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Characterization of Human Mucin Gene *MUC4* Promoter

IMPORTANCE OF GROWTH FACTORS AND PROINFLAMMATORY CYTOKINES FOR ITS REGULATION IN PANCREATIC CANCER CELLS*

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The human mucin gene *MUC4* encodes a large transmembrane mucin that is thought to play important roles in tumor cell biology and that is overexpressed in human pancreatic carcinomas. In this report, we describe the structure and functional activity of the 5'-flanking region, including 1.0 kilobase of the promoter. The long 5'-untranslated region (2.7 kilobases) is characterized by a high content of GC in its 3'-end. The first TATA box was located at -2672/-2668. Multiple transcription start sites and a high density of putative binding sites for Sp1 (GC and CACC boxes), AP-1/-2/-4, cAMP-responsive element-binding protein, GATA, GR, and STAT transcription factors were found within the 5'-flanking region. Transcriptional activity of the promoter was assessed using pGL3-luciferase deletion mutants in two *MUC4*-expressing (CAPAN-1 and CAPAN-2) and one nonexpressing (PANC-1) pancreatic cancer cell line. Two highly active fragments (-219/-1 and -2781/-2572) that drive *MUC4* transcription in CAPAN-1 and CAPAN-2 cells were identified. Gel retardation assays indicated that Sp1 and Sp3 bind to cognate *cis*-elements found in the 5'-flanking region and that Sp1 transactivates, whereas Sp3 inhibits the GC-rich region (-464/-1) in CAPAN-2 cells. Activation of protein kinase C with phorbol ester and treatment of cells with epidermal growth factor and transforming growth factor- α resulted in up-regulation of the promoter. Tumor necrosis factor- α and interferon (IFN)- γ inflammatory cytokines had no or mild effect on *MUC4* transcriptional activity when used alone. However, a very strong synergistic effect (10–12-fold activation) between IFN- γ and tumor necrosis factor- α or IFN- γ and transforming growth factor- α was obtained in CAPAN-2 cells. Altogether these results demonstrate that the 5'-flanking region of *MUC4* contains epithelial cell-specific, positive, and negative regulatory *cis*-elements, that Sp1/Sp3 are important regulators of *MUC4* basal expression, and that its regulation in pancreatic cancer cells involves complex interplay between several signaling pathways.

Mucins are large, highly O-glycosylated proteins that are encoded by at least 10 genes in humans (1, 2). The common structural feature among all mucins is the presence of tandemly repeated amino acid regions that serve as a docking site for O-glycans (1). Mucins are now classified in two main categories: secreted mucins including gel-forming (*MUC2*, *MUC5AC*, *MUC5B*, and *MUC6*) and non-gel-forming (*MUC7*) and membrane-bound mucins (2). This last group includes *MUC1*, *MUC3*, *MUC4*, and *MUC12* (3).

Human *MUC4* has been extensively studied in our laboratory. Its first partial cDNA was isolated from a human tracheobronchial library (4), and the gene was located on chromosome 3q29 (5). Recently its complete genomic organization has been established. The 5'-region of the gene is characterized by a single exon (exon 2) made of a 48-bp¹ minimal unit repeated in tandem that encodes a large Ser/Thr-rich domain (6). The tandem repeat varies in size between 7 and 19 kb because of a variable number of tandem repeat polymorphism. The 3'-end region of *MUC4* mainly consists of two EGF-like domains, a transmembrane domain, and a short cytoplasmic tail (7) and shows extensive alternative splicing downstream of the tandem repeat that generates a family of putative secreted and membrane-associated *MUC4* isoforms (8).

The human *MUC4* displays extensive similarities in the Nt and Ct regions with the rat sialomucin complex (SMC) previously isolated from mammary adenocarcinoma ascites cells (9). SMC is a heterodimer glycoprotein composed of an O-glycosylated mucin subunit ASGP-1 tightly bound to a N-glycosylated transmembrane subunit ASGP-2 (10). Interestingly, ASGP-2 contains two EGF-like domains in its extracellular part, one of which acts as a ligand for ErbB2/Neu oncogene (11). The whole SMC molecule is transcribed from a single gene as a 9-kb mRNA, which is translated into a single polypeptide that is secondary cleaved to yield the heterodimer complex ASGP-1/ASGP-2 (12). In the same way, *MUC4* may also be cleaved in two subunits: the mucin subunit *MUC4 α* and a transmembrane subunit with two EGF-like domains *MUC4 β* (7). *MUC4* is therefore considered to be the human homologue of rat *SMC*.

In situ hybridization studies have shown that *MUC4* is expressed in numerous normal tissues such as trachea, lung, stomach, colon, uterus, and prostate (13, 14) as well as very

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF241535.

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¹ The abbreviations used are: bp, base pair(s); ASGP, ascites sialoglycoprotein; AP, activating protein; EGF, epidermal growth factor; EMSA, electrophoretic mobility shift assay; IFN- γ , interferon- γ ; NF- κ B, nuclear factor- κ B; PCR, polymerase chain reaction; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RT, reverse transcriptase; SMC, sialomucin complex; STAT, signal transducers and activators of transcription; TGF- α , transforming growth factor- α ; TNF- α , tumor necrosis factor- α ; UTR, untranslated region; kb, kilobase(s).

TABLE I
Oligonucleotides used in PCR

Sequences of the pairs of oligonucleotides used in PCR to produce deletion mutants covering *MUC4* 5'-flanking region (see Fig. 5). *Kpn*I (GGTACC), *Sac*I (GAGCTC), *Mlu*I (ACGCGT), and *Bgl*II (AGATCT) sites were added at the end of the primers to direct subcloning and are italicized and underlined. Positions of the DNA fragments relative to the first ATG are indicated. S, sense; AS, antisense.

pGL3 deletion mutant	Position of the DNA fragment	Orientation	Oligonucleotide used for PCR (5' → 3')
1919	-144/-1	S	CGC <u>ACG CGT</u> CTT CCC AGG TTC CCT GGC
		AS	CGC <u>ACG CGT</u> GGC TGC GGC AAA AGT CCC
1809	-219/-1	S	CGC <u>ACG CGT</u> TTC CTG GTG GGG TAG TGG
		AS	CGC <u>ACG CGT</u> GGC TGC GGC AAA AGT CCC
1555	-461/-1	S	CGC <u>GAG CTC</u> GTA CAG CCC CAA GGT CGC
		AS	CGC <u>ACG CGT</u> GGC TGC GGC AAA AGT CCC
2038	-899/-1	S	CGC <u>ACG CGT</u> CTG GGG TCT GCC
		AS	CGC <u>GAG CTC</u> CTG GGG TCT GCC
2150	-1187/-1	S	CGC <u>GAG CTC</u> AAC TGG GGT GAG CAG AGC
		AS	CGC <u>GAG CTC</u> GGC TGC GGC AAA AGT CCC
1641	-3135/-2572	S	CGC <u>GAG CTC</u> CTG TCT CTT CAA GCC AAG
		AS	CGC <u>ACG CGT</u> CCC TGA AGG GAG ACA CAA
1959	-2781/-2572	S	CGC <u>GAG CTC</u> ATA TTG AGG GGA GCT GGA
		AS	CGC <u>ACG CGT</u> CCC TGA AGG GAG ACA CAA
2095	-3135/-2614	S	CGC <u>GAG CTC</u> CTG TCT CTT CAA GCC AAG
		AS	CGC <u>GAG CTC</u> GGA GTC CGG AAG TGA ATT
1718	-3135/-2837	S	CGC <u>GAG CTC</u> CTG TCT CTT CAA GCC AAG
		AS	CGC <u>ACG CGT</u> TCA TCT GGG TTG GGT CAC
1744	-3713/-3059	S	CGC <u>GGT ACC</u> ACT ATA GGG CAC GCG TGG
		AS	CGC <u>AGA TCT</u> AGT GTG GAT GTG GGG TGT

early during the development of the primitive gut (6.5 weeks of gestation) (15, 16). No *MUC4* mRNAs are detected in normal pancreas (17), gall bladder, liver, biliary epithelial cells, or intrahepatic bile ducts (18). Interestingly, abnormal expression of *MUC4* was demonstrated to occur in several human epithelial cancers such as lung and pancreas carcinomas (17, 19, 20). Furthermore high levels of *MUC4* mRNAs have been found in several differentiated pancreatic tumor cell lines (21–23). The dysregulation of *MUC4* expression, often dramatic, together with the homology to *SMC/rMuc4*, which is considered to promote tumor cell metastasis (24), points out an important role for *MUC4* in human tumor biology, especially in pancreatic tumors. Knowledge of the molecular mechanisms underlying the dysregulation of *MUC4* observed in pancreatic cancer is thus necessary to understand the role and contribution of *MUC4* altered expression during carcinogenesis. However, very few data concerning the factors involved in the control of *MUC4* expression have been published so far. Gollub *et al.* (25) have shown that estrogen and dexamethasone up-regulate *MUC4* mRNA levels in the endometrial Ishikawa epithelial cell line. In bronchial cells, Bernacki *et al.* (26) showed that *MUC4* is regulated at the transcriptional level by retinoic acid. More recently, Choudhury *et al.* (27) have demonstrated that all-*trans*-retinoic acid induces *MUC4* expression in CD18/HPAF pancreatic carcinoma cells and is mediated by TGF- β_2 .

