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Multiple Biochemical Activities of NM23/NDP Kinase in Gene Regulation

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NM23/NDPk proteins play critical roles in cancer and development; however, our understanding of the underlying biochemical mechanisms is still limited. This large family of highly conserved proteins are known to participate in many events related to DNA metabolism, including nucleotide binding and nucleoside triphosphate synthesis, DNA binding and transcription, and cleavage of DNA strands via covalent protein–DNA complexes. The chemistry of the DNA-cleavage reaction of NM23-H2/NDPk is characteristic of DNA repair enzymes. Both the DNA cleavage and the NDPk reactions are conserved between *E. coli* and the human enzymes, and several conserved amino acid side chains involved in catalysis are shared by these reactions. It is proposed here that NM23/NDP kinases are important regulators of gene expression during development and cancer via previously unrecognized roles in DNA repair and recombination, and via previously unrecognized pathways and mechanisms of genetic control.

KEY WORDS: NM23; NDP kinase; nuclease; DNA transcription; DNA recombination; DNA repair.

BACKGROUND

Although the NM23/NDPk protein family has been known to play a role in development and cancer for at least a decade, the underlying mechanisms by which these proteins contribute to various physiological and pathological functions is unclear. The first *nm23* gene was isolated from mouse melanoma cells on the basis of a negative correlation between metastasis and expression levels (Steeg *et al.*, 1988). Later, the same correlation was observed in experimental systems of human melanoma and breast carcinoma cells, suggesting that NM23 may play the role of metastasis inhibitor in these tumors (review in Hartsough and Steeg, 2000). However, the evidence is contradictory regarding a global metastasis inhibitory effect of NM23, and, indeed, *NM23-H1* RNA levels are elevated in aggressive neuroblastoma (Hailat *et al.*, 1991), and, in colorectal cancers, *NM23-H2* mRNA levels are among the most abundant (Zhang *et al.*, 1997). Mutations in *NM23* genes are rare in cancer. Thus, *NM23* may be an example of a family of cancer genes that become dysregulated not through mutations (like oncogenes and tumor suppressor genes), but through expression changes at the RNA level (Sager, 1997). This mode of regulation would explain why both underexpression of *NM23* (e.g., in metastatic breast cancer), and its overexpression (e.g., in colorectal cancers) are oncogenic.

For decades, NM23 proteins have been characterized by enzymatic means as nucleoside-diphosphate kinases (NDPk) (Lascu, 2000), although not all members in the NM23 family exhibit NDPk activity (e.g., H5; Lacombe *et al.*, 2000). NDP kinases (EC 2.7.4.6) were originally described as housekeeping enzymes needed for the synthesis of nucleoside triphosphates, and for the maintenance of nucleotide pools. The NDPk reaction involves transfer of a γ-phosphoryl group from nucleoside triphosphates to nucleoside diphosphates, in a pingpong mechanism that uses a conserved histidine residue as the intermediate (review in Postel, 1998; Lascu *et al.*, 2000). However, through all these years and in all organisms, the true biological significance of this enzymatic activity has remained elusive. In *E. coli*, the gene encoding NDPk is not essential for viability, suggesting that the primary function of NM23/NDP kinase is not in housekeeping. Interestingly, deletion of the *ndk* gene in *E. coli* produces a mutator phenotype, which

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has been attributed to an imbalance in the nucleotide pools caused by the absence of NDPk (Bernard *et al.*, 2000; Lu *et al.*, 1995; Miller *et al.*, 2002). NM23/NDP kinases are composed of identically folded subunits of about 16–17 kDa each, which assemble into hexamers or tetramers, their native conformations (Lascu *et al.*, 2000). Many of these structures, with and without bound nucleotide substrates, have been described in the literature (review in Janin *et al.*, 2000).

A variety of regulatory activities have been attributed to NM23/NDPk. These include DNA binding and transcription (Arnaud-Dabernat *et al.*, submitted; Berberich and Postel, 1995; Cho *et al.*, 2001; Ji *et al.*, 1995; Lee *et al.*, 1997; Ma *et al.*, 2002; Postel *et al.*, 1993), and cleavage of DNA (Levit *et al.*, 2002; Ma *et al.*, 2002; Postel, 1999, Postel *et al.*, 2000a, 2002; Singh *et al.*, submitted). Many of these properties have been described in detail in a previous review in this journal (Postel *et al.*, 2000b). It has also been suggested that NM23-H2/NDPk has DNA helicase activity, but no experimental evidence has been presented (Hildebrandt *et al.*, 1995). During the past few years, it has been reported that the high-energy phosphorylated intermediate of NM23/NDP kinase exhibits protein kinase activity towards a number of proteins (review in Levit *et al.*, 2002). However, some of these data (e.g., Lu *et al.*, 1996; Wagner *et al.*, 1997) have been found to be artifactual (Levit *et al.*, 2002). The present review will mainly concentrate on activities of NM23/NDPk that are related to its interactions with DNA.

