Ku Entry into DNA Inhibits Inward DNA Transactions in Vitro*

Received for publication, May 19, 2000, and in revised form, July 7, 2000
Published, JBC Papers in Press, August 16, 2000, DOI 10.1074/jbc.M004315200

Philippe Frit†, Ruo-Ya Li§, Doriane Arzel‡, Bernard Salles‡‡, and Patrick Calsou‡‡

From the †Institut de Pharmacologie et de Biologie Structurale, CNRS UMR 5089, 205 Route de Narbonne, 31077 Toulouse and the ‡Société Française de Recherches et d’Investissements, Berganton, 33127 Saint Jean d’Illac, France

Association of the DNA end-binding Ku70/Ku80 heterodimer with the 460-kDa serine/threonine kinase catalytic subunit forms the DNA-dependent protein kinase (DNA-PK) that is required for double-strand break repair by non-homologous recombination in mammalian cells. Recently, we have proposed a model in which the kinase activity is required for translocation of the DNA end-binding subunit Ku along the DNA helix when DNA-PK assembles on DNA ends. Here, we have questioned the consequences of Ku entry into DNA on local DNA processes by using human nuclear cell extracts incubated in the presence of linearized plasmid DNA. As two model processes, we have chosen nucleotide excision repair (NER) of UVC DNA lesions and transcription from viral promoters. We show that although NER efficiency is strongly reduced on linear DNA, it can be fully restored in the presence of DNA-PK inhibitors. Simultaneously, the amount of NER proteins bound to the UVC-damaged linear DNA is increased and the amount of Ku bound to the same DNA molecules is decreased. Similarly, the poor transcription efficiency exhibited by viral promoters on linear DNA is enhanced in the presence of DNA-PK inhibitor concentrations that prevent Ku entry into the DNA substrate molecule. The present results show that DNA-PK catalytic activity can regulate DNA transactions including transcription in the vicinity of double-strand breaks by controlling Ku entry into DNA.

DNA double-strand breaks (DSBs) are generated by agents such as ionizing radiation or as intermediates in certain recombination reactions (for review see Ref. 1). In response to the deleterious consequences associated with DSBs, highly efficient mechanisms have evolved for their recognition and repair. Mammalian cells appear to rejoin DSBs primarily by a mechanism of non-homologous recombination (reviewed in Refs. 6–8). Insights into the proteins involved in DSBs repair have been gained from analysis of the molecular defects in mutant rodent cells that are hypersensitive to ionizing radiation and unable to carry out the V(D)J recombination process in the immunoglobulin and T-cell receptor genes. Recent evidence shows that a multiprotein complex, the DNA-dependent protein kinase (DNA-PK), plays a central role in DSBs repair in mammalian cells (reviewed in Refs. 6–8).

The DNA-PK is a heterotrimeric enzyme composed of a large catalytic subunit of ~460 kDa (DNA PKcs, p460), a serine/threonine kinase that belongs to the phosphatidylinositol 3-kinase family (9) and a dimeric regulatory component consisting of the Ku80 and Ku70 proteins (10, 11).

Ku was originally identified as a heterodimeric autoantigen consisting of ~80 and 70-kDa subunits (Ku80 and Ku70, respectively) (12). It binds specifically to double-strand breaks and, more generally, to any transition from single- to double-strand DNA, without demonstrating sequence specificity (12–14). Ku is able to translocate along the DNA in an ATP-independent manner allowing several Ku dimers to bind to a single DNA molecule and form a multimeric complex (15, 16). Ku has been found to constitute the DNA-dependent protein kinase (DNA-PK) activating subunit since, once bound to DNA, it recruits and activates the p460 catalytic subunit (10, 11).

Although the DNA-binding properties of Ku protein have been well characterized with purified native or recombinant proteins, the question of the influence of the catalytic subunit on Ku interaction with DNA was poorly documented. We have shown recently by assessing Ku DNA end binding activity in human nuclear cell extracts by electrophoretic mobility shift assays that DNA-PK, including Ku and p460, is blocked at DNA ends in the presence of a phosphatidylinositol 3-kinase inhibitor such as wortmannin and prevents end processing by either DNA polymerization, degradation, or ligation. On the opposite, the kinase activity is required for Ku translocation along the DNA helix when the whole Ku-DNA-PKcs complex assembles on DNA ends (17).

The question of the function of Ku entry into the DNA helix is still open. For example, it is known that Ku rigidifies the DNA structure (18), is able to form a DNA loop by contact of distant Ku dimers (19), and to transfer from one DNA molecule to another (20). Alternatively, multiple Ku proteins bound to a single dsDNA may serve the recruitment of additional proteins. Thus, the spreading of several Ku dimers possibly integrated in multiprotein complexes bound to DNA around the breaks may have various consequences on local DNA processes like replication or transcription. Here, we have addressed this question by using human nuclear cell extracts incubated in the presence of linearized plasmid DNA. As two model processes, we have chosen nucleotide excision repair (NER) of UVC DNA lesions and transcription from viral promoters.

NER is the major DNA repair pathway for numerous DNA lesions including UVC photoproducts (for review see Refs. 21–
23. The reaction is carried out by the coordinated action of about 30 proteins that are involved in the two main steps, recognition/incision-excision of the lesion and DNA synthesis/ligation to restore strand continuity. The human cell lines of complementation group A to G representative of the cancer-prone disease xeroderma pigmentosum (XP) exhibit a deficiency in the early steps of the reaction.

