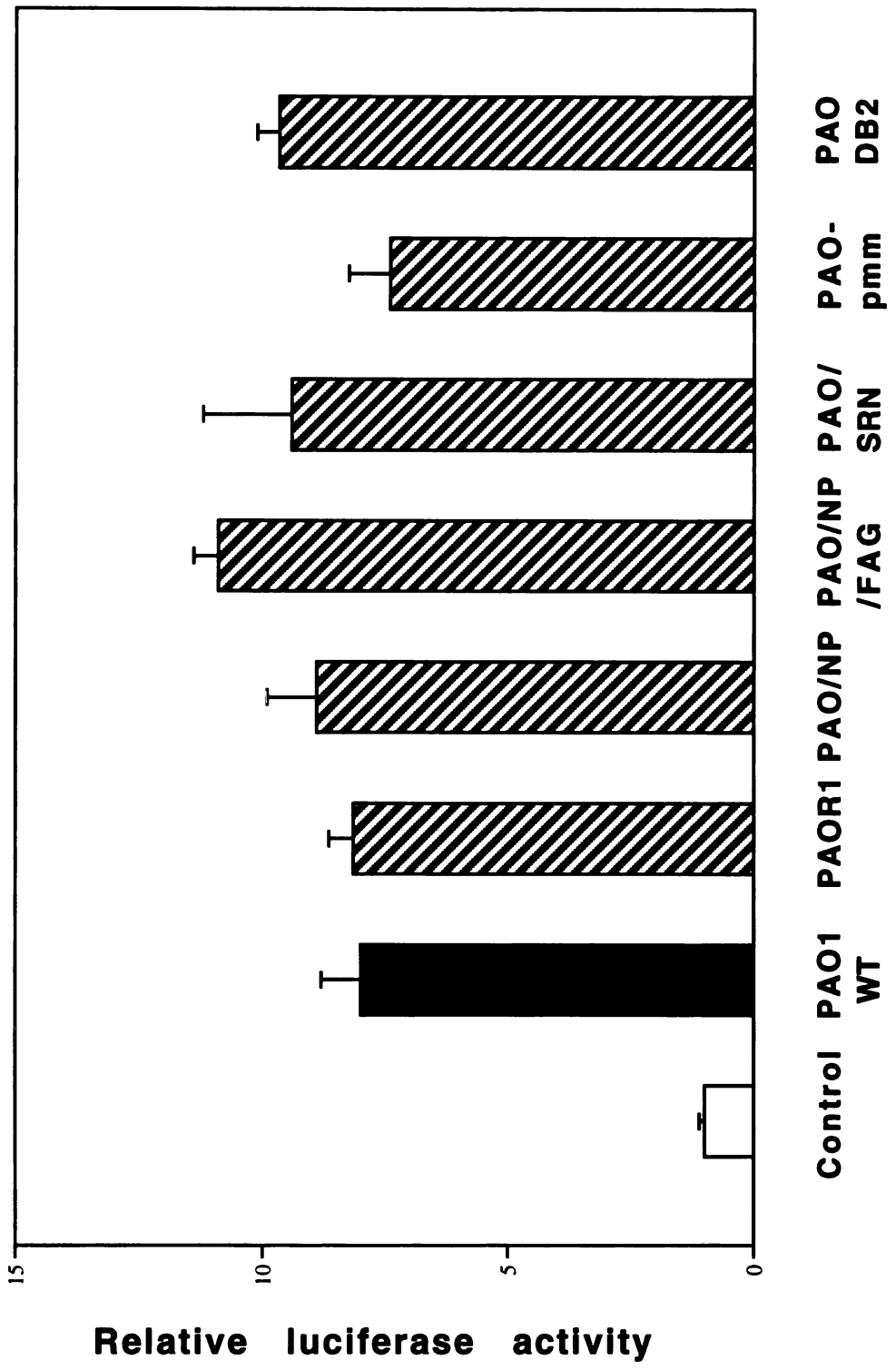

Relative luciferase activity
15
10
5
0
Control
PAO1 WT
PAOR1
PAO/NP
PAO/NP /FAG
PAO/ SRN
PAO- pmm
PAO DB2

Figure 4

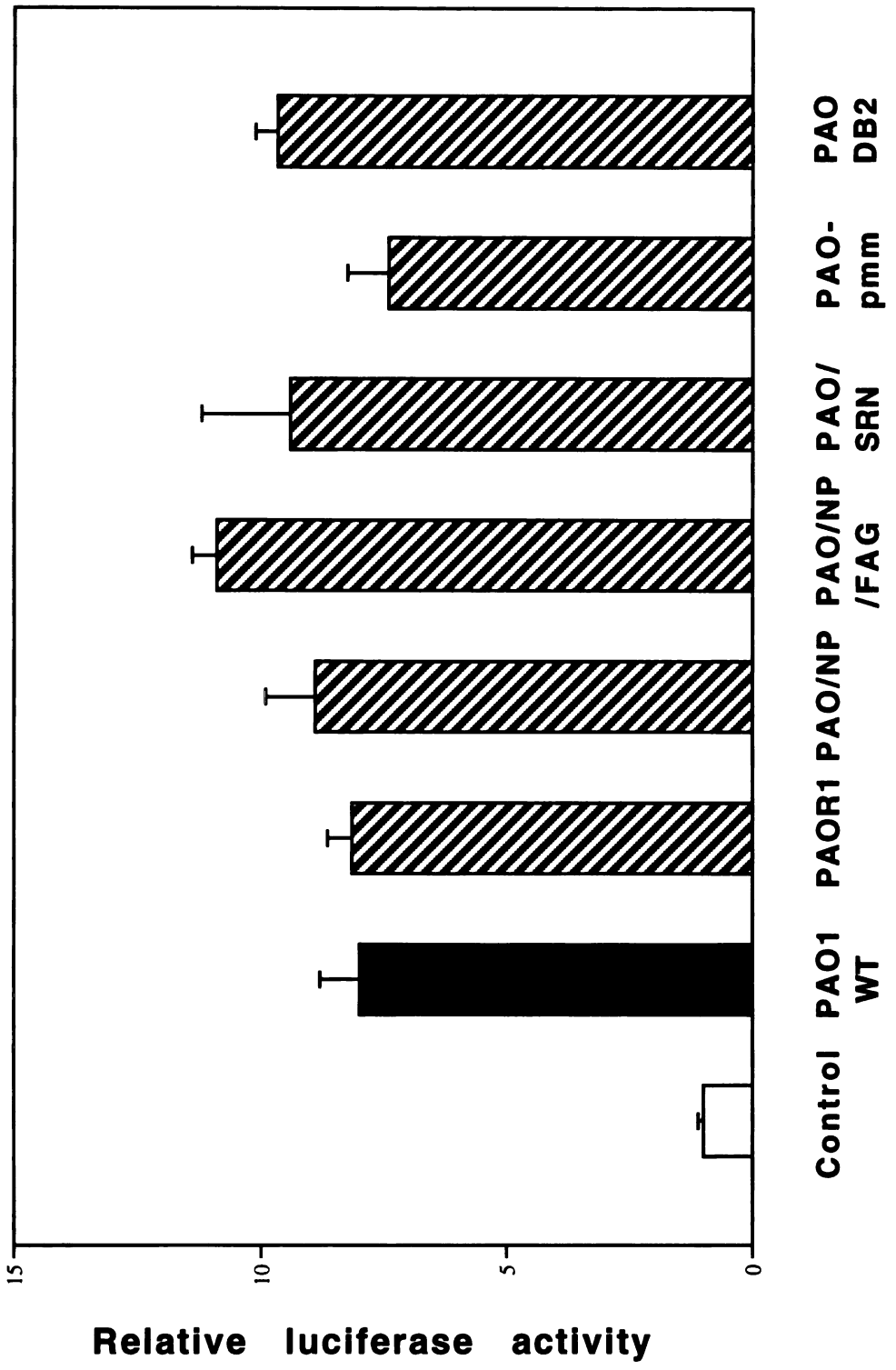

Relative luciferase activity
15
10
5
0
Control
PAO1 WT
PAOR1
PAO/NP
PAO/NP /FAG
PAO/ SRN
PAO-pmm
PAO DB2

Figure 4

Fig. 5. Characterization of the virulence factor of *P. aeruginosa* responsible for *MUC 2* upregulation based on molecular weight using spin column. HM3 cells were transfected with p-2864luc. After 42 hours the cells were exposed to different fractions of wild-type culture supernatant fractionated using centricon filters with graded molecular weight cut-offs. After 6 hours the cells were harvested for luciferase activity measurement. All transfections were carried out in triplicate. Values are the means ± SD; n = 3. Luciferase activity was normalized with respect to ß-galactosidase. Mix: mixed fractionated supernatant.

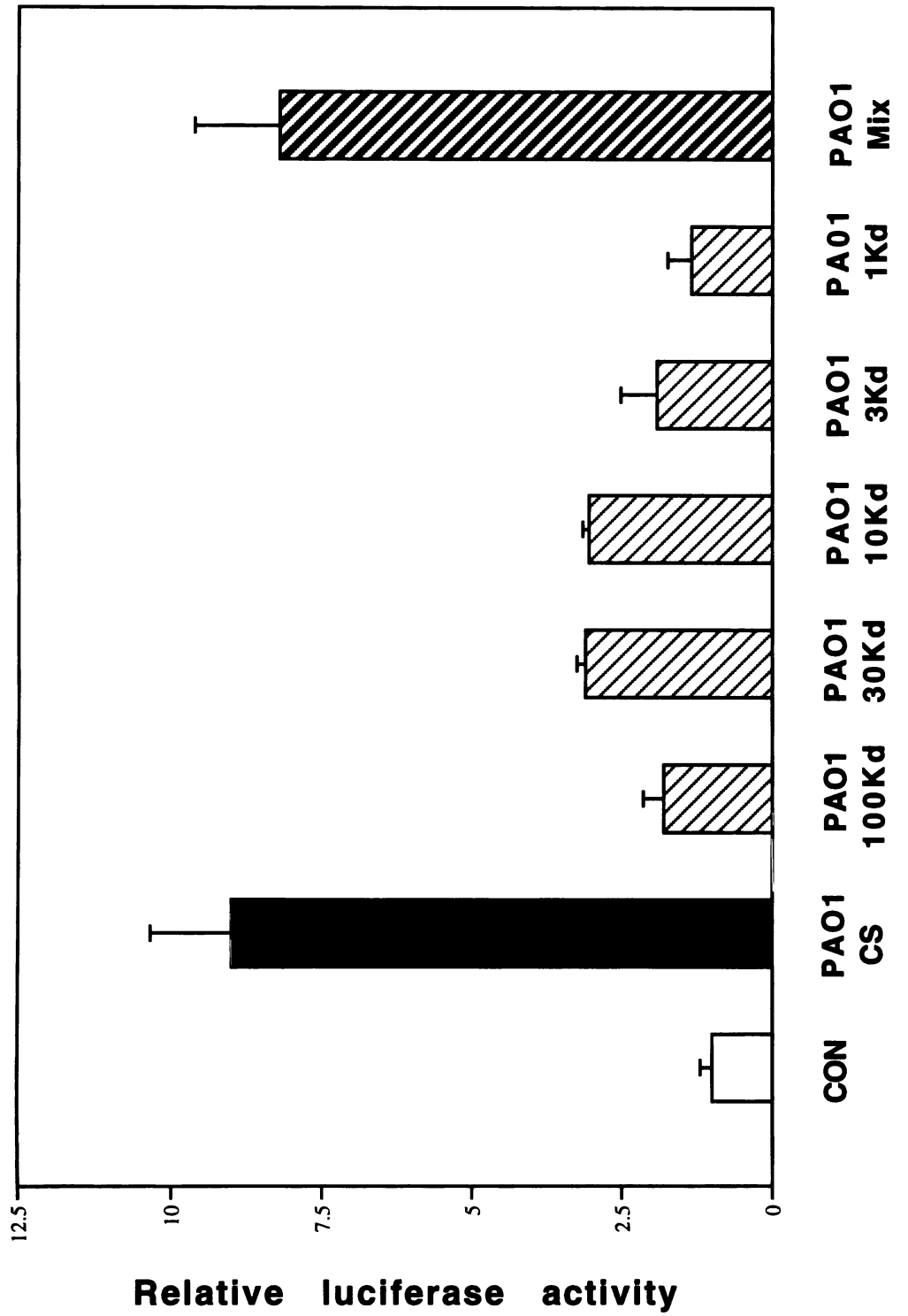

Relative luciferase activity
12.5
10
7.5
5
2.5
0
CON
PAO1 CS
PAO1 100Kd
PAO1 30Kd
PAO1 10Kd
PAO1 3Kd
PAO1 1Kd
PAO1 Mix

Figure 5

Fig. 6. Characterization of the virulence factor of *P. aeruginosa* responsible for *MUC 2* upregulation using biochemical approach. HM3 cells were transfected with p-2864luc. After 42 hours the cells were exposed to culture supernatants (CS) from wild-type PAO1 strain treated with heat, protease and DNAse I, respectively. After 6 hours the cells were harvested for luciferase activity measurement. All transfections were carried out in triplicate. Values are the means ± SD; n = 4. Luciferase activity was normalized with respect to ß-galactosidase.

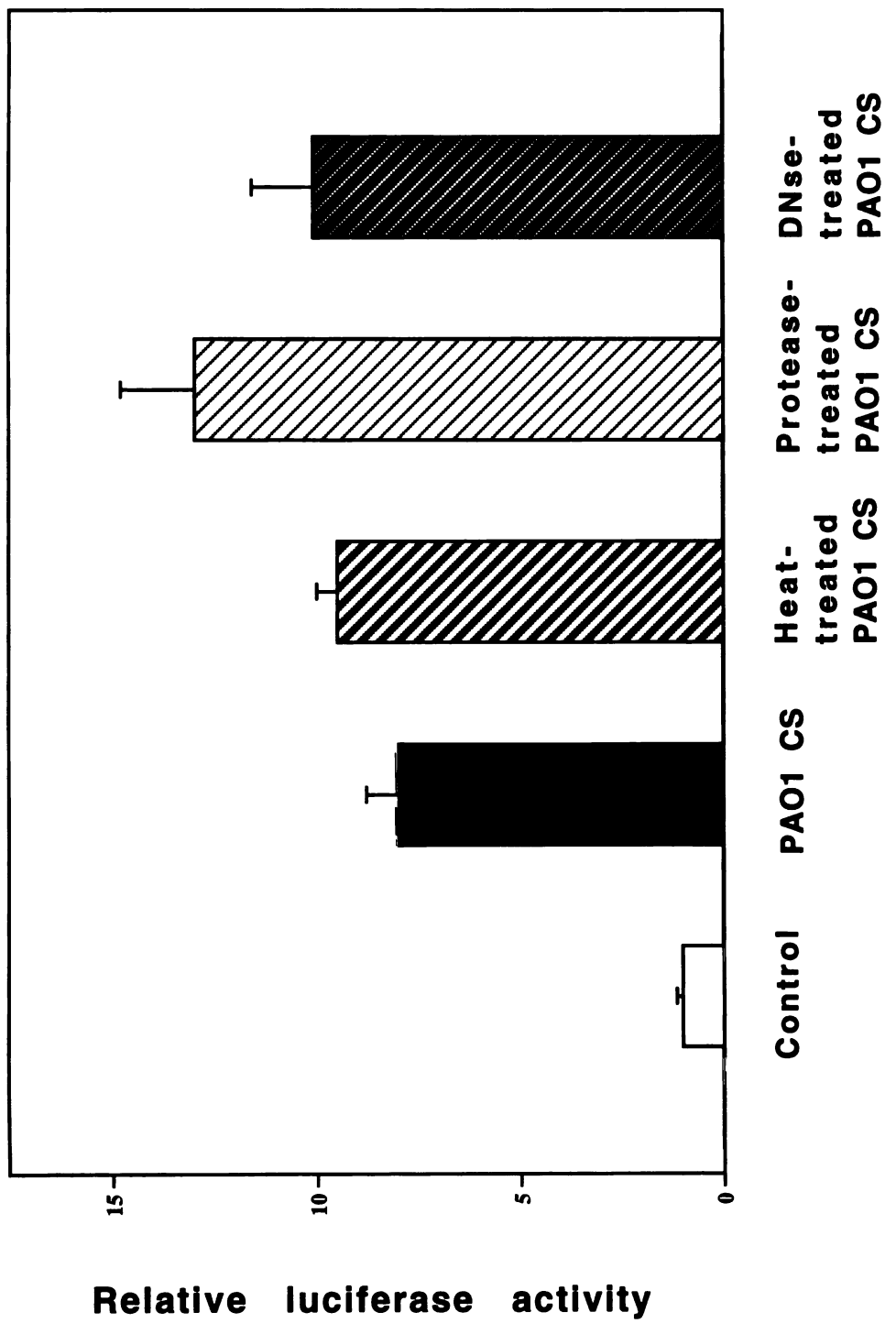

Relative luciferase activity
15
10
5
0
Control
PAO1 CS
Heat-treated PAO1 CS
Protease-treated PAO1 CS
DNse-treated PAO1 CS

Figure 6

chemical findings are inconsistent with properties of protein and DNA but consistent with properties of polysaccharides and lipids.

Up-regulation of *MUC 2* by *P. aeruginosa* LPS

Preeminent among bacterial polysaccharides is lipopolysaccharide (LPS), the major outer membrane component of Gram-negative bacteria and a potent activator of host defense responses (18). Based on the results of the physico-chemical assays described above, we hypothesized that LPS is involved in *MUC 2* upregulation. Indeed, when we tested the effects of LPS (obtained commercially or purified from *P. aeruginosa*) on steady-state levels of *MUC 2* mRNA (data not shown) and *MUC 2* transcriptional activity in cells transfected with the *MUC 2*-luciferase expression vector, we observed a strong response (Fig. 7). Taken together, our results indicate that LPS is a major factor mediating *P. aeruginosa* upregulation of *MUC 2* transcription.

LPS core polysaccharide and lipid A is sufficient to induce *MUC 2* up-regulation.

Structurally, LPS is composed of a variable polysaccharide domain covalently linked to an invariable diglucosamine based acylated phospholipid, lipid A. Although many biological effects of LPS are mediated by the lipid A and the core oligosaccharide portion of the molecule, others require a complete LPS complex (19, 20). To examine this with respect to mucin upregulation, we obtained culture supernatant from a *P. aeruginosa* mutant (PAO-pmm) deficient in synthesis of the LPS variable polysaccharide domain and compared its activity to that of wild-type (6). The two supernatants, and LPS purified from each of them, were equipotent (Fig. 8 A, B), suggesting that lipid A and/or its linked core oligosaccharide is sufficient to induce LPS-mediated *MUC 2* transcriptional upregulation.

Fig. 7. Effect of *P. aeruginosa* LPS on *MUC 2* transcriptional activity. HM3 cells were transfected with p-2864luc. After 42 hours the cells were exposed to culture supernatants (CS) and LPS (5 μg/ml) purified from PAO1 and PA10 (serotype 10) (Sigma Chemical Co., St. Louis, MO). After 6 hours the cells were harvested for luciferase activity measurement. All transfections were carried out in triplicate. Values are the means ± SD; n = 4. Luciferase activity was normalized with respect to ß-galactosidase. In *Proc. Natl. Acad. Sci. USA* 94, 967-972, 1997. Reproduced with permission.

