Cleavage of DNA by Human NM23-H2/Nucleoside Diphosphate Kinase Involves Formation of a Covalent Protein-DNA Complex*

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The NM23 gene family in humans is implicated in differentiation and cancer, but the biochemical mechanisms are unknown. Most NM23 proteins have phosphotransferase (nucleoside diphosphate kinase) activity, and the second human isoform, NM23-H2, also binds to a nuclease-hypersensitive c-MYC promoter element through which it activates c-MYC transcription. It is shown here that this DNA binding can result in double-stranded breaks. The DNA breaks occur within repeated sequence elements in the linear nuclease-hypersensitive duplex and leave staggered ends with 5'-nucleotide-long 3'-extensions. The enzyme also cleaves supercoiled plasmid DNA to yield nicked circular and unit length linear products. The cleavage reaction requires only NM23-H2, DNA, Mg2+, and buffer, occurs in the absence of denaturing conditions, and can be reversed by EDTA. The cleaved DNA strands have free 3'-OH groups, and protein is attached to the 5'-phosphoryl ends. Transfer of 32P radioactivity from DNA to NM23-H2 has been observed, and a covalent polypeptide-DNA complex has been isolated and identified by Western blotting as NM23-H2. Since covalent protein-DNA complexes are known to serve the role of breaking and rejoining DNA strands, the present findings suggest that NM23-H2 is involved in DNA structural transactions necessary for the activity of the c-MYC promoter.

The NM23 gene was originally identified as a potential metastasis suppressor gene by virtue of its reduced expression in highly metastatic melanoma and breast carcinoma cells (1–3). It was found subsequently that NM23 expression varies in a wide spectrum of human cancers and that up-regulation of NM23 may play a role in the pathogenesis of tumors (4–6). The gene family is large and highly conserved (7, 8) and, in a wide variety of animal species, functions in normal cellular proliferation and development (3, 9, 10). Of the five distinct human genes identified to date (2, 8, 11–13), four are implicated in differentiation and development (3, 9, 10). Of the five distinct human NM23 isoforms (14), two are 88% identical in sequence and map 4 kb apart on chromosome 17q21–22 near the BRCA1 locus (17).

NM23-H1 and -H2 each encode 152 amino acid peptides that are the subunits of the A and B nucleoside diphosphate kinases (NDP kinase; see Ref. 18), respectively. NDP kinases are phosphotransferases that reversibly catalyze phosphoryl group transfers between nucleoside diphosphates and nucleoside triphosphates, exhibiting little specificity toward either the donor or the acceptor nucleotides. The intermediate in the reaction is a highly conserved histidine that becomes autokatalytically phosphorylated (see Ref. 19, for review). Crystallographic evidence indicates that NM23/NDP kinase is a hexamer of six identically folded subunits, enclosing a large (25 Å) central cavity. The hexamer has dipheral 3-fold (D3) symmetry and can be viewed as a dimer of trimers or a trimer of dimers, with the dimers exhibiting 2-fold symmetry (20, 21).

NM23-H2 was purified from overexpressing bacteria by ammonium sulfate fractionation and hydroxyapatite chromatography as described (23), with the addition of an ion exchange chromatography step prior to hydroxyapatite fractionation. The DEAE column retains the DNA and...
most bacterial proteins, including bacterial NDI kinase, whereas NM23-H2 is eluted with 50 mM Tris buffer, pH 8 (19). Protein preparations were apparently homogeneous as assessed by SDS-PAGE and hexameric as determined by size exclusion chromatography (28). Affinity-purified NM23-H2 was prepared by applying the 60–90% ammonium sulfate fraction to a Blue Sepharose (19) or Reactive Yellow agarose column (27) and subsequent elution with a NaCl gradient. NM23-H1 was purified by ammonium sulfate fractionation (60–90% saturation), elution from DEAE-cellulose with a NaCl gradient, followed by hydroxyapatite and size exclusion chromatography (23, 26).