We have previously shown that the incision activity exhibited by human cell extracts in vitro was decreased by about 80% on a linear damaged plasmid DNA when compared with the efficiency on a supercoiled plasmid DNA substrate (18). By using an assay allowing us to monitor directly the interactions of proteins with damaged DNA (24), we have shown a dramatic inhibition of the binding of key NER proteins to linear damaged DNA. NER efficiency and NER proteins binding to damaged DNA were concomitantly regained when the reaction was performed with extracts lacking Ku activity, whereas addition of purified human Ku complex to these extracts restored the inhibition (25).

Here, we show that NER efficiency can be fully restored in the presence of DNA-PK inhibitors. This restoration is accompanied by an increased binding of NER proteins to the UVC-damaged linear DNA and by a mirror decreased binding of Ku heterodimer. Similarly, the poor transcription efficiency exhibited by viral promoters on linear DNA is enhanced in the presence of wortmannin concentrations that prevent Ku entry into the DNA substrate molecule.

The present results support a model in which DNA-PK catalytic activity can regulate DNA transactions in the vicinity of double-strand breaks by controlling Ku entry into DNA.

**EXPERIMENTAL PROCEDURES**

**Cell Extracts and Proteins**—The HeLa S3 cell line was obtained from the stock of European Molecular Biology Laboratories (Heidelberg, Germany). Cells were grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 2 mM glutamine, 125 units/ml penicillin, and 125 μg/ml streptomycin at 37 °C, in a humidified atmosphere containing 5% CO₂.

Nuclear protein extracts were prepared from HeLa cells as described previously (26) except that the final dialysis was performed for 3 h at 4 °C in 25 mM Hapes-KOH, pH 7.9, 17% glyceral, 100 mM potassium glutamate, 2 mM EDTA, 1 mM EGTA, and 2 mM dithiothreitol. After preparation, extracts were immediately frozen and stored at −80 °C.

After cell lysis and centrifugation to pellet the nuclei, the supernatant was removed, extracted in parallel, and dialyzed in the same buffer as the nuclear extracts. It was designed thereafter as cytoplasmic extracts. After preparation, extracts were immediately frozen and stored at −80 °C.

The purified Ku complex and the p60 DNA-PK catalytic subunit were generous gifts of Dr. J. D. Chen (Lawrence Berkeley National Laboratory, Berkeley, CA). Ku heterodimer was baculovirus-expressed recombinant proteins, and p460 was purified from HeLa cells, as described previously (19).

**Plasmids and DNA Fragments**—Closed circular 2959-bp pBluescript KS− (pBS, Stratagene) and the related 3738-bp pHM14 plasmid (gift from Dr. R. D. Wood, ICRF, Herts EN6 3LD, UK) were prepared by the standard alkaline lysis method and cesium chloride gradient centrifugation from Esherichia coli JM109 (relevant genotype: recA1, endA1, gyrA96, hsdR17) followed by two neutral sucrose gradient centrifugations as described (27). pBS plasmid was irradiated UVC light (peak wavelength 254 nm) under conditions where 100 J/m² produced an average of 2.7 cyclobutane pyrimidine dimers per molecule as described (28).

Plasmids used in transcription assays were kind gifts of Dr. M. S. Satoh, (Université Laval, Quebec, Canada). Dowstream from a strong viral promoter, they contained a G-less cassette that does not have any guanine residue in the non-transcribed strand (29). pGFl contained the adenovirus major late promoter (AdMLP), and pGFl was prepared from pGFl plasmid by removing AdMLP (30). pCMV contained the cytomegalovirus promoter. The sequence of the non-transcribed strand of the G-less cassette is as follows: 5′-TCCTCAGCTCCTCTCTCCTCCTCTATATATATATACTCA-

CTCCCCCTACCATATACACACTCTTACCCATCCACACTCTTACCCATCCACACTTAA-

-3′, where the +1 start site for transcription is shown in boldface and the last 3′ dinucleotide belongs to a HindIII restriction site. Supercoiled plasmids were prepared as described above.

When necessary, damaged and undamaged plasmids were linearized by digestion with restriction enzyme as mentioned, and a reaction with or without enzyme was performed in parallel. The resulting plasmids were purified by phenol/chloroform extraction and ethanol-precipitated. In the presence of restriction enzyme, the linearization was complete as judged by eye after migration on agarose gel. The linearized plasmid was designed thereafter as linear DNA (L), and the product of the reaction without enzyme was named supercoiled or circular plasmid (C).

