Nm23/PuF Does Not Directly Stimulate Transcription through the
CT Element in Vivo*

(Received for publication, November 13, 1996, and in revised form, May 19, 1997)

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The nm23 gene family was identified on the basis of its reduced expression in highly metastatic murine melanoma cell lines (1). In human cells, three members of the family, nm23-H1 (2), nm23-H2 (3), and DR-nm23 (4) have been identified. nm23 expression has been linked with suppression of tumor metastasis, differentiation, apoptosis, proliferation, DNA mutation rate, and other phenotypes (reviewed in Ref. 5). Considerable investigation has focused on the biochemical mechanism of nm23 in mediating these diverse phenotypes. Although only 17 kDa in size, a number of biochemical functions have been ascribed to Nm23, including a nucleoside diphosphate kinase (NDPK)† activity (6–8), serine phosphorylation (9–12), a histidine protein kinase activity (13), and a transcriptional stimulatory activity (14–17). Interactions of Nm23 with small or heterotrimeric G proteins, GTP proteins, or microtubules have been postulated, but have been subject to considerable debate (reviewed in Ref. 5).

A transcriptional regulatory activity for Nm23 was first proposed by Postel et al. (14, 15) based principally upon in vitro observations. Protein from Escherichia coli expressing a cDNA clone encoding nm23-H2 bound a probe derived from the nucleoside-hypersensitive element (NHE site, also termed PuF site and CT element) derived from positions −142 to −115 of the c-myc promoter. Recombinant Nm23-H2/PuF bound to reporter fragments containing the GGTTGCG PuF recognition sequence and amplified c-myc transcription supported by a DEAE column fraction in vitro. Others have suggested that recombinant Nm23-H2/PuF recognizes a variety of single-stranded polypyrimidine-rich DNA and RNA sequences (18), including the NHE of the c-myc gene. Although structure of Nm23/NDPK has been determined and mutations that diminish interactions with nucleic acid have been engineered (19), the Nm23 sequence-specific DNA binding domain has not yet been delimited. The relative molar amounts of recombinant Nm23 protein versus probe necessary to study binding in vitro would indicate either that the DNA binding species in Nm23-H2/PuF preparations is a biochemically a minor fraction or alternatively that the nucleic acid-Nm23 interaction in vitro is weak. In vivo, an nm23-H2/PuF expression vector was reported to augment the expression of a c-myc reporter plasmid bearing the NHE by 3–4-fold in transient transfections (16), but these studies leave unresolved the mechanism(s) through which Nm23-PuF might modify transcription. Given the broad specificity of Nm23-H2 in DNA binding assays in vitro, the high molar ratio of protein to DNA required to study nucleic binding, and the magnitude of its in vitro and in vivo effects on gene activation, we have designed a series of experiments to test for a direct role for the Nm23-H1 and Nm23-H2 proteins in gene expression.

EXPERIMENTAL PROCEDURES

Immunoprecipitation and Enzymatic Assay of Gal4-Nm23 Fusion Proteins—Lysates of transiently transfected COS cells were prepared in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1% Triton, 0.2 mM phenylmethylsulfonfluoride, 10 μM leupeptin, 1 μM pepstatin, 1 mM EDTA, 100 mM NaF, 2 mM Na2VO4, and 10 mM Na2H2P2O7. Lysates were clarified by centrifugation and stored at −70 °C. To immunoprecipitate Gal4 fusion proteins, the lysates were diluted 4-fold with TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) and an anti-Gal4 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added to a final concentration of approximately 30 μg/ml. After an overnight incubation at 4 °C, 50 μl of GammaBind G-Sepharose suspension (Pharmacia Biotech Inc., Uppsala, Sweden) were added and incubated at 4 °C for 1 h, followed by centrifugation. The precipitates were resuspended in 2× SDS sample buffer and analyzed by SDS-PAGE.

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for 2 h. The immune complexes were recovered by centrifugation, washed twice with 0.5% Triton in TBS, twice with TMD buffer (20 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM dithiothreitol), and finally resuspended in 30 µl of TMD buffer. Six microliters of the suspension were incubated with 1 µl [γ-32P]ATP and 0.1 mM GDP in a final volume of 8 µl at room temperature for 30 min. This reaction was stopped with 1 volume of 50 mM EDTA, and 2 µl were applied to a 20 × 20-cm polyethyleneimine-cellulose thin layer chromatography (TLC) plate (J. T. Baker Inc., Phillipsburg, NJ) and resolved by capillary action with 0.75 M KH₂PO₄, pH 3.65. The TLC plate was dried and exposed to autoradiography.

