A nuclear factor that binds purine-rich, single-stranded oligonucleotides derived from S1-sensitive elements upstream of the CFTR gene and the MUC1 gene

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ABSTRACT

We have identified two regions of non-random purine/pyrimidine strand asymmetry that were nearly identical in sequence in the 5' flanking (promoter) regions of the human cystic fibrosis transmembrane conductance regulator (CFTR) gene and the human MUC1 gene. These regions contain perfect mirror repeat elements, a sequence motif previously found to be associated with the formation of H-DNA conformations. In this report we demonstrate that a single-stranded non-B DNA conformation exists at low pH in supercoiled plasmids containing the similar mirror repeat elements, and that S1 nuclease digestion maps the single-stranded region to the position of the mirror repeats. In addition, we identify a nuclear protein of approximately 27 kD that binds to single-stranded oligonucleotides corresponding to the purine-rich strand of this region, but not to the pyrimidine-rich strands or to double-stranded oligonucleotides with corresponding purine/pyrimidine strand asymmetry.

INTRODUCTION

In the course of evaluating the sequence of the 5' flanking (promoter) region of the human cystic fibrosis transmembrane conductance regulator (CFTR) gene, we noted the presence of two regions of non-random purine/pyrimidine strand asymmetry that were nearly identical in sequence. Both of these regions contain perfect homopurine mirror repeat elements, wherein a homopurine sequence displays symmetry about a bisecting plane. These sequence motifs have previously been found to be associated with H-DNA conformations involving intramolecular triple helix formation under certain *in vitro* assay conditions (1–4) under certain *in vitro* assay conditions. The presence of non-B conformation DNA with single-stranded character has been detected upstream and downstream from a number of genes (5–9) using assays that demonstrate sensitivity to S1 nuclease. A few trans-acting factors that might bind to these regions have

been described (5–7). It has been postulated that non-B DNA conformations and factors that bind to them play a role in the regulation of transcription of some genes (10); however, little direct evidence supporting this hypothesis has been reported.

Our interest in the CFTR promoter mirror repeat regions was heightened when we identified two sequences sharing 80–85% identity in the 5' flanking (promoter) region of the human MUC1 gene, which is expressed in some (though not all) of the same epithelial cell types as the CFTR gene in different organs and at different stages of development (11). In this report we identify a nuclear protein of approximately 27 kD that binds to single-stranded oligonucleotides corresponding to the purine-rich strand of this region, but not to the pyrimidine-rich strands or to double-stranded oligonucleotides with corresponding purine/pyrimidine strand asymmetry. We also demonstrate for both CFTR and MUC1 that a single-stranded non-B DNA conformation exists at low pH in supercoiled plasmids containing the similar mirror repeat elements, and that S1 nuclease digestion maps the single-stranded region to the position of the mirror repeats.

MATERIALS AND METHODS

Cell lines

Human pancreatic adenocarcinoma cell lines HPAF (13) and the human colon adenocarcinoma cell line HT 29 (ATCC), were grown in Eagle's minimal essential media plus 10 % fetal calf serum, 2 mM L-glutamine, 100 units penicillin G/mL, and 0.1 mg/mL streptomycin.

Preparation of nuclear extracts

All extraction steps were performed at 4°C. The indicated cell lines were washed five times with phosphate buffered saline (PBS), and then harvested by scraping into the following nuclei preparation buffer (NPB) at 5°C: 10 mM HEPES pH 7.2, 50 mM NaCl, 500 mM sucrose, 0.1 mM EDTA, 0.5% (w/v) Triton X-100, 5 mM MgCl₂. Nuclei were removed by centrifugation at 1000 × g for 20 min, then resuspended at 7 × 10⁷ nuclei/ml in

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NPB, followed by the addition of spermidine to 5 mM and NaCl to 500 mM. The mixture was incubated with gentle stirring for 60 min. Following a 10 min centrifugation at 5000×g, the supernatant was dialyzed overnight against at least 3 changes of 100 volumes of 10 mM HEPES pH 7.2, 1 mM MgCl₂, 50% (w/v) glycerol, 50 mM NaCl. Nuclear extracts were stored frozen in aliquots at -80°C until use.

Oligonucleotides

Oligonucleotides were synthesized with an Applied Biosystems model 380B synthesizer using standard β -cyanoethyl phosphoramidite chemistry.

Plasmids

Plasmids pMAH3 and pMAH5 contain restriction fragments from the 5' flanking region of the human MUC1 gene cloned into pUC20sr (a derivative of pUC18 with an *RsrII* site inserted into the polylinker between the *HindIII* and *XbaI* sites, kindly provided by Solon Rhode, Eppley Institute, University of Nebraska Medical Center). The insert in plasmid pMAH3 contains 385 bp of MUC1 promoter DNA, positions -790 to -405, shown in Fig. 1B. The insert in plasmid pMAH-5 contains 487 bp of MUC1 promoter DNA, positions -404 to +84, shown in Fig. 1B (GenBank accession number X69118). Fragments of the 5' flanking region of the human CFTR gene (GenBank accession number M55106) were amplified by PCR and cloned into the pCR vector (Invitrogen, San Diego, CA) to produce plasmids p2001 and p2002. The insert in plasmid p2001 contains 313 bp of CFTR promoter DNA (positions -696 to -383, see Fig. 1A). The insert in plasmid p2002 contains 300 bp of CFTR promoter DNA (positions -228 to +71).