**Chemiluminescent Detection of DNA Repair Synthesis in Microplates**—Repair synthesis assay was performed essentially as described previously (24). Briefly, 50 ng of damaged or untreated plasmid DNA closed-circular or linear were adsorbed on sensitized 96-well microtiter plates (Microlite II, Dynatech) in 10 mM phosphate buffer, pH 7, for 30 min at 30 °C with shaking. No significant difference in DNA adsorption was found between linear versus circular or damaged versus undamaged DNA, as quantified with fluorescence detection (picogreen dye, Molecular Probes) or with radiolabeled DNA. The standard repair reaction was carried out in 50 μl (final volume/per well) and contained typically 150 μg of protein extract in reaction buffer (70 mM KCl) (or 70 mM KCl, 0.1 mM MgCl₂, 1 mM EGTA, and 0.5 mM dithiothreitol, 10 mM potassium glutamate, 2.5 mM creatine kinase, and 0.5 mM 4-iodophenol (both from Aldrich), 30% H₂O₂ (w/v) diluted 1/25,000) was added for 5 min at 30 °C. Emitted light was measured with a luminometer (Lumax SA, SFRI, France) and expressed as a relative light unit. When necessary, extracts were preincubated in the presence of DNA-PK inhibitor (wortmannin or LY294002 from Sigma) in reaction buffer without plasmids and dNTP for 10 min at 30 °C. After a 2-h incubation time at 30 °C, the wells were washed three times with PBS (phosphate-buffered saline solution, pH 7.4, plus 0.01% Tween 20). Biotin-21-DUMP incorporated into DNA was detected by incubation with ExtrAvidin conjugated to peroxidase (Sigma) diluted 1/10,000 in PBS and incubated for 30 min at 30 °C. Wells were washed three times with PBS and 0.1 μl of chemiluminescent substrate mixture (10 mM Tris-HCl, pH 8.5, 1.25 mM luminol and 145 μM 4-iodophenol (both from Aldrich), 30% H₂O₂ (w/v) diluted 1/25,000) was added for 5 min at 30 °C. Emitted light was measured with a luminometer (Lumax SA, SFRI, France) and expressed as a relative light unit. Experiments were performed in duplicate or triplicate. DNA repair synthesis activity was expressed as the mean value of relative light unit/pmol treated minus the value in untreated plasmid DNA

**DNA Repair Synthesis Assay in Microplates**—Standard 50-μl reactions mixtures contained 200 ng of each of damaged and untreated closed-circular or linear plasmids, 74 kBq of [32P]dCTP (110 TBq/mmol, PerkinElmer Life Sciences), cell extract as indicated, and 60 mM potassium glutamate in reaction buffer containing 45 mM Hapes-KOH, pH 7.8, 7.4 mM MgCl₂, 0.9 mM dithiothreitol, 0.4 mM EDTA, 2 mM ATP, 20 μM dCTP, dGTP, dATP, and biotin-21-dUTP, 40 μM dUTP, and 10 μM dCTP, 40 μM dGTP, 2.5 μM of creatine phosphokinase (type I, Sigma), 3.4% glycerol, and 18 μg of bovine serum albumin as described (28). When necessary, extracts were preincubated in the presence of DNA-PK inhibitor (wortmannin or LY294002 from Sigma) in reaction buffer without plasmids and dNTP for 10 min at 30 °C. Complete reactions were carried out at 30 °C for 3 h. Plasmid DNA was purified from the reaction, linearized with KpnI, and electrophoresed overnight on a 1% agarose gel containing 0.5 μg/ml ethidium bromide. Radiolabel incorporation was visualized by exposure to a storage phosphor screen (Molecular Dynamics) followed by processing with a PhosphorImager (Molecular Dynamics, Storm System™). Data were quantified by scintillation counting of excised DNA bands.

For data presentation, gels and phosphorimaging of the gels were processed with Adobe Photoshop 3.0 software.

**DNA Transcription Assay in Microplates**—Standard 25-μl reactions contained 150 μg of HeLa nuclear extracts, 1 μg of plasmid DNA, 370 kBq of [32P]dCTP (111 TBq/mmol, PerkinElmer Life Sciences), and 100 mM potassium glutamate in buffer containing 22 mM Hapes-KOH, pH 7.8, 6.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.2 mM EDTA, 0.1 mM CTP, 0.15 mM GTP, and 50 μM each of dGTP, dATP, dCTP, and dTTP, 20 μM phosphocreatine, and 1 μg of creatine phosphokinase (type I, Sigma), 1.7% glycerol, 9 μg of bovine serum albumin, and 100 units of RNase T1 (Life Technologies, Inc.). When necessary, protein extracts were preincubated in the presence of wortmannin for 5 min at 30 °C and then plasmid DNA was added by a further 5-min preincubation period; then the other components were added and the reactions were carried out for 90 min at 30 °C. The reactions were...
terminated by addition of 175 μl of 0.3 M Tris-HCl, pH 7.4, 0.3 M sodium acetate, 0.5% SDS, 2 mM EDTA, 3 μg/ml trNA. RNase T1-resistant transcripts were purified by phenol/chloroform extraction, precipitated with ethanol, dissolved in gel loading buffer (98% formamide, 10 mM EDTA, 0.1% xylene cyanol, 0.1% bromphenol blue), denatured at 85 °C for 5 min, and fractionated on a 6% acrylamide, 7 M urea gel. Radiolabel incorporation was visualized by storage phosphor screen (Molecular Dynamics) followed by processing with a PhosphorImager (Molecular Dynamics, Storm System™). The regions of the gel containing the RNase T1-resistant transcripts were quantitated by the ImageQuaNT software, version 4.2A (Molecular Dynamics). For data presentation, phosphorimaging of the gels was processed with Adobe Photoshop 3.0 software.

