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Identification of a 42-kDa Nuclear Factor (NF1-MUC5B) from HT-29 MTX Cells That Binds to the 3′ Region of Human Mucin Gene *MUC5B*

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MUC5B gene is one of the four human mucin genes mapped to chromosome 11p15. The identification of three potential Sp1 binding sites located between the tandem repeat and the 3′ end of *MUC5B* suggests a possible regulatory role for this region. In this report we show by electrophoretic mobility shift assay that only one potential Sp1 binding site (NAU62) leads to a specific interaction with a nuclear factor from HT-29 MTX cells which does not exist in parental HT-29 cells. By using mutated versions of NAU62, an 18 mer sequence within this later was shown to be directly involved in the interaction. The nuclear factor called NF1-MUC5B which binds to this element has a M_r of 42000 and is not Sp1. These results suggest that *MUC5B* contains a sequence in its 3′ region that might act as a cis-element. This report opens the field of transcriptional regulation of human mucin genes encoding secreted mucins.

Mucins are highly O-glycosylated proteins normally produced by epithelial cells mainly as protective agents. Several mucin cDNA clones have been isolated and all exhibit an organization made of tandem repeats (1,2). To date eight human mucin genes have been identified and designated *MUC1* to *MUC7*. Each of these genes, except *MUC1* whose main product is a membrane-bound mucin, is expressed in a tissue-specific manner although it has been shown that several mucin genes can be co-expressed in the same tissue in physiological situations (3). Furthermore, altered mucin gene expression is often associated with epithelial tumors and some inflammatory diseases (4–6). So far, nothing is known about the transcriptional regulation of human secreted mucin genes. *MUC5B* which belongs to the 11p15 mucin gene cluster, as well as *MUC2*, *MUC6* and *MUC5AC* (1), is mainly expressed in submaxillary and bronchus glands (3) and also in gallbladder cells (7). Several cDNA clones corresponding to *MUC5B* have been isolated in our laboratory (8) and their study showed that *MUC5B* is somehow distinct from the other genes since its tandem repeat structure is degenerated and is not as highly polymorphic as the other mucin genes (unpublished data). Its whole genomic organization is being elucidated from three overlapping clones and the first results showed that *MUC5B* contains a large central exon made of numerous copies of degenerate tandemly repeated sequences. The 5′ and 3′ ends of the gene have been defined by the presence of a CpG island and two polyadenylation sequences, respectively (9). Three potential Sp1 binding sites (-GGGCGG-) were located between the tandem repeat and the 3′ end of *MUC5B*. HT-29 MTX are a mucus-secreting subclone selected by adaptation to methotrexate from parental HT-29 cells. This subclone expresses high levels of *MUC1*, *MUC2*, *MUC3*, *MUC5AC* (10) and *MUC5B* mRNAs (11). On the other hand, the parental cell line displays very low levels of expression of these human mucin gene mRNAs (10,12).

In this report we identify a DNA stretch in *MUC5B*, located between the tandem repeat and the 3′ end of the gene, capable of specifically binding to a nuclear factor present in HT-29 MTX cells.

Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EMSA, electrophoretic mobility shift assay; fpu, footprinting unit; M_r, relative molecular mass; MTX, methotrexate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TBE, Tris-borate-EDTA.

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This protein that we called NF1-MUC5B is different from the nuclear factor Sp1 and has a M_r of 42000.

MATERIALS AND METHODS

Oligonucleotides. The oligonucleotides were synthesized from *MUC5B* sequences containing Sp1-like sequences (underlined): **NAU62:** 5′ TTCGGT-GAGTGG*GGGCGG*CCCCGGGCCCCCAGACC 3′ **NAU63:** 5′ TTACGCAGA-GCTCTG*CCGCCCC*CCGGGGAGTGTGCAGTGAC 3′ **NAU64:** 5′ CCCTGG-GGTCCC*CCGCCG*CCCGCATGCACG-CACGACGC 3′. Five mutated versions of NAU62 were also used to check the specificity of the binding (mutated sequence is underlined): **NAU94:** 5′ TTCGGTGAGTGG*AAAAGA*CCCCGGGCCCCCAGACC 3′; **NAU95:** 5′ TTCGGT-GAGTGGGGGCGGCCCCGG*TTA*CCCAGACC 3′; **NAU108:** 5′ T*ATAT*GAGTGGGGGCGGCCCCGGGC-CCCCAGACC 3′; **NAU109:** 5′ TTCGGTGAGTGGGGGCGG*TATTCA*GCCCCCAGACC 3′; **NAU110:** 5′ TTCGGT-GAGTGGGGGCGGCCCCGGGCC*TAT*AGACC 3′. Single-stranded oligonucleotides were synthesized by Eurogentec (Belgium). Oligonucleotides were radiolabeled using T4 polynucleotide kinase (Promega, Madison, WI) and [γ^{32}P] ATP from Amersham, France. Probes were purified on Sephadex G-25 columns (Quick Spin, Boehringer Mannheim, France).

