NM23-H1 and NM23-H2 Repress Transcriptional Activities of Nuclease-hypersensitive Elements in the Platelet-derived Growth Factor-Α Promoter*

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The platelet-derived growth factor (PDGF)-A promoter is regulated by a number of GC-rich regulatory elements that possess non-B-form DNA structures. Screening of a HeLa cDNA expression library with the C-rich strand of a PDGF-A silencer sequence (5′-SHS nuclease-hypersensitive site (SHS)) yielded three cDNA clones encoding NM23-H1, a protein implicated as a suppressor of metastasis in melanoma and breast carcinoma. Recombinant human NM23-H1 cleaved within the 5′-portions of both 5′-SHS strands in either single-stranded or duplex forms. In contrast, NM23-H2, known as a transcriptional activator with a DNA cleavage function, cleaved within the 5′-portions of both strands, revealing that NM23-H1 and NM23-H2 cleave at distinct sites of the 5′-SHS and by different mechanisms. NM23-H1 and NM23-H2 also cleaved within the PDGF-A basal promoter region, again exhibiting preferences for cleavage within the 5′- and 3′-portions of the element, respectively. Transient transfection analyses in HepG2 cells revealed that both NM23-H1 and -H2 repressed transcriptional activity driven by the PDGF-A basal promoter (~82 to +8). Activity of the negative regulatory region (~1853 to ~883), which contains the 5′-SHS, was also inhibited modestly by NM23-H1 and NM23-H2. These studies demonstrate for the first time that NM23-H1 interacts both structurally and functionally with DNA. They also indicate a role for NM23 proteins in repressing transcription of a growth factor oncogene, providing a possible molecular mechanism to explain their metastasis-suppressing effects.

The platelet-derived growth factor (PDGF) family consists of three structurally similar glycoproteins (M, 30,000) that induce proliferation and other growth-related effects in cells of mesenchymal origin. These proteins arise from covalent dimerization of two PDGF subunits, designated the A-chain and B-chain, yielding the heterodimer PDGF-AB and two homodimers, PDGF-AA and PDGF-BB (1, 2). PDGF was implicated in tumorigenesis following the discovery of high sequence homology between the PDGF B-chain (PDGF-B) and the viral oncogene, v-sis (for a review, see Ref. 3). Other studies suggest that both PDGF-A and PDGF-B may also mediate tumor progression to the metastatic phenotype (4, 5).

Transcription of the PDGF-A gene is regulated by several enhancer and silencer elements that are poly-purine/pyrimidine-rich and possess a high degree of single-stranded, non-B DNA structure. Other laboratories (6, 7) as well as our own (8) have demonstrated that a highly GC-rich and nuclease-hypersensitive element (PDGF-A NHE) in the proximal 5′-flanking sequence of the PDGF-A promoter (~82 to ~40) contributes most of the basal transcriptional activity of the gene. This activity is mediated by the binding of members of the Sp1 family of transcription factors and can be induced in vascular endothelial cells by phosphol ester treatment through displacement of Sp1 and Sp3 by the early growth response factor Egr-1 (7, 9) or repressed by binding of the Wilms' tumor gene product WT1 (10). More recently, we localized a GC-rich, nuclease-hypersensitive silencer element in a more 5′-distal region of the PDGF-A promoter (~1488 to ~1388), which we designated the 5′-s1 nuclease-hypersensitive silencer or 5′-SHS, that represses transcription in a variety of normal and transformed cell lines (11). The noncoding (pyrimidine-rich) strand of the 5′-SHS has been shown by Southwestern blot analysis to bind specifically to proteins of M, 97,000, 87,000, 44,000, and 17,000, and binding of these proteins is well correlated with silencer function.

The current study was undertaken to identify these 5′-SHS binding proteins and better characterize their potential transcriptional functions. To that end, a HeLa cell cDNA expression library was screened with a radiolabeled probe derived from the pyrimidine-rich strand of the 5′-SHS. This approach yielded multiple cDNA clones, each encoding the 17-kDa protein, NM23-H1. The nm23-H1 gene belongs to a family of eight human members that have been identified to date (reviewed in Ref. 12), including nm23-H1, nm23-H2, DR-nm23, nm23-H4, and nm23-H5. These genes appear to play critical roles in

NHE, nuclease-hypersensitive element; NRR, negative regulatory region; Pu, pyrimidine; Py, pyrimidine; SHS, 5′ nuclease-hypersensitive site.