Gel mobility-shift assays

Oligonucleotides were radiolabeled using T4 polynucleotide kinase (Gibco BRL, Gaithersburg, MD), γ [³²P]-ATP (New England Nuclear), and reaction conditions as suggested by the suppliers. Unincorporated radiolabel was removed by ethanol precipitation of the oligonucleotide or by Sep-Pak C-18 chromatography. Some experiments included double-stranded oligonucleotides, which, were prepared by annealing and purification from single-stranded oligonucleotides by agarose gel electrophoresis. Radiolabeled probe (5000 cpm) was added to nuclear extracts containing 10 μ g-100 μ g protein in a buffer containing 10 mM Tris-HCl (pH 7.5), 5mM MgCl₂, 2 mM dithiothreitol, 0.1% (w/v) Triton X-100, 0.1 mg/ml bovine serum albumin, 0.1 mg/ml poly dI:dC, and 6% (w/v) glycerol. In some cases, five- to fifty-fold excess unlabeled competitor oligonucleotide was also added to these reactions. Reactions were incubated for 15-30 min at 25°C, loaded directly onto 5% polyacrylamide gels (29:1 acrylamide:bis) that were buffered by 0.5×TBE. Electrophoresis was performed for 500 V-h, the gels were dried, and exposed to X-ray film (Kodak X-OMAT) or a phosphor screen for use on a Molecular Dynamics phosphorimager.

UV cross-linking

Samples treated exactly as described above for gel mobility-shift assays were exposed to ultraviolet light (UV; Stratagene Stratalinker model 2400, 254 nm) for varying times up to 15 min, to determine if radiolabeled oligonucleotides could be covalently cross-linked to specific proteins. These reactions were resolved by electrophoresis on 12.5% SDS polyacrylamide gels

(13) after adding 1/3 volume of 3×sample buffer [125 mM Tris-HCl, pH 6.8; 15% (v/v) β -mercaptoethanol; 9% (w/v) SDS; 30% (w/v) glycerol; 0.4 mg/ml Bromophenol Blue] and heating to 95°C for ten min.

S1 nuclease analysis

Nuclease S1 was obtained from Pharmacia, Piscataway, NJ. For analysis of susceptibility to S1 nuclease, samples of supercoiled p2002 DNA (1.9 μ g) were added to 50 μ l digestion reactions containing 30 mM sodium acetate (pH 4.5), 50 mM sodium chloride, 1 mM zinc chloride, and 5% (v/v) glycerol. In some cases, reactions were supplemented with magnesium chloride (4 mM). DNA samples were incubated on ice for 30 min, followed by the addition of S1 nuclease (75 units). After digestion for 30 min on ice, reactions were terminated by the addition of 10 μ l of a stop solution (0.4 M Tris base, 0.25 M EDTA). Reactions were extracted once with an equal volume of phenol: chloroform:isoamyl alcohol (25:24:1 v/v/v), and precipitated from ethanol. For mapping experiments, portions (0.7 μ g) of the resulting plasmid samples were digested with restriction endonuclease *PstI* or *ScaI* for linearization at a defined site. The resulting DNA was analyzed by electrophoresis in 0.8% (w/v) agarose gels containing ethidium bromide in TAE buffer. DNA fragment lengths were calculated by using an exponential equation derived by least-squares fitting of the relationship between molecular weight and mobility for *HindIII* fragments of phage λ DNA.

RESULTS

Identification of mirror repeat elements

Figure 1A presents 696 bp of sequence from the 5' flanking region of the human CFTR gene. Two regions of sequence that display purine/pyrimidine strand asymmetry and mirror symmetry (purine/pyrimidine mirror repeat element—PMR), and that are highly similar in sequence (see below), are indicated by the boxes designated C-PMR1 and C-PMR2. The positions of these elements in relation to several other putative cis element sequences that have been identified by other laboratories are presented (14-16). Figure 1B shows 805 bp of the sequence of the 5' flanking region of the human MUC1 gene. The MUC1 promoter region was found to contain one PMR sequence element (M-PMR1) with ~85% identity to the PMR elements found in the CFTR promoter (Figure 2, discussed below). Another PMR element composed of distinct sequence was found in the MUC1 promoter (M-PMR2). A third PMR element with >90% mirror repeat sequence is highlighted by the box marked M-PMR3. The sequence of M-PMR3 is very similar to that of C-PMR1 (Figure 2, discussed below). The relative positions of several putative and known cis element sequences that have been identified in the MUC1 promoter in previous publications are also indicated in Figure 1B (17,18).

Figure 2 presents an alignment between four of the PMR elements found in the CFTR and MUC1 promoters. The alignment presented in this figure depicts the mirror character within each of the elements, and highlights sequence similarities between the different elements. The boundaries of the elements and the central points of mirror symmetry presented here have been set so that element length is maximized with minimal sequence imperfections. Different lengths of perfect mirror symmetry exist for some of the elements: The C-PMR1 element contains a central perfect mirror repeat of 19 residues, and the

A.