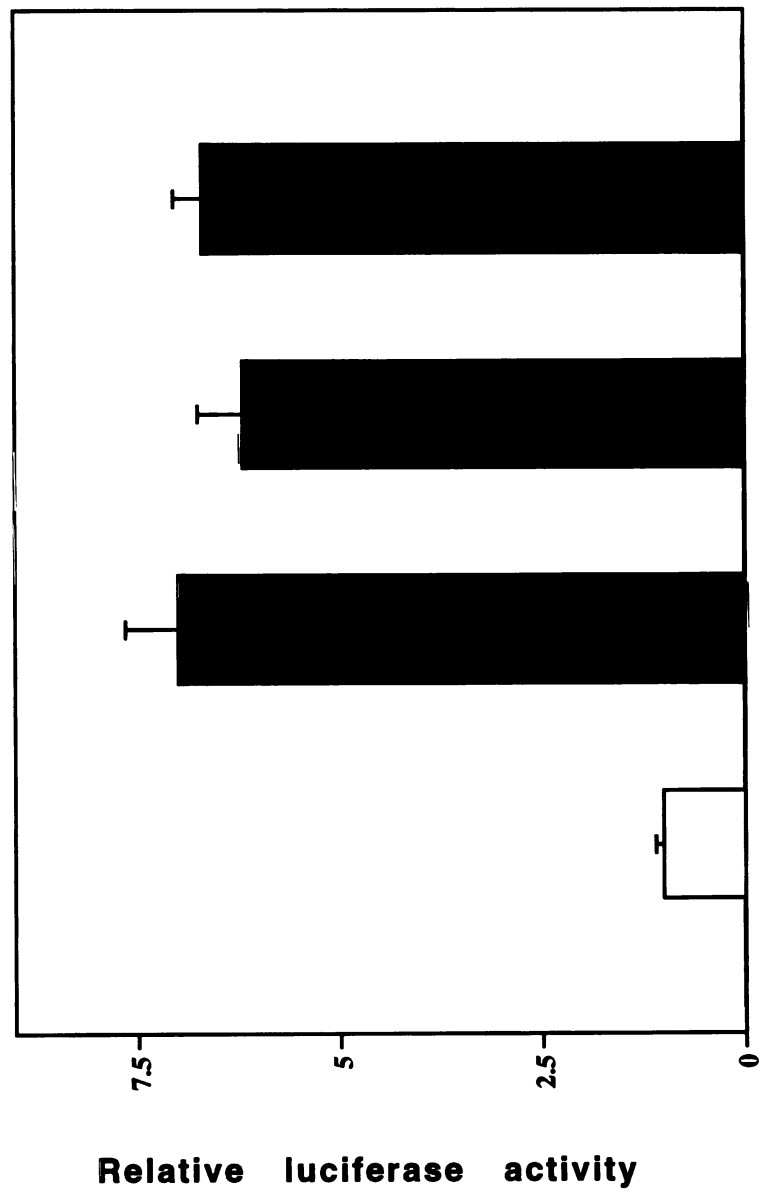

7.5
5
2.5
0
Relative luciferase activity
CONTROL
CS(PAO1)
LPS(PAO1)
LPS(PA10)

Figure 7

Fig. 8. Effect of *P. aeruginosa* LPS (MT) and (WT) on *MUC 2* transcriptional activity. HM3 cells were transfected with p-2864luc. After 42 hours the cells were exposed to culture supernatants (CS) and LPS (5 μg/ml) purified from PAO1 (wild-type) or PAO-pmm (algC) mutant (LPS mutant), which were characterized using SDS-PAGE (A). After 6 hours the cells were harvested for luciferase activity measurement (B). All transfections were carried out in triplicate. Values are the means ± SD; n = 4. Luciferase activity was normalized with respect to ß-galactosidase. In *Proc. Natl. Acad. Sci. USA* 94, 967-972, 1997. Reproduced with permission.

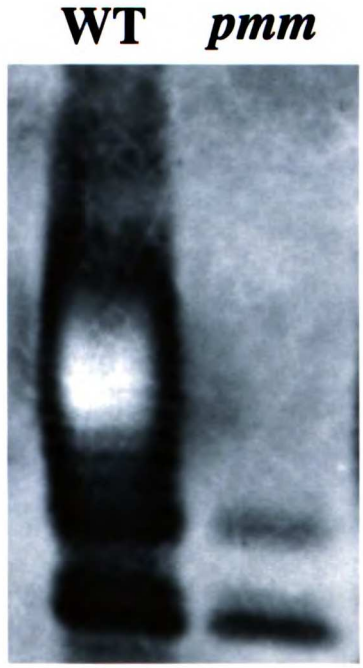


Figure 8A

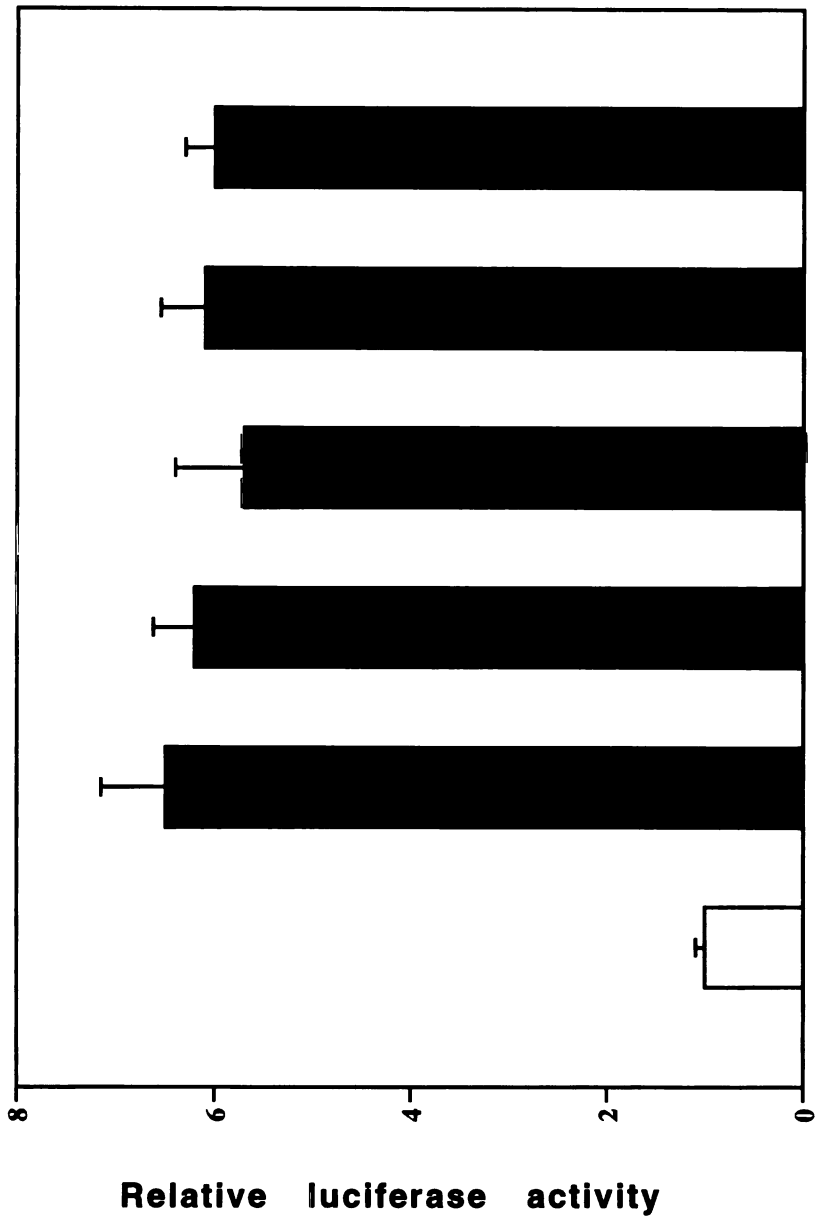

Relative luciferase activity
8
6
4
2
0
CON
CS
LPS
CS
LPS
LPS
(PAO1WT) (PAO1WT) (PAOpmm) (PAOpmm) (PA1OWT)

Figure 8B

Lipid A is a major inducer.

An experiment showing that lipid A (purified from E. coli) could essentially mimic the LPS effect (Fig. 9) indicates that lipid A is a key factor in the *MUC 2* stimulation and that lipid A from diverse bacterial species is potent in this respect.

LPS effect on MUC-2 transcription is generalizable for most gram-negative bacteria.

To determine whether the effect of *P. aeruginosa* LPS on *MUC 2* upregulation is generalizable for other gram-negative bacteria LPS, we studied the effect of LPS from several gram-negative bacteria including *E. coli*, *S. enteritidis* and *K. pneumonia*. LPS from all bacteria tested showed upregulation of *MUC 2* transcription. (Fig. 10).

Fig. 9. Effect of Lipid A on *MUC 2* transcriptional activity. HM3 cells were transfected with p-2864luc. After 42 hours the cells were exposed to E. coli lipid A (5 μg/ml) (Sigma Chemical Co., St. Louis, MO). After 6 hours the cells were harvested for luciferase activity measurement. All transfections were carried out in triplicate. Values are the means ± SD; n = 3. Luciferase activity was normalized with respect to ß-galactosidase. In *Proc. Natl. Acad. Sci. USA* 94, 967-972, 1997. Reproduced with permission.

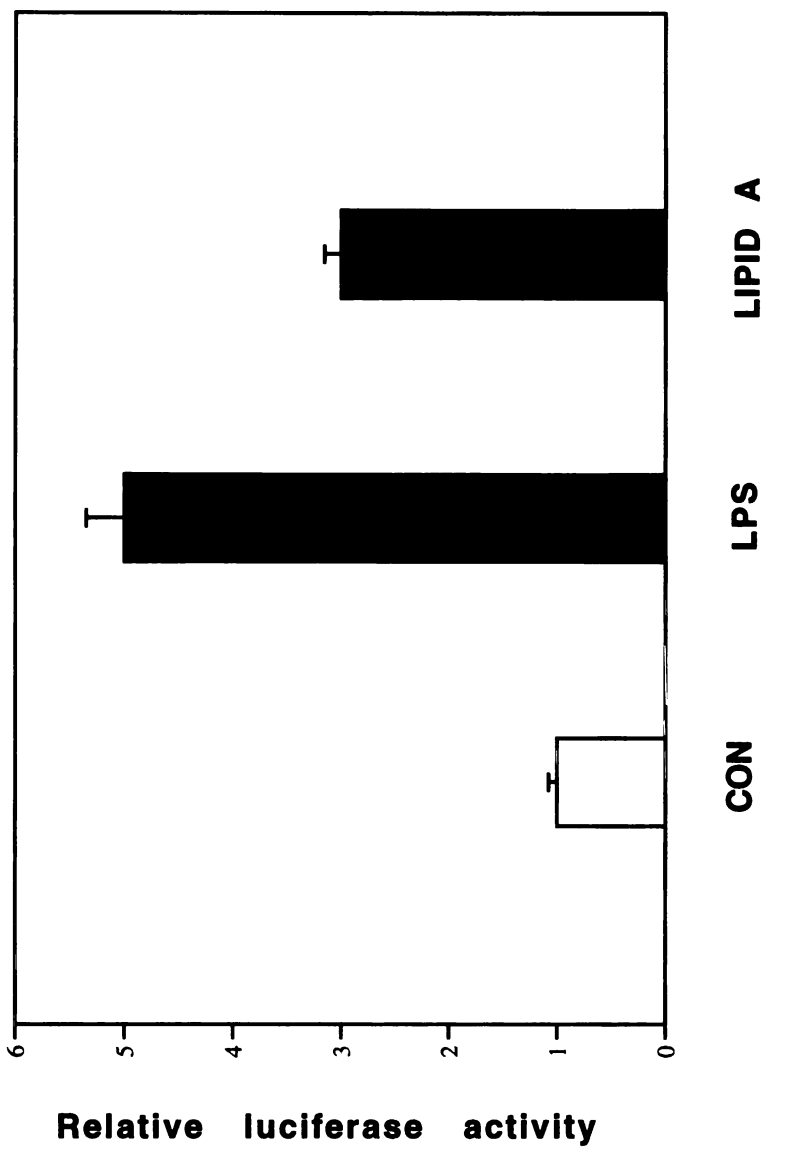


Figure 9

Fig. 10. Effect of LPS from other gram-negative bacteria on *MUC 2* transcriptional activity. HM3 cells were transfected with p-2864luc. After 42 hours the cells were exposed to LPS (5 μg/ml) purified from PAO1, E. coli, S. enteritidis and K. pneumonia (Sigma Chemical Co., St. Louis, MO). After 6 hours the cells were harvested for luciferase activity measurement. All transfections were carried out in triplicate. Values are the means ± SD; n = 4. Luciferase activity was normalized with respect to ß-galactosidase.

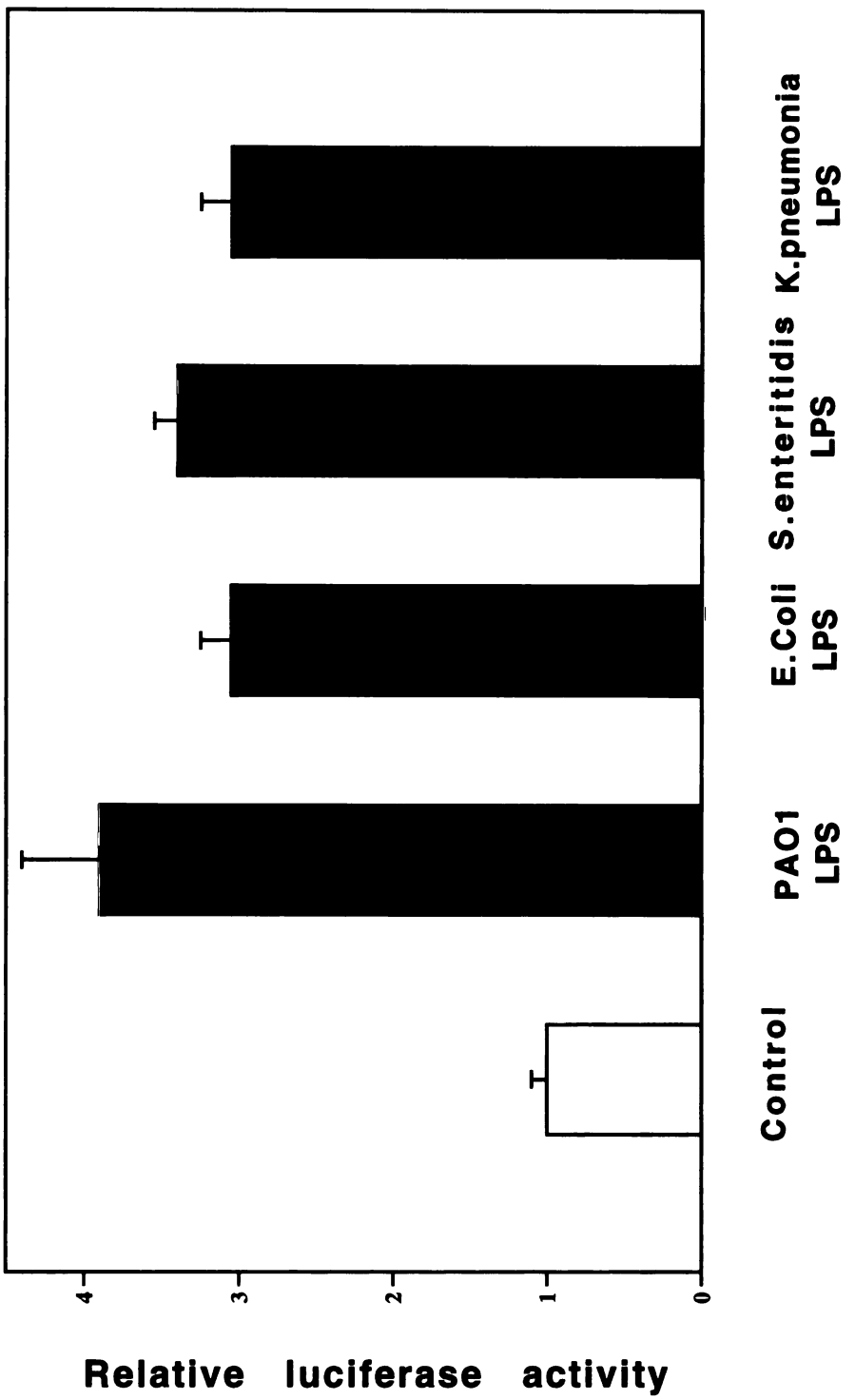

Relative luciferase activity
0
1
2
3
4
Control
PAO1 LPS
E.Coli LPS
S.enteritidis LPS
K.pneumonia LPS

Figure 10

SPECIFIC AIM III. DETERMINE WHETHER *MUC2* IS HYPERINDUCIBLE BY *P. AERUGIONSA* IN CF EPITHELIAL CELLS.

MUC 2 gene is not hyperinducible by *P. aeruginosa* in CF epithelial cells.

Previously reported data have revealed that IL-8 is hyperinducible by *P. aeruginosa* in CF epithelial cells (13). To examine the possibility that this is also true of *MUC 2* transcriptional upregulation, we compared the magnitude of *P. aeruginosa* responses in CF epithelial cells vs non-CF epithelial cells (Fig.3). The data indicated that *MUC 2* is not hyperinducible by *P. aeruginosa* in CF epithelial cells. To determine whether or not mutant CFTR is directly involved in *MUC 2* induction by *P. aeruginosa* in CF epithelial cells, we compared the magnitude of *P. aeruginosa* responses in CFTR mutant cells that had vs those that had not been complemented by transfection with wild-type CFTR expression plasmid. No difference was found between these two groups (Fig.11).

Fig.11. Effect of *P. aeruginosa* culture supernatant on *MUC 2* transcriptional activity in CFTE 29O ΔF508 cells co-transfected with p-2864luc +/- the CFTR wild-type expression plasmid pREP4.7kbCFTR. 42 hours after transfection, PAO1 culture supernatant (CS) was added to cells. 6 hours later the cells were harvested for luciferase activity measurement. All transfections were carried out in triplicate. Values are the means ± SD; n = 4. Luciferase activity was normalized with respect to ß-galactosidase. In *Proc. Natl. Acad. Sci. USA* 94, 967-972, 1997. Reproduced with permission.

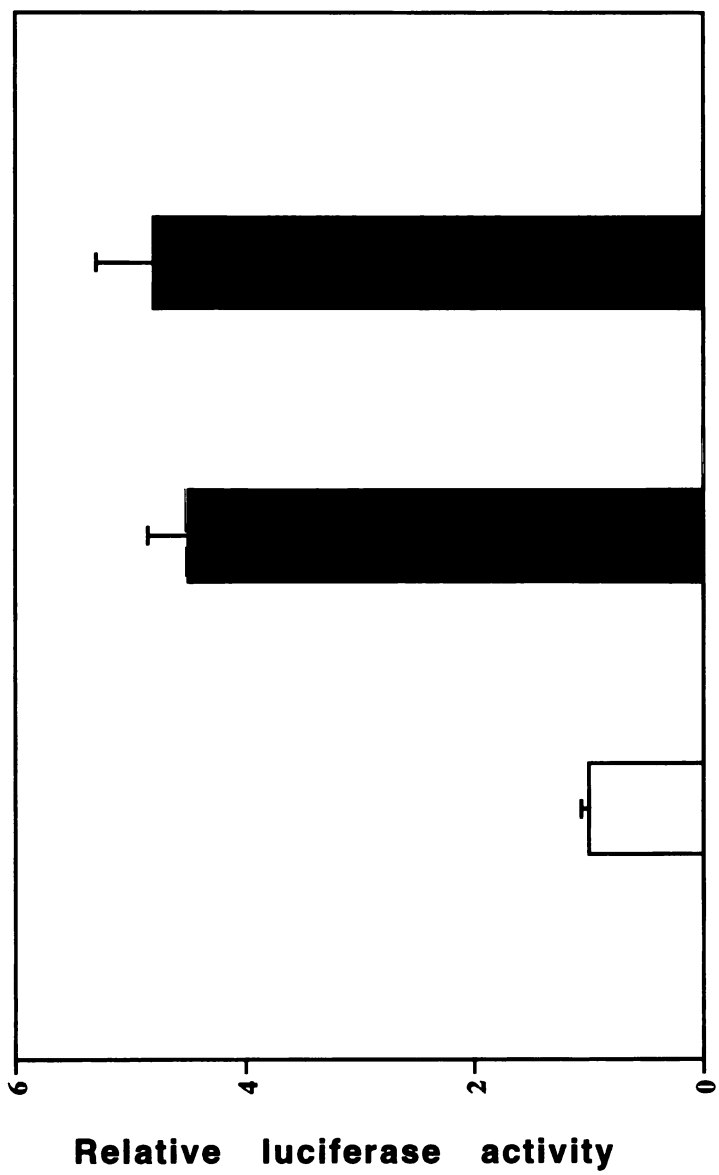

Relative luciferase activity
0
2
4
6
CFTEΔF508
Non-treated
CFTEΔF508
+
CS(PAO1)
CFTEΔF508
+
CS(PAO1)
+
CFTR(WT)

Figure 11

SPECIFIC AIM IV. IDENTIFY *P. AERUGINOSA*-INDUCIBLE DNA RESPONSE ELEMENTS IN THE *MUC 2* MUCIN GENE REGULATORY REGION AND COGNATE TRANSCRIPTION FACTORS.

