Human c-*myc* Transcription Factor PuF Identified as nm23-H2 Nucleoside Diphosphate Kinase, a Candidate Suppressor of Tumor Metastasis

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A human gene encoding the c-*myc* purine-binding transcription factor PuF was identified by screening of a cervical carcinoma cell complementary DNA library with a DNA fragment containing PuF binding sites. The 17-kilodalton bacterially produced PuF was shown to have biological activity and properties similar to that of human PuF. DNA sequence analysis of recombinant PuF revealed perfect identity with the human *nm23-H2* nucleoside diphosphate kinase gene, a potential negative regulator of cancer metastasis. These results provide a link between *nm23* and the c-*myc* oncogene and suggest that the nm23 protein can function in vitro in the transcriptional regulation of c-*myc* expression.

Myc, the protein product of the c-*myc* proto-oncogene, functions in cellular proliferation, differentiation, and tumor formation, presumably by modulating the expression of genes involved in these processes. Expression of the c-*myc* gene is subject to complicated regulatory mechanisms mediated by positive and negative cis-acting elements and a number of trans-acting factors (*1*, *2*). A dozen DNA binding proteins have been identified for the human c-*myc* gene (*2*), although only one, PuF, is known to regulate c-*myc* transcription in vitro (*2*, *3*).

PuF was identified in our laboratory as a partially purified HeLa cell (human cervical carcinoma) factor that binds to a nuclease-hypersensitive element (NHE) at positions −142 to −115 of the human c-*myc* P1 promoter and is necessary for efficient P1 and P2 transcription initiation in vitro (*3*, *4*). The c-*myc* NHE is a homopurine-homopyrimidine sequence with potential to form DNA secondary structures (*5*). That a site-specific oligonucleotide that forms a triplex with NHE inhibited c-*myc* transcription in vitro (*4*, *6*) and in vivo (*7*) suggests that a conformational alteration at NHE interferes with PuF binding. Thus, PuF may regulate human c-*myc* activity by interaction with structural elements of NHE.

To clone PuF, we screened a HeLa cell plasmid expression library (cDNA library) (*8*) with a c-*myc* NHE promoter fragment (*3*). Positive colonies from the initial screening (*8*) were subjected to four rounds of colony purification from which a single clone, c19, was selected for further analysis. DNA sequencing of c19 (*9*) showed that the cDNA contains 675 nucleotides and an open reading frame (ORF) capable of encoding a 152–amino acid polypeptide with a molecular mass of ~17 kD. A search of GenBank DNA sequences showed that the 0.7-kb cDNA has 99% homology to the human *nm23-H2* gene (*10*). Aside from partial homology in the 5′ untranslated leader region, both the protein coding and 3′ untranslated sequences of c19 (*11*) and *nm23-H2* are identical.

Complementary DNA from the c19pSPORT plasmid was subcloned into the T7 expression vector PET3c and subsequently expressed in bacteria to produce soluble protein (*12*). SDS–polyacrylamide gel electrophoresis (SDS-PAGE) of the c19 nm23-H2 protein after purification showed that the protein has a mass of ~17 kD and was >95% homogeneous (Fig. 1A, lane 7). A ~17-kD protein was also present in the partially purified HeLa cell fraction D.4, which was used previously as the source of PuF (*3*) (Fig. 1A, lane 9).

To establish whether the 17-kD polypeptide encoded by the c19 ORF and presumed to be recombinant PuF (rPuF), and HeLa cell PuF (hPuF) were identical, we raised antibodies against the product of the cloned gene. The resulting antiserum reacted with a polypeptide of ~17 kD in the purified bacterial fraction, as well as with a ~17-kD protein in the human D.4 fraction (Fig. 1B, lanes 3 and 4). Preimmune serum did not react with either polypeptide (Fig. 1B, lanes 1 and 2). These results, combined with the DNA sequence data, indicate that the c19 nm23-H2 protein is present in the human D.4 chromatographic fraction containing PuF activity.

