Characterization of a Ku86 Variant Protein That Results in Altered DNA Binding and Diminished DNA-dependent Protein Kinase Activity*

(Received for publication, January 24, 1996, and in revised form, April 1, 1996)

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Three proteins known to play a critical role in mammalian DNA double-strand break repair and lymphoid V(D)J recombination are the autoantigens Ku86 and Ku70 and a 465-kDa serine/threonine protein kinase catalytic subunit (DNA-PKcs). These proteins physically associate to form a complex (DNA-PK) with DNA-dependent protein kinase activity. In this study, we demonstrate using electrophoretic mobility shift assays (EMSAs) that the nuclear DNA end-binding activity of Ku is altered in the human promyelocytic leukemia HL-60 cell line. Western blot and EMSA supershift analyses revealed that HL-60 cells expressed both full-length and variant Ku86 proteins. However, a combined EMSA and immunoanalysis revealed that the Ku heterodimers complexed with DNA in HL-60 cells contained only the variant Ku86 proteins. Finally, UV cross-linking experiments and DNA-PK assays demonstrated that the Ku complexes containing variant Ku86 had a greatly reduced ability to interact with DNA-PKcs, and that consequently HL-60 cells had severely diminished DNA-PK activity. These data provide important insights into the interaction between Ku and DNA-PKcs and into the role of DNA-PK in DNA double-strand break repair.

The exposure of mammalian cells to ionizing radiation (IR) induces lesions in chromosomal DNA such as strand scissions, single-stranded breaks, double-stranded breaks (DSBs), and base cross-links (1, 2). In particular, DNA DSBs appear to be the predominant cytotoxic lesions as even a single unrepaired DNA DSB can be a lethal event (3, 4). IR-sensitive (IRs) mutants have been isolated from hamster ovary (CHO) or lung (V79) cell lines, and in approximately half of these cell lines, IR sensitivity correlated with a greatly decreased ability to repair DNA DSBs (reviewed in Ref. 5). Thus, the DSB repair capacity of a cell appears to be a critical, although not the sole, factor in determining cellular IR sensitivity.

Lymphoid V(D)J recombination is a site-specific reaction that involves the assembly of noncontiguous genomic segments that encode the variable (V), diversity (D), and joining (J) elements of immunoglobulin and T cell receptor genes (for a recent review, see Ref. 6). Elegant analyses of V(D)J recombination products in vivo and in vitro strongly suggest that DNA DSBs are an essential intermediate in the V(D)J reaction mechanism (7-11). Thus, it is plausible that DNA DSB repair and mammalian V(D)J recombination may share some common factors. Consistent with this hypothesis, mutants have been identified that have defects in both pathways. Mice homozygous for the murine severe combined immune deficiency (scid) mutation, exhibit a profound immune deficiency in vivo, that is caused by a defect in V(D)J recombination coding junction formation, and they are also severely IRs due to a DNA DSB repair defect (reviewed in Refs. 6, 12, and 13). In addition, several Chinese hamster IRs mutants (V3, xrs-6, XR-1, xsi-1, xsi-2, xsi-3, and xsi-4) that are impaired in DSB repair were also shown to be defective for V(D)J recombination (14-19). Therefore, the genes defective in these mutant cell lines are strong candidates to encode factors which are involved both in DNA DSB repair and V(D)J recombination.

