Distinct Roles for Ku Protein in Transcriptional Reinitiation and DNA Repair*

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Transcriptional reinitiation is a distinct phase of the RNA polymerase II transcription cycle. Prior work has shown that reinitiation is deficient in nuclear extracts from Chinese hamster ovary cells lacking the 80-kDa subunit of Ku, a double-strand break repair protein, and that activity is rescued by expression of the corresponding cDNA. We now show that Ku increases the amount or availability of a soluble factor that is limiting for reinitiation, that the factor increases the number of elongation complexes associated with the template at all times during the reaction, and that the factor itself does not form a tight complex with DNA. The factor may consist of a preformed complex of transcription proteins that is stabilized by Ku. A Ku mutant, lacking residues 687–728 in the 80-kDa subunit, preferentially suppresses transcription in Ku-containing extracts, suggesting that Ku interacts directly with proteins required for reinitiation. The Ku mutant functions normally in a DNA end-joining system, indicating that the functions of Ku in transcription and repair are genetically separable. Based on our results, we present a model in which Ku is capable of undergoing a switch between a transcription factor-associated and a repair-active state.

The ability of a cell to produce multiple copies of a particular mRNA requires the recycling of template, general transcription factors, and RNAP II. This process, termed reinitiation, is a key determinant of the overall transcription rate. Reinitiation at a given promoter occurs in vivo as frequently as every few seconds (1, 2). The ability to maintain such high, sustained transcription rates suggests the existence of a facilitated reinitiation pathway in which many sequential events are coordinated so that the transcription cycle can proceed at a rapid overall rate (reviewed in Ref. 3).

Mechanisms that promote rapid reinitiation can be divided broadly into those that operate at the template level, influencing the availability of a particular DNA for reinitiation, and those that operate at the protein level, influencing the availability of RNAP II and transcription factors. A number of examples of mechanisms that promote reinitiation have been characterized. One of the mechanisms that operates at the template level is the persistent binding of transcription factors to the promoter. The binding of TFIID and TFIIA is commonly the rate-limiting step in de novo transcription complex formation (4–6). After the first round of initiation is complete, TFIID and TFIIA remain bound to the DNA and nucleate the assembly of another transcription complex (7). Because this bypasses a potentially rate-limiting step, the overall rate of reinitiation is increased. Consistent with this interpretation, mutations that destabilize the TFIID-TFIIA-DNA complex selectively decrease the reinitiation rate in vitro (8, 9). Another example of a mechanism that promotes the availability of templates for reinitiation is the suppression of pausing during elongation. The presence of a stable, paused elongation complex limits the ability of successive RNAP II molecules to transit the template. The elongation factor TFII S also known as SII, which suppresses pausing, stimulates reinitiation in vitro (10).

Although template availability is important, it is not sufficient, in itself, to assure a high reinitiation rate, because reinitiation also requires that certain events occur at the protein level. For example, the C-terminal domain of the largest RNAP II subunit is phosphorylated at the time of initiation and must be dephosphorylated to allow re-entry of RNAP II into the transcription cycle (reviewed in Ref. 11). This dephosphorylation is mediated by a specific phosphatase, FCP-1 (12, 13), which is in turn regulated by an interaction with the C-terminal region of the 74-kDa subunit of TFII F (12). Mutations in TFII F decrease reinitiation efficiency in vitro (14). Several other examples of proteins that affect reinitiation have been reported, although their mechanisms of action have not been characterized in detail. These include estrogen receptor (15) and the TATA-binding protein-associated factors, also known as TAFs (16).

In addition to specific protein-protein interactions, overall spatial organization of the transcription apparatus is likely to be important in determining reinitiation rate. Cytological studies reveal that the mRNA synthesis apparatus is not distributed evenly throughout the nucleus but rather occurs in hundreds or thousands of discrete clusters termed “transcription factories” (Refs. 17 and 18; reviewed in Ref. 19). Complementary biochemical studies show that a portion of the RNAP II in the mammalian cell is associated with discrete sets of transcription factors in so-called “holoenzyme” complexes (Refs. 20 and 21; reviewed in Refs. 22 and 23). Proteins that preserve the spatial organization of the transcription apparatus in a cell-free system or that promote the formation of stable holoenzyme complexes could exert a major influence on reinitiation by increasing the local concentration of transcription proteins in the vicinity of the promoter.

In previous work, we made an unexpected observation that
expression of the Ku protein, a double-strand DNA break repair factor, affects transcription efficiency in vitro (24). Extracts from mutant CHO cells that lack the 80-kDa Ku subunit (25, 26) show a transcription deficit of up to 5-fold, compared with extracts from isogenic control cells rescued by expression of the corresponding cDNA (24). The effect is seen with several unrelated promoters, indicating that it involves the general transcription machinery and is seen only in a multiple-round transcription assay, that is, when reinitiation is permitted. Activity in extracts from the mutant cells can be rescued by addition of small amounts of extract from Ku-expressing cells but not by the addition of purified Ku protein, RNAP II, or general transcription factors. The inability to rescue activity with purified Ku suggests either that the role of Ku is indirect or that the endogenous Ku differs in some way from the exogenous protein. Interestingly, a different CHO cell mutant that lacks the DNA-dependent protein kinase catalytic subunit, a protein in the same repair pathway as Ku, also exhibits a decrease in transcription activity (24).