Preparation and Radiolabeling of DNAs

Double-stranded oligonucleotides were prepared by mixing equimolar concentrations of each DNA strand in 10 mM Tris buffer, pH 7.0, 200 mM NaCl and by heating to 95 °C and slow cooling. After 5′-end labeling with [γ-32P]ATP and T4 polynucleotide kinase (Amersham Pharmacia Biotech), the oligonucleotides were gel-purified by electrophoresis and ethanol-precipitated. The 57-bp-long DNA substrate was obtained by exonuclease III (Amersham Pharmacia Biotech) overnight at 37 °C; this treatment was repeated for an additional 6 h. One sample was further treated for 60 min with 500 μg/ml proteinase K. The reaction mixtures were precipitated with 10% trichloroacetic acid by incubation on ice for 30 min. The centrifuged pellets were rinsed with trichloroacetic acid, and resuspended in SDS-PAGE sample buffer. After boiling for 5 min the samples were resolved on 4–15% SDS-PAGE gels. The gels were stained with Coomassie Brilliant Blue for 1 h, destained overnight in 20% acetic acid, 10% methanol, and vacuum-dried prior to autoradiography. The gels were stained with 0.5 μg/ml ethidium bromide for 30 min, destained in water, and photographed under short wave UV illumination.

Analysis of Covalent Protein-DNA Complexes

Transfer of DNA 32P Label to NM23-H2—Uniformly labeled plasmid (25 ng, specific activity 1–2 × 107 cpm/μg) was mixed with an equal amount of unlabelled plasmid and with 4 μg of NM23-H2 and incubated in Reaction Buffer containing 40 mM KCl and 1.5 mM MgCl2 for 30 min at room temperature. The reaction was terminated by heating for 10 min at 90 °C, and the bulk DNA was exhaustively digested with 30 units of Dnase I (Roche Molecular Biochemicals) and 145 units of RNase A (Amersham Pharmacia Biotech) overnight at 37 °C; this treatment was repeated for an additional 6 h. One sample was further treated for 60 min with 500 μg/ml proteinase K. The reaction mixtures were precipitated with 10% trichloroacetic acid by incubation on ice for 30 min. The centrifuged pellets were rinsed with trichloroacetic acid and ethanol, dried, and resuspended in SDS-PAGE sample buffer. After boiling for 5 min the samples were resolved on 4–15% SDS-PAGE gels. The gels were stained with Coomassie Brilliant Blue for 1 h, destained overnight in 20% acetic acid, 10% methanol, and vacuum-dried prior to photography and exposure to x-ray film. Molecular mass standards were purchased from Life Technologies, Inc.

Western Analysis of Protein-DNA Adducts Isolated from Mobility Shift Reaction Products—Reactions of 57-bp DNA with 105-bp DNA fragments (26) 5′-end-labeled at both ends with [γ-32P]ATP (10:1 protein to DNA ratio) were first separated on native 5% acrylamide gels. The radiolabeled protein-DNA complexes were excised and eluted into buffer containing 50 mM Tris, pH 8, 0.1% SDS, 0.1 mM EDTA, 150 mM NaCl, and 0.1 mg/ml bovine serum albumin with gentle rolling for 6 h at room temperature. The eluted complexes were acetone-precipitated, dissolved in SDS sample buffer, boiled, and loaded onto 4–15% SDS-PAGE gels. The wet gels were exposed to film at 4 °C overnight, followed by immunoblotting as described previously (26). After the initial DNA-binding reaction, some of the samples were treated with 0.01% glutaraldehyde for 20 min in order to produce dimers and higher order NM23 oligomers (26) as markers for migration.

Determination of the Polarity of the Protein-DNA Linkage—Uniformly end-labeled 57-bp DNA fragments were incubated in standard reactions with varying amounts of KCl, followed by the addition of SDS, EDTA, and, as indicated, proteinase K treatment. The reactions were diluted 2-fold with sequencing gel-loading buffer, heated for 5 min at 90 °C, and loaded onto 5 or 8% sequencing gels.