Recently, the 5'-flanking region as well as 2.4 kb of the promoter of the rat *Muc4* was described (10, 28). The promoter is TATA-less and is characterized by the presence of an initiator consensus sequence located 27 bp upstream of the unique transcription start site. The cell-specific activity of the promoter is thought to be under the control of *cis*-elements present upstream from the initiator (28).

The purpose of our study was to isolate and characterize the promoter of the human mucin gene *MUC4* and identify *cis*-elements as well as define the molecular mechanisms involved in the regulation of its expression in pancreas cancer cells. To this end, three human well differentiated pancreatic duct cancer cell lines CAPAN-1 (*MUC4* positive), CAPAN-2 (*MUC4* positive), and PANC-1 (*MUC4* negative) were chosen (21, 29–31). We have identified regulatory *cis*-elements and *trans*-acting factors involved in the control of *MUC4* expression. Moreover, this study shows that *MUC4* promoter regulation is

complex and involves many signaling pathways that may be responsible for the overexpression of *MUC4* expression in carcinoma of the pancreas and hepatobiliary system.

EXPERIMENTAL PROCEDURES

Cloning—Inserts were prepared using the restriction map of a cosmid clone called LEA 51 (6), which covers the 5'-proximal region of *MUC4*. Gel-purified fragments (Qiaquick gel extraction kit, Qiagen) were subcloned into the promoterless pGL3 Basic vector (Promega). Internal deletion mutants were generated by PCR using pairs of primers bearing specific restriction sites at their 5'- and 3'-ends (Table I). PCR products were digested, gel-purified, and subcloned into the pGL3 vector that had been previously cut with the same restriction enzymes. Fragments 1488 (1.43 kb, *Kpn*I-*Sac*I) and 1489 (1.32 kb, *Kpn*I-*Sac*I) were generated using the restriction sites present within the LEA 51 genomic clone. Fragment 1744 was generated using the Genome walker kit (CLONTECH). The *Eco*RV 650-bp PCR fragment was first subcloned into pCR 2.1 vector (Invitrogen) before being subcloned into pGL3 basic vector. All clones were sequenced on both strands on an automatic LI-COR sequencer (ScienceTec, France) using infrared labeled RV3 and GL2 primers (Promega). Plasmids used for transfection studies were prepared using the Endofree plasmid Mega kit (Qiagen).

Cell Culture—Pancreatic cancer cell lines CAPAN-1 and CAPAN-2 were purchased from American Type Culture Collection, and PANC-1 cells were from European Collection of Cell Cultures. CAPAN-1 and CAPAN-2 cells were cultured in RPMI 1640 medium supplemented with 2 mM glutamine and 15 and 10% fetal calf serum (Roche Diagnostics), respectively. PANC-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine and 10% fetal calf serum. The cells were maintained in a 37 °C incubator with 5% CO₂.

RT-PCR—Total RNAs from pancreatic cancer cells were prepared using the RNeasy® midi-kit from Qiagen. The cells were harvested at confluence, and 1.5 μ g of total RNA was used to prepare cDNA (Advantage™ RT-for-PCR kit, CLONTECH). PCR was then performed on 5 μ l of cDNA as described by Van Seuningen *et al.* (32) using specific pairs of primers for *MUC4* mucin gene (*MUC4* forward primer, 5'-CGCGGTGGTGGAGGCGTTCTT-3'; *MUC4* reverse primer, 5'-GAAGAATCCTGACAGCCTTCA-3'; positions 3094–3114 and 3670–3690; accession number AJ242549) (7). The PCR product expected size is 596 bp. Single-stranded oligonucleotides were synthesized by MWG-Biotech. Glycer-aldehyde-3-phosphate dehydrogenase was used as an internal control. PCR reactions and PCR product analyses were carried out as described previously (32, 33).

Oligonucleotide Probes—The oligonucleotides used for gel shift assays are indicated in Table II. They were synthesized by MWG-Biotech. Equimolar amounts of single-stranded oligonucleotides were annealed and radiolabeled using T4 polynucleotide kinase (Promega) and [γ -³²P]dATP. The radiolabeled probes were then separated from free

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

Antisense complementary oligonucleotides were also synthesized and annealed to the sense oligonucleotide to produce double-stranded DNA. Positions of the DNA fragments relative to the first ATG are indicated.

Oligonucleotide name (sense orientation)	Sequence (5' → 3')
T16 (Sp1 site), -2723/-2703	AGGAGTGCACGCCAGTTTC
T30 (Sp1 site), -173/-150	TAGCGTGGGGCCCCGCTCTTTT
T31 (CACCC box), -214/-188	GGTGGGGTAGTGGGGTGGGGCTGAGGA

nucleotides on Bio-Gel P-6 columns (Bio-Rad).

Primer Extension—Primer extension reactions were performed using 20 μ g of total RNAs prepared from CAPAN-1 and CAPAN-2 cells as above. Annealing and labeling of the primers and extension reactions were performed as described previously (32). The reverse primer NAU 979 (5'-CCTGTTGTGGAGGACACCTGTG-3') is located downstream of the TATA box, and the reverse primer NAU 1035 (5'-CAGCAGCTG-CAGTGTGAGGAG-3') is located in the 3'-end of the 5'-UTR. ϕ X174 DNA/*Hinf*I dephosphorylated markers (Promega) were radiolabeled with [γ -³²P]dATP just before use. Manual sequencing of M13mp18 control DNA was performed using the T7 Sequenase version 2.0 kit (Amersham Pharmacia Biotech). The samples were denatured for 10 min at 90 °C before loading on a 8% sequencing gel (Sequagel-8; National Diagnostic, Prolabo, France). The gel was then vacuum-dried and autoradiographed for 3–4 days at -80 °C.

Transfections—The transfections were performed using Effectene® reagent (Qiagen) as described previously (33). Briefly, the cells were passed at 0.5×10^6 cells/well the day before the transfection. Total cell extracts were prepared after 48 h of incubation at 37 °C using 1 \times reagent lysis buffer (Promega) as described in the manufacturer's instruction manual. The results were corrected for transfection efficiency by co-transfecting 0.1 μ g of pSV- β Gal vector (Promega). β -Galactosidase and luciferase activities were measured as described by Perrais *et al.* (33). The luciferase activity is expressed as fold of induction of the test plasmid activity compared with that of the corresponding control vector (pGL3 control vector, Promega) after correction for transfection efficiency by dividing by β -galactosidase activity. Each plasmid was assayed in triplicate in at least three separate experiments. Co-transfection studies with pCMV-Sp1 and pCMV-Sp3 expression vectors were performed as described previously (32). PMA (100 nM, 24 h), cholera toxin (1 μ g/ml, 24 h), and calcium ionophore A23187 (250 nM, 30 min) were added to the transfected cells as indicated, and the cells were then harvested to measure luciferase activity. The cells were also treated with EGF (25 ng/ml, 24 h) and TGF- α (25 ng/ml, 24 h) growth factors and with TNF- α (40 ng/ml, 24 h) and IFN- γ (25 ng/ml, 24 h) inflammatory cytokines. All reagents were from Sigma unless otherwise indicated. In some experiments, the cells were pretreated for 30 min with tyrosine-kinase inhibitor tyrphostin AG 1478 (10 μ M) (Calbiochem) before adding the growth factor of interest.

Nuclear Extract Preparation—Nuclear extracts from cell lines of interest were prepared as described by Van Seuning *et al.* (34) and kept at -80 °C until use. Protein content (5 μ l of cell extracts) was measured using the bicinchoninic acid method in 96-well plates as described in the manufacturer's instruction manual (Pierce).

Electrophoretic Mobility Shift Assays (EMSA)—Nuclear proteins (5 μ g) were preincubated for 20 min on ice in 20 μ l of binding buffer with 2 μ g of poly(dI-dC) (Sigma) and 1 μ g of sonicated salmon sperm DNA. Radiolabeled DNA probe was added (120,000 cpm/reaction), and the reaction was left for another 20 min on ice. For supershift analyses, 1 μ l of the antibody of interest (anti-Sp1 and anti-Sp3, Santa Cruz Laboratories, Tebu, France) was added to the proteins and left for 1 h on ice before adding the radiolabeled probe. The reaction was stopped by adding 2 μ l of loading buffer and loaded onto a 4% nondenaturing polyacrylamide gel, and electrophoresis conditions were as described by Van Seuning *et al.* (32). The gels were vacuum-dried and autoradiographed overnight at -80 °C.