To investigate the DNA binding activity of the recombinant c19 nm23-H2 protein, we carried out electrophoretic mobility-shift assays (EMSAs) with NHE promoter fragments that contain the PuF recognition sequence GGGTGGG (*3*, *8*). Human PuF (D.4) displayed a complicated DNA binding pattern on addition of increasing amounts of protein, forming three major complexes at the titration end point (A, B, and C) (Fig. 2A, lanes 2 to 4). DNA binding was eliminated when D.4 was immunodepleted with antibody to c19 nm23-H2 (lane 9), suggesting that the c19 nm23-H2 protein was present in all three complexes. EMSAs with the recombinant c19 nm23-H2 protein (HTP fraction) indicated that at the appropriate protein concentration, c19 nm23-H2 binds to the NHE fragment strongly, producing one major shifted complex in the B configuration (Fig. 2A, lane 6). When the immunodepleted D.4 fraction, lacking detectable NHE binding activity, was reconstituted with the c19 nm23-H2 protein, all three shifted complexes were observed (Fig. 2A, lane 10).

Like many DNA binding proteins, both human and rPuF bind DNA nonspecifically and to a degree that is determined by the stringency of the assay (for example, carrier DNA used and Mg^{2+} concentration). Under the conditions used here to detect DNA binding, neither hPuF nor c19 nm23-H2 bound significantly to either a 100–base pair (bp) pBS-polylinker fragment or a mutated NHE fragment lacking PuF binding sites (Fig. 2A, lanes 11 to 16). Addition of immune but not preimmune serum resulted in a supershift in both hPuF and c19 nm23-

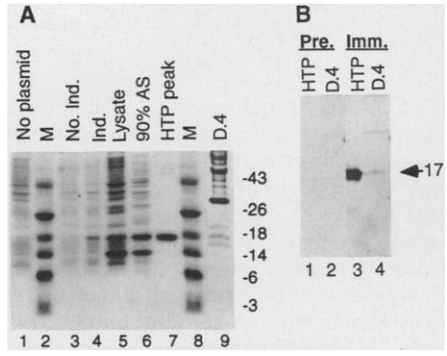

Fig. 1. (**A**) SDS-PAGE of *E. coli*–expressed PuF as visualized by Coomassie blue staining. Samples were boiled in loading buffer and electrophoresed in 5 to 15% SDS-PAGE gels (*23*). Lanes 1, 3, and 4, bacteria without and with p19PET3c before and after induction; lane 5, 5 μl of lysate; lane 6, 10 μl of $NH_4(SO_4)_4$ (AS) fraction; lane 7, 20 μl (1 μg) of HTP peak fraction; and lane 9, 25 μl (19 μg) of D.4. Lanes 2 and 8 have molecular size markers (BRL, prestained) (shown on the right in kilodaltons). The 14-kD protein present in lanes 5 and 6 is lysozyme used in the treatment of cells. (**B**) For immunoblotting, proteins were fractionated by SDS-PAGE, transferred to nitrocellulose membrane, and incubated with a 1:1000 dilution of polyclonal mouse antiserum (Imm.) or preimmune serum (Pre.). Bound antibodies were visualized with horseradish peroxidase–conjugated secondary antibodies (Vector). Lanes 1 and 3 contain 0.5 μg of HTP, and lanes 2 and 4 contain 11 μg of D.4.

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H2–containing complexes (Fig. 2B, lanes 3 and 9). When similar EMSA gels were immunoblotted, antibody bound specifically to the supershifted complex (lanes 6 and 12), indicating that sequence-specific PuF binding activity is recognized by antibody to c19 nm23-H2. These results clearly identify the 17-kD bacterially produced protein encoded by c19 *nm23-H2* as PuF (rPuF) and indicate that hPuF and rPuF have similar DNA binding specificities. Our data also demonstrate that the nm23-H2 polypeptide has DNA binding activity.

In vitro assays were done to test the transcriptional activity of rPuF. When supplemented with an RNA polymerase–containing fraction (CL.25), hPuF (D.4) supported accurate initiation from the c-*myc* promoters P1 and P2 (*3, 4*) (Fig. 3, lane 4). Antibodies against rPuF inhibited hPuF-dependent transcription, whereas preimmune sera had negligible effect (Fig. 3, lanes 4 to 6). Recombinant PuF (HTP), together with polymerase, induced accurate c-*myc* transcription, but as in DNA binding assays, rPuF was less active than hPuF (Fig. 3, lanes 4, 8, and 9). Addition of hPuF-depleted D.4 fraction, which alone showed no activity (lane 7), to rPuF-containing fractions caused a large increase in activity (lane 10). Thus, rPuF is functional, but may require an additional factor (or factors) present in the immunodepleted D.4 fraction to attain maximal transcriptional activity. Whether this factor (or factors) participates in activation or stabilization of rPuF is unknown. These data also demonstrate that the nm23-H2 protein plays a direct role in transcriptional activation of the human c-*myc* gene.