-696 CCACCCCTGGAGTTCACTACCTAAACCTAACTAATAAGCTTGGTTCTTTCTCCGA -637
 GTGGGAACCTCAAGTAGTGGATTGGAGTTGATATTTCGAACCAAGAAAGAGGCT
 -636 CACGCAAGGAAGCGCTAAGGTAAATGATCAGACCCACTGCCGGAACCTTTTCGGC -577
 GTGCGTTTCCTTCGCGATTCCATTACGTAGTCTGGGTGTGACGGCGCTTGAAGAGCGC
 -576 TCTCTAAGGCTGTATTGTATATACGAAGGCACATTTCTCCCTTTCAAATGCAC -517
 AGAGATTCCGACATAAAATATATGCTTCCGTGTAAAGGAAGGAAAGTTTACGTG
 -516 CTTCGAACGTAACAGGAGCCGCTAGGATCATCGGAAGAGGAGGAGGAGGAGGAGG -457
 GAACGTTTGCATTGCTCGCTGGCTGATCTAGTACCCCTTTCTCTCTCTCTCTCTCTCTC
 -456 CAGGCTCCGGGGAAGCTGTGTGACGCGGCTCTGGGTCTGGCGGACCCCTGACGCGAAGGA -397
 GTCCGAGGCCCTTTCGACACCGCTCGCCAGGACCCAGACCGCTGGGACTGCGCTTCTCT
 -396 GGGTCTAGGAAGCTCTCCGGGAGCCGCTTCTCCGCGGTGGCTTCTTGTCTCCAGC -337
 CCCAGATCTCTCGAGAGGCCCTCGGCAAGAGGGCGGCCACCGAAGAGACAGGAGGTG
 -336 GTTGCAACTGACCTAAAGAGAGCGCGACTGTGCGCCACCTGCGGGATGGCGCTGTG -277
 CACGGTTGACCTGGATTCTCTCCGCGCTGACAGCGGGTGGACGCCCTACCCGACCA
 -276 GCTGGCGGTAAAGACACGACCTGGAAGGAGCGCGCGAGGAGGAGGAGGCTGGAGTC -217
 CGACCGGCATTCTCTGCTGCGACCTTCTCGCGCGCTCCCTCCCTCGACCCCTCAG
 -216 AGAATCGGGAAGGAGGTGCGGGGCGCGAGGAGCGGAAGGAGGAGGAGGAGGAGGAGC -157
 TCTTAGCGCTTTCCTCGACCGCCGCGCTCTCCCTCGCTCTCTCTCTCTCTCTCTCTC
 -156 GGGAGGGTCTGCGGGGGTGCCTAGTGGGTGGAGAAAGCCGCTAGAGCAATTTGGGG -97
 CCTCTCCACGACCGCCCGACCGATCACCCACCTCTTTCGGCGATCTCGTTTAAACCCC
 -96 CCGGACGAGGACGACCTCGGCTTTTAACTGGGCGAGTGAAGCGGGGGAAGAGCAAAA -37
 GGCGTGGTCGCTGTCGAGCGGAGTGAAGCCGCTACTTCCGCGCCCTTCTCGTTTTC
 -36 GAAAGGGTGGTGTGCGGAGTAGGGTGGGTGGGGGAATTGGAAGCAAAATGACATACA +24
 CTTCGCCACCAACGCGCTCATCCCAACCCACCCCTTAACCTTCGGTTTACTGTAGTGT
 +25 CGAGGTGAGAGAAAGGGTTAGCGGCGAGGACCCAGAGTAGTAGG +71
 CGTCAGCTCTCTTTTCCCAACTCGCGCTCGGTGGGTCTCATCATCC

B.

-805 TACTCTCTCCGCGCGGTCGAGCGGCGCCCTCAGCTTGCAGCGGCCAGCCCGCAAGGCT -748
 ATGAGGAGAGGCGGCGGCTCGCGCGGGAGTCGAACGCGCGGTGCGGGGCTTCCGA
 -747 CCGGCTGACCACTAGAGCGGAGGAGGAGCTCTGGCAGTGGTGGAGTGGCAAGGAAG -688
 GGGCCACTGTGATCTCCCGGCTCTCGAGGACCGGTCAACACCTCTCACCGTCTCTTC
 -687 GACCCATAGGTTTCATCGGAGCCAGGTTTACTCCCTTAAGTGAATTTCTTCCCGCACT -628
 GTGATGCCAAGTAGGCTCGGGTCAAGTAGGGAATTCACCTTTAAGAGGGGGTGA
 -627 CTTCTTGGCTTTCTCAAGGAGGAACCCAGGCTGCTGGAAGTCCGCTGGGGCGGGG -568
 GAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG
 -567 ACTGTGGTTCAGGGGAGAGCGGGGTGTGAACGGGACAGGAGCGGTTAGAAGGTGGG -508
 TGACACCAAGTCCCTCTTGGCCCAACACCTTGCCCTGCTCTGCGCAATCTCCCAACC
 -507 GCTATTCGGGAAGTGGTGGGGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG -448
 CGATAAGGCGCTTCAACACCGCCCTCCCTCGGTTTGTATCGTGGATCAGGTGAGTAATA
 -447 CCAGCCCTCTTATTCTCGGCGCTCTGCTCAGTGGACCGGGGAGGCGGGGAAAGTGG -388
 GGTGCGGAGAAATAAGAGCGCTCGGCGAGCAAGTCACTTGGGCGCCCTCCGCGCCCTCAC
 -387 AGTGGAGACCTAGGGGTGGCTTCCGACCTTCTGTACAGGACCTCGACCTAGCTGGC -328
 TCACCTCTGTGATCCCAACCGAGGGCTGGAACGATGTCTGAGCTGGATCGACCG
 -327 TTTGTTCCCACTCCCACTAGTTAGTTGTTGCCCTGAGGCTAAATAGAGCCAGGGGCC -268
 AAACAAGGGGTAGGGGTCAATCAACACCGGAGTCCGATTGATCTCGGGTCCCGGG
 -267 CAAGTCCAGACTCCCTCCCGCTCCCGCGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG -208
 GTTCAAGTCTGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG
 -207 AGGCGAGCTGGAGAACAAACGGGTAGTCAAGGGGTTGAGCGATTAGAGCCCTTGTACCTT -148
 TCGGTGCACTCTTGTGTTGCGCATAGTCCGCAACTCGCTAATCTCGGGAACATGGGA
 -147 ACCAGGAATGTTGGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG -88
 TGGGTCTTACCACTCCCT
 -87 TGTCACTGTCACTGCTGCTGTGCTAGGCGGGCGGGCGGGGAGTGGGGGACCGGT -28
 ACAGTGGACAGTGGACGACGACGAGATCCCGCGCCCGCCCTCACCCCGCTGGCCA
 -27 ATAAAGCGGTAGGCGCTGTGCCCTCCACCTCTCAAGCAGCCAGCGCTGCTGTAATC +33
 TATTTCCCATCCCGGACGCGGAGGTGGAGAGTTCGTGCTGCGGAGCGGACTTAG
 +34 TGTTCTGCCCCCTCCCAACCACTTCAACCAACCATGACACCGGGCACCCAGTCTCTCT -93
 ACAAGACGGGGAGGGGAGGTTAAAGTGGTGGTACTGTGCGCCGTGGGTGAGAGGA