RESULTS

NM23-H2 Cleaves Linear Duplex DNA—NM23-H2 was previously identified in our laboratory as a DNA-binding protein with affinity for a polyuridine/polyuridimidine sequence of the human c-MYC promoter, termed NHE (see Ref. 25; Fig. 1). In the context of these studies we noted that purified NM23-H2 preparations also cleaved the substrate DNA, provided it contained the NHE sequence (Fig. 2, lanes 2 and 3). Unrelated DNA or oligonucleotides that did not bind NM23-H2 (23) were not cleaved by the enzyme (Fig. 2, lanes 4 and 5), indicating that the cleavage of DNA, like the DNA binding, is sequence-
DNA Cleavage by NM23-H2/NDP Kinase

Fig. 2. DNA binding and cleavage by NM23-H2. Reactions in lanes 1–3 and 6–7 contained 10 mM 34-bp c-MYC oligonucleotide and lanes 4 and 5 the same amount of nonspecific 34-mers. Lane 2 reaction had 8 mM; lanes 3, 5, and 7 had 16 mM hexameric wild type NM23-H2; and lane 6 had 16 mM R34A mutant protein. MgCl₂ was 2 mM in each reaction and KCl was 70 mM. Arrows on the side panel point to positions of the complexed, free, and cleaved DNAs separated on 5% native acrylamide gels.

dependent. NM23-H2 mutant proteins known to be defective in DNA binding (26) showed significantly reduced cleaving activity in comparison with wild type (Fig. 2, lanes 6 versus 7). Additional experiments indicated that NM23 proteins purified by affinity chromatography through Blue Sepharose (19) or Reactive Yellow (27) also contained DNA binding and cleaving activity. These observations suggested that the cleavage of DNA by NM23-H2 depends on an intrinsic component of the wild type protein and that the cleavage is functionally related to the DNA binding activity.

Experiments examining the role of metal ions on DNA cleavage indicated that cleavage was stimulated by Mg²⁺ ions (Fig. 3A), whereas KCl was inhibitory (Fig. 3B). When EDTA was included in the reaction, the cleavage was virtually abolished (Fig. 3C, lanes 3 versus 2 and 8 versus 7). However, when EDTA was added at the end of the cleavage reaction followed by proteinase K treatment, the cleavages were restored almost completely in both the uniquely end-labeled 3' and 5' end DNA fragments (Fig. 3C, lanes 4 versus 2 and 9 versus 7). These results demonstrated that 1) DNA breaks occur in both strands and 2) that the breaks can be restored upon removal of divalent cations by EDTA treatment; the latter suggests that the cleavage reaction is mediated by a covalent protein-DNA complex.

The high energy cofactor ATP was not required for the cleaving activity; in fact, 1 mM ATP was inhibitory (Fig. 3C, lanes 5 versus 2 and 10 versus 7). Because the inhibition depended, to some extent, on the order of addition of ATP and MgCl₂, it was probably due in part to tying up Mg²⁺ ions. Since KCl or EDTA did not significantly affect the DNA binding, it seems likely that the binding and cleavage of DNA are separate steps and that the binding is a prerequisite for the cleaving activity.

Mapping of the Cleavage Sites on DNA Induced by NM23-H2—To confirm that the breaks occur in both DNA strands and to determine the size of the newly produced fragments, the products of the cleavage reaction were analyzed on sequencing gels (29). The use of denaturing gels allows the visualization of a break produced in a single strand that is uniquely end-labeled. With the 57-bp c-MYC DNA fragment labeled at the EcoRI restriction site, we located a strong cleavage site at 12 nucleotides from the 3'-end on the top strand (Fig. 4A, lane 6). Several additional but less prominent cleavages were also noted in the top strand, all appearing between the first and second cytosine residues, counting from the 3'-end, of the CTC-CCCA directly repeated sequence (indicated by small arrows on the right side panel). A prominent break also occurred in the 5'-end-labeled bottom strand between the fifth and sixth guanines in the first repeat element, at 17 nucleotides from the 5'-end (Fig. 4A, lane 3). This indicated that the fragment that was cleaved off at the 5'-end was longer, by 5 nucleotides, than the fragment released from the 3'-end. Of several minor cleavages on the bottom strand, at least one other occurred between the fifth and sixth position in the next repeat element (lane 3, marked by a small arrow on the right side panel). These and several other independent experiments established the following: 1) DNA is cleaved in both strands; 2) the cleaved DNA ends are staggered with single-stranded 3' termini; and 3) the strand breaks occur ~5 bp apart. A schematic representation of these findings is shown in Fig. 4B. Chemical sequencing (29) of gel-purified DNA cleavage products confirmed the major cleavage sites on both strands.²