Cell Culture, Transfection, and Chloramphenicol Acetyltransferase (CAT) Assays—HeLa and COS cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells (6 × 10⁶) were suspended in 225 µl of 1 x phosphate-buffered saline (without calcium and magnesium) and incubated on ice 10 min with plasmid DNA. Electroporation was performed with a cellporator (Life Technologies, Inc.) at 180 V, 1180 microfarads setting. Following electroporation, cells were incubated on ice for an additional 10 min. Transfected cells were added to 10 ml of complete growth medium and incubated for 24 h before harvesting for CAT assays (20).

Constructs—The G/Nm23/VP16 fusion protein expression vectors were generated by cloning nm23 PCR products into EcoRI and BamH1 digested pG4VP16 (21). This vector expresses a G4VP16 fusion protein. 5'-oligonucleotides used for PCR of nm23-H1 were AGCCGGT-GCGAATTCATGACCGCACTGTTGAGTACATGATTCCAGTTCTGAGCACAGCT, respectively. 5'-oligonucleotides used for PCR of nm23-H2 were AGCCGTT-GCGAATTCATGACCGCACTGTTGAGTACATGATTCCAGTTCTGAGCACAGCT, respectively. The G/Nm23 fusions were constructed similarly to the triple fusions except that a stop codon was introduced following the completion of the nm23 sequences. PCR products were cleaved with EcoRI and BamH1 and gel purified before cloning. All clones were verified by double-stranded sequencing. nm23-H1/VP16 and nm23-H2/VP16 were constructed as follows: The BglII-PF1M fragments (encoding the entire Gal4 DBD and the amino termini of nm23-H1 and nm23-H2) were removed from G4/Nm23/H1/VP16 and G4/Nm23/H2/VP16 and the amino termini of nm23-H1 and nm23-H2 were restored by portions of their respective cDNAs, extending from the 5'-untranslated region through the PF1M sites.

RESULTS

Test for Intrinsic Transactivation by Nm23/PuF Proteins Coupled to the Gal4 DNA Binding Domain—Nm23 expression vectors have been reported to stimulate modestly expression of the appropriate cotransfected reporters (16). Because of the various biochemical and physiological properties proven or ascribed to reside in this 17-kDa protein, a series of experiments were designed to test in vivo whether Nm23 could directly modify transcription. To assess the intrinsic transcriptional stimulatory capability of Nm23, it was directed to an upstream promoter region by fusion to the Gal4 DBD. Expression vectors encoding the Gal4 DBD fused with the H1 or H2 nm23 isoforms (G/H1 and G/H2) were cotransfected into HeLa cells with a CAT reporter containing five Gal4 upstream activation sequences (UASs), each capable of binding a dimer of the Gal4 DNA binding domain alone (G, compare Fig. 1a, lanes 7–10 with lanes 5 and 6). Several possible artifactual explanations for lack of activity by the/or nm23 constructs were investigated. Immunoblot analysis of extracts from transfected cells demonstrated proper expression of the full-length chimeric protein (data not shown). Nuclear localization and proper function of the Gal4 DBD when fused to the nm23 isoforms were preserved, since addition of the VP16 activation domain to the carboxyl terminus of the Gal4-Nm23 fusion proteins (G/H1/VP16 and G/H2/VP16) greatly increased levels of CAT activity (lanes 11–14). These experiments suggest that when tethered upstream of a promoter in vivo, Nm23 lacks an activation domain capable of interacting with the basal machinery and stimulating transcription.

Test for Targeting of Nm23 to PuF/NHE/CT Elements in Vivo—If the in vitro DNA binding by Nm23 to the PuF site is relevant in vivo, then the G/Nm23/VP16 constructs described above should bind to and transactivate a CAT reporter driven by the PuF/CT site. As a positive control, G/HnRNP K/VP16 was also transfected (21). The hnRNP K protein binds sequence specifically to the top single strand of the PuF site in the c-myc promoter and is able to transactivate PuF/CT containing reporters with or without an artificially attached additional activation domain (21–25). Two elements of the experimental design are noteworthy. First, to prove nuclear localization and quantitate expression levels, constructs were also tested for activation of Gal4 site containing reporters. Stimulation through Gal4 UASs but absence of activation of the PuF/CT dependent reporter would indicate an in vivo failure to bind the PuF/CT element. Second, the VP16 activation domain would amplify any transcriptional stimulation resulting from binding as has been employed previously with hnRNP K (21).

