The Binding of Ku Antigen to Homeomain Proteins Promotes Their Phosphorylation by DNA-dependent Protein Kinase*

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The Ku antigen (70- and 80-kDa subunits) is a regulatory subunit of DNA-dependent protein kinase (DNA-PK) that promotes the recruitment of the catalytic subunit of DNA-PK (DNA-PKcs) to DNA ends and to specific DNA sequences from which the kinase is activated. Ku and DNA-PKcs plays essential roles in double-stranded DNA break repair and V(DJ) recombination and have been implicated in the regulation of specific gene transcription. In a yeast two-hybrid screen of a Jurkat T cell cDNA library, we have identified a specific interaction between the 70-kDa subunit of Ku heterodimer and the homeomain of HOXC4, a homeomain protein expressed in the hematopoietic system. Unexpectedly, a similar interaction with Ku was observed for several additional homeomain proteins including octamer transcription factors 1 and 2 and Dlx2, suggesting that specific binding to Ku may be a property shared by many homeomain proteins. Ku-homeomain binding was mediated through the extreme C terminus of Ku70 and was abrogated by amino acid substitutions at Lys596/Lys596. Ku binding allowed the recruitment of the homeomain to DNA ends and dramatically enhanced the phosphorylation of homeomain-containing proteins by DNA-PK. These results suggest that Ku functions as a substrate docking protein for signaling by DNA-PK to homeomain proteins from DNA ends.

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1 The abbreviations used are: Ku70, Ku antigen 70-kDa subunit; Ku80, Ku antigen 80-kDa subunit; DNA-PK, DNA-dependent protein kinase, DNA-PKcs, DNA-dependent protein kinase catalytic subunit; Oct-1 and -2, octamer transcription factor 1 and 2, respectively; DBD, DNA binding domain; GST, glutathione S-transferase; HD, homeomain; EMSA, electromobility shift assay; PCR, polymerase chain reaction; aa, amino acid; nt, nucleotide; CREB, cAMP-response element-binding protein.

and specific DNA sequences (4, 5). Ku-deficient mice are proportional dwarfs that are immunodeficient and sensitive to DNA-damaging agents (7–10). They also suffer from genomic instability, undergo chromosome rearrangements, and develop T cell lymphoma (7, 11–14), while fibroblasts from Ku−/− mice undergo premature senescence in culture (8, 15).

Ku is an integral regulatory component of DNA-dependent protein kinase (DNA-PK), and many of its actions in the nucleus, including DNA repair, recombination, and transcriptional regulation, occur in concert with the catalytic subunit of DNA-PK (DNA-PKcs) (4, 16). DNA-PKcs is one of a family of large phosphatidylinositol 3-kinase-related nuclear kinases that also includes the ataxia telangiectasia gene product, ATM, and the TRAPP/PAF400 transcriptional cofactor (4, 17, 18).

DNA-PKcs binds nonspecifically to DNA and is activated from the ends of double-stranded DNA (19, 20). The interaction of DNA-PKcs with Ku promotes recruitment of the kinase to DNA ends, thereby enhancing its activation (4, 21). Ku also promotes the recruitment and activation of DNA-PKcs from specific DNA sequences (3, 4, 22). However, even when associated with Ku, DNA-PKcs is an inefficient kinase with a k_m for peptide substrates in excess of 200 μM (21, 23, 24). DNA-PK also displays only a modest specificity for substrates in vitro, with a preference for (S/T)Q motifs. How these in vitro properties of DNA-PK translate to what is presumed to be the specific and targeted phosphorylation of substrates in vivo, remains to be elucidated.