DNA Sequence and Transcription Factor Binding Site Analyses—DNA sequences were analyzed using PC-GENE software, and the Transfac 4.0 data base was used to localize putative transcription factor binding sites within the 5'-flanking region of MUC4. The search was conducted using MatInspector V2.2 software (35).

RESULTS

Cloning and Characterization of the MUC4 Promoter—To isolate the promoter of the human mucin gene MUC4, a human

genomic pWE15 library was screened with RAC3 probe (183 bp), which spans 106 bp of the 5'-UTR and 77 bp of the first exon of MUC4 (6). One positive clone, called LEA 51, was isolated and contains an insert of 40 kb (Fig. 1). *Eco*RI digestion of the insert generated a 2.2-kb fragment that was sequenced and showed 100% homology in its 3'-end region with the previously published 5'-flanking cDNA sequence of MUC4 (6). To obtain more clones containing the promoter region, a PCR-based DNA walking technique was used. A 2.8-kb fragment was obtained from the *Pvu*II library using the two primers NAU 376 and NAU 357 that are located in the 5'-end of the 5'-UTR of MUC4. The sequence of the 2.8-kb fragment perfectly aligned with the 3'-end of the cosmid clone LEA51, which indicated that we had most likely isolated the promoter of MUC4. A second round of PCR was then carried out using two primers (NAU 598 and NAU 599) located in the 5'-end of the 2.8-kb fragment. A 650-bp-long PCR product was isolated from the *Eco*RV library. The 3'-end of that fragment matched the 5'-end of the 2.8 kb. Altogether 3.7 kb of the MUC4 5'-flanking region upstream of the first ATG was isolated using this strategy.

The sequence of the promoter and of the 5'-UTR is presented in Fig. 2. The first typical TATA box (TATAA) was found at -2672/-2668 upstream of the translational start site. The 5'-UTR is mainly composed of GC-rich domains that are potential binding sites for Sp1 but also for the CACCC box-binding protein. This region is also characterized by numerous putative binding sites for transcription factors of the AP family (AP-1, AP-2, and AP-4), PEA3 transcription factor (Ets family), glucocorticoid receptor element, and two sites for the progesterone receptor. A very high density of binding sites for factors known to initiate transcription in TATA-less promoters (Sp1, CACCC box, glucocorticoid receptor element, AP-2, PEA3, and Med-1) (36–40) was found in the sequence encompassing the -330/-29 nucleotides. Finally, two putative STAT-binding sites, a transcription factor activated by IFN- γ (41), were found in the 3'-end of the 5'-UTR. Upstream of the first TATA box, numerous putative binding sites for Sp1, glucocorticoid receptor element, AP-1, AP-4, GATA, and cAMP-responsive element-binding protein transcription factors were found.

Characterization of the Transcription Start Sites—The expression of MUC4 in CAPAN-1 (lane 4) and CAPAN-2 (lane 5) and its absence in PANC-1 (lane 6) cells was confirmed by RT-PCR (Fig. 3). One can note that MUC4 expression is higher in CAPAN-1 (lane 4) cells compared with CAPAN-2 (lane 5). To localize transcription initiation sites, primer extension experiments were then carried out with total RNAs from the two cell lines expressing MUC4 (CAPAN-1 and CAPAN-2) (Fig. 4). Three extension products of 89, 90, and 91 bp were produced when using NAU 979 reverse primer located downstream of the TATA box (Fig. 4B), indicating the existence of three consecutive transcription initiation sites at positions -2603, -2604, and -2605, respectively (Fig. 4A). Intensity of the extension products was much more important in CAPAN-1 cells (lane 2) compared with CAPAN-2 (lane 1), which indicates that cell-specific transcription factors are involved in the transcriptional activity of that region of the promoter and for the higher expression of MUC4 in these cells as shown by RT-PCR (Fig. 3, compare lanes 4 and 5). Because it is widely known that transcription initiation sites are found in GC-rich regions of mammalian promoters, several oligonucleotides localized in the 3'-end of the 5'-UTR were used. One additional extension product of 153 bp was obtained when using NAU 1035 (Fig. 4C) and corresponds to a thymidine residue located at position -199 from the translational start site (Fig. 4A). One can note that the intensity of the extension product is the same in both cell

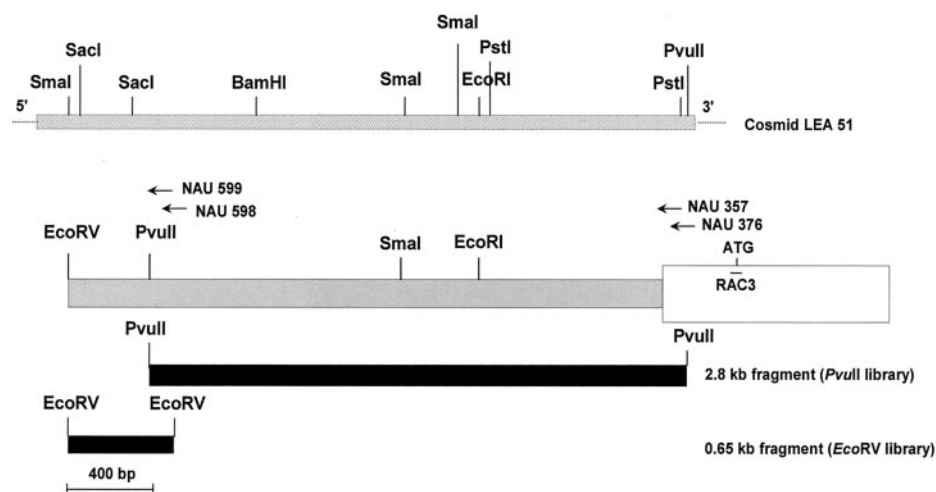


FIG. 1. Schematic representation of the genomic clones covering the 5'-flanking region of human mucin gene *MUC4* upstream of the ATG. The 3'-end of the cosmid LEA 51 is shown and aligned with the *PvuII* 2.8-kb clone and the *EcoRV* 0.65-kb clone obtained with the Genome walker kit.

lines (lanes 2 and 3). RT-PCR experiments performed with pairs of primers covering the region stretching from the TATA box down to the first ATG confirmed the fact that this region is transcribed in CAPAN-1 and CAPAN-2 but not in PANC-1 cells (not shown). Because previous results from our laboratory determined the first ATG to be located 2.67 kb downstream of the TATA box described in this report (6), one can conclude that a rather long 5'-UTR lays between the first TATA box and the translation initiation site of *MUC4*.

Transcriptional Activity of the Promoter and of the 5'-UTR of *MUC4* in Pancreatic Cancer Cells—From the above experiments, two main zones can be distinguished in the *MUC4* 5'-flanking region located upstream of the first ATG: a long 5'-UTR rich in GC and the region containing the promoter upstream of the TATA box. A series of deletion mutants covering 3712 nucleotides upstream of the first ATG were made in pGL3 basic vector to characterize the DNA sequences involved in *MUC4* promoter activity (Fig. 5A).

The luciferase diagram shown on Fig. 5B clearly indicates that two highly active regions (–219/–1 and –2781/–2572) are responsible for *MUC4* transcription in CAPAN-1 and CAPAN-2 cell lines. On the contrary, the activity of these two regions was kept to a minimal level in the *MUC4* negative PANC-1 cells. Fragments covering the –1187/–1 region of the 5'-UTR (fragments 1555, 2038, and 2150) have about the same range of activity in the three cell lines, indicating that cell-specific elements are not present in that region of the 5'-UTR. The minimal sequence with the highest luciferase activity covers the first 219 nucleotides upstream of the ATG. When the bases –219/–145 are deleted the luciferase activity dramatically drops (fragment 1919). This result indicates that region –219/–145 contains *cis*-elements that are essential to drive transcription of *MUC4*. Fragment 1489 (–1708/–387), which does not contain the first 386 nucleotides, does not have any luciferase activity. This points out again, in accordance with the primer extension data (Fig. 4C), that initiation of transcription in the GC-rich region occurs within the first 386 nucleotides upstream of the ATG.

Interestingly, the region covering the –3135/–1703 nucleotides (1488), which is 1.43 kb long and which contains the first TATA box, is also inactive. This may indicate that inhibitory *cis*-elements are present in that region of the 5'-UTR downstream of the TATA box. This hypothesis was confirmed when cells were transfected with the next set of fragments in which a large part of the sequence downstream of the TATA box was deleted. The most active fragment covering the –2781/–2572 nucleotides (fragment 1959) is 2- and 4-fold more active in CAPAN-1 cells than in CAPAN-2 and PANC-1 cells, respec-

tively. This results indicates that this domain contains regulatory elements that confer cell specificity to the expression pattern of *MUC4*. This is in good agreement with the results obtained by primer extension in which transcription was shown to be more important in that region of the promoter in CAPAN-1 cells (Fig. 4B, lane 2). The other two fragments, covering the –3135/–2572 (fragment 1641) and –3135/–2614 (fragment 2095) nucleotides, although much less active, showed also a higher activity in CAPAN-1 cells. The removal of the TATA box sequence to obtain a fragment covering the –3135/–2837 region (1718) resulted in a total loss of the luciferase activity, confirming the fact that this TATA box is essential and drives transcription of *MUC4* promoter. A fragment covering the –3713/–3059 nucleotides (1744) was generated because our computational studies indicated that it contained two putative TATA boxes. This region, however, does not have any luciferase activity in any cell line tested. One can conclude from these results that the –219/–145 and –2781/–2572 DNA regions contain essential cell-specific *cis*-elements that drive *MUC4* transcription in CAPAN-1 and CAPAN-2 cells.

