The C Terminus of Ku80 Activates the DNA-Dependent Protein Kinase Catalytic Subunit

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Ku is a heterodimeric protein with double-stranded DNA end-binding activity that operates in the process of nonhomologous end joining. Ku is thought to target the DNA-dependent protein kinase (DNA-PK) complex to the DNA and, when DNA bound, can interact and activate the DNA-PK catalytic subunit (DNA-PKcs). We have carried out a 3′ deletion analysis of Ku80, the larger subunit of Ku, and shown that the C-terminal 178 amino acid residues are dispensable for DNA end-binding activity but are required for efficient interaction of Ku with DNA-PKcs. Cells expressing Ku80 proteins that lack the terminal 178 residues have low DNA-PK activity, are radiation sensitive, and can recombine the signal junctions but not the coding junctions during V(D)J recombination. These cells have therefore acquired the phenotype of mouse SCID cells despite expressing DNA-PKcs protein. Interaction between DNA-PKcs and Ku, involving the C-terminal region of Ku80, is required for DNA double-strand break rejoining and coding but not signal joint formation.

To gain further insight into important domains in Ku80, we report a point mutational change in Ku80 in the defective xrs-2 cell line. This residue is conserved among species and lies outside of the previously reported Ku70–Ku80 interaction domain. The mutational change nonetheless abrogates the Ku70–Ku80 interaction and DNA end-binding activity.

DNA-dependent protein kinase (DNA-PK) is a complex comprising subunits of 70 and 80 kDa (Ku70 and Ku80), and a large catalytic subunit, DNA-PKcs (7, 14). Ku has non-sequence-specific double-stranded DNA (dsDNA) end-binding activity and, when DNA bound, can interact with DNA-PKcs, enhancing its kinase activity. DNA-PK functions in a major pathway for rejoining DNA double-strand breaks (DSBs) in mammalian cells termed nonhomologous end joining (NHEJ) (for reviews, see references 18 and 23). This process is additionally used to recombine the site-specific DNA DSBs introduced during the process of V(D)J recombination. Cell lines defective in components of DNA-PK are both radiation sensitive, since the major lethal lesion induced by ionizing radiation is a DNA DSB, and defective in their ability to carry out V(D)J recombination (19). Compatible with these cellular studies is the finding that mice with disrupted components of DNA-PK exhibit a severe combined immunodeficiency (SCID) phenotype and display radiation sensitivity (1, 3, 11, 17, 31, 49).

However, SCID mice, which are defective in DNA-PKcs, have a less severe V(D)J recombination defect than do Ku-defective mice and cell lines. Mutants with mutations in Ku are defective in recombining the two distinct types of junctions that arise during the process, namely, signal and coding joints. In contrast, signal joint formation can proceed largely unpained in mutants defective in DNA-PKcs, although the possibility remains that there is some residual kinase activity in the DNA-PKcs-defective mutants analyzed to date which is sufficient for signal joint but not coding-joint formation. This caveat notwithstanding, it appears that Ku has a function independent of its role as a component of DNA-PK (for reviews, see references 12, 23, and 43).

Neither Ku70 nor Ku80 cDNA has any obvious motifs (28, 37, 48). Ku80 appears to require association with Ku70 for DNA binding, although Ku70 has been reported to show Ku80-dependent and -independent DNA binding (45). Ku has been reported to have ATP-dependent helicase activity, but both Ku70 and Ku80 lack a helicase domain (44). Ku has been reported to have ATPase activity following autophosphorylation, but mutations in putative ATP-binding sites do not impair the ability of Ku70 or Ku80 to correct defective cell lines (4, 24, 39). Several approaches can provide insight into the identification of functional domains within Ku70 and Ku80. Multiple rodent cell lines defective in the causal mutational changes could potentially provide insight into functionally important domains. To date, however, such analysis has shown that either the mutants lack Ku80 expression or the mutations result in large deletions or truncations due to a changed reading frame (9, 29, 39).

Although important in verifying that Ku80 is the gene defective in group 5 mutants, these studies have provided little insight into important functional domains. Other studies have examined the regions of Ku70 and Ku80 required for heterodimer formation by using the two-hybrid system and by analysis of in vitro-expressed cDNA fragments (5, 32, 46). The results show some discrepancies but, taken together, define a minimal domain of 28 amino acids from the central region of Ku80 (residues 449 to 477) that is required for Ku70–Ku80 interaction. Additionally, amino acid residues 334 to 449 of Ku80 appear to be required for DNA end-binding activity (46). Two studies have reported aberrant Ku80 proteins in human cell extracts that apparently lack the C terminus and have DNA end-binding but not DNA-PK activity, suggesting that the C-terminal region of Ku80 might be involved in interacting with DNA-PKcs (16, 30). Ku has been widely recognized as the DNA-
binding component of DNA-PK that activates the catalytic subunit (DNA-PKcs) when DNA bound. The lack of DNA-PK activity in both Ku-defective and DNA-PKcs-defective cell lines has substantiated these in vitro findings (2, 10, 35). Recent studies, however, have challenged this view, showing that DNA-PKcs can bind to linear DNA fragments and function as a protein kinase in the absence of Ku and that Ku merely stimulates DNA-PK activity up to eightfold (47). Hammarslet and Chu (15) have also reported similar findings and concluded that Ku stabilizes the recruitment of DNA-PKcs to DNA ends and that a cooperative interaction between Ku, DNA-PKcs, and DNA efficiently activates the DNA-PKcs activity.

