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Purification of Nuclear Proteins That Potentially Regulate Transcription of the MUC1 Mucin Gene Induced by a Soluble Factor[1]

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Transcriptional regulation of the MUC1 mucin gene in KM12C human colon carcinoma cells, which is induced by a soluble stimulatory factor derived from normal colonic connective tissues, was investigated. The minimum responsive element that was sufficient for this upregulation by the soluble factor is the upstream sequence of the MUC1 mucin gene from −531 to −488. Several factors in nuclear extracts of KM12C cells bound to this sequence in gel retardation assays. Neither the quantities nor the mobilities of the retarded bands changed on treatment with the soluble factor. Mutagenesis within the region from ACAGGGAGCGGTTAGAAGGGTGGGGCTATTCCGGGAAGTGGTGG to ACAGGGAGCGGTTAGAATTTTGGGGCTATTCCGGGAAGTGGTGG (underlined letters were mutated) substantially decreased the induction of the MUC1 mucin gene by the soluble factor. Two retarded bands were observed when the unmutated sequence was used as a probe; the bands disappeared when the mutated sequence was used as a probe. These results indicate that factors corresponding to each band were responsible for the upregulation of the MUC1 mucin gene, although the quantities of these proteins and their affinity to the nucleotide sequence did not change during the induction. Purification of the protein components comprising each band by a combination of column chromatographies indicated that one band contained four proteins (111, 106, 101, and 95 kDa) and the other consisted of two proteins (66 and 64 kDa).

Key words: colon carcinoma, MUC1, mucin, transcriptional regulation, tumor antigen.

Mucins, major epithelial luminal surface glycoproteins, are characterized by their high molecular weights (>200 kDa) and high contents of carbohydrate side chains (50-90%). The gene for the core polypeptide of human milk fat globule membrane mucins is designated as *MUC1*. MUC1 mucins are expressed in the epithelial cells of mammary glands, salivary glands, pancreatic ducts, lungs, kidneys, and stomachs (*1-3*). Adenocarcinoma cells often express MUC1 mucins, regardless of their origins, and the cell surface distributions and patterns of glycosylation are aberrant (*2-5*). MUC1 mucins are thought to protect the apical surface of the mucosa, through their anti-adhesive character, by interfering with cell-cell and cell-matrix interactions (*6-8*). Because sialyl Lewis X and sialyl Lewis a carbohydrate antigens are found in MUC1 mucins, these molecules might also function as ligands for carbohydrate-binding adhesion molecules such as selectins (*9-14*). MUC1 mucin expression makes tumor cells resistant to cytotoxic T lymphocytes, lymphokine-activated killer cells, natural killer cells, and macrophages (*6, 15*). Whereas the normal colonic mucosa contains very low levels of mature MUC1 mucins, colorectal carcinoma cells, particularly at the advanced stages, and metastases have been found to express higher levels of mature MUC1 mucins (*16*). Thus, it is important to determine how the expression of MUC1 mucins is regulated. The mechanism of constitutive expression of MUC1 mucins in breast and pancreatic carcinoma cell lines was elucidated previously (*17, 18*). Little is known, however, about the mechanism of induction of MUC1 mucins.

We previously demonstrated that a novel soluble factor (mucomodulin) present in normal colon conditioned medium (NCCM) stimulates sialomucin biosynthesis in colon carcinoma cells *in vitro* (*15*). The polypeptide core of the sialomucins was shown to be the product of the MUC1 mucin gene, and upregulation of MUC1 mucin mRNA was detected following sialomucin biosynthesis (*19*). We also demonstrated that the 5′-flanking sequence of the MUC1 mucin gene from −531 to −520 was required but not sufficient for the upregulation of the MUC1 mucin gene by NCCM (*20*).

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Abbreviations: CAT, chloramphenicol acetyltransferase; NCCM, normal colon conditioned medium.

