Advances in Brief

Identification of a Second Human nm23 Gene, nm23-H2

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Abstract

Reduced RNA and/or protein levels corresponding to the murine nm23-1 and human nm23-H1 complementary DNA clones have been correlated with high tumor metastatic potential in several rodent model systems and human breast carcinomas. We report the identification of a second human nm23 gene, designated nm23-H2. The pNM23-H2S complementary DNA clone predicted a Mr, 17,000 protein 88% identical to nm23-H1. nm23-H2 also shared a significant homology with nucleoside diphosphate kinases and a Drosophila development gene. Southern blots containing BglI-restricted genomic DNA, which exhibited an allelic restriction fragment length polymorphism for nm23-H1, contained nonallelic bands upon rehybridization to the nm23-H2 probe. Thus, nm23-H1 and nm23-H2 are distinct genes. Northern blot hybridization of nm23-H1- and nm23-H2-specific probes to breast tumors and cell lines indicated that nm23-H1 expression was reduced in high metastatic potential tumor cells to a greater extent than nm23-H2. The data indicate the existence of a family of independently regulated nm23 genes.

Introduction

Tumor metastasis is the major cause of death for cancer patients (1). Even without evidence of metastatic disease at the time of primary tumor diagnosis, patients face a 50% chance that clinically occult micrometastases exist and will become manifest (2). Accordingly, considerable investigation has focused on the genetic events that regulate tumor metastasis, in an effort to identify better diagnostic and therapeutic strategies. In this regard, the nm23 gene was identified by differential colony hybridization between related low and high metastatic murine K-1735 melanoma lines (3). Quantitative reductions in nm23 RNA and/or protein levels have been observed in the more highly metastatic tumor cells in four rodent metastasis model systems: murine K-1735 melanomas (3); N-nitrosomethylurea-induced rat mammary tumors (3); mouse mammary tumor virus induced tumors (4); and rasG12V adenovirus 2 E1α cotransfected rat embryoblasts (5). In a limited series of primary human infiltrating ductal carcinomas, tumors from patients with metastases to the lymph nodes at surgery contained lower nm23 RNA levels than did nonmetastatic primary tumors (6). Two nm23 cDNA clones have been characterized, murine pNM23-1-3 (7) and human pNM23-H1 (7), which both encode Mf, 17,000 nuclear and cytoplasmic proteins. The function(s) of nm23 are unconfirmed, but significant homologies have been noted between nm23 and the Drosophila abnormal wing discs developmental gene (7) and NDP kinases from a variety of species (8–10). We report the identification of a second, highly homologous human nm23 gene, nm23-H2.

Materials and Methods

Probes. The 450-base pair HpaII restriction fragment of murine pNM23-1, the 419-base pair Fnu4HI-HindIII fragment of pNM23-H2, the 900-base pair BamHI fragment of pNM23-H1, the 700-base pair EcoRI fragment of pNM23-H2S, and the 1.5-kilobase PstI insert of chick β-actin were eluted from polyacrylamide gels. All probes were labeled with a [α-32P]dCTP using a random primer translation kit (Amersham, Arlington Heights, IL) according to the manufacturer's directions. For Northern and Southern blot hybridizations (Fig. 4), specific probes to the 3' untranslated regions of pNM23-H1 and pNM23-H2S were amplified by PCR (Perkins Elmer Cetus, Emeryville, CA). A 141-base pair nm23-H2 probe, corresponding to bases 529–669 of the pNM23-H2S cDNA clone, and a 189-base pair nm23-H1 probe, corresponding to bases 558–746 of the pNM23-H1 cDNA clone (7), were synthesized. An aliquot of each of the PCR-amplified DNA fragments was electrophoresed on a 1.5% agarose gel to confirm that the products were of the predicted size. The probes were labeled with a random primer translation kit, as described above, with the exception that synthetic oligonucleotide primers used for PCR amplification were used in place of random primers, in order to obtain full-length probes.

Identification of the pNM23-H2 cDNA Clone. Approximately 2 x 106 clones from a normal human fibroblast Okayama-Berg cDNA library (gift of H. Okayama) were hybridized to a murine pNM23-1 probe. Hybridization was conducted at 41°C in 5 x SSC, 40% (v/v) formamide, 0.75 M sodium citrate, 5 × Denhardt's solution, and 50 μg/ml denatured salmon sperm DNA. Filters were washed to 0.1 x SSC, 0.2% SDS at 42°C. A pNM23-H2 cDNA clone was identified that contained only the COOH terminal of a novel nm23-like protein.