In this study, we have used two approaches to study Ku80 function. First, to gain further insight into the role of the Ku80 C terminus, we have examined C-terminally truncated Ku80 proteins for their ability to interact with Ku70, to bind dsDNA ends, and to interact with and activate DNA-PKcs. We examined the activity of the proteins generated following cotranslation with full-length Ku70 and, to gain insight into the significance of these in vitro findings, introduced truncated Ku80 cDNAs into Ku80-defective xrs-6 cells. Our results suggest that the C-terminal region of Ku80 is dispensable for interaction with Ku70 and for DNA end binding but is required for optimal DNA-PK activity. Cells lacking this region of Ku80 acquire the phenotype of Ku-deficient DNA-PKcs and are impaired in rejoining signal but not coding junctions during V(D)J recombination. We also report the identification of a point mutation change Ku80-defective xrs-2 cells. This is the first Ku80 mutant harboring a point mutation change, and it identifies a site outside of the previously described Ku70-Ku80 minimal interaction domain that is nonetheless required for heterodimer formation.

MATERIALS AND METHODS

Cell culture, DNA transfections, and protoplast fusions. The xrs cell lines were derived from the CHO-K1 cell line on the basis of their sensitivity to ionizing radiation (21). Cells were cultured in minimal essential medium (Gibco) supplemented with nonessential amino acids, penicillin, streptomycin, glutamine, and 10% fetal calf serum as described previously (21). Transfection was carried out by the Polybrene transfection method as described previously (22). Transfectants were selected by using 600 \( \mu \)g of G418 per ml alone or with 50 \( \mu \)g of zeocin per ml and subsequently maintained in 300 \( \mu \)g of G418 per ml alone or with 50 \( \mu \)g of zeocin per ml. Yeast artificial chromosomes (YACs) were transfected to rodent cells by the protoplast fusion protocol described previously (2). Isolation and purification characterization of the DNA-PKcs YAC have been described previously (2, 36). Experiments to assess survival in response to ionizing radiation were described as previously (21). V(DJ) recombination assays were described as previously (40, 41).

Analysis of DNA transfectants. Between 6 and 15 clones were picked per transfection and analyzed by PCR for the presence of the construct. PCR was carried out in 25 \( \mu \)l containing 2.5 \( \mu \)l of 10× PCR buffer (100 mM Tris [pH 9.0], 500 mM KCl, 15 mM MgCl\(_2\), 0.1% gelatin, 1% Triton X-100), 1.25 \( \mu \)l of pcDNA3.1/HisC (Invitrogen). The deletion constructs were generated as site-directed mutagenesis at the residues indicated. The activity of the proteins generated following cotransfection with full-length Ku70 and for DNA end binding but is required for optimal DNA-PK activity. Cells lacking this region of Ku80 acquire the phenotype of Ku-deficient DNA-PKcs and are impaired in rejoining signal but not coding junctions during V(D)J recombination. We also report the identification of a point mutation change Ku80-defective xrs-2 cells. This is the first Ku80 mutant harboring a point mutation change, and it identifies a site outside of the previously described Ku70-Ku80 minimal interaction domain that is nonetheless required for heterodimer formation.

RESULTS

Analysis of Ku80 3′ deletion constructs. To investigate the function of the C-terminal region of Ku80, a series of Ku80 cDNAs having 3′ deletions of different sizes were generated (Table 1). Some of the deletions were derived with CH Ku80 cDNA in the mammalian expression vector pcDNA3. Subsequently, additional deletions were generated with Hs Ku80 cDNA in a pcDNA3 vector containing an Xpress epitope tag. The former has the advantage that the full-length Hs Ku80 cDNA correctly the Ku80-defective xrs-6 cells but has the disadvantage that the Hs Ku80 antibodies that cross-react with CH Ku80 do not recognize the C-terminally truncated proteins (the proteins show 80.3% homology at the amino acid level). The use of Hs cDNA constructs therefore enabled a wider range of antibodies as well as the anti-Xpress antibody to be used.