In the study reported here, we identified the minimum domain required for the induction of the MUC1 mucin gene by NCCM. Two bands observed in gel retardation assays appeared to be responsible for the upregulation. DNA-binding proteins corresponding to the two bands were isolated by a combination of S-Sepharose chromatography, DNA affinity chromatography with specific nucleotide sequences, heparin-Sepharose affinity chromatography, and gel permeation HPLC on Superose 6.

MATERIALS AND METHODS

Cell Lines and Cell Culture—Human colon carcinoma cell line KM12C was obtained from Dr. I.J. Fidler, The University of Texas M.D. Anderson Cancer Center (Houston, TX, USA). Human mammary carcinoma cell line MRK-nu-1 was obtained from the Japanese Cancer Research Resources Bank (Tokyo). Cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium, supplemented with 10% heat-inactivated fetal calf serum under a humidified atmosphere containing 5% CO_2 at 37°C.

MUC1-Chloramphenicol Acetyltransferase (CAT) Reporter Plasmids—An expression vector, sh4,12-*Sma*I-CAT, was generated as previously described (*20*). sh4,13-*Sma*I-CAT and sh4,14-*Sma*I-CAT were generated as indicated below. The polymerase chain reaction products obtained with primers sh4 (5′-TTAAGCTTACAGGGAGCGGTTAG-3′) and sh13 (5′-TTTTGGGCTCCCTCC-3′) or sh4 and sh14 (5′-CCACCACTTCCCGGA-3′) were digested with *Hin*dIII, and then cloned between the *Hin*dIII and *Sma*I sites of p3C. sh4m,14-*Sma*I-CAT or sh4,14m-*Sma*I-CAT was also generated by the same method except that primers sh4m (5′-TTAAGCTTACAGGGAGCGGTTAGAATTTTGGGG-3′) and sh14 or sh4 and sh14m (5′-CCACCACTTCCATTAATAGCCC-3′) (underlined letters were mutated) were used. The nucleotide sequences of the inserted DNA fragments in all MUC1-CAT constructs were confirmed by the dideoxy chain-termination method (*21*).

Transient Transfection and CAT Assays—Transient transfection and CAT assays were performed as previously described (*20*). Briefly, KM12C cells, NCCM-responsive as previously described (*20*), were cotransfected with a series of MUC1-CAT deletion reporter plasmids and pRSV-β-Gal. Cells were incubated in the absence or presence of 5% NCCM for 2 days following transfection. Lysates of these cells were assayed for β-galactosidase and CAT activity. Transfection efficiency was normalized as to β-galactosidase activity. The reproducibility of the results was confirmed by at least three independent experiments.

Gel Retardation Assays—Nuclear proteins of the transfected cells were extracted by the method of Dignam and co-workers (*22*) using 0.35 M NaCl. A double-stranded probe corresponding to the sequence from −531 to −488 was labeled by polymerase chain reaction in the presence of [α-^{32}P]dCTP using primers sh4 and sh14. Mutated probes were also labeled by polymerase chain reaction with the mutated primers. The nuclear extracts were incubated at room temperature for 15 min with 2 μg of poly(dI-dC)-poly(dI-dC) (Pharmacia, Uppsala, Sweden) and ^{32}P-labeled probes (20,000 cpm) in 20 μl of binding buffer (10 mM HEPES, pH 8.0, 4.0 mM $MgCl_2$, 1.0 mM dithiothreitol, 5% glycerol). The reaction mixtures were electrophoretically separated in 5% polyacrylamide native gels in 0.5×TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0). The gels were analyzed with a BioImaging analyzer, BAS2000 (Fuji Film, Tokyo). In competition experiments, nuclear extracts were incubated with excess amounts of oligonucleotides prior to the addition of a ^{32}P-labeled probe. When fractions in the purification processes were used in gel retardation assays, 0.1 mg/ml bovine serum albumin was added as a carrier.