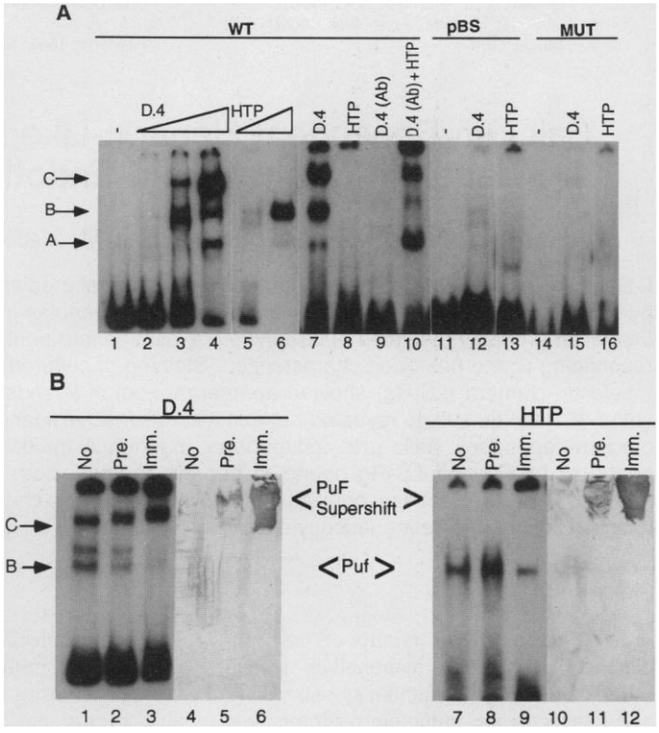

Fig. 2. DNA binding properties of rPuF. (**A**) Reaction volumes of 15 μl were analyzed by EMSAs as described (*3*), except that nonspecific carrier DNA was sonicated salmon sperm (10 ng/μl for D.4 and 2.5 ng/μl for HTP) and HTP was supplemented with BSA (5 mg/ml; Sigma). Specific DNA binding probe (WT) was the 105-bp NHE fragment described (*8*). Nonspecific probes were a 100-bp restriction fragment from the polylinker region of the plasmid pBS (PBS; Stratagene) and an 83-bp mutant fragment of NHE (MUT) lacking PuF binding sequences (*3*). Immunodepletion was carried out by incubation of D.4 with antiserum to c19 nm23-H2 for 1 hour at 0°C and then by removal of immune complexes bound to protein G–agarose (Sigma) by centrifugation. Lanes 1, 11, and 14, probe alone; lane 2, 1.5 μg of D.4; lane 3, 4 μg of D.4; lanes 4, 7, 12, and 15, 8 μg of D.4; lanes 5, 8, and 10, 0.2 μg of HTP; lanes 6, 13, and 16, 2 μg of HTP; and lanes 9 and 10, 8 μg of antibody-depleted D.4. Lanes 1 to 6 and lanes 7 to 16 represent independent experiments. In a control EMSA, antiserum to c19 nm23-H2 did not inhibit DNA binding by E2F binding factor to E2F binding-site oligonucleotide (*24, 25*). (**B**) EMSA supershift and immunoblotting. Protein fractions were incubated on ice for 20 min with 1 μl of a 1:100 dilution of preimmune or immune serum before addition of probe. Immunoblotting was done as described in Fig. 1. Lanes 1 to 6, 8 μg of D.4; lanes 7 to 12, 2 μg of HTP. Preimmune serum produced cross-reacting signals in lanes 5 and 11.