Cell culture. Human colon adenocarcinoma parental HT-29 and 10^{-6} M methotrexate-treated (HT-29 MTX) cell lines were a kind gift of Dr A. Zweibaum (INSERM U178, Villejuif, France). Cells were cultured in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% fetal calf serum as previously described (10).

Electrophoretic mobility shift assays. Nuclear protein extractions were carried out as previously described (13), and nuclear proteins were stored frozen in aliquots at −80°C before use. Protein content was measured using the Bradford method. Nuclear extracts (5μg of proteins) were pre-incubated for 10 min at room temperature (RT) in 20 μl binding buffer (10 mM Tris-HCl pH 7.6, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 4% glycerol) with 2 μg of poly dI-dC (Sigma, St-Louis, MO) and 0.5 μg of salmon sperm DNA. Radiolabeled probe (≈ 60,000 cpm/lane) was then added and reaction was left at RT for 20 min. Cold competitor DNA at different concentrations was pre-incubated with nuclear proteins before addition of the radiolabeled probe. For super shift analysis, 1 μl of anti-human Sp1 polyclonal antibody (PEP 2, Santa Cruz Biotechnology, Santa Cruz, CA) was added and reaction was left at 4°C for 12 h. Human recombinant Sp1 protein used as a positive control was purchased from Promega (Madison, WI). Reaction was stopped by adding loading buffer (25 mM Tris-HCl pH 7.5, 0.02% bromophenol blue, 0.02% xylene cyanol, 4% glycerol). Reaction mixtures were then loaded on 4% polyacrylamide gels and electrophoresis run for 3h at 130V in 0.5X TBE buffer. Gels were dried and analysed by autoradiography.

UV cross-linking and immunoblotting. Samples treated exactly as above were exposed to UV light (Fluolink 312nm, Combilight, France) for varying times up to 10 min. Proteins were resolved on 10% SDS polyacrylamide gels as previously described (14) and transferred to 0.45 μm nitrocellulose membrane (Schleicher & Schuell, CéraLabo, France). Immunoblotting was performed by incubating the membrane with a 1:1,000 dilution of anti-human Sp1 antibody (PEP2), then washed and probed with horseradish peroxydase conjugated goat anti-rabbit IgG (Caltag Laboratories, San Francisco, CA) at a dilution of 1:1,000. Once stained, the membrane was autoradiographed. Molecular weight standards (prestained low range) were purchased from Biorad (Hercules, CA).

RESULTS AND DISCUSSION

MUC5B gene sequencing revealed the presence of three potential Sp1 nuclear factor binding sites, two of them (NAU62 and 64) located in an intron whereas NAU63 is in an exon, downstream the tandemly repeated central exon and about 5kb upstream the two polyadenylation signal sequences found so far (Laine A., manuscript in preparation). HT-29 derived cell-lines display variable differentiated phenotypes depending on the treatment and hence provide a very good tool to study the regulation of intestinal cell differentiation. Parental HT-29 are not mucus-secreting cells but treatment of these cells with methotrexate (MTX) allows them to differentiate into intestinal mucus-secreting cells (15). *MUC5B* is poorly expressed in parental HT-29 but has its expression increased after MTX treatment (11). Studies of *MUC5B* mRNA expression over a period of 21 days of culture showed a constant increase of expression with time: almost no expression at day 7, the presence of one transcript at day 14 which becomes maximal at day 21 (11). In this report, HT-29 and HT-29 MTX were cultured in the same conditions and nuclear extracts were prepared at days 7, 14 and 21 and then studied for the presence of nuclear factors which would bind to the three oligonucleotides NAU62, NAU63 and NAU64 representative of the three potential Sp1 binding sites found in the 3′ region of *MUC5B.*