Antibodies—Anti-PuF/NM23-H2 antibody was kindly provided by Dr. Narimichi Kimura of the Department of Molecular Biology, Tokyo Metropolitan Institute of Gerontology (Tokyo). Anti-Sp-1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Purification of DNA-Binding Proteins—Nuclear extracts were prepared from MRK-nu-1 mammary carcinoma cell lines. Purification of DNA-binding proteins was monitored by gel retardation assays. The nuclear extracts (330 mg of protein) were diluted to 0.1 M NaCl with buffer A (10 mM HEPES, pH 8.0, 4 mM $MgCl_2$, 1 mM dithiothreitol, 5% glycerol), and then loaded onto a column of S-Sepharose (10 ml bed volume; Pharmacia) at the flow rate of 10 ml/h and washed with 5 column volumes of buffer A. The column was eluted with a stepwise increase in the NaCl concentration (0.1, 0.2, 0.4, and 1.0 M) in buffer A. The fractions containing proteins forming bands A and B on the gels were recovered primarily with 0.4 M NaCl.

These fractions were then pooled and diluted to 0.1 M NaCl with buffer A and loaded onto the first DNA affinity column. This column (500 μg of DNA/3.4 ml resin) was prepared by coupling annealed oligonucleotides corresponding to the upstream sequence of the MUC1 mucin gene from −527 to −495 to cyanogen bromide-activated Sepharose 4B (Pharmacia) as described previously (*23*). The column was eluted with 0.1, 0.2, 0.4, and 1.0 M NaCl in buffer A. The fractions containing proteins forming bands A and B in gel retardation assays were recovered primarily with 0.4 M NaCl. These fractions were pooled and dialyzed against buffer A, and then mixed with 10 mg/ml of poly(dI-dC)-poly(dI-dC) for 15 min before loading for the rechromatography on the same DNA affinity column. This second column was eluted with a linear gradient of NaCl, from 0.1 M to 0.6 M (10 ml/h). The fractions containing proteins forming bands A and B were pooled and diluted to 0.1 M NaCl with buffer A, and then loaded onto a Heparin-Sepharose column (1 ml bed volume). This column was eluted with a linear gradient of NaCl, from 0.1 to 0.6 M. The fractions containing proteins corresponding to bands A and B were then pooled and concentrated with Centricon-10 (Amicon, Beverly, MA, USA), and diluted with buffer A containing 0.35 M NaCl. The solution was re-concentrated to 200 μl and loaded onto a Superose 6 gel filtration column (10×300 mm) equilibrated with buffer A containing 0.35 M NaCl at a flow rate of 24 ml/h.

RESULTS

Minimum Element Required for Upregulation of the MUC1 Mucin Gene by NCCM—We previously showed that the 5′-flanking sequence from −531 to −520 was required for the upregulation of the MUC1 mucin gene by NCCM and that factors binding to this region were present in nuclear extracts of KM12C cells when the region was

used as a probe in gel retardation assays (*20*). Subsequently, this protein proved to be a nonspecific DNA-binding protein, Ku (p70/p80) (*24*), and specific binding to the region was not reproduced. Thus, other proteins should be considered as candidates for the upregulation. The previous study also showed that another region extending more to the 3′-flanking sequence was also required.

These two observations led us to search for the minimum element responsive to NCCM. The differences in CAT activity of lysates of untreated cells and NCCM-treated cells are shown in Fig. 1. When the responses of deleted vectors were compared, it appeared that the minimum responsive element was located between bases −531 and −488.

DNA-Binding Factors to Upstream Sequence of the MUC1 Mucin Gene from −531 to −488—Nuclear extracts of untreated and NCCM-treated KM12C cells were prepared and examined for their reactivity with the nucleotide sequence from −531 to −488. Using double-stranded DNA corresponding to this region as a probe in gel retardation assays, as shown in Fig. 2, two major retarded bands and three minor bands were observed. The nuclear extracts exhibited no differences with regard to their relative intensities and mobilities on the gels. Increased amounts of nuclear proteins resulted in more intense bands.