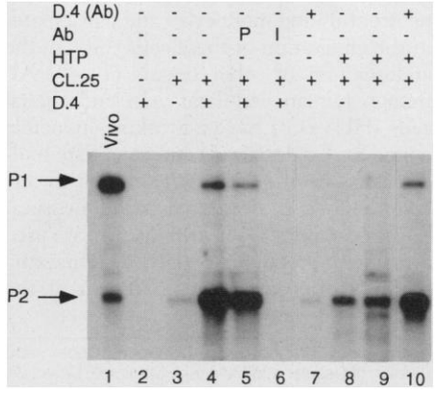

Fig. 3. In vitro transcriptional activity of rPuF. Transcription analysis using RNAse protection was done as described (*3*). Reaction volumes (75 μl) included 0.6 μg of supercoiled c-*myc* template DNA (*3*) and where indicated, 32 μg of CL.25 (RNA polymerase fraction) (*3*). Lanes 2, 4, 5, and 6, 1.5 μg of D.4 (hPuF); lanes 8 and 10, 0.4 μg of HTP (rPuF); and lane 9, 1 μg of HTP (rPuF). Lanes 7 and 10 represent 1.5 μg of rPuF-antibody–treated D.4. D.4 in lanes 5 and 6 was preincubated on ice for 20 min with 1 μl of a 1:100 dilution of preimmune (P) or immune (I) polyclonal antisera. Arrows point to transcripts initiated from the P1 and P2 c-*myc* start sites. Lane 1 shows control HeLa cell P1 and P2 RNA transcribed in vivo (*7*), the ratio of which varies between 0.5:1 to 2:1, depending on the growth state. P1 RNA synthesis relative to P2 is less efficient in vitro (lanes 4, 5, and 10) (*3, 4*).

nm23 is a family of independently regulated genes implicated in the metastatic potential of mammalian cancers (*10, 13*) and in normal tissue development and differentiation (*14*). Mammalian *nm23* genes bear significant homology to the *Drosophila abnormal wing discs* gene *awd*, shown to be essential for normal fly development (*15*). The *nm23-awd* genes encode 17-kD proteins with nucleoside diphosphate kinase (NDK) activity (*15, 16*). Whether the NDK activity of nm23-awd plays a regulatory role in development and metastasis is presently controversial because hypotheses concerning nm23 involvement in GTP-binding protein phosphorylation have recently been disproved (*17*). On the basis of its nuclear localization and potential leucine zipper motif, nm23-H2 was proposed to function as a transcription factor (*10*). Here we present evidence that nm23-H2 is a transcription factor and that, moreover, one of its targets is the c-*myc* gene.

Although no clear correlation has yet been established between overexpression of c-*myc* and tumor metastasis, the inverse relation between c-*myc* expression and cell differentiation is well documented (*2*). In this context, the recent identification of a differentiation inhibiting factor in mouse myeloid leukemia cells as the murine homolog of *nm23-H2* (*18*) suggests that one point of interaction between *nm23* and c-*myc* may occur in a developmental pathway. Data presented here suggest new hypotheses to examine regarding the physiological roles of *nm23* and c-*myc* in the cell.

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8. For cloning of PuF, cDNA was synthesized from HeLa cell, and polyadenylated RNA was size-fractionated and ligated into the Not I–Sal I–restricted plasmid expression vector pSPORT (SuperScript Plasmid System, BRL) and introduced into *Escherichia coli* by electroporation. The resulting cDNA library (L7) contains 2.5×10^5 independent recombinants with an average insert size of ~3 kb. For screening, 1×10^6 cells of the amplified library were plated onto nitrocellulose filters (150 mm, Triton-free, Millipore) laid on agar plates containing ampicillin (100 μg/ml), and incubated overnight at 37°C (*19*). Filters were replicated and transferred to fresh plates containing ampicillin and 1 mM isopropylthio-β-D-galactoside (IPTG) and incubated for 3 hours at 37°C to induce expression of fusion proteins. Colonies were lysed in situ in a chloroform-saturated chamber (*20*) and then transferred to lysis buffer [100 mM tris (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 1.5% bovine serum albumin (BSA), lysozyme (40 μg/ml), and deoxyribonuclease I (2 U/ml)] for 2 hours at room temperature. After being washed in lysis buffer, filters were incubated for 1 hour each in Blotto (*21*) and then in binding buffer [10 mM tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.25% nonfat milk powder (Carnation), and heat-denatured sonicated salmon sperm DNA (5 μg/ml)] containing ^{32}P-labeled 105-bp DNA probe [100 ng/ml (6×10^6 cpm per 150-mm filter)] comprising nucleotides −200 to −96 of the c-*myc* P1 promoter. This probe contains NHE, including the PuF recognition sites GGGTGGG between nucleotides −142 to −115 (*3*). Filters were washed in binding buffer and exposed to X-AR film (Kodak).
9. Plasmids of two independent subclones of c19 were sequenced (*22*) with T7 and T3 primers and Sequenase version 2.0 kit (USB). Computer analysis was done with the GCG (Wisconsin) sequence analysis package.
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11. The nucleotide sequence of PuF cDNA has been deposited with GenBank (accession number L16785).
12. For purification of *E. coli*–expressed PuF, the DNA sequence adjacent to the initiator methionine of c19pSPORT cDNA was mutated by polymerase chain reaction to introduce an Nde I restriction site. The resulting 625-bp fragment of the protein coding sequence and the 3′ untranslated region of c19 were subcloned into the Nde I–Bam HI site of the vector PET3c (Novogen). p19PET3c was transformed into *E. coli* BL21 (DE3), expressed, and the soluble protein purified as follows: Cells from a 1-liter culture harvested 3 hours after IPTG induction were resuspended in 50 ml of 50 mM tris (pH 8)–2 mM EDTA and lysed by sonication and treatment with lysozyme (100 μg/ml). After dialysis into 20 mM tris (pH 7.4), 1 mM EDTA, 1 mM DTT, the 60 to 90% $NH_4(SO_4)_4$ fraction was applied to a hydroxylapatite column (HTP, Bio-Rad), which was developed with a 0 to 300 mM potassium phosphate gradient (*16*). HTP fractions containing c19 nm23-H2 were identified by SDS-PAGE, pooled, concentrated (Centricon), and equilibrated in 0.1 HM buffer [20 mM Hepes (pH 7.9), 5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, 20% glycerol, 0.1 M KCl] (*3*), and portions were frozen at −80°C. D.4 fractions containing hPuF were obtained by sequential chromatography of nuclear extracts on heparin-agarose and DEAE-Sepharose (*3*).
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Calcium-Dependent Heparin-Like Ligands for L-Selectin in Nonlymphoid Endothelial Cells