Identification of the pNM23-H2S cDNA Clone. A Fnu4HI-HindIII restriction fragment of pNM23-H2S was used as a probe to screen a human lung fibroblast Lambda Zap II phage library (Stratagene, La Jolla, CA) as described previously (3). Briefly, filters containing DNA from approximately 106 phage plaques were hybridized at 40°C in 50% (v/v) formamide, 5 x SSC, 5 x Denhardt's solution, 0.5% (w/v) SDS, 50 mm Tris (pH 7.5), 10% (w/v) dextran sulfate, and 50 μg/ml sheared denatured salmon sperm DNA. Filters were washed to a stringency of 0.25 x SSC, 0.5% (w/v) SDS, 1 mm EDTA at 65°C. Twenty-nine positive clones were identified, plaque purified, and characterized.

Bluescript phagemid containing the cDNA insert and amp' gene was produced from single positive plaques according to the manufacturer's protocol and infected into XL-1 Blue bacteria. Restriction analysis of the Bluescript clones with EcoRI indicated cDNA inserts of varying sizes, with a clone designated pNM23-H2S containing a 700-base pair insert.

DNA Sequencing. The pNM23-H2 and pNM23-H2S cDNA clones were sequenced using Sequenase Version 2.0 (U. S. Biochemical Corp., Cleveland, OH) by the Sanger dideoxy chain termination method.

Southern Blot Hybridizations. Genomic DNA was isolated and restricted with BglII, and Southern blotting was performed as described.

Received 10/23/90; accepted 11/12/90.

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4 The abbreviations used are: cDNA, complementary DNA; NDP, nucleoside diphosphate; RFLP, restriction fragment length polymorphism; SSC, 3.0 M sodium chloride-0.3 M sodium citrate, pH 7.0; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction.
Previously, Blots were hybridized with either the nm23-H1 or nm23-H2 probes at 40°C, in 5x SSC, 50 mm Tris, pH 7.5, 5x Denhardt’s, 0.2% (w/v) SDS-50 μg/ml sheared, denatured salmon sperm DNA for 16 h. Blots were washed sequentially in 2x, 1x, 0.5x, and 0.25x SSC; 0.2% SDS; and 1 mm EDTA at 55°C and then again in the last buffer for 30 min, air dried and autoradiographed. For multiple hybridizations, probes were removed by incubation in 0.1x SSC, 100°C for 5 min. Blots were autoradiographed overnight to ensure that all control hybridizations were stripped and hybridized to a chick β-actin probe (13).

Fig. 1. Nucleotide and predicted amino acid sequences of pNM23-H1, pNM23-H2, and pNM23-H2S cDNA clones. The pNM23-H2 cDNA clone was identified by screening a human fibroblast cDNA library with the HpaII restriction fragment of murine pNM23-l. The sequence of this cDNA clone extends from the asterisk (*) to the polyadenylate (Poly A) tail of pNM23-H2S. A Fnu4Hl///III restriction fragment of pNM23-H2 was used to screen a human lung fibroblast Lambda Zap II phage library, resulting in the identification of the pNM23-H2S cDNA clone. The sequence of the pNM23-H2S insert, determined by dye sequencing of both strands, is compared to the previously identified pNM23-H1 (7). Methionines with an optimal translation initiation sequence are boxed; for pNM23-H1, this methionine has been demonstrated to be the initiation point for translation (7). Predicted amino acids which are identical in pNM23-H1 and pNM23-H2S are dotted.

Results
Identification of the pNM23-H2 and pNM23-H2S cDNA Clones. A human fibroblast cDNA library was screened with A 419-base pair Fnu4Hl-HindIII restriction fragment of pNM23-H2 was used to screen a human lung fibroblast Lambda ZAP II phage library, resulting in the identification of 29 positive clones. The cDNA clone with the longest EcoRI insert, designated pNM23-H2S, was sequenced by the dyeoxy chain termination method (Fig. 1). The pNM23-H2S cDNA clone predicted a M, 17,000 protein, 88% identical to nm23-H1 (Fig. 1). Amino acid differences between nm23-H1 and nm23-H2 were highly conservative in hydrophobicity and charge, bringing the total homology of the two proteins to 98%. Identity in nucleotide sequence in the 3’ and 5’ untranslated regions was less than 50%, indicating an evolutionary pressure for conservation of the protein sequences.