While the exact enzymology of mammalian DNA DSB repair is still unknown, a complex, which has DNA-dependent serine/threonine protein kinase activity (DNA-PK) (20, 21), and which consists of at least two components, the 465-kDa catalytic subunit (DNA-PKcs) and Ku protein (22-24), has been shown to be intimately involved in DNA DSB repair (reviewed in Refs. 13, 25-28). DNA-PKcs has been shown recently to very likely be the product of the scid gene (29-33), and it has long been known that animals homozygously defective at this locus were profoundly IRs and defective in DNA DSB repair (34-36). Ku is a heterodimeric protein of 70- and 86-kDa subunits which binds tightly to the ends of a variety of double-stranded DNA (37-40). Ku is thus thought to provide the DNA binding component for the DNA-PK holoenzyme. Recently, it was shown that members of the fifth x-ray cross-complementation group (XRC5) (5, 41), which are very IRs and DNA DSB repair-defective, lack Ku DNA end-binding activity (19, 42-44). Since the Ku86 gene maps to human chromosome 2q33-35 (45) and XRC5 group cell lines could be rescued by the same region (46). Ku86 was a strong candidate for the XRC5 gene. Consistent with this hypothesis, several groups independently reported that transfection of a Ku86 cDNA was able to rescue the defects of XRC5 mutants (18, 19, 47-49), and molecular defects in the Ku86 gene in XRC5 group cell line have been identified (49). Therefore, DNA-PK has been unequivocally identified as an impor-
tendant mammalian DNA repair complex and mutations in either the DNA-PKcs or the 86-kDa subunit of Ku result in severe IR and V(D)J recombination and DNA DSB repair defects.

We have recently demonstrated that the human promyelocytic leukemic HL-60 cell line was IR-resistant, but DNA DSB repair-proficient (50) and thus, in this latter regard, differed significantly from scid or XRCC5 mutant cell lines. In this study, we demonstrate that although HL-60 cells expressed both a full-length and a variant truncated version of Ku86, only the heterodimer of Ku70-variant Ku86 was complexed with DNA. This altered Ku complex had a greatly reduced ability to interact with DNA-PKcs and this resulted in severely diminished DNA-PK activity. These data provide important insights into the interaction between Ku and DNA-PKcs and into the role of DNA-PK in DNA double-strand break repair.

**EXPERIMENTAL PROCEDURES**

**Materials—** DNase I and RPMI 1640 medium were purchased from Life Technologies, Inc. Fetal bovine serum was purchased from Sigma.

**Cells—** HL-60 cells were purchased from American Type Culture Collection (Rockville, MD). Isolation of the HCW-2 cell mutant from HL-60 cells has been described (54). All cells were cultured in RPMI 1640 medium (Life Technologies, Inc.) supplemented with fetal bovine serum (20% for HL-60 and 10% for HCW-2 cells), 100 units/ml penicillin, 100 μg/ml streptomycin, and 5% CO2 at 37°C.

**Preparation of Nuclear and Cytoplasmic Extracts—** Cells were washed three times in phosphate-buffered saline. The cells were lysed in 10 ml of ice-cold lysis buffer A (10 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 0.25 mM spermine, 0.5 mM spermidine, 0.25% Nonidet P-40) on ice, and then briefly vortexed. The lysate was layered on top of 30 ml of ice-cold sucrose buffer (buffer A with 0.25 M sucrose) in a 50-ml centrifuge tube and centrifuged at 15,000 relative centrifugal force in an MSE MISTRAL 3000i centrifuge at 4°C for 5 min. The supernatant was removed by aspiration. The pellet was resuspended in lysis buffer A, and the nuclei were purified through sucrose medium twice. Nuclei were then lysed in NaCl extraction buffer (10 mM Tris-HCl, pH 8.0, 0.5 mM NaCl, 100 mM EDTA, 1 mM phenylmethylsulfonfluryl fluoride, and the lysate was incubated on ice for 10 min. An equal volume of 50% polyethylene glycol 8000 buffer was added to the lysate, and the incubation continued for another 10 min. The lysate was then centrifuged at 12,500 × g for 10 min at 4°C. The clarified supernatant was used as the native nuclear extract. To prepare cytoplasmic extract, nuclei were lysed in lysis buffer A in the presence of 1 mM phenylmethylsulfonfluryl fluoride. The lysate was immediately centrifuged at 12,500 × g for 10 min at 4°C. The clarified supernatant was used as native cytoplasmic extract.

**Digestion of DNA-Protein Complexes by DNase I—** A protocol used by Gottlieb and Jackson (23) to digest DNA-protein complexes was employed. The DNA-protein complexes were treated with 5 μl of DNase I solution (500 units of DNase I, 50 mM NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonfluryl fluoride, and 30 mM MgCl2) were added, and the mixtures were incubated at 37°C for 3 min and then subjected to electrophoresis in a 5% polyacrylamide gel under the conditions described for the gel mobility shift assay. Each sample was prepared in duplicate and an electrophoretic mobility shift assay was performed. One half of the gel containing one set of the samples was dried on a piece of Whatman paper and exposed for autoradiography. The duplicate samples were electroblotted into a Bio-Rad Trans-Blot chamber and detected as described previously (50, 54).