At least two mechanisms offer the potential for increasing the specificity and efficiency of substrate phosphorylation by DNA-PK. First, since the affinity of Ku/DNA-PKcs for DNA ends and sequences is several orders of magnitude higher than the k_m of DNA-PK for substrates, the colocalization of substrates with DNA-PK in cis on DNA can dramatically enhance their phosphorylation (3, 21, 22, 25). In one specific example, the glucocorticoid receptor is efficiently phosphorylated by DNA-PK in the presence of DNA molecules containing binding sites for both the receptor and Ku/DNA-PKcs. However, when the receptor binding sites are transferred to a second, covalently closed circular DNA molecule to which Ku and DNA-PKcs are not attracted, glucocorticoid receptor phosphorylation is strongly decreased (3, 22). Phosphorylation was also abrogated by introduction of a point mutation that disrupts the binding of glucocorticoid receptor to DNA (22). The consequence of this DNA sequence-specific phosphorylation is the specific regulation of promoters containing sequences from which the DNA-PK is activated (22, 26).

A second possibility is that substrates may be recruited to Ku/DNA-PKcs through specific protein–protein interactions with components of the DNA-PK complex. Recently, several proteins have been identified as interacting with Ku antigen.
For the most part the functional consequences of these interactions remain to be established. At least some of these Ku binding factors (e.g. hGCN5, the progesterone receptor, XRCC4, and c-Abl) are phosphorylated by DNA-PK in vitro (27–32). However, the contribution of Ku binding to their phosphorylation has not been evaluated.

In the present study, we have identified a specific protein-protein interaction between the C terminus of the Ku antigens and the homeodomains of a series of homedomain proteins that occurs in solution and leads to the recruitment of homedomain proteins to DNA ends. This interaction contributed to a greater than 50-fold enhancement of the phosphorylation of homedomain-containing proteins by DNA-PK. These results demonstrate that Ku serves a molecular scaffold for the recruitment of homodomain proteins to DNA ends for phosphorylation by DNA-PK and suggests that DNA-PK-mediated phosphorylation regulates the function of at least some homedomain proteins in response to DNA damage.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs—**Full-length human Ku70 cDNA and Ku80 cDNA were obtained from Dr. J. G. Parker (University of California, Santa Cruz). The two S. pombe mutant clones Ku70mt595–600-pAS1 and Ku70mtK595–6N-pAS1 were obtained by cloning the mutated Ku70 cDNA generated by PCR into pAS1. The Jurkat T-cell cDNA library in pGAD-10 was purchased from CLONTECH. Plasmid HOXC4-pACT2 was generated by cloning HOXC4 cDNA (nt 600–1421) isolated from a λgt11 cDNA library (see below) into pACT2. The T7-Met Gal4 activation domain was able to activate transcription sufficiently to induce detectable Gal4 activation domain fused to the T7 promoter and an oligonucleotide complementary to the 3′-end of the multiple cloning site of both vectors. To generate C-terminal Ku70 truncation or point mutants from the Ku70-pACT2 plasmid, the T7-Met Gal4 AD primer described above was used as forward primer in combination with Ku70-specific primers (clone N3H10; NeoMarkers) followed by incubation with protein A-Sepharose. To release Ku80 from the complex, beads were incubated in 100 μl of BC0 buffer (20 mM Tris (pH 8.0), 0.5 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin) containing 1 mM KCl and 1% sodium deoxycholate for 20 min and washed extensively in binding buffer (25 mM Hepes (pH 7.9), 60 mM KCl, 0.5 mM EDTA, 0.2 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.1% Nonidet P-40, 12% glycerol). Binding of 35S-labeled in vitro translated proteins to Ku dimer or Ku70 immobilized on beads was performed in binding buffer for 2 h at 4°C. Complexes were washed three times in WCE buffer with 0.1% Nonidet P-40, resuspended in SDS sample buffer, and resolved on SDS-polyacrylamide gel electrophoresis. Filters were washed and dried, and 35S-labeled proteins (typically 0.2–2 μCi of TnT reactions) were added to 100 μg of Jurkat whole cell extract diluted to 60 mM NaCl, and 0.1% Nonidet P-40 and extracts were incubated at 4°C for 2 h before the addition of the Ku70 antibody. Samples were then processed as above. To test Ku binding to the homeodomain, 0.5–1 μg of GST-Oct-1 POU or GST-HOXC4 HD (as indicated in the figure legends) was mixed with 35S-labeled in vitro translated Ku in binding buffer and processed as above.