Alteration of the NCCM-Responsive Element by Point Mutations—To confirm that the region from base −531 to −488 is essential for the upregulation of the MUC1 mucin gene by NCCM, we introduced point mutations into the nucleotide sequence. The resulting reporter plasmids were transfected into KM12C cells, and then lysates of untreated and NCCM-treated cells were assayed for CAT activity. As shown in Fig. 3A, NCCM-treated cells transfected with sh4m,14-*Sma*I-CAT (mutated at −514 to −512) or sh4,14m-*Sma*I-CAT (mutated at −501 to −499) did not exhibit increased CAT activity. These results strongly suggest that both regions are critical for upregulation. Oligonucleotides corresponding to these mutated nucleotides were used as probes in gel retardation assays, as shown in Fig. 3B. When the oligonucleotide mutated at −514 to −512 was used as a probe, bands A and B, lanes 4 to 6, disappeared. When the oligonucleotide mutated at −501 to −499 was used as a probe, however, bands A and B were observed, lanes 7 to 9.

From these results, we concluded that the sequence from −514 to −512 was critical for both the upregulation of MUC1 mucin gene expression observed in CAT assays, and for the DNA-binding of proteins yielding bands A and B. The sequence from −501 to −499 was critical for the upregulation of MUC1 mucin gene expression but not for the DNA-binding. The specificity of the DNA-binding as to the formation of bands A and B was confirmed by competition experiments (data not shown).

Purification of Factors Comprising Bands A and B—Previously, it was reported that the sequence, GGGTGGG, was the consensus binding site of Sp-1 and PuF/NM23-H2 (*25-27*). The same sequence, GGGTGGG, located in the upstream sequence of the MUC1 mucin gene from −514 to −508, was shown in this study to be critical for both the upregulation of the MUC1 mucin gene, and the binding of proteins comprising of bands A and B. Specific antibodies against Sp-1 or PuF/NM23-H2, however, did not cause any change in the migration of the nucleotide (−531 to −488) in gel retardation assays (data not shown).

We attempted to isolate the DNA-binding proteins corresponding to bands A and B from nuclear extracts of MRK-nu-1 mammary carcinoma cells. This cell line was previously shown to respond to NCCM to upregulate MUC1 mucin production and suitable for a large-scale culture (unpublished observation). Purification was monitored by gel retardation assaying with a nucleotide corresponding to the −531 to −488 region. Starting with 330 mg of nuclear extract, purification was performed in five steps, S-Sepha-

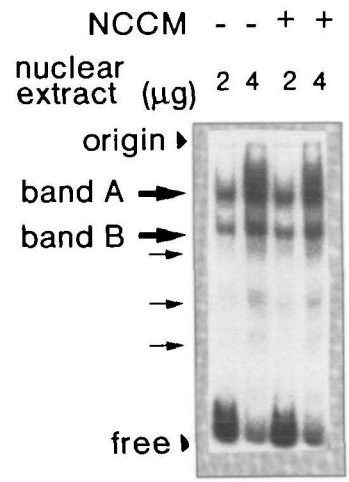

Fig. 2. **Binding of nuclear proteins of KM12C cells to double-stranded oligonucleotides (−531 to −488).** A ^{32}P-labeled probe (−531 to −488) was incubated with nuclear extracts of untreated KM12C cells and treated KM12C cells with 5% NCCM for 2 days. Each mixture was electrophoretically separated on a 5% polyacrylamide nondenaturing gel. Nuclear extracts of the untreated cells are in the first and second lanes; nuclear extracts of the treated cells are in the third and fourth lanes. Bands A and B are indicated by thick arrows; minor bands are indicated by thin arrows.