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cellular proliferation (13, 14), embryonic development (15, 16), and differentiation (reviewed in Ref. 17) and have also received attention for their potential roles in oncogenesis and tumor metastasis (13, 18–21). All NM23 proteins possess nucleoside diphosphate kinase activities, which direct the transfer of γ-phosphates between nucleoside triphosphates and diphosphates (22). In addition, NM23-H2 was shown to be identical to PuF, a transcriptional activator of the c-myc protooncogene that binds to the nuclease-hypersensitive element of the c-myc promoter (c-myc NHE) (23). NM23-H2/PuF has recently been identified as an activator of several genes required for early myeloid differentiation through recognition of c-myc NHE-like elements (24). However, no DNA binding or transcriptional function has yet been ascribed to NM23-H1.

In the current study, we find that NM23-H1 recognizes both the 5′-SHS silencer and the NHE basal promoter element of the PDGF-A gene as substrates for DNA cleavage, directing its activity to the 3′-portions of the individual strands of these nuclease-hypersensitive elements. Interestingly, NM23-H2 also cleaves both elements, but its activity is directed to their respective 5′-terminal regions. NM23-H1 and NM23-H2 also repress transcriptional activity of the PDGF-A promoter via functional interactions with both elements, suggesting a potentially important role for these proteins in dampening expression of an oncogenic and metastasis-promoting growth factor.

**EXPERIMENTAL PROCEDURES**

**Screening of a HeLa cDNA Expression Library for 5′-SHS Silencer Element-binding Proteins**—A HeLa S3 cDNA expression library constructed in the expression vector Uni-Zap XR (Stratagene) was employed for DNA recognition site cloning. An oligodeoxyribonucleotide corresponding to the noncoding strand of the 5′-SHS silencer element was end-labeled with T4 polynucleotide kinase and γ-[32P]ATP (3000 Ci/mmol), purified by polyacrylamide gel electrophoresis, and used for screening of protein replica filters as described (25). Clones (5 × 106) were screened on 150-mm plates containing 0.7% agarose at a density of 5 × 105 plaque-forming units/plate. Three to four cycles of limiting dilution and screening were conducted to obtain purified plaques containing cDNAs of interest. cDNA inserts were sequenced by the Sequenase procedure (U.S. Biochemicals, Inc.).

**Expression and Purification of Recombinant Human NM23-H1 and NM23-H2—Recombinant NM23-H1 and NM23-H2 were expressed under lacZ induction in the Escherichia coli strain BL21 (DE3) using the vector pET3C (Novogen). Both proteins were purified by ammonium sulfate precipitation (60–90% fraction) and chromatography over anion exchange (DEAE-Sephacel) and hydroxyapatite (HTP, Bio-Rad) columns, as described (23, 26). NM23-H1 bound to the DEAE column (25 mg; 5.4 × 2.4 cm) in a low salt condition and eluted at −100 mM NaCl in a gradient of 0–400 mM NaCl. NM23-H2 did not bind to the DEAE-Sephacel column and was collected in the fall-through fraction. NM23-H1 and NM23-H2 were applied to HTP column (12 ml; 6.5 × 1.5 cm) and eluted with a 60-mL gradient of 10–400 mM potassium phosphate. The peak of NM23-H1 was found at ~150 mEq phosphate, whereas NM23-H2 was eluted much later in the gradient (peak, ~350 mEq). NM23-H1 obtained from the most enriched HTP fraction was employed for further analysis. NM23-H1 and NM23-H2 prepared by this procedure was >95% pure.

**DNA Binding and Cleavage Assays—**DNA cleavage assays and mapping of DNA cleavage sites were performed as described (26), using 10–50 fmol of [32P]-labeled oligodeoxyribonucleotide/15 μl of incubation mixture. To promote DNA cleavage activities of the NM23 proteins, 2.7 mM Mg2+ and 27 mM KCl were included in the incubation buffer. Double-stranded probes were prepared as indicated in the text by either (a) radiolabeling at both 5′-termini after annealing or (b) radiolabeling the strand of interest followed by annealing to the unlabeled opposite strand. Annealing of single-stranded oligodeoxyribonucleotides into double-stranded forms was performed by denaturation of individual strands for 2 min at 94 °C, followed by a gradual decrease to 23 °C over a 4-h time span. Protein-DNA complexes, radiolabeled probes, and cleavage products were resolved by electrophoresis through 6% polyacrylamide gels in 0.5× TBE buffer (45 mM Tris borate, pH 8.3, 1 mM EDTA), whereas mapping of DNA cleavage products was performed on denaturing 16% sequencing gels. Sequencing ladders were generated by established procedures (27). Radiolabeled bands were visualized with a PhosphorImager (Molecular Dynamics). The nucleotide sequences of oligodeoxyribonucleotides employed (only sense strand sequences are shown) were: 5′-SHS, 5′-CTAGAGACCGGGGGGCCTTAGGG-TGGTTG-3′; and PDGF-A HNE, 5′-CTAGAGGCGGGGCCTTGGGGGGG-CTCCAGGGG-GTG-3′.