Cleavage of Negatively Supercoiled DNA by NM23-H2—Because the natural DNA-binding substrate of NM23-H2 is a potentially distorted DNA region that may be under superhelical stress, we considered the possibility that NM23 may have DNA topoisomerase-like activity, binding and cleaving this structure in order to relax it. To test for such an activity, NM23-H2 protein was incubated with supercoiled pUC19 plasmid DNA containing the 57-bp c-MYC, NHE sequence; the reaction was terminated with detergent and the protein removed by proteinase K treatment, and the products were monitored by horizontal agarose gel electrophoresis. Under standard DNA-binding conditions, i.e. in the presence of 120 mM KCl and saturating (stoichiometric) amounts of protein (greater than 1:2 hexamer to plasmid ratio), NM23-H2 had no relaxation activity and yielded products that comigrated with nicked (open circular) and cleaved unit length (linear) plasmids (Fig. 5A). In most cases, however, when the presumed “open circular” plasmids were gel-purified and subjected to a second round of electrophoresis, more than half of these structures comigrated with the linear products, indicating that the open circular products are a mixed population. Nonetheless, the conversion of negatively supercoiled plasmids to nicked circular DNA suggests the production of single-stranded breaks, whereas the presence of linear molecules signify double-stranded cleavage. The structure of these plasmid products, and the relationship of the nicking and cleaving, remain to be addressed. It should be noted here that the cleavage of plasmid DNA, like that of linear DNA fragments, occurs in the absence of detergent, although SDS can liberate additional fragments from one DNA end (see Fig. 7B). Therefore, SDS, EDTA, and proteinase K were routinely added at the end to terminate the reactions and remove NM23.

Plasmid cleavage, like the cleavage of linear DNA, was also inhibited by high (>200 mM) KCl concentrations. In the absence of KCl, however, and with limiting amounts of protein (less than 1:2 NM23 hexamer to plasmid ratio), NM23-H2 relaxed supercoiled plasmids in a stepwise manner, although the extent of the relaxation appeared to be limited to superhelical densities in between the supercoiled and linear population. Additional experiments will be needed to understand this activity.

Cleavage of plasmid DNA was inhibited by the addition of EDTA at the start of the reaction (Fig. 5B, lane 4 versus lane 3), indicating a requirement for divalent cations. When EDTA was added at the end of the reaction followed by proteinase K

² Molecular Biology Corelab students, Princeton University, and E. H. Postel, unpublished results.
³ E. H. Postel, unpublished results.
⁴ E. H. Postel and M. Feld, unpublished results.
treatment, the linear plasmid band disappeared, and most, but not all, of the nicked plasmids were reverted to supercoiled forms (lane 5). The presence of 1 mM ATP in the reaction inhibited the cleaving activity, although it allowed some nicking to take place (lane 6). These findings suggest that the cleavage of plasmid DNA is due to the same activity of NM23-H2 as that which cleaved linear DNA and that the cleavage of plasmid DNA is also a reversible reaction. When NM23-H2 was incubated with the monoclonal antibody 3E4 known to inhibit DNA binding (26), the cleaving and nicking activities were diminished, and the plasmid showed partial relaxation (lane 7). This interesting result implies that there may exist more than one region on the protein for DNA interaction. The DNA-binding defective mutant K135H was also defective in the cleaving activity (lane 8), whereas the related NM23-H1 protein behaved similarly to the wild type NM23-H2 (lane 9). Apparently, NM23-H1 either recognizes negatively supercoiled NHE DNA better than linear NHE DNA (to which it binds poorly; see Ref. 26) or it recognizes a different DNA sequence embedded in the pUC19 plasmid. Indeed, DNA binding by NM23-H1 to an upstream sequence in the platelet-derived growth factor promoter has recently been identified.5