HeLa cell nuclear extracts were prepared essentially as described (36). For co-immunoprecipitation, about 1 mg of nuclear extract was diluted down to 100 μM KCl with binding buffer and incubated with either Oct-1 antibody (YL15; Upstate Biotechnology, Inc., Lake Placid, NY) or nonspecific antibody (anti-glucocorticoid receptor (BuGR2)). Proteins were then transferred on membrane and hybridized successively with antibodies to Ku70 (NH310; NeoMarkers), Ku80 (clone 111; NeoMarkers) and Oct-1 (C-21; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). EMSA—EMSA was performed as described (26) using 32P-labeled oligonucleotides corresponding to the H2B Oct-2 binding site (5′-AGCT-AAACCTTTG-3′) as a probe. DNA-protein complexes were resolved on 4% polyacrylamide gels in 0.5× Tris borate buffer.

**DNA-PK Phosphorylation Assay—**Phosphorylation of recombinant proteins by purified DNA-PK (Promega) was performed essentially as described (26). Samples were denatured for 5 min at 95 °C and 10% of the total protein substrate was incubated for 20 min at 30 °C in kinase buffer (50 mM HEPES, pH 7.5, 100 mM KCl, 10 mM MgCl2, 0.2 mM Na2EGTA) in the presence of 20 ng of HaeIII-restricted calf thymus DNA, 5 μCi of 32P-ATP (6000 Ci/mmol; Amersham Pharmacia Biotech), and 8 units of DNA-PK (Promega).

**RESULTS**

Ku Antigen Interacts Physically with Several Homedomain Proteins through Determinants within the 70-kDa Subunit—To identify proteins that interact with the Ku70, we screened a Jurkat T cell cDNA library using full-length Ku70 fused to the Gal4 DNA-binding domain as the bait for interacting peptides. Expression of the Gal4p52Ku70 construct alone or together with Gal4 activation domain was able to activate transcription sufficiently to induce detectable β-galactosidase activity upon overnight incubation (Fig. 1B). This low level of transcriptional activity was consistent with previous reports of a weak acidic activator domain within the N terminus of Ku70 and was abrogated by deletion of the first 57 amino acids. However, this low level of activation did not prevent the detection of interactions with peptides expressed from the Jurkat cDNA.

C. Schild-Poulter and R. J. G. Haché, unpublished observation.
library that led to a strong induction of β-galactosidase activity through a specific interaction with Ku70.

Approximately 300 strongly positive clones were isolated in the original library screen. 80% of these induced β-galactosidase activity in the absence of the Ku70 bait, while a further 15% were completely negative. DNA sequence analysis of one of the remaining positive clones identified it as encoding a 94-amino acid peptide encompassing the homeodomain of HOXC4 (Fig. 1A). Interestingly, this HOXC4 peptide also displayed a significant ability to activate β-galactosidase expression in the absence of Ku70, with blue color being detectable after 6–7 h (Fig. 1B). Thus, this clone was almost missed in the secondary screen. Nevertheless, co-expression of this HOXC4 peptide with GalDBDKu70 led to a strongly enhanced activity, with the blue color being detectable after 6–7 h; (+), light blue by 24 h; −, white after 24 h.

To test the specificity of binding of Ku to these homeodomain proteins, we used a library that led to a strong induction of β-galactosidase activity through a specific interaction with Ku70. The library was screened for many protein-protein interactions that are crucial to homedomain protein function (39, 40). Since the Ku70-HOXC4 interaction mapped to the HOXC4 homeodomain, we sought to determine whether Ku70 could interact with other homedomain proteins.

Strikingly, immunoprecipitated Ku70 was bound specifically by all four of the additional homeodomain proteins tested (Fig. 2B): HOXD4 (41), a close orthologue to HOXC4 (lanes 1–3); zebrafish Dlx2 (42), a more distant family member (lanes 3–6); the POU homeodomain factor Oct-2 (43) (lanes 7–9); and the divergent homeodomain factor Oct-60 (44) (lanes 10–12). By contrast, CREB (45), a basic region/leucine zipper (bZip) transcription factor, did not interact with Ku70 (lanes 13–15). These homeodomain proteins interacted similarly with the Ku heterodimer, while CREB and a regulatory subunit of protein phosphatase 2A (PP2Aβ) did not interact with Ku dimer (data not shown). Last, the stringency of the washes employed in preparing the Ku70 for binding, which was sufficient to strip away Ku80 from Ku70, suggested that the interaction between Ku and the homeodomain proteins was likely to be direct.