INTERACTIONS BETWEEN HUMAN NM23 AND DNA

DNA Binding and Transcriptional Activity of NM23-H2

The DNA binding and in vitro transcriptional activities of NM23-H2/NDPk were discovered in the author's laboratory, after cloning of PuF, a c-*MYC* transcription factor, as NM23-H2 (Postel *et al.*, 1993). These in vitro findings have been confirmed in living cells by cell transfection assays (Berberich and Postel, 1995; Ji *et al.*, 1995). NM23-H2/PuF activates transcription by binding to a nuclease-hypersensitive element in the c-*MYC* promoter, termed NHE. The NHE contains an asymmetric pattern of repeated sequences, consisting mostly of C's in one strand and G's in the other, that are capable of forming unusual structures, including "slipped DNA," loops, triplexes, and G-quadruplex structures (Boles and Hogan, 1987; Postel *et al.*, 2000b; Siddiqui-Jain *et al.*, 2002; Simonsson *et al.*, 2000). From early on, our studies have suggested that PuF/NM23-H2 is a DNA transactional protein that recognizes and interacts with such structural elements, and that their alteration or removal from the promoter may be necessary for activation of the c-*MYC* gene (Postel, 1998, 1999; Postel *et al.*, 1993, 2000b). Recent observations of transcriptional repression of the c-*MYC* gene by a G-quadruplex structure formed within the NHE promoter (Siddiqui-Jain *et al.*, 2002) support this idea (see below).

Transcriptional activation of the c-*MYC* gene by NM23 has been confirmed in both human and murine cells by other laboratories (Arnaud-Dabernat *et al.*, submitted; Cho *et al.*, 2001; Ji *et al.*, 1995; Lee *et al.*, 1997). NM23-H2 is also capable of negative regulation of transcription, as demonstrated by its silencing effect on the *PDGF-A* chain gene in cell transfection experiments (Ma *et al.*, 2002; see below). Negative results on transactivation of the c-*MYC* gene by NM23-H2 (Michelotti *et al.*, 1995), may have been due to the use of fusion constructs that would not have been expected to yield properly folded native hexameric structures essential for biological activity (Lascu *et al.*, 2000). Additionally, even if a transactivation domain does exists on NM23, it may not be important, as has been shown for other transactivators (e.g., Pongubala and Atchinson, 1997).

Interactions Between Human NM23-H1 NDP Kinase and DNA

NM23-H1 was first identified as a DNA-binding protein with transcriptional activity on the *PDGF-A* chain promoter in the laboratory of D. Kaetzel (Ma *et al.*, 2002). The *PDGF-A* chain promoter is regulated by several enhancer and silencer elements, which are GC-rich and possess non-B DNA structures. To identify proteins that bind and mediate the function of a silencer element in the 5′-flanking sequence of the *PDGF-A* gene termed 5′ SHS, Ma *et al.* (2002) screened a HeLa cell cDNA expression library using the DNA recognition site approach. Three cDNA clones encoding NM23-H1 were isolated. Transient transfection analyses in HepG2 cells revealed that both H1 and H2 repressed transcriptional activity driven by the *PDGF-A* proximal promoter, another NHE element. Activity of the negative regulatory region (−1853 to −883), which contains the 5′SHS, was also inhibited by H1 and H2. These studies by Ma *et al.* (2002) demonstrated for the first time that NM23-H1 also interacts, both structurally and functionally, with DNA. They also indicated a role for both NM23 proteins in repressing transcripion of an oncogene, and a possible explanation for their metastasis-suppressing effects.

Binding of NM23 to Single Stranded DNA

In the author's laboratory, NM23-H2/NDPk binds to both single- and double-stranded c-*MYC* NHE DNA oligonucleotides with approximately similar stoichiometries, but with different specificities (Postel *et al.*, 2000b, 2002). With respect to *PDGF* NHE oligonucleotide substrates, NM23-H2 behaved similarly in D. Kaetzel's laboratory (Ma *et al.*, 2002). These studies employed electrophoretic mobility shift assays (EMSAs) to assess DNA-binding. In M. Veron's laboratory (Hildebrandt *et al.*, 1995), binding of NM23-H2 to single-stranded c-*MYC* NHE DNA by the EMSA was specific and significantly more favored than binding to duplex DNA. Using a filter-binding assay, the Veron group again observed poor binding to duplex oligonucleotides; however, the binding to single-stranded c-*MYC* NHE oligonucleotides in this assay was nonspecific (Agou *et al.*, 1999). As the tertiary structure of DNA oligonucleotides is a key determinant in protein–DNA interactions, a possible source of variability could be the fact that single-stranded DNA is rarely just a rod, as it can fold back into annealed double-stranded structures in a sequence and length-dependent manner. Differences in tertiary structures can also explain why NM23-H1 binds poorly to duplex c-*MYC* NHE oligonucleotides (Hildebrandt *et al.*, 1995; Postel *et al.*, 1996), whereas it binds with high affinity to both duplex and single-stranded *PDGF* NHE oligonucleotides (Ma *et al.*, 2002). The physiological significance of recognition of different DNA structures by NM23 is discussed below.