Several novel plasmid bands were also produced by the reaction of NM23 with DNA, as indicated by asterisks on the side panels in Fig. 5, A and B. These new bands move more slowly and are not present in the reactions with DNA only (lanes 1). Since the mobilities of these plasmid forms are not affected by proteinase K treatment, they are unlikely to be caused by retardation through protein binding and may thus be catednated multimers or other products of recombination. Conditions are under investigation that will permit higher frequencies of such NM23-induced plasmid multimerizations.

Transfer of Radioactivity from Uniformly 32P-Labeled Plas-
mid DNA to NM23-H2—The reversibility of DNA cleavage by NM23-H2 implied that a covalent protein-DNA intermediate is involved in these reactions. Such covalent intermediates are common energy sources for strand breakage and reunion by DNA topoisomerases and site-specific recombinases (30–35).

To determine if NM23-H2 indeed becomes covalently linked to DNA during the cleavage reaction, the enzyme was reacted with uniformly $^{32}$P-labeled plasmid DNA, heat-denatured, the bound DNA hydrolyzed, and the products analyzed on denaturing SDS-PAGE gels. The Coomassie Brilliant Blue stained gel is shown on the left panel of Fig. 6A. Autoradiography of the same gel indicates that transfer of radioactive phosphate to the protein has taken place, as radiolabel is present only in the NM23-H2 17-kDa peptide (Fig. 6A, lane 3, right-hand panel).

In control experiments without NM23, nuclease treatment of the plasmid completely eliminated the acid-precipitable radiolabel (lane 2), whereas proteinase K treatment of the complex digested NM23-H2 and caused the radiolabel to become acid-soluble (lane 4). This experiment indicates that transfer of radioactive phosphate to the protein has taken place, as radiolabel is present only in the NM23-H2 17-kDa peptide (Fig. 6A, lane 3, right-hand panel).

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Western Analysis of NM23-H2-linear DNA Complexes Purified from Mobility Shift Gels—To probe the covalent interaction further, we examined the stability of the linkage between NM23-H2 and radiolabeled DNA in the complexes resolved on native polyacrylamide gels (e.g. Figs. 2 and 3). The shifted complexes were excised from the gel, purified, and subjected to electrophoresis in the second dimension on denaturing SDS-PAGE gels. Autoradiography of the SDS-PAGE gel identified the labeled DNA component (Fig. 6B, left panel), and subsequent Western blotting identified the NM23-H2 peptide (Fig. 6B, right panel). Clearly, NM23 and DNA purified from the complex have the same mobility on denaturing SDS-PAGE gels (~26 kDa). This mobility appears to be in between that of monomeric NM23-H2 (17 kDa) that has not been exposed to DNA treatment (lanes 3) and dimeric NM23-H2 (~34 kDa), produced by chemical cross-linking (lanes 2 and 4; see Ref. 26). These results confirm that the polypeptide in the purified protein-DNA complex is NM23-H2 covalently bound to the radiolabeled DNA fragment.