(i) Analysis with in vitro-expressed proteins. First, the truncated Ku80 proteins of human origin were examined for their ability to interact with Ku70 following their coexpression with full-length Hs Ku70 cDNA in an in vitro translation system with rabbit reticulocyte lysates. The expression of Ku80 protein was assessed by Western blotting with anti-Xpress antibody (Fig. 1A). To examine the Ku70-Ku80 interaction, Ku70 protein was immunoprecipitated with anti-Ku70 antibodies and communoprecipitation of Ku80 was determined with anti-Xpress antibody.
Xpress antibodies (Fig. 1B). In the absence of Ku80 cDNA in the translation system, no anti-Xpress cross-reacting protein was detectable (Fig. 1A, lane pcDNA3 vector). Ku80 truncated proteins lacking up to 178 amino acid residues interacted as efficiently with Ku70 as did the full-length protein, while Hs Ku80Δ510 (where Δ510 denotes deletion of C-terminal residues 510 to 732) displayed slightly reduced Ku70 interaction, as determined by the amount of Ku80 coimmunoprecipitated relative to that expressed (compare Fig. 1B with Fig. 1A). The same results were obtained by examining the ability of Ku80 antibodies to coimmunoprecipitate Ku70 protein (data not shown). The smaller size of the Ku80 protein verifies that the site-directed mutations generated yield the anticipated truncated proteins. Next, we determined whether the in vitro-translated proteins had DNA end-binding activity by examining them in an electrophoretic mobility shift assay (EMSA) (Table 1; Fig. 1C). A low background level of DNA end-binding activity was obtained in the absence of Ku70 and Ku80 cDNA or in the presence of either cDNA alone, probably due to Ku homologues present in the reticulocyte lysates (Fig. 1C). Full-length Hs Ku80 and deletions up to Hs Ku80Δ554 had similar DNA end-binding activity, while the protein with the larger truncation (Hs Ku80Δ510) gave no end-binding activity above DNA end-binding activity, while the protein with the larger length Hs Ku80 and deletions up to Hs Ku80Δ510 (where Δ510 denotes deletion of C-terminal residues 510 to 732) gave no end-binding activity above background.  

To measure DNA-PK activity, DNA-binding proteins were first microfractionated from whole-cell extracts by using DNA-cellulose beads and then assayed for kinase activity with a pS3-derived peptide as substrate (10). The phosphorylated activity due to the presence of an inactivating missplicing mutation close to the N terminus of the Ku80 open reading frame (29, 39). Individual stable transfectants were selected with the neomycin marker present in pcDNA3 and screened by PCR with primers that amplify the Ku80 cDNA construct used. A minimum of two transfectants from each transfection were analyzed for the presence of Ku70 and Ku80 protein by Western blot analysis, for DNA end binding and DNA-PK activity. The clones derived from these transfections are named to reflect the construct used: e.g., XCH80Δ560 represents a stably transformed clone of xrs-6 with hamster Ku80Δ560. Normal levels of truncated Ku80 protein are expressed in XHs80Δ554 cells (Fig. 2A). Expression of Ku80 protein in clones derived with the other constructs could not be assessed since the truncated CH proteins do not cross-react with the anti-Ku80 antibodies. xrs-6 cells, as well as Ku80 knockout cell lines, have little or no Ku70 protein, demonstrating that Ku70 is unstable in the absence of Ku80 (6, 31, 39, 41). The faint residual signal seen in Western blot analysis of xrs-6 cells with anti-Ku70 antibodies could be due to residual Ku70 protein in the absence of Ku80 or to some other cross-reacting protein (Fig. 2A). The recovery of Ku70 protein in the transfected clones assessed by Western blot analysis was therefore used to monitor the interaction of Ku80 with Ku70 in vivo. Ku70 protein was clearly stabilized in XCH80Δ560 and XHs80Δ554 cells (Fig. 2A). Thus, it is likely that Ku80 is expressed in XCH80Δ560 clones, although it is not seen in the Western blots due to lack of cross-reactivity of the Ku80 antibodies. When the constructs with larger deletions were used, only low residual Ku70 protein levels were seen, comparable to that observed in xrs-6 cells (Fig. 2A). Taken together, these results suggest that the Hs80Δ554 and CH80Δ560 truncated proteins are stably expressed and interact efficiently with Ku70 in vivo. The lack of Ku70 protein in clones bearing the larger deletions probably reflects the reduced ability of the truncated Ku80 proteins to interact with Ku70, although we have not verified that Ku80 is expressed in these clones. The DNA end-binding activity of the clones is closely correlated with the stabilization of Ku70 protein (Fig. 2B). Thus, XHs80Δ554 and XCH80Δ560 had wild-type levels of DNA end-binding activity, while the two larger deletions resulted in loss of such activity. To measure DNA-PK activity, DNA-binding proteins were first microfractionated from whole-cell extracts by using DNA-cellulose beads and then assayed for kinase activity with a pS3-derived peptide as substrate (10).
product was separated by polyacrylamide gel electrophoresis and quantified by scanning. This procedure considerably enhanced the sensitivity of the assay by separating DNA-PK independent, nonspecific phosphorylated products from the p53 peptide (36). Only clones with DNA end-binding activity were examined for kinase activity. Phosphorylation of the p53 peptide with extracts of xrs-6 cells was not significantly above background (the signal obtained in the absence of p53 peptide). Clones expressing full-length CH Ku80 have levels of activity comparable to that obtained with CHO-K1 cells, which is three- to fivefold above background (data not shown). The activity obtained with clones expressing Hs Ku80 cDNA was approximately half of that obtained with wild-type cells (data not shown). XHs80A554 and XCH80A560 clones reproducibly had decreased DNA-PK activity compared to that of clones expressing the corresponding full-length Ku80 protein, which by scanning was estimated to be 4% (XHs80A554) and 28% (XCH80A560) of the signal obtained with clones expressing full-length Ku80 of the same species (data not shown). However, although the decrease in kinase activity was reproducible, the quantitative estimation of the decrease is highly inaccurate due to lack of sensitivity of the kinase assay in the rodent cells. These limitations notwithstanding, these results raise the possibility that clones with normal DNA end-binding activity have reduced kinase activity and suggest that the C-terminal region of Ku80 is required for kinase activation.