A Nuclear Factor Specific to HT-29 MTX Cells Binds to the 3′ Region of MUC5B

Considering the difference in mucin gene expression between parental HT-29 and HT-29 MTX

cells, we first looked at whether this was reflecting the absence in the parental cell line of nuclear factor(s) involved in the regulation of *MUC5B.* As shown in Fig. 1A, no shift was obtained when the radiolabeled oligonucleotide NAU62 was incubated with nuclear proteins prepared from parental HT-29 cells (lanes 1–3). Conversely, when the same experiment was carried out with nuclear proteins from HT-29 MTX, a shift occurred (lanes 4–6). The shift is different depending on the stage of the culture. At day 7, a unique band of low mobility is visualized (lane 4) whereas at days 14 and 21 of culture, the observed complex shows a higher mobility and a second minor complex becomes visible (lanes 5 and 6, respectively). The intensity of these two complexes becomes also more intense at day 21 (lane 6). These two bands may be the reflection of two complexes either made of two non-related nuclear factors or two different forms of the same factor, for example a factor with different phosphorylation degrees. Furthermore, it should be stressed that the EMSA profile agrees with the HT-29 MTX *MUC5B* expression pattern which has been shown to be a growth-related phenomenon (10,11). This shows that this region of *MUC5B* interacts with a nuclear factor(s) only present in HT-29 MTX cells.

We then checked whether the three potential Sp1 binding sites were giving the same shifting activity. As shown in Fig. 1B, a specific shift is only obtained when nuclear proteins from HT-29 MTX cells were incubated with the oligonucleotide NAU62 (lanes 1–3). Neither the oligonucleotide NAU63 (lanes 4–6) nor NAU64 (lanes 7–9) led to any shift. As in Fig. 1A, only one complex exists at day 7 (lane 1) whereas two complexes are present at days 14 and 21 (lanes 2,3). We can conclude from these experiments that only NAU62 represents a binding site for a HT-29 MTX nuclear factor(s).

Localization and Specificity of the Interaction between NAU62 and HT-29 MTX Nuclear Factor(s)

After showing that NAU62 contains a binding site, we checked whether this interaction was due

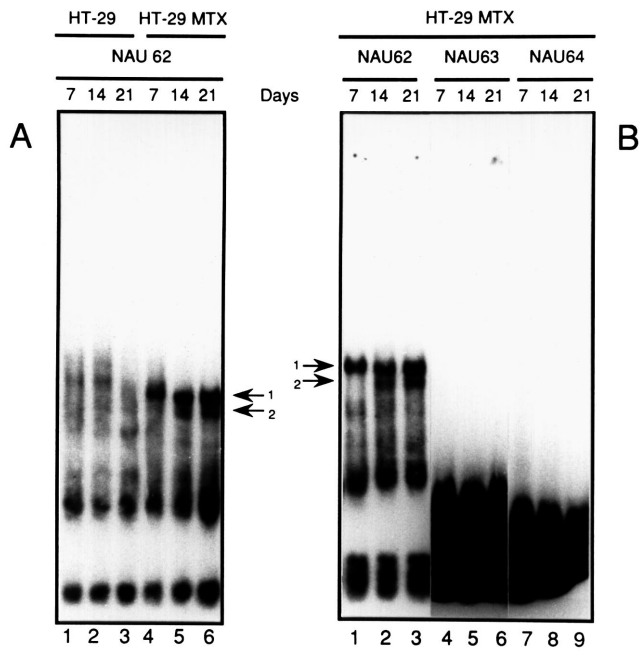

FIG. 1. A HT-29 MTX nuclear protein specifically binds to NAU62. (A) EMSA of nuclear proteins (5 μg) prepared from parental HT-29 and HT-29 MTX cell lines at days 7 (lanes 1,4), 14 (lanes 2,5) and 21 (lanes 3,6) were incubated with radiolabeled oligonucleotide NAU62. (B) Binding activity of HT-29 MTX nuclear proteins (5 μg) to the three oligonucleotides derived from *MUC5B* containing a potential Sp1 binding site: NAU62 (lanes 1–3) NAU63 (lanes 4–6) NAU64 (lanes 7–9). Arrows indicate the low (1) and high(2) mobility complexes of NAU 62 and a HT-29 MTX nuclear factor.