Binding of Sp1 and Sp3 to the Promoter and the 5'-UTR of *MUC4*—The region adjacent to the first ATG is characterized by a high amount of GC-rich domains as well as numerous putative binding sites for Sp1. Two Sp1 binding sites were also found right upstream of the TATA box. To confirm the binding of Sp1 in these regions, EMSA studies were carried out with nuclear proteins from the three cell lines and radiolabeled double-stranded oligonucleotide probes each containing a Sp1-binding site present either in the –219/–1 (T30 and T31) or –2781/–2572 (T16) region (Fig. 6). A very faint binding occurs with the T16 probe (–2723/–2703) representative of a Sp1-binding site located upstream of the TATA box (lane 2). Supershift with anti-Sp1 antibody was only visualized in CAPAN-1 and CAPAN-2 cells (lane 3). The addition of Sp3 antibody did not produce any supershift (lane 4). The strongest binding was obtained with the T30 probe (Sp1 binding site at –173/–150). Three main shifted bands were visualized (complexes 1, 2, and 3) in the three cell lines (lane 6). Complex 3 corresponds to Sp1 binding because a supershift occurred upon addition of anti-Sp1 antibody in the mixture to produce complex 4 (lane 7). Complexes 1 and 2 correspond to the two forms of Sp3 (70 and 105 kDa) (lane 6) because these two bands were supershifted upon addition of anti-Sp3 antibody to produce complex 5 (lane 8). The same profile was obtained with T31 probe representative of another Sp1/Sp3 binding site at –214/–188 in the 5'-UTR (lanes 10–12), although binding was not as intense. Altogether, it can be stressed from these data that Sp1 and Sp3 engage with two of their cognate *cis*-elements (–214/–188 and

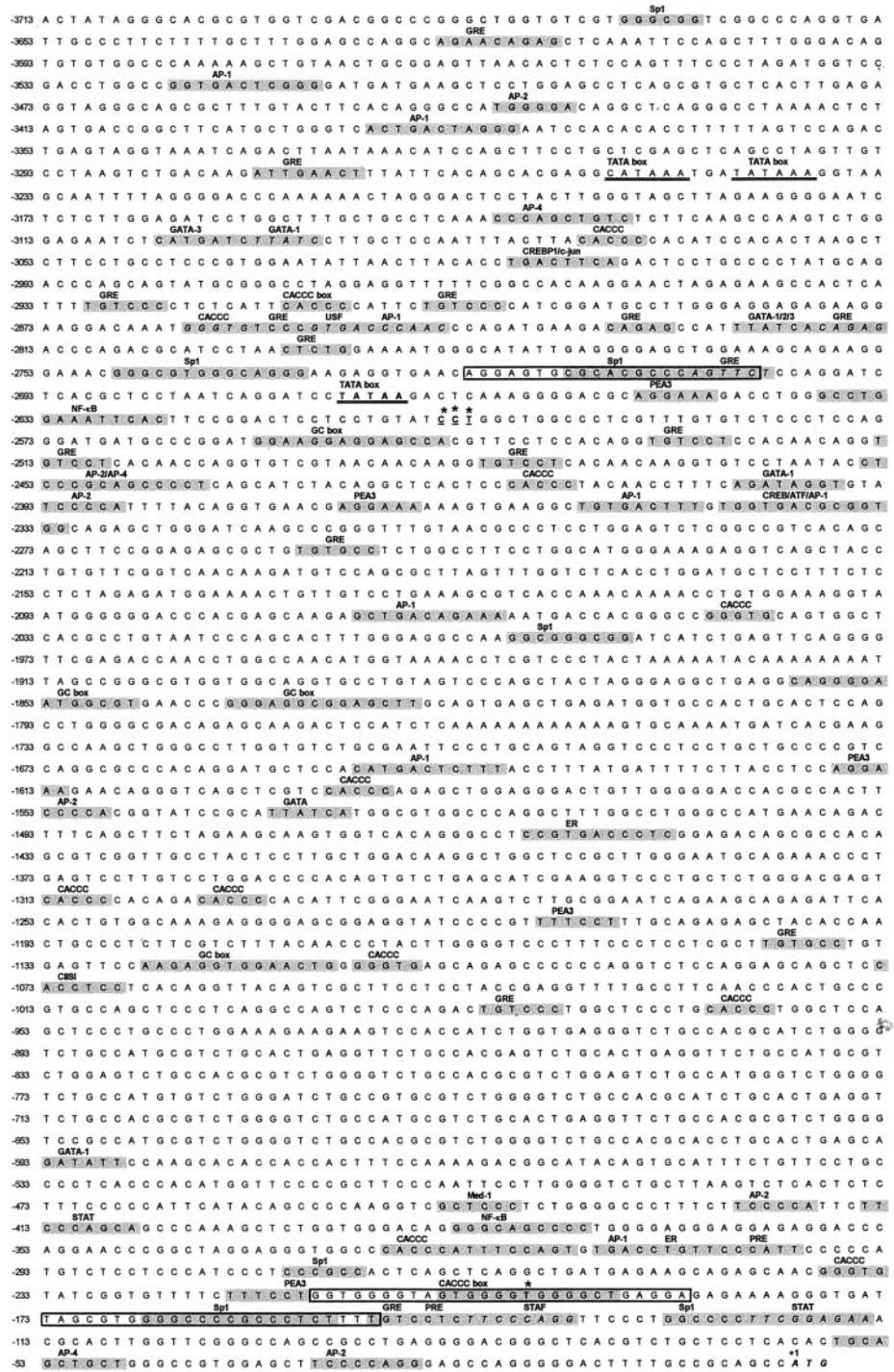


FIG. 2. Sequence of the promoter region and 5'-UTR of the human mucin gene MUC4. The three putative TATA boxes are double-underlined, and putative binding sites for known transcription factors are in gray. The transcription start sites at -199, -2603, -2604, and -2605 are in bold type and marked with an asterisk. The sequences of the oligonucleotides used for EMSA studies are boxed. The first ATG is in bold type and italicized. Numbering refers to the first ATG designated +1.

-173/-150) in the 5'-UTR of MUC4 in the three cell lines, whereas Sp1 specifically binds to a remote Sp1-element (-2723/-2703) in CAPAN-1 and CAPAN-2 cells.

Role of Transcription Factors Sp1 and Sp3 on MUC4 Promoter Activity—Having shown that Sp1 and Sp3 simultaneously bind to two cognate cis-elements in the GC-rich region of the 5'-UTR and that Sp1 engages with a binding site upstream of the TATA box, co-transfection studies were performed to study their biological effect on MUC4 transcription rate (Fig. 7). Deletion mutants from the GC-rich region (fragments 1809 and 1555) and from the TATA region (fragments 1641 and 1959) were transfected in the presence of an expression vector carrying the coding sequence of Sp1 (pCMV4-Sp1) or Sp3 (pCMV4-Sp3). The luciferase diagram indicates that

Sp1 efficiently co-transactivates the -461/-1 region (fragment 1555) in CAPAN-2 and to a lesser extent in PANC-1 cells. No effect was seen on fragments 1809, 1641, and 1959 in either cell line. This result indicates that cis-elements in the -461/-1 region are sufficient to the up-regulation of MUC4 transcription by Sp1 in CAPAN-2 cells, whereas other elements are required in CAPAN-1 and PANC-1 cells despite the efficient binding shown in Fig. 6. Sp3 effect on MUC4 promoter was also studied, and, as expected, inhibition of the transcription activity of the different fragments was observed. Sp3 inhibition was rather strong (loss of 75% of the activity) in CAPAN-1 and CAPAN-2 cells except for the 1555 fragment (25% inhibition). No significant effect was observed in PANC-1 cells.

Involvement of PKC, PKA, and Intracellular Calcium Signal-

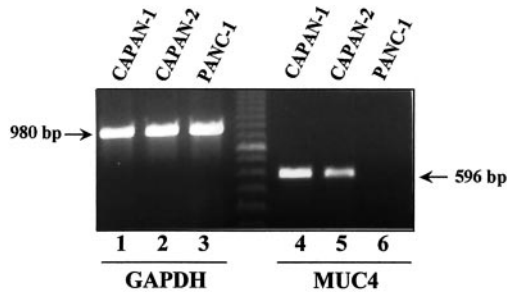


FIG. 3. Expression of MUC4 in pancreatic cancer cells by RT-PCR. RT-PCR on 1.5 μ g of RNA from CAPAN-1 (lanes 1 and 4), CAPAN-2 (lanes 2 and 5), and PANC-1 (lanes 3 and 6) cells used for cDNA synthesis. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, lanes 1–3) and *MUC4* (lanes 4–6) PCR products are 980 and 596 bp long, respectively. PCR products were separated on a 1.5% agarose gel run in 1 \times Tris borate-EDTA buffer.