We have shown transcriptional regulation mechanisms to be involved in *P. aeruginosa*-induced *MUC 2* upregulation. To localize DNA response elements participating in these mechanisms, we transfected the three epithelial cell lines used above with deletion mutants of the 2.8 kb *MUC 2* luciferase reporter gene and tested transcriptional activity in the presence and absence of *P. aeruginosa* culture supernatant. We detected *P. aeruginosa* response elements between -2864/-73bp in all three cell lines (Fig. 12 A). To further define these, we analyzed a larger panel of deletion mutants in HM3, the cell line yielding the strongest *P. aeruginosa* response. This revealed inducible response elements in the proximal region -343/-73 and distal region -2864 /-1308 bp (Fig. 12 B).

Characterization of Proximal element.

To accurately identifying the proximal response element, we constructed more deletional mutants and transfected them into HM3 cells. As shown in Fig.13 A, the proximal *P. aeruginosa*-response element was inferred at -91/-74 bp. This finding was also confirmed in NCIH292 cells (Fig. 13 B).

To characterize the DNA consensus sequence and the cognate transcription factors responsible for the *P. aeruginosa* response, we performed gel-shift assays using oligonucleotides corresponding to the region from -91 to -74 bp and nuclear proteins

Fig.12. Characterization of P. aeruginosa-inducible DNA response elements in the MUC 2 5'-flanking region. DNA fragments in different size (deletion mutant) (indicated below) of the 5'-flanking region of the human *MUC 2* gene cloned into a luciferase reporter gene were transfected into NCIH292, HM3 and CFTE29O cells. (**A**) Comparison of the *P. aeruginosa* responsiveness of p-2864luc (-2864 to +14 bp) and p-73luc (-73 to +14 bp) in NCIH292, HM3 and CFTE29O cells. Response elements reside between -2864 and -73 bp. (**B**) Comparison of the *P. aeruginosa* responsiveness of p-73luc, p-343luc (-343 to + 14 bp), p-621luc (-621 to +14 bp), p-1308luc (-1308 to + 14 bp) and p-2864luc in HM3 cells. Luciferase activity was then assessed in *P. aeruginosa* treated- and non-treated cells. Induction by *P. aeruginosa* was detected in all cell lines. Transfected cells were treated with either *P. aeruginosa* culture supernatants or vehicle for 6 hours prior to cell lysis. All transfections were carried out in triplicate. Values are the means ± SD; n = 5. Luciferase activity was normalized with respect to ß-galactosidase activity. Reproduced with permission.

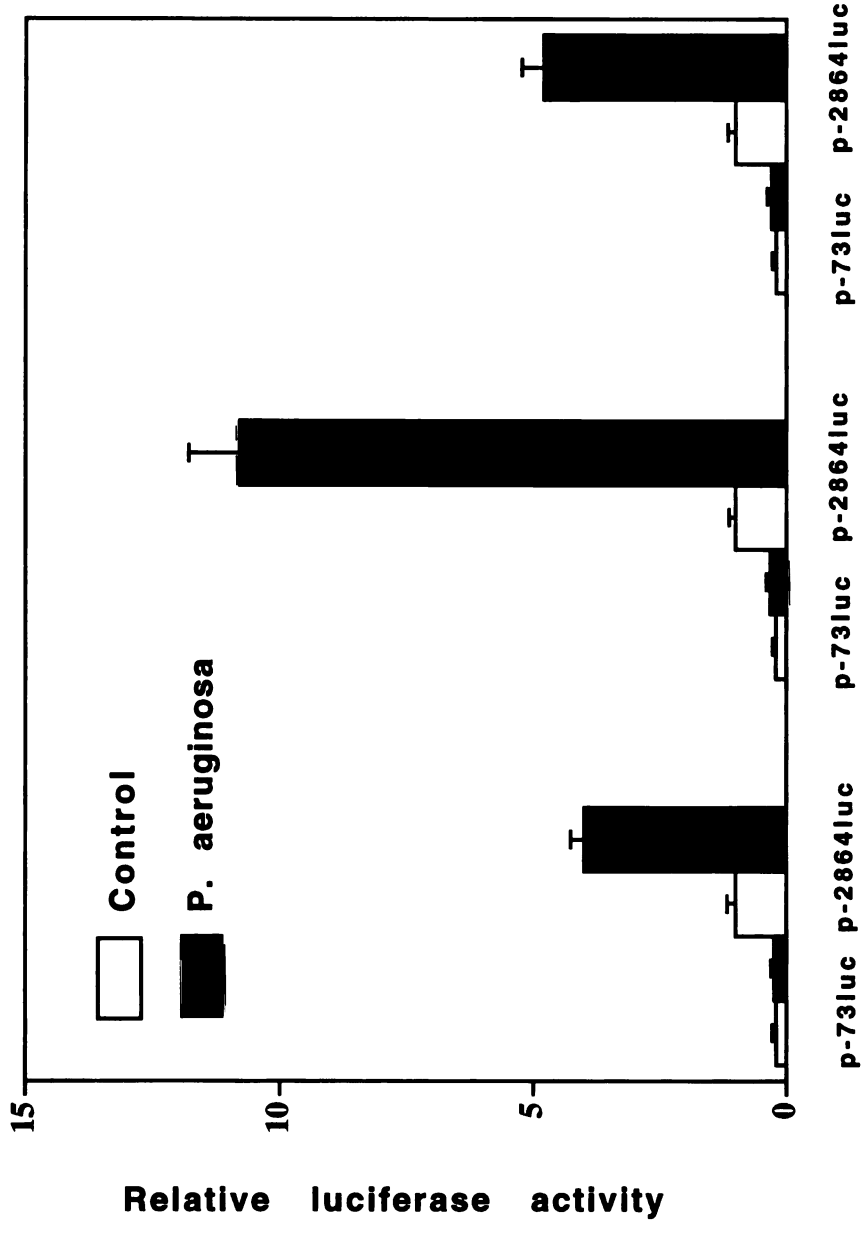

Relative luciferase activity
15
10
5
0
Control
P. aeruginosa
p-73luc
p-2864luc
NCIH292
HM3
CFTE29O

Figure 12A

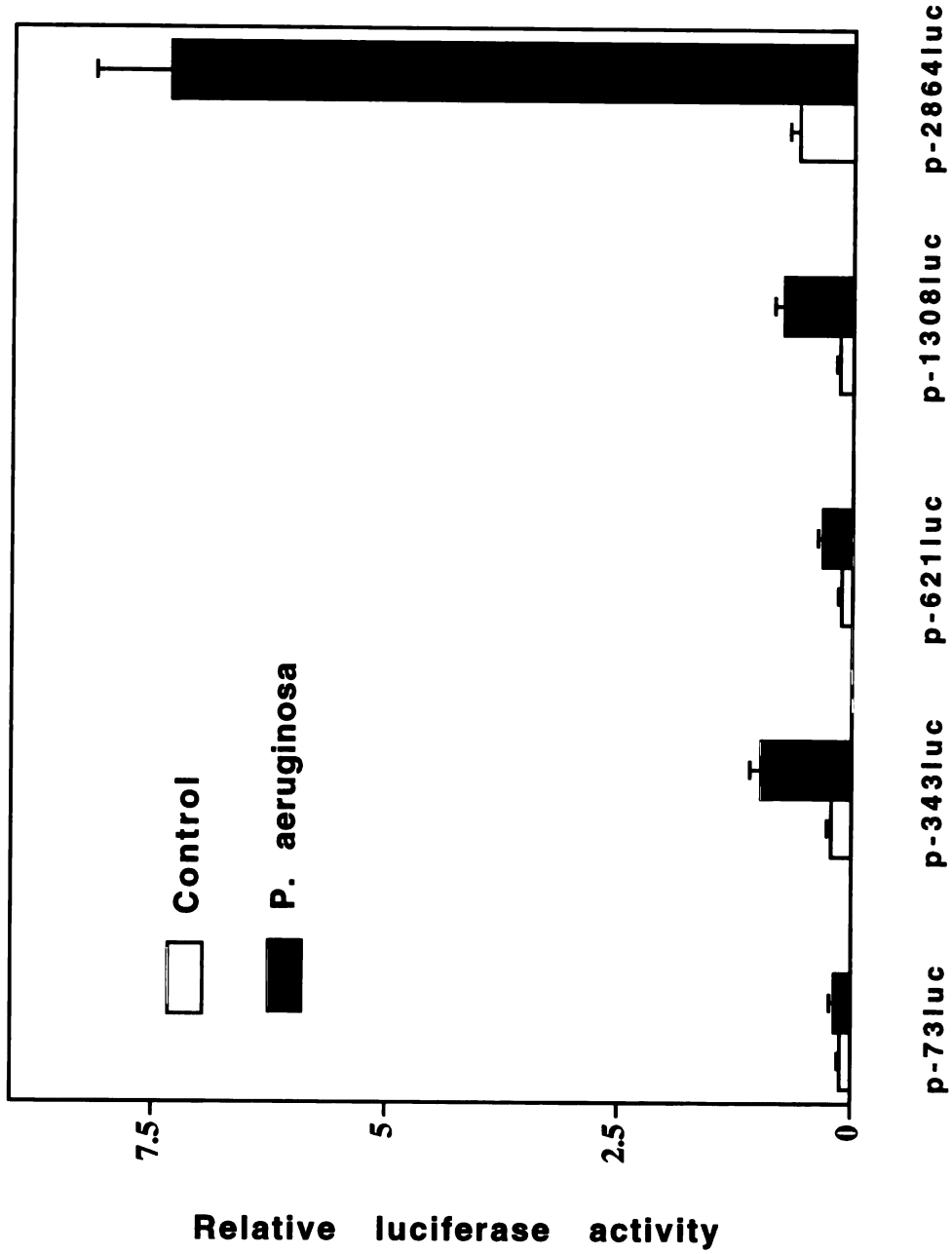

Control
P. aeruginosa
7.5
5
2.5
0
Relative luciferase activity
p-73luc
p-343luc
p-621luc
p-1308luc
p-2864luc

Figure 12B

Fig.13. Characterization of the proximal P. aeruginosa response element. (A) Human MUC 2 promoter regions (-343 to +14, -114 to +14, -102 to +14, -91 to +14 and -73 to +14 bp) were subcloned upstream of luciferase reporter gene in pGL2 basic vector and transfected into HM3 cells. (B). Human MUC 2 promoter regions (-343 to +14, -91 to +14 and -73 to +14 bp) subcloned upstream of luciferase reporter gene in pGL2 basic vector were also transfected into NCIH292 cells. Transfected cells were treated with either *P. aeruginosa* culture supernatants or vehicle for 6 hours prior to cell lysis. Luciferase activity was then assessed in *P. aeruginosa* treated- and non-treated cells. All transfections were carried out in triplicate. Values are the means ± SD; n = 4. Luciferase activity was normalized with respect to ß-galactosidase activity.

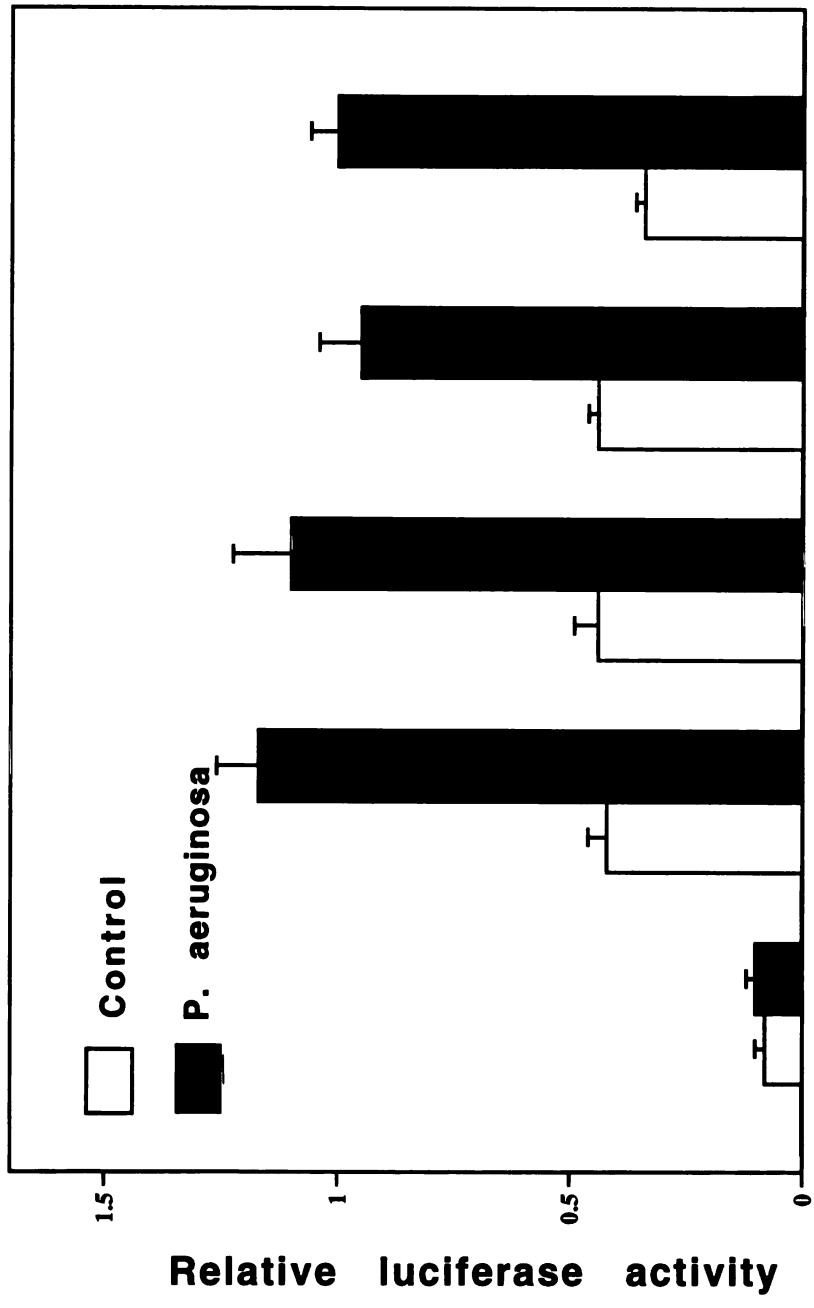

Control
P. aeruginosa
1.5
1
0.5
0
Relative luciferase activity
p-73luc
p-91luc
p-102luc
p-114luc
p-343luc

Figure 13A

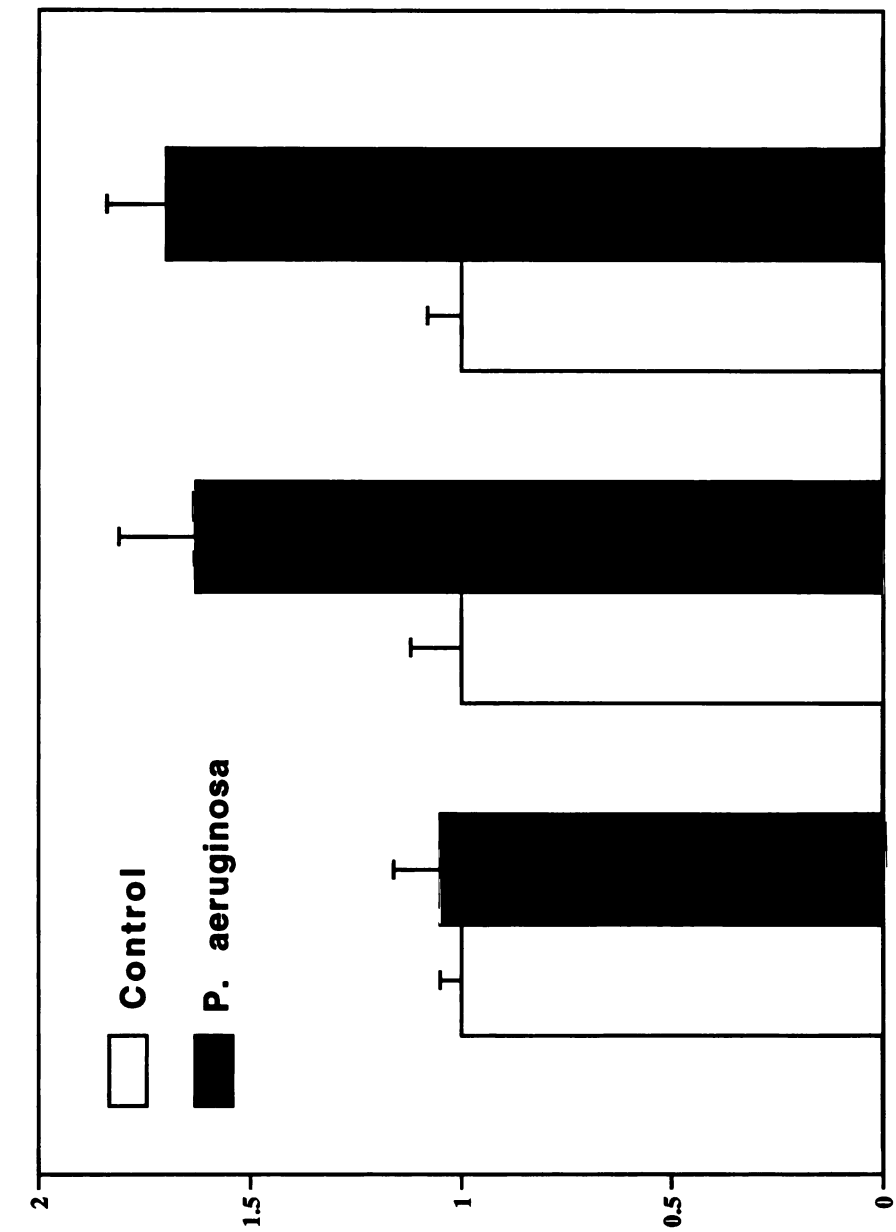

Control
P. aeruginosa
2
1.5
1
0.5
0
p-73luc
p-91luc
p-343luc
Relative luciferase activity (Fold increase over control)

Figure 13B

from *P. aeruginosa*-treated and non-treated epithelial cells. As shown in Fig.14 A, two DNA-protein binding complexes became stronger when nuclear proteins from *P. aeruginosa*-treated cells were used. More accurate localization of the protein binding sites was achieved by mutation analysis of oligonucleotides corresponding to the -91 to -74 bp region. Results indicated that these two DNA-protein complexes were not changed by M1, and were abolished by M2 and M3 (Fig. 14 B). Sequence analysis showed that the CCCACC motif was mutated in both M2 and M3. These two complexes were not supershifted by the antibodies against either AP2 or SP1, although it has been shown that transcription factor AP2 and SP1 can bind to this motif. When a deletion mutant containing no CCCACC was transfected into HM3 cells or NCIH292 cells (Fig. 13 A and B), a significant decrease of luciferase activity was observed, indicating that this CCCACC motif is important for the P. aeruginosa-induced MUC2 up-regulation.