to Sp1 since NAU62 contains a potential Sp1 binding site (GGGCGG). To this end, a series of mutated versions of NAU62 were synthesized. NAU94 represents an oligonucleotide lacking the Sp1 binding site. The other ones were mutated around the Sp1 binding site. Competition studies were carried out as described in Materials & Methods. Total inhibition was achieved with 150 fold excess of NAU62 (Fig. 2A, lane 3). Among the mutated versions, NAU94 where the Sp1 binding site was abolished had no competition effect (lanes 8,9). NAU95 (lanes 12,13) and NAU110 (lanes 4,5) equally competed away the interaction between nuclear factor(s) and radiolabeled NAU62. NAU109, where the mutation is next to the Sp1 binding site, had no effect at 150 fold excess (lane 10) but showed some inhibitory activity with the highest concentration (lane 11). Finally, NAU108 which was mutated in the 5′ region of NAU62 almost totally abolished the interaction (lanes 6,7). These results indicate that i) the Sp1 binding site is involved in the interaction ii) the adjoining sequence on the 3′ side is also involved but engages the protein with a lower affinity. We then checked whether the factor Sp1 was the one involved in the interaction. The absence of a supershift when a polyclonal anti-Sp1 antibody was used, suggests that Sp1 is not involved (Fig. 2B, lane 2). The activity of the antibody was checked as it gave a supershift when added to the recombinant