**Combined Electrophoretic Mobility Shift and Western Analyses—** Approximately 500,000 cpm of a 32P-radiolabeled double-stranded DNA (dsDNA) probe was mixed with 5 μg of nuclear extract and incubated on ice for 5 min under conditions described in the electrophoretic mobility shift assay. Each sample was prepared in duplicate and an electrophoretic mobility shift assay was performed. One half of the gel containing one set of the samples was dried on a piece of Whatman paper and exposed for autoradiography. The duplicate samples were electroblotted into a Bio-Rad Trans-Blot chamber and detected as described previously (50, 54).

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**UV Cross-linking—** A protocol used by Gottlieb and Jackson (23) to UV cross-link Ku70 and DNA-PKcs proteins to DNA was slightly modified. Briefly, approximately (10 ng) 500,000 cpm of a 32P-radiolabeled double-stranded 25-mer double-stranded DNA probe was mixed with 25 μg of nuclear extract in the presence or absence of 500 ng of unlabeled DNA in a final volume of 50 μl. The final mixture also contained 1 μg of pRSV-neo plasmid DNA, 200 μM NaCl, 2 mM EDTA, and 10 mM Tris-HCl, pH 8.0. The mixture was incubated on ice for 10 min, followed by UV irradiation at 254 nm in a UV Stratalinker (Stratagene) for 5 min at room temperature. Then, 15 μl of a DNase I solution (500 units of DNase I, 50 mM MgCl2, 10 mM CaCl2) was added, and the DNA was digested at 37°C for 20 min. Five volumes of acetic acid were added to each sample, and the precipitates were precipitated at −20°C for 15 h. The precipitated proteins were pelleted by centrifugation at 12,500 × g, dissolved in a urea-glycerol-SDS solution, and subjected to electrophoresis in a 5% polyacrylamide gel. The gel was electroblotted onto a nitrocellulose filter and exposed to x-ray film. DNA-PKcs on the same filter was identified subsequently by Western analyses.

**Downstream "P" IP Assays—** Briefly, 100 μg of nuclear extract protein was incubated with 50 μl of dsDNA cellulose (30 mg/ml) in 1 ml K buffer (25 mM Tris-HCl, pH 7.9, 10 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol, 2.5% glycerol) containing 60 mM NaCl for 30 min at 4°C (55). The DNA cellulose was then washed by centrifugation 3 times in 1 ml of 20 μl buffer (55). The DNA cellulose was then resuspended in 50 μl of potassium buffer. Using synthetic DNA-PK peptide substrates derived from the N-terminal transcriptional activation domain of murine p53 (wild-type peptide, EPPLSQEAFDLLKK; mutated peptide, EPPLSQEAFLD(L)KK), DNA-PK activity in an aliquot (13 μl) of the DNA cellu-lose was assayed as described elsewhere (55). To determine the status of Ku and DNA-PKcs in the kinase reaction mixture, aliquots of the reaction mixture were electrophoresed in 6% (for DNA-PKcs) or 10% SDS-polyacrylamide gel for (for Ku) under reducing conditions, proteins were transferred onto nitrocellulose filters which were subjected to Western blot analysis for the presence of DNA-PKcs and Ku70 and Ku86 proteins.