**Cell Culture, Transient Transfection, and CAT, β-Galactosidase Assays—**HepG2 cells were obtained from the American Type Culture Collection. Construction of plasmids pAC1800, pAC880, pAC261, pAC-F11, pAC-F47, 5′-SHS/pAC261 (8, 11), and pCMV/βgal (28) has been described. To construct the NM23-H1 expression plasmid pL2-H1, a 700-bp EcoRI-KpnI DNA fragment containing the full coding sequence for NM23-H1/PuF was released from Bluescript SK+ (HeLa λ-ZAP clone 25–121) and inserted into the EcoRI site of pL2. The NM23-H2 expression plasmid pL3-H2 was constructed by insertion of a 650-bp EcoRI/HindIII NM23-H2 cDNA fragment from pSPORT (Invitrogen) into the corresponding site in pL3. Expression of both proteins was driven by the cytomegalovirus (CMV) promoter/enhancer. The cells were transfected in 60-mm dishes by the calcium phosphate/DNA co-precipitation method (29). The precipitates were prepared using 2 μg of promoter/CAT reporter plasmid, 4 μg of NM23 expression plasmid, and 2 μg of the β-galactosidase-expressing plasmid pCMVβ. CAT and β-galactosidase assays were performed as described (11).

**RESULTS**

**Screening of a HeLa Cell cDNA Expression Library with the Pyrimidine-rich Strand of the 5′-SHS Yields Three cDNA Clones, Each Encoding nm23-H1—**Our previous studies indicated that the noncoding, pyrimidine-rich strand of the 5′-SHS silencer element (5′-SHS Py) bound to a number of proteins with specificity for single-stranded DNA and that binding by those proteins appeared to mediate silencer function (11). To identify those proteins, the method of DNA recognition site screening described by Singh et al. (25) was undertaken using a HeLa cDNA expression library (λ-Zap II, Stratagene) and a single-stranded, [32P]-labeled oligodeoxyribonucleotide 5′-SHS Py probe. Screening of ~5 × 105 independent plaques yielded three clones that exhibited strong retention of the radiolabeled 5′-SHS Py probe. DNA sequencing and a search of the human genome revealed that all three contained a 456-bp open reading frame encoding NM23-H1 (for a review see Ref. 30). Two of the clones (HL1 and HL7) expressed identical 784-bp cDNA inserts containing a short segment of 5′-untranslated sequence (7 bp) and 321 bp of 3′-untranslated sequence (Fig. 1). The 3′-untranslated segment was 122 bp longer than that previously reported (31), suggesting a novel pattern of processing of the primary NM23-H1 transcript. The third clone (HL2) contained a 660-bp insert that contained shorter 5′-untranslated (2 bp) and 3′-untranslated (189 bp) segments, of which the latter was identical to that described previously (31).

**NM23-H1 Cleaves the 5′-SHS Silencer Sequence, but with a Pattern of Nucleotide Specificity Distinct from NM23-H2—**To study the interactions of NM23-H1 with the 5′-SHS element in detail, the protein was expressed in E. coli and was purified to near homogeneity, using sequential steps of anion exchange and HTP column chromatography (see “Experimental Procedures”). NM23-H1 eluted as a doublet of ~19 kDa from the HTP column with a gradient of increasing potassium phosphate (Fig. 2A). Resolution of NM23-H1 into a doublet is related to the presence of phosphate, because a single 19-kDa band is observed after phosphate removal (data not shown). Using a 32P-labeled, single-stranded 5′-SHS Py strand as the DNA substrate and conditions shown previously for NM23-H2 to promote its DNA cleavage activity and to reduce formation of stable DNA complexes with DNA (see “Experimental Procedures”; Ref. 26), strong cleaving activity was demonstrated in NM23-H1-containing fractions (Fig. 2B). The peak in DNA cleaving activity co-eluted precisely with NM23-H1 protein at ~150 mEq of the phosphate gradient, strongly suggesting that NM23-H1 was indeed the active species.