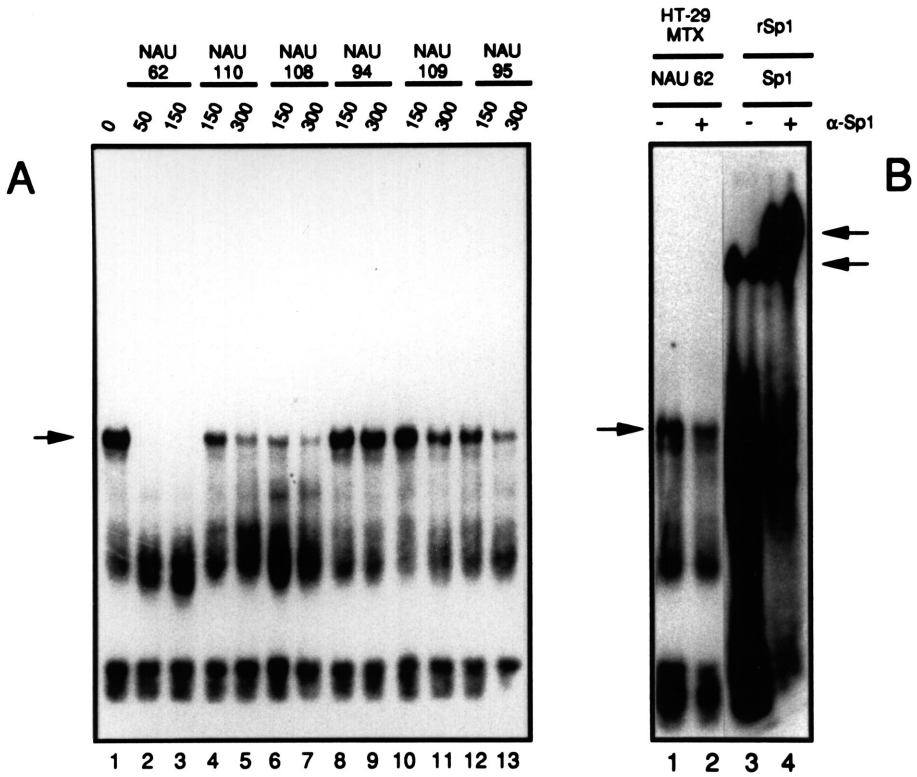

FIG. 2. Localization and specificity of the interaction between NAU62 and HT-29 MTX nuclear factor. (A) EMSA of nuclear proteins (5μg) prepared from HT-29 MTX cells at day 14 were incubated with radiolabeled oligonucleotide NAU62. Binding reactions were carried out without (lane 1) or with a 50-, and 150-fold excess of wild type NAU 62 (lanes 2–3, respectively). 150-, and 300-fold excess were used for mutated versions of NAU62: NAU 110 (lanes 4,5), NAU 108 (lanes 6,7), NAU 94 (lanes 8,9), NAU109 (lanes 10,11), and NAU 95 (lanes 12,13). (B) Supershift studies with a polyclonal anti-Sp1 antibody. Binding of HT-29 MTX nuclear proteins (5 μg) with NAU 62 (lanes 1,2) and of recombinant Sp1 protein (rSp1: 2 fpu) with Sp1 oligonucleotide (lanes 3,4) were done in the absence (lanes 1,3) or presence (lanes 2,4) of 1 μl of the anti-Sp1 antibody. Arrows on the left indicate the complex of NAU 62 and the nuclear factor. On the right hand side is indicated the complex of the Sp1 oligonucleotide with the recombinant Sp1 protein (lower arrow) and the supershift after addition of the anti-Sp1 antibody to the mixture (upper arrow).

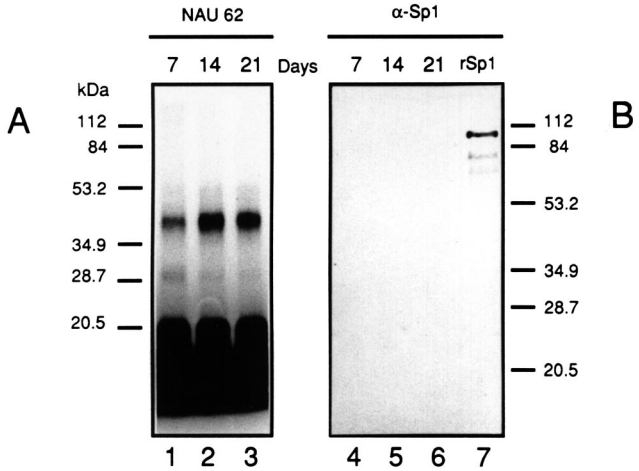

FIG. 3. Characterization of the NAU62 binding nuclear factor by UV cross-linking and 10% SDS-PAGE. (A) Autoradiography of NAU62-bound nuclear proteins (5 μg) after UV cross-linking. (B) Immunostaining with anti-Sp1 antibody of proteins electrotransferred onto nitrocellulose after UV cross-linking and electrophoresis. Proteins were prepared at days 7 (lanes 1,4) 14 (lanes 2,5) and 21 (lanes 3,6) of the cell culture. Positive control with recombinant Sp1 protein (2 fpu) (lane 7).

human Sp1 protein incubated with Sp1 oligonucleotide (compare lane 4 to lane 3). In conclusion, *MUC5B* contains a sequence spanning 18 nucleotides including a Sp1 binding site which interacts with a nuclear factor capable of recognizing GC boxes but that is not Sp1.

MUC5B Binds to a Nuclear Factor of 42 kDa

In order to characterize the protein(s) involved in the binding with NAU62, UV cross-linking experiments were performed using the same stringency as for EMSA experiments. After UV cross-linking, proteins were resolved on a 10% SDS-polyacrylamide gel (Fig. 3). The major band visualized has a M_r of 42000. Again, the intensity increases at days 14 and 21 compared to day 7 of culture (compare lanes 2 and 3 to lane 1). This is in good agreement with EMSA experiments shown in Fig. 1 where intensity of the shift also increased as the cells were older. In panel B, is shown an immunostaining of the same experiment where proteins were blotted on a nitrocellulose membrane and stained with the same anti-Sp1 antibody used for supershift analysis. The absence of positive staining at days 7, 14 and 21 is again in favor of the involvement of a nuclear factor different from Sp1 (lanes 4–6). A positive control with the recombinant Sp1 is shown in lane 7.

In conclusion we demonstrate in this report that *MUC5B* contains a sequence located between the central exon and the 3′ region that is a binding site for a 42 kDa nuclear factor different from Sp1, that we called NF1-MUC5B. This interaction becomes more important at the later stages of mucus-secreting HT-29 MTX cell culture and is absent in parental HT-29 cells. These last two results might indicate a possible regulatory role for this region. Moreover, according to NF1-MUC5B capacity of binding to single strand DNA representative of the 3′ region of *MUC5B*, the hypothesis that this factor could alter the stability of *MUC5B* mRNA also needs to be evaluated. Transfection studies are currently being done in the laboratory to show whether this sequence acts as a cis-element in transcriptional regulation of *MUC5B.*

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