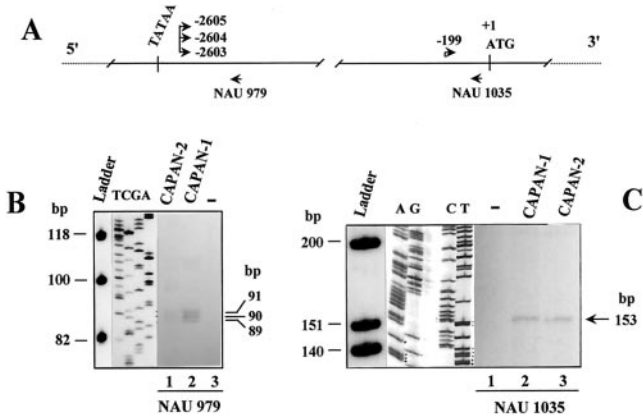


FIG. 4. Identification of multiple transcription start sites within the 5'-flanking region of MUC4 using primer extension. A, positions of the transcription start sites are indicated on the schematic view of the 5'-flanking region of *MUC4* and numbering refers to the translational start site of *MUC4*. The positions of the reverse primers used in primer extension experiments are indicated. B, primer extension on 20 μ g of total RNA from CAPAN-2 (lane 1) and CAPAN-1 (lane 2) cells. Lane 3, no RNA added. Three extension products of 89, 90, and 91 bp (lanes 1 and 2) were produced when using NAU 979 reverse primer located downstream of the TATA box. C, primer extension on 20 μ g of total RNA from CAPAN-1 (lane 2) and CAPAN-2 (lane 3) cells. Lane 1, no RNA added. One extension product of 153 bp was produced (lanes 2 and 3) when using NAU 1035 reverse primer located in the 3'-end of the 5'-UTR. ϕ X174 DNA/*Hinf*I dephosphorylated markers previously radiolabeled and denatured are indicated on the left side of the gels. The sequence of the M13mp18 plasmid control is shown.

ing Pathways in MUC4 Promoter Activity in Pancreatic Cancer Cells—The proximal and the distal regions of the promoter of *MUC4* are characterized by the presence of numerous potential binding sites for transcription factors of the AP family (AP-1, AP-2 and AP-4) as well as the PEA3 transcription factor of the Ets family. These factors can be activated by PKC signaling pathway and heterodimerize with c-Jun or c-Fos transcription factors to induce transcription of the target gene (42, 43). To study PKC signaling pathway, the transfected cells were treated with PMA for 24 h before harvesting cells. As expected, the luciferase diagram (Fig. 8A) shows that PMA strongly induces the activity of all the fragments tested whether they are located in the 5'-UTR region (fragments 1809, 1555, and 2150) or in the region containing the TATA box (fragments 1641 and 1959). The activation is, however, more important in the fragments containing the TATA box (3.5–4-fold activation compared with 2.0–2.5-fold, respectively). From these data, one can conclude that PKC is a strong activator of *MUC4* transcription and that PMA-sensitive *cis*-elements are present throughout the 5'-flanking region of *MUC4*.

A few putative binding sites for cAMP-responsive element-binding protein/activating transcription factor transcription factors were found in the promoter. Because these factors are activated by PKA (44), involvement of PKA signaling pathway was tested with cholera toxin. At the most, cholera toxin induced a 2-fold activation of the $-219/-1$ region (fragment 1809) in CAPAN-1 cells (Fig. 8B). Thus, it seems that *MUC4* up-regulation by PKA is not very strong and is confined to the $-219/-1$ region in CAPAN-1 cells.

MUC4 is one of the major mucins expressed in the respiratory tract. Moreover, in bronchial epithelial cells, mucin secretion is influenced by inositol 1,4,5-triphosphate-sensitive intracellular calcium release (45). When we studied the effect of intracellular calcium increase in the three cell lines using calcium ionophore A23187, the diagram profile of luciferase activity mimicked that of cholera toxin with a 2-fold activation of the $-219/-1$ region (fragment 1809) in CAPAN-1 cells (Fig. 8C).

From these results, it can be postulated that PKC-sensitive *cis*-elements present throughout the region upstream of the first ATG are responsible for the up-regulation of *MUC4* and that PKA and calcium signaling pathways induce a mild activation of the $-219/-1$ region of the 5'-UTR.

Involvement of Growth Factors (EGF and TGF- α) in the Regulation of MUC4 Promoter Activity in Pancreatic Cancer Cells—The high density of binding sites for transcription factors activated by growth factors in the 5'-flanking region of *MUC4* is in favor of regulatory mechanisms involving these molecules. Moreover, mucin secretion and/or expression has already been shown to be regulated by growth factors (46, 47), and PKC, which was shown in this report to up-regulate *MUC4* transcription (Fig. 8), is also known to be activated by EGF. In our study, biological effect of EGF as well as TGF- α , an EGF-related growth factor, were tested on *MUC4* promoter activity. As shown in Fig. 9, the activating effect of these two growth factors in CAPAN-1 cells was directed toward the $-219/-1$ region (fragment 1809) of the 5'-UTR (2-fold activation). In CAPAN-2 cells, EGF and TGF- α strongly induced the activity of $-3135/-2572$ region (fragment 1641) (3-fold activation) and to a lesser extent that of fragments 1809, 2150, and 1959 (approximately 2-fold activation). Because EGF receptor is known to be up-regulated by the proinflammatory cytokine TNF- α , which is increased in hypersecretory diseases (48), the synergistic effect between TNF- α and EGF or TGF- α was tested. In our studies, TNF- α alone had no effect on *MUC4* promoter in either cell line. Cell treatment with EGF and TNF- α did not result in a synergistic up-regulation of *MUC4* transcriptional activity. Finally, when cells were pretreated with the tyrosine kinase inhibitor tyrphostin AG1478 before adding EGF or TGF- α , the luciferase activity dramatically dropped (Fig. 9), indicating that EGF- and TGF- α -mediated up-regulation of *MUC4* transcription involves downstream intracellular tyrosine kinases. As expected, EGF and TGF- α had no significant effect on *MUC4* transcriptional activity in PANC-1 cells.

Involvement of Interferon- γ in the Regulation of MUC4 Promoter Activity in Pancreatic Cancer Cells—INF- γ , a proinflammatory cytokine, is a potent antitumoral, antiviral, and antibacterial molecule that exerts its effects through the stimulation of a signaling cascade that activates transcription factors of the STAT family (41). Because two putative STAT-binding sites were found in the 3'-end of *MUC4* 5'-UTR (Fig. 2), the biological effect of IFN- γ was tested on the *MUC4* 5'-flanking region. Opposite effects were seen when cells were treated with IFN- γ (Fig. 10). In CAPAN-1 cells, IFN- γ led to a slight decrease of the activity of all fragments. In CAPAN-2 cells, a 2-fold activation of the fragments covering the GC-rich