Why a DNA double-strand break repair protein should influence the apparently unrelated process of transcriptional reinitiation is not known. Ku is a heterodimer, composed of 70- and 80-kDa subunits, referred to as Ku70 and Ku80, respectively. Ku binds avidly to DNA ends and recruits DNA-PKcs to form a complex with a protein kinase activity. The assembly of this complex initiates a repair pathway (reviewed in Refs. 27 and 28). The effect of Ku on transcription does not require free ends in the template, and it is not dependent on the catalytic activity of DNA-PKcs. This suggests that the effect may involve protein-protein interactions rather than DNA binding per se. Consistent with this, Ku and DNA-PKcs have been found to physically associate with an RNAP II-containing complex in vitro (20).

There is a precedent for functional interaction between DNA repair proteins and the transcription apparatus. The general transcription factor TFIIH has several subunits in common with a multiprotein complex required for nucleotide excision repair. Mutations in at least two of these proteins, the XPB and XPD helicases, have pleiotropic effects on transcription and repair processes (Refs. 29–31; reviewed in Ref. 32). Mutations in two other repair proteins, the Cockayne Syndrome A and B proteins, also affect general transcription capacity, although the mechanism is not fully understood (33, 34).

The studies described here address two major questions. First, what is the underlying reason for the greater reinitiation activity in extracts from Ku-expressing cells? Second, and more generally, what insights can we obtain into the physiological interaction between double-strand break repair proteins and the RNA polymerase II transcription apparatus? In the course of this work, we identified a dominant mutant form of Ku that preferentially suppresses transcription activity in extracts from Ku-expressing cells. The mutation lies outside the DNA-binding domain of Ku and has no apparent effect on the function of Ku in a cell-free DNA end joining assay. Our data suggest that Ku interacts directly with a set of proteins required for transcriptional reinitiation and that this function is genetically separable from the role of Ku protein in DNA double-strand break repair.

MATERIALS AND METHODS

Templates, Nuclear Extracts, and Transcription Reactions—The xrs-6 cell line lacks detectable Ku80 protein and has a stable, radiation-sensitive phenotype. One allele of the Ku80 gene contains a 13-base pair insertion that results in a frameshift; the other allele is silent (26). The xrs-6cKu80 and xrs-6cvec cell lines were derived by transfection of xrs-6 cells with a human Ku80 cDNA expression vector and with an empty vector, respectively (35). We reconfirmed the presence of a Ku80 cDNA in the xrs-6cKu80 line by polymerase chain reaction amplification (data not shown). Nuclear extracts were prepared from each cell line and in vitro transcription was performed using supercoiled DNA templates as described (24). In some experiments, immobilized templates were used. The template for these experiments consisted of an hsp70 promoter fused to a 190-base pair G-less cassette (24). This DNA was linearized by digestion with XhoI, and the DNA ends were biotinylated in a fill-in reaction before annealing to 1 μM unlabeled fragment of Escherichia coli DNA polymerase I and 0.025 mCi each of dATP, dCTP, dGTP, and biotin-11-dUTP (CLONTECH). The reaction was allowed to proceed for 30 min at 30 °C. The product was digested with SphI to remove the biotinylated end upstream of the hsp70 promoter and subjected to spin column chromatography to remove the released fragment and unincorporated nucleotides. The biotinylated DNA was then allowed to bind to streptavidin-coated paramagnetic beads (Dynal, Dynabeads M-280). Template and beads were incubated at a ratio of 5 pmol DNA/mg beads for 1 h at 43 °C in the presence of 5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 2 mM NaCl. Following incubation, the supernatant was removed, and its DNA content was quantitated to provide an estimate of the fraction of DNA that bound to the beads. The beads were washed twice in 5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 2 mM NaCl, followed by extensive water washes. The beads were resuspended in a final volume of water to a concentration of 40 μg DNA/ml.

Expression and Purification of Ku Protein—Recombinant wild type Ku protein was produced by coinfection of SF9 cells with VB2B–68Ku and VB2B–70Ku and was purified by sequential Superdex-200, single-stranded DNA-agarose, and heparin-agarose chromatography as described (36, 37).

To produce the Ku80Δ681–728 mutant, polymerase chain reaction-based mutagenesis was used to replace codons 681–728 of the human Ku80 cDNA with an XhoI linker. Two divergent primers with sequences d(CGCCGTCGAGCTGGACAACTTTCCGACAG) and d(CCCCGTCGAGTTGGACATGTATAGTCGAC) were annealed to a human Ku80 cDNA subcloned in pGEM3zf(+) (Promega). Polymerase chain reaction was performed, and the resulting fragment was digested with XhoI and allowed to recircularize. The resulting clone was digested with BamHI and SalI to release a Ku-encoding fragment, which was inserted into the pCTET4(+) vector (Novagen) to provide additional flanking restriction sites. This plasmid was digested with BamHI and NotI, and a fragment containing the Ku gene was inserted into the pVL1393 baculovirus transfer vector (PharMingen). This plasmid was cotransfected into SF9 cells with linearized AcNPV baculovirus DNA (PharMingen), and viral stocks were produced. Recombinant Ku80Δ681–728 protein was produced by coinfection of SF9 cells with the Ku80Δ681–728 baculovirus and VB2B–70Ku. Mutant protein was purified by the same method as wild type (37).