Cotransfection of the G/K/VP16, G/H1/VP16, and G/H2/VP16 constructs with the Gal reporter showed that comparable amounts of functional nuclear fusion proteins were made in vivo (Fig. 1a, compare lanes 3 and 4 with lanes 11–14). As expected, G/K/VP16 protein bound to and transactivated a cotransfected CT-dependent reporter (as compared with the inactive G/VP16 fusion protein, which lacks a CT binding domain, Fig. 1b, compare lanes 1 and 2 with lanes 3 and 4). In contrast, neither G/Nm23/VP16 fusion protein activated transcription of this CT-dependent reporter and hence showed no evidence of binding in vivo (Fig. 1b, lanes 5–8). Experiments were performed to test the possibility that the lack of functional activity by the triple-fusion constructs was due to misfolding or steric hindrance of the Nm23/PuF portion of the fusion protein. Such misfolding would be expected also to inhibit the NDPK activity of Nm23 in the triple fusion constructs. Extracts of cells transfected with the G/VP16, G/Nm23/VP16, and G/Nm23 expression vectors were immunoprecipitated with anti-Gal4 antibodies. The pellets were then washed and tested for the NDPK activity of Nm23 (Fig. 2B). Formation of [γ-32P]GTP from [γ-32P]ATP and cold GDP was stimulated by G/Nm23/VP16 and G/Nm23, but not by G/VP16 (the lower levels of NDPK activity in extracts of cells transfected with VP16 fusions paralleled lower protein levels of these chimeras, indicating that they were either less stable or expressed at lower levels (Fig. 2B)). Thus, the Nm23/PuF moieties in the triple-fusion expression constructs met the conformational stability requirements for enzymatic activity.

These experiments indicate that the G/Nm23/VP16 fusion proteins fail to transactivate the PuF/CT-dependent reporter in vivo. Although Nm23 transcriptional activation has been described as requiring the greater context of the human c-myc promoter, the powerful VP16 transactivating domain should bypass this prerequisite. Therefore, lack of transcription stimulation by the G/Nm23/VP16 fusion proteins of the CT-dependent reporter most likely was due to an inability to bind sequence specifically to the CT element and was not due to either misfolding of an otherwise high affinity DNA binding domain or an inability to interact with cooperating proteins. Nevertheless, a requirement by Nm23 for the intact c-myc promoter to activate transcription was directly investigated.

The inability of the Gal4/Nm23/VP16 triple chimeras to stimulate CT element-dependent reporter gene expression indicated that the triple fusion protein did not bind with CT elements in vivo. Therefore, either these molecules intrinsi-
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Fig. 1. A, Nm23 does not stimulate transcription when tethered upstream of a promoter in vivo. Expression vectors for G/H1 and G/H2 were cotransfected in duplicate with a CAT reporter containing five Gal sites upstream of a basal promoter (G5-CAT, lanes 7–10). Cells were harvested and assayed for CAT activity 24 h later as described under "Experimental Procedures." As positive controls, cells were also transfected with expression vectors for G/VIP16 (lanes 1 and 2) and G/K/VIP16 (lanes 3 and 4). A Gal alone expression vector (G) was used as the negative control (lanes 5 and 6). To verify functional expression and localization of Nm23-bearing proteins, cells were also transfected with expression vectors for Nm23-H2/PuF (lanes 7 and 8) and Nm23-H2/PuF (lanes 11 and 12). In all cases, the G5-CAT reporter was cotransfected with the CT reporter as the positive and negative controls, respectively.

B, Nm23 does not bind to the CT element in vivo. Expression vectors for G/H1/VIP16 (lanes 3 and 4) and G/H2/VIP16 (lanes 7 and 8) were cotransfected with a CT-dependent reporter (CT4ElB-CAT). Transfectants were assayed 24 h later. G/K/VIP16 (lanes 3 and 4) and G/VIP16 (lanes 1 and 2) were also cotransfected with the CT reporter as the positive and negative controls, respectively.

C: top, description of the reporters used in transfection experiments. Not shown is the myc-CAT construct containing 2.3 kb of myc promoter sequences, which has been described previously. Bottom, chimeric constructs. The construct names used in the text are shown in the center portion of the panel and are described by schematic diagrams roughly drawn to scale to the left and by a more detailed molecular description to the right.

DNA on c-myc promoter activity, expression vectors for both Nm23-H2/VIP16 and Nm23-H2/PuF were cotransfected with a reporter plasmid containing 2.3 kb of c-myc upstream sequences. Despite the linkage to the powerful VIP16 activation domain, neither isoform was able to enhance c-myc transcription relative to the G/VIP16 control (Fig. 4, compare lanes 3–6 with lanes 1 and 2). Strong stimulation of a cotransfected Gal4 UAS-driven reporter verified the synthesis of similar levels of active G/Nm23/VIP16 and G/VIP16 (data not shown).