The HTP-purified preparation of NM23-H1 was next ana-
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Fig. 1. cDNA inserts obtained by DNA recognition site screening of a HeLa expression library with a single-stranded oligonucleotide corresponding to the Py-rich strand of the PDGF-A gene silencer, 5′-SHS. Represented are NM23-H1 cDNA inserts isolated from clones HL1 and HL7 (top) and clone HL2 (bottom). The numbering system is based on the assignment of +1 to the A residue of the translation start codon, as determined previously (31). 5′- and 3′-untranslated sequences (UT) are shaded, with the open reading frame (ORF) left open.

Fig. 2. NM23-H1 co-elutes with DNA cleaving activity during hydroxyapatite column chromatography. 1-ml fractions were collected, individually concentrated 40–50-fold by Centricon ultrafiltration (Amicon), and analyzed by SDS-PAGE (A) and EMSA (B). Shown are the results obtained with individual fractions in the vicinity of the NM23-H1 peak (lane 4), which occurred at approximately 150 mM of the phosphate gradient. Both SDS-PAGE and EMSA analyses were conducted on 0.04% aliquots of each concentrated fraction (equivalent to ~2 μg of NM23-H1 in B, lane 4). The proteins resolved by SDS-PAGE were visualized by Coomassie staining. For EMSA, NM23-H1 was incubated with single-stranded 32P-labeled 5′-SHS Py probe in the presence of 2.7 mM Mg2+ and reduced ionic strength (27 mM KCl), conditions previously shown to promote DNA cleaving activity of NM23-H2 (26). Mobilities of cleaved DNA species are identified within the bracket.

also evaluated. NM23-H1 cleaved both the single-stranded 5′-SHS Pu and 5′-SHS Py DNAs in a dose-dependent manner (Fig. 3A, lanes 1–12). Increasing amounts of NM23-H1 protein (0.1–2.5 μg) yielded bands of progressively faster electrophoretic mobility from both individual 5′-SHS strands, suggesting a progressive nuclelease activity. The ability of NM23-H1 to cleave such distinct single-stranded DNA sequences (Pu-rich versus Py-rich) indicated recognition of structural features distinct from the primary nucleotide sequence per se. Co-incubation of NM23-H1 with double-stranded 5′-SHS that was radiolabeled at both 5′-termini yielded a pattern of bands similar to those obtained from the individual strands alone (lanes 13–18), suggesting that the signals for cleavage were the same in either single-stranded or duplex DNA forms.

Based on the previously characterized ability of NM23-H2 to cleave the c-myc NHE sequence (26, 32), we next examined the extent to which NM23-H2 could cleave the structurally similar 5′-SHS sequence. Indeed, NM23-H2 cleaved the 5′-SHS sequence in both its single-stranded and double-stranded forms (Fig. 3B). Most of the 5′-SHS Py strand was converted into a single fragment with high mobility, although small amounts of three fragments of lesser mobility were also observed (lanes 1–6). Co-incubation of increasing amounts of NM23-H2 with the 5′-SHS Pu strand yielded increasing levels of one rapidly migrating species and lesser amounts of two others (lanes 7–12). The pattern of cleavage obtained with a 5′-SHS double-stranded DNA substrate was consistent with that seen with the single-stranded 5′-SHS substrates (lanes 13–18). NM23-H2 appeared to be somewhat more potent than NM23-H1 under the experimental conditions employed, with 5′-SHS cleaving activity appearing at concentrations as low as 100 ng/reaction as compared with 250–500 ng with NM23-H1. Interestingly, NM23-H2 yielded a small number of DNA fragments that in general were not converted to progressively smaller sizes with increasing NM23-H2 concentration in the manner seen with NM23-H1. Cleavage of single- and double-stranded forms of the 5′-SHS by NM23-H1 and NM23-H2 were of relatively high affinity, because cleavage of each was inhibited with low concentrations of unlabeled, homologous DNAs (IC50 = 0.5–5 nM; data not shown).