**Antibodies**—Anti-Ku70 (N3H10), anti-Ku80 (111), anti-Ku70/80 (162) and anti-DNA-PKcs (18.2 and 25.4) were from NeoMarkers (Fremont, CA). Polyclonal antibodies anti-XPA (31) were generously provided by Dr. C. J. Jones (University of Wales, College of Medicine, Cardiff, UK). Monoclonal IgM antibody H14 against the large subunit of RNA polymerase II (pol II L5) (32) was a kind gift from Dr. D. B. Bregman (Albert Einstein College of Medicine, Bronx, NY). For Ku or DNA-PKcs immunodepletion, anti-Ku70/80 (162) or anti-DNA-PKcs (25-4) monoclonal antibodies were coupled to magnetic anti-mouse IgG beads (Dynabeads M-450, Dynal), according to the manufacturer's recommendations. Under 20 μl final volume, 250 μg of HeLa nuclear protein extracts were incubated for 60 min with 20 μl of wet control, anti-Ku or anti-DNAPK beads in dialysis buffer under gentle agitation. The supernatant was removed over a magnet (Dyna MPC, Dynal). A second depletion was performed immediately under the same conditions.

**Analysis of DNA-associated Proteins**—50 ng of closed circular or linear plasmid DNA was adsorbed on sensitized 96-well microtiter plates as described above. DNA repair reactions were carried out as described above for the chemiluminescent detection of DNA repair synthesis in microplates. RNA transcription reactions were performed as described above for assays in microtubes, except that the reaction volume was 50 μl and radiolabeled CTP was omitted. After incubation, the protein fraction bound to DNA was analyzed after four washes with PBST and then incubated 30 min at 30 °C with room temperature and proteins immuno- blotted with monoclonal antibodies overnight at 4 °C. After incubation, membrane secondary antibody conjugated to horseradish peroxidase, specific proteins were visualized as immunoreactive bands by the enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech). For data presentation, films were scanned and processed with Adobe Photoshop 3.0 software.

DNA-PK Assay—Kinase activity was assessed under DNA repair conditions in microtiter wells. After DNA adsorption and washing as described above, 150 μg of HeLa protein extract were incubated for 1 h at 30 °C in standard repair buffer but without ATP. The wells were washed three times with PBST and then incubated 30 min at 30 °C with 185 kBq of [γ-32P]ATP (PerkinElmer Life Sciences), 2 mM ATP, wortmannin, when necessary, and 10 μg of DNA-PK-specific peptide substrate (SQE peptide, EPPLSqEAFADLWKK (34)) in 50 μl of buffer (25 mM Hepes-KOH, pH 7.9, 50 mM KCl, 1 mM MgCl2, 1 mM dithiothreitol, 0.1% Nonidet P-40, and 20% glycerol). The supernatant was then resolved by SDS-PAGE (22%) followed by processing with a PhosphorImager (Molecular Dynamics, Storm System™). The regions of the gel containing the radiolabeled peptide were quantitated by the ImageQuaNT software, version 4.2A (Molecular Dynamics). The DNA-binding fraction was analyzed by SDS-PAGE (10%) and Western blotting as described above.

RESULTS

**Regulation of Ku Binding to Linear DNA by DNA-PK Catalytic Activity**—In order to investigate in human nuclear cell extracts the influence of DNA-PK catalytic activity on Ku binding to DNA, we have taken advantage of an assay developed in our group using plasmid DNA quantitatively adsorbed on sensitized microplate wells, which has allowed us to reproduce DNA repair reactions (35) and to analyze the DNA-bound protein fraction (24).

First, we have adapted this assay to DNA-PK activity. As shown in Fig. 1A, the phosphorylation of a consensus peptide for DNA-PK activity was dependent on the presence of free DNA ends in microplates, as expected for true DNA-PK activity. In addition, this free DNA ends-dependent phosphorylation activity was inhibited by increasing concentrations of known inhibitors of DNA-PK-like wortmannin (9) or the less efficient LY 294002 (36, 37) (Fig. 1A)

![Fig. 1. Effect of wortmannin on protein kinase and DNA binding activities of DNA-PK in HeLa protein extracts as assessed with DNA bound to microwells.](image)

**Fig. 1. Effect of wortmannin on protein kinase and DNA binding activities of DNA-PK in HeLa protein extracts as assessed with DNA bound to microwells.** A, DNA-PK activity of HeLa protein extracts in the presence of DNA-PK-specific peptide substrate, [γ-32P]ATP, closed circular (C), or linear plasmid DNA (L) adsorbed in microwells and increasing concentrations of wortmannin as indicated (added along with ATP). The picture shows the radiolabel incorporation in the peptide substrate resolved under a 22% polyacrylamide gel. B, Ku DNA binding activity of HeLa protein extracts in the presence of closed circular (C) or linear plasmid DNA (L) adsorbed in microwells. Extracts were preincubated in reaction buffer in the presence of increasing concentration of wortmannin as indicated, before contacting DNA in microwells. After incubation, the wells were washed, and the proteins bound to the plasmids were recovered and analyzed by Western blotting. mAbs 18.2, 111, and N3H110 were used to detect p460, Ku80, and Ku70, respectively. C, Western blotting analysis of HeLa nuclear extracts immunodepleted by control magnetic beads (–) or anti DNA-PKcs-coupled beads (monoclonal antibody 25.4, +). D, Ku DNA-binding activity of HeLa protein extracts in the presence of closed circular (C) or linear plasmid DNA (L) adsorbed in microwells as in B but with p460 immunodepleted extracts.

DNA repair reactions (35) and to analyze the DNA-bound protein fraction (24).