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L-Selectin is a calcium-dependent mammalian lectin that mediates lymphocyte trafficking by recognizing sialylated ligands on high endothelial venules in lymph nodes. Although L-selectin probably mediates neutrophil extravasation into nonlymphoid tissues, no corresponding ligand has been characterized. Staining of cultured endothelial cells with an L-selectin chimera (LS-Rg) showed an internal pool of ligands. Metabolic labeling with sulfur-35–labeled sulfate revealed heparin lyase–sensitive ligands that bound LS-Rg in a calcium-dependent, sialic acid–independent manner. A fraction of commercial heparin bound to LS-Rg and LS-Rg bound to heparin-agarose, both in a calcium-dependent manner. Thus, L-selectin recognizes endothelial heparin-like chains, which could be physiological ligands mediating leucocyte trafficking.

The selectins are a family of calcium-dependent (C-type) mammalian lectins found on cells of the vascular system (*1*). P- and E-selectin are inducible receptors that recognize certain sialylated fucosylated ligands on leukocytes. They participate in the trafficking of these cells to areas of inflammation and injury (*1*). In contrast, L-selectin is expressed on leukocytes and recognizes specific carbohydrate ligands on endothelial cells. L-Selectin mediates the trafficking of lymphocytes by binding to specific sialylated sulfated ligands on the high endothelial venules (HEVs) of lymph nodes (*2, 3*). L-Selectin is also expressed on neutrophils and monocytes and participates in the emigration of these cells through the endothelium of other organs (*1, 4*). Although human umbilical vein endothelial cells (HUVECs) have a cytokine-inducible ligand for L-selectin (*5*), no candidate molecule has been positively identified in any endothelial cells from nonlymphoid tissues.

To search for L-selectin ligands, we used a chimeric probe (LS-Rg) (*6–8*) consisting of the entire extracellular domain of the L-selectin molecule attached to the Fc domain of an immunoglobulin G2 (IgG2) COOH-terminus. Ligands were not detectable in the postcapillary endothelium of normal rat tissues other than in the lymph nodes (*6–8*). However, this probe detected ligands in cultured calf pulmonary artery endothelial (CPAE) cells. When the cells were fixed under specific conditions (*9*), we observed divalent cation–dependent intracellular staining (Fig. 1). In contrast with previous findings with lymph node HEV (*8*), this staining was not enhanced by mild periodate oxidation (*10*). Cells grown on glass slides instead of plastic slides had reduced staining (*11*). This, together with the intracellular location of the ligands, may account for the previous inability to detect them.

The L-selectin ligands isolated from lymph node HEV are mucin-like and carry sialylated, fucosylated, sulfated, O-linked oligosaccharides that are best labeled with $^{35}SO_4$ (*2, 3*). We therefore metabolically labeled CPAE cells, HUVECs, and AGO8132 cells (a fetal bovine aortic endothelial cell line) with $^{35}SO_4$ and looked for radioactive macromolecules recognized by LS-Rg. Similar to $^{35}SO_4$-labeled material from lymph nodes (*2, 3*), a portion of the radioactivity from each cell type bound to

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