Figure 1. A. Sequence of the 5' flanking region of the human CFTR gene. Two elements that have purine/pyrimidine strand asymmetry and mirror symmetry are enclosed in boxes labeled C-PMR1 and C-PMR2. Several different transcription start sites have been reported for this gene. We have arbitrarily selected one described by Chou *et al.* (11) for presentation here. Several other putative cis elements as described by Yoshimura *et al.* (10), Chou *et al.* (11), and Koh *et al.* (12) are also labeled. **B.** Sequence of the 5' flanking region of the human MUC1 gene. Three elements that have purine/pyrimidine strand asymmetry and sequence mirror symmetry are enclosed in boxes labeled M-PMR1, M-PMR2, and M-PMR3. Several other putative cis-acting elements identified previously by other investigators (13,14) are also labeled.

C-PMR2 element is a perfect 18 residue mirror repeat if the central point of symmetry is drawn between the two guanine residues in the center of the element.

Distinct factors in nuclear extracts bind to single-stranded purine-rich strands or pyrimidine-rich strands of the mirror repeat elements

Oligonucleotides corresponding to several of the PMR elements (Table 1) were synthesized and evaluated for binding by nuclear factors in gel mobility-shift assays. Control oligonucleotides included those with scrambled sequences based on the PMR elements, and other oligonucleotides with distinct sequences and varying amounts of purine/pyrimidine strand asymmetry. Initial studies revealed the presence of distinct factors in nuclear extracts from several cell lines that bind to either the purine-rich strand or the pyrimidine-rich strand of both of the mirror repeat elements from the CFTR promoter region and the similar sequence found in the MUC1 promoter. Interestingly, double-stranded oligonucleotides of the appropriate sequences were not shifted. An experiment demonstrating this observation is shown in Figure 3. These data show that oligonucleotides containing the purine-rich strand of the C-PMR1, C-PMR2, M-PMR1 and M-PMR2 elements are shifted to a similar position (indicated by gel-shift position 1, Figure 3) following incubation with nuclear extracts. A second complex is also seen with the purine-rich strands, with a gel mobility that is slightly greater than that shown as position 2 in Figure 3. Pyrimidine-rich strands were shifted to at least two different positions that were distinct from the shifted positions of the purine-rich strands (indicated by gel-shift positions 2 and 3, Figure 3). Double-stranded oligonucleotides corresponding to these sequences were not shifted. Control oligonucleotides that did not have extensive purine/pyrimidine strand asymmetry also did not show gel-shift activity in these assays. However, control single-stranded oligonucleotides (both purine-rich and pyrimidine-rich) with the same base composition as the PMR elements but scrambled sequences were shifted to the same positions as the those with the conserved PMR sequences. Curiously, the pyrimidine-rich strand (and to a much lesser extent the purine-rich strand) of control oligonucleotide E yielded a distinct complex with a gel mobility-shift position that was slightly greater than that seen designated by position 2. The pyrimidine-rich strands of control oligonucleotides C and D also showed a similar complex with greater mobility.

UV cross-link experiments and nature of the *trans*-acting factor

UV cross-linking experiments showed that single-stranded oligonucleotides derived from all of the purine-rich strand PMR elements could be cross-linked to a molecule of approximately 27 kD when analyzed by SDS-PAGE (Figure 4). The corresponding pyrimidine-rich oligonucleotides (and other control oligonucleotides) did not cross-link to this molecule. Instead, the pyrimidine-rich strands cross-link to at least two distinct proteins of 60 kD and 83 kD (Figure 4). The molecular weights reported here do not include a contribution by the cross-linked oligonucleotides (which have molecular weights of approximately 6000), since previous reports have suggested that cross-linking of small oligonucleotides to proteins does not significantly alter their mobility in SDS-PAGE (19).

A previous report demonstrated that a ribonucleoprotein interacts with an H-DNA element in the *c-myc* promoter (7). We therefore tested the sensitivity of the elements being evaluated

Figure 2. The mirror repeat sequence character within and between PMR elements found in the CFTR and MUC1 promoters. One potential central point of mirror symmetry is indicated by the vertical line that bisects the outward facing arrows, and this is indicated in the sequences at the underlined positions. This presentation emphasizes sequence similarities among some of the elements. Mismatches in perfect mirror repeats are shown in lower case. The numbers in parentheses to the right of the PMR element names indicate number of perfect mirror repeat sequence matches/total sequence length. Note that C-PMR2 has perfect mirror symmetry if the central point is drawn between the two central G residues, and that the central 22 residues of C-PMR1 have perfect mirror symmetry.

Figure 3. Gel mobility-shifts using oligonucleotides based on the PMR elements (see Materials and Methods for experimental conditions). The sequences of the oligonucleotides are described in Table 1. Lanes that contain both purine-rich and pyrimidine-rich strands contained gel-purified double-stranded oligonucleotides that had been previously annealed. Other lanes contained single-stranded oligonucleotides. Single stranded oligonucleotides without nuclear extract do not show a mobility shift (data not shown). Three gel-shift positions are evident: 1, gel mobility-shift position of purine-rich oligonucleotides; 2 and 3 indicate two distinct gel mobility-shift positions obtained with pyrimidine-rich oligonucleotides. The amount of radioactivity in the first two lanes was improperly estimated for this experiment; longer autoradiographic exposure of these lanes confirmed that they were negative.

here to RNase A treatment and found that both gel-mobility-shift and UV cross-link properties were unaffected by prior RNase A digestion of nuclear extracts (data not shown).