Cell-free End Joining Assay—Whole cell extracts were prepared as described (38) using 4 × 108 human lymphoblasts (GM00558 cell line, Coriell Cell Repositories). Cells were harvested by centrifugation, resuspended, and lysed by homogenization in 10 ml of hypotonic lysis buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 5 mM dithiothreitol) using 20 strokes of an A pestle. Phenylmethylsulfonyl fluoride (0.17 mM), pepstatin A (1 μg/ml), leupeptin (1 μg/ml), and soybean trypsin inhibitor (10 μg/ml) were added. After 20 min of incubation on ice, 3.33 ml of high salt buffer (50 mM Tris-HCl, pH 7.5, 1 mM KCl, 2 mM EDTA, 2 mM dithiothreitol) was added. Cell lysate was centrifuged for 3 h at 200,000 g. The supernatant was dialyzed against 20 mM Tris-HCl, pH 8.0, 0.1 mM KCl, 20% glycerol, 0.5 mM EDTA, 1 mM dithiothreitol. Aliquots were prepared and stored at −70 °C.

DNA substrate for the end joining assay was prepared by digestion of pBluescript II KS (+) vector (Stratagene) with BamHI. DNA substrate was labeled using T4 polynucleotide kinase and [γ-32P]ATP (6000 Ci/mmol). End-joining reactions were performed essentially as described (39). Briefly, reaction mixtures contained 10 mM Tris-HCl, pH 7.9, 65 mM KCl, 0.25 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 50 mM triethanolamine, pH 7.5, 0.7 mM MgOAc, 100 μM bovine serum albumin, 2 mM ATP, 100 ng of purified, recombinant DNA Ligase IV/XRC4C (39), 4 μl of immunodepleted lymphoblast extract (see below), and purified recombinant human Ku protein (40) or DNA-PKcs protein (41). The reaction mixture was incubated for 10 min at 37 °C. DNA (10 ng) was added, bringing the final volume to 20 μl. Incubation was continued for 1 h at 37 °C. The reaction was stopped by adding 150 μl of a solution containing 0.2 mM NaCl, 0.03% Triton X-100, and 100 μl of 10 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.1 mM NaCl. Reactions were extracted with 300 μl of phenol: chloroform:isoamyl alcohol (50:49:1 v/v/v). A solution of 0.5 mM NH4OAc in ethanol was added (500 μl), the nucleic acid precipitate was collected by centrifugation, and reaction products were analyzed by 0.6% agarose gel electrophoresis. Ligated products were detected by PhosphorImager analysis.
Immunodepletion of Ku and DNA-PKcs from End Joining Extracts—

500 μl of IPP buffer (0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, 0.1% Nonidet P-40) was added to 5 mg of protein A-Sepharose beads and incubated for 30 min at 4 °C. Transcription was initiated by the addition of ribonucleoside triphosphates. One reaction in each set was limited to a single round of transcription by the addition of 10 μg/ml heparin. Following incubation, 5 μl of a normal human serum or a human serum (TT) containing autoantibodies directed against Ku and DNA-PKcs was added to the beads and incubated overnight at 4 °C. The beads were washed 3 times in IPP buffer and twice in DB buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM KOAc, 1 mM dithiothreitol, 0.5 mM EDTA, 20% glycerol). 12–15 μl of GM00558 cell extract was added to the washed beads and incubated for 2 h at 4 °C. The supernatant was removed and set aside for use in end joining assays and for analysis by SDS-PAGE and immunoblotting. The beads were washed four times with IPP buffer and analyzed by SDS-PAGE and immunoblotting.

RESULTS

Expression of the Ku80 cDNA in xrs-6 Cells Increases the Amount or Availability of a Limiting Reinitiation Factor—We have defined a system for studying transcription that is based on nuclear extracts prepared from two isogenic CHO cell lines (24). As described under “Materials and Methods,” the xrs-6cvec cell line lacks Ku80 expression, whereas the xrs-6cKu80 cell line has been rescued with a human Ku80 cDNA (35). Extracts from the latter cell line exhibit a much higher level of in vitro transcription under multiple-round conditions (24).

Many different mechanisms can influence reinitiation rate, and in an initial attempt to understand the mechanism used by the Ku-dependent reinitiation activity, we performed an experiment in which the amount of template was varied while the amount of nuclear extract was held constant. Transcription was performed using supercoiled templates containing the hsp70 promoter fused to a G-less cassette. At each concentration of template, transcription was measured under conditions that permit either single or multiple-round transcription (Fig. 1A).