To test the specificity of binding of Ku to these homeodomain proteins under conditions that more closely resembled the cellular milieu, we mixed the in vitro translated homeodomain proteins with the Jurkat whole cell extract prior to immunoprecipitation with the Ku70 antibody (Fig. 2C). Under these more stringent conditions, Oct-60 binding to Ku was no longer detected, demonstrating that at least some degree of specificity existed for Ku binding within the homeodomain protein family.
and compared with 10% of input proteins.

**Lane blot analysis showing the preparation of Ku70 and Ku70/80 dimer**

**Lane** 1, 25 μg of Jurkat T cell whole cell extract. **Lane** 2, the Ku dimer immunoprecipitated from Jurkat T cell extract with Ku70 antibody N3H10. **Lane** 3, removal of Ku80 subunit following KCl/deoxycholate treatment of the immunoprecipitate. **A** (right), 35S-labeled in vitro translated HOXC4 homeodomain (clone 18–27) was tested for binding to immunoprecipitated Ku70 (lanes 5), the immunoprecipitated Ku dimer (lane 6), or protein-Sepharose beads alone (lane 7) and compared with 10% of input proteins (lane 4). **B**, 35S-labeled in vitro translated proteins HoX4 (lanes 1–3), Dlx2 (lanes 4–6), Oct-2 (lanes 7–9), Oct-60 (lanes 10–12), and CREB (lanes 13–15) were tested for binding to protein A-Sepharose-bound Ku70 (Ku70) or protein-A-Sepharose alone (−). 10% of input proteins. **C**, 35S-labeled in vitro translated proteins HoX4, Dlx2, Oct-2, Oct-60, and CREB were incubated in whole cell extract prepared from Jurkat T cells and immunoprecipitated with Ku70 N3H10 antibody (Ku) or with protein A-Sepharose alone (−). **Lanes** 1 show 5% of input proteins.

**Fig. 2. Ku interacts with homeodomain proteins.**

**A** (left), Western blot analysis showing the preparation of Ku70 and Ku70/80 dimer used in binding assays. **Lane** 1, 25 μg of Jurkat T cell whole cell extract. **Lane** 2, the Ku dimer immunoprecipitated from Jurkat T cell extracts with Ku70 antibody N3H10. **Lane** 3, removal of Ku80 subunit following KCl/deoxycholate treatment of the immunoprecipitate. **A** (right), 35S-labeled in vitro translated HOXC4 homeodomain (clone 18–27) was tested for binding to immunoprecipitated Ku70 (lanes 5), the immunoprecipitated Ku dimer (lane 6), or protein-Sepharose beads alone (lane 7) and compared with 10% of input proteins (lane 4). **B**, 35S-labeled in vitro translated proteins HoX4 (lanes 1–3), Dlx2 (lanes 4–6), Oct-2 (lanes 7–9), Oct-60 (lanes 10–12), and CREB (lanes 13–15) were tested for binding to protein A-Sepharose-bound Ku70 (Ku70) or protein-A-Sepharose alone (−). 10% of input proteins. **C**, 35S-labeled in vitro translated proteins HoX4, Dlx2, Oct-2, Oct-60, and CREB were incubated in whole cell extract prepared from Jurkat T cells and immunoprecipitated with Ku70 N3H10 antibody (Ku) or with protein A-Sepharose alone (−). **Lanes** 1 show 5% of input proteins.

**Ku Interacts with Homeodomain Proteins**

To begin to examine the requirements within Oct-1 for Ku binding, we tested the ability of in vitro translated C-terminally truncated Oct-1 peptides (Fig. 4A) to bind the immunoprecipitated Ku heterodimer. Full-length in vitro translated Oct-1 and an N-terminal Oct-1 peptide truncated to the edge of the homeodomain bound specifically to immunoprecipitated Ku (Fig. 4B, lanes 1–6). However, truncation of the Oct-1 by a further 28 amino acids into the homeodomain abrogated binding (lanes 7–9), demonstrating the requirement of the homeodomain for Ku binding by Oct-1.