Sequence specificity and competition experiments

To determine the nucleotide sequence requirements for interaction between the trans-acting factors and the purine-rich oligonucleotides, we tested the series of oligonucleotides described in Table 1 in competition experiments using both mobility shift and UV cross-link assays. Examples of the results of oligonucleotide competition in mobility shift assays are presented in Figure 5. Similar competition experiments were performed with the UV-cross link assays. All purine-rich oligonucleotides that showed gel-shift mobilities corresponding to position 1 in Figure 3A were cross-linked to the 27 kD protein upon UV irradiation and their patterns of competition in UV cross-link assays were identical to those seen in the mobility-shift assays. A summary of the results of these experiments are presented in Table 2. These results demonstrate that a single nuclear factor binds to the purine-rich strands of the elements M-PMR1, C-PMR1 and C-PMR2 (oligonucleotides A+, B+, and G+ respectively), which share

substantial sequence similarity. Interestingly, the same factor binds to single-stranded oligonucleotides with identical compositions to these but with scrambled sequences (oligonucleotides C+ and D+), and it also binds to the purine-rich strand of a PMR with distinct sequence (M-PMR2, oligonucleotide I+); however, the protein factor does not exhibit a general single-stranded oligonucleotide binding activity. Binding was not observed for several oligonucleotides with high purine content and stretches of contiguous purine sequence with lengths similar to those that are bound. For example, oligonucleotides E+ and F+ did not show the same mobility shifts, they did not UV cross-link to the 27 kD protein, and they did not compete for these activities. Moreover, the factor showed very weak binding with oligonucleotide H (as evaluated by the gel-shift assays), which contains approximately half of the sequence of the C-PMR2 element. The factor does not bind to the complementary pyrimidine-rich strands of any of the oligonucleotides tested, nor does it bind to double-stranded oligonucleotides of the same sequences.

Similar studies were performed with the pyrimidine-rich oligonucleotides. The results of these experiments are complicated by the fact that there appears to be more than one protein species

Figure 4. 12.5% SDS-PAGE of single-stranded oligonucleotides UV cross-linked to nuclear proteins (see Materials and Methods for experimental conditions). The sequences of the oligonucleotides are described in Table 1. Unlabeled competitor was a 50-fold (purine-rich strand) or 500-fold (pyrimidine-rich strand) excess of unlabeled single-stranded oligonucleotide of the same sequence used for the radiolabeled probe. Control experiments in which probe alone was UV cross-linked in a buffer control were negative (see Figure 6 for representative experiments).

involved in oligonucleotide binding, and further experiments are required to adequately characterize these factors.

Based on these results we hypothesize that a 27 kD protein detected in these assays binds to a subset of single-stranded oligonucleotides that are purine rich. The factor does not exhibit a precise sequence binding specificity beyond its preference for purines. A similar 'generic' specificity has been previously described for a protein (termed nuclease-sensitive element protein-1, or NSEP-1) that binds to pyrimidine-rich promoter elements, (6).

Nuclear factor expression in different cell lines

We evaluated nuclear extracts from different cell lines for the presence of the 27 kD protein by using gel-mobility-shift assays (data not shown) and UV cross-linking assays. Results from one UV cross-linking experiment are shown in Figure 6. The two cell lines shown in this figure display differential expression patterns of MUC1 and CFTR (20). HPAF expresses high levels of MUC1 transcripts, but no CFTR. HT-29 expresses moderate levels of CFTR and very low levels of MUC1. The fact that both of these cell lines express the 27 kD protein demonstrates that its expression does not directly correlate with expression patterns of CFTR or MUC1 in these cells.

S1 nuclease sensitivity of PMR elements

Because certain PMR elements show the propensity to adopt local non-B DNA structures characterized by sensitivity to S1 nuclease, we investigated the effect of S1 nuclease on PMR elements identified in the 5' flanking regions of the CFTR and MUC1 genes under conditions of supercoiling and acidic pH. Four recombinant plasmids were created for these experiments.

Table 1. Description and sequence of oligonucleotides used in this study

Oligonucleotide	Description	Oligonucleotide Sequence
A+	(M-PMR1, 90% purine strand)	5' CAAGGAGGAGTGGGGGAAG 3'
A-	(M-PMR1, 90% pyrimidine strand)	3' GTTCCTCCTCACCCCTTC 5'
B+	(C-PMR1, purine strand)	5' GAAGGAGGAGAGGAGGAAG 3'
B-	(C-PMR1, pyrimidine strand)	3' CTCCTCCTCTCCTCCTTC 5'
C+	(Scrambled A+)	5' GAGGTAGGACGAGGGAGA 3'
C-	(Complement to C+)	3' CTCATCCCTGCTCCCTCT 5'
D+	(Scrambled B+)	5' AGGGGAAGGAAGGGGAGAA 3'
D-	(Complement to D+)	3' TCCCTTCTCTCCCTCTT 5'
E+	(Control, 61% purine)	5' AGGGAGTAAACCTGGGCTCCGA 3'
E-	(Control, 61% pyrimidine)	3' TCCCTCATTTGGACCCGAGGCT 5'
F+	(Control, 75% purine)	5' GGCAAGGAAGGACCT 3'
F-	(Control, 75% pyrimidine)	3' CCGTTCCTTCTGGGA 5'
G+	(C-PMR2, purine strand)	5' AAAAGGAGGAGGAGGAGGAAG 3'
G-	(C-PMR2, pyrimidine strand)	3' TTTTCTCCTCTCTCTCTCC 5'
H+	(Partial G+ purine strand)	5' AGGAGGAGGAAG 3'
H-	(Partial G- pyrimidine strand)	3' TCCTCTCTTC 5'
I+	(M-PMR2, purine strand)	5' GGGGAGGGGGAGGGG 3'
I-	(M-PMR2, pyrimidine strand)	3' CCCCTCCCCCTCCCC 5'