When transcription was restricted to a single round by the addition of 10 μg/ml heparin, 2 min after the NTPs, the amount of RNA synthesis was similar with both extracts. Moreover, the amount of transcription remained constant as the amount of template increased (Fig. 1, B and C, †heparin lanes). In all cases, two closely spaced RNA bands were seen, corresponding approximately to the expected size of the full-length RNA (Fig. 1B). Shorter RNAs were not present in significant amounts (Ref. 24 and data not shown). The observation that the amount of first round transcription remained constant as the amount of template was varied indicates that overall RNA synthesis is limited by a component in the extract, presumably a protein, and not by the amount of DNA. Because the levels of RNA synthesis were similar with both extracts, we infer that they are well matched with respect to the amount of this limiting component.

When transcription was allowed to proceed for multiple rounds, the xrs-6cKu80 extract was much more active than the xrs-6cvec extract (Fig. 1, B and C, †heparin lanes). Moreover,
the amount of RNA synthesis with the xrs-6cKu80 extract increased as the amount of template increased. By contrast, the amount of RNA synthesis with the xrs-6vec extract showed little change as the amount of template increased. We conclude that the introduction of a functional Ku80 gene in the xrs-6cKu80 cell line increases the amount or availability of a component in the extract that is specifically required for multiple-round transcription. This component is evidently different than the component that limits single-round transcription, and we provisionally refer to it as the “reinitiation factor.”

We use the term “reinitiation” for convenience and because the process presumably reflects facilitated recycling or reuse of some component that was limiting for the first round. It should be understood, however, that our use of the term embraces any form of initiation that occurs after the first 2 min, regardless of the underlying mechanism.

The results of the template titration experiment help to discriminate between different possible mechanisms of action for the reinitiation factor. The finding that an increase in the amount of template leads to an increase in multiple-round transcription, whereas the amount of first-round transcription remains constant, strongly suggests that the factor promotes the utilization of individual template molecules that were not used for productive initiation in the first round. It appears that the reinitiation factor facilitates the redistribution of some limiting transcription component between templates rather than the reuse of templates per se. This is consistent with the results of a colliding polymerase assay performed previously (24), which showed that few, if any, templates are reused in the course of the reaction.

The Reinitiation Factor Increases the Number of Transcription Complexes Associated with the Template at All Times after Addition of NTPs—It was important to confirm the results of the preceding experiment using an independent experimental design. Heparin is a polyanion that interrupts the transcription cycle by binding to free RNAP II and transcription factors and blocking their incorporation into transcription complexes. We have assumed that the addition of heparin affects only reinitiation and does not substantially interfere with the rate or efficiency of elongation. This assumption is supported by previous observations that heparin does not affect RNAP II elongation in a purified system (42). However, it is possible that heparin may affect elongation in a crude extract, where additional components are present. It would be of particular concern if there were a special class of heparin-sensitive elongation complexes formed in the xrs-6cKu80 extract, because if so, the difference in transcription in the presence and absence of heparin would not reflect reinitiation, but rather some other property of the system.

To address this concern, we measured reinitiation in a more direct way. We carried out two sets of transcription reactions in parallel, using different protocols, as diagrammed in Fig. 2A. In pathway 1, transcription was allowed to proceed for a variable length of time, heparin was added, and incubation was continued for an additional 30 min to complete previously initiated chains. Pathway 1 measures accumulated RNA chains as a function of time. In pathway 2, transcription was allowed to proceed for the same amounts of time as in pathway 1, but EDTA was then added to chelate divalent cations, halting RNA synthesis immediately. The difference in the amount of RNA synthesized in pathway 1 versus pathway 2 allows the estimation of the relative number of transcription complexes that are associated with the template at a given time.

Results are shown in Fig. 2B, with quantitation in Fig. 2 (C and D). Under the conditions where chain elongation was allowed to proceed to completion in the presence of heparin (pathway 1), there was an approximately linear increase in the number of RNA chains with time, consistent with a constant reinitiation rate throughout the course of the reaction. Although this linear increase was observed with both extracts, the slope of the line, which is proportional to the reinitiation rate, was 4-fold greater with xrs-6cKu80 than with the xrs-6vec extracts (Fig. 2C). By plotting the differences between the amount of synthesis in the two pathways (Fig. 2D), it becomes apparent that more transcription complexes are associated with the template at all times in the presence of the xrs-6cKu80 extract. The number of transcription complexes actually appears to increase as the reaction proceeds, perhaps reflecting the fact that NTPases in the crude extract reduce the substrate concentration at later times, decreasing the rate of elongation, and thus prolonging the lifetime of individual elongation complexes. These results provide independent support for the conclusion that xrs-6cKu80 extracts have a higher reinitiation rate. They appear to exclude the possibility that the original results were attributable to the existence of a special class of heparin-sensitive elongation complexes in the xrs-6cKu80 extracts.