**Ku-Homeodomain Binding Is Abrogated by Site-directed Mutations in the Extreme C Terminus of Ku70**—To delimit the determinants of Ku required for homeodomain binding, we examined the binding of in vitro translated Ku to a GST-HOXC4 homeodomain fusion protein in pull-down assays (Fig. 5A). Full-length Ku70, but not Ku80, bound efficiently to the immobilized GST-HOXC4 homeodomain (lanes 2 and 5), while neither peptide bound to GST alone (lanes 3 and 6). Truncation of 26 amino acids from the C terminus of Ku70 eliminated HOXC4 binding (lanes 7–9) and 13–15). By contrast, binding was unaffected by truncation of Ku70 from the N terminus as far as amino acid 538 (lanes 10–12 and 16–21).

Alignment of the Ku70 C-terminal peptide sequence from several species revealed a six-amino acid motif, KKKQELL, that was highly conserved between vertebrate Ku70s (Fig. 5B). Further, although deletion of Ku70 C-terminal peptide to amino acid 603 had no effect on binding to GST-HOXC4, truncation to amino acid 594 eliminated Ku70 binding (Fig. 5C). Replacement of the KKKQELL motif with NNNMAHH abrogated the binding of full-length Ku70 to GST-HOXC4 (Fig. 5D, lanes 1–6). Substitution of Lys595 and Lys596 with Asn also eliminated binding of the C terminus of Ku70 to GST-HOXC4 (lanes 7–12), whereas E598Q had no effect on binding (lanes 13–15).

To confirm that Lys595 and Lys596 were required for the binding of Ku70 to homeodomain proteins, we compared the interaction of WTKu70 and Ku70K595N/K596N with full-length HOXC4 and Oct-2 in a yeast two-hybrid assay (Fig. 6). Both full-length Oct-2 and HOXC4 when expressed as fusion proteins with the Gal4 activation domain interacted strongly with the Gal4p-Gal4Ku70 construct to induce β-galactosidase activity, while Gal4p-Gal4Ku70 Rab was again negative. In contrast to WTKu70, however, both the m595–600 and the K595N/K596N substituted Ku70 Gal-DBD construct failed to interact with either HOXC4 or Oct-2. Thus, the interaction of Ku70 with homeodomain proteins is mediated through a conserved element and can be disrupted by a substitution of only two amino acids.

**Ku Binding Promotes the Phosphorylation of Homeodomain Proteins**

**By DNA-PK from DNA Ends**—To begin to examine the influence of DNA binding on the interaction between the homeodomain proteins, we compared the association of recombinant Ku with a GST-Oct-2 peptide containing the complete DNA binding domain of Oct-2 in EMSA (Fig. 7). GST-Oct-2 bound specifically to the octamer motif of the histone H2B gene but did not associate with a nonspecific oligonucleotide (lanes 2 and 10). By contrast, recombinant Ku bound to both oligonucleotides in the presence of 1 μg of calf thymus competitor DNA but did not bind when the competitor DNA was increased to 2.5 μg (lanes 3, 8, 11, and 16–18). Co-incubation of GST-Oct-2 with Ku resulted in the appearance of an additional complex on the nonspecific DNA that was specifically competed by an Oct-2 antibody (lanes 4–7), demonstrating that Oct-2 could associate with DNA end-bound Ku. Similarly, on the octamer motif, a Ku-Oct-2 complex was detected under conditions where Ku also bound to DNA ends (lanes 12–15). However, at 2.5 μg of competitor DNA, the Oct-2-octamer motif complex remained, but
Fig. 3. Co-immunoprecipitation of Ku and Oct-1 in HeLa cells. Proteins from HeLa nuclear extracts were incubated with an Oct-1 antibody (lane 2) or a nonspecific antibody (lane 3), precipitated with protein A-Sepharose and resolved on a 10% SDS-polyacrylamide gel. Proteins were transferred on membrane and hybridized with a Ku70 antibody (top). The blot was successively stripped and rehybridized with antibodies to Ku80 (middle) and Oct-1 (bottom). Immunoprecipitates are compared with Ku and Oct-1 found in 15 μg of HeLa nuclear extract (lane 1).