NM23 as a Nuclease

Through a series of mutational and functional studies between 1994 and 2002, our laboratory has identified the NDPk phosphorylation site of NM23-H2/PuF as His118, the key residue for the kinase, and showed that mutation of His118 had no effect on DNA binding and in vitro transcription (Postel and Ferrone, 1994). We have subsequently identified a sequence-dependent DNA-binding surface, involving Arg34, Asp69, and Lys135, on the equator of the hexameric protein (Postel *et al.*, 1996). This finding suggested that, because the NDP kinase reaction does not require sequence-dependent DNA binding, NM23-H2 must possess both a nonspecific metabolic activity associated with the catalytic domain (NDPk), and a regulatory function (PuF), that requires sequence-specific DNA-binding. This DNA binding activity has been shown to be a nuclease (Postel, 1999).

DNA Cleavage by NM23-H2 Involves a Covalent Protein–DNA Complex

The nuclease activity of NM23-H2 cleaves both DNA strands of a duplex oligonucleotide substrate comprised of the c-*MYC* NHE, leaving double-stranded breaks within the repeated sequence elements. The breaks produce staggered ends, with five nucleotide-long 3′-extensions. The enzyme also cleaves supercoiled, plasmid DNA containing the NHE, yielding unit length linear plasmid DNA, as well as nicked circular (relaxed) products. The cleavage reaction is reversible with EDTA, suggesting that the enzyme remains attached to the DNA throughout the reaction. The cleaved DNA strads have free 3′-OH groups, and 5′-phosphoryl ends, and the protein–DNA covalent link is via a 5′-phosphoester to Lysine 12 (see below). A covalent polypeptide–DNA complex was confirmed by Western blotting as containing NM23-H2 (Postel, 1999). Because the polypeptides linked to DNA are recognized by monoclonal antibodies raised against NM23-H2, this covalent interaction obviously involves NM23-H2, and not a contaminating enzyme. Moreover, since covalent protein–DNA complexes are known to serve the roles of breaking and rejoining DNA strands, it was concluded that NM23-H2 is involved in DNA structural transactions that are necessary for the activity of the c-*MYC* promoter (Postel, 1999).

Identification of the Catalytic Residue of the Cleavage Activity and of a Second DNA-Binding Surface

Sequencing of a DNA-linked peptide and site-directed mutagenesis identified Lys12 as the amino acid responsible for the covalent cleavage by NM23-H2 (Postel *et al.*, 2000a). In particular, the ε-amino group of Lys12 was the critical nucleophile, since substitution with glutamine, but not arginine, completely abrogated covalent adduct formation and DNA cleavage, whereas the sequence-specific DNA-binding properties were not affected by these changes. Indeed, the K12Q mutation may prove to be a powerful tool for functional studies on the role of NM23 in cancer and development as a potentially dominant-negatively acting enzyme. The identification of lysine as the catalytic residue, combined with chemical modification data obtained with sodium borohydride, indicated, furthermore, that phosphodiester bond cleavage by NM23-H2 occurs via a combined DNA glycosylase/lyase like cleavage mechanism (Dodson *et al.*, 1994; Postel *et al.*, 2000a). This mechanism is known as the signature of bifunctional DNA repair enzymes in the base excision-repair (BER) pathway, containing both

DNA glycosylase and lyase activities (Cunningham, 1997; McCullough *et al.*, 1999; Mol *et al.*, 1999; Nash *et al.*, 1997).

Lys12 is a conserved amino acid that, in the X-ray crystallographic structure of NM23-H2, lies in the catalytic pocket of the NDP kinase phosphorylation reaction. Not surprisingly, therefore, Lys12 is also essential for the NDP kinase activity of NM23-H2 (Postel *et al.*, 2000a). This observation provided the first link between the nuclease and the NDPk activities, and the implication that the NDPk phosphorylation reaction may be somehow involved in DNA-repair mechanisms. Further mutational and functional analyses identified additional amino acid residues that are shared, supporting the idea that the two activities are components of a single mechanism (Postel *et al.*, 2002; see below). In addition to having established a connection between two seemingly disparate enzymatic activities, the nuclease and the phosphotransferase, these studies also revealed the existence of a second, covalent DNA-binding site located within the nucleotide-binding region (Postel *et al.*, 2000a,b, 2002).

NM23-H1 was also found to cleave both strands of the c-*MYC* NHE (Postel, 1998), and both strands of the *PDGF* 5′SHS silencer sequence, yielding an array of DNA fragments (Ma *et al.*, 2002). NM23-H2 also cleaved the 5′SHS, yielding a more limited set of fragments, suggesting that the mechanism of cleavage by these two enzymes, or of the processing of cleavage sites, may be different. Moreover, NM23-H2 can also cleave *PDGF* NHE oligonucleotides in the single-stranded forms (Ma *et al.*, 2002), in contrast to its ability to cleave the c-*MYC* NHE only as double-stranded (Postel *et al.*, 2002). High resolution mapping of the cleavage sites revealed that NM23-H1 and NM23-H2 have different recognition specificities, both within the c-*MYC* NHE (E. Postel, unpublished results), and in the *PDGF* silencer regions (Ma *et al.*, 2002).