Fragments of the 5' flanking region of the CFTR gene were amplified by PCR and cloned into the pCR vector (Invitrogen, San Diego, CA) to produce plasmids p2001 and p2002. The insert in plasmid p2001 contains 313 bp of CFTR promoter DNA (positions -696 to -383, see Fig. 1A) including the C-PMR2 element. The insert in plasmid p2002 contains 300 bp of CFTR promoter DNA (positions -228 to +71, see Fig. 1A) including the C-PMR1 element. Fragments of the 5' flanking region of the MUC1 gene were subcloned into plasmid pUC20 to produce plasmids pMAH-3, or into pBluescript(KS+) (Stratagene, La

Figure 5. Competition among purine-rich oligonucleotides for binding to a nuclear factor, analyzed by gel mobility-shift assays (see Materials and Methods for experimental conditions). The sequences of the oligonucleotides are described in Table 1. Unlabeled competitor consisted of a 10-fold molar excess of the indicated single-stranded oligonucleotide.

Table 2. Summary of data using purine oligonucleotides in gel mobility-shift assays, UV cross-linking assays, and excess unlabelled oligonucleotide competition in those assays

Oligonucleotide designation ¹	Gel-shift position 1 ²	UV cross-link to 27 kD protein ³	Oligonucleotides that show competition in gel-shift and cross-link experiments ⁴
A+	+ ⁵	+	A+, B+, C+, D+, G+, (H+) ⁶
B+	+	+	A+, B+, C+, D+, G+, (H+)
C+	+	+	A+, B+, C+, D+, G+, (H+)
D+	+	+	A+, B+, C+, D+, G+, (H+)
E+	—	—	—
F+	—	—	—
G+	+	+	A+, B+, C+, D+, G+, (H+)
H+	+/-	—	—
I+	+	+	A+, B+, C+, D+, G+, (H+)

¹See Table 1 for sequences of the oligonucleotides.
²Position 1 as shown in Figure 3. This table does not include data on gel shifts to positions 2 and 3, Double stranded oligonucleotides did not show gel mobility shifts to any of these positions.
³As seen in Figure 4.
⁴Competition experiments such as those shown in Figure 4 for the cross-linking, and Figure 5 for the gel shifts.
⁵+ is a positive result, — is a negative result.
⁶The parentheses indicate that oligonucleotide H showed weak (incomplete competition in these experiments, similar to that seen in Figure 5).

Figure 6. Expression of the 27 kD protein in cell lines HPAF and HT29 (see Materials and Methods for experimental conditions). 12.5% SDS-PAGE of single-stranded oligonucleotides UV cross-linked to proteins in nuclear extracts from the human tumor cell lines HPAF and HT29. The sequences of the oligonucleotides are described in Table 1. The specificity of the interaction between radiolabeled oligonucleotides A and B and the same 27 kD protein was confirmed by experiments that included 50× unlabeled competitor (A competed with B and vice versa, but F did not compete with either A or B), using extracts from both cell lines (data not shown).

Jolla, CA) to produce plasmid pMAH-5. The insert in plasmid pMAH3 contains 385 bp of MUC1 promoter DNA (positions -790 to -405, see Fig. 1B) including the M-PMR1 element. The insert in plasmid pMAH-5 contains 487 bp of MUC1 promoter DNA (positions -404 to +84, see Fig. 1B) including the M-PMR2 and M-PMR3 elements.

The four plasmids (native superhelical density) containing fragments of the CFTR and MUC1 promoters were individually tested for sensitivity to S1 nuclease digestion. The inserts in two of the plasmids, p2001 and pMAH-3, did not show sensitivity to S1 nuclease (data not shown). This result suggests that neither C-PMR2 nor M-PMR1 adopt structures accessible to S1 nuclease under these experimental conditions.

In contrast, the inserts in plasmids p2002 and pMAH-5 both showed strong sensitivity to digestion by S1 nuclease. Results of an S1 nuclease digestion experiment for plasmid p2002 (containing the C-PMR1 element) are shown in Fig. 7A. Exposure to S1 nuclease results in substantial linearization of this plasmid (Fig. 7A, compare lanes 1 and 5). Subsequent treatment with *Pst*I (which cleaves at a single site in the vector) revealed the pattern seen in lane 6 of Fig. 7A. As indicated in the schematic map, the resulting fragments correspond to partial S1 cleavage of the 3205 bp plasmid p2002 at two distinct sites. A minor cleavage site (A) maps to vector sequences ca. 1502 bp counterclockwise from the *Pst*I site, corresponding to the position of a well-documented cruciform structure that encodes hairpin I of the RNAI transcript of the *colEI* replication origin (21–23). This was the only observed cleavage site when vectors lacking promoter inserts were treated with S1 nuclease (data not shown). Interestingly, a major cleavage site (B) maps to a position within the CFTR promoter insert of plasmid p2002, 1029 bp clockwise from *Pst*I. The resolution of this experiment is sufficient to place the major site of S1 nuclease sensitivity in or near the C-PMR1 element in the CFTR promoter. Subsequent high resolution mapping experiments have confirmed this assignment (manuscript in preparation).