Fig. 4. The integrity of the homeodomain is required for Ku interaction. A, schematic representation of human Oct-1 indicating the position of the restriction sites used to prepare the Oct-1 homeodomain deletion mutants. B, binding of full-length tritiated in vitro translated Oct-1 (lanes 1–3) or Oct-1 deletion mutants (lanes 4–9) to immunoprecipitated Ku dimer from Jurkat T cell extracts (Ku) or to protein A-Sepharose (-) is compared with 10% of input (I).
no additional Ku-containing complex was detected (lanes 16–18), indicating that Ku did not associate with the DNA-bound Oct-2 fusion protein under these conditions. Last, the inclusion of 10 mM Mg²⁺, which has previously been shown to induce changes in the interaction between Ku and DNA (26), had no further influence the effect of DNA on the Ku-Oct-2 interaction (lanes 5, 13, and 17). These results were consistent with our subsequent observation that the presence of Ku did not significantly affect the activation of transcription by Oct-1 or Oct-2 from octamer motifs in transient transfection experiments (data not shown).

These results suggested that the basis for the functional consequence of Ku-homeodomain binding was likely to lie in the recruitment of homeodomain proteins to DNA ends. One possibility was that Ku-homeodomain binding could promote the phosphorylation of homeodomain proteins by DNA-PK. Full-length Oct-1 and Oct-2 are phosphorylated by DNA-PK in vitro, and Oct-1 contains at least two phosphorylation sites for DNA-PK, one of which is in the POU-specific domain (data not shown). To assess whether the interaction of Oct-1 to Ku bound to DNA ends could promote its phosphorylation by DNA-PK, we evaluated the contribution of the Oct-1 homeodomain for phosphorylation within the POU-specific domain peptide resulted in a proportional increase in phosphorylation (lanes 3–7). However, even at a 40-fold higher concentration, phosphorylation of this peptide remained 2.3-fold lower than obtained when the homeodomain was included.

To provide additional evidence that this difference in phosphorylation was due to recruitment of the POU-specific domain of Oct-1 to Ku by the homeodomain, we repeated the in vitro phosphorylation experiment with chimeric proteins in which we substituted the homeodomain of HOXC4 into Oct-1 (Fig. 8A). The HOXC4 homeodomain peptide was not appreciably phosphorylated by DNA-PK (Fig. 8C, lane 1), while the POU-specific peptide of Oct-1 was weakly phosphorylated (lanes 2–4). However, fusion of the homeodomain of HOXC4 to the N terminus of the POU-specific domain of Oct-1 resulted in a strong induction of phosphorylation within the POU-specific domain to a level equivalent to that observed with the WT Oct-1 peptide (lanes 5–8). Thus, Ku-homeodomain binding appears to strongly potentiate protein phosphorylation by DNA-PK.

**DISCUSSION**

In this study, we provide insight into two aspects of the mechanism of action of DNA-dependent protein kinase. First, our results demonstrate that the Ku antigen may serve as a scaffold or adapter protein for the attraction of DNA-PK substrates. Second, our results suggest that Ku binding may be a property of many homeodomain proteins. Together these results highlight the potential for the regulation of homeodomain protein function by DNA-PK in vivo.