NM23-H2 Is Attached to the 5' Terminus of the Broken DNA Strand—To determine which of the DNA termini at the cleavage site formed the covalent linkage, we analyzed the effect of protein association, with and without proteinase K treatment, on the electrophoretic mobility under denaturing conditions of uniquely end-labeled DNA fragments. Such experiments are used commonly to demonstrate the polarity of the covalently linked DNA strand. However, interpretation of the data with NM23 may be complicated by the fact that significant DNA breakage already occurs in the absence of added detergent,
FIG. 5. Cleavage of negatively supercoiled plasmid DNA by NM23-H2. A, reactions were assembled in 120 mM KCl, 1 mM MgCl₂, 2.4 mM pUC19myc plasmid, and 0, 2.5, 5, 7.5, 10, and 15 nM NM23-H2 hexamers (lanes 1–6). Marker in lane 7 is BamHI-linearized, and in lane 8, DNase I-nicked (28) plasmids. Gel was run at 100 V for 1.5 h in 0.5 × TBE, 0.1% SDS. B, Ten nm each of wild type NM23-H2 (lanes 1 and 3–7), K135H mutant (lane 8), and NM23-H1 proteins (lane 9) were reacted with 2.4 nM pUC19myc plasmid. KCl was 120 mM and MgCl₂ 1 mM. Lane 4 had 10 mM EDTA at the start of the reaction, and in lane 5 EDTA was added after the reaction, followed by proteinase K treatment. Lane 6 had 1 mM ATP. In lane 7 NM23-H2 was incubated overnight at 0 °C with monoclonal antibody 3E4 (26) before plasmid addition. Gel was run at 100 V for 1.5 h in 0.5 × TAE buffer. Arrows on side panels indicate plasmid states: SC, supercoiled; Lin, linear; OC, open circle/nicked circular. Species migrating behind the monomeric OC are supercoiled and nicked dimers intrinsic to the plasmid preparations. Asterisks mark high molecular weight DNA of unknown origin. All lanes are from the same gel, although not necessarily adjacent.

FIG. 6. Covalent complex formation between NM23-H2 and DNA. A, transfer of 32P radioactivity from plasmid to NM23-H2. Left panel shows photograph of SDS-PAGE gel after Coomassie staining, and the right panel shows autoradiogram of the dried gel. Lane 1 has pure unreacted NM23-H2 without any DNA or treatment, except it was autophosphorylated with γ-[32P]ATP. Lane 2, reaction without NM23; lane 3, result of NM23 incubation with DNA; lane 4, same as lane 3 after proteinase K digestion. Faint Coomassie-stained bands at ~34 kDa in lanes 2 and 3 are DNase I used for plasmid digestion. The strong band in lane 4 is proteinase K. Left lane shows molecular mass standards in kDa. Arrow on the right points to the 17-kDa NM23-H2 peptide. B, complexes formed between NM23-H2 and 32P-linear DNA fragments were first separated on native acrylamide gels, purified, and then subjected to denaturing electrophoresis in SDS-PAGE gels. Left panel is autoradiogram of the wet gel; right panel shows the immunoblot of the same gel. Reactions in lanes 2 and 4 contained, in addition to the 17-kDa NM23-H2 monomeric peptide, dimers and higher order oligomers generated by glutaraldehyde cross-linking (26). Left panel shows the molecular mass in kDa of monomeric, DNA-complexed, and dimeric NM23-H2.

DISCUSSION

Numerous observations in the past have caused speculation that the NM23/NDP kinase family of proteins may perform a more sophisticated role in cell physiology than merely catalysis of nonspecific phosphoryl group transfer (3, 19, 38). Buttressing these ideas was our discovery that NM23-H2/NDP kinase-B is a DNA-binding and transcriptionally active protein (23, 39). The present study demonstrates that human NM23-H2 indeed has additional activities and that these are related to DNA
It is shown here that NM23-H2 cleaves the c-MYC NHE promoter element, its natural duplex DNA substrate, site-specifically and within the directly repeated 5'-CCTC-CCCA motifs. These cleavages have staggered, 5-nucleotide-long 3'-overhangs. The enzyme also cleaves negatively supercoiled plasmid DNA containing the NHE element, yielding linear, unit length fragments and nicked circular products, and, at a low frequency, generating plasmid multimers.

DNA cleavage is stimulated by divalent cations and is inhibited by EDTA, salt, and ATP. The cleaving activity requires stoichiometric rather than catalytic amounts of NM23, suggesting that two or more protein molecules are necessary to effect a double-stranded DNA break. Cleavage is not dependent on the addition of detergent, proteinase K, or drugs, unlike the breaks induced by topoisomerases, suggesting that NM23 is not holding the DNA strands together during the reaction. However, the cleavage reaction can be reversed by EDTA, indicating that NM23-H2 has the ability to reseal the broken DNA strands.