**RESULTS**

**IR Responses of HL-60 and a Clonal Variant, HCW-2—** We have recently demonstrated that the human promyelocytic leukemic HL-60 cell line was hypersensitive to x-irradiation, whereas a stable clonal variant of the HL-60 cell line, HCW-2, was IR-resistant (50). Interestingly, both cell lines were proficient for DNA DSB repair and appeared to repair DSB lesions with identical kinetics (50). In addition, both HL-60 and HCW-2 cells progressed to G2 following x-irradiation; however,
A Ku86 Variant Protein and Altered DNA-PK Activity

The Ku-DNA binding complexes formed with HL-60 nuclear extract exhibit a different mobility than those formed with HCW-2 nuclear extract. A, a 250-bp radiolabeled dsDNA probe was incubated with either no (0X), 0.2 μg (0.5X), 0.4 μg (1X), 0.8 μg (2X), 1.2 μg (3X), 1.6 μg (4X), 2.0 μg (5X), or 2.4 μg (6X) of HL-60 or HCW-2 nuclear extract as described under "Experimental Procedures" and then subjected to EMSA analysis on a 5% polyacrylamide gel. The positions of the free probe (f.p.) and some of the complexes formed with HL-60 nuclear extract (L1, L2, L3, and L4) and HCW-2 nuclear extract (H1, H2, H3, and H4) are shown with arrows. B, HL-60 extracts display altered Ku-DNA mobility even on a DNA substrate of minimal length. A 25-bp radiolabeled dsDNA probe was incubated with either 0.4 μg (1X) or 0.8 μg (2X) of HL-60, HCW-2, Jurkat, or HT-H9 nuclear extract and analyzed by an EMSA. The positions of the free probe (f.p.) and of the complex formed with HL-60 nuclear extract (L1) and HCW-2, Jurkat, or HT-H9 nuclear extract (H1) are shown with arrows.

HL-60 cells, unlike HCW-2 cells which eventually resided cyclin cycling, arrested at G2 for at least 48 h before they underwent apoptosis (50). Since much of the mammalian cellular response to x-irradiation appears to be mediated by the DNA-PK complex (reviewed in Refs. 13, 25–28), we investigated the status of this activity in HL-60 and HCW-2 cells. These results are presented below.

Ku-DNA Complexes of HL-60 and HCW-2 Cells Have Different Electrophoretic Mobilities—The DNA binding component of the DNA-PK complex is provided by the Ku70 and Ku86 autotigens and this binding activity can easily be detected by using dsDNA fragments in an electrophoretic mobility shift assay (EMSA) (43, 44). Thus, nuclear extracts were prepared from HL-60 and HCW-2 cells, mixed with a radiolabeled 250-bp dsDNA fragment, and then analyzed for Ku-DNA complex formation by an EMSA (Fig. 1A). The results consistently showed two features. First, as the concentration of nuclear extract in the binding assay was increased, the mobility of the protein-DNA complexes became slower. At least four complexes of different mobility were observed for both HL-60 (L1–L4) and HCW-2 (H1–H4). The slowest moving complex for both cells appeared when the protein concentration in the binding assay was approximately 2.0 μg. This mobility change in association with increased protein concentration has been observed in many laboratories and has been interpreted as multiple Ku heterodimers binding to the same DNA probe (37, 40, 56). This is presumed to occur because, once bound to the end of a dsDNA fragment, Ku has the ability to translocate internally on that fragment, thus freeing up the end for another Ku heterodimer to bind (37, 40, 57). More interestingly, however, was the observation that the mobility of HL-60 complexes was always faster than the corresponding HCW-2 complexes (Fig. 1A, compare L1 with H1, L2 with H2, etc.). These results suggested that the DNA-DNA complexes of HL-60 were different from those of HCW-2.

It has been demonstrated that a 25–30-bp dsDNA fragment is the minimum length required for the binding of a single Ku heterodimer (38). Thus, a 25-mer dsDNA probe was prepared and used for EMSA analysis with HL-60 and HCW-2 nuclear extracts. The resulting HL-60 complex (L1) still migrated faster than the corresponding HCW-2 complex (H1) (Fig. 1B). Thus the size differences observed on a larger fragment (Fig. 1A) could be observed with a minimal DNA binding site (Fig. 1B), and this was suggestive that the difference in DNA binding between the two cell lines was an inherent property of the Ku heterodimer and not due to additional factors.

We next surveyed a large number of human cell lines for their Ku DEB activity. Jurkat and HT-H9 T cell lymphoma cell lines showed the higher HCW-2 pattern (Fig. 1B) as did a variety of transformed and nontransformed cell lines derived from different tissues (data not shown). Thus, it appeared as if it was the lower (HL-60) Ku DEB pattern which was abnormal.