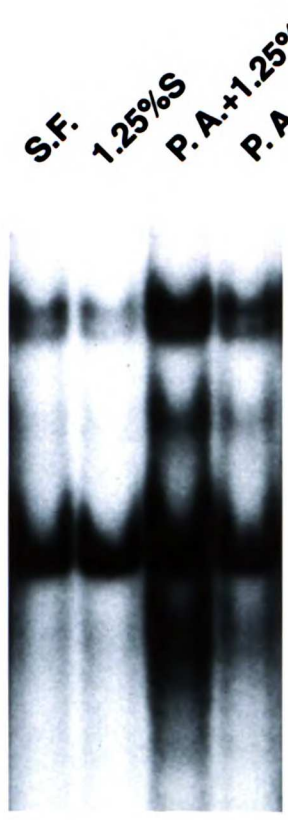
Characterization of distal element.

To define the distal *P. aeruginosa*-response element, we constructed more deletional mutants and transfected them into HM3 cells. Luciferase activity was then measured in *P. aeruginosa*-treated vs non-treated cells. The distal element was inferred at -1627/-1307 bp (Fig.15).

To more precisely define the distal element, we made heterologous constructs in which human *MUC 2* promoter -1628/-1307, -1528/-1307 and -1430/-1307 bp regions were subcloned upstream of the TK promoter. As shown in Fig. 16 A and B, this suggested that the distal element may reside in the -1528/-1430 bp region.

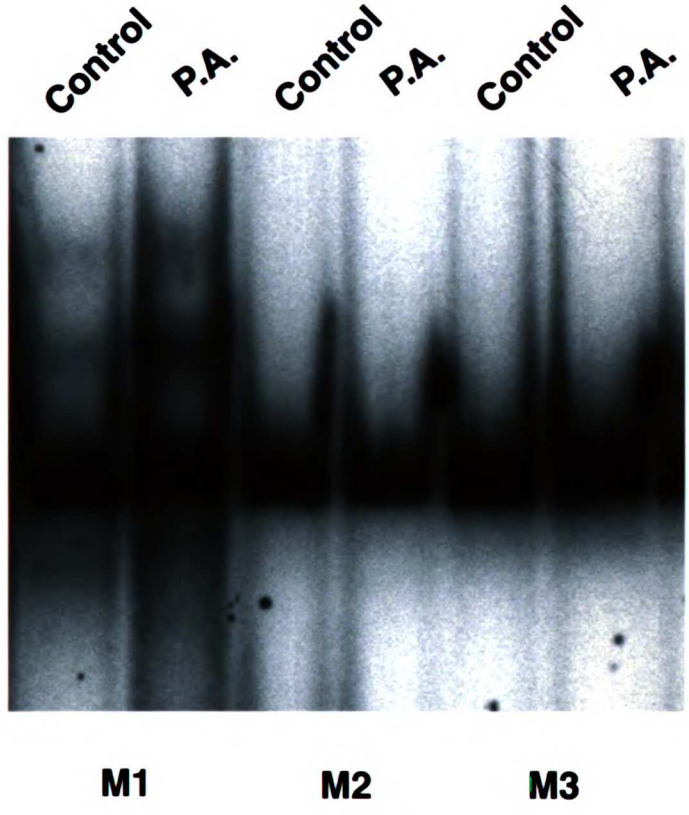
To more accurately define the distal P. aeruginosa response element, we performed gel-shift assays using oilgonucleotides corresponding to -1528/-1486 (A1) and -1485/-

Fig.14. Electrophoretic mobility shift assay (EMSA) analysis of human MUC 2 promoter region (-91 to -74 bp) binding proteins. Nuclear proteins were incubated with a probe consisting of the ^{32}P-labeled 18-bp double-stranded oligonucleotides corresponding to human MUC 2 promoter region -91/ -74 bp WT (A), mutant M1, 2, and 3 (B) and subjected to EMSA as described in the Material & Methods. Probe incubation were with nuclear extracts (10 μg) from HM3 cell either treated or non-treated with P. aeruginosa culture supernatant as indicated. S.F.: serum free medium; 1.25% S: 1.25% serum medium; P.A.: P. aeruginosa culture supernatant.



WT GATGCCACACCCACCCTT

Figure 14A



WT GATGCCACACCCACCCTT

M1 GAT*TAACTC* CCCACCCTT

M2 GATGCCACA*AAACTAA* TT

M3 GAT*TAACTCAAACTA* CTT

Figure 14B

Fig.15. Characterization of the distal P. aeruginosa response element. Human MUC 2 promoter regions (-2864 to +14, -1627 to +14 and -1308 to +14 bp) were subcloned upstream of luciferase reporter gene in pGL2 basic vector and transfected into HM3 cells. Transfected cells were treated with either *P. aeruginosa* culture supernatants or vehicle for 6 hours prior to cell lysis. Luciferase activity was then assessed in *P. aeruginosa* treated- and non-treated cells. All transfections were carried out in triplicate. Values are the means ± SD; n = 4. Luciferase activity was normalized with respect to ß-galactosidase activity.

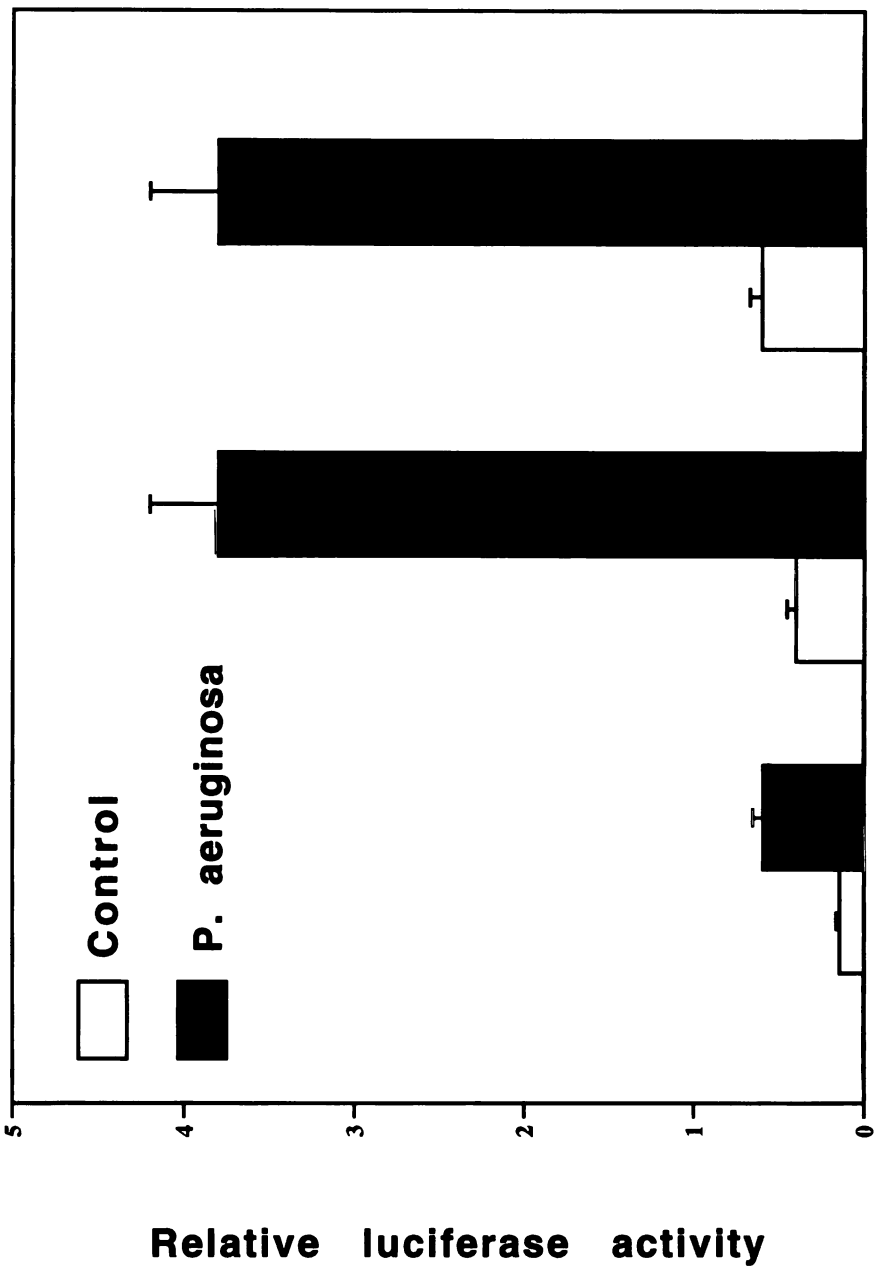

Control
P. aeruginosa
0
1
2
3
4
5
Relative luciferase activity
p-1308luc
p-1627luc
p-2864luc

Figure 15

1430 bp (A2) and nuclear proteins from P.aeruginosa-treated and non-treated epithelial cells. A new protein complex was formed with probe A2, but not with A1. When we further performed gel-shift assay using oligonucleotides corresponding to -1485/-1459 (B1) and -1458/-1430 bp (B2), this protein complex was formed with probe B2, but not B1 (Fig. 17A). Sequence analysis showed that this region contains overlapping C/EBP and NF-kappa B motif. This finding is consistent with the recent finding that LPS induces the binding of C/EBP and NF-kappa B to the promoter of serum amyloid gene expression in liver. We further performed EMSAs in the absence or presence of non-labled oligonucleotide competitors containing different mutated transcription factor binding sites (for sequence information, please see the figure legend). As shown in Fig. 17B, the protein complexes formed with nuclear proteins from P. aeruginosa-treated epithelial cells were competed away completely by oligonucleotides M1 and M4, partially by M2, but, however, not affected at all by M3. These data indicated that either NF-kappa B or C/EBP motifs may be involved in *MUC 2* induction. Inclusion of NF-kappa B antibody, but not C/EBP antibody in EMSA supershifted the major protein complex, suggesting that NF-kappa B may be involved in *MUC2* induction (Fig. 17C).

Fig.16. Characterization of the distal P. aeruginosa-inducible enhancer element. Human MUC 2 promoter regions (-1628 to -1307, -1528 to -1307 and -1430 to -1307 bp) were subcloned upstream of TK-32 promoter luciferase vector (named as -1.6/-1.3TK, -1.5/-1.3TK and -1.4/-1.3TK, respectively) and transfected into HM3 cells (A) and NCIH292 cells (B). Transfected cells were treated with either *P. aeruginosa* culture supernatants or vehicle for 6 hours prior to cell lysis. Luciferase activity was then assessed in *P. aeruginosa* treated- and non-treated cells. All transfections were carried out in triplicate. Values are the means ± SD; n = 3. Luciferase activity was normalized with respect to ß-galactosidase activity.

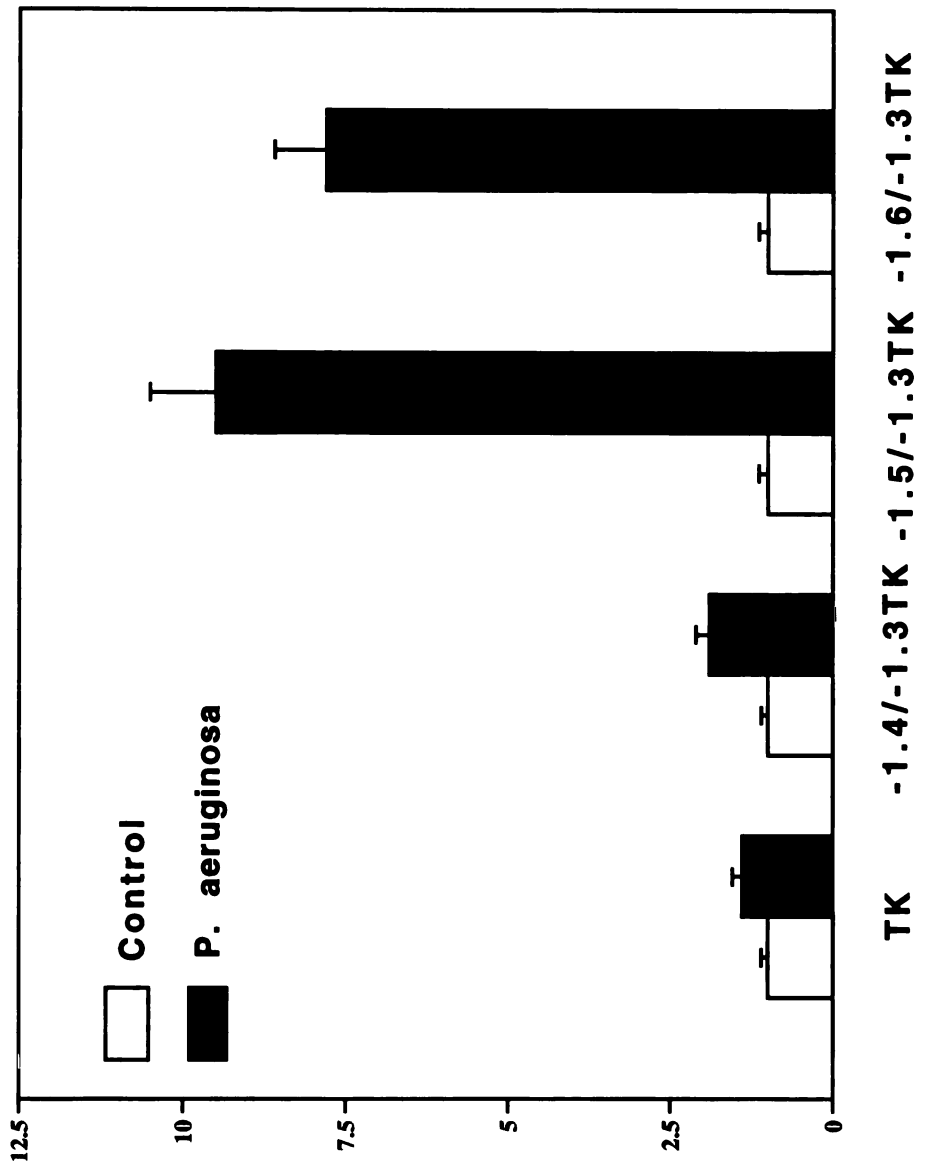

Control
P. aeruginosa
12.5
10
7.5
5
2.5
0
TK
-1.4/-1.3TK
-1.5/-1.3TK
-1.6/-1.3TK
Relative luciferase activity (Fold increase over control)

Figure 16A

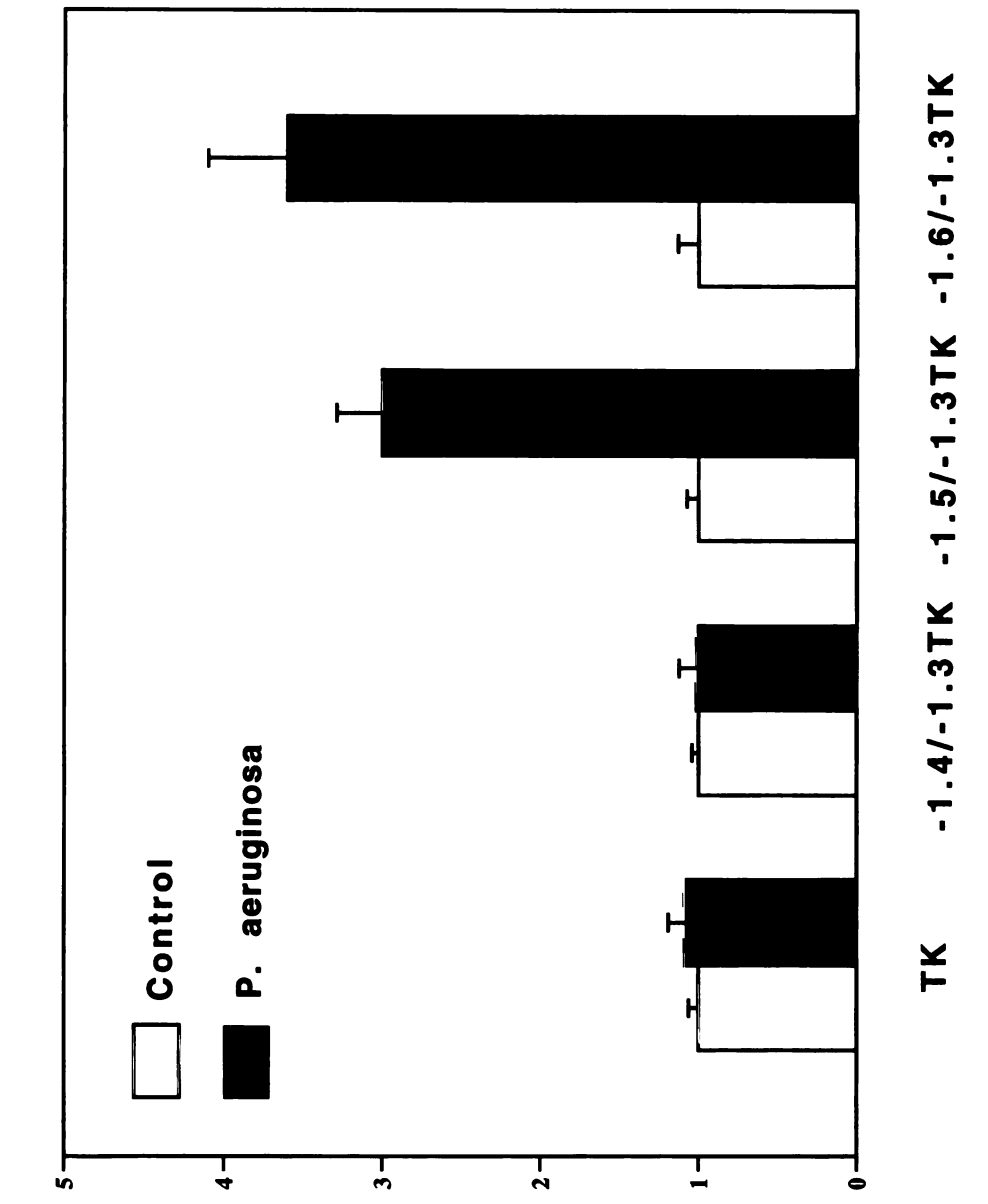
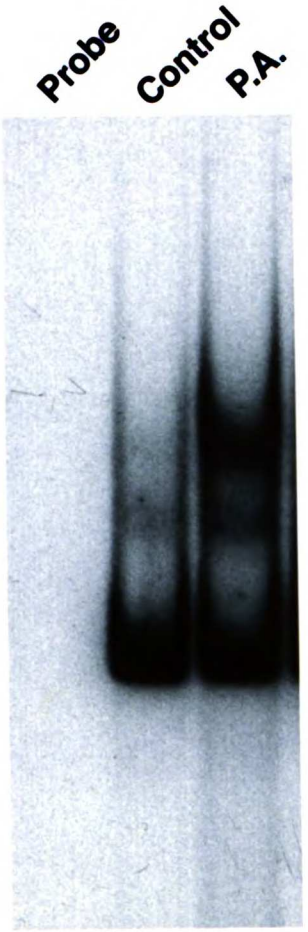

Control
P. aeruginosa
5
4
3
2
1
0
TK
-1.4/-1.3TK
-1.5/-1.3TK
-1.6/-1.3TK
Relative luciferase activity (Fold increase over control)

Figure 16B

Fig.17. EMSA analysis of human MUC 2 promoter region (--1458/-1430 bp) binding proteins. Nuclear proteins were incubated with a probe consisting of the ^{32}P-labeled double-stranded oligonucleotides corresponding to human MUC 2 promoter region -1458/-1430 bp in the absence (**A**) or presence of different mutant unlabeled oligonucleotides (M1 to M4) as indicated (**B**), or preincubated either with anti-NF-kappa B p65 or anti-C/EBP antibodies (**C**), and were subjected to EMSA as described in the Material & Methods. Probe incubation were with nuclear extracts (10 μg) from HM3 cell either treated or non-treated with P. aeruginosa culture supernatant as indicated.