A reversible DNA cleavage reaction requires breakage and rejoining of DNA strands through a covalent enzyme-DNA complex (31, 32, 40, 41), and these data suggest that NM23-H2 is capable of forming such covalent bonds. This was confirmed by the following experiments. First, transfer of 32P radiolabel from DNA to protein was detected. Second, the retarded complex in mobility shift gels contained NM23-H2, identified by antibody, bound to DNA in a covalent complex. Third, linkage to the 5'-end and not to the 3'-end of the cleaved DNA strand of the SDS-trapped protein was demonstrated, leaving the 3'-hydroxyl groups free. All of these findings are consistent with the conclusion that NM23-H2 can break and reseal the phosphodiester bond of DNA through a covalent enzyme-DNA complex. Since covalent protein-DNA complexes are known to serve the role of breaking and rejoining DNA strands, the present findings support our earlier proposal (23, 39) that NM23-H2 is involved in modifying the structure of the promoter necessary for the activity of the c-MYC gene. We have taken steps to confirm the covalent bond formation by identifying the nucleotide and amino acid residues in the complex and by determining the chemical nature of their linkage.

A combined set of criteria strongly suggests that the activities described in this paper are not caused by a trace bacterial contaminant. First, DNA cleavage by the wild type protein is independent of the enzyme purification procedure. Second, mutant proteins deficient in DNA binding activity, which were purified by the same procedure as the wild type protein, were defective in the cleaving activity. Third, NM33-H1, an acidic protein, also cleaves plasmid DNA, although it is purified differently from NM23-H2 (which is basic; see Ref. 19), and would, therefore, be expected to copurify with a different set of contaminants. Most important, however, is the observation that the broken DNA strands contained NM23-H2 polypeptides in a covalently bound form, strongly suggesting that the properties of NM23-H2 described in this paper are intrinsic and that the same activity that breaks DNA is the one responsible for its resealing.

Although these data are still too preliminary to suggest a mode of interaction of NM23 with DNA, the inhibition by ATP and the stimulation by Mg2+ of DNA cleavage is interesting in light of the inherent NDP kinase activity. NM23 binds ATP in

**Fig. 7.** NM23-H2 binds to cleaved DNA at the 5'-phosphoryl end and has free 3'-hydroxyl groups. A, reactions were assembled in 120 mM KCl as for mobility shift assays (Fig. 3B) but were stopped with SDS, and in lanes 3 and 6 were further treated with proteinase K. Lanes 1 and 4 had no NM23-H2. Reactions contained 120 mM KCl and were run in a 5% sequencing gel. DNA substrate on the left panel was 5'-end-labeled and on the right panel was a 3'-end-labeled 57-bp fragment. B, procedures were as in A, except the reactions contained 50 mM KCl and were run in an 8% sequencing gel. Note that the NM23-H2-cleaved fragment on the 3'-end is shorter than the fragment from the 5'-end (see Fig. 4). C, labeling of the 3'-OH end of the NM23-H2 cleaved DNA strand with [α-32P]ddATP and terminal deoxynucleotidyltransferase. Panel is an autoradiographic exposure of the dried down gel. Arrows on the left point to NM23-H2-cleaved and control BamHI-digested linear DNA fragments. Both lanes contained the same amount (50 ng) of DNA.
its NDP kinase active site, where ATP donates a phosphate to the autocatalytic histidine (19). The active site of NDP kinase is shared during the catalytic cycle both by the donor and the acceptor nucleotides, as well as by Mg$^{2+}$, and Mg$^{2+}$ greatly stimulates the turnover rate of the phosphotransferase activity (42). Thus, the inhibition of DNA cleavage by ATP suggests that, at least in part, ATP controls the cleavage through the NDP kinase active site and that Mg$^{2+}$ competes for the active site with ATP. In addition, ATP may tie up Mg$^{2+}$ ions directly, and the phosphorylation of the catalytic His-118 may also be inhibitory.