Altered Immunoreactivity of the HL-60 and HCW-2 Ku-DNA Complexes—We sought to identify the nature of the alteration in Ku-DNA complexes in HL-60 cells. As a first approach we attempted to confirm the presence or absence of Ku70 and Ku86 proteins in the HL-60 Ku-DNA complexes. This was achieved by using mAbs in EMSA "supershift" experiments. Thus, nuclear extracts were prepared from HL-60 and HCW-2 cells and incubated with a 250-bp radiolabeled probe in the presence or absence of mAbs specific for Ku polypeptides before being subjected to electrophoresis. Ab 162 (52, 53), was recently shown to recognize an epitope defined by the heterodimer of Ku70/Ku86. In the presence of this antibody, both the HL-60 (L1, L2, L3, and L4) complexes and the HCW-2 (H1 and H2) complexes were completely supershifted and retarded in the gel (Fig. 2). A different result, however, was obtained using mAb 111, which recognizes an epitope on the C terminus (defined by amino acids 610–705) of the Ku86 protein (51). This antibody clearly supershifted the HCW-2 Ku-DNA complexes although not as completely as mAb 162. The lower reactivity of mAb 111 in supershift assays has been previously observed in other laboratories and is probably an inherent property of this mAb (18). In contrast to HCW-2 extracts, however, mAb 111 did not affect the migration of the HL-60 complexes at all. A control monoclonal antibody specific for the human bcl-x proteins similarly did not affect the migration of the Ku complexes (Fig. 2). From these results we concluded that although both Ku70 and Ku86 were present in the HL-60 Ku-DNA complexes, the Ku86 C terminus was either altered or missing, such that mAb 111 could no longer react with it.

Detection of a Variant Truncated Ku86 in Nuclei of HL-60 Cells—To pursue the above observation, we next performed Western blot analyses of nuclear Ku proteins from HL-60 and HCW-2 cells using additional monoclonal antibodies that react against different epitopes of Ku. mAb N3H10 is specific for an epitope (defined by amino acids 506–541) in the C terminus of Ku70 (51). This mAb detected equal amounts of Ku70 proteins in nuclear extracts from HL-60 and HCW-2 (Fig. 3A). Not surprisingly, mAb 111 (specific for the C terminus of Ku86) which had failed to supershift HL-60 Ku-DNA complexes (Fig. 2), also failed to detect full-length Ku86 protein in standard HL-60 nuclear extracts (Fig. 3B, lane 1). Only by overloading the gel was Ku86 detected (Fig. 3B, lane 2). This was in sharp contrast to nuclear extracts prepared from HCW-2 cells, which...
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had easily detectable levels of full-length Ku86 (Fig. 3B, lanes 1 and 3) or 40 μg (lanes 2 and 4) of nuclear extracts were subjected to electrophoresis on a 10% SDS-polyacrylamide gel under reducing conditions. The proteins were then electrophoresed onto a nitrocellulose filter and detected using mAbs. A, mAb N3H10 (Ku70, amino acids 506–541); B, mAb 111 (Ku86, amino acids 610–705); and C, mAb S10B1 (Ku86, amino acids 8–221). The position of a 66-kDa marker protein electrophoresed in an adjacent lane is shown.

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under the conditions described for the EMSA. Before being subjected to electrophoresis in a 5% polyacrylamide gel, I were added, and the mixtures were then incubated at 37°C for 3 min; 150-bp DNA fragment and nuclear extracts were incubated on ice for 10 min, followed by UV irradiation and then DNase I digestion. The proteins were then precipitated with acetone, dissolved in a ureaglyceraldehyde buffer, and subjected to electrophoresis in a 5% polyacrylamide gel. The gel was electroblotted onto a nitrocellulose filter and exposed to autoradiography. Under these conditions, other laboratories have observed that significant cross-linking of the DNA probe occurs to DNA complexes. A level of cross-linked Ku70 in HCW-2 cells, since the former could be competed by excess unlabeled probe (Fig. 7A, HCW-2, + lane). When the same filter, from which the autoradiogram shown in Fig. 7A was derived, was subjected to a Western analysis using a monoclonal antibody directed against DNA-PKcs (20), a signal in all lanes was detected that precisely corresponded to the >205-kDa species (Fig. 7B). From these results we conclude several things. First, the >205-kDa species observed in the UV-cross-linking experiment is indeed DNA-PKcs. Second, that the Ku70 in HL-60 cells is not bound as tightly to DNA as the Ku70 in HCW-2 cells, since the former could be competed by excess DNA, whereas the latter could not. This conclusion is consistent with the altered DNase I specificity we previously observed (Fig. 6). Last, and most importantly, we conclude that the HL-60 Ku complex is greatly diminished in its ability to interact with DNA-PKcs.