The Reinitiation Factor Is Diffusible Rather Than Template-associated—To further investigate the mechanism of action of the Ku-dependent reinitiation factor, we tested whether the reinitiation activity became stably associated with the template during the course of the reaction. The design of these experiments is shown in Fig. 3A. A promoter-containing DNA fragment was prepared with a single biotin group downstream of the transcription unit, the DNA was allowed to bind to streptavidin-coated paramagnetic beads, and the beads were incubated with nuclear extracts to allow preinitiation complex formation. In some reactions, NTPs were added, and RNA synthesis was measured directly, and in others, the supernatant was removed, the beads were washed, and the same or a different supernatant was added back. NTPs were then added, and RNA synthesis was measured.

In reactions where RNA synthesis was measured directly, without washing or otherwise manipulating the DNA beads, there was a substantial, 4.2-fold difference between xrs-6cKu80 and xrs-6vec extracts (Fig. 3B, lanes 1–4). These results are comparable with those obtained with soluble, supercoiled template (Fig. 1). In contrast, when the supernatant was removed, and beads were gently washed in transcription buffer, the overall level of transcription was greatly reduced and the difference between extracts was virtually eliminated (lanes 5–8). The present results clearly demonstrate that the components remaining on the template following the wash are not, in themselves, sufficient to support the greater level of transcription characteristic of xrs-6cKu80 extracts. This was a surprising result, given that prior work in our laboratory and elsewhere has shown that RNAP II preinitiation complexes are stable to washing (43). However, the initial studies validating the immobilized template method focused on single-round transcription (43) and are thus not directly comparable with the experiment presented here.

When supernatant fractions were added back to the washed beads, the xrs-6cKu80 supernatant was able to partially restore activity (lanes 11 and 12), and, interestingly, the same level of transcription was obtained regardless of the source of preinitiation complex (compare lanes 13 and 14 with lanes 11 and 12). By contrast, the xrs-6vec supernatant restored very little activity to either type of preinitiation complex (lanes 9, 10, 15, and 16). Thus, in these add-back experiments, the washed preinitiation complexes showed a level of transcription characteristic of the supernatant rather than the extract used for the initial complex formation. Qualitatively similar results were

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obtained when the wash step was omitted and supernatant was added back directly to unwashed preinitiation complexes (lanes 17–24). The results of these experiments are quantitated in Fig. 3C.

These experiments appear to rule out the possibility that the difference in activity between the two extracts can be explained by differential binding of transcription factors to the DNA at the time of preinitiation complex formation. It seems very unlikely that the template is "marked" as reinitiation-compe-
tent during the preincubation period, for example, through differential formation of TFIID-TFIIA-DNA complexes or through stable binding of Ku protein to a region of DNA melting at the promoter. The finding that the Ku-dependent reinitiation factor is diffusible rather than template-associated provides additional evidence that the factor works by influencing the availability of limiting transcription proteins rather than template.

Addition of Purified Ku Protein Preferentially Suppresses Activity of the xrs-6cKu80 Extract—The preceding experiments leave open the question of whether the effect of Ku on transcription is direct or indirect. We have previously reported an inability to correct the defect in xrs-6cvec extracts by addition of exogenous Ku protein, but this negative result does not rule out a direct role for Ku, because it could reflect a difficulty in assembling exogenous Ku into an active multiprotein complex.

To address whether Ku is directly involved in reinitiation, we turned to an alternative approach, which was to investigate whether addition of excess wild type or mutant Ku protein could suppress reinitiation activity in vitro.

Several recent reports have focused on potential functions of sequences in the C-terminal region of the Ku80 subunit (44–46). Partial proteolysis suggests that this region forms a distinct structural domain that increases in protease sensitivity...
upon DNA binding (47). This region is not required for DNA binding but may be involved in contacts with DNA-PKcs (44–46). Interestingly, expression of a natural variant of Ku80, which lacks the C terminus, correlates with a phenotype where cells are competent for repair but are radiation-sensitive because of an inability to pass a G2/M cell cycle checkpoint (44, 48). Because the C-terminal region of Ku80 appears to be involved in signaling rather than repair per se, it was a plausible target for our initial mutagenesis. A mutant, Ku80D681–728, was constructed that removes 45 amino acids near the C terminus of Ku80. This disrupts the C-terminal domain without impinging on the DNA binding and dimerization domains.

We coexpressed this mutant with Ku70 using baculovirus vectors in Sf9 cells and purified the resulting heterodimeric Ku protein as described under “Materials and Methods.” SDS-PAGE analysis with silver staining showed that the mutant protein was homogeneous and that the concentrations of mutant and wild type Ku protein were accurately matched (Fig. 4A).

Purified wild type and mutant Ku proteins were added to in vitro transcription assays containing DNA template immobilized on streptavidin-coated paramagnetic beads (Fig. 4B). Both the wild type and mutant Ku proteins inhibited transcription in a multiple-round reaction. The mutant had a greater effect, however, and showed more selectivity for the xrs-6cKu80 extract. The Ku80Δ681–728 mutant, unlike wild type Ku, nearly abolished the difference in transcription between the two extracts (Fig. 4C). Qualitatively similar inhibition was obtained using different amounts of wild type and mutant Ku, ranging from 150 to 450 ng/reaction (data not shown). These are relatively modest amounts of Ku, representing only a 2–6-fold molar excess of Ku over DNA ends. Although greater than the amount of Ku typically found in rodent cell extracts, they are comparable with the levels of endogenous Ku present in human cell extracts (see immunoblot in Fig. 7A).