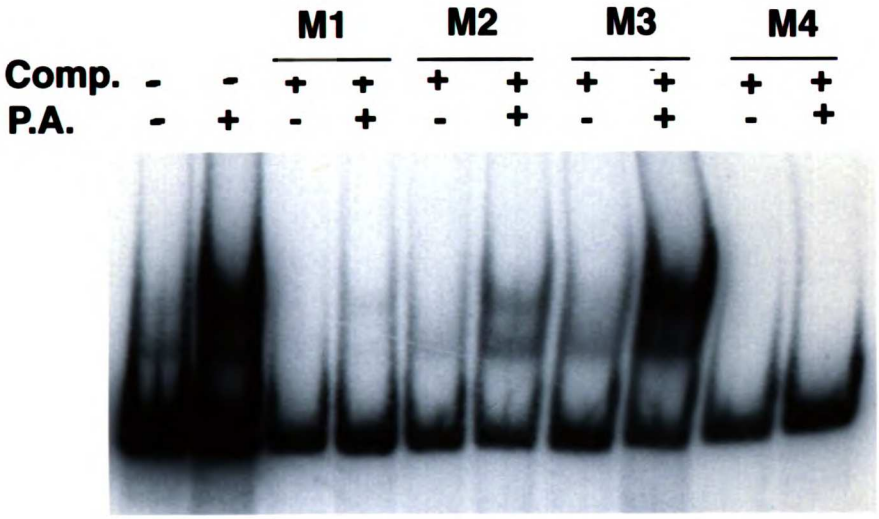


C G T C C T T G G G T T T C C C C A G G G G C T

NF-kappaB

C/EBP

Figure 17A



WT C G T C C T T G G G T T T C C C C A G G G C T

NF-kappaB

C/EBP

MT1 C G T C C T *G T A* G T T T CC C C A G G G C T

MT2 C G T C C T T G G *A G C* T C C C C A G G G C T

MT3 C G T C C T T G G G T T *T C C C C* A G G G C T

MT4 C G T C C T T G G G T T T C C C C *C T T A G* T

Figure 17B

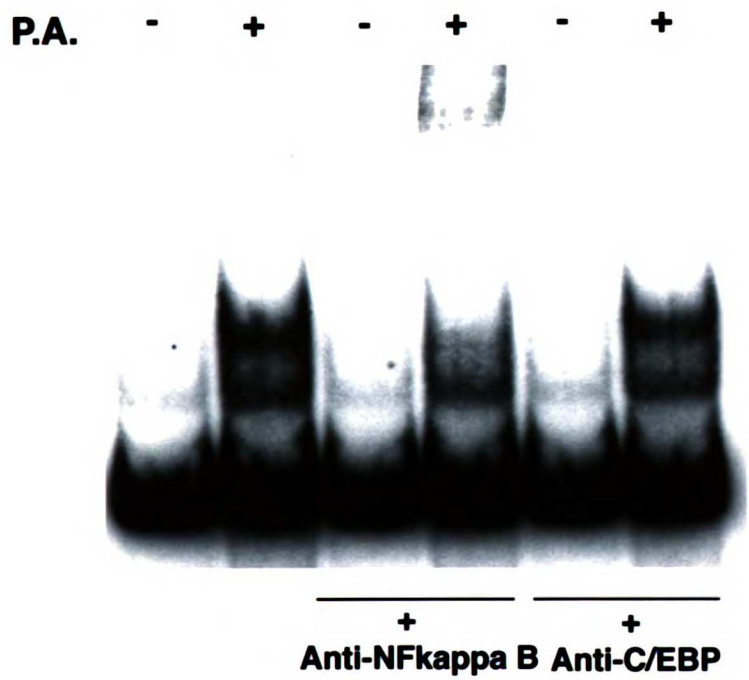


Figure 17C

SPECIFIC AIM V. IDENTIFY INTRACELLULAR SIGNAL TRANSDUCTION PATHWAYS INVOLVED IN *P. AERUGINOSA*-INDUCED *MUC 2* INDUCTION.

Tyrosine kinase inhibitors abolished *MUC 2* induction by *P. aeruginosa*. Having identified LPS as a bacterial "trigger" for *MUC 2* transcriptional upregulation and having localized *P. aeruginosa*-inducible elements of the *MUC 2* 5' flanking region directly mediating this effect, we next sought information regarding the intracellular signaling pathway linking these elements. In experiments aimed at assessing the role of protein kinase activity, we found that preincubation of cells with the protein tyrosine kinase inhibitor genistein for 2 hours prior to *P. aeruginosa* exposure abolished increases in *MUC 2* steady-state mRNA and transcriptional activity (Fig 18 A and B). This suggested that protein tyrosine phosphorylation is required for the response.

Classical MEK1/2 and MAPK (ERK1/2) pathway is required for *P. aeruginosa*-induced *MUC 2* upregulation.

Since the tyrosine kinase MAP kinase (ERK1/2) has been shown to be activated by LPS in macrophages, we tested the possibility that it is involved in *P. aeruginosa*-induced *MUC 2* upregulation using specific chemical inhibitors. Preincubation of cells with tyrphostin AG 126 abolished *P. aeruginosa*- (and LPS-) induced upregulation of *MUC 2* transcription (Fig.19), confirming the requirement for tyrosine phosphorylation and focusing interest on the best known substrate for tyrphostin AG 126, the LPS-activated tyrosine kinase p42MAPK (21). Taken together with our data implicating LPS in *MUC 2* upregulation, the tyrphostin AG 126 data suggest that the LPS-activated

Fig. 18. Inhibition of *P. aeruginosa*-induced *MUC 2* upregulation by tyrosine kinase inhibitors. (**A**) HM3 cells were pretreated with genistein (Genist.) (100 μg/ml) (Sigma Chemical Co., St. Louis, MO) or vehicle for 2 hours and then exposed to *P. aeruginosa* PAO1 culture supernatants (PA) or vehicle (CON) for 6 hours before RNA extraction and RPA analysis. The results are typical of 4 separate experiments. (**B**) HM3 cells were transfected with p-2864luc. After 40 hours the cells were pretreated with genistein (100 μg/ml) for 2 hours and then exposed to *P. aeruginosa* culture supernatants (PAO1) for 6 hours before harvesting for luciferase analysis. Luciferase activity was measured as described above. All transfections in (B) were carried out in triplicate. Values are the means ± SD; n = 4. Luciferase activity was normalized with respect to ß-galactosidase. In *Proc. Natl. Acad. Sci. USA* 94, 967-972, 1997. Reproduced with permission.

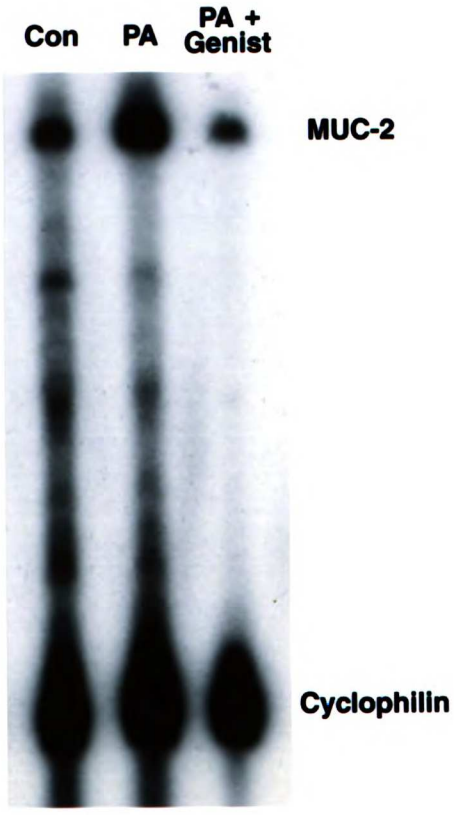


Figure 18A

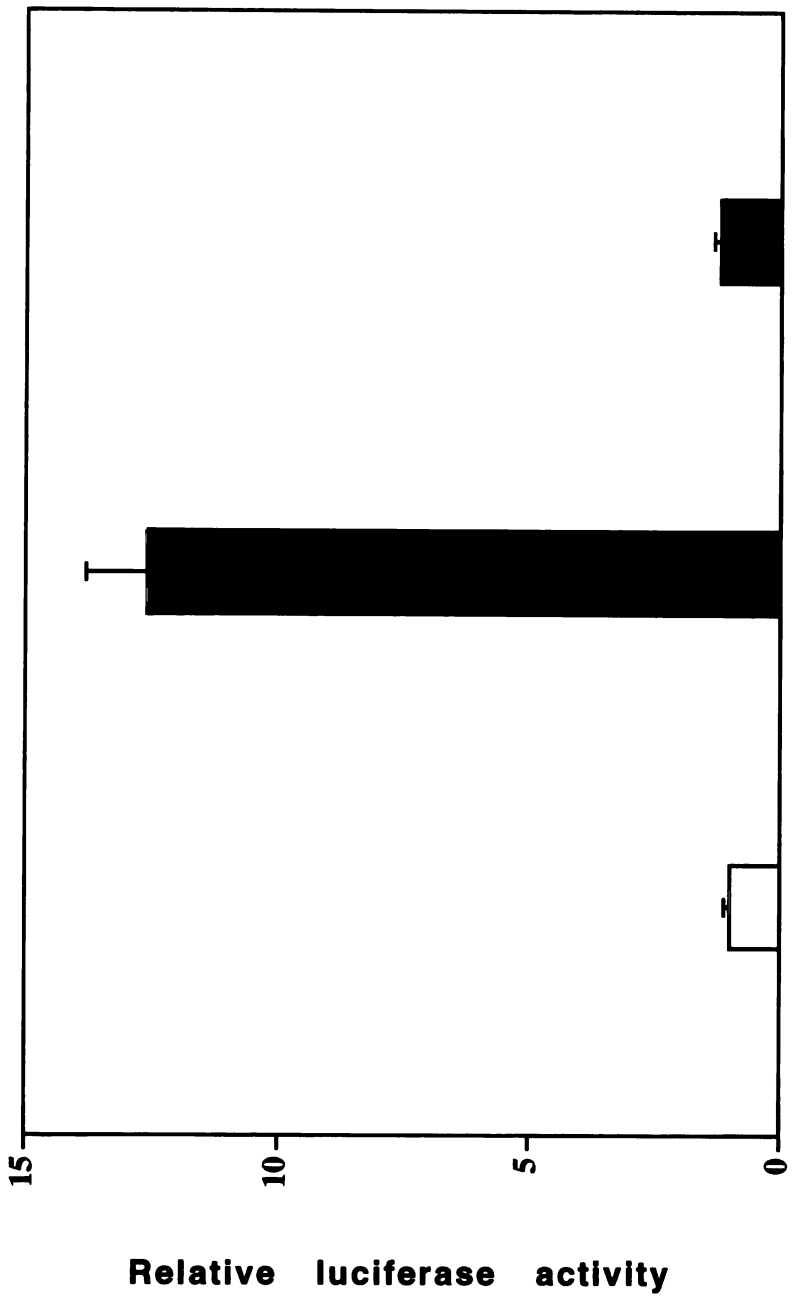

15
10
5
0
Relative luciferase activity
CONTROL
PAO1
PAO1+GENISTEIN

Figure 18B

Fig. 19. Inhibition of *P. aeruginosa*-induced *MUC 2* upregulation by Tyrphostin AG126. HM3 cells were transfected with p-2864luc. 40 hours after being transfected with p-2864luc, HM3 cells were pretreated with Tyrphostin AG 126 (25 μM) (CALBIOCHEM, La Jolla, CA) for 3 hours and then exposed to PAO1 culture supernatant (CS) or LPS from PA10 (serotype 10) (5 μg/ml) for 6 hours before harvesting. Luciferase activity was measured as described above. All transfections were carried out in triplicate. Values are the means ± SD; n = 4. Luciferase activity was normalized with respect to ß-galactosidase. In *Proc. Natl. Acad. Sci. USA* 94, 967-972, 1997. Reproduced with permission.

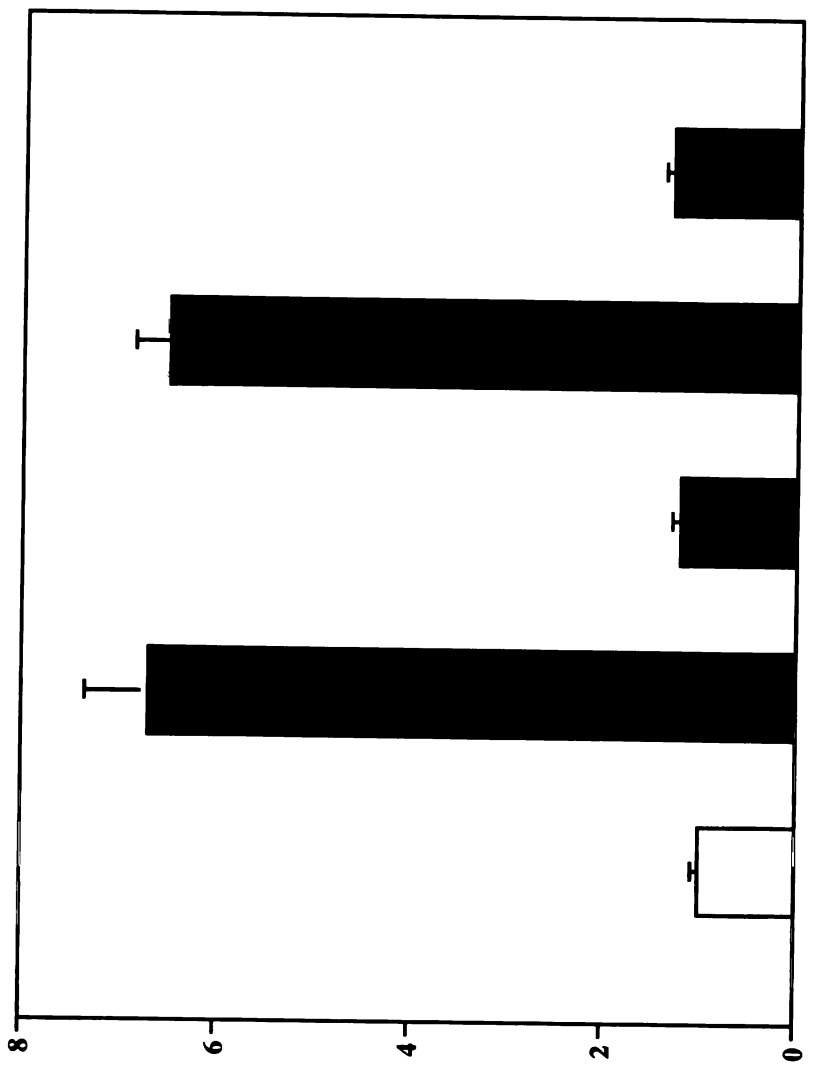

Relative luciferase activity
0
2
4
6
8
CON
CS (PAO1)
CS (PAO1) +AG126
LPS (PA10)
LPS (PA10) +AG126

Figure 19

tyrosine kinase p42MAPK is an integral part of the signal transduction pathway upregulating *MUC 2* transcription.

To determine whether activation of classical MAPK pathway is required for *P. aeruginosa*-induced *MUC 2* upregulation, we evaluated the effect on *MUC 2* induction of a specific chemical inhibitor of MAPK kinase (MEK1/2), PD98059. Preincubation of cells with PD98059 abolished *P. aeruginosa*-induced *MUC 2* upregulation (Fig.20), indicating that activation of MAPK (ERK1/2) is required for *MUC 2* induction. To confirm requirement of activation of MAPK(ERK1/2) pathway in *MUC 2* induction, we also investigated the effect of co-expressing a dominant-mutant form of MEK1/2 and the wild-type MEK1/2 on *P. aeruginosa*-induced *MUC 2* upregulation. Co-expression of dominant-negative mutant from of MEK1/2 inhibited *P. aeruginosa*-induced *MUC 2* induction by approximately 60% whereas overexpression of wild-type MEK1/2 greatly enhanced the upregulation of *MUC 2* induced by *P. aeruginosa* (Fig. 21). Taken together, these data suggest that the classical MEK1/2-MAPK(ERK1/2) pathway is involved in upregulation of *MUC 2* induced by *P. aeruginosa*.

Ras is also required in *P. aeruginosa*-induced *MUC 2* upregulation.

In the classical MAPK signal transduction pathway, the binding of growth factors such as EGF to their receptors first activates Ras, which in turn activates c-Raf, MEK1/2 and ERK1/2. Here we confirmed that Ras is also involved in *P. aeruginosa*-induced *MUC 2* upregulation by co-expressiing one MUC 2 promoter luciferase construct with a dominant-negative mutant form of Ras. This inhibited *P. aeruginosa*-induced *MUC 2* upregulation by approximately 80%, suggesting that Ras activation is also required for *MUC 2* induction (Fig. 22).

Fig. 20. Inhibition of *P. aeruginosa*-induced *MUC 2* upregulation by MEK1/2 inhibitor PD98059. HM3 cells were transfected with p-2864luc. 40 hours after being transfected with p-2864luc, HM3 cells were pretreated with PD98059 (37 μM and 75 μM) (CALBIOCHEM, La Jolla, CA) for 30 min. and then exposed to PAO1 culture supernatant (CS) 6 hours before harvesting. Luciferase activity was measured as described above. All transfections were carried out in triplicate. Values are the means ± SD; n = 4. Luciferase activity was normalized with respect to ß-galactosidase.

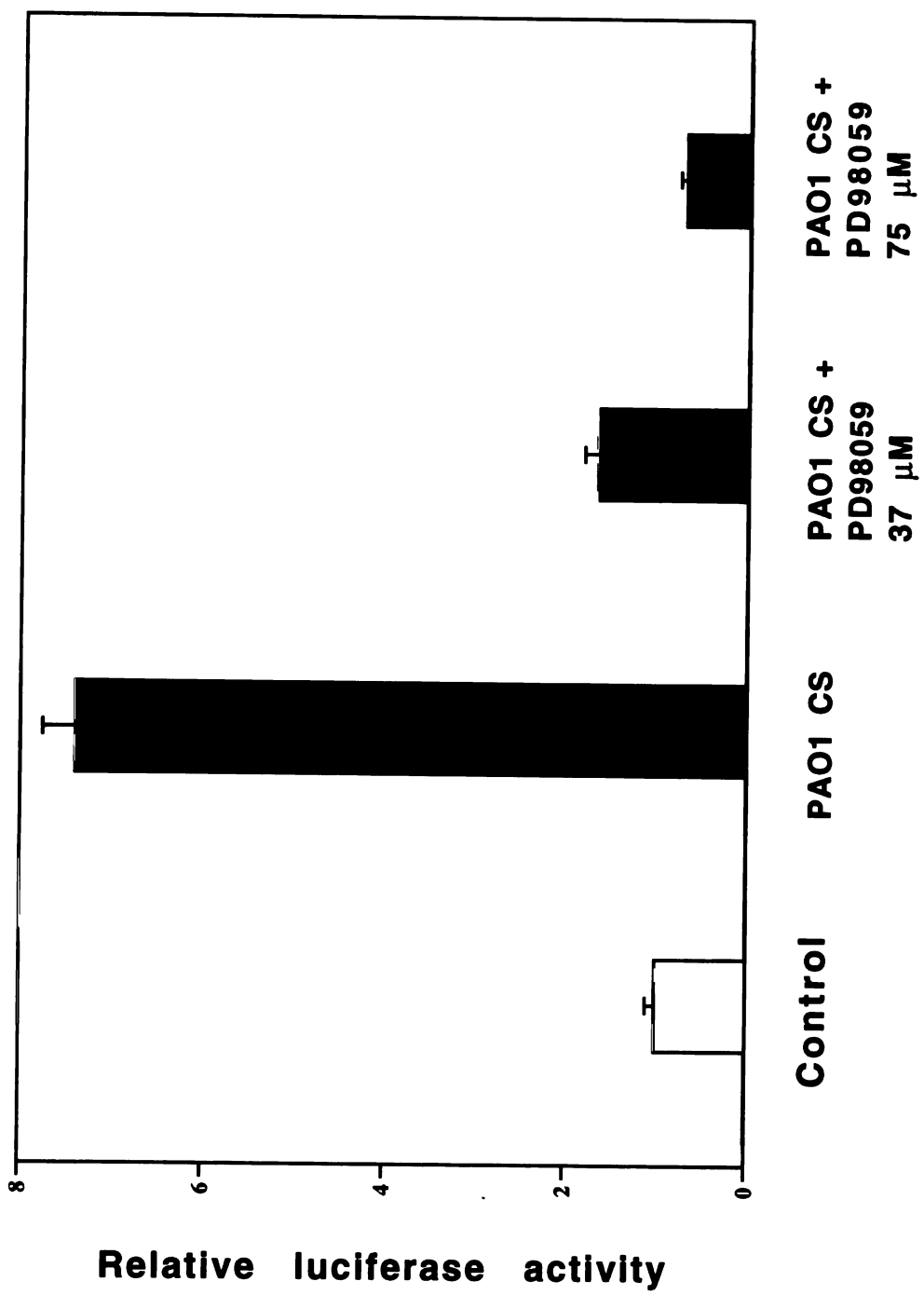

Relative luciferase activity
0
2
4
6
8
Control
PAO1 CS
PAO1 CS + PD98059 37 µM
PAO1 CS + PD98059 75 µM

Figure 20

Fig. 21. Effect of co-expressing a dominant-negative mutant form and wild-type form of MEK1/2 on *P. aeruginosa*-induced *MUC 2* upregulation. p-2864luc (15 μg) was co-transfected with either a dominant-negative mutant form (3 μg) or a wild-type form (3 μg) of MEK1/2 HM3 cells were transfected into HM3 cells. 40 hours after being transfected with, HM3 cells were exposed to PAO1 culture supernatant (CS) 6 hours before harvesting. Luciferase activity was measured as described above. All transfections were carried out in triplicate. PD98059 treatment was used as a control for inhibition of MUC2 transcription. Values are the means ± SD; n = 4. Luciferase activity was normalized with respect to ß-galactosidase.