Expression of Variant Ku86 in HL-60 Cells Results in Diminished DNA-PK Activity—Since the available evidence suggests that interaction of DNA-PKcs with Ku activates the protein kinase (29, 55, 58), we anticipated that nuclear extract derived from HL-60 cells would be deficient in DNA-PK activity. To experimentally test this hypothesis, DNA-cellulose was added to HL-60 and HCW-2 nuclear extracts and the DNA-PK complex was “pulled down” (55) by centrifugation. The resulting pellets were then incubated with a synthetic peptide (wild-type) derived from the N-terminal transcriptional activation domain of murine p53 (EPPLSQEAFADLLKK) (55), in the presence of [γ-32P]ATP. As negative controls, either no peptide or a peptide (mutant; EPPLSQEAFADLLKK), which is a poor DNA-PK substrate were also used to ensure the specificity of DNA-PK-catalyzed phosphorylation. As expected, the nuclear pull-down preparation of HCW-2 cells exhibited a significant amount of DNA-PK activity, whereas there was no detectable DNA-PK activity in the nuclear pull-down of HL-60 cells (Fig. 8A). To verify the status of Ku and DNA-PKcs, the proteins associated with the DNA cellulose were eluted and subjected to Western analysis. Under these conditions, other laboratories have observed significant cross-linking of proteins of 70 and >205 kDa (the approximate size of DNA-PKcs) (20, 21) in HCW-2 nuclear extracts (Fig. 7A, HCW-2, + lane). The cross-linking of both proteins could be diminished, but not abolished, by preincubating the nuclear extracts with an excess of unlabeled DNA probe (Fig. 7A, HCW-2, + lane). A level of cross-linked Ku70 similar to that observed in HCW-2 extracts was detected in the nuclear extracts of HL-60 cells (Fig. 7A, compare HL-60, + lane with HCW-2, + lane). This cross-linking could also be completely competed away with excess probe (Fig. 7A, HCW-2, + lane). Most impressively, however, was that only a minute amount of the >205-kDa species was cross-linked in HL-60 nuclear extracts. This cross-linking could also be completely competed away with excess unlabeled probe (Fig. 7A, HCW-2, + lane). When the same filter, from which the autoradiogram shown in Fig. 7A was derived, was subjected to a Western analysis using a monoclonal antibody directed against DNA-PKcs (20), a signal in all lanes was detected that precisely corresponded to the >205-kDa species (Fig. 7B). From these results we conclude several things. First, the >205-kDa species observed in the UV-cross-linking experiment is indeed DNA-PKcs. Second, that the Ku70 in HL-60 cells is not bound as tightly to DNA as the Ku70 in HCW-2 cells, since the former could be competed by excess DNA, whereas the latter could not. This conclusion is consistent with the altered DNase I specificity we previously observed (Fig. 6). Last, and most importantly, we conclude that the HL-60 Ku complex is greatly diminished in its ability to interact with DNA-PKcs.

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Western analyses. HCW-2 cell pull-downs contained full-length Ku86, Ku70, and DNA-PKcs (Fig. 8, B, C, and D, lane 2, respectively). In contrast, the pull-down of HL-60 cells contained full-length Ku70 (Fig. 8C, lane 1), only variant Ku86 (Fig. 8B, lane 1), and lacked DNA-PKcs (Fig. 8D, lane 1). These results demonstrate that DNA-PKcs is incapable of interacting with a DNA-bound Ku heterodimer containing a variant Ku86 subunit and this results in the loss of DNA-PK activity detectable by the pull-down assay.