Similar results were also obtained in transcription assays using a circular template in solution, although the effect was not as dramatic as when the template was immobilized (data not shown). The dominant-negative effect of the exogenous, purified Ku protein strongly suggests that Ku is directly involved in the reinitiation pathway, either as a component of the reinitiation factor or as a protein that interacts with the reinitiation factor.

**Preincubation with Exogenous Ku Causes Loss of Template Activity**—To further investigate the ability of exogenous Ku to suppress transcription activity, we performed an experiment in which exogenous Ku was preincubated with DNA bead tem-
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Ku80, mutant Ku80, and Ku70 are indicated. Proteins were resolved, and molecular mass standards were present, purified wild type human Ku80 or Ku80D687–728 protein (450 ng) was added prior to preinitiation complex formation. C, transcription reactions (inset), performed using extracts from xrs-6cvec (vec) or xrs-6cKu80 (Ku80) cells with or without exogenous purified Ku as indicated. Assays were performed in duplicate. RNA was isolated, analyzed, and visualized as in Fig. 1. Graph represents quantitation of transcription data. Error bars denote standard deviation.

Plate. This preincubation was performed in the presence of xrs-6cKu80 extract. Beads were then washed, and fresh xrs-6cKu80 supernatant was added from a parallel incubation containing no exogenous Ku protein. NTPs were added, and RNA synthesis was measured in a multiple-round assay (Fig. 5A). The results (Fig. 5B) show that exposure to exogenous Ku during the preincubation activates the template such that it can no longer support multiple rounds of transcription. As in the preceding experiment, the effect is more pronounced with the Ku80D681–728 mutant than with the wild type Ku protein. Because the inhibitory activity becomes associated with the template, the results suggest that both the exogenous mutant and the exogenous wild type Ku are capable of DNA binding, as expected, because the mutation lies outside the DNA-binding domain. The DNA binding activity of the mutant Ku was confirmed directly in a subsequent experiment (see below).

The results point to the existence of a key functional difference between endogenous and exogenous Ku protein. Endogenous Ku stimulates transcription through a mechanism that does not involve tight binding to the template. Exogenous Ku inhibits transcription and at the same time exhibits the expected ability to bind to DNA. One hypothesis to reconcile these observations is that Ku is capable of undergoing a switch between a latent form, which is sequestered in a complex with transcription factors, and a repair-active form, which binds DNA ends, recruits DNA-PKcs, and initiates the repair pathway. This model will be explored in more detail under “Discussion.” We suggest that endogenous Ku exists in the latent form, whereas biochemically purified Ku, which has been dissociated from other proteins, exists predominantly in the repair-active form. The balance may be further shifted toward the repair-active form by the Ku80D687–728 mutation.

Sequestration of DNA-PKcs in a Non-DNA Binding Form in the xrs-6cKu80 Extracts—To investigate this model further, it was of interest to investigate the status of the double-strand break repair apparatus in the xrs-6cKu80 extracts. For these experiments, we sought to compare the DNA binding properties of the endogenous and exogenous Ku, as well as the endogenous DNA-PKcs. DNA binding was measured under the same conditions used for transcription. Nuclear extract was incubated with DNA beads in the presence or absence of exogenous Ku, and the bound and unbound fractions were collected and analyzed by SDS-PAGE and immunoblotting.

The available anti-Ku80 antibodies were not sensitive enough to allow direct detection of the endogenous Ku, which is typically present at very low levels in rodent cells (Fig. 6A, lanes 1–4, 13, and 14). We were able to draw inferences about its behavior based on the DNA binding properties of endogenous DNA-PKcs (see below). The larger amounts of exogenous Ku were readily detected (lanes 5–12), and, as expected, a substantial proportion of the exogenous Ku was in the DNA-bound fraction. The wild type and Ku80D687–728 mutant bound to the DNA beads equivalently, consistent with the fact that the mutation lies outside the DNA-binding domain.

Fig. 6B shows an analysis of the distribution of DNA-PKcs in the same experiment. Endogenous DNA-PKcs is readily detected using a sensitive, cross-species reactive monoclonal antibody, 18-2 (49). A substantial fraction of the DNA-PKcs in the xrs-6cvec extract associates with the DNA beads, whether or not exogenous Ku is present (lanes 1 and 2). This is consistent with recent findings that DNA-PKcs has some intrinsic in vitro DNA binding activity, independent of Ku (50, 51). By contrast, the DNA-PKcs in the xrs-6cKu80 extract appears to be sequestered in a non-DNA binding form (lanes 3 and 4) and is recruited to the DNA beads only when the reinitiation factor is disrupted by addition of exogenous Ku. In some lanes, the non-DNA bound form of DNA-PKcs appears as a doublet; whether this relates to functional properties of the protein is unknown. These results establish a correlation between the behavior of endogenous DNA-PKcs and reinitiation factor activity. When reinitiation activity is present, in the xrs-6cKu80 extract, the DNA-PKcs is sequestered in a non-DNA binding form. When activity is absent in the xrs-6cvec extract or when it is suppressed by exogenous Ku, the DNA-PKcs associates with the DNA.