Mapping of NM23-catalyzed Cleavage Sites in the 5′-SHS and NHE Sequences—Although non-denaturing polyacrylamide gels proved useful in the analysis of NM23-generated cleavage products, they did not provide the resolution necessary to localize specific sites of cleavage. Thus, we next analyzed the 5′-SHS cleavage products on denaturing sequencing gels, restricting our focus to the duplex form. To obtain an independent analysis of the individual strands within the 5′-SHS duplex, substrates were prepared for which either the Py or the Pu strand was selectively radiolabeled (see “Experimental Procedures”). In addition, we extended our analyses to the

- **SDS-PAGE:**
  - Lane 1: Control
  - Lane 2: 100 ng NM23-H1
  - Lane 3: 250 ng NM23-H1
  - Lane 4: 500 ng NM23-H1
  - Lane 5: 1 μg NM23-H1
  - Lane 6: 2 μg NM23-H1
  - Lane 7: 5 μg NM23-H1
  - Lane 8: 10 μg NM23-H1
  - Lane 9: 20 μg NM23-H1
  - Lane 10: 50 μg NM23-H1

- **EMSA:**
  - DNA cleaved
  - DNA intact

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**Fig. 1 Diagram:**
- HL1, HL7
- ORF: ATG (start) and TGA (stop)
- Poly A: 456, 646, 777

**Fig. 2 Diagram:**
- A. SDS-PAGE
  - Lane 1: 1 molar fraction
  - Lane 2: 2 molar fraction
  - Lane 3: 3 molar fraction
  - Lane 4: 4 molar fraction
  - Lane 5: 5 molar fraction
  - Lane 6: 6 molar fraction
  - Lane 7: 7 molar fraction
  - Lane 8: 8 molar fraction
  - Lane 9: 9 molar fraction
  - Lane 10: 10 molar fraction

- B. EMSA
  - DNA cleaved
  - DNA intact
  - 150 mM PO4

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**Table:**
- NM23-H1 cDNA inserts
- ORF (open reading frame)
- Poly A

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**Legend:**
- **SDS-PAGE:**
  - Lanes 1-10: Increasing amounts of NM23-H1
  - Lane 1: Control
  - Lane 2: 100 ng NM23-H1
  - Lane 3: 250 ng NM23-H1
  - Lane 4: 500 ng NM23-H1
  - Lane 5: 1 μg NM23-H1
  - Lane 6: 2 μg NM23-H1
  - Lane 7: 5 μg NM23-H1
  - Lane 8: 10 μg NM23-H1
  - Lane 9: 20 μg NM23-H1
  - Lane 10: 50 μg NM23-H1

- **EMSA:**
  - DNA cleaved
  - DNA intact
  - 150 mM PO4
cleavage site was mapped to 9 nucleotides from the 5' terminus of the Pu strand (lane 6) but again with minor amounts of cleavage in the 3'-region. Interestingly, the cleavage sites mapped for NM23-H1 and NM23-H2 were not localized to the CCCCCTCCCC motif shared by the 5'-SHS, c-myc NHE, and the PDGF-A NHE. The observation that NM23-H2 cleaved the Py and Pu strands at similar distances from the 5'-terminus but within very different sequence contexts strongly suggests that cleavage of the 5'-SHS by NM23-H2 is position-dependent rather than strictly sequence-specific.

**Cleavage of the PDGF-A NHE**—The patterns of NHE cleavage by NM23-H1 were quite similar to those observed with the 5'-SHS sequence, with cleavage directed to the 3'-portions of both strands. The Py strand appeared to be a better cleavage substrate than the Pu strand, however, being characterized by a more extensive array of 3'-truncated products (lanes 8 and 11). NM23-H2 produced a single Py strand fragment resulting from cleavage at a site 9 nucleotides from the 5'-terminus (lane 9). The Pu strand was also a poorer substrate for NM23-H2 than the Py strand, although minor amounts of cleavage products were observed (lane 12).

Overexpression of either NM23-H1 or NM23-H2 repressed each of the PDGF-A promoter constructs studied (Fig. 5A). These repressive effects were selective for the PDGF-A promoter, with little or no effect seen on a co-transfected β-galactosidase reporter plasmid (pCMV-βgal, data not shown). Although the inhibition obtained with NM23-H1 and NM23-H2 appeared to repress pAC1853 more strongly (33 and 26% of the activity of pAC883, from which the NRR has been deleted (Fig. 5A). These trends were consistent with previous observations of NRR activity in BSC-1 (African green monkey epithelial cells; Ref. 8) and HeLa (6) cell lines. Also, as seen previously (11), relocation of the 33-bp 5'-SHS silencer sequence to a site just upstream of the basal A-chain promoter (−261 to +8) reduced CAT activity to 55% of that seen with the basal promoter alone.