Then we have assessed the binding of Ku to linear DNA under the same conditions by analyzing the DNA-bound pro-
tein fraction by Western blotting. As shown in Fig. 1B, we observed a strong free ends-dependent Ku binding to DNA (compare the 1st to 2nd lanes). However, a strong binding inhibition was detected when the extracts were preincubated with wortmannin in the absence of DNA and ATP (lanes 0–30 μM wortmannin). Preincubation of the extracts in the presence of inhibitor was necessary. Indeed, when increasing wortmannin concentrations were added to the reaction at the time of incubation with DNA bound to the wells, no effect was observed on Ku binding to DNA (data not shown), more likely due to the competing effect of ATP toward wortmannin at the time when DNA-PK contacts DNA. In addition, although Ku binding to DNA decreased in the presence of wortmannin, binding of the p460 DNA-PK catalytic subunit to DNA increased (Fig. 1B). However, p460 DNA binding activity was still Ku-dependent since extracts immunodepleted of Ku dimer exhibited no p460 binding activity to linear DNA (data not shown).

A Western blotting experiment was run with several dilutions of recombinant Ku heterodimer and purified p460 preparations in order to standardize the chemiluminescence detection. A simultaneous detection by Western blotting of Ku and p460 bound to linear DNA in an experiment identical to the one described above (Fig. 1B) allowed us to calculate that the molar ratio Ku/p460 varied from 43 without wortmannin to ~1 at 30 μM wortmannin. In addition, since each well contained a mean of 17 fmol of pBS plasmid molecule, we calculated that the molar ratio protein/DNA end was 8.7 and 0.2 without wortmannin for Ku and p460, respectively, and was close to 1 for both proteins at 30 μM wortmannin.

Since the effect of wortmannin on Ku binding to DNA could conceivably rely on a phosphatidylinositol 3-kinase different from DNA-PKcs, we have depleted HeLa extracts of p460 subunit by two successive immunoprecipitations with monoclonal antibody 25-4 which removed more than 90% of the DNA-PKcs, unit by two successive immunoprecipitations with monoclonal antibody for Ku and p460, respectively, and was close to 1 for both of 17 fmol of pBS plasmid molecule, we calculated that the molar ratio protein/DNA end was 8.7 and 0.2 without wortmannin for Ku and p460, respectively, and was close to 1 for both proteins at 30 μM wortmannin.

The present data clearly visualize the regulation of Ku binding to DNA by DNA-PK catalytic activity and allow us to ask the question of the consequence of Ku binding to DNA on local DNA processes.

Effect of the DNA-PK Inhibitors on NER Activity and Binding of NER Proteins to Linear UV-damaged DNA—As a model reaction involving DNA, we have first chosen NER that can be easily driven in vitro by cell-free extracts operating on damaged plasmid DNA (28). We have previously reported an adaptation of the fluid-phase repair synthesis assay set up by Wood et al. (28) into a solid-phase repair assay using plasmid DNA adsorbed on sensitized microplate wells. Incorporation of biotin-dUMP takes place during the repair synthesis step and is quantified by chemiluminescence detection in an enzyme-linked immunosorbent assay-like reaction (35). Moreover, in Western blotting experiments on the DNA-binding fraction, we have quantitatively detected repair complexes formed at damage sites and containing XPA and p62-TFIH, indispensable for the incision step of the reaction (24).

We have previously reported an inhibition of the NER activity at the level of the incision step when human cell extracts operate on linear DNA compared with circular plasmid DNA (18, 25). Similarly, a strong repair inhibition was observed on linear UV-irradiated DNA compared with the counterpart circular plasmid DNA, as quantified by biotin-dUMP incorporation (Fig. 2A). However, when the extracts were preincubated in the presence of increasing concentrations of wortmannin, we observed a striking increase in the repair synthesis activity on linear DNA (Fig. 2A) which was restored up to the yield obtained on the circular substrate for 3 μM wortmannin. No significant variation in repair activity was observed on circular damaged DNA over the range of wortmannin concentrations tested. The increase in biotin-dUMP incorporation in linear DNA can be confidently ascribed to repair synthesis activity since no increase was observed on the control undamaged linear DNA under the same conditions.

In order to investigate the interaction between Ku and NER proteins with damaged DNA, the DNA-bound protein fractions were analyzed by Western blotting experiments. To focus on the early steps of the NER process, XPA and one subunit of TFIH (p62-TFIH) were chosen as reporter proteins.

In agreement with our previous report (25), XPA and p62-TFIH bound specifically to circular damaged DNA, and in contrast, a strong decrease in binding activity was observed with linear damaged DNA (Fig. 2B). With circular DNA, no binding of Ku was observed, but in sharp contrast, both Ku subunits were detected on linear DNA, either undamaged or UV-damaged (Fig. 2B). When the repair reaction was carried out with extracts preincubated in the presence of wortmannin, a strong decrease in Ku binding to linear DNA was observed (Fig. 2B), as expected from the experiments described above (Fig. 1B). Moreover, wortmannin induced a striking restoration...
of XPA and p62-TFIIH-specific binding to UV-damaged linear DNA, whereas no effect was observed on circular DNA.