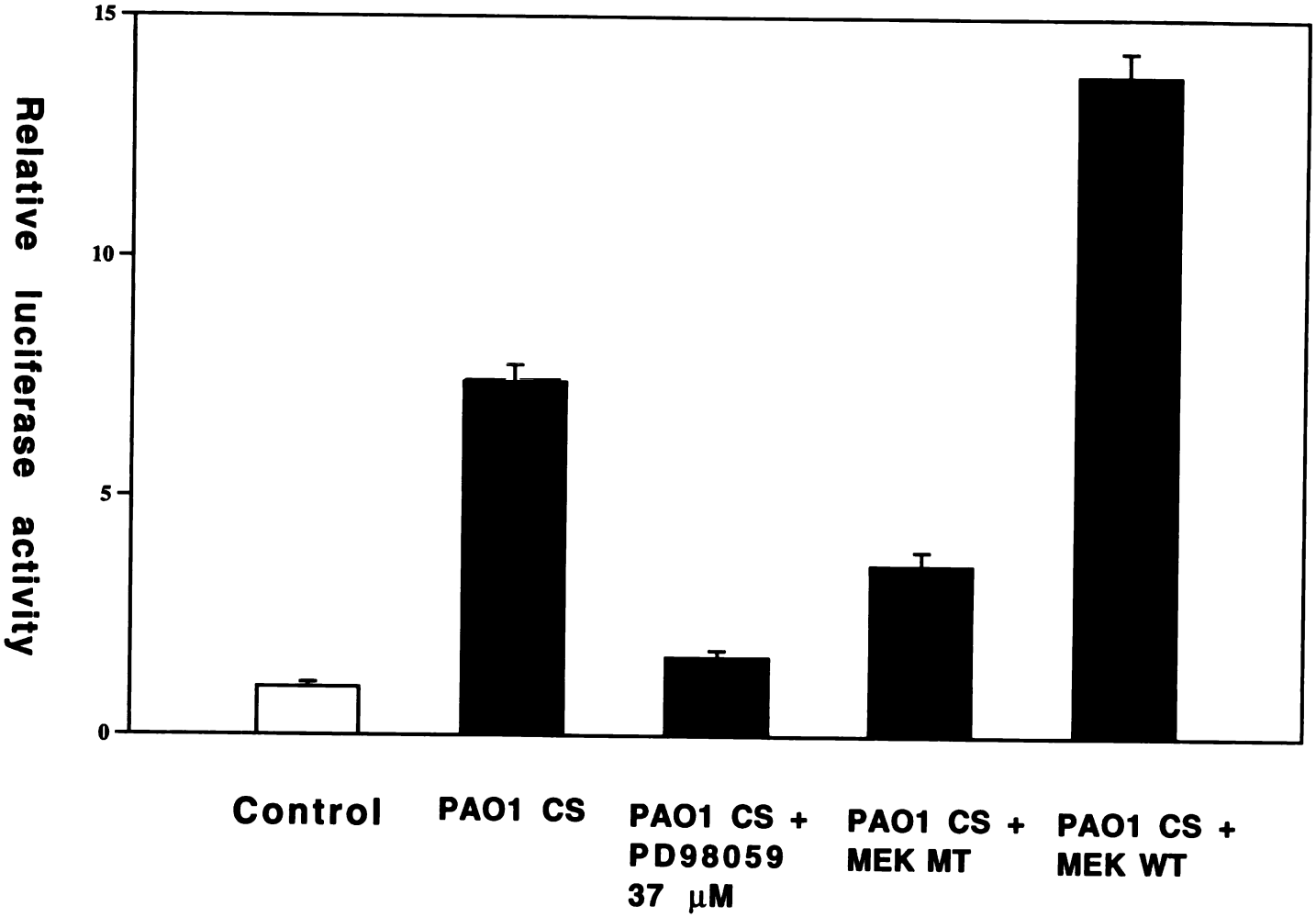

Relative luciferase activity
15
10
5
0
Control
PAO1 CS
PAO1 CS + PD98059 37 μM
PAO1 CS + MEK MT
PAO1 CS + MEK WT

Figure 21

Fig. 22. Effect of co-expressing a dominant-negative mutant form of Ras (RasN17) on *P. aeruginosa*-induced *MUC 2* upregulation. p-2864luc (15 μg) was co-transfected with a dominant-negative mutant form of Ras into HM3 cells. 40 hours after being transfected with, HM3 cells were exposed to PAO1 culture supernatant (CS) 6 hours before harvesting. Luciferase activity was measured as described above. All transfections were carried out in triplicate. Values are the means ± SD; n = 4. Luciferase activity was normalized with respect to ß-galactosidase.

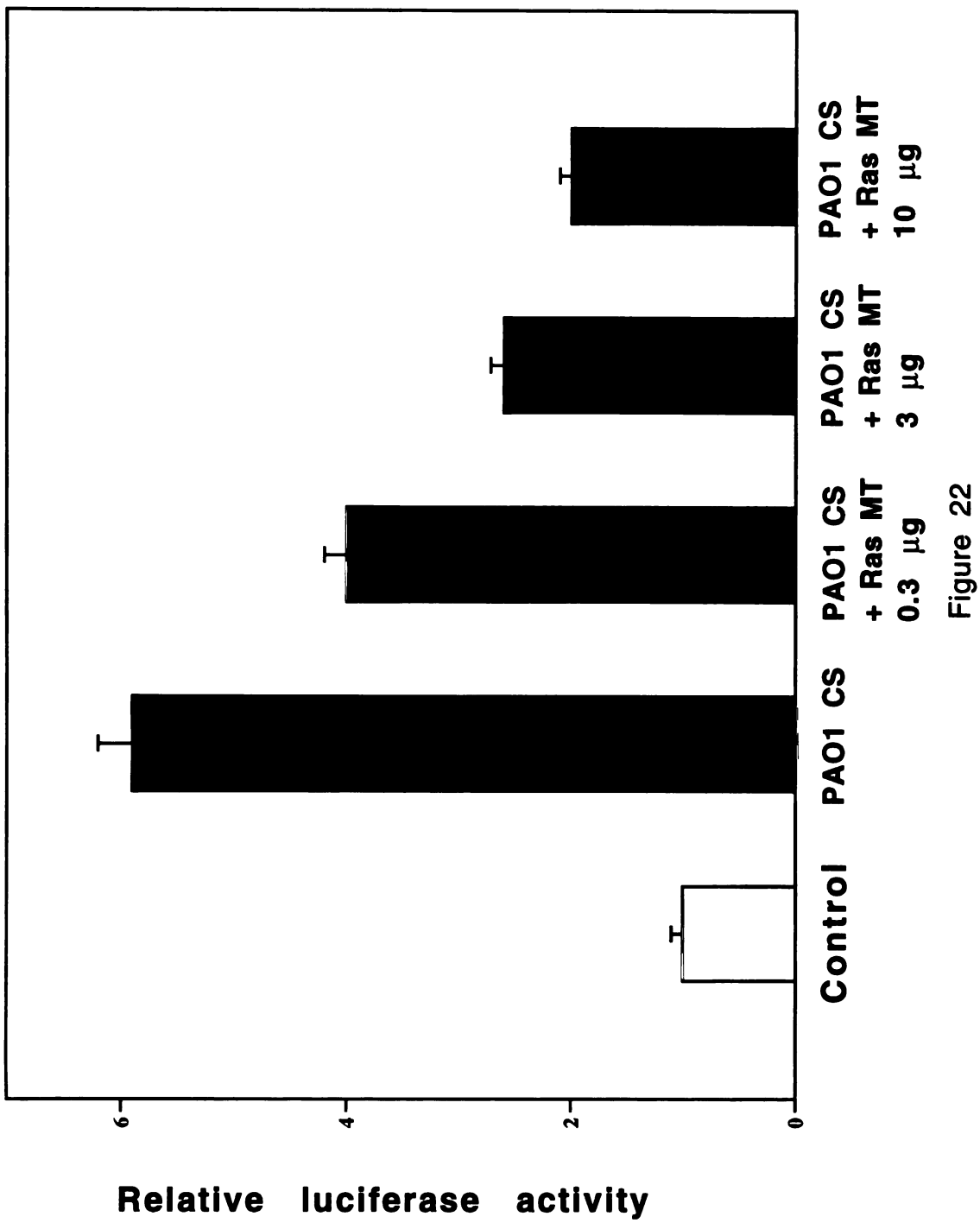

Relative luciferase activity
0
2
4
6
Control
PAO1 CS
PAO1 CS + Ras MT 0.3 µg
PAO1 CS + Ras MT 3 µg
PAO1 CS + Ras MT 10 µg

Figure 22

Src is involved in *P. aeruginosa*-induced *MUC 2* induction.

In addition to its role in cell growth and tumorigenesis, Src has also been shown to be involved in oxygen radical H_2O_2-induced activation of the Ras, Raf-1, MEK1/2 and MAPK (ERK1/2) pathway which ultimately leads to activation of ICAM transcription. We investigated the possibility that Src is also involved in *P. aerginosa*-induced *MUC 2* upregulation by studying the effect on *MUC 2* induction of a newly developed Src-specific chemical inhibitor, PP1. Preincubation of cells with PP1 inhibited *P. aeruginosa*-induced *MUC 2* upregulation by approximately 80% (Fig.23). To confirm the role of Src in *MUC 2* induction, we also investigated the effect of co-expression of V-Src, the constitutively active form of $pp60^{c\text{-}Src}$ on *MUC 2* transcription. Co-expression of V-Src greatly upregulated *MUC 2* transcription (Fig. 24), indicating that activation of Src is sufficient to mimic *P. aeruginosa*-induced *MUC 2* upregulation.

Src activates *MUC 2* transcription via activation of Ras, Raf-1, MEK1/2 and MAPK(ERK1/2) pathways.

To determine whether Src activates *MUC 2* transcription via activation of Ras, Raf1, MEK1/2 and MAPK(ERK1/2) pathway or other pathways, we observed the effect of co-expressing dominant negative mutant forms of Ras and MEK1/2 and the chemical inhibitor of MEK1/2 PD98059 on V-Src-induced *MUC 2* upregulation. Src inhibitor was used as a control for inhibition of Src activity. Upregulation of *MUC 2* induced by V-Src was inhibited by approximately 80 - 90% by MEK1/2 specific inhibitor PD98059 and co-expression of dominant negative mutant forms of Ras and MEK1/2 (Fig.25). When P. aeruginosa supernatnt was applied to the epithelial cells co-expressed with v-Src, no further significant up-regulation of MUC2 was observed (Fig. 26). Taken together, these results suggest that V-Src upregulates *MUC 2* transcription at least partially via activation of Ras, MEK1/2 and MAPK(ERK1/2) pathways.

Fig. 23. Inhibition of *P. aeruginosa*-induced *MUC 2* upregulation by Src inhibitor PP1. HM3 cells were transfected with p-2864luc. 40 hours after being transfected with p-2864luc, HM3 cells were pretreated with PP1 (5, 14 and 28 μM) (CALBIOCHEM, La Jolla, CA) for 30 min. and then exposed to PAO1 culture supernatant (CS) 6 hours before harvesting. Luciferase activity was measured as described above. All transfections were carried out in triplicate. Values are the means ± SD; n = 3. Luciferase activity was normalized with respect to ß-galactosidase.

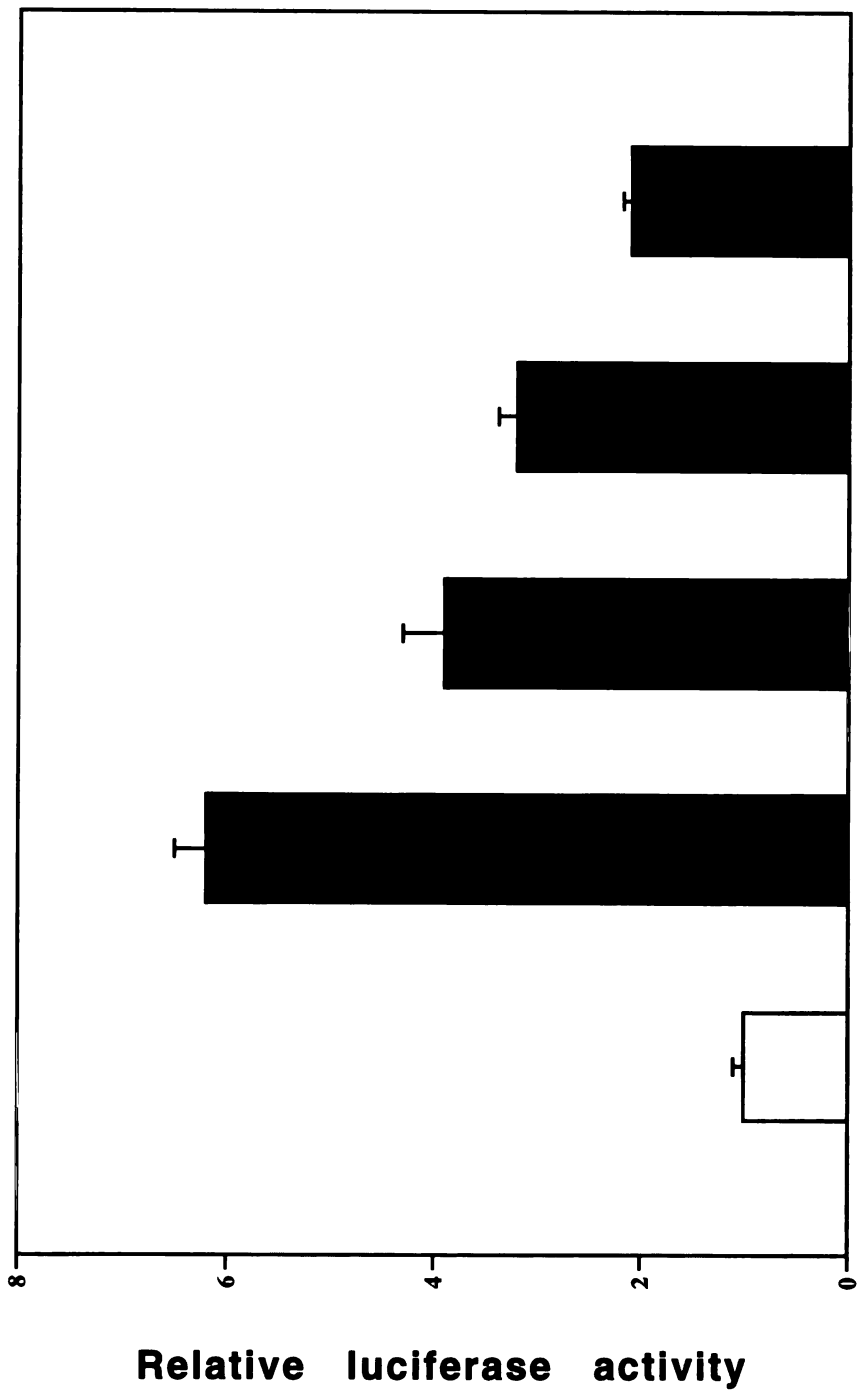

Relative luciferase activity
0
2
4
6
8
Control
PAO1 CS
PAO1 CS + PP1 5 µM
PAO1 CS + PP1 14 µM
PAO1 CS + PP1 28 µM

Figure 23

Fig. 24. Effect of co-expressing V-Src on *MUC 2* transcription. p-2864luc (15 μg) was co-transfected with V-Src (0.3, 1, 3 and 5 μg) into HM3 cells. 40 hours after being transfected with, HM3 cells were exposed to PAO1 culture supernatant (CS) 6 hours before harvesting. Luciferase activity was measured as described above. All transfections were carried out in triplicate. Values are the means ± SD; n = 4. Luciferase activity was normalized with respect to ß-galactosidase.

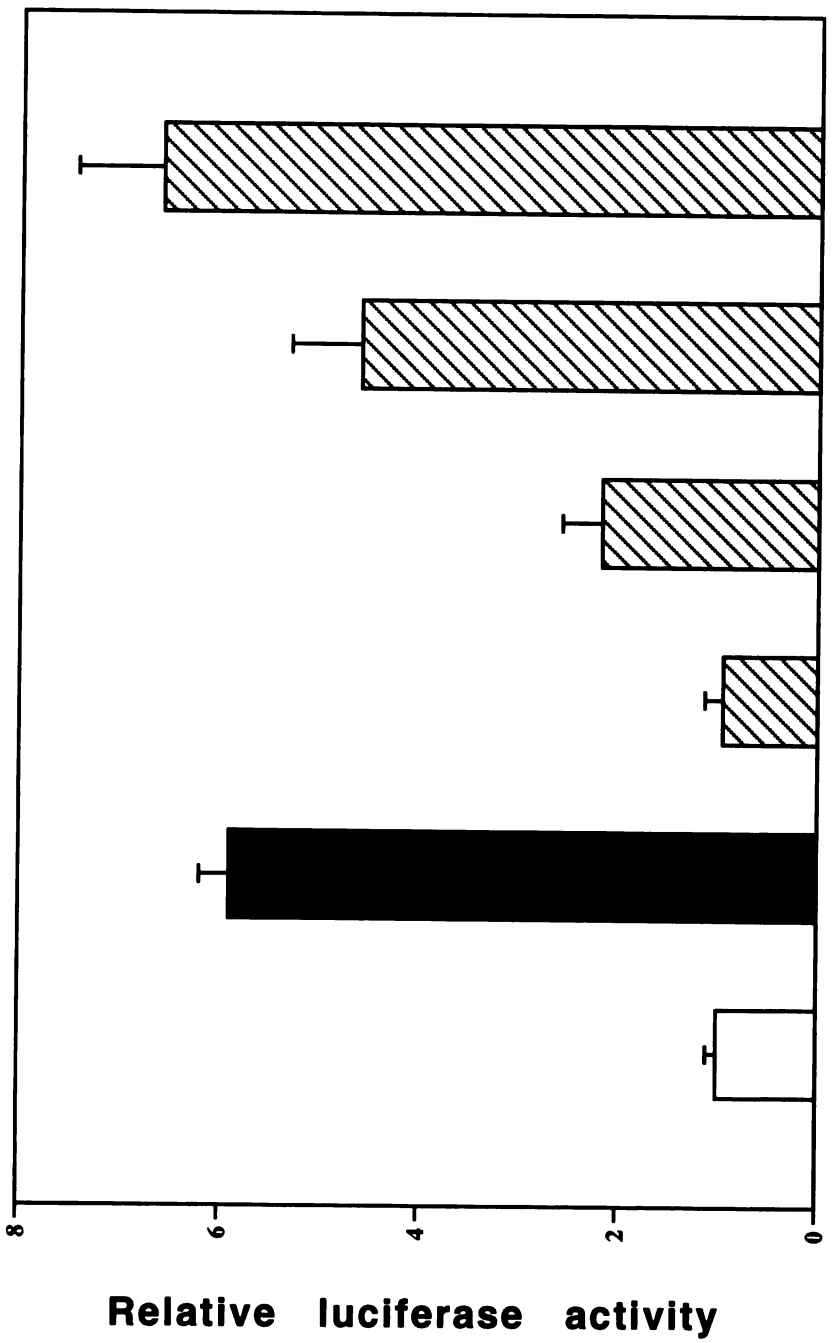

Relative luciferase activity
0
2
4
6
8
Control
PAO1
CS
v-src 0.3 µg
v-src 1 µg
v-src 3 µg
v-src 5 µg

Figure 24

Fig. 25. Effect of co-expressing a dominant-negative mutant form of Ras and MEK1/2 on V-Src-induced *MUC 2* upregulation. p-2864luc (15 μg) was co-transfected with V-Src (3 μg) and a dominant-negative mutant form of either Ras or MEK1/2 or both into HM3 cells. 48 hours after being transfected, luciferase activity was measured as described above. All transfections were carried out in triplicate. Values are the means ± SD; n = 3. Luciferase activity was normalized with respect to ß-galactosidase.