Overexpression of either NM23-H1 or NM23-H2 repressed each of the PDGF-A promoter constructs studied (Fig. 5A). These repressive effects were selective for the PDGF-A promoter, with little or no effect seen on a co-transfected β-galactosidase reporter plasmid (pCMV-βgal, data not shown). Although the inhibition obtained with NM23-H1 and NM23-H2 appeared to repress pAC1853 more strongly (33 and 26% of the activity of pAC1853), suggesting that additional repressive activity might be directed to the NRR. The presence of the 5'-SHS silencer (pAC261/5'-SHS) did not result in additional repression, possibly because of relocation of this element from its natural upstream location to a position much closer to the basal promoter.

The previous localization of transcriptional activity of pAC261 to the −82 to −40 region (8, 33), taken together with the current demonstration of efficient cleavage of this region in vitro by NM23-H1 and NM23-H2, strongly suggested that the repressive effects of NM23-H1 and NM23-H2 on pAC261 were directed to this element. To address this question more directly, a plasmid (pAC-F11) bearing the −82 to +8 promoter fragment that contains only the NHE and TATA motifs of the PDGF-A NHE located between −82 and −42, which has been shown to be critical for basal transcriptional activity. The PDGF-A NHE was considered a likely target of NM23 because of its considerable paranemic character (i.e. hypersensitivity to nuclease and chemical modification) and its high degree of nucleotide sequence identity with both the 5'-SHS silencer and the c-myc NHE, most notably the perfect conservation of a GGGGAAGGGG motif at its 3'-terminus.

**NM23-catalyzed Cleavage of the 5'-SHS silencer**—As expected from the nondenaturing gel analyses (Fig. 3), NM23-H1 and NM23-H2 cleaved both the Pu-rich and Py-rich strands of the duplex 5'-SHS sequence, but the cleavage patterns obtained with the two proteins were quite different (Fig. 4A; results summarized in Fig. 4B). NM23-H1 generated an array of Pu strand fragments (lane 2), with cleavage occurring predominantly at two sites located 4 and 5 nucleotides from the 3'-terminus. Cleavage of the Pu strand by NM23-H1 was similar to that of the Py strand, characterized by multiple fragments with varying lengths of 3' truncation (lane 5). In contrast, NM23-H2 produced a more limited set of fragments resulting from cleavage within the 5'-portions of both 5'-SHS strands (Fig. 4A, lanes 3 and 6). The Py strand was cleaved predominantly between nucleotides located 7–11 residues from the 5'-terminus (lane 3), although minor cleavage sites were detected within the 3'-terminal portion. A single predominant
basal promoter was analyzed. Both NM23-H1 and NM23-H2 reduced CAT activities of F11 by ~50% (Fig. 5B), localizing the inhibitory effects to within the minimal ~82 to ~8 region of the PDGF-A promoter. Co-expression of NM23-H1 and NM23-H2 consistently resulted in less CAT reporter gene repression than was seen with either protein alone; however, this apparent repressive effect was cancelled by a concomitant inhibition of the transfection efficiency control plasmid, pCMV-βgal (data not shown), presumably because of a cooperative transcriptional effect of NM23-H1 and NM23-H2 on the CMV promoter.

**Fig. 4.** Mapping of NM23-induced cleavage sites within the 5'-SHS and NHE sequences of the PDGF-A gene *in vitro*. A, analysis of NM23-generated DNA fragments by denaturing polyacrylamide gel electrophoresis. Duplex oligodeoxyribonucleotides corresponding to the 5'-SHS silencer element (lanes 1–6) or the NHE basal promoter element (lanes 7–12) of the PDGF-A gene were end-labeled selectively with 32P on either the Pu-rich or Py-rich strand prior to incubation with 1 μg of NM23-H1 or NM23-H2. Also included in the figure were the individual radiolabeled DNAs with no NM23 treatment (lanes 1, 4, 7, and 10). Shown adjacent to each lane are nucleotide sequences corresponding to sequencing ladders generated by the Maxam and Gilbert procedure (not shown and Ref. 27). B, summary of DNA cleavage data shown in A. Sites of NM23-H1-induced cleavage are summarized in the top half of the panel, and NM23-H2 cleavage sites are in the bottom half. Cleavage sites are identified using arrows, with arrow size representing the relative intensity of cleavage.
CAT activity of a minimal −42 to +8 promoter fragment was below the limit of detection (data not shown), consistent with previous studies (8).