Enzymes that structurally modify DNA using a covalent protein-DNA complex are DNA topoisomerases (31, 32, 40, 41), and “conservative” site-specific recombinases (33–35). It is noteworthy that topoisomerase II and site-specific recombinases also require stoichiometric amounts of protein for their DNA cleaving activities (32–35). In cleaving both DNA strands, NM23 resembles a type II-like topoisomerase activity, although a type I-like action is also a possibility since these enzymes can also yield double-stranded breaks opposite nicks in the other strand (32). Clearly, though, NM23-H2 is not a typical DNA topoisomerase, for it does not seem to allow the plasmid to remain a covalently closed circle throughout the relaxation reaction. In terms of the chemistry of the cleavage site and the unusual DNA-binding surface of the protein, NM23 resembles resolvase-like recombination enzymes. A search in the data base, however, failed to detect significant amino acid sequence homologies between NM23-H2, topoisomerases, and recombinases.

It is an interesting question as to whether the NDP kinase and the DNA cleaving activities of NM23 are related to each other in vivo. It has been generally assumed that NDP kinases are of paramount importance to cell survival. As highly efficient phosphotransferases (the term “kinase” is a misnomer), they are thought to function in maintaining critical intracellular nucleotide concentrations, although the precise role of this activity in metabolism has not been demonstrated. Interestingly, in the case of the Drosophila NM23 protein AWD, it has been observed that although the catalytic histidine is necessary, it is not sufficient for AWD biological activity. This suggests that AWD/NM23 has another activity that is distinct from its role in the NDP kinase catalytic cycle, which also requires this histidine (38). One might suppose that this “other” activity is related to the DNA transactions demonstrated herein. Since covalent bond formation involves phosphoryl group transfer from DNA to protein, the phosphotransferase (NDP kinase) active site of NM23-H2 may well be involved in this reaction. For example, the conserved autocatalytic His-118 of NM23-H2 (18, 36) may play the role of activating nearby potential nucleophiles (e.g. Tyr-52 or Ser-120), in a reaction similar to that used by type I topoisomerases and Int recombinases (35, 43). Another possibility is, of course, that a functional relationship between the NDP kinase and the DNA transactions properties of NM23 does not exist and that the enzyme performs multiple and independent functions in DNA metabolism.

Chromatin structure is inextricably linked to transcription (32, 40). Specific topological changes and chromatin rearrangements are known to occur during different stages of both development and cancer and are presumed to modulate stage-specific transcription (44). A progression to the metastatic state in the case of human breast tumors, for example, involves structural modifications in DNA that are considerably different from the topological alterations associated with the formation of primary tumors (45). However, enzymes that can carry out such stage-specific topological changes in mammalian systems have not been identified. The human NM23 family of proteins is a good candidate for such a role, for there exist at least five isoforms that are differentially expressed (see Introduction and Ref. 39) and that may also form mixed oligomers in vivo (18).

The ability of NM23 (so far restricted to NM23-H2 and H1), to specifically recognize regulatory sequences in DNA and to specifically alter these sequences, might well be brought into play for a particular gene and at a certain step in the activation process. The nuclear localization of NM23-H2 in several tissues, including breast cancer cells (46), and its association with chromatin (47), have already been demonstrated.

As a key regulator of growth and differentiation and as a major factor in oncogenesis, the c-MYC gene is a likely target for selective regulation by sequence-specific DNA rearrangements. The c-MYC promoter, a natural substrate of NM23-H2, is comprised of a sequence (NHE), which is structurally distorted and may be inhibitory to transcription. This repression may be counteracted by NM23-H2 through alterations or removal of the inhibitory sequence, converting the NHE into a more typical B conformation, thereby providing access of conventional transcription factors to the promoter. The ability of NM23-H2 to alter the structure of the c-MYC promoter is consistent with the observations that in tumors where NM23 is overexpressed, alterations in the c-MYC gene structure are common (4–6, 16, 48–50).

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