The DNA Substrate Specificity of NM23-H2

The DNA substrates used for NM23-H2 have been derived from naturally occuring promoter sequences, either as excised from plasmid DNA, or synthesized in vitro and used as oligonucleotide duplexes. The c-*MYC* NHE contains four tracts of the repeated nucleotide motif CCCTCCCCA, which are NM23-H2 cleavage and binding sites (Postel, 1999). What aspect of this motif does NM23-H2 recognize in the c-*MYC* and in the various other nuclease-hypersensitive elements (NHE; summarized in Postel *et al.*, 2000b), and what else does NM23 recognize in nature? Is it structural, as have been proposed earlier (Cooney *et al.*, 1988; Postel *et al.*, 1993, 2000b), and/or is it also chemical? We have determined that sequence-dependent DNA binding and cleavage require a minimum length of about 18 bp of duplex DNA, and that, the longer the DNA fragment, the stronger the binding and cleaving activity, indicating that the tertiary structure is a key determinant of its nuclease susceptibility.

The NHE has been known for a long time to be critical in the control of the c-*MYC* gene (review in Postel *et al.*, 1989; Siddiqui-Jain *et al.*, 2002). Over the years, several different structures have been proposed for it, including CGG triplexes (Boles and Hogan, 1987; Cooney *et al.*, 1988), the looping out of nucleotides and slip mispairing (Postel *et al.*, 2000b), and G-quadruplexes (Siddiqui-Jain *et al.*, 2002; (Simonsson *et al.*, 2000). Although some of these structures were shown to be relevant for c-*MYC* transcription in vitro (Cooney *et al.*, 1988), and in cell culture analyses (Siddiqui-Jain *et al.*, 2002), none have yet been shown to form in vivo. Nevertheless, of the various structures proposed for the c-*MYC* NHE, the chair-form G-quadruplex structure appears to be the most relevant as has been demonstrated both by mutational (single nucleotide replacements), and by functional (cell transfection) analyses (Siddiqui-Jain *et al.*, 2002). Thus, NM23-H2 may be considered as a quadruplex-binding protein.

A Model for DNA Binding by NM23-H2

On the basis of mutational and functional analyses, it was suggested earlier that the sequence-dependent DNA-binding surface located on the equator (Postel *et al.*, 1996) is required for the purpose of positioning the target DNA into the nucleotide-binding site, in order for DNA cleaving to occur (Postel *et al.*, 2000a). As of now, there is no evidence as to how DNA binds to the nucleotide-binding site during the cleavage reaction. We have previously proposed a base-flipping mechanism to explain both the structural and biochemical findings (Postel *et al.*, 2002). The structural data have indicated that, in the absence of significant alterations, there is no space in the nucleotide-binding site for duplex DNA binding (Morera *et al.*, 1995). Moreover, phosphodiester bond cleavage by all DNA glycosylases, both simple and bifunctional, and of many recombinases, occurs exclusively via a base-flipping mechanism (Bruner *et al.*, 2000; David and Williams, 1998; House *et al.*, 2001; McCullough *et al.*, 1999). In the bifunctional glycosylase/lyase mehanism, the catalytic lysine acts both to remove the base and to cleave the DNA strand. A base-flipping mechanism, initiated by the attack of a specific base by a lysine amine, would be consistent with the observed chemistry of the cleavage reaction of NM23-H2 (Postel *et al.*, 2000a, 2002). Structural studies

on NM23/NDPk interactions with DNA should clarify the existence of such a mechanism, as well as confirm the identity of any excised bases.

STRUCTURE BASED MUTATIONAL AND FUNCTIONAL ANALYSIS HAS IDENTIFIED HUMAN NM23-H2 AS A MULTIFUNCTIONAL ENZYME

Although direct proof is still lacking, the evidence accumulated thus far strongly suggests that the modus operandi of NM23 as a transcriptional regulator is through its ability to bind and cleave DNA. The role of the NDPk activity in this process does not appear to be required, even though the nuclease and the NDPk share amino acid side chains within the nucleotide-binding region. To understand the role of the nucleotide-binding site in the DNA-cleavage reaction, we used the known crystal structure of NM23-H2 complexed with GDP (Morera *et al.*, 1995) as a basis for site-directed mutations. Several functionally important amino acid residues were identified, some of which (Lys12, Arg88, Arg105) are shared by the covalent DNA binding, DNA cleavage, and NDP kinase activities. Other mutations have separated these functions (Gln17 is important only for the cleavage, while Tyr52, Asn115, and His118, are important only for the NDP kinase activity) (Postel *et al.*, 2002). With the exception of Gln17, all of the other catalytically critical amino acids of NM23-H2 identified thus far are fully conserved, signifying their biological importance. In other experiments, nucleoside triphosphates, but not nucleoside diphosphates inhibited the covalent DNA-binding and -cleavage reactions, independently of phosphoryl transfer and NDPk activity, suggesting that nucleoside diphosphates and DNA nucleosides bind to the nucleotide-binding site in a different manner.