The ability of the Ku80 deletion constructs to complement the radiation sensitivity of xrs-6 cells was also examined (Fig. 2C). XCH80A560 clones that retained approximately one-third of the kinetic activity found in control cells had elevated γ-ray survival compared with xrs-6 cells or cells transfected with an empty vector but less than that observed with wild-type CH Ku80 cDNA or parental cells. In contrast, the larger deletions were unable to complement the radiation sensitivity of xrs-6 cells. Clones expressing Hs Ku80A554 cDNA resemble cell lines defective in DNA-PKcs (e.g., the mouse SCID cell line) in having DNA end-binding activity but lacking DNA-PK activity (2, 13). DNA-PKcs-defective cell lines (V-3, 1rs20, and the mouse SCID line) differ from Ku80-defective lines in the nature of their V(D)J recombination defect (26, 27, 34, 40, 42). Ku80-defective mutants have major defects in both signal joint and coding-joint formation, whereas the DNA-PKcs-defective lines can rejoin signal sequences with only a slightly impaired frequency. Additionally, while signal junctions are normally accurate in control cells, those formed in DNA-PKcs-defective lines have an elevated frequency of deletions. We therefore examined the ability of the XHs80A554 cells to carry out signal joint and coding-joint formation. XHs80A554 cells significantly recovered the frequency of signal joint formation to 50% of the level seen in clones expressing full-length Hs Ku80, although the defect in coding-joint formation remained (Table 2). The accuracy of signal joint formation also increased, although some junctions had deletions. Therefore, cells expressing this Ku80 deletion construct have the V(D)J recombination phenotype of cells lacking DNA-PKcs, even though they do express DNA-PKcs protein. XH80A560 clones showed considerable recovery of coding-joint formation, suggesting that their higher level of DNA-PK activity is sufficient to carry out coding joint formation effectively. This is consistent with their elevated survival (Fig. 2C).

Overexpression of DNA-PKcs in xrs-6 cells. Rodent cells have approximately 50-fold-lower levels of DNA-PK proteins than do human cells. While human cells have high levels of DNA-PK activity, we routinely observed levels of DNA-PK activity in parental CHO-K1 cells only three- to fivefold above background. This made it difficult to monitor accurately the decrease in kinase activity in transfectants expressing the Ku80 deletion constructs. Previously, we have shown that rodent cells harboring a YAC expressing Hs DNA-PKcs express human levels of the protein and have high DNA-PK activity compared with wild-type rodent cells (2, 36). This suggests that DNA-PKcs is rate limiting for DNA-PK activity and provided a tool to overexpress DNA-PKcs and enhance the sensitivity of the DNA-PK assay in the rodent cells. First, we examined the effect of overexpression of DNA-PKcs in xrs-6 cells by introducing YACs encoding Hs DNA-PKcs into CHO-K1 and xrs-6.
cells by protoplast fusion. As previously reported, fusion clones (designated CHO-K1Y or xrs-6Y) derived from both CHO-K1 and xrs-6 expressed the higher levels of DNA-PKcs expected (36) (Fig. 3A). Cell extracts from the fusion clones were next examined for DNA-PK activity. While CHO-K1Y clones had approximately 20- to 30-fold-higher levels of activity than control CHO-K1 cells, the DNA-PK activity in xrs-6Y cells expressing DNA-PKcs YACs (xrs-6Y) was only marginally increased (Fig. 3B). It is also notable that xrs-6Y clones expressing full-length CH Ku80 protein had DNA-PK activity 50- to 100-fold above that present in xrs-6Y clones, demonstrating the significant increase in kinase activity when Ku is present (Fig. 3B). The overexpression of DNA-PKcs in these clones did not affect the phenotype of either CHO-K1 or xrs-6 cells and did not affect their level of radiosensitivity (36). The high levels of DNA-PKcs in these fusion clones, however, allowed us to examine the effect of Ku on the ability of DNA-PKcs to bind to DNA. To assess DNA-PKcs/DNA binding, half the microfractionated DNA-binding proteins obtained during the DNA-PK assay were boiled to remove the bound proteins and examined for the presence of DNA-PKcs by Western blotting (Fig. 3A, Pd). High levels of DNA-PKcs were recovered from extracts of
Table 2. Analysis of signal joint and coding-joint formation by transient V(D)J recombination assay

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PJH200 (signal joints)</th>
<th>PJH209 (coding joints)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. Amp/ no. Chl</td>
<td>% recombed</td>
</tr>
<tr>
<td>XHs80</td>
<td>220/7,650</td>
<td>2.9</td>
</tr>
<tr>
<td>XHs80Δ554</td>
<td>27/1,770</td>
<td>1.5</td>
</tr>
<tr>
<td>XCH80</td>
<td>120/3,275</td>
<td>3.2</td>
</tr>
<tr>
<td>XCHΔ560</td>
<td>471/13,950</td>
<td>3.4</td>
</tr>
<tr>
<td>xrs-6</td>
<td>20/15,000</td>
<td>0.15</td>
</tr>
</tbody>
</table>

a Mouse SCID and V-3 cells have approximately a 50-fold defect in coding-joint formation and a 2-fold defect in signal joint formation. SCID and V-3 cells rejoin signal joints with 60 to 80% fidelity (34, 40).

b The percent correct RS joints screened by ApaLI digestion of the PCR product by using the recombination substrate pH200. The results represent the mean of at least three independent transfection experiments.