In order to test the possibility that these observations resulted from the particular conditions used, i.e. DNA adsorbed onto microwells, we have performed a similar experiment under standard fluid-phase NER conditions (28). A typical result is shown in Fig. 3A where DNA repair synthesis inhibition yields ~70% on a linear UV-irradiated plasmid DNA (compare lanes 1 and 2). When the extracts were preincubated in the presence of increasing concentrations of wortmannin, we observed again an increase in repair synthesis activity only devoted to linear UV-damaged DNA (Fig. 3A), up to the yield obtained on the circular damaged substrate for 3 μM wortmannin (Fig. 3B). When the same experiment was performed with extracts preincubated with increasing LY294002 concentrations up to 200 μM, we observed the same progressive restoration of NER activity on linear UV-damaged DNA (data not shown). We have shown elsewhere that NER was inhibited at the incision step on linear DNA (18), as assessed by an in vitro assay on plasmid DNA (39). When we measured the incision activity exhibited by extracts preincubated with wortmannin, we observed also a restoration of this activity on UV-damaged linear DNA (data not shown).

Under these DNA repair conditions, part of the linear DNA was converted to higher molecular weight species (Fig. 3A, lanes L) that we have identified as homo- or mixed plasmid dimers (18). Interestingly, NER restoration on linear DNA correlated with the disappearance of this end-joining activity with extracts preincubated in the presence of wortmannin (Fig. 3A) or LY294002 (not shown).

**RNA Transcription on Linear DNA Substrate and Effect of Wortmannin**—Since the nuclear protein extracts that we employed were competent for transcription in vitro from viral promoters (28), we have analyzed the consequence of Ku binding to DNA on this activity.

We used the pGf1 plasmid that contained the AdMLP and a G-less cassette (29) consisting of a stretch of DNA without guanine residues in the non-transcribed strand. Thus the transcripts do not contain guanine residues and are resistant to RNAS-T1 in the reaction mixture. After fractionation by urea gel electrophoresis, the RNA transcription activity of RNA pol II is determined by quantitation of radioactivity incorporated in 106 nucleotides transcripts. pGf1 was either closed circular or linearized with HindIII at a site 104 bp downstream from the +1 site of transcription initiation. A negligible amount of RNAS-T1-resistant transcripts was generated from pΔGf1, which lacked AdMLP but contained the G-less cassette (not shown). As shown in Fig. 4A, transcription activity from...
AdMLP was efficient with closed circular pGf1 but was very low with HindIII-linearized pGf1 (95% inhibition of transcription activity, Fig. 4B). In addition, transcription was also greatly decreased when pGf1 was linearized with NdeI at a site 281 bp upstream from the +1 site of transcription initiation (99% inhibition of transcription activity, Fig. 4C).

When the extracts were preincubated in the presence of increasing concentrations of wortmannin before transcription reaction, a significant inhibition of transcription activity on closed circular plasmid was observed (Fig. 4A), especially for the highest wortmannin concentrations (80% inhibition of transcription activity for 1.3 μM wortmannin, Fig. 4B). Strikingly, the opposite effect was observed with both HindIII or NdeI-linearized pGf1 plasmids for which wortmannin stimulated transcription in a dose-dependent manner (7–8- and 4-fold stimulation, respectively, of transcription activity for 1.3 μM wortmannin, Fig. 4A and C). In consequence, similar yields of transcription activity were observed for 1.3 μM wortmannin with linear or circular pGf1 template (Fig. 4C). Identical results were obtained in transcription experiments with pCMV plasmid containing the cytomegalovirus promoter, either closed circular or linearized with EcoRI at a site 614 bp upstream from the +1 site of transcription initiation (data not shown).

We then analyzed the DNA-bound protein fraction under conditions identical to those used for transcription reaction in fluid phase. The binding to DNA of Ku, DNA-PKcs, and the large fragment of RNA pol II was checked in parallel. As shown in Fig. 5, Ku showed the typical pattern of preferential binding to linear DNA and decreased DNA end-dependent binding with extracts preincubated with wortmannin, whereas DNA-PKcs binding to linear DNA increased, as already shown above (Fig. 1B). In the absence of wortmannin, pol II LS bound strongly to closed circular pGf1 but in contrast was hardly detectable on HindIII linearized pGf1. According to results obtained with transcription activity, preincubation of the extracts with wortmannin had the opposite effect with linear and circular plasmid templates since pol II LS DNA binding activity increased on linear pGf1 but decreased on closed circular DNA (Fig. 5). Similar results were obtained with pCMV plasmid adsorbed on microplates (data not shown).

**DISCUSSION**

First, the present data substantiate the model that we have proposed recently in which DNA-PK catalytic activity regulates Ku entry into the DNA molecule (17). This model was inferred from electrophoretic mobility shift assays using human nuclear extract that closely mimic the physiological conditions of molar ratios of the DNA-PK components and of the protein environment. Here, using similar nuclear protein extracts, we report that the strong free end-dependent Ku binding to DNA is reversed when the extracts are preincubated with kinase-inhibiting concentrations of DNA-PK inhibitors. On the opposite, no effect was obtained when the inhibitors were added after DNA-PK has contacted DNA. Since no effect was observed with extracts devoid of catalytic subunit, a side effect of these inhibitors on other protein kinases is unlikely.

The strong Ku binding to linear DNA that we observed under kinase-permissive conditions more likely corresponds to entry of Ku into DNA plasmid molecules that we have previously demonstrated (17). Accordingly, we calculated that the molar ratio Ku/DNA end was >8 under these conditions. A recent report confirmed that recruitment of DNA-PKcs at a DNA end induces inward translocation of Ku protein (40), although no effect of ATP was found on protein-DNA contacts, probably due to the short length of the DNA fragment used in this study.