Underlying Biochemistry of the Nuclease Activity

N-glycosylic/AP Lyase Activity

The suggestion that NM23-H2 cleaves DNA via a combined N-glycosylase/AP lyase activity, is based on the identification of lysine as the active site, and on experiments with sodium borohydride ($NaBH_4$), which interacts irreversibly with a Schiff base formed between Lys-12 and C1′ of the ribose moiety of the nucleotide (Postel *et al.*, 2000a). Enzymes that use a lysine-amine to cleave DNA belong to a subtype of base excision repair (BER) enzymes known as bifunctional N-glycosylase/AP lyases. These enzymes are termed bifunctional, because, unlike simple glycosylases, they act sequentially to both remove a base and to cleave the phosphodiester backbone using a single lysine nucleophile, which forms a covalent intermediate during the reaction. Database searches, however, have not revealed significant amino acid sequence similarities between NM23-H2 and known N-glycosylase/AP lyases, although, given the limited homology even among BER enzymes, this fact is not very surprising. Still, five of the NM23 residues surrounding the active site Lys12 are identical with the HhH DNA binding motifs of MutM/OGG1 and Endo III/NTH (Nash *et al.*, 1997), residues that, by mutational analyses, also affected the DNA-cleaving mechanism (Postel *et al.*, 2000a, 2002). Nonetheless, because NM23-H2 does form covalent complexes with DNA that can be further stabilized by $NaBH_4$-trapping, it is highly likely that NM23-H2 proceeds via the glycosylase/AP lyase like mechanism. Moreover, we have independent evidence for both a glycosylase (base excision) and for a lyase (cleavage) reaction (E. Postel, and B. Abramczyk, unpublished observations).

The Chemical Nature of the Cleaved DNA Termini

In the N-glycosylase/AP lyase reaction, the enzyme cleaves the C—O bond 3′ to the AP site by β-elimination, generating a 5′-phosphoryl end and a base-free 3′-unsaturated sugarphosphate attached on the 3′-end. This product is termed the "dangling sugar-phosphate" and must be removed in vivo by either a 3′-phosphodiesterase, a 3′- to 5′-exonuclease, or another β-elimination step (called δ-elimination) in order to produce a proper 3′-OH end necessary for DNA repair during synthesis. The final products of the N-glycosylase/AP lyase reaction mechanism are 3′-OH and 5′-P termini, and a single nucleotide gap (Dogliotti *et al.*, 2001). Thus, remarkably, a single protein can possess three (or more) different activities: a glycosylase, a lyase, and a 3′-processing activity, which can be either a 3′-phosphodiesterase, a 3′- to 5′-exonuclease, or a phosphatase, or all three. Pure glycosylases, without the AP lyase activity, and conventional endonucleases, do not catalyze these reactions, and they cannot form covalent complexes (Cunningham, 1997; David and Williams, 1998; Dodson *et al.*, 1994).

The cleaved DNA produced by an NM23-H2 reaction also possesses 3′-OH and 5′-P ends, because the cleaved fragments can be labeled with α-^{32}P and terminal deoxynucleotidyltransferase (Postel, 1999), but not with γ-^{32}P [ATP] and T4 polynucleotide kinase, unless the DNA was previously treated with alkaline phosphatase (Postel, unpublished results). The covalent protein-DNA

link, which is transient and can be captured in a small fraction of the population, is at the 5′-end (Postel, 1999). The free 3′-OH end produced by NM23-H2 could have resulted from a β-lyase action (Postel *et al.*, 2000a, 2002), consisting of base excision, phosphodiester bond cleavage by β-elimination, followed either by a δ-elimination step, or by an activity that removes the dangling 3′-phosphate left after the first two reactions. Whether this 3′-end trimming activity of NM23-H2 is a 3′-phosphatase, a 3′-phosphodiesterase, or a 3′- to 5′-exonuclease, remains to be determined. Preliminary data also suggest that NM23 is capable of strand discrimination, although the discrimination signal is not yet known.

IS NM23/NDP KINASE A NOVEL FAMILY OF DNA REPAIR ENZYMES?

More than 70 human genes, involved in five major pathways of DNA repair, have been described (Cunningham, 1997; David and Williams, 1998; Dogliotti *et al.*, 2001; Leadon, 1999; Lloyd and Linn, 1993; Modrich and Lahue, 1996). The activities of these enzymes often overlap and most are involved in other DNA metabolic processes including replication, transcription, and recombination, each recognizing a different family of substrates and a different subset of lesions. The most common lesions are due to oxidative stress from normal metabolism, alkylation, or ionizing radiation, that can induce damage to both purines and pyrimidines, lesions that, if not removed, are mutagenic. Large numbers of mutations, if not repaired, can lead to the development of cancer (Jiricny, 1996; Kolodner, 1995; Krokan *et al.*, 2000; Lindahl and Wood, 1999; Loeb, 1998; Modrich and Lahue, 1996).