We have determined in immunoprecipitation and two-hybrid experiment. 10% of input was loaded on the gel to compare the levels of binding. The arrows indicate the position of the complete in vitro translated proteins. B, alignment of the C-terminal amino acid sequence of Ku70 from several species. Amino acids displayed in boldface letters outline the highly conserved motif targeted for mutation. C, 35S-labeled in vitro translated Ku70 C-terminal peptides with C-terminal deletions of six amino acids (301–603) or 15 amino acids (301–594; lanes 1–7) were tested for binding to GST-HOXC4 HD as described for A. D, point mutations in the C-terminal domain of Ku70 abrogate homeodomain binding. GST pull-down assay using GST-HOXC4 HD was performed to test the binding of 35S-labeled in vitro translated full-length wild-type Ku70 (lanes 1–3) compared with Ku70 containing mutations of amino acids 595–600 (KKQELL to NNMAHH; lanes 4–6). Binding of the C-terminal Ku70 peptide (301–609, lanes 7–9) was compared with Ku70 peptides bearing a double point mutation of amino acids 595 and 596 (KK to NN, lanes 10–12) or a single point mutation of amino acid 598 (E to Q, lanes 13–15). In vitro translated products bound to GST-HOXC4 HD (GST-HD) or to GST alone (GST) were compared with 10% of input proteins (Input). Proteins were run on a 10% polyacrylamide gel.

**FIG. 5.** Ku70 determinants for homeodomain interaction. A, 35S-labeled in vitro translated Ku70 (lanes 1–3), Ku80 (lanes 4–6), and deletion mutants of Ku70 (lanes 7–21) were tested for binding to GST-HOXC4 homeodomain (GST-HD) or to GST alone (GST) by a pull-down assay. 10% of input was loaded on the gel to compare the levels of binding. The arrows indicate the position of the complete in vitro translated proteins. B, alignment of the C-terminal amino acid sequence of Ku70 from several species. Amino acids displayed in boldface letters outline the highly conserved motif targeted for mutation. C, 35S-labeled in vitro translated Ku70 C-terminal peptides with C-terminal deletions of six amino acids (301–603; lanes 1–3) or 15 amino acids (301–694; lanes 4–6) were tested for binding to GST-HOXC4 HD as described for A. D, point mutations in the C-terminal domain of Ku70 abrogate homeodomain binding. GST pull-down assay using GST-HOXC4 HD was performed to test the binding of 35S-labeled in vitro translated full-length wild-type Ku70 (lanes 1–3) compared with Ku70 containing mutations of amino acids 595–600 (KKQELL to NNMAHH; lanes 4–6). Binding of the C-terminal Ku70 peptide (301–609, lanes 7–9) was compared with Ku70 peptides bearing a double point mutation of amino acids 595 and 596 (KK to NN, lanes 10–12) or a single point mutation of amino acid 598 (E to Q, lanes 13–15). In vitro translated products bound to GST-HOXC4 HD (GST-HD) or to GST alone (GST) were compared with 10% of input proteins (Input). Proteins were run on a 10% polyacrylamide gel.
experiments that several homeodomain proteins interact through their homeodomains to Ku antigen through a motif contained within the extreme C terminus of Ku70. Binding was unaffected by dimerization of Ku70 with Ku80 and the binding of Ku to DNA ends but appeared to be exclusive of the sequence-specific DNA binding of the homeodomain protein.

Docking proteins that function as scaffolds to promote the assembly of protein complexes for phosphorylation are a common feature of phosphorylation signaling cascades. For example, SH2 and SH3 domains and specific adapter proteins determine the phosphorylation of downstream effector proteins by signal-dependent tyrosine kinases (47). Similar types of interactions have also been shown to be important for signaling through Ser/Thr phosphorylation (48). Our results indicate that substrate phosphorylation by DNA-PK may also be regulated through related protein-protein interactions employing Ku as the attractant or adapter protein for DNA-PK substrates.

The affinity and specificity of DNA-PK for its substrates described to date is modest. While a large number of proteins have been shown to have the potential to be phosphorylated by DNA-PK in vitro when incubated at high concentration with the kinase (21), the search for in vivo phosphorylation targets of Ku to DNA ends but appeared to be exclusive of the sequence-specific DNA binding of the homeodomain protein.