The dramatic decrease of Ku amount that we detected on DNA in the presence of wortmannin correlated with an enhanced p460 association with DNA, which was still Ku-dependent. In addition, the molar ratio Ku/p460 was close to 1 under these conditions, and the molar ratios of Ku or p460 per DNA end were also close to 1. This can be fully explained by a blocked whole DNA-PK complex at DNA termini, which we have already inferred from data obtained under identical kinase-preventive conditions (17). On the opposite, p460 tends to detach from DNA under kinase-permissive conditions, more likely due to autophosphorylation (41). We observed also a strong inhibition of the intrinsic ligase activity of the extracts on the linear DNA molecules in the presence of wortmannin (Fig. 3A). This cannot be attributed to an inhibition of DNA ligase IV-dependent end joining in which DNA-PK activity is necessary since multimerization of linear DNA is ligase IV-independent in such protein extracts (42, 43). Rather, this ligation inhibition relies more likely on a complete prevention of end processing by either DNA polymerization, degradation, or ligation by the blocked DNA-PK complex at DNA ends (17).

We then asked the question of the consequence of Ku binding to DNA on cis-acting DNA processes. NER and transcription were chosen as representative inward DNA transactions. The present results establish that Ku binding at internal sites in broken DNA is responsible for inhibition of local DNA metabolism. Indeed, a strong NER and transcription inhibition correlated with inward Ku DNA binding, whereas the release of Ku from internal sites by DNA-PK kinase inhibition paralleled the restoration of NER and transcription activities. Identical results were obtained by monitoring the enzymatic DNA repair synthesis and RNA transcription activities in vitro on the one hand and by analyzing the binding of key proteins to broken DNA substrate on the other hand (damage recognition proteins XPA and p62-TFIH or RNA pol II LS for NER or transcription activities, respectively). The effect of DNA-PK inhibitors can confidently be attributed to the modulation of Ku binding to DNA rather than a regulation of NER or transcription activities by DNA-PK for several reasons. (i) DNA-PK inhibitors did not reverse inhibition when they were added to the reaction after DNA-PK has contacted DNA (Fig. 1B and data not shown). (ii) Stimulation of transcription by wortmannin on linear DNA was at the opposite to its effect on closed circular template. The latter inhibiting effect of wortmannin on transcription with closed circular template could correspond to the facilitating role that has been described for DNA-PK in basal transcription on circular DNA in vitro (44); the precise underlying mechanism of this inhibition is still unknown but can conceivably involve phosphorylation of a transcription factor.

Although the cause has not been precisely established, sim-
ilar repressing of transcription by nuclear protein extracts has been reported (26). An identical inhibitory property of Ku has been described since in electrophoretic mobility shift assays, purified Ku complex was found to compete strongly with transcription factors for their sequence specific binding (45). In addition, in an in vitro transcription assay, Ku was found to inhibit transcription from linear but not from circular template DNA (45).

How might Ku translocation into DNA regulate internal DNA transactions? Apart from regulating inward Ku translocation when the whole DNA-PK complex assembles at DNA termini, DNA-PK kinase activity is unlikely to contribute to the inhibition of these DNA processes. We have observed an inhibition of NER activity or NER protein binding in the presence of broken DNA with extracts devoid of p460 activity (18, 25). We could reconstitute NER inhibition on linear DNA by adding recombinant Ku in extracts from Ku-deficient rodent cells which otherwise did not exhibit this inhibition (25). Accordingly, several functions of Ku independent of DNA-PKc have been described including, for example, a specific processing devoted to signal joint in VDJ recombiniation (46–49) or double-strand DNA breaks induced by topoisomerase II inhibitors (50). Also, Ku may play different role per se; for example, Ku rigidifies DNA structure (51), is able to form a DNA loop by contact of distant Ku dimers (19), and is able to transfer from one DNA molecule to another (20).

Here, we report a transcription inhibition on DNA substrate linearized at a site up to ~600 bp upstream from the +1 site of transcription initiation. We have already reported NER inhibition on lesions at a distance ~1.5 kilobase pairs from a DNA end (18). Although inhibition of internal DNA processes actually relies at least on inward Ku DNA binding, we cannot exclude that other partners are required for this effect to take place. For example, multiple Ku dimers spread on DNA may serve the recruitment of additional proteins in order to build multiprotein complexes. Indeed, the yeast Ku homolog has been shown to interact with Sir4 protein (52), a regulatory factor in telomere transcriptional silencing, which is believed to rely on a condensed heterochromatin-like DNA organization. It has been demonstrated that Sir proteins are also necessary for Ku-dependent non-homologous end-joining (52, 53). Conversely, in Saccharomyces cerevisiae, Ku is constitutively present at telomere and subtelomeric regions and is essential in maintaining chromosome integrity (54–57). In addition, Ku has also been shown to be localized to mammalian telomeric repeats, and significantly, DNA-PKc is not required in vivo for this association (58). Recently, it has been demonstrated that a single double-strand break in the yeast genome is sufficient to promote Sir proteins and Ku dissociation from subtelomeric heterochromatin complexes and disruption of telomeric silencing (59–61). Interestingly, a significant amount of Ku and Sir proteins relocalized at or near the double-strand breaks within a few hours after the damage occurrence (59).