It appears that the specific DNA binding affinity of Nm23 for the CT element is insufficient to promote cis-element binding in vivo and hence that Nm23, alone, is not likely to be a CT-element-dependent transcription factor. Our data do not eliminate the possibilities that Nm23-H2/PuF might indirectly participate in transcription via a mechanism independent of the CT/PuF site or that special conditions or cofactors might be re-
required to activate latent transcription factor activity. The inclusion of multiple positive controls in the experimental design has enabled a distinction between inconclusive and negative data. The biochemical features and mechanisms conferring a regulatory role upon Nm23 remain to be elucidated.

**DISCUSSION**

The nm23 family of genes have been associated with diverse biological processes. Reduced Nm23 expression has been correlated with poor patient survival or other indicators of high tumor metastatic potential in cell line model systems as well as cohort studies of human breast, gastric, cervical, ovarian, and hepatocellular carcinomas and melanoma, although it does not represent an independent prognostic factor (reviewed in Ref. 5). Over expression of transfected nm23 reduced the in vivo tumor metastatic potential of melanoma and breast carcinoma cell lines, indicating that this gene suppresses metastasis (26–30). Also, a role for the nm23/awd/ndpk gene family has been described in differentiation. Mutation or reduced expression of Drosophila awd resulted in altered morphology, aberrant differentiation, and cell necrosis in the wing disc and other organs postmetamorphosis, leading to death (31, 32). Immunohistochemical staining of Nm23 expression in mouse embryogenesis correlated increased protein expression with the functional differentiation of most epithelial tissues (33). Transfection experiments have documented morphological, growth regulatory, and biosynthetic changes indicative of mammary differentiation following nm23-H1 transfection of human MDA-MB-435 cells (34). Neuroendocrine differentiation followed transfection of the murine phaeochromocytoma PC12 cell line with nm23 (35). Furthermore, nm23 influences genome stability as a mutator phenotype is present in knockout strains of bacteria (36, 37). In addition, particularly in unicellular lineages of cells, Nm23 expression often correlates with proliferation and lack of differentiation (reviewed in Ref. 5). For some of these effects Nm23 was reported to function as a cytokine.

If a transcription factor, then Nm23 might direct this broad spectrum of phenotypic changes through multiple target genes. Alternatively, its biological effects may be due to a series of protein targets for its enzymatic activity or through metabolic effects indirectly modifying the expression of other genes. The experiments presented in this report examined the hypothesis that a direct transcriptional role for Nm23 would most simply explain its biological properties. This role would impose two molecular requirements on the protein. First, in vivo Nm23 should have the capability of docking upon a cis-element, and second, that from this DNA based platform, Nm23 should pro-
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Engrafting a strong activation domain, VP16, to any form of Nm23 failed to create an in vivo transactivator of a reporter plasmid driven by the sequence proposed to bind Nm23, NHE/CT element. The same triple chimera, Gal4/Nm23/VP16, which failed to activate thru a CT element, successfully stimulated a reporter otherwise identical except that the NHE/CT element was substituted with a Gal4 UAS. Therefore this artificial transactivator was expressed, localized to the nucleus, and when bound near a promoter, stimulated gene expression. Removing the Gal4 DBD (yielding Nm23/VP16), which might otherwise obstruct Nm23 DNA binding or re-direct the transactivator to alternative sequences, still failed to create a transactivator of NHE/CT elements.

An important issue is the preservation of the native structure and activity of Nm23-H2/PuF in the double and triple fusion proteins. Following transfection, all of the chimeric proteins examined retained the best characterized enzymatic activity associated with Nm23, NDPK activity. Usually multifunctional proteins are formed by the linkage of structurally separate molecules used in this study should serve an accurate monitor of structural integrity. Unless alterations in Nm23-H2’s secondary, tertiary, or quaternary structure due to fusion cleanly abrogates the DNA binding activity of this small protein (17 kDa), without similarly effecting its enzymatic activity, the fusion proteins are formed by the linkage of structurally functional proteins are formed by the linkage of structurally.

These experiments indicate that Nm23 is unlikely to be a CT-dependent transcription factor in vivo. Our data do not eliminate the possibility that Nm23-H2/PuF might indirectly participate in transcription. Other known biochemical activities for the Nm23 protein family include a nonspecific nucleoside diphosphate kinase (Ndpk) activity (6–8), protein histidine kinase activity (13), and under reducing conditions, protein serine phosphorylase activity (38), any of which might be linked obliquely to gene regulation, but directly to other processes. We have recently correlated Nm23-H1 structure and function to suppression of tumor motility. Mutations to either proline 96 or serine 120 abrogated motility suppressive activity (39), and histidine-dependent protein phosphorylase activities in vitro (40), suggesting that this biochemical pathway may underlie some of the biological effects of Nm23.

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