CHO-K1Y cells in this pull-down assay, and lower but measurable levels were obtained from control CHO-K1 cells expressing only endogenous DNA-PKcs (Fig. 3A). No detectable DNA-PKcs was recovered from xrs-6 cells in this pull-down assay, even though DNA-PKcs was present in the whole-cell extract (Fig. 3A), but low levels have been recovered in other experiments (data not shown). The level of DNA-PKcs recovered from the pull-down fraction by using extracts from xrs-6Y cells was variable but was always markedly lower than that recovered from CHO-K1Y cells (Fig. 3B). However, despite the recovery of DNA-PKcs from extracts of xrs-6Y cells, only low DNA-PK activity was observed (Fig. 3B). The response to radiation and the V(D)J recombination phenotypes of xrs-6 and CHO-K1 cells were unaffected by the presence of the DNA-PKcs YAC (for data on the survival of xrs-6 cells, see Fig. 4C). Taken together, these results show that Ku significantly enhances the ability of DNA-PKcs to bind to DNA and to function as a DNA-dependent protein kinase.

Transfection of Ku80 C-terminal deletion constructs into DNA-PKcs-overexpressing xrs-6 cells. The low level of DNA-PKcs activity in rodent cells limited our ability to assess the magnitude of the defect in DNA-PKcs interaction resulting from the C-terminal truncations of Ku80. We were also unable to assess efficiently whether the Ku80 deletion constructs impaired the ability of DNA-PKcs to interact with DNA. We therefore introduced the two most informative 3' deletion constructs (Hs Ku80Δ554 and CH Ku80Δ560) and full-length Hs or CH Ku80 cDNA into xrs-6Y cells, yielding transfectants designated XHs80Δ554, XCH80Δ560, XYHs80, and XCHΔ560, respectively. First, the constructs were subcloned into a pZeoSV vector, since the YACs encoded the neomycin selectable marker, and subsequently they were transfected into xrs-6Y cells by DNA transfection and selection for resistance to zeocin. All the clones expressed a truncated Ku80 protein, and Ku70 protein was stabilized, indicating efficient Ku70-Ku80 interaction (Fig. 4A; not visible for CH80Δ560 due to the inability of Ku80 antibody to cross-react with rodent protein).

Cell extracts from these clones also had DNA end-binding activity, as assessed by Western blotting (Fig. 4C). Taken together, these results show that Ku significantly enhances the ability of DNA-PKcs to bind to DNA and to function as a DNA-dependent protein kinase.

Figure 3. Interaction of DNA-PKcs with DNA and DNA-PK activity in the absence of Ku. (A) Recovery of DNA-PKcs from DNA cellulose beads. (Top) Protein (50 μg) from whole-cell extracts (WCE) of CHO-K1 and xrs-6 cells either expressing DNA-PKcs YACs (lanes CHO-K1Y and xrs6Y) or not carrying DNA-PKcs YACs (lanes CHO-K1 and xrs6) was subjected to Western blotting with the monoclonal anti-DNA-PKcs antibody, 18-2. (Bottom) DNA-binding proteins from 150 or 300 μg of protein from whole-cell extracts were microfractionated with DNA-cellulose beads and then analyzed for DNA-PKcs by Western blotting. Pd represents extracts from the pull-down fraction by using extracts from xrs-6Y cells in this pull-down assay, even though DNA-PKcs was present in the whole-cell extract (Fig. 3A), but low levels have been recovered in other experiments (data not shown). The level of DNA-PKcs recovered from the pull-down fraction by using extracts from xrs-6Y cells was variable but was always markedly lower than that recovered from CHO-K1Y cells (Fig. 3B). However, despite the recovery of DNA-PKcs from extracts of xrs-6Y cells, only low DNA-PK activity was observed (Fig. 3B). The response to radiation and the V(D)J recombination phenotypes of xrs-6 and CHO-K1 cells were unaffected by the presence of the DNA-PKcs YAC (for data on the survival of xrs-6 cells, see Fig. 4C). Taken together, these results show that Ku significantly enhances the ability of DNA-PKcs to bind to DNA and to function as a DNA-dependent protein kinase.
activity with the predicted altered mobility (Fig. 4B). *xrs-6Y* cells expressing full-length CH Ku80 protein had DNA-PK activity 20- to 50-fold above that obtained with CHO-K1 cell extracts and similar to that found in CHO-K1Y cells (Fig. 3B) and human cells (36). In contrast, DNA-PK activity was decreased in clones expressing the 3′ deletion constructs, although some residual activity was detectable, which was lower with the larger deletion (Fig. 3B; Table 1). Although XYHs80Δ554 has markedly lower kinase activity than XYCH80Δ560 did, this is more likely to be due to the different species origin of the proteins than to a specific functional significance of the extra 6 amino acids deleted. An additional deletion construct (Hs Ku80Δ543) was introduced into *xrs-6Y* cells, and the transfectants derived had an almost identical phenotype to XYHs80Δ554 cells, with 6% of the parental kinase activity (data not shown). Thus, deletion of an additional 11 amino acid residues does not decrease the residual kinase activity. These results substan-