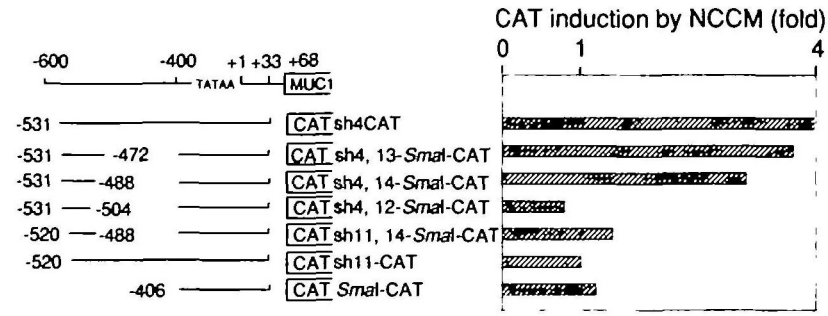

Fig. 1. **Effects of NCCM on the expression of MUC1-CAT reporter plasmids.** In the left half of the figure are shown the structures of nucleotides modified by deleting specific portions, as indicated by the numbers upstream of the 5′-flanking sequence of the MUC1 mucin gene. In the right half of the figure the CAT activity of lysates of NCCM-treated cells relative to the activity of lysates of untreated cells is indicated as the relative degree of activity induced by NCCM (fold).

rose cation exchange chromatography, DNA affinity chromatography, DNA affinity chromatography in the presence of carrier DNA, heparin-Sepharose affinity chromatography, and Superose 6 gel permeation column chromatography. Prior to the Superose 6 column separation, the proteins comprising bands A and B were recovered in the same fraction at each step, but after this step, they were separated. As shown in Fig. 4A, band A was eluted as a fraction corresponding to approximately 400 kDa (fraction 29), while band B was eluted as a fraction corresponding to 230 kDa (fraction 31). The fractions after the Superose 6 column step were analyzed by electrophoresis on 10% polyacrylamide gels in the presence of sodium dodecyl sulfate, as shown in Fig. 4B. The amounts of the four proteins (111, 106, 101, and 95 kDa) in fractions 28-30 were in accordance with the intensity of band A. The amounts of the two proteins (66 and 64 kDa) in fractions 30-32 corresponded to the intensity of band B.