DISCUSSION

Unwound or non-B-form regions of DNA, termed paranemic structures by Watson and Crick (34), are often associated with both enhancement and repression of transcription in eukaryotic genes (35). Our studies have demonstrated that NM23-H1 (H1) interacts with two such structures in the PDGF-A gene, the 5′-SHS silencer and the NHE basal promoter element, recognizing them as binding/cleavage substrates and repressing their transcriptional activity in cultured cells. These sequences can be added to a rapidly growing list of elements shown to be targets of NM23 proteins, including the NHE of the c-myc gene (23), as well as elements located within five different genes involved in myeloid-specific differentiation (myeloperoxidase, CD11b, CD11c, CD54, and CCR5) (24).

Although considerable nucleotide sequence identity is seen among the NM23 target sequences identified to date (e.g. the GGCGAAGGGG motif found in the c-myc NHE, and the NHE and 5′-SHS of PDGF-A), a clear consensus sequence for DNA cleavage is not apparent. However, evidence of sequence selectivity has been observed; for example, NM23-H2 binds a 104-bp fragment of the c-myc NHE better than 50- or 34-bp duplex oligonucleotides (24). Furthermore, methylation interference footprinting has revealed specific contacts between NM23-H2 and the repeated palindromic sequence GGCTGGG of the c-myc NHE (36, 37). On the other hand, NM23-H2 binds similarly to both the Pu-rich and Py-rich strands of the c-myc NHE, as well as to single- and double-stranded DNA forms (24). Furthermore, cleavage of the PDGF-A NHE and 5′-SHS appears to be determined by distance from the 3′-terminus (H1) or 5′-terminus (H2) rather than by specific nucleotide sequence. What do NM23 proteins recognize as the signal for cleavage in DNA, if not a strict nucleotide sequence per se?

NM23 target sequences were originally identified by virtue of their hypersensitivity to nucleases, suggesting that these proteins may recognize paranemic DNA structures. Consistent with this notion is the efficient binding and cleavage of single-stranded DNA substrates by NM23-H1 and NM23-H2 (Fig. 3 and Ref. 38).

The repressive effects of NM23-H1 and NM23-H2 upon PDGF-A transcriptional elements contrast with the enhancement of c-myc NHE and myeloid differentiation gene activities by NM23-H2 (39). The repressive activities observed in the current study are not exceptions to a general rule, however, because NM23-H1 and NM23-H2 also inhibit the SV40 early promoter/enhancer and the Rous sarcoma virus 3′-long terminal repeat in the HepG2 cell line (data not shown). The abilities of these NM23 proteins to both repress and enhance transcription, depending on cell type and promoter context, would seem to indicate an accessory function rather than strictly defined on/off switches.

The NM23-induced repression observed in the current study was unlikely to be nonspecific, because the cytomegalovirus promoter used for constitutive β-galactosidase expression was unaffected by either NM23-H1 or NM23-H2 overexpression. The specificity of this activity is further indicated by more recent observations that a DNA binding-deficient form of myc (Arg34 to Ala) (59) exhibits impaired repression of PDGF-A regulatory elements. Moreover, a relationship between the DNA cleaving and transcription-regulating activities of NM23-H1 and NM23-H2 has been demonstrated through a dominant-negative effect of DNA cleavage-deficient NM23-H1 and NM23-H2 on the transcription of myeloid differentiation genes (24). In this regard, an important goal of future studies will be to better understand the relationship between DNA cleavage and transcriptional repression. These proteins may play a role in recognizing and converting paranemic structures into relaxed B-form conformations, structural transitions that

2 D. Ma, et al., unpublished observations.
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could have profound implications for the types of multiprotein complexes that would assemble at such locations. Our studies suggest that such transitions may be favorable for the recruitment of repressor complexes, possibly containing histone deacetylase activities. An elegant example of how structural transitions in DNA can affect transcriptional activity is illustrated by a 30-bp nucleosome-hypersensitive element in the vascular smooth muscle α-actin gene (40). The transcriptional activity of this element is repressive in non-α-actin-expressing cell types (e.g., fibroblasts) but enhancing in vascular smooth muscle cells, and this switch in activity is closely correlated with DNA conformation. Two single-stranded DNA-binding proteins termed VACssBF-1 and VACssBF-2 repress transcription by stabilizing a local single-stranded conformation, whereas activation in vascular smooth muscle cells is mediated by the double-stranded DNA binding protein TEF-1. The contributions of NM23 proteins to DNA conformation and transcriptional activity should be facilitated through the study of well-characterized elements as the PDGF-A basal promoter region, whose DNA structure and individual protein components (e.g., the SP-1 family, WT-1, and EGR-1) have been well characterized.