FIG. 4. Analysis of extracts of *xrs-6Y* clones. (A) Protein (50 μg) from whole-cell extracts was separated by SDS-PAGE through 8% polyacrylamide gels and subjected to Western blotting with the Ku70 and Ku80 antibodies, N3H10 and Ku80-4. (B) EMSA analysis. Whole-cell extracts (30 μg) from the *xrs-6Y* clones indicated were subjected to EMSA. (C) Survival of *xrs-6Y* clones in response to ionizing radiation. (D) Recovery of DNA-PKcs from DNA-cellulose beads. (Top) Protein (15 μg) from whole-cell extracts (WCE) of CHO-K1Y cells, *xrs-6Y* cells expressing full-length Hs Ku80, *xrs-6Y* cells expressing CH Ku80Δ554, and *xrs-6Y* cells was subjected to Western blotting with anti-DNA-PKcs antibody, 18-2. (Bottom) DNA-binding proteins derived from 150 μg of protein from the same whole-cell extracts were first microfractionated with DNA-cellulose beads and then analyzed for DNA-PKcs with antibody 18-2. Pd represents extracts microfractionated for DNA-binding proteins by pull-down assays with DNA-cellulose beads. Autoradiographic images were scanned on an AGFA StudioStar scanner with FotoLook32 software.
tiated those obtained with the xrs-6 parental cells and verified that the C-terminal region of Ku is required for optimal DNA-PK activity. To assess whether DNA-PKcs can interact with DNA in the presence of the Ku80 truncated proteins, we used Western blotting to examine the microfractionated DNA-bound proteins obtained in XYHs80D554 cells for DNA-PKcs levels. The level of DNA-PKcs protein recovered from extracts of XYHs80D554 cells was markedly lower than that recovered from xrs-6Y cells expressing full-length Ku80 protein and similar to that obtained with xrs-6Y cells (Fig. 4D). These results show that the major impact of the C-terminal Ku80 deletions is to impair DNA-PKcs binding to DNA. Decreased but measurable kinase activity was detected with these extracts, however. The survival of these clones after exposure to γ-irradiation was also examined. While XCH80Δ560 cells were partially complemented for radiation sensitivity (Fig. 2C), XCH80Δ560 cells had a level of resistance close to that of parental CHO-K1 cells (Fig. 4C). In contrast, expression of the Hs Ku80Δ554 protein resulted in only minor complementation of the radiation sensitivity of the xrs-6Y cells.

**Identification of a point mutation in Ku80-deficient xrs-2 cells.** xrs-2 is a Ku80-defective mutant that displays slightly less radiation sensitivity compared to other xrs mutants (21). Like other xrs mutants, xrs-2 lacks Ku70 and Ku80 proteins, detectable dsDNA end binding, and DNA-PK activity, but the Ku80 transcript levels are normal (data not shown). Sequence analysis of Ku80 cDNA from xrs-2 cells by RT-PCR identified a C-to-T base change at position 1229 that results in the substitution of a leucine for a proline at residue 410. Although a proline at this position is conserved between human, hamster, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae* Ku80 proteins, the GenBank sequence (U68181) for mouse Ku80 showed a leucine at this position. We sequenced this region from mouse Ku80 cDNA and found, contrary to the published sequence, a proline at residue 410. This amino acid residue is therefore conserved between species, enhancing the likelihood that the mutational change identified in *xrs-2* at this residue represents the causal inactivating mutation.

To verify the significance of this mutational change, we generated the mutation in CH Ku80 cDNA by site-directed mutagenesis and coexpressed the mutated cDNA (designated *xrs-2* cDNA) with full-length Ku70 in the reticulocyte lysate in vitro translation system. The *xrs-2* Ku80 protein generated cross-reacted with anti-Ku80 antibodies but was unable to co-immunoprecipitate Ku70 (data not shown), and the coexpressed proteins lacked DNA end-binding activity (Fig. 5A). *xrs-2* cDNA was also introduced into xrs-6 mutants by DNA transfection, and individual clones were analyzed for radiation sensitivity, for DNA end binding, and for the presence of Ku70 and Ku80 protein by Western blotting (data not shown). The level of radiation sensitivity varied between clones generated from three independent transfection experiments, but none of the clones had detectable Ku70 protein or DNA end-binding activity.
activity (Fig. 5B shows the DNA end-binding activity). Ku80 protein was also undetectable in the majority of clones. We also showed that wild-type CH Ku80 cDNA fully corrected xrs-2 cells (Fig. 5C shows the DNA end-binding activity). Taken together, these results support our contention from the sequence analysis that the likely causal mutation in xrs-2 sequence analysis that the likely causal mutation in xrs-2 represents the mutation in position 410. This impairs the ability of Ku80 to interact with Ku70 and thus abolishes DNA end-binding activity. Potentially, however, some interaction remains in vivo, allowing some level of complementation. However, the aberrant Ku complex appears to be unstable following our extraction procedure.