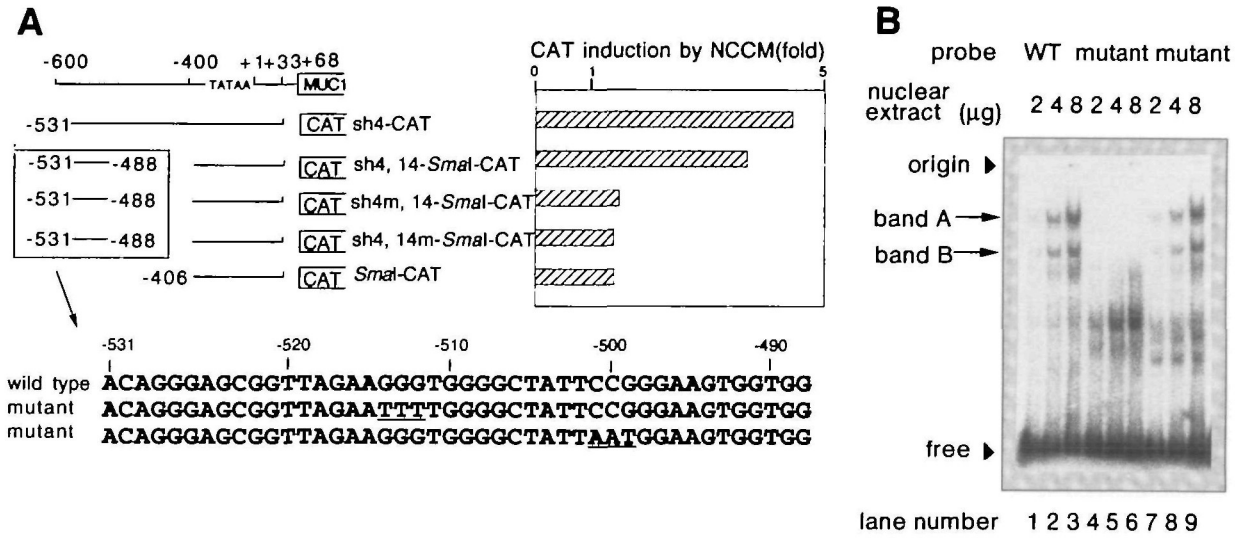

Fig. 3. **Mutation analysis of NCCM responsive elements.** (A) Effects of mutations on NCCM responsiveness, as indicated by CAT assays. The sequences of the mutated reporter plasmids are shown at the bottom. CAT induction by NCCM, shown in the right half of the panel, was defined as in the legend to Fig. 1. (B) Gel retardation assays with mutated nucleotide probes. Unmutated oligonucleotides, from −531 to −488 (lanes 1 to 3), and mutated oligonucleotides, from −514 to −512 (lanes 4 to 6) and from −501 to −499 (lanes 7 to 9), were used as probes. Nuclear extracts of KM12C cells were incubated with the ^{32}P-labeled oligonucleotide probes and then electrophoretically separated on 5% polyacrylamide nondenaturing gels.

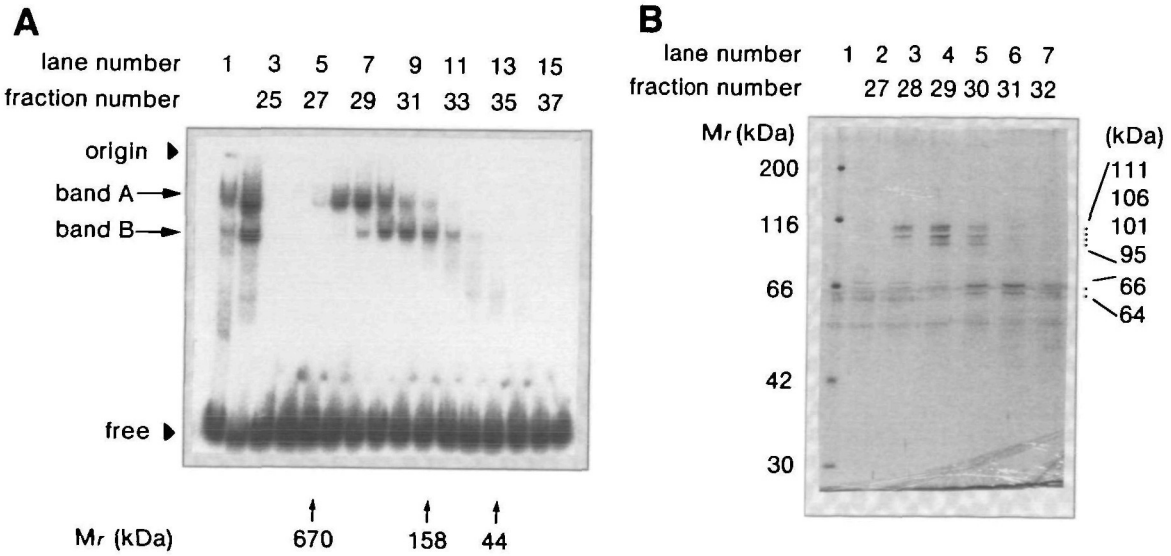

Fig. 4. **Results of gel retardation assays and sodium dodecyl sulfate polyacrylamide gel electrophoresis of fractions after Superose 6 gel permeation column chromatography.** (A) DNA-binding activity of the fractions after the Superose 6 column step was examined by gel retardation assaying (lanes 3 to 16). Crude nuclear extracts (lane 1) and pooled fractions after the heparin-Sepharose column step (lane 2) were also assayed. Molecular weights were estimated from the standard markers shown at the bottom. (B) Fractions after the Superose 6 column step were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 10% gels and then visualized by silver staining (lanes 2 to 7). A mixture of M_r markers was separated in lane 1.