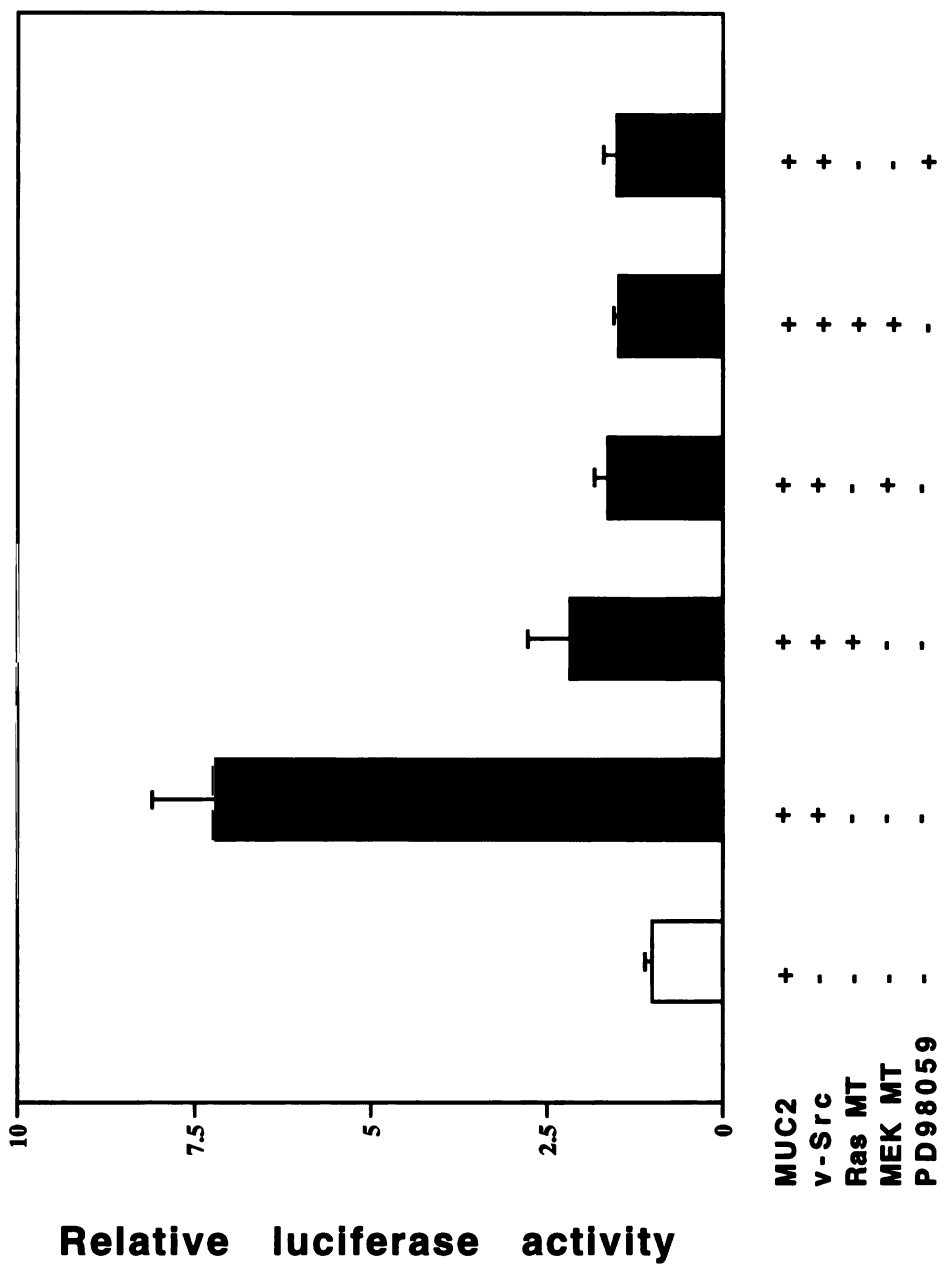

10
7.5
5
2.5
0
Relative luciferase activity
MUC2
v-Src
Ras MT
MEK MT
PD98059
+ - - - -
+ + - - -
+ + + - -
+ + - + -
+ + + + -
+ + - - +

Figure 25

Fig. 26. Effect of *P. aeruginosa* on V-Src-induced *MUC 2* upregulation. p-2864luc (15 μg) was co-transfected with V-Src (3 μg) into HM3 cells. 40 hours after being transfected with, HM3 cells were either exposed non-exposed to PAO1 culture supernatant (CS) 6 hours before harvesting. Luciferase activity was measured as described above. All transfections were carried out in triplicate. Values are the means ± SD; n = 3. Luciferase activity was normalized with respect to ß-galactosidase.

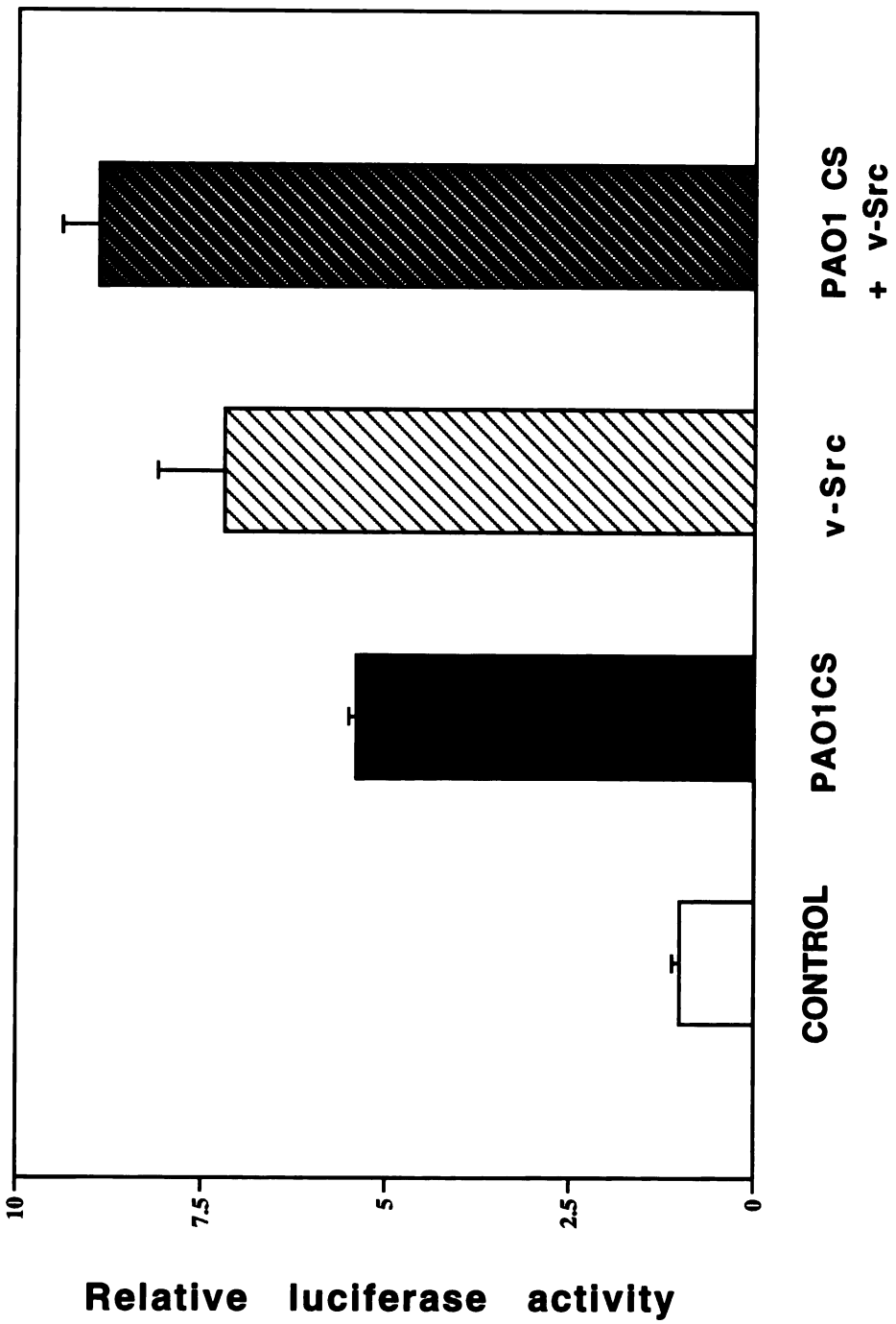

10
7.5
5
2.5
0
Relative luciferase activity
CONTROL
PAO1CS
v-Src
PAO1 CS
+ v-Src

Figure 26

Inhibition of *P. aeruginosa*-induced *MUC 2* induction by the oxygen radical scavenger DMTU and antioxidant NAC

Oxygen radical species (ROS) have been shown to be involved in LPS-induced TNF-a expression in airway epithelial cells. To determine whether or not ROS are also involved in *P. aeruginosa*-induced *MUC 2* upregulation, we studied the effects of oxygen radical scavengers DMTU and NAC. Both DMTU and NAC abolished *P. aeruginosa*-induced *MUC 2* upregulation (Fig. 27 and 28), indicating that ROS are also involved in *P. aeruginosa*-induced *MUC 2* upregulation.

Fig. 27. Inhibition of *P. aeruginosa*-induced *MUC 2* upregulation by oxygen radical scavenger DMTU. HM3 cells were transfected with p-2864luc. 40 hours after being transfected with p-2864luc, HM3 cells were pretreated with dimethylthiourea (DMTU) (4, 20, 40 and 80 mM) (CALBIOCHEM, La Jolla, CA) for 30 min. and then exposed to PAO1 culture supernatant (CS) 6 hours before harvesting. Luciferase activity was measured as described above. All transfections were carried out in triplicate. Values are the means ± SD; n = 4. Luciferase activity was normalized with respect to ß-galactosidase.

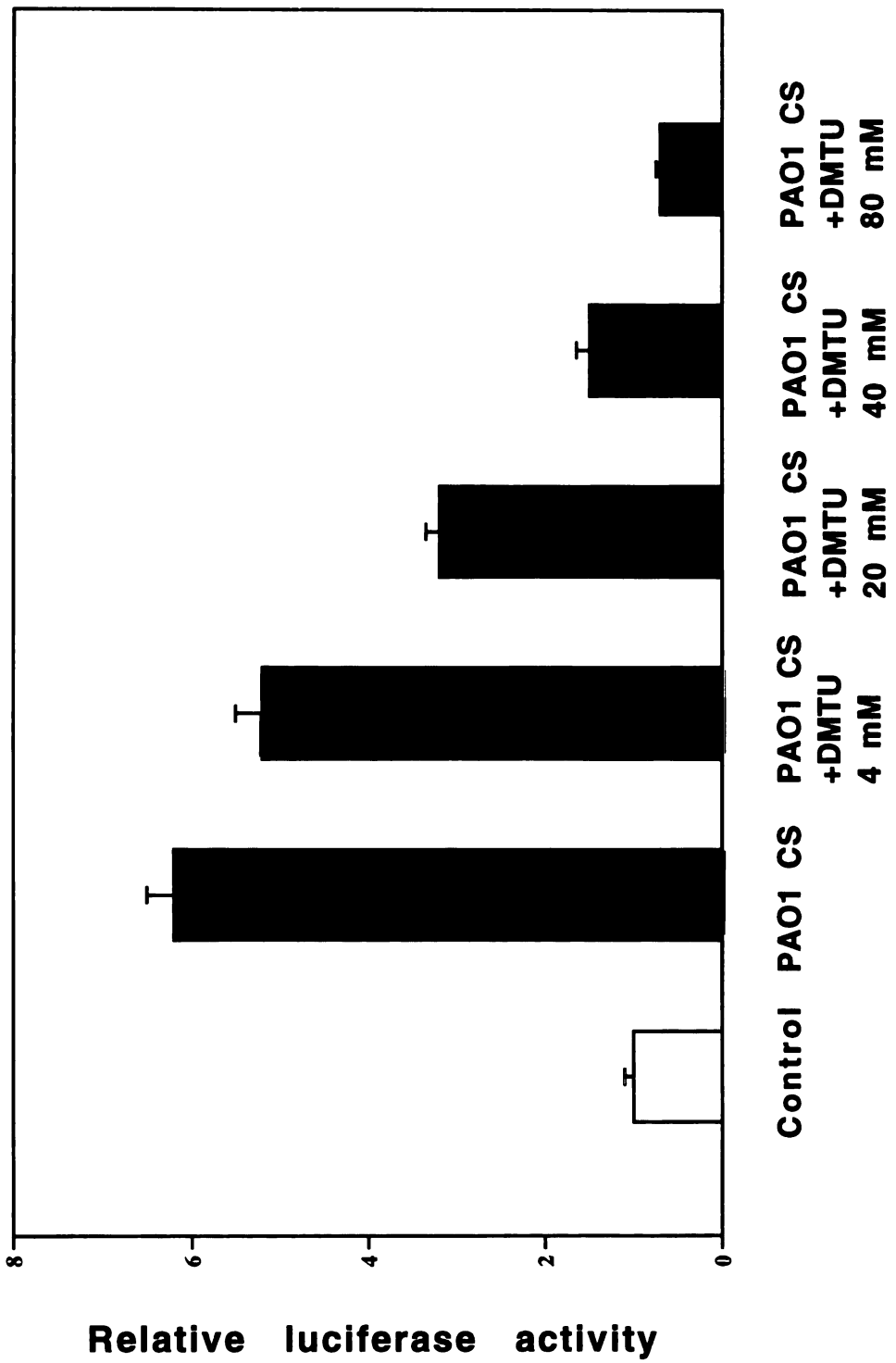

Relative luciferase activity
0
2
4
6
8
Control
PAO1 CS
PAO1 CS +DMTU 4 mM
PAO1 CS +DMTU 20 mM
PAO1 CS +DMTU 40 mM
PAO1 CS +DMTU 80 mM

Figure 27

Fig. 28. Inhibition of *P. aeruginosa*-induced *MUC 2* upregulation by antioxidant NAC. HM3 cells were transfected with p-2864luc. 40 hours after being transfected with p-2864luc, HM3 cells were pretreated with NAC (80 mM) (CALBIOCHEM, La Jolla, CA) for 30 min. and then exposed to PAO1 culture supernatant (CS) 6 hours before harvesting. Luciferase activity was measured as described above. All transfections were carried out in triplicate. Values are the means ± SD; n = 3. Luciferase activity was normalized with respect to ß-galactosidase.

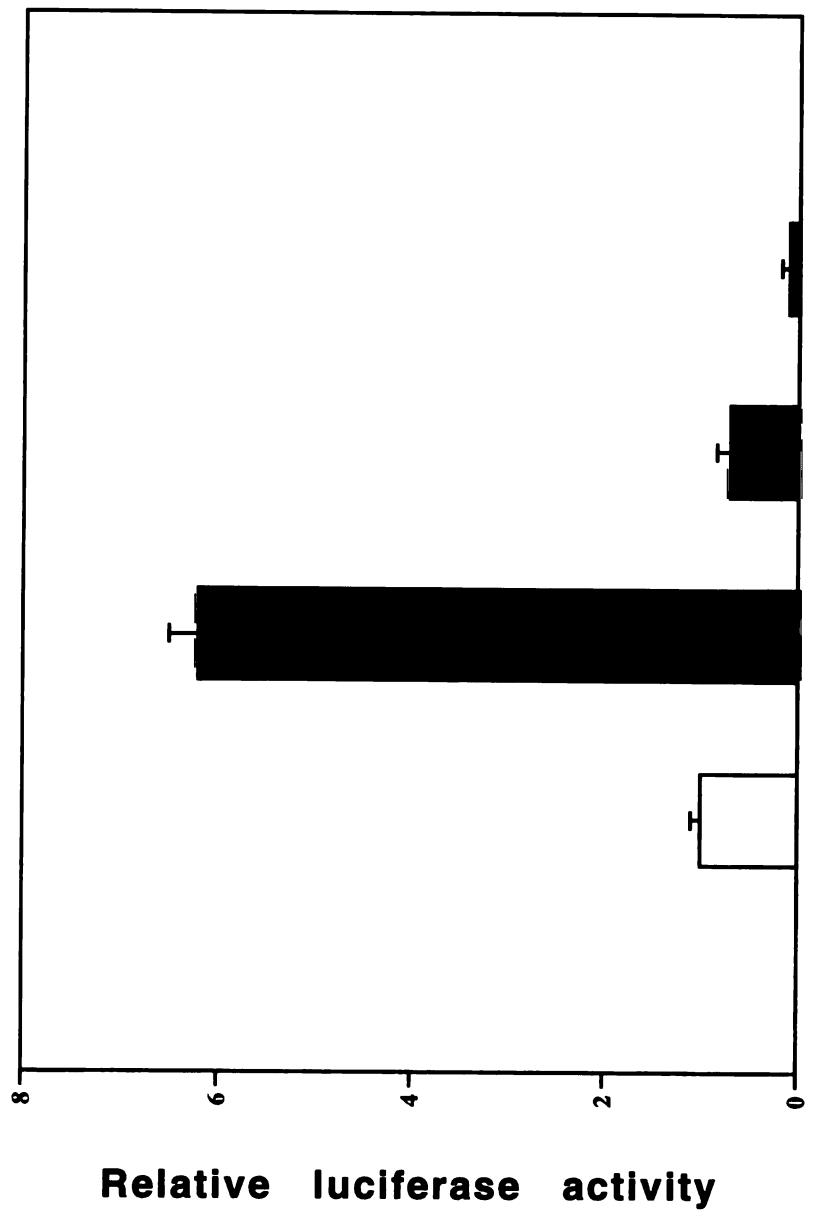

Relative luciferase activity
8
6
4
2
0
Control
PAO1 CS
PAO1 CS +DMTU 75 mM
PAO1 CS +NAC 80 mM

Figure 28

DISCUSSION

Since the isolation of the cystic fibrosis gene CFTR in 1989, considerable progress has been made towards understanding the metabolic abnormality in cystic fibrosis. Defective Cl^- transport across affected epithelial cells is the hallmark of the disease, although numerous other abnormalities have been also reported (52). Airway disease leads to progressive lung dysfunction, which is the major cause of morbidity and is responsible for 95 % of mortality. CF airway disease is characterized by chronic lung infection and mucus overproduction. Recently, some studies have linked CFTR mutation to the onset and persistence of P. aeruginosa, the most common opportunistic pathogen in CF airways, infecting more than 80 % of CF patients. However, how a defective CFTR causes mucus overproduction remains unclear.