Several hypotheses have been proposed for possible roles of Ku association with special chromatin structures at DNA breaks (reviewed in Ref. 62). The major role does not seem to be a protection of DNA ends against degradation since in yeast the absence of Ku causes a minor increase in resection of DNA at double-strand ends (63). Another possibility is that Ku and Sir proteins participate in chromatin refolding after repair of the breaks. Similarly, the chromatin assembly factor CAF1 has been shown to be involved in nucleosome assembly following repair of UV DNA damage by NER (64). It has not yet been established if Sir and Ku protein relocation may serve to shut down temporarily essential nuclear processes such as DNA replication and transcription in the vicinity of the DNA break or to prevent deleterious recombinational events, while the repair of potentially lethal damage takes place (59, 61).

Our present results in vitro are fully in agreement with the latter hypothesis since we actually demonstrated a Ku-dependent suppression of DNA-dependent processes like transcription in a broken DNA template. However, we have not yet established if under our experimental conditions, Ku internal binding to DNA is sufficient to mediate NER or transcription repression or if the association with other proteins, possibly equivalent to yeast Sir proteins, is required. Current experiments are in progress in our laboratory in order to assess the association of chromatin proteins with Ku on linear DNA under kinase-permissive conditions.

From the above reports and the present data, a general model for DNA-PK functions at DNA breaks can be proposed. Most probably, Ku binding to DNA ends serves in recruiting the kinase subunit to the break since it needs to contact DNA to be activated. The transient binding of p460 subunit at the ends might help its postulated role as structural framework by attracting protein actors of subsequent steps for end processing (e.g. the 5′-nuclease FEN1(65) or the XRCC4/DNA ligase IV complex (66)) or for signaling pathways. Under kinase-permissive conditions, interactions between DNA and DNA-PK lead to efficient activation of the kinase that in turn allows Ku entry into DNA and possibly dissociates DNA-PKc from the break. Thus, assembling of new DNA-PK subunits at the ends and/or further DNA processing in the route to the break repair can take place. Then, Ku inward translocation may serve one or several of the possible roles discussed above. Further characterization of these special Ku functions inside DNA possibly independent from direct end joining will help to resolve the paradox that the same proteins are required for protecting telomere from fusing or recombining and for joining broken DNA ends.

Acknowledgments—We thank Drs. J. M. Egly, C. J. Jones, and D. B. Bregman for their generous gift of antibodies; Dr. M. S. Satoh for the gift of plasmids; and Dr. D. J. Chen for the gifts of Ku and DNA-PK preparations. We thank Dr Catherine Muller for comments on the manuscript.

REFERENCES

1. Pastink, A., and Lohman, P. (1999) Mutat. Res. 428, 141–156
2. Karran, P. (2000) Curr. Opin. Genet. & Dev. 10, 144–150
3. Chu, G. (1997) J. Biol. Chem. 272, 24097–24100
4. Weaver, D. T. (1995) Adv. Immunol. 58, 29–85
5. Zhu, C., and Roth, D. B. (1996) Cancer Surv. 28, 295–309
6. Featherstone, C., and Jackson, S. P. (1999) Mutat. Res. 434, 3–15
7. Jegg, P. A. (1997) Mutat. Res. 384, 1–14
8. Jin, S. F., Inoue, S., and Weaver, D. T. (1997) Cancer Surv. 29, 221–261
9. Hartley, K. O., Gell, D., Smith, G. C., Zhang, H., Dvekha, N., Connely, M. A., Adimno, A., Lees-Miller, S. P., Anderson, C. W., and Jackson, S. P. (1995) Cell 82, 849–856
10. Dvir, A., Peterson, S. R., Knuth, M. W., Lu, H., and Dynan, W. S. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11292–11294
11. Gottlieb, T. M., and Jackson, S. P. (1993) Cell 72, 131–142
12. Mimori, T., and Hardin, J. A. (1986) J. Biol. Chem. 261, 10375–10379
13. Blier, P. R., Griffin, A. J., Craft, J., and Hardin, J. A. (1993) J. Biol. Chem. 268, 7594–7601
14. Falzon, M., Fewell, J. W., and Kuff, E. L. (1993) J. Biol. Chem. 268, 10546–10552
15. De Vries, E., van Driel, W., Bergsma, W. G., Arnberg, A. C., and van der Vliet, P. C. (1989) J. Mol. Biol. 208, 65–78
16. Zhang, W. W., and Yanev, M. (1992) Biochem. Cell Biol. 60, 574–579
17. Calsou, P., Frit, P., Humbert, O., Muller, C., Chen, D. J., and Salles, B. (1999) J. Biol. Chem. 274, 7748–7756
18. Calsou, P., Frit, P., and Salles, B. (1996) J. Biol. Chem. 271, 27601–27607
19. Cary, R. B., Peterson, S. R., Wang, J. T., Bear, D. G., Bradbury, E. M., and Chen, D. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4267–4272
20. Bliss, T. M., and Lane, D. P. (1997) J. Biol. Chem. 272, 5765–5773
21. de Luat, W. L., Jaspers, N., and Hoeijmakers, J. (1999) Genes Dev. 13, 768–785
22. Batty, D. P., and Wood, R. D. (2000) Gene Umanst. 241, 193–204
23. de Boer, J., and Hoeijmakers, J. (2000) Carcinogenesis 21, 453–460

Inward DNA Processes around a DNA Double-strand Break