DISCUSSION

In this study, we have shown that the nuclear DNA binding activity of Ku is altered in the human promyelocytic leukemic HL-60 cell line. Western blot and EMSA supershift analyses revealed that although HL-60 cells expressed both a full-length and a variant truncated version of Ku86, only the variant Ku86 was found to be complexed with DNA. The variant Ku86 complexes had a greatly reduced ability to interact with DNA-PKcs which resulted in severely diminished DNA-PK activity. The significance of these results is discussed below.

On the Nature of the Variant Ku86 Protein—There are multiple possible explanations for the appearance of the faster migrating species which we have termed variant Ku86. First, it is possible that the variant Ku86 is not Ku86 at all, but an immunologically related protein(s). However, the observation that three different monoclonal antibodies, 162 (Fig. 2), S10B1 (Figs. 3 and 4), and N9C1 (data not shown), which have been rigorously characterized as recognizing human Ku86 protein (52, 53), cross-react with the faster migrating Ku86 protein(s) argues strongly against this interpretation. Second, it is possible that the variant truncated Ku86 arises from an unusual spliced form of Ku86. While we cannot rule out this possibility, we have seen no evidence for a smaller Ku86 mRNA on Northern blots (data not shown). In addition, the observation that cytoplasmic extracts from HL-60 cells express full-length Ku86 (Fig. 5 and data not shown) argues strongly against the possibility that HL-60 cells contain an alternatively spliced or mutated Ku86 allele that simply expresses a truncated Ku86 protein. Instead, these results suggest that HL-60 cells express wild-type, full-length Ku86, but that either as the protein enters, or once it is inside, the nucleus it is proteolytically processed to a smaller form(s).

Last, it should pointed out that proteolytic cleavage of the C terminus of Ku86 has been previously observed. Paillard and Strauss (61) have shown that in vitro under certain incubation conditions that preparations of simian Ku86 could be proteolytically cleaved to a single species of 69 kDa. This truncated Ku86 resulted in an altered EMSA Ku-DNA binding profile that was similar or identical to the profile we observed with nuclear extracts from HL-60 cells. However, these authors showed that the protease inhibitors leupeptin and chymostatin or buffers with an elevated pH (8.0) would completely abrogate the cleavage event. We have made HL-60 nuclear extracts under all these conditions individually and in combination and we have not detected any inhibition of the cleavage event (data not shown). Resolution of this issue may come with the purification of the protease responsible for the cleavage event.

Multiple Roles for the C Terminus of Ku86—The observation that HL-60 cells contain a variant truncated Ku86 provides many interesting clues to the role of this protein in DNA repair. First, Ku has been identified to contain DNA-dependent ATPase (64) as well as ATP-dependent helicase (57) activities. In photoaffinity labeling experiments with [γ-32P]ATP both Ku subunits were radiolabeled (57). In particular, the putative ATP binding site in Ku86 was postulated (62) to reside within amino acids 677–689, which is in the portion of Ku86 that is apparently missing in the variant form. It can easily be envisioned that the loss of this ATP binding site may significantly affect either ATPase or the helicase activities. Second, we observed that a long (250 bp) dsDNA fragment complexed with variant Ku heterodimers was much more susceptible to DNase I digestion than full-length heterodimers (Fig. 6). This suggests that the variant Ku complexes do not bind as tightly to DNA or they are less capable of cooperative interaction. The observation that the variant Ku complexes, in comparison to full-length complexes, could be more efficiently competed away with excess competitor DNA in the UV cross-linking experiments suggests that DNA binding may indeed be affected (Fig. 7). Therefore, we favor a model where the presence of the C terminus of Ku86 is necessary for generating cooperative interactions between Ku heterodimers. Since it has long been known that the DNA end-binding activity of Ku resides solely or exclusively in the Ku70 subunit (Fig. 7) (23, 39, 56, 63, 64), the predominant role of Ku86 may be in mediating protein–protein interactions. The loss of interaction between Ku heterodimers could easily explain the increased DNase I sensitivity of the variant Ku-DNA complexes. Third, the greatly diminished levels of DNA-PKcs that can be UV cross-linked to DNA (Fig. 7) and the greatly reduced DNA-PKcs levels in DNA pull-down assays implies that the truncated Ku complexes fail to interact with DNA-PKcs. A simple interpretation would be that the C terminus of Ku86 directly interacts with DNA-PKcs, though we cannot rule out at this time that the loss of Ku86 sequences is affecting the conformation of the heterodimer and it is some other domain that is actually making contact with DNA-PKcs.