Fig. 2 compares the predicted amino acid sequences of nm23-H2 to other nm23 proteins, the Drosophila and developmental protein, and NDP kinases from several species. The nm23-H2 protein bears a 97% amino acid identity to rat NDP kinase, which was purified from membranous NDP kinase involved in G-protein signal transduction (8). A lesser degree of identity, 42–61%, was observed in relation to NDP kinases from lower eukaryotes and prokaryotes.

Both the nm23-H1 and nm23-H2 predicted amino acid se-
quences also contain 2–3 leucines in a periodic repeat suggestive of a leucine zipper (14). A comparable leucine zipper-like structure is present in the other nm23, Drosophila aww, and rat NDP kinase proteins but not in the lower eukaryotic and prokaryotic forms (Fig. 2). Consistent with a possible leucine zipper motif; (a) functional leucine zippers containing two or three leucines have been previously reported (15); (b) computer modeling of the nm23-H1, nm23-H2, and aww protein secondary structures using the Novotony program under PC Gene indicated an α-helical conformation throughout this region (data not shown); (c) a region of basic amino acids was consistently observed NH2-terminal of the leucine zipper-like motif; and (d) substitution of a methionine for a leucine, as shown for nm23-H1, has also been reported for human L-myc (14).

nm23-H1 and nm23-H2 Are Distinct Genes. The degree of nucleotide identity between nm23-H1 and nm23-H2 within their translated regions suggested that the two cDNA clones represented distinct genes. Southern blot hybridizations of the pNM23-H1 and pNM23-H2 cDNA probes to human genomic DNA were utilized to confirm whether nm23-H1 and nm23-H2 were alleles of the same gene or, alternately, were two distinct genes. A restriction fragment length polymorphism (RFLP) was identified previously for nm23-H1 using BglII-digested human chromosomal DNA on Southern blots. (Fig. 3A shows representative Southern blots containing BglII-digested DNA, hybridized to the entire pNM23-H1 cDNA insert. As shown, nm23-H1 bands at 2.3 and 7.6 kilobases demonstrated an allelic pattern of expression, with individuals homozygous for the 7.6-kilobase band (lanes 1 and 2), homozygous for the 2.3-kilobase band (lanes 3 and 4), and heterozygous (lanes 5 and 6). After hybridization of the blots to the pNM23-H2 insert, the blots were stripped and rehybridized to the entire pNM23-H2 cDNA insert (Fig. 3B). In each lane a set of distinct bands was observed at 2.4, 2.8, 4.2, 13.8, and 17.5 kilobases. The data demonstrate that nm23-H1 and nm23-H2 are distinct genes.

Expression of nm23-H1 and nm23-H2 RNAs in Human Breast Tumors and Cell Lines. Due to the high degree of identity between nm23-H1 and nm23-H2 throughout their translated regions, it was possible that hybridizations with a full length probe could detect both genes simultaneously. In fact, some cross-hybridization of the full length nm23-H1 and nm23-H2 probes was detectable on the Southern blots in Fig. 3. A system was therefore established for the specific detection of nm23-H1 and nm23-H2 RNAs, using PCR-amplified probes corresponding to the 3′ untranslated regions of the cDNA clones (which were less than 50% identical in nucleotide sequence), and stringent washing conditions. In the control experiment shown in Fig. 4A duplicate Southern blots were prepared, each containing restricted pNM23-H1 and pNM23-H2 cDNAs. One blot was hybridized to the 3′ nm23-H1 probe, and the other hybridized to the 3′ nm23-H2 probe. In each case, specific hybridization was observed.

nm23-H1 and nm23-H2 RNA levels were determined in a limited series of human primary infiltrating ductal breast car-

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Fig. 2. Amino acid sequence similarities between nm23-H2, other nm23 proteins, NDP kinases and Drosophila aww. The predicted amino acid sequences of pNM23-H2S, human pNM23-H1 (7), murine pNM23-1 (3), Drosophila abnormal wing discs (awd) developmental gene (7), rat NDP kinase (8), Dictyostelium NDP kinase (9), and Myxococcus NDP kinase (10) are listed. The percentages of nm23-H2 amino acid identity to each protein, calculated based on the number of amino acid residues which overlap, are listed below. Leucines in a configuration consistent with a leucine zipper (14) are boxed.