The idea that NM23-H2 might be a DNA repair protein followed our discovery that a conserved lysine residue, the signature nucleophile of a family of DNA repair enzymes, is the active site amino acid of the nuclease (Postel *et al.*, 2000a). We also recognized that many previously puzzling and unexplained properties of NM23/NDPk described in the literature, could be understood in the DNA repair context (Postel *et al.*, 2001a). For examples (1) The *ndk* gene of *E. coli* exhibits a mutator function in vivo (Bernard *et al.*, 2000; Lu *et al.*, 1995; Miller *et al.*, 2002), which is a phenotype of mismatch repair (MMR) defects (Jiricny, 1996; Loeb, 1998; Modrich, 1991; Modrich and Lahue, 1996). (2) *NM23-H1* transfected cell lines exhibit greater sensitivity to cisplatin, an alkylating agent, than control cells (Ferguson *et al.*, 1996), this being typically a phenotype of DNA repair defects (Aebi *et al.*, 1996). (3) Disruption of *nm23-M1* in mice, which is 88% homologous to *nm23-M2* and *NM23-H2*, leads to an array of phenotypes (Dabernat *et al.*, 1999; this volume), known to be associated with DNA repair defects. Although involvement of NM23 in DNA repair has not been shown directly yet, on the basis of the active site chemistry, and of the known phenotypic properties listed above, an obvious candidate mechanism is MMR and/or BER. Therefore, the important questions to be resolved are whether the inactivation of *NM23/NDPk*, like that of *E. coli ndk*, also leads to increased spontaneous mutation rates in mammalian cells, and whether NM23/NDPk is a DNA mutator by repair action in living cells.

The *E. coli* NDPk is a DNA Mutator

It has been suggested that the increased spontaneous mutation rates in *E. coli* strains lacking NDPk can be explained on the basis of nucleotide pool changes, with elevations of $>$20-fold in dCTP and $>$7-fold in dGTP levels (Bernard *et al.*, 2000; Lu *et al.*, 1995; Miller *et al.*, 2002). Indeed, the control of DNA precursor concentrations is known to be essential for the maintenance of genetic stability, and an imbalance in nucleotide pools can be mutagenic (Kunz *et al.*, 1994). The possibility that the mutator effect of NDPk deficient strains, and of the imbalance in the nucleotide pools, may be due to a defective mismatch repair (MMR) system, has been considered by Miller *et al.* (2002); however, they found no evidence of a mismatch glycosylase activity by *E. coli* NDPk, or by human NM23-H2, with respect to a number of different MMR substrates (Miller *et al.*, 2002).

An additional and interesting result, reported by Bernard *et al.*, (2000) and Miller *et al.* (2002), is that although the observed nucleotide imbalance was in the dCTP and dGTP pools, the mutations were inexplicably AT$\rightarrow$TA transversions that could not have resulted directly from elevated dCTP and dGTP pools. However, the AT$\rightarrow$TA mutations could have resulted from a defective DNA repair pathway, one of many associated with BER, which may require NDPk, and which is specific for AT$\rightarrow$TA transversions. It is possible, therefore, that in addition to the maintenance of nucleotide pool concentrations, NDPk in *E. coli* may be be able to prevent these types of mutations in DNA. Miller *et al.* (2002) also reported that deletion of *ndk* in a *mutS E. coli* strain resulted in a greater than 20-fold potentiation of AT$\rightarrow$GC transitions, rather than the AT$\rightarrow$TA transversions observed in the absence of NDPk alone. Miller *et al.* (2002) suggested that NDPk deficiency leading to these additional uncorrected mispairs is due to polymerase errors and affects on the editing capacity of cells. However, these results could also be interpreted to mean that NM23/NDPk acts as a cofactor in

MMR, or as a component of another pathway that overlaps with MMR, and that one reason why *MutS* may exacerbate the *ndk* deletion effect is that NM23/NDPk, a moderately sequence specific DNA-binding activity (Postel *et al.*, 1993), may require MutS, a more specific DNA recognition factor (Modrich and Lahue, 1996), to direct, or enhance, the binding activity of NM23/NDPk to its target DNA.

Is NM23 a DNA Mutator in mammalian Cells?

Another observation suggesting the involvement of NM23 in DNA repair is the cisplatin sensitivity of NM23-H1 transfected cells. Cisplatin is a known alkylating agent that induces both intra- and interstrand adducts between two adjacent purines, that are usually repaired by the excision repair process (David and Williams, 1998; Lloyd and Linn, 1993). Ferguson *et al.* (1996) observed that when K-1735 TK murine melanoma, MDA-MB-435 human breast carcinoma, and OVCAR-3 human ovarian carcinoma cells are transiently transfected with *NM23-H1*, they show increased sensitivity to cisplatin. It is possible that in these cells, ectopically expressed NM23 recognizes and binds to cellular DNA modified by alkylation and intercalation. MMR proteins are known to mediate the cellular response to cisplatin damage; however, paradoxically, they seem to sensitize, rather than protect the cell (Aebi *et al.*, 1966; Zdraveski *et al.*, 2002, and refs. therein). In the case of *NM23* transfected cells, this could result in excessive repair, or misrepair, of chromosomal damage by the inappropriately high levels of the cleaving activity.

