

Decreased origin usage and initiation of DNA replication in haploinsufficient HCT116 Ku80^{+/-} cells

Sahar Sibani^{1,2}, Gerald B. Price^{1,*} and Maria Zannis-Hadjopoulos^{1,2,‡}

¹McGill Cancer Center and ²Department of Biochemistry, McGill University, Montreal, Quebec H3G 1Y6, Canada

*This paper is dedicated to the memory of G. B. Price

‡Author for correspondence (e-mail: maria.zannis@mcgill.ca)

Accepted 14 March 2005

Journal of Cell Science 118, 3247-3261 Published by The Company of Biologists 2005

doi:10.1242/jcs.02427

Summary

One of the functions of the abundant heterodimeric nuclear protein, Ku (Ku70/Ku80), is its involvement in the initiation of DNA replication through its ability to bind to chromosomal replication origins in a sequence-specific and cell cycle dependent manner. Here, using HCT116 Ku80^{+/-} cells, the effect of Ku80 deficiency on cell cycle progression and origin activation was examined. Western blot analyses revealed a 75% and 36% decrease in the nuclear expression of Ku80 and Ku70, respectively. This was concomitant with a 33% and 40% decrease in chromatin binding of both proteins, respectively. Cell cycle analysis of asynchronous and late G₁ synchronized Ku80^{+/-} cells revealed a prolonged G₁ phase. Furthermore, these Ku-deficient cells had a 4.5-, 3.4- and 4.3-fold decrease in nascent strand DNA abundance at the lamin B2, β-globin and *c-myc* replication

origins, respectively. Chromatin immunoprecipitation (ChIP) assays showed that the association of Ku80 with the lamin B2, β-globin and *c-myc* origins was decreased by 1.5-, 2.3- and 2.5-fold, respectively, whereas that of Ku70 was similarly decreased (by 2.1-, 1.5- and 1.7-fold, respectively) in Ku80^{+/-} cells. The results indicate that a deficiency of Ku80 resulted in a prolonged G₁ phase, as well as decreased Ku binding to and activation of origins of DNA replication.

Supplementary material available online at <http://jcs.biologists.org/cgi/content/full/118/15/3247/DC1>

Key words: Chromatin immunoprecipitation, Human, Replication origins, Nascent DNA abundance, Ku protein, Haploinsufficient

Introduction

DNA replication is regulated at the level of initiation, and more specifically, at the level of pre-replication complex (pre-RC) formation at replication origins (reviewed by Dutta and Bell, 1997; Bell and Dutta, 2002; Sharova and Abramova, 2002; Li et al., 2003). Although several replication origins have been mapped to specific DNA sequences, indicating that the process of initiation of DNA replication does not occur randomly throughout the genome (Huberman and Riggs, 1968; Hand, 1978; Goldman et al., 1984; Pierron et al., 1984; Jalouzet et al., 1985; Gilbert, 1986; Zannis-Hadjopoulos and Price, 1998; Zannis-Hadjopoulos and Price, 1999), to date, no human DNA initiator protein has been identified that binds to replication origins in a sequence-specific manner (Dhar et al., 2001a; Bell and Dutta, 2002). Analyses in model organisms and in human cells have shown that the assembly of the pre-RC proceeds in a stepwise manner (Hua and Newport, 1998; Rowles et al., 1999). The earliest origin-binding protein identified in the pre-RC assembly has been the hexameric origin recognition complex (ORC) (Li and Herskowitz, 1993; Quintana et al., 1998; Moon et al., 1999; Okuno et al., 2001). In vitro analyses, however, have shown that the ORC complex alone does not possess sequence-specific DNA binding activity (Dhar et al., 2001a; Bell and Dutta, 2002), with the exceptions of those of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. In *S. pombe*, the AT hook of the Orc4 subunit of the complex allows sequence-specific binding to AT rich regions (Chuang

and Kelly, 1999; Moon et al., 1999), whereas in *S. cerevisiae*, ATP-bound Orc1p is responsible for ScORC attachment to origins (Klemm et al., 1997). Thus, it was postulated that ORC might interact with a sequence-specific binding protein that recruits ORC to the origins (Bell and Dutta, 2002; Zannis-Hadjopoulos et al., 2004).