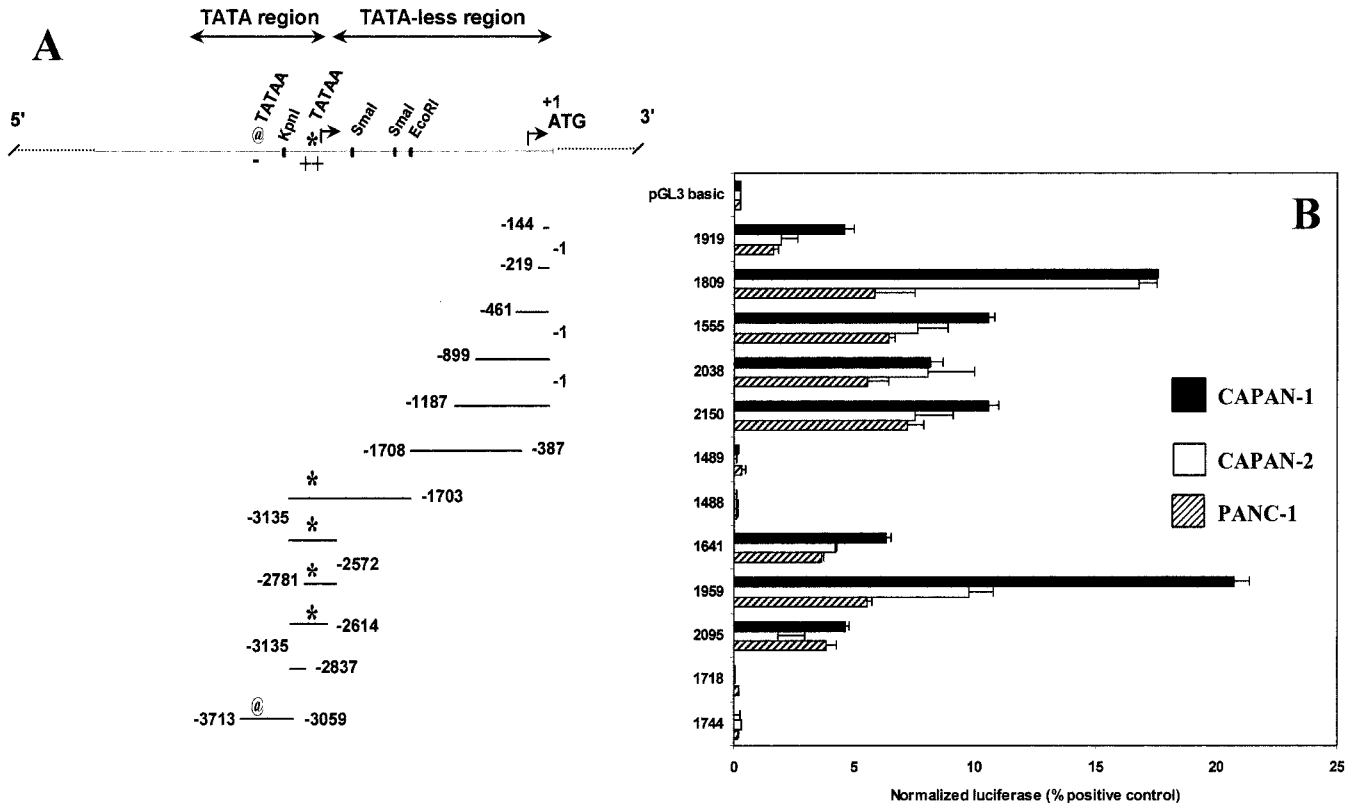
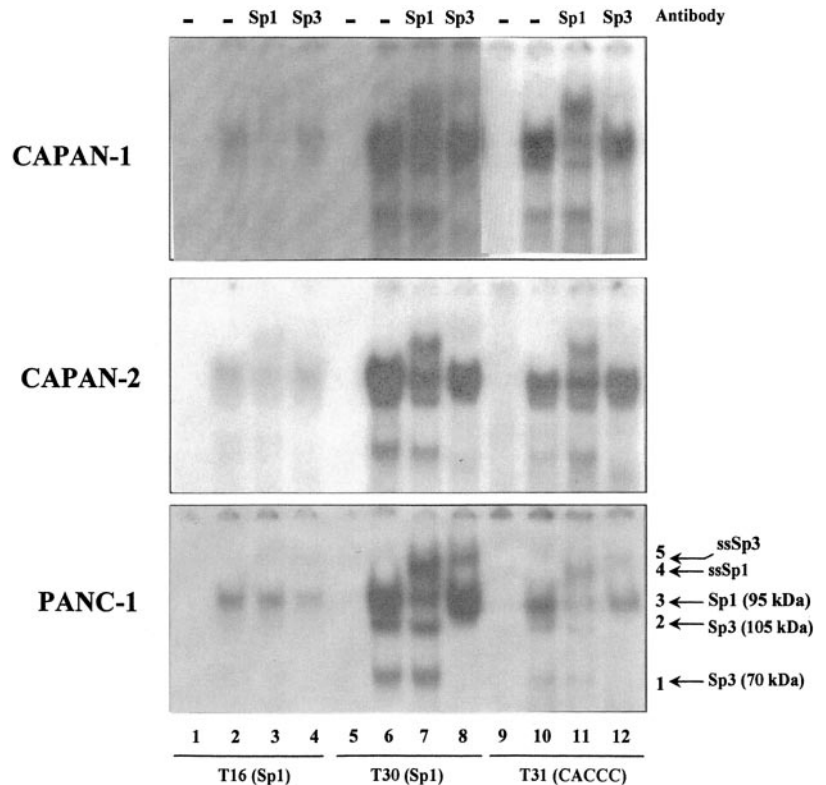


FIG. 5. Position and transcriptional activity of MUC4 pGL3 deletion mutants in pancreatic cancer cell lines. A, schematic representation of the localization of the different pGL3 deletion mutants covering 3721 nucleotides upstream of the first ATG. The numbering refers to the translational start site designated +1. The TATA box locations are indicated as well as some restriction sites found in the 5'-flanking region of the gene. B, transcriptional activity of the deletion mutants was studied in CAPAN-1 (black bars), CAPAN-2 (white bars), and PANC-1 (hatched bars) cell lines. The background activity of pGL3 Basic promoterless vector used to subclone MUC4 fragments is shown. The results are the means \pm S.D. and represent more than three different experiments in triplicate for each fragment.

FIG. 6. Binding of Sp1 and Sp3 to three different binding sites in MUC4 promoter using EMSA. Autoradiograms of EMSAs performed with 5 μ g of nuclear proteins isolated from CAPAN-1, CAPAN-2, and PANC-1 cell lines. Nuclear proteins were incubated with the radiolabeled DNA probes as indicated. Lanes 1-4, T16, Sp1-binding site at -2723/-2703; lanes 5-8, T30, Sp1-binding site at -173/-150; lanes 9-12, T31, CACCC box at -214/-188. Supershift experiments were carried out by adding 1 μ l of the antibodies of interest (anti-Sp1, lanes 3, 7, and 11; anti-Sp3, lanes 4, 8, and 12). Radiolabeled probe alone were loaded in the first lane of each series (lanes 1, 5, and 9). DNA-protein complexes with Sp1 (complex 3) and Sp3 (complexes 1 and 2) and supershifted complexes with anti-Sp1 (complex 4) and anti-Sp3 (complex 5) are indicated on the right side of the figure with arrows.



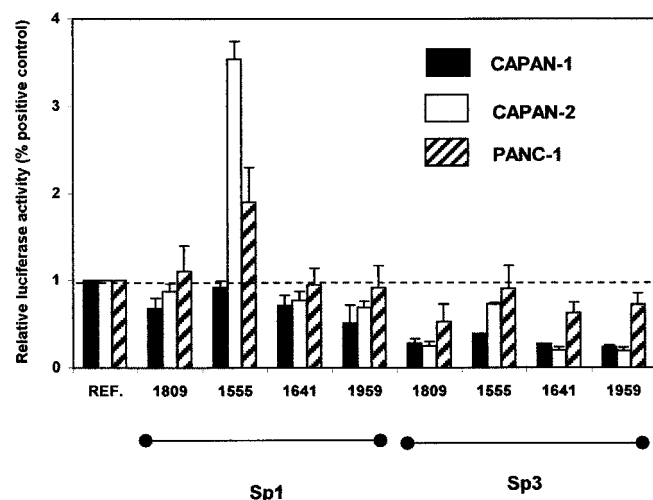


FIG. 7. Transactivating effect of Sp1 and Sp3 on different regions of the promoter of *MUC4*. Co-transfection experiments were performed with 1 μ g of *MUC4* deletion mutants (fragments 1809, 1555, 1641, and 1959) and 0.25 μ g of pCMV4, pCMV-Sp1 or pCMV-Sp3 expression vectors in CAPAN-1 (black bars), CAPAN-2 (white bars), and PANC-1 (hatched bars) cells. The results are the means \pm S.D. and represent more than three different experiments in triplicate for each fragment.

region of the 5'-UTR (fragments 1809 and 1555) was observed, whereas it had no significant effect on the two fragments containing the TATA box (fragments 1641 and 1959). In PANC-1 cells, no effect was observed.

Because IFN- γ effects on *MUC4* transcription activity were not dramatic, we hypothesized that it could act in synergy with TGF- α or TNF- α , as it has already been shown for numerous other genes involved in cell differentiation and proliferation during inflammatory processes (49, 50) or carcinogenesis (51). As shown in Fig. 10, IFN- γ and TNF- α did not synergize to induce *MUC4* transcription in CAPAN-1 cells. On the contrary, in CAPAN-2 cells, a strong synergistic effect was visualized on the region covering the first 1.2 kb of the 5'-UTR (fragments 1809, 1555, and 2150; 4-, 10-, and 6-fold activation, respectively). No synergistic effect was seen on the two fragments containing the TATA box (fragments 1641 and 1959). In CAPAN-1 cells, IFN- γ and TGF- α led to a decrease of the activity of all the fragments but 1809. In CAPAN-2 cells, the results were opposite because IFN- γ strongly potentiated the effect of TGF- α (from a 3-fold activation on fragments 1555 and 1959 and a 4-fold activation on fragment 1809 to a 8-fold activation on fragment 2150) on all fragments. In PANC-1 cells, IFN- γ , TNF- α , and TGF- α did not have any regulatory effects on *MUC4* promoter activity, nor did they have synergistic effects.