Implications for DNA DSB Repair—Cells defective in DNA-PKcs (scid) and Ku86 (sxi-3) cells are defective in DNA DSB repair (17, 34, 35), which suggested that a functional DNA-PK holoenzyme was essential for DNA DSB repair activity. Thus, it is surprising that HL-60 cells, which have been shown to be proficient for DNA DSB repair (50, 59), contain an altered Ku86 and greatly diminished DNA-PK activity (Fig. 8). One explanation for this could be that the sxi-3 mutant is completely null for Ku86 expression (18, 19), and while the molecular basis of the sxi mutation is unknown, scid cells contain undetectable amounts of DNA-PK activity (29, 30). In HL-60 cells, some nuclear full-length Ku86 is expressed (Fig. 3), and while we could not detect DNA-PK activity in DNA pull-down assays (Fig. 8) there was some, albeit greatly reduced, DNA-PKcs detectable in the UV cross-linking experiments (Fig. 7). This agrees with an earlier study which, using a different assay
system, suggested that HL-60 cells had very low, but detectable, DNA-PK activity (60). This implies that even low levels of DNA-PK may be sufficient to carry out DNA DSB repair. This interpretation is supported by the observation that, in comparison to human cells, rodent cell lines normally contain 50-fold less DNA-PK activity (55) and yet are perfectly capable of carrying out DNA DSB repair (17, 34, 35). Thus, in terms of DNA DSB repair, human cell lines may be more tolerant of alterations in DNA-PK activity.

Alternatively, the DNA DSB repair-proficient phenotype of HL-60 cells might also be explained if the presumed proteolysis of Ku86 is DNA-dependent and only occurs after the DNA-PK complex forms on DNA. In vivo then, once the DNA-PK complex is assembled, there could be a competition between the complex performing its function (i.e. DNA DSB repair) and inactivation of the complex through proteolysis of Ku86. This would contrast with the case of scid or xsi-3 cells, where there would be no opportunity for DNA repair since the complete DNA-PK complex cannot form.

A Novel Function for DNA-PK?—The original motivation for performing the experiments described above was the observation that following X-irradiation HL-60 cells arrested permanently at the G2 checkpoint whereas x-irradiated HCW-2 cells eventually resumed cell cycling (50). The subsequent demonstration that HL-60 cells contain reduced DNA-PK activity due to an alteration in Ku86 suggests that permanent G2 arrest may be a common feature of cells defective in DNA-PK (65). This observation would suggest that DNA-PK may provide an activity, distinct from its activity required for DNA DSB repair, that is necessary for DNA-damaged cells to traverse a G2 checkpoint. DNA-PK is clearly required for the efficient rejoining of broken chromosomes following DNA damage (17, 34, 35, 66, 67). Consequently, it could have been argued that DNA-PKcs or Ku mutant cells arrest in G2 not because DNA-PK activity is needed to progress through the G2 checkpoint, but simply because the cells arrive at that checkpoint with a significant amount of DNA damage. This, however, is unlikely to be the case for HL-60 cells because we (50) and others (59) have shown that HL-60 cells are proficient for DNA DSB repair. Together, these observations argue that DNA-PK may have at least two roles in mammalian cells: a function required for DNA DSB repair and a second function required for G2 checkpoint transition following DNA damage.

Acknowledgments—We thank Dr. J. ohn T. Leith for granting us unlimited access to his laboratory and his x-ray equipment.
Characterization of a Ku86 Variant Protein That Results in Altered DNA Binding and Diminished DNA-dependent Protein Kinase Activity
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J. Biol. Chem. 1996, 271:14098-14104.
doi: 10.1074/jbc.271.24.14098

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