Figure 7. S1 nuclease sensitivity of PMR elements. **A)** Sensitivity of the C-PMR1 element in plasmid p2002. The plasmid was incubated under slightly acidic pH in the absence (lanes 1–4) or presence (lanes 5–8) of S1 nuclease. Certain reactions (lanes 3, 4, 7, 8) were supplemented with additional magnesium chloride. For agarose gel electrophoresis, plasmid samples were either analyzed without further treatment (lanes 1, 3, 5, 7) or linearized by digestion with *Pst*I (lanes 2, 4, 6, 8). Mobilities of supercoiled (SC) and linear (L) plasmid forms are indicated. The mobilities of phage λ *Hind*III fragments are shown (M). A schematic map showing S1-sensitive sites A (at the plasmid replication origin) and B (at the C-PMR1 element) is shown below. Fragments (2176 bp and 1029 bp) generated by cleavage at both site B and the *Pst*I site are indicated by asterisks. **B)** S1 sensitivity of the M-PMR3 element in plasmid pMAH-5. The plasmid was incubated as in panel A, except that linearization (lanes 2, 4, 6, 8) was accomplished by digestion with *Sca*I. Plasmid fragments (2226 bp and 1225 bp) generated by simultaneous S1 nuclease digestion at both sites A and B are indicated by white dots adjacent to lane 7. A schematic map showing S1-sensitive sites A (at the plasmid replication origin) and B (at the M-PMR3 element) is shown below. Fragments (2025 bp and 1426 bp) generated by cleavage at both site B and the *Sca*I site are indicated by asterisks. **C)** Dependence of S1-sensitivity on supercoiling. As an example, the S1-sensitivity of M-PMR3 is shown. S1-sensitivities of supercoiled DNA are shown in lanes 3–4. Lane 5 depicts the insensitivity of linearized pMAH-5 to S1 nuclease digestion.

The data in Fig. 7A also indicate that magnesium ions (4 mM) enhance the overall susceptibility of plasmid p2002 to S1 nuclease digestion (Fig. 7A, compare lanes 5 and 7). One interpretation of this result is that magnesium ions stabilize non-B DNA structures at sites A and/or B.

Results of S1 nuclease digestion of plasmid pMAH-5 (containing the M-PMR2 and M-PMR3 elements) are shown in Fig. 7B. Exposure to S1 nuclease again results in substantial linearization of this plasmid (Fig. 7B, compare lanes 1 and 5). Subsequent treatment with *ScaI* (which recognizes a single site in the vector) revealed the pattern seen in lane 6 of Fig. 7B. As indicated in the schematic map, the resulting fragments correspond to partial S1-cleavage of the 3451 bp plasmid pMAH-5 at two sites. The minor cleavage site (A) maps to vector sequences ca. 801 bp counterclockwise from the *ScaI* site, again corresponding to the cruciform structure in the *colEI* replication origin. A second cleavage site (B) maps to a position within the MUC1 promoter insert of plasmid pMAH5, 1426 bp clockwise from *ScaI*. This low resolution mapping establishes the existence of an S1-sensitive site within the MUC1 insert, mapping in or near the M-PMR3 element (see Fig. 1B). Magnesium ions again are observed to increase the S1-sensitivity of the plasmid (Fig. 7B, compare lanes 5 and 7). Under these conditions, evidence of simultaneous cleavage at both sites A and B can be observed (bands indicated by white dots in lane 7).

To study the dependence of the observed S1-sensitivity of pMAH-5 on plasmid superhelicity, plasmid samples were linearized before or after S1 treatment. The results of such an experiment are shown in Fig. 7C. Lanes 3 and 4 correspond to lanes 7 and 8, respectively, of Fig. 7B, indicating the presence of two S1-sensitive sites, one of which maps to the M-PMR3 insert. In contrast to the S1-sensitivity of supercoiled plasmid DNA, linearized DNA shows no detectable S1-sensitivity (Figure 7C, compare lanes 4 and 5). This result suggests that formation of S1-sensitive structures at sites A and B of pMAH-5 requires superhelical tension.

DISCUSSION

We have employed an endonuclease that detects distortions in the DNA phosphodiester backbone to demonstrate that a site in the human CFTR promoter adopts a non-B form under conditions of negative supercoiling and acidic pH. A similar site is found in the proximal portion of the MUC1 promoter that likewise adopts a non-B form. A variety of non-B DNA structures can account for S1-sensitivity of plasmid DNA. However, both the presence of purine/pyrimidine strand asymmetry in or near the S1 sensitive sites, and the dependence of reactivity on negative supercoiling suggest the involvement of H-DNA (1–4). Definitive characterization of the S1-sensitive structures awaits the results of high resolution chemical modification studies that are currently in progress. The sites of S1 nuclease sensitivity in these promoter regions map to PMR elements that share substantial sequence homology, which we term C-PMR1 and M-PMR3. A similar element (C-PMR2) exists further upstream in the CFTR promoter, and two other similar elements exist in the promoter of the MUC1 gene. Of the 5 elements that have been assayed (C-PMR1, C-PMR2, M-PMR1, M-PMR2, and M-PMR3), only C-PMR1 and M-PMR3 exhibit S1-sensitivity. Perhaps the unique reactivity of the C-PMR1 and M-PMR3 elements reflects their size and sequence. When imperfect mirror repeat sequences are considered outside of the core perfect repeat, these are the largest and most nearly perfect PMR elements in

the two promoters. Certainly, shorter length is one factor that may explain the lack of S1 nuclease sensitivity for the other PMR elements in these assays. The sequence and length of homopurine mirror repeats have previously been shown to be important in determining the stability of H-DNA (24, 25). It is also important to remember that the conditions of these *in vitro* S1 nuclease assays do not accurately reflect *in vivo* conditions. For example, high negative supercoiling levels may transiently exist in promoters during transcription, and protein factors might also stabilize non-B DNA structures.