Although NM23-H1 and NM23-H2 overexpression resulted in a significant loss in activities of the NHE and upstream negative regulatory region (∼1853 to ∼881) of the PDGF-A gene, they did not affect 5′-SHS activity when this silencer was relocated much closer to the basal promoter from its natural upstream location (approximately ∼1400). If NM23s do indeed play an important role in determining DNA conformation, it seems plausible that such an artificial relocation of the 5′-SHS could affect its dependence on these proteins for proper orientation with downstream positive regulatory elements. In fact, the 5′-SHS element, like many other silencers (for a review, see Ref. 41), does exhibit some orientation dependence, functioning in both orientations when placed upstream of the promoter but only in one orientation when placed downstream of a CAT reporter gene (11).

The differences observed in the cleavage characteristics of NM23-H1 and NM23-H2 suggest they may have complementary functions in transcriptional regulation. Consistent with that notion, co-expression of NM23-H1 and NM23-H2 inhibited CAT reporter activity more than either protein alone, although the effect could not be verified because of concomitant effects on the CMV-driven β-galactosidase plasmid used for a transfection efficiency control. The repressive effects of NM23 overexpression were modest, possibly because of the high and potentially saturating NM23-H2 protein levels in the HepG2 cell line studied (data not shown). Nevertheless, even modest effects on individual elements could have a significant cumulative impact on PDGF-A transcription, considering the multiplicity of NHEs identified thus far in this gene. Moreover, small changes in PDGF expression resulting from alterations in NM23 status might potentially have profound implications for cellular growth and malignancy.

The DNA cleaving activity of NM23-H2 was recently suggested to be mediated by a glycosylase/lyase-like mechanism (32), a feature of base excision enzymes that function in DNA repair. The relationship of NM23 to both transcriptional regulation and DNA repair represents an interesting parallel with BRCA1, the breast- and ovarian-specific tumor suppressor protein to which both transcriptional and repair functions have also been recently ascribed (for a review, see Ref. 42). A repair function for BRCA1 is strongly suggested by its enrichment at sites of DNA repair and its direct association with such DNA repair factors as Rad51, Rad50, and proliferating cell nuclear antigen (43, 44), whereas a role in transcriptional regulation is strongly suggested by its association with the RNA polymerase II holoenzyme (45) and histone deacetylase complex (46). These functional parallels between the structurally distinct BRCA1 and NM23 proteins are intriguing in light of their shared roles as suppressors of malignant progression, and further investigation may reveal the relative importance of the transcriptional and repair activities to their suppressor functions.

The inhibitory interactions of NM23-H1 and NM23-H2 with the basal promoter and upstream silencer regions of the PDGF-A gene shown in the current study suggest a molecular mechanism that underlies the metastasis-suppressing function of NM23. The PDGFs were the first secreted polypeptides to be characterized as oncogenic (47, 48), with PDGF overexpression being a hallmark of many different cancers (for reviews, see Refs. 3 and 49). Moreover, PDGF has been directly linked to neoplastic growth properties in sarcoma and astrocytoma (50–52), as well as progression to the metastatic phenotype (53). Of particular relevance are the autocrine and paracrine actions of PDGF-A in melanoma (54–56), in which the metastasis-suppressing activity of NM23-H1 was first observed (57, 58). Our observations suggest the possibility that NM23 proteins may contribute to repression of PDGF-A and perhaps other growth-related genes. Conversely, loss of NM23 expression could lead to derepression of growth-related genes, an event that could favor progression to increasingly malignant phenotypes, including metastasis. In this regard, we have observed that PDGF-A silencer activity is compromised in two human metastatic cell lines (WRO82, thyroid carcinoma; NKM-1, neuroblastoma) that express very low levels of NM23-H1 and NM23-H2 (data not shown). Additional studies will be directed to experimental testing of this molecular model and whether it can be extended to other growth-promoting genes whose derepression could also contribute to malignant progression.

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