**DISCUSSION**

In this and a previous study we have used two approaches to gain insight into the function of the Ku protein (39). One approach involves the identification of mutations in Ku80-defective rodent cells, and the other involves a structure-function analysis of Ku80 by site-directed mutagenesis. Others have also carried out a structural analysis of Ku80 and identified domains required for interaction with Ku70 and for DNA end-binding activity (5, 32, 46). Here we describe the analysis of C-terminally truncated Ku80 proteins following coexpression with Ku70 in vitro and following expression in Ku80-deficient xrs-6 cells. Our results, summarized in Fig. 6, show that the C-terminal region of Ku80 is involved in an interaction with DNA-PKcs. To facilitate the examination of this interaction, we introduced human YACs encoding DNA-PKcs into xrs-6 cells. Rodent cell-YAC fusion hybrids express levels of DNA-PKcs similar to that found in human cells and, in the presence of full-length Ku80 protein, have high DNA-PK activity (36). The introduction of the 3'-deleted Ku80 cDNAs into these DNA-PKcs-overexpressing cells therefore increased our ability to monitor the interaction between the truncated Ku80 proteins and DNA-PKcs. The results we have obtained in vivo are generally in agreement with our in vitro findings. Taken together, our results show that the terminal 178 amino acid residues are dispensable for interaction with Ku70 and for DNA end-binding activity. In contrast, amino acid residues from 474 to 531 are required for DNA end-binding activity. The loss of DNA end-binding activity is probably due to reduced Ku70-Ku80 interaction as demonstrated for Hs Ku80x510. Unfortunately, this could not be verified with the constructs generated with CH Ku80 cDNA since our Ku80 antibodies do not cross-react with the truncated CH proteins. The results of previous studies have defined a minimal region of 28 amino acids from 449 to 477 required for heterodimerization, although this minimal region alone has not been shown to be sufficient for Ku70-Ku80 interaction (5, 32, 46). Therefore, some aspects of our results differ from these previous studies. First, although the C-terminal 178 amino acid residues are dispensable for heterodimerization, loss of further amino acids to residue 531 impairs interaction. Additionally, we show that changing residue 410 from a proline to a leucine impairs heterodimerization. Thus, in contrast to previous results, we show that the presence of the minimal region of 28 amino acids from 449 to 477 is not sufficient for Ku70-Ku80 interaction. A likely explanation is that the larger protein context of our truncated or mutated proteins may hinder protein-protein interactions if the correct protein conformation is not achieved, a feature that may not arise in the analysis of short protein fragments. In a separate collaborative study in which mutational changes have been constructed at various sites in full-length Ku80, we have obtained further evidence that changes at multiple sites in the N-terminal two-thirds of Ku80 can impinge upon Ku70-Ku80 heterodimerization (39a). Furthermore, there are many sites in Ku70 at which mutations abolish heterodimerization (24).

Previously it has been reported that DNA-PKcs has Ku-independent DNA-binding and kinase activity, and Ku was believed merely to stabilize DNA-PKcs/DNA binding, enhancing the kinase activity no more than eightfold (15, 47). Here, using xrs-6 cells that overexpress DNA-PKcs, we show that Ku can stimulate DNA-PK activity 50- to 100-fold. Our deletion analysis shows that the C-terminal region of Ku80 is required for interaction with DNA-PKcs and stimulation of the kinase activity. Thus, Ku80 proteins truncated at positions 554 and 560 produce a Ku heterodimer efficient for DNA end binding but with decreased ability to stimulate DNA-PK kinase activity. XYH80A554 clones were not complemented for γ-ray sensitivity and were defective in coding-joint formation but were able to recombine signal junctions. These cells have thus acquired the phenotype of mouse SCID cells. Although we cannot verify that the sole function of the C-terminal region of Ku80 required for DSB rejoining is the recruitment and/or activation of DNA-PKcs, the fact that cells lacking this region of Ku80 but having high DNA-PKcs protein acquire the phenotype of cells lacking DNA-PKcs is highly supportive of this notion. The decreased kinase activity of cells expressing Ku80 truncated proteins could arise because the C-terminal region of Ku80 facilitates or stabilizes binding of DNA-PKcs to DNA and/or because it activates DNA-PKcs catalytic activity, for example by inducing a conformational change in the protein. The presence of the C-terminal region of Ku80 markedly increased the recovery of DNA-PKcs from DNA cellulose beads, showing that Ku enhances DNA-PKcs binding to DNA. While the recovery of DNA-PKcs protein from DNA cellulose beads

**FIG. 6. Summary of results with deletion constructs analyzed.** The solid box represents the minimal Ku70-Ku80 interaction domain previously identified (32); P at position 410 represents the mutation in xrs-2 cells; ND, not done; S and R, sensitive and resistant to ionizing radiation, respectively; Ku70/80, heterodimerization; DB, DNA end-binding activity; DNA-PK, DNA-PK activity.
was similar in extracts expressing the Ku80Δ554 protein and those lacking Ku (Fig. 4D, lanes XCH80Δ554 and xrs6Y), greater residual kinase activity was detected when the truncated protein was present (Fig. 3B). This is consistent with the notion that Ku enhances DNA-PKcs/DNA binding and activates DNA-PKcs catalytic activity and that the truncated proteins retain some residual DNA-PKcs activation activity. However, lack of sensitivity of this analysis precludes a firm conclusion about the relative contribution of these two potential aspects of Ku80 function. These limitations notwithstanding, we conclude that the C terminus of Ku80 interacts with DNA-PKcs, enhancing its ability to bind to DNA and to function as a DNA-dependent protein kinase.