That the onset of P. aeruginosa infection in the CF airway presages airway mucus obstruction led us to hypothesize that the pathogenesis of CF lung disease proceeds in two stages: first, the increased susceptibility of P. aeruginosa infection as a direct consequence of CFTR gene mutation and second, the overproduction of mucin as a consequence of P. aeruginosa infection.

Mucin glycoprotein is the major component of airway mucus. Several mucin genes have been shown to be expressed in airway, mucin MUC2 and MUC5 genes (8, 53). Molecular cloning of the cDNA and 5'-flanking region of the human MUC2 gene provided reagents with which to investigate the molecular mechanisms of mucin overproduction in CF airways of CF patients (9, 11). Using a probe for the human MUC2 mucin gene, we showed that a greater proportion of cells expressed MUC2 in CF than in non-CF airways and that the level of expression per cell was also higher. We

next directly tested the hypothesis that P. aeruginosa up-regulates mucin mRNA by exposing human bronchial explants to P. aeruginosa culture supernatant by performing MUC2 in situ hybridization. We found that P. aeruginosa greatly increased MUC2 mRNA levels in both the surface epithelium and submucosal glands of the explants, confirming our hypothesis that P. aeruginosa up-regulates mucin expression. Our findings provided the first evidence to directly link the infection of P. aeruginosa to mucin overproduction and explain why CF patients have abnormally high level of mucus.

The bronchial explants contain multiple cell types. The up-regulating effect of P. aeruginosa on MUC2 mRNA could have resulted from either a direct effect of bacterial exoproducts on epithelial cells or an indirect effect mediated by resident inflammatory cells. It has been shown that TNF-alpha and IL-4 upregulate MUC2 expression in airway epithelial cells (54). Therefore we could not rule out a role for cytokines released from other inflammatory cells. To test the hypothesis that the effect is direct, we applied P. aeruginosa culture supernatant to cultured epithelial cells and measured MUC2 mRNA by RPA. P. aeruginosa greatly increased MUC2 mRNA, indicating that P. aeruginosa exoproducts act directly on epithelial cells to up-regulate MUC2 mRNA. To test the possibility that MUC2 mRNA is up-regulated by cytokines released from epithelial cells due to P. aeruginosa stimulation, we studied the effect of TNF-alpha, IFN-gamma, IL-1, IL-4, IL-6 and IL-8 on MUC2 expression and none of these upregulated MUC2 expression, supporting the view that the effect of P. aeruginosa on MUC2 expression is direct.

To determine whether MUC2 mRNA regulation is controlled at the level of transcription, we transfected epithelial cells with an expression vector containing the MUC2 5'-flanking region fused to a luciferase reporter gene. The luciferase activity was

increased several fold when P. aeruginosa culture supernatant was applied to the transfected cells, indicating that P. aeruginosa up-regulates MUC2 at the transcriptional level.

P. aeruginosa expresses numerous gene products that could contribute to the pathogenesis of P. aeruginosa lung infection (6, 13, 20, 55-57). The adhesins are responsible for binding to specific receptors on epithelial surfaces. Exoenzymes modify eukaryotic targets. Mucoexopolysaccharide alginate has the capacity to suppress neutrophil and lymphocyte function. Pilin and flagellin are responsible for stimulation of IL-8 release from airway epithelial cells. Lipopolysaccharide (LPS) plays a role in the causation of fever, hypertension, disseminated intravascular co-agulation (DIC), septic shock and also stimulates the production of arachidonic acid metabolites, including prostaglandins and TNF-alpha. Exotoxin A appears to mediate both local and systemic disease processes. Its necrotizing activity in locally exposed tissue probably contributes to the pathologic lesions at primary and metastatic sites of infection as well as bacterial dissemination. Elastase and alkaline protease are most clearly associated with virulence such as tissue destruction and bacterial invasion. Both are necrotizing in the skin, lung and cornea, whereas elastase is capable of producing hemorrhage. To identify those components of P. aeruginosa responsible for MUC2 up-regulation, we used several approaches. Using a genetic approach, we ruled out several common protein virulence factors including pilin, flagellin, elastase, alkaline protease, PLC-, alginate and exotoxin A. Biochemical analysis showed that the virulence factors responsible for MUC2 up-regulation are resistant to heat, proteolysis and DNA digestion. A physical approach revealed that either a single virulence factor with a wide range of molecular sizes or multiple components are responsible for MUC2 up-regulation. Taken together, these findings are consistent with the active factor being lipopolysaccharide (LPS). When we directly tested the effect of LPS on MUC2 transcription, we confirmed this. Since no

efficient approach is currently available for completely removing LPS from P. aeruginosa culture supernatant, we can not rule out a role for additional virulence factors (58, 59). This seems unlikely, however, because purified LPS administered at a similar concentration present in P. aeruginosa supernatant produces an equivalent effect.

The finding that the LPS sugar core and lipid A is sufficient to induce the up-regulation of MUC2 transcription is interesting. This differs from results showing that the complete LPS complex including O-side chain, sugar core and lipid A is required to up-regulate IL-8 mRNA expression in airway epithelial cells (13). This disparity indicates that LPS interacts with host epithelial cells to stimulate expression of various genes via multiple mechanisms.

Much effort has been put into studying the interaction of LPS with macrophages (18, 29). This study, together with the IL-8 study mentioned above is among the few reports describing the interaction LPS with host epithelial cells. It had been thought that LPS effects on epithelial cells are indirect. Thus, it has been known for some time that LPS interacts with macrophages and stimulates the production of cytokines. These, in turn, are known to modify the host response of epithelial cells (18, 24, 29, 61). By contrast, our findings indicated that LPS can directly interact with airway epithelial to induce a host response. As the first barrier of the host defense system, surface epithelial cells are the first to interact with bacteria. We have observed that this direct interaction quickly stimulates the production of mucin. The bacteria are entrapped in the overproduced mucin and are cleared by the beating of the epithelial cell cilia, which moves mucus movement towards the throat where it is swallowed. Mucin production thus normally promotes host defense. Due to defect in hydration and ciliary beat in CF patients, the overproduced mucin induced by P. aeruginosa likely accumulates in the airways rather than being cleared. This is likely to causes airway mucus obstruction over time. In

addition to impaired airflow, accumulated mucin in the airway may also serve to protect the bacteria from attack by the host immune defense system and antibiotics, likely contributing to the persistence of the bacteria infection in the airways of cystic fibrosis patients.

We showed transcriptional control mechanisms to be involved in the up-regulation of MUC2 gene expression. We further identified DNA response elements in the 5'-flanking region of the MUC2 gene in two regions, -91/-74 bp (proximal enhancer) and -1528/-1430 bp (distal enhancer). Sequence analysis showed that a CCCACC box in the proximal region and a NF-kappa B element in the distal region are involved in P. aeruginosa-mediated up-regulation of MUC2 transcription. Interestingly, a recent study demonstrated that the CCCACC box is also involved in controlling basal transcription of MUC2 in MUC2-expressing epithelial cells (60). That NF-kappa B is involved in the P. aeruginosa-induced MUC2 upregulation is consistent with previous findings showing a NF-kappa B element to be involved in LPS-induced serum amyloid gene expression in the liver (36).

The classical MAP kinase pathway has been shown to participate in the signal transduction pathways activated by a variety of extracellular ligands (31, 33, 62). Analysis of the intracellular signal transduction pathways mediating the P. aeruginosa-induced MUC2 up-regulation indicated that P. aeruginosa directly upregulates mucin transcription via activation of the MAPK(ERK1/2) pathway. This is consistent with the findings in macrophages. Additional experiments provided evidence, for the first time, to suggest the involvement of Src in bacterial product-induced cellular response (63, 64).

The finding that P. aeruginosa-induced MUC2 upregulation is inhibited by certain tyrosine kinase inhibitors is of interest for the treatment of CF lung disease. Recently, much effort has been put into developing pharmaceutical tyrosine kinase inhibitors for the treatment of tumors, diabetes, macular degeneration, diabetic retinopathy and psoriasis. For example, antagonists for the PDGF receptor, VEGF receptor Flk-1, Raf-1 and GRB2 have been developed for treatment of brain, breast, prostate and lung cancers (23, 65, 66). Understanding of the intracellular signal transduction pathway mediating P. aeruginosa-induced MUC2 upregulation may be helpful for developing new therapeutic strategies to reduce mucus overproduction in CF patients.

We found no hypersusceptibility of CF vs non-CF epithelial cells with respect to MUC2 up-regulation by P. aeruginosa. Consistent with this, we also found no reduction of up-regulation of MUC2 in CF epithelial cells complemented with wild-type CFTR expression plasmid. This is also consistent with findings in CFTR (-/-) mice. No mucus overproduction was found in airways until they were switched from a pathogen-free to a pathogen-containing environment (67-71). These findings, taken together, strongly suggest an indirect rather than a direct relationship between mutant CFTR and mucus overproduction.

From what we know now about the CF lung it is evident that pathogenesis does not emanate directly from the CFTR mutation by a linear sequence of events. Instead, by interfering with a variety of physiological mechanisms (2-4), the mutation increases the susceptibility of P. aeruginosa infection via three possible mechanisms, including (a) undersialylated cell surface glycolipids that act as *P. aeruginosa* binding sites (2), (b) impaired capacity for bronchial epithelial cells to clear *P. aeruginosa* by endocytosis (3) and (c) decreased activity of bronchial bacteriolytic substances due to abnormal airway surface liquid (4). Infection, by the mechanisms outlined above, triggers mucin

overproduction. The latter, in concert with dehydration (5) also engendered by the CFTR mutation, leads to airway mucus obstruction and lung failure. The data shown here for the first time reveal the capacity for *P. aeruginosa* to directly stimulate mucin production and thereby offer a rational explanation for why mucus plugging and deterioration of lung function occur relentlessly subsequent to the onset of infection (12). These data predict that by directly upregulating mucin transcription in airway epithelial cells via activation of Src, Ras, Raf-1, MEK1/2 and MAPK(ERK1/2) pathways, *P. aeruginosa* may greatly increase the mucin load discharged into the CF airway lumen (Fig.29). This is especially true in light of the fact that we have recently observed profound upregulation of the human *MUC 5* as well as the *MUC 2* gene by *P. aeruginosa* (8).

Mucus not only impairs airflow and O_2/CO_2 exchange, but by physically enmeshing bacteria (13) as well as providing nutrition and protection from bactericidal agents, mucus also promotes chronic infection. Thus, the upregulation of mucin transcription by *P. aeruginosa* is at the crux of a vicious cycle of chronic infection and mucus obstruction that kills CF patients. The inhibition of this upregulation by LPS antagonists and/or specific tyrosine kinase inhibitors and oxygen radical scavengers would constitute a new therapeutic strategy to reduce morbidity and mortality in CF patients (22-23).

Fig. 29. Schematic diagram of up-regulation of human mucin MUC2 gene by P. aeruginosa in the pathogenesis of Cystic fibrosis airway disease. As indicated in the diagram, mutant CFTR engenders two major abnormalities: (i) dehydration in the airway lumen due to the defective Cl^- flux across the cell membrane; (ii) increased susceptibility of P. aeruginosa infection due to three abnormalities: (a) undersialylated cell surface glycolipids that act as *P. aeruginosa* binding sites (2), (b) impaired capacity for bronchial epithelial cells to clear *P. aeruginosa* by endocytosis (3) and (c) decreased activity of bronchial bacteriolytic substances due to abnormal airway surface liquid (4). P. aeruginosa releases LPS in the airway, which in turn triggers mucin overproduction via activation of Src, Ras, Raf-1, MEK1/2 and MAPK (ERK1/2) pathways. The overproduced mucin, in concert with dehydration, leads to airway mucus obstruction and lung failure.

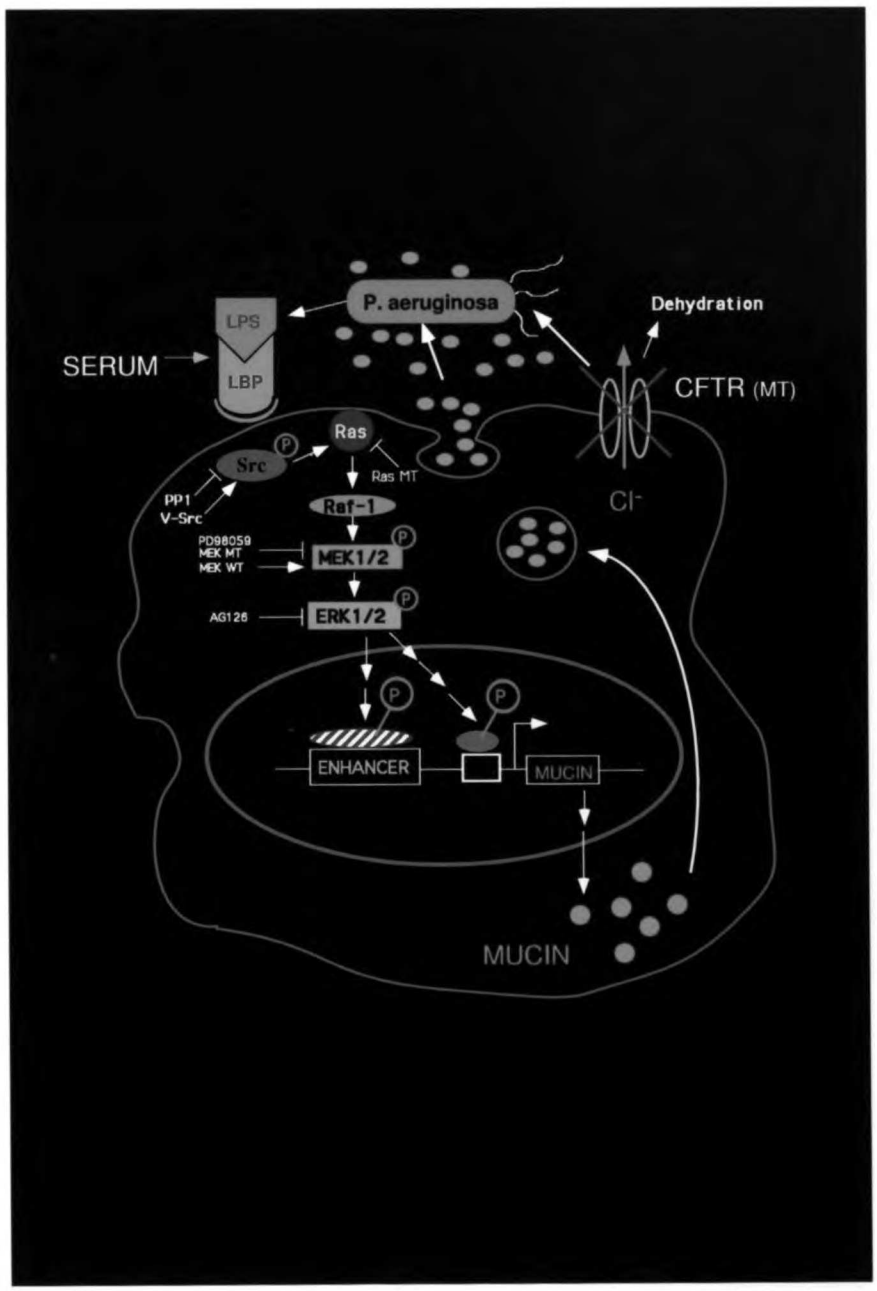

P. aeruginosa
Dehydration
LPS
SERUM
LBP
CFTR (MT)
Ras
Src
Ras MT
PP1
V-Src
Raf-1
PD98059
MEK MT
MEK WT
MEK1/2
AG126
ERK1/2
ENHANCER
MUCIN
Cl-
MUCIN

Figure 29

FUTURE DIRECTIONS

Future studies will be focused on the following questions:

1. Which receptors on the epithelial surface interact with P. aeruginosa LPS ? How does the LPS receptor activate Src pathway with respect to mucin up-regulation? Understanding these mechanisms will help developing therapeutics to disrupt LPS signaling and inhibit mucin overproduction induced by LPS.

2. How are other airway mucin genes such as MUC5 regulated by P. aeruginosa ? If other mucin genes are also regulated by P. aeruginosa, the increased mucin load discharged into the CF airway lumen will be more significant to the pathogenesis of CF lung diseases.

3. We have observed that airway mucin genes are also regulated by gram-positive bacteria. Since Gram- positive bacteria do not produce LPS, it will be interesting to know how this occurs.

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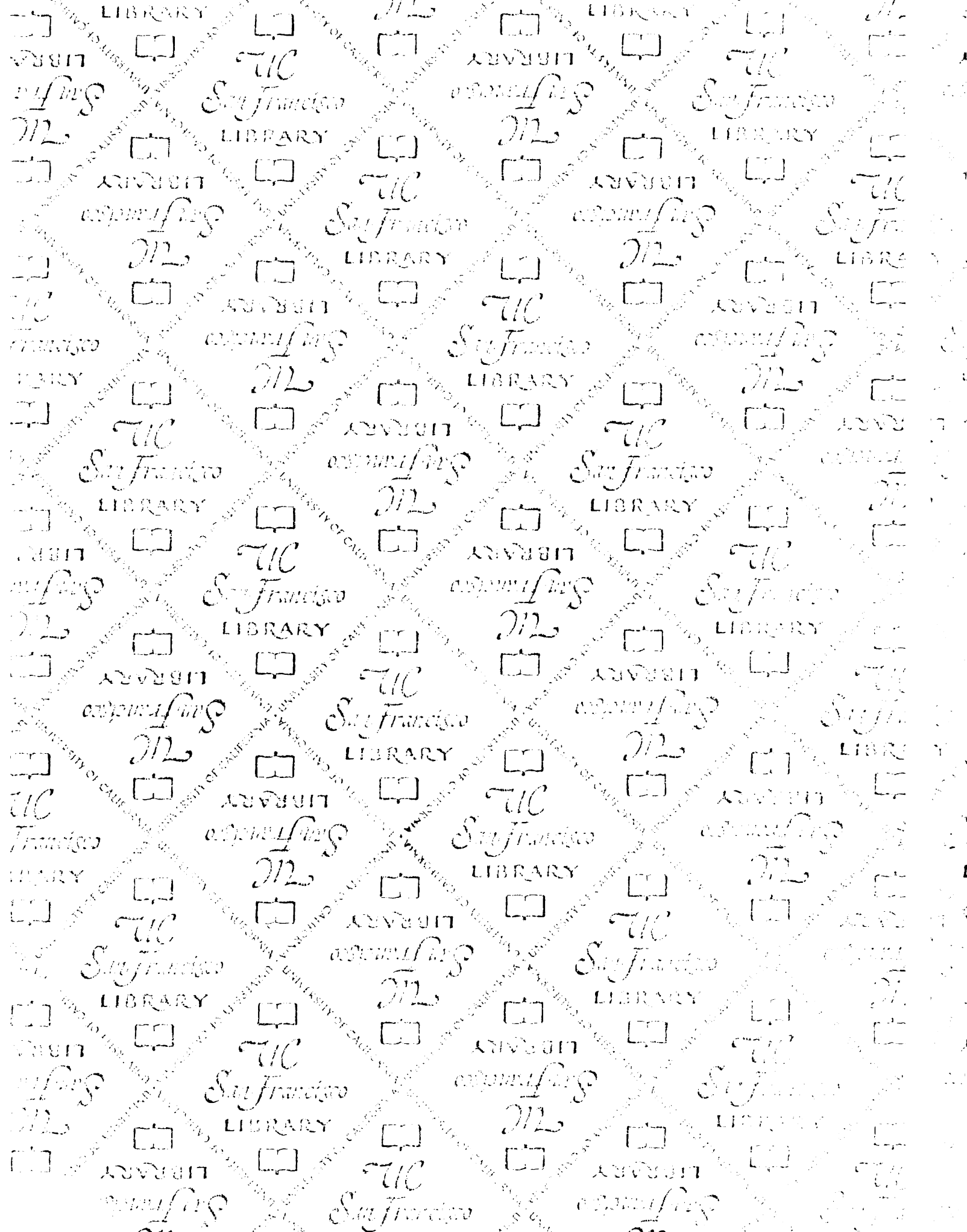
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