DISCUSSION

Human mucin gene *MUC4* belongs to the subfamily of mucin genes encoding transmembrane mucins like *MUC1*, *MUC3*, and *MUC12* (1–3). Several reports show that the cytoplasmic tail of these transmembrane mucins is involved in cell signaling events. *MUC1* possesses multiple tyrosine phosphorylation sites in its C-terminal part that interact with either the adaptor protein Grb2 and then the Sos-Ras pathway (52) or β -catenin (53). The frequent aberrant expression of *MUC1* in cancer cells over the entire cell surface also may promote metastasis *via* the anti-adhesive effects of the extracellular mucin domain (54, 55). Studies on *rMuc4* also demonstrated its anti-adhesive and anti-immune recognition effects when overexpressed (10) and its link to ErbB2 signaling pathway (11), pointing out an important role for *MUC4* in tumor progression. In this paper, we describe the structure of the 5'-flanking region of *MUC4*

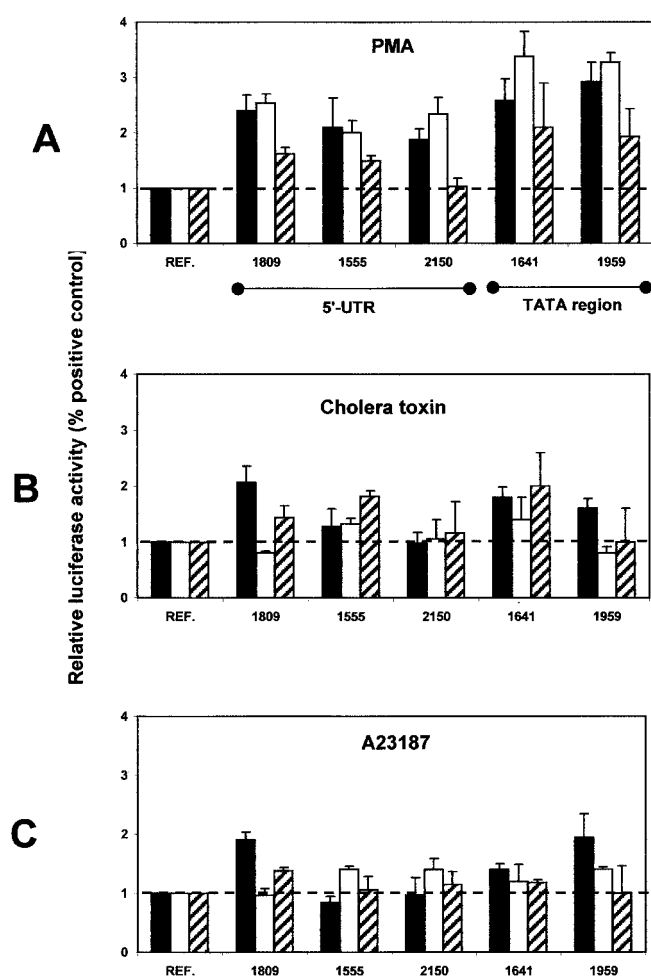


FIG. 8. Effect of PMA, cholera toxin, and A23187 on the activity of *MUC4*-pGL3 deletion mutants. Transfected cells (CAPAN-1, black bars; CAPAN-2, white bars; and PANC-1, hatched bars) were treated as described under "Experimental Procedures" before measurement of the luciferase activity of the deletion mutants of interest. REF. refers to the activity of the fragments of interest without any treatment. The results are the means \pm S.D. and represent more than three different experiments in triplicate for each fragment.

and its transcriptional regulation in pancreatic cancer cells. This model was chosen because *MUC4* is not expressed in normal pancreas (17), whereas it is aberrantly expressed in pancreatic cancer (17, 21). The identification of regulatory regions and responsive elements to growth factors and proinflammatory cytokines involved in tumorigenesis provides new insights as to how *MUC4* is regulated and its potential role as a tumor marker in pancreatic cancer.

Sequencing of the 5'-flanking region of *MUC4* shows that a long 5'-UTR (2.5 kb) lays between the first ATG and the first active TATA box (−2672/−2668). When compared with the recently published promoter sequence of the *rMuc4* (28), the human sequence shows ~70% homology over the first 464 nucleotides upstream of the first ATG, and then both sequences diverge. The absence of TATA box and the presence of a transcription start site in the close vicinity of the first ATG was also found in the rat (28). Functional studies of the 5'-flanking region of *MUC4* in pancreatic cancer cells show that *MUC4* transcription is controlled by two regulatory regions (−219/−145 and −2781/−2572). Cell-specific elements are present in these two transcription units because their activity was kept to a minimum in PANC-1 cells, which do not express *MUC4*. The residual activity found in these cells, however, indicates that other *cis*-elements or mechanisms are necessary to completely

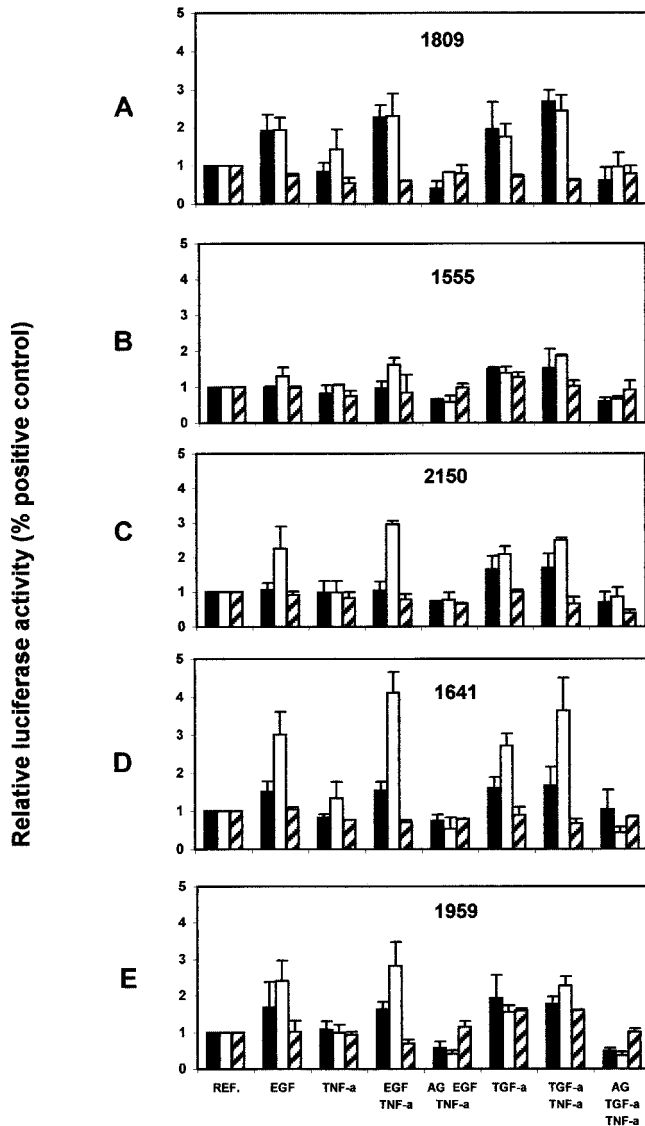


FIG. 9. Effect of EGF, TGF- α , and TNF- α on the activity of *MUC4*-pGL3 deletion mutants. Transfected cells (CAPAN-1, black bars; CAPAN-2, white bars; and PANC-1, hatched bars) were treated as described under "Experimental Procedures" before measurement of the luciferase activity of the deletion mutants of interest. REF. refers to the activity of the fragments of interest without any treatment. AG refers to the tyrosine kinase inhibitor tyrphostin AG1478. The results are the means \pm S.D. and represent more than three different experiments in triplicate for each fragment.

abrogate *MUC4* expression as shown by RT-PCR.