Ku80D687–728 Supports DNA End Joining in an in Vitro Assay—We have proposed that the Ku80D687–728 mutation favors a repair-active conformation of Ku and that the presence of an excess amount of this repair-active form disrupts interactions between endogenous Ku and other proteins, including DNA-PKcs, transcription factors, or both. One of the predictions of the model is that the mutant form of Ku should be active in DNA end joining and be capable of productive interaction with DNA-PKcs in a repair complex. To test this prediction, we measured repair activity in a Ku-dependent cell-free DNA end-joining system. The system is based on human whole cell lymphoblast extracts (38) and has been supplemented with...
recombinant DNA ligase IV-XRCC4 complex. This increases ligation efficiency and makes the system less sensitive to small changes in buffer composition, which was important for the immunodepletion experiments described below. Purification of DNA ligase IV-XRCC4 and characterization of its ability to function synergistically with endogenous repair factors has been described in detail elsewhere (39).

For the purpose of this experiment, it was necessary to have a way to quantitatively remove endogenous Ku and DNA-PKcs without unduly diluting the extract or introducing interfering components. To accomplish this, we used a human autoimmune serum (serum TT) that contains a very high titer of autoantibodies against both Ku and DNA-PKcs. An immunoblot, shown in Fig. 7A, indicates that the TT serum depleted essentially all of the endogenous Ku and DNA-PKcs from the lymphoblast extract. By contrast, normal human serum (NS) did not deplete significant amounts of either protein.

Results of a functional end joining assay are shown in Fig. 7B, with quantitation in Fig. 7C. Mock-immunodepleted cell extract showed readily detectable end joining activity (lane 2). The activity was dependent on as-yet-unidentified extract components, because activity was not seen with a mixture of re-
Ku protein in transcriptional reinitiation and in double-strand break repair are genetically separable.

FIG. 7. Ku80Δ681–728 supports DNA end-joining in an in vitro assay. 12–15 μl of the GM00558 lymphoblast extract was immunodepleted with 5 μl of normal human serum (NS) or of TT serum containing autoantibodies directed against the Ku and DNA-PKcs. 4 μl of immunodepleted extract, 10 ng of 32P-labeled DNA substrate and the indicated quantities of purified Ku or DNA-PKcs protein were incubated for 1 h at 37 °C to allow for end joining. A, immunoblot showing purified protein markers (100 ng of Ku, 150 ng of DNA-PKcs), undepleted cell extract, and the proteins precipitated in the immune complex (Bead). B, substrate and products of end joining reaction, resolved by 0.6% agarose gel electrophoresis, and visualized by PhosphorImager analysis. Positions of unligated monomer and dimer and trimer ligation products are indicated. C, quantitation of results in B. Ratio of product (dimer plus trimer) to total DNA was determined. All values were then normalized to the result obtained with normal serum-depleted extract (lane 2).

DISCUSSION

In the present work, we have compared RNAP II transcription in extracts from Ku-deficient CHO cells with transcription in extracts from genetically matched cells that have been rescued by transfection of a human Ku80 cDNA. We found that expression of Ku increased the amount or availability of a soluble reinitiation factor. The factor appears to work by facilitating the recycling or redistribution of limiting transcription factors to new template molecules subsequent to the first round of initiation. We have shown that it increases the number of elongation complexes loaded on the template at all times after addition of NTPs. The factor itself does not form a tight complex with DNA, ruling out various models based on the DNA binding properties of Ku or the differential binding of transcription factors to DNA during the first round of transcription.

Addition of a mutant form of Ku lacking the C-terminal region of Ku80 preferentially suppresses transcription in the extract from Ku-expressing cells. Addition of wild type Ku also suppresses transcription, but to a lesser extent. The finding that mutant and wild type Ku differ in their effect on transcription, although they have similar DNA binding properties, provides evidence that Ku influences reinitiation through protein-protein rather than protein-DNA interactions.

We used a recently developed cell-free double-stranded break repair system (38, 39) to further investigate the functional behavior of the mutant Ku. The system is based on lymphoblast whole cell extracts and mimics the in vivo double-strand break repair pathway, in that its activity is dependent on Ku, DNA-PKcs, and the DNA ligase IV-XRCC4 complex (39). In vitro cooperation between Ku and DNA ligase IV-XRCC4 has also been reported by others in recent studies (52, 53). We used a high titer human autoantiserum to deplete the cell extracts of endogenous Ku and DNA-PKcs and reconstituted end joining activity with recombinant or highly purified preparations of these proteins. The results indicate that the mutant Ku is functional in repair and capable of interaction with DNA-PKcs. Thus, the C-terminal region of Ku80, although phylogenetically conserved, is not directly required to promote DNA end joining. We suggest that this region of Ku80 may serve to interact with other nuclear proteins, including components of the transcription apparatus, to coordinate repair with other cellular processes.