Initially isolated as an autoantigen in patients with systemic lupus (Mimori et al., 1981), the heterodimeric Ku protein, consisting of the Ku70 and Ku80 subunits, has been implicated in various DNA-related cellular processes, including DNA repair, transcription, recombination, telomere maintenance and DNA replication (reviewed by Tuteja and Tuteja, 2000; Zannis-Hadjopoulos et al., 2004). Purified as an origin binding activity (OBA) from HeLa cells (Ruiz et al., 1995; Ruiz et al., 1999) through its ability to bind to the minimal functional sequence of the monkey replication origin *ors8* (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991; Pearson et al., 1991; Todd et al., 1995), OBA was identified by microsequencing as Ku80 (Ruiz et al., 1999). Since then, several studies implicated Ku in the initiation of DNA replication (Novac et al., 2001; Matheos et al., 2002) [Matheos et al. (Matheos et al., 2003), and refs therein]. In human (HeLa) cells, Ku was shown to co-fractionate with the 21S multiprotein complex competent for DNA synthesis (Vishwanatha and Baril, 1990) and to bind replication origins in a sequence-specific manner (Toth et al., 1993; Ruiz et al., 1999; Novac et al., 2001; Matheos et al., 2002; Matheos et al.,

2003). Southwestern analysis showed that Ku80 binds specifically to A3/4 (Ruiz et al., 1999), a version of a mammalian origin consensus sequence (Price et al., 2003). When HeLa cell extracts were immunodepleted of Ku, a 70% decrease in in vitro DNA replication was observed (Matheos et al., 2002), whereas Ku's association with replication origins was found to be cell cycle regulated in CV1 cells, being maximal in late G₁ (Novac et al., 2001). Ku80^{-/-} mice were smaller than their heterozygous littermates (Nussensweig et al., 1996) and displayed an early onset of senescence (Vogel et al., 1999). Although they were predisposed to a higher incidence of lymphomas, these knockout mice had a 13-fold decreased incidence of all other cancer examined relative to wild-type mice (2.2% vs 27.6%) (Vogel et al., 1999).

Evidence implicating Ku in DNA replication is not limited to human or mammalian cells, but has also been identified in lower eukaryotes. In the budding yeast, *S. cerevisiae*, OBF2, the yeast Ku homologue, was found to be necessary in the stable formation of an ORC-like complex at *ARS121* (Shakibai et al., 1996). Ku has also been implicated in determining the time of activation of telomeric DNA replication origins in *S. cerevisiae*, although this was interpreted as due to a potential role of Ku in chromatin remodeling (Cosgrove et al., 2002). It was found to associate with viral DNA replication proteins, including the herpes simplex (HSV)-encoded UL9 protein, which binds OriS of HSV and is required for HSV DNA replication (Baker et al., 2000; Murata et al., 2004). It also coprecipitates with ICP8, a multifunctional protein important for HSV DNA replication, among other things (Taylor and Knipe, 2004).

In this study, we examined the role of Ku in the initiation of DNA replication, using HCT116 Ku80^{+/-} haploinsufficient cells. Their homozygous counterparts (HCT116 Ku80^{-/-}) are nonviable, dying of massive apoptosis (Li et al., 2002). We found that the Ku80^{+/-} cells have a prolonged G₁ phase and a decreased frequency of activation of three early-firing DNA replication origins, the lamin B2, β -globin and *c-myc* origins.

Materials and Methods

Cell culture, synchronization and FACS analysis

HCT116 Ku80^{+/-} cells (clone 70-32; a generous gift from Eric A. Hendrickson, University of Minnesota, Minneapolis, MN), and their parental cell line, HCT116, were cultured in McCoy's 5A medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) (hereafter termed complete medium) at 37°C, 5% CO₂. Synchronization to the late G₁ phase was carried out by a modification of previously published methods (Stephens et al., 1977; Novac et al., 2001). In brief, cells were cultured in complete medium in the presence of 2 mM thymidine (Sigma, St Louis, MO) for 12 hours, released for 10-11 hours in pre-warmed complete medium without thymidine, and then incubated for 12-14 hours in complete medium containing either 400 μ M mimosine or 2 mM hydroxyurea (Sigma). For synchronization to S phase, the cells were released