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Docking proteins that function as scaffolds to promote the assembly of protein complexes for phosphorylation are a common feature of phosphorylation signaling cascades. For example, SH2 and SH3 domains and specific adapter proteins determine the phosphorylation of downstream effector proteins by signal-dependent tyrosine kinases (47). Similar types of interactions have also been shown to be important for signaling through Ser/Thr phosphorylation (48). Our results indicate that substrate phosphorylation by DNA-PK may also be regulated through related protein-protein interactions employing Ku as the attractant or adapter protein for DNA-PK substrates.
for DNA-PK continues unsatisfied. Previously it has been demonstrated that colocalization of DNA-PK and potential substrates to the same double-stranded DNA molecule as a result of high affinity DNA binding can dramatically enhance their phosphorylation (3, 22, 25). We have recently extended these results to demonstrate that DNA-PK can also efficiently phosphorylate substrates from single-stranded DNA structures when these proteins are co-localized to the same DNA molecules.3 Our results here demonstrate that specific binding to Ku can also dramatically promote substrate phosphorylation by DNA-PK. This mechanism of promoting substrate phosphorylation alleviates the requirement for adjacent substrate DNA-binding sites and broadens the potential for DNA-PK phosphorylation to non-DNA-binding proteins.

The homeodomain is a 60-aminoc acid helix-turn-helix DNA binding domain whose primary sequence and secondary structure is highly conserved within the extended homeodomain gene family (37, 39, 40). In addition to participating in DNA binding, homeodomains have also been reported to participate extensively in protein-protein interactions with transcriptional modifiers and other factors (49–51). However, there is enough variation within the homeodomain to allow for discrimination in protein-protein interactions between even closely related family members. For example, although the homeodomains of Oct-1 and Oct-2 differ by only seven amino acids, including four conservative substitutions, only Oct-1 is targeted by the herpes simplex virus protein 16 and host cell factor (52). Nonetheless, there are also at least two precedents for the broadly based specific interactions between nuclear factors and the homeodomain. It has been reported that the transcriptional repressor N-CoR can interact with a diverse series of homeodomain proteins (53, 54). Second, a motif within the glucocorticoid hormone receptor has also been reported to interact with a series of homeodomain proteins in transfect ed cells, and ectopic expression of this motif in one-cell stage embryos dramatically affected embryonic development (55).

On the basis of the results reported here, it would be premature to extrapolate Ku70 binding and DNA-PK phosphorylation throughout the homeodomain protein family, particularly since Oct-60 binding was lost under stringent conditions. However, our results have demonstrated specific interactions between full-length HOXC4, Oct-1, Oct-2, and either the Ku binding domain whose primary sequence and secondary structure is highly conserved within the extended homeodomain gene family (37, 39, 40). In addition to participating in DNA binding, homeodomains have also been reported to participate extensively in protein-protein interactions with transcriptional modifiers and other factors (49–51). However, there is enough variation within the homeodomain to allow for discrimination in protein-protein interactions between even closely related family members. For example, although the homeodomains of Oct-1 and Oct-2 differ by only seven amino acids, including four conservative substitutions, only Oct-1 is targeted by the herpes simplex virus protein 16 and host cell factor (52). Nonetheless, there are also at least two precedents for the broadly based specific interactions between nuclear factors and the homeodomain. It has been reported that the transcriptional repressor N-CoR can interact with a diverse series of homeodomain proteins (53, 54). Second, a motif within the glucocorticoid hormone receptor has also been reported to interact with a series of homeodomain proteins in transfected cells, and ectopic expression of this motif in one-cell stage embryos dramatically affected embryonic development (55).

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How might Ku binding and DNA-PK phosphorylation affect homeodomain protein function? Homeodomain proteins are transcriptional regulators that direct specific gene expression programs (38, 40, 56, 57), whereas DNA-PK is activated from DNA ends in response to DNA damage (4). DNA damage induces multiple changes in cellular regulation including many changes in specific gene transcription. These changes contribute to arresting cell growth until the DNA damage is repaired or to directing irretrievably damaged cells into apoptosis (58, 59). Thus, our results suggest that one way in which DNA-PK may act from DNA ends subsequent to DNA damage is to modify the transcriptional regulatory potential of at least some homeodomain proteins.

Continuing experiments suggest that this may be the case at

3 S. Soubeyrand, H. Torrance, W. Giffin, W. Gong, C. Schild-Poulter, and R. J. G. Haché, submitted for publication.
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