The fact that the PMR elements occur in similar positions in the proximal promoter regions of these two genes is intriguing. It seems unlikely that two such non-random sequences would appear in these positions by chance, raising obvious questions about their potential structural or functional roles in promoter activity. Denamur and Chehab (26) have recently described a large region (297 bp) with purine/pyrimidine strand asymmetry in the murine CFTR promoter. These authors have shown that this element is also sensitive to S1 nuclease under conditions of acid pH and supercoiling. Moreover, it is interesting that a three base pair deletion (AGG) at position –471 (Figure 1A) on one allele has been found in a non-CF female while screening for CF associated mutations in this gene (Dr Klaus Grade, personal communication). It remains to be established whether this is truly a disease-associated mutation in a CF carrier. The full characterization of this and other naturally occurring mutations in these elements may significantly aid in determining their biological function.

Careful studies of the developmental expression patterns of CFTR and MUC1 have only recently been performed in a number of tissues (11,12). These studies suggest that CFTR and MUC1 are co-expressed in some but not all of these tissues at different developmental stages and in the adult. Thus, it is possible that the PMR elements shared by these promoters play a role in regulating the temporal and spatial expression of these genes. Several other laboratories have employed relatively low resolution deletion analyses to study either the human CFTR or the human MUC1 promoter regions using reporter gene constructs (14–18). The results of these studies showed that there were fluctuations in reporter gene activity in constructs that had broad deletions involving the PMR elements we identify here; however, these studies did not provide detailed information on the influence of specific elements on promoter activity. Denamur and Chehab (26) have presented evidence that the element with purine/pyrimidine strand asymmetry in the murine CFTR promoter region may act as a negative regulator of expression of CFTR in murine cells.

Several potential functions have been proposed for PMR elements. It has been proposed that PMR sites may isomerize to H-DNA and play a structural role in altering the flexibility of DNA to facilitate the simultaneous binding of transcription factors or other DNA binding proteins. There are other possibilities. Some investigators (19) have noted the presence of non-B DNA sites near DNA origins of replication, and it has been suggested that these may play a role in initiating the process of DNA replication. It is also conceivable that these sites play a structural role in the tethering of DNA to other nuclear components.

Regarding the potential stabilization of non-B DNA structures by proteins, it is provocative that we have detected a single-strand-specific DNA binding activity that recognizes purine-rich sequences such as those in the C-PMR and M-PMR elements. It is noteworthy that the 27 kD protein recognizes only single stranded oligonucleotides and not the corresponding double-

stranded oligonucleotides. It is possible that such a single-strand-specific binding activity could influence equilibration between duplex and single-stranded conformers, and thus play a role in stabilizing DNA structures that form a single-stranded structure. Further experiments are in progress to clarify this point.

It is clear that the single-stranded purine binding protein reported here does not exhibit exquisite sequence specificity, since oligonucleotides with scrambled purine-rich sequences were bound equally well by the factor (Table 2). In addition, the data with scrambled oligonucleotides suggest that protein binding does not require mirror symmetry. The fact that a shorter oligonucleotide (oligonucleotide H, Table 2) showed greatly reduced binding to the protein suggests that there may be a length requirement for binding (eg, greater than 12 purine nucleotides). The fact that the nuclear protein bound oligonucleotides A+ and C+ suggests that it can tolerate interruption of a homopurine sequence with one or two pyrimidine residues (Table 2). The lack of binding by oligonucleotides E+ and F+ (Table 2) suggest that protein binding is adversely affected by the presence of four or more pyrimidine bases interrupting the homopurine sequence (25% pyrimidine content). Further studies are required to more completely discern the sequence preferences of this protein.

Although it is clearly distinct in size and binding specificity, the generic specificity of the protein factor described here for purine oligonucleotides is similar, in principle, to that reported previously for a pyrimidine oligonucleotide binding protein termed NSEP-1 (6). Indeed, NSEP-1 shows an apparent molecular weight similar to the 60 kD protein that was cross-linked to the pyrimidine-rich strand oligonucleotides evaluated in this study (see Figure 4), and these proteins may be identical; however, the data presented here clearly shows that the purine-rich and pyrimidine-rich strands of these oligonucleotides are bound by distinct proteins.

Several other single-strand-specific binding proteins have been described in a variety of biological systems (5,27–29). These are all apparently distinct from the purine-binding protein described here on the basis of molecular weight, binding specificity, or other considerations, with one exception. A protein factor termed Pur (19) may be similar or identical to the factor we describe here. Pur was identified in Hela cell extracts as a 28 kD protein (by UV cross-linking) that binds to a specific purine rich sequence element that is found in gene flanking regions and near origins of DNA replication. The binding specificity of the Pur factor has been evaluated and it is found to bind to a limited set of purine-rich oligonucleotides. Methylation analysis revealed points of contact between the Pur protein and bound oligonucleotides at the following guanine bases (bold) GNNNGNNGNGG. Mutations (G→A) of those positions caused a loss of protein binding activity as measured by gel mobility-shift assays. Comparison of the motif defined by these contact points with the sequences of the oligonucleotides we tested shows that the presence of this motif was incompletely correlated with binding by the protein reported here. These results suggest that Pur factor may be distinct from the protein we are evaluating, however further characterization of both proteins is necessary to establish this conclusion.

Currently, the relationship between the oligopurine-specific single-stranded binding activity of the 27 kD nuclear protein and the single-stranded character of the C-PMR1 and the M-PMR3 elements is unknown. The fact that this 27 kD protein is expressed in cell types that both express and do not express CFTR and MUC1 transcripts shows that expression of this protein is not directly correlated with expression of those genes. Moreover,

the lack of exquisite sequence specificity for the binding activity suggests that the protein may play a general role in binding single-stranded, purine-rich DNA sequences. This does not rule out the possibility that this protein plays a role in regulating transcription. Studies to evaluate these and other hypotheses related to the biological significance of the PMR elements defined here and the 27 kD nuclear protein are currently in progress.

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