Our results also provide evidence for Ku-independent DNA-PKcs/DNA binding and kinase activity. Since rodent cells have levels of DNA-PK activity only three- to fivefold above background, it was not possible previously to assess whether Ku80-defective mutants retain low DNA-PK activity. When cell extracts that lack Ku80 protein but have high DNA-PKcs activity (xrs-6/YAC fusion cells) are used, the level of DNA-PKcs protein recovered from DNA cellulose beads is similar to that found in wild-type hamster cells. This may represent Ku-independent DNA-PKcs binding to DNA but may also result from nonspecific recovery due to the elevated DNA-PKcs activity in these extracts. However, these same Ku-defective extracts have low but detectable DNA-PK activity, indicative of Ku-independent kinase activity. However, although the mutation in xrs-6 cells inactivates the Ku80 protein, we cannot exclude the possibility that there is some expression from a methylation-silenced allele that we have shown to be present in these cells and capable of being activated (20, 39). Furthermore, we cannot assess whether the low activity has functional significance, since such cells lack Ku DNA end-binding activity. Taken together, however, our results support the model proposed by Hammarsten and Chu (15), that Ku stabilizes an interaction between DNA-PKcs and DNA and that this interaction serves to stimulate DNA-PK activity.

Although XCH80Δ560 and XCH80Δ560 clones retain residual kinase activity and were partially complemented for γ-ray sensitivity, no complementation was observed with the larger truncated protein (Hs Ku80Δ554), even though, in the DNA-PKcs-overexpressing background, the cells retained some kinase activity and indeed had DNA-PK activity greater than that observed in CHO-K1 cells. In this context, it should be noted that the in vitro kinase assay uses a p53 peptide substrate and may not represent the constraints imposed upon the physiologically relevant in vivo substrate. The ability to phosphorylate the relevant in vivo substrate may therefore be more strongly impaired than is evident from the in vitro kinase assay.

We and others have observed the presence of aberrant Ku80 proteins in some human cell extracts (references 16 and 30 and our unpublished observations). These smaller Ku80 proteins were similar in size to our Hs Ku80Δ554 protein, did not cross-react with a Ku80 antibody specific for a C-terminal epitope, and retained DNA end binding but had decreased kinase activity. One particular extract had 90% smaller Ku80 protein and barely detectable DNA-PK activity (our unpublished data). Proteolytic degradation of Ku80 has been previously reported (33), and, in our hands at least, the aberrant, smaller Ku80 product appears to arise during extraction. Therefore, for example, when cells that appeared to have a smaller Ku80 protein were lysed in SDS and immediately subjected to Western blotting, a normal-sized Ku80 protein was obtained. Our results would therefore caution that the variant Ku80 proteins may not represent the in vivo Ku80 state and may not be related to radiation sensitivity. Nonetheless, these results support our findings with defined Ku80 truncated proteins and raise the possibility that such modifications of Ku have some function in the regulation of the NHEJ process.

The C-terminal region of Ku80 is less highly conserved between species than is the rest of the Ku80 cDNA. For example, the S. cerevisiae Ku80 cDNA is 102 amino acid residues shorter than its human homologue and most of these nonconserved residues are located in the C-terminal region. It is possible significant, therefore, that a DNA-PKcs homologue has not been detected in yeast. In contrast, there is greater conservatism between the C. elegans and mammalian sequences in this region, raising the possibility that these cells will have a functionally homologous DNA-PKcs protein. It has also been reported that Ku70 and Ku80 are evolutionarily related (8). Significantly, Ku70 lacks the C-terminal region present in Ku80, raising the possibility that only Ku80 functions in interactions with DNA-PKcs. Further work, including additional mutational analysis of Ku80 and Ku70, is required to characterize this interaction further.

A second strategy for the analysis of functionally important residues in Ku80 is the identification of mutations in Ku80-defective mutants. Previously, such analysis has not been informative, since the changes identified resulted in large deletions or truncations. Here we report the identification of a point mutational change in the xrs-2 mutant. Additionally, we report that the mouse GenBank sequence (U68181) has an error at position 410 and that a proline at this position is completely conserved between species. The proline-to-leucine change at this site in xrs-2 impairs the ability of the Ku80 protein to interact with Ku70, possibly by affecting protein conformation, as discussed above. Our results also show that this mutational change does not act as a dominant-negative mutation since the defect in xrs-2 cells can be corrected by wild-type Ku80 cDNA.

In conclusion, we show here that the C-terminal region of Ku80 significantly enhances the binding of DNA-PKcs to DNA and DNA-PK activity. Significantly, xrs-6 cells expressing Ku80 constructs lacking the C-terminal 178 amino acids acquire the phenotype of SCID cells even though they express wild-type DNA-PKcs protein. These results suggest that an interaction between DNA-PKcs and Ku80 is required for DSB repair and coding joint formation and that this interaction requires the C-terminal region of Ku80.

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