Fig. 3. nm23-H1 and nm23-H2 are distinct genes. Human genomic DNA from six individuals was digested with BglII, and a Southern blot was prepared. The blot was hybridized with the BamHI restriction fragment of pNM23-H1 (A). An allelic pattern of hybridizing nm23-H1 bands was obtained, including homozygotes for the 7.6-kilobase band (Lanes 1 and 2), homozygotes for the 2.3-kilobase band (Lanes 3 and 4), and heterozygotes (Lanes 5 and 6). The probe was removed from the Southern blot, and it was rehybridized to the EcoRI restriction fragment of pNM23-H2 (B). Bands distinct from the alleles of nm23-H1 were observed at 17.5, 13.8, 4.2, 2.8, and 2.4 kilobases. Sizes of DNA bands were calculated from the electrophoretic mobility of DNA λ HindIII markers.
The predicted amino acid sequences of \( nm23-H1 \) and \( nm23-H2 \) have prompted at least two hypotheses concerning their biochemical functions. First, \( nm23-H2 \) exhibited a 97% amino acid sequence identity to the rat NDP kinase. Barring the possibility that the four amino acids changed between \( nm23-H2 \) and rat NDP kinase abrogated its activity, it is anticipated that \( nm23-H2 \) is a NDP kinase. We are currently in the process of expressing and purifying the \( nm23-H1 \) and \( nm23-H2 \) proteins from bacteria to confirm their NDP kinase activities. The NDP kinases are thought to provide intracellular pools of nucleoside triphosphates (excepting ATP), regulate polymerization of microtubules in the mitotic spindle and cytoskeleton, and supply GTP to G-proteins in signal transduction (16). It is not known if \( nm23-H1 \) and \( nm23-H2 \), if proven to be NDP kinases, participate in different functions.

The second proposed functional domain for \( nm23-H2 \) (and \( nm23-H1 \)) is a leucine zipper, a motif proposed to mediate protein:protein binding and found on nuclear oncogenes and transcription factors (14). The \( nm23 \) proteins, awd, and rat NDP kinases exhibit 2–3 leucines in a characteristic arrangement, short of the 4 leucines in the classic definition of a leucine zipper (14). However, leucine zippers of 2–3 residues have been reported in other proteins (15). It is noteworthy that the leucine repeat is an evolutionary addition, in that the lower eukaryotic and prokaryotic NDP kinases do not exhibit this motif. This observation also suggests that protein dimerization via a leucine zipper is not necessary for NDP kinase activity. A region containing basic amino acids was identified to the NH\(_2\) terminus of each leucine repeat, although the placement of basic amino acids within this region did not fit a proposed DNA binding site model (15). The data suggest a possible function of \( nm23-H1 \) and \( nm23-H2 \) as transcription factors. Consistent with this hypothesis is the localization of \( nm23 \) protein in both the cytoplasm and nucleus (7). Proof of this hypothesis awaits evidence of homo- or heterodimerization of \( nm23 \) and identification of a DNA sequence that is bound and regulated.

On the basis of the high degree of identity between \( nm23-H1 \) and \( nm23-H2 \) throughout their translated regions, we conclude that previously published data using full length probes may have measured the composite RNA level for both genes. In this report we have quantitated specific expression of \( nm23-H1 \) and \( nm23-H2 \) in a limited series of breast carcinomas and cell lines, using probes to their 3' untranslated regions and stringent washing conditions. On these Northern blots, \( nm23-H2 \) RNA levels were not reduced in the more highly metastatic specimens to the extent that \( nm23-H1 \) RNA levels were. The data indicate that the two genes are not identically regulated. The data should not be generalized to indicate that \( nm23-H2 \) expression is not inversely correlated with tumor metastatic potential. In a series of v-Ki-ras-transfected, immortalized human bronchial epithelial cell lines (17), the expression of \( nm23-H2 \) was decreased with increasing malignant behavior to a greater extent than that of \( nm23-H1 \). The data indicate the independent regulation of the two \( nm23 \) genes and suggest that factors such as cell type and oncogene expression may be relevant to further dissection of this complicated regulatory network.

Discussion

The data presented herein identify a second human \( nm23 \) gene, \( nm23-H2 \). \( nm23 \) must therefore be considered a family of closely related genes.


Identification of a Second Human nm23 Gene, nm23-H2


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