It is of interest that certain hematopoietic cells contain a natural variant of Ku80 that lacks the C-terminal region, and the presence of this form is correlated with a phenotype where cells are capable of repairing DNA damage but defective in their ability to recover and resume normal growth (44, 48). This observation appears to be analogous to our in vitro findings, where mutant Ku is able to participate in repair but not in other functions. It is also of interest that threonine 715 of Ku80, which falls within the putative C-terminal regulatory region, is a site of phosphorylation by DNA-PKcs in vitro (54). Phosphorylation at this site in vivo could help mediate the switch between alternative functions of Ku.

To exert its effect on reinitiation, the Ku-dependent reinitiation factor must be capable of at least transient interaction with promoter DNA or promoter-bound proteins, but these interactions do not lead to creation of a stable, bound complex that can be recovered in association with immobilized template. It seems paradoxical that endogenous Ku protein would not bind to the template, given the avid DNA binding properties of isolated Ku protein, and our findings have led us to hypothesize that the endogenous Ku in the xrs-6cKu80 extracts may be sequestered in a form that is incapable of assembling combinator and purified repair proteins alone (lane 1). To make the in vitro system dependent on exogenous Ku- and DNA-PKcs, we depleted the extract with serum TT. This greatly reduced end joining activity (lane 3). Addition of wild type Ku protein and DNA-PKcs together, but not separately, restored activity to the depleted extract (lanes 6–8). Addition of Ku80Δ681–728 mutant protein, together with DNA-PKcs, also restored activity, to a level comparable to that seen with wild type (lanes 9–12). The restoration of activity by wild type and mutant Ku showed a similar dose dependence.

These results demonstrate that both the wild type and mutant Ku proteins are capable of directing DNA end joining and that the mutant retains the ability for functional interaction with DNA-PKcs in the context of a repair complex. The finding that Ku80Δ687–728 behaves differently than wild type Ku in an in vitro transcription inhibition assay but identically in an in vitro end joining assay demonstrates that the functions of Ku protein in transcriptional reinitiation and in double-strand break repair are genetically separable.
A. Ku and DNA-PKcs are sequestered in a complex with transcription factors. The presence of Ku and DNA-PKcs stabilizes the higher order structure of the transcription machinery, which promotes efficient recycling of limiting protein components in the reinitiation phase of the transcription reaction. When sequestered in this form, Ku and DNA-PKcs are not capable of forming a high affinity complex active at DNA ends. In the presence of DNA damage signals, Ku and DNA-PKcs are released and become active for DNA end binding and repair. Simultaneously, the transcription factor complex is disrupted, limiting its ability to facilitate rapid reinitiation. Upon recovery, the system returns to its original state.

FIG. 8. Model for the regulation of Ku and DNA-PKcs activity. A. Ku and DNA-PKcs are sequestered in a complex with transcription factors. The presence of Ku and DNA-PKcs stabilizes the higher order structure of the transcription machinery, which promotes efficient recycling of limiting protein components in the reinitiation phase of the transcription reaction. When sequestered in this form, Ku and DNA-PKcs are not capable of forming a high affinity complex active at DNA ends. In the presence of DNA damage signals, Ku and DNA-PKcs are released and become active for DNA end binding and repair. Simultaneously, the transcription factor complex is disrupted, limiting its ability to facilitate rapid reinitiation. Upon recovery, the system returns to its original state.

B. DNA-PKcs-deficient cells show a defect in the ability to carry out sustained high levels of hsp70 mRNA transcription, consistent with a reinitiation defect (58). Interestingly, Ku-deficient mice are smaller than their wild type littermates (59), which is consistent with the existence of a mild but general transcription defect. Similar dwarfism has been observed in mice with a small deletion in the C-terminal domain of RNA polymerase II (60).

We have not yet been able to identify the Ku-dependent reinitiation factor at the molecular level. Although this remains an important goal, activity appears to be labile to column chromatography, and we have been unsuccessful in reconstituting the activity from fractionated extracts. One possibility is that the complex between Ku and transcription factors, if it exists, is based on low affinity interactions, because we have not been able to demonstrate specific high affinity interactions between Ku and general transcription factors by biochemical methods. It may not be accurate to characterize such a complex as a “holoenzyme,” comparable with the well-defined E. coli RNA polymerase holoenzyme, but even if it is short-lived, heterogeneous, or easily disrupted, it could have profound effects on reaction kinetics.

Since the initial molecular characterization of Ku protein in 1986, there have been a number of reports that Ku and its partner, DNA-PKcs, are involved in some way in transcription, either through binding to DNA, through phosphorylation of RNA polymerase II and transcription factors, or through participation in multiprotein complexes. Although these observations are suggestive, it has been difficult to demonstrate a direct functional role for Ku or DNA-PKcs in transcription. In the present study, we have investigated this role through an assessment of the phenotype of Ku80 mutant cells, making no prior assumptions based on biochemical studies. We conclude that expression of Ku80 has dramatic effects on in vitro reinitiation rate and that this is genetically separable from its role in end joining. We put forward a testable model that the enhanced rate of reinitiation is a consequence of sequestration of double-strand break repair proteins in a network of low affinity molecular interactions with transcription proteins in the living cell.

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