DISCUSSION

We determined the minimum responsive element for NCCM and identified the region from −531 to −488 as the *cis*-element sufficient to produce the response seen on CAT assays in the 5′-flanking sequence of the MUC1 mucin gene. When gel retardation assays were performed with the nucleotide sequence from −531 to −488 as a probe, two major bands (A and B) and three minor bands resulted from the affinity of this nucleotide with nuclear proteins. CAT assays and gel retardation assays with mutated nucleotides showed that both the responsiveness to NCCM and the binding of the nuclear proteins were disrupted when the sequence from −514 to −512 was mutated from GGG to TTT. Thus, the DNA-binding proteins comprising bands A and B were suggested to be responsible for the upregulation of the MUC1 mucin gene. The quantities and mobilities of bands A and B in gel retardation assays did not change after NCCM treatment. It is possible that the upstream sequence is constitutively occupied by DNA-binding proteins and that NCCM induces modifications of them, thus causing the upregulation of the MUC1 mucin gene. Examples of the transcriptional regulation of proteins by phosphorylation independent of the effects on DNA-binding have been reported (*28*). In our additional mutation experiments, the mutation of the sequence from −523 to −521 or the sequence from −499 to −501 resulted in disruption of CAT activity. However, these nucleotides gave the same bands as the wild-type nucleotides in gel shift assays. Thus, these sequences are apparently necessary for transcriptional upregulation but not required for the binding of nuclear proteins. These results, however, did not absolutely prove that these binding proteins are responsible for the inducible transcription.

From the results of their CAT assays, Abe and Kufe (*17*) suggested that the upstream sequence of the MUC1 mucin gene from −592 to −479 was required for constitutive gene expression in MCF-7 human breast carcinoma cell lines. On footprint analysis, they detected DNA-binding proteins corresponding to two regions, from −519 to −507 and from −499 to −479. The former includes the region from −514 to −511 which was suggested in our study to be the binding site for the proteins comprising bands A and B. However, they did not present any information on the nature of the binding proteins. The present report is the first to show the presence of potentially novel DNA-binding proteins specific to this upstream gene sequence responsible for the cytokine-induction of the MUC1 mucin gene. More studies will be required to clarify the cellular events leading to the transcriptional upregulation of the MUC1 mucin gene after cells' exposure to soluble factors derived from cells in connective tissues.

Judging from the molecular weights of the corresponding proteins, band A appears to contain four polypeptides (111, 106, 101, and 95 kDa), and band B appears to contain a tetramer of two polypeptides (66 and 64 kDa). Although the molecular weight of the Sp-1 protein is about 100 kDa, such a component was not observed in fraction 29 after the Superose 6 column step, as determined with an anti-Sp-1 antibody. The specific activities and the degrees of purification of binding proteins corresponding to bands A and B after each chromatographic step are shown in Table I.

It has been suggested that MUC1 mucin antagonizes the homotypic aggregation of tumor cells (*7*), which renders tumor cells resistant to natural immunity (*6, 15*). It is also suggested that MUC1 mucin induces the apoptosis of human T cells (*29, 30*). The upregulation of MUC1 mucins during the progression of carcinomas may be due in part to the effects of soluble proteins derived from the surrounding connective tissues. Braga and co-workers (*31*) have suggested that the mouse *Muc1* gene is under both spatial and temporal control during the development of epithelial cells. The regulatory mechanism for the *Muc1* gene during development may also be related to soluble factors derived from connective tissues. Our study provides insight into the biological role of MUC1 mucins, and their regulation during the development of epithelial tissues and carcinomas.

TABLE I. **Activities and purification of binding proteins corresponding to bands A and B.**

	Protein (mg)	Activity (U)[a]	Specific activity (U/mg)	Purification (fold)
		Band A		
Nuclear extract	330	141,180	458	1
S-Sepharose column	52	98,784	1,900	4.44
First DNA affinity column	4	12,527	3,130	7.32
Second DNA affinity column	< 0.56	4,316	>7,707	>18.0
Heparin-Sepharose column	< 0.28	2,177	>7,775	>18.2
Superose 6 column (fractions 28-30)	0.00052	2,087	401,000	9,380
		Band B		
Nuclear extract	330	60,905	185	1
S-Sepharose column	52	44,192	850	4.6
First DNA affinity column	4	10,983	2,750	14.9
Second DNA affinity column	<0.56	3,528	>6,300	>34.1
Heparin-Sepharose column	<0.28	1,560	>5,570	>30.2
Superose 6 column (fractions 30-32)	0.00028	1,845	7,450,000	40,373

[a]Defined as the relative radioactivity of the retarded band.

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