In order to delineate the biological significance of NM23 enzymes, Dabernat *et al.* (1999; this volume), have directed considerable effort toward preparing mice lacking *NM23* genes. *Nm23-M1* disrupted mice exhibit high mortality rates (60%) after birth, premature senescence, reduced birth size, and defects in the immune system (Dabernat *et al.*, 1999; this volume), typical phenotypes of DNA repair defects. Moreover, *nm23-H1* null mice show predisposition to cancer, suggesting elevated mutation rates (Jiricny, 1996; Kolodner, 1995; Krokan *et al.*, 2000; Lindahl and Wood, 1999; Loeb, 1998; Modrich and Lahue, 1996), and the hypothesis that the role of NM23-H1 in these mice may be to prevent DNA damage from happening, as was suggested above to be the case for the *E. coli* enzyme.

Is NM23-H4 a Uniquely Mitochondrial DNA Repair Enzyme?

Mutations are known to increase in mitochondrial DNA during cancer, particularly in the so-called D-loop region, which contains the sequence CCCTCCCCCC, a frequent target for mutations, and a hot spot for deletions (Sanchez-Cespedes *et al.*, 2001). Interestingly, the D-loop sequence is virtually identical to the CCCTC-CCC repeated elements in the c-*MYC* NHE. In most cases in which mitochondrial DNA repair has been documented, oxidative change, or alkylation has been identified (Dianov *et al.*, 2001); in fact, mitochondrial DNA contains higher levels of oxidative DNA base damage than does nuclear DNA. Surprisingly, however, no *uniquely* mitochondrial BER/MMR enzyme has yet been isolated. NM23-H4 is a *uniquely* mitochondrial NM23 (Milon *et al.*, 2000). Aside from a 33 amino acid N-terminal sequence which targets H4 to the mitochondria, NM23-H4 is more than 60% identical to NM23-H2 in sequence, and has the same fold and hexameric structure. Most important, all of the catalytic residues essential for DNA cleavage and for NDP kinase catalysis are present, and we have observed a DNA binding and an associated DNA cleavage activity in NM23-H4 as well (E. Postel and M.-L. Lacombe, unpublished observations).

NM23 AND THE GENERATION OF GENOMIC ALTERATIONS VIA A RECOMBINATIONAL MECHANISM

Today DNA repair is no longer thought of only as the mechanism responsible for the correction of replication errors and of damages to DNA. Repair enzymes have been implicated in other DNA metabolic functions, including mitotic and meiotic recombination, recombinational repair, transcription, and transcription-coupled repair (Jiricny, 1998). For example, the tumor suppressor *BRCA1* is involved in repairing oxidative damage to DNA, in recombinational repair, and in transcription coupled repair (Leadon, 1999). Interestingly, *BRCA1*, *NM23-H1*, and *NM23-H2*, all map to the same chromosomal location (17q21), together with some other genes implicated in development and cancer, suggesting involvement in the same pathways. Indeed, in colon, lung, and breast cancer cells, *BRCA1* acts as a downstream effector of *NM23-H1* and *H2*, and of other DNA damage response genes (MacLachlan *et al.*, 2000).

Evidence suggests that NM23 also performs multiple and overlapping roles in transcription, repair, and recombination. First, NM23-H2 is required for transcriptional activation of the c-*MYC* gene both in vitro and in vivo (Berberich and Postel, 1995; Ji *et al.*, 1996; Postel *et al.*, 1993). In addition, NM23 is involved in the control of early myeloid development (Postel *et al.*, 2000b), and in the repression of a growth factor gene implicated in metastasis

control (Ma *et al.*, 2002).). Preliminary in vitro analyses have shown that the reaction between NM23-H2 and plasmid DNA containing NM23 target sequences yields accurately spliced recombinant DNAs from which segments of the c-*MYC* NHE have been deleted, suggesting that transcriptional activation of the c-*MYC* gene occurs via excisional recombinational (Postel, 1999; S. Gursky and E. Postel, unpublished results). Such a mechanism would be necessary for the removal of an inhibitory G-quadruplex structure from the c-*MYC* NHE, in order to activate transcription (Siddiqui-Jain *et al.*, 2002). Preliminary evidence also suggests that NM23 is involved in immunoglobulin class-switch recombination (CSR), which requires GC rich NHE-like sequences (Zou and Perlmutter, 2001). Moreover, were NM23 to function as a DNA mutator in mammalian cells, it could also direct somatic hypermutation (SHM) during antibody development, since SHM requires both a DNA cleaving activity, as well as the capacity for nonspecific binding to nucleotides. Moreover, CSR and SHM are known to act temporally, both require DNA cleavage, and both depend on transcription; indeed, they are believed to be catalyzed by the same mechanisms (e.g., Muramatsu *et al.*, 2000).

WHY SO MANY MAMMALIAN NM23/NDPK ISOFORMS?

The existence of multiple isoforms of NM23/NDP kinases in mammalian cells has been a constant source of puzzlement over the years. The human *NM23* family consists of eight genes, termed *NM23-H1* through *NM23-H8*, but only 1–4 are closely related and known to play critical roles in development (Lacombe *et al.*, 2000; Lombardi *et al.*, 2000; Venturelli *et al.*, 1995). The various isoforms are differentially distributed, with NM23-H1 and H2 being the most abundant, ubiquitous, and located both in nuclei and cytoplasm, while H4 is located in mitochondria (Lacombe *et al.*, 2000). There are several tiers of homology, e.g., *H1* and *H2* are 88% identical, and are localized to chromosome 17, while *H3* and *H4* are less homologous and less abundant, and are localized to chromosome 16. *NM23-H5* is located in the sperm axoneme (Munier *et al.*, 2001). The *NM23-H5–H8* isoforms are more distant relatives, and are less well characterized (Lacombe *et al.*, 2000).