CAPAN-1 and CAPAN-2 both express *MUC4*, with a slightly more abundant amount of mRNAs in CAPAN-1 cells. Higher expression of *MUC4* in CAPAN-1 cells shown by RT-PCR was confirmed by primer extension and transfection experiments and allowed us to pin down CAPAN-1-positive *cis*-elements in the $-2781/-2572$ distal region. Besides goblet cells, *MUC4* is also expressed in non-mucus-producing cells such as ciliated and basal epithelial cells of the lung and intestine absorptive cells (56). The proximal region, extremely rich in GC and CACC boxes, was shown to bind both Sp1 and Sp3 ubiquitous factors and to be transactivated by Sp1 in CAPAN-2 cells but inhibited by Sp3 in CAPAN-1 and CAPAN-2 cells. Sp1/Sp3 are thus important regulators of *MUC4* basal expression and may be responsible for *MUC4* wide expression in normal cells from various tissues (15, 16, 56, 57). *MUC4* is not the only mucin gene to be regulated by Sp1 (58). This factor indeed has already

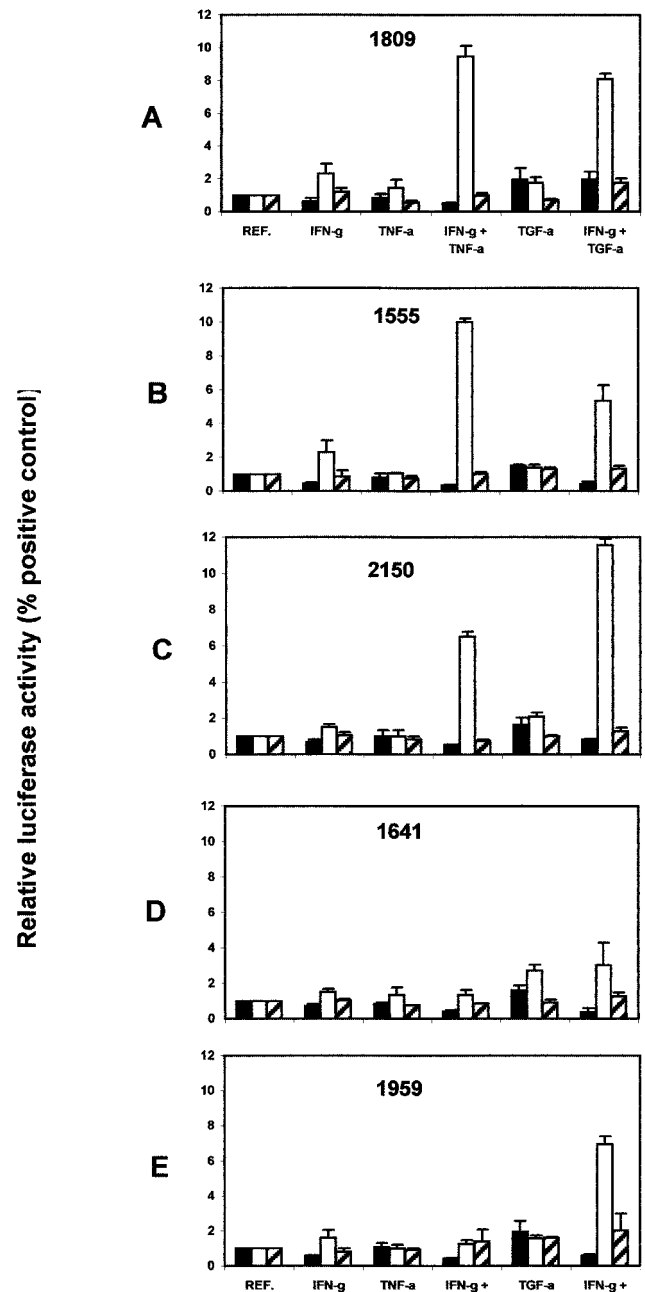


FIG. 10. Study of the synergistic effect of IFN- γ , TNF- α and TGF- α . Transfected cells (CAPAN-1, black bars; CAPAN-2, white bars; and PANC-1, hatched bars) were treated as described under "Experimental Procedures" before measurement of the luciferase activity of the deletion mutants of interest. REF. refers to the activity of the fragments of interest without any treatment. The results are the means \pm S.D. and represent more than three different experiments in triplicate for each fragment.

been shown to regulate human *MUC1* (59), *MUC2* (60), *MUC5AC* (data not shown), and *MUC5B* (32, 33), and rat (61) and mouse (62) *Muc2* mucin genes. Mucin gene regulation by Sp1/Sp3 thus appears as a common mechanism and most likely is responsible for the basal expression of mucins in epithelial cells.

The very high density of putative binding for transcription factors activated by growth factors (NF- κ B and AP-1) and cytokines (NF- κ B and STATs) and for hormone receptors (progesterone and estrogen) indicates that the transcription of *MUC4* is tightly regulated and involves many signaling pathways that may be responsible for the aberrant expression of

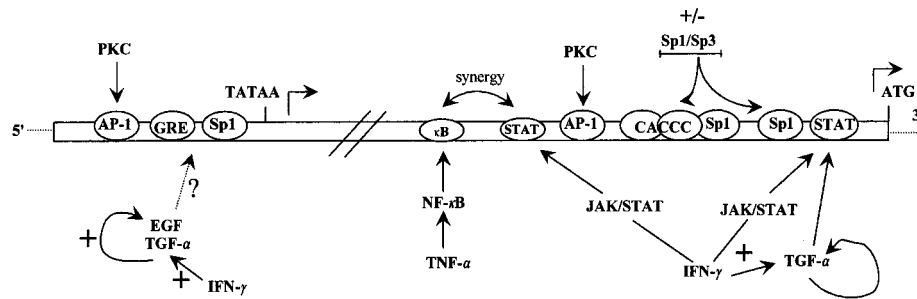


FIG. 11. Schematic representation of the different molecules and signaling pathways involved in *MUC4* promoter regulation. Positions of regulatory AP-1, glucocorticoid receptor element, Sp1, CACCC, NF- κ B, and STAT binding sites within *MUC4* 5'-flanking region are indicated. The signals leading to the activation of these transcription factors and that of *MUC4* thereafter are shown.

MUC4 described previously in inflammatory diseases of the lung (16), gastrointestinal tract (57), or epithelial tumors (17, 19, 20). Growth factors are thought to be involved in mucin-secreting cell production, because hypersecretory diseases are associated with abnormal epithelial cell growth and proliferation (48). Among growth factors, EGF seems a good candidate because it has already been shown to activate *MUC5AC* production in the human lung mucoepidermoid carcinoma NCI-H292 cell line (47). In this report, we now show that *MUC4* transcription is activated by EGF and TGF- α via the activation of downstream tyrosine kinases. PKC-mediated activation of *MUC4* shown in this report could also be another pathway used by growth factors to increase *MUC4* expression in cancer cells.

Mucins have long been shown to be target molecules during inflammatory reactions. Inflammatory diseases of the epithelium are often characterized by mucin up-regulation and mucus hypersecretion in response to the aggression (63–67). In previous reports, TNF- α was shown to activate *MUC2* (68) and *MUC5AC* (47). In this report, *MUC4* does not seem to respond to that proinflammatory cytokine, nor does it respond to IFN- γ . Up-regulation of *MUC4* was thus hypothesized to be the result of synergistic mechanisms between TNF- α and IFN- γ . Redundancy and synergy are fundamental and ubiquitous phenomena in the immune system are needed to elicit specific and efficient response of the body toward infection (69). Synergy between IFN- γ and TNF- α or TGF- α has been extensively studied and occurs in the regulation of numerous genes involved in cell differentiation and proliferation during inflammatory processes (49, 50) as well as in carcinogenesis (51). In this report, we show that *MUC4* is strongly up-regulated by IFN- γ /TGF- α or IFN- γ /TNF- α in CAPAN-2 cells. Synergy between IFN- γ and TNF- α is mediated through the simultaneous activation of STATs (IFN- γ) and NF- κ B (TNF- α) transcription factors. Interaction between STAT and NF- κ B once bound to their cognate *cis*-elements then induces the promoter of the target gene (69, 70). In the 5'-UTR of *MUC4* such a proximity between NF- κ B and STAT binding sites was found. Regarding IFN- γ and TGF- α , the mechanism is different and goes through an autocrine loop in which IFN- γ induces the transcription and expression of TGF- α that will then bind to the EGF receptor and consequently activates the gene of interest (71).

From our results, it is clear that *MUC4* promoter is regulated by growth factors that affect cell growth and proliferation and by proinflammatory cytokines, which besides their important role in inflammation have anti-tumoral activities. Thus, it may be hypothesized that *MUC4*, which is a transmembrane mucin aberrantly expressed in pancreas and gall bladder adenocarcinomas or overexpressed in mucoepidermoid cancers (lung, esophagus, and gall bladder) and thought to participate in ErbB2/Neu signaling, plays important roles in cell proliferation, tumorigenesis, and metastasis. Moreover, being up-regulated by molecules involved in chronic inflammatory diseases,

MUC4 could be considered to be a molecule participating to the host defense against pathogens like other adhesion molecules such as β -catenin and ICAM-1 (72–74).

In conclusion, we have isolated the promoter of the human mucin gene *MUC4* and showed that it is characterized by a typical TATA box flanked by a very long 5'-UTR. The 3'-end of the 5'-UTR is characterized by a GC-rich region that acts as a second transcription unit. From our results was drawn a schematic representation of *MUC4* 5'-flanking region in which signaling pathways that mediate *MUC4* regulation in pancreatic cancer cells identified in this paper are shown (Fig. 11). Regulation by transcription factors involves Sp1, Sp3, and probably factors from the AP family as well as NF- κ B and STATs. *MUC4* transcription is up-regulated by PKC, and EGF- and TGF- α -mediated up-regulation involves activation of downstream tyrosine kinase cascades. The most dramatic effect was the cell-specific up-regulation because of a synergistic effect between IFN- γ and TNF- α and IFN- γ and TGF- α . Investigations are now in progress to demonstrate whether the inactivation of these pathways represses *MUC4* expression and affects the behavior of human pancreatic cancer cells.

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