One might predict that the various NM23 isoforms will have different DNA binding specificities, because the sequence-dependent DNA binding residues of NM23-H2 are not conserved, not even between H1 and H2 (Postel, 1998; Postel *et al.*, 1996). In fact, these two enzymes cleave DNA at different sites in the c-*MYC* NHE (E. Postel, unpublished), and in the *PDGF* NHE (Ma *et al.*, 2002). On the other hand, the amino acids participating in the catalytic events (NDPk, nuclease) are conserved. It is possible that the different isoforms have slightly different roles, e.g., H1 and H2 could be ubiquitous DNA metabolic enzymes in charge of cleaving different DNA sequences, or could perhaps be different DNA base mutators (GC vs AT, for instance), while H4 may be performing similar functions in mitochondria. Another possible reason for the existence of this many isoforms of NM23 may be to provide a redundancy of functions, which are critical to the cell.

INTERACTIONS BETWEEN BACTERIAL NDP KINASE AND DNA

E. coli NDP kinase, like its human homologues, binds duplex DNA oligonucleotides and supercoiled plasmid DNA and cleaves them in both strands (Levit *et al.*, 2002). This ability suggests a mechanism whereby *E. coli* NDP kinase might function in the enhancement of *OmpR*-dependent gene expression, which has previously been attributed to a protein phosphotransferase activity (Lu *et al.*, 1996). NM23-H2 and the *E. coli* enzymes are 45% identical, and share, besides the catalytic lysine and histidine, all of the active site residues important for DNA cleavage and NDPk activity. A DNA cleavage activity associated with a repair mechanism may also provide insight into the observed DNA mutator phenotype of ndk$^-$ *E. coli* strains (Bernard *et al.*, 2000; Lu *et al.*, 1995; Miller *et al.*, in press), discussed above. In preliminary studies, *M. tuberculosis* NDPk has also been observed to bind and cleave DNA (Singh, personal communication.).

A MODEL FOR THE BIOLOGICAL ACTIVITIES OF NM23/NDP KINASE

From the above discussions, a model emerges for the biological functions of NM23. *At the biochemical* level, NM23/NDPk is clearly multifunctional and participates in many events related to DNA metabolism, including nucleotide binding, nucleoside triphosphate synthesis (NDP kinase), transcriptional activation/repression, DNA binding, and DNA cleavage, which may itself be trifunctional (glycosylase, lyase, and a 3′-trimming activity). Whether these are separate activities, or whether they are integrated into a single mechanism, remains to be determined. *At the cellular level*, the normal function of NM23/NDPk is proposed to be the role of a transcriptional regulator of specific genes during specific stages of

development, using mechanisms of DNA recombination and repair. Normal levels of NM23 would be sufficient to prevent mutations and DNA alterations through normal damage repair. In cancer cells, NM23 can have opposing effects, depending on its expression levels. For example, when *NM23* is downregulated (e.g., in metastatic breast carcinoma and melanoma), there may be a deficiency in DNA repair, causing accumulation of chromosomal mutations and genomic alterations that accompany tumor progression (Malins *et al.*, 1996, 1998). Steps in tumorigenesis are often associated with elevated NM23 levels (e.g., Zhang *et al.*, 1997), suggesting that high levels of NM23 are mutagenic, due to excessive cleavage, too much repair, or misrepair. This may explain in part why NM23 levels must be so highly regulated.

CONCLUSIONS

For more than 40 years, NDP kinases have been known as housekeeping enzymes central to nucleotide metabolism (Lascu, 2000). Their recently demonstrated involvement in metastasis, carcinogenesis, and development, however, prompted a new round of experiments looking for additional biochemical activities. Despite hundreds of papers on this topic, the true function of NM23/NDPks has remained elusive. In 1993, our laboratory discovered a DNA-binding and transcriptional activation function for NM23-H2 (Postel *et al.*, 1993), and more recently, an associated DNA-cleaving activity that breaks double-standed DNA via a covalent protein-DNA interaction (Postel, 1999). Because covalent protein-DNA complexes are known to serve the role of breaking and rejoining DNA strands, we hypothesized that the function of NM23 during oncogenesis and development may be to regulate gene expression through structural transactions on DNA. Recent experiments aimed at understanding the underlying mechanisms of this novel NM23 function suggest that this mechanism consists of DNA recombination and/or repair. It is proposed here that NM23/NDPk is a multifunctional regulatory enzyme essential for gene expression *and* for the cellular defense against pathological accumulation of damage to DNA. Through a repair function, NM23/NDPk is involved in the generation and prevention of mutations, and, mutations being carcinogenic, it is also involved in the development and prevention of cancer. In addition to single nucleotide changes brought about by a mutator activity, the recombinational activity of NM23 could be a significant factor in the sequence alterations that occur in DNA during normal development and during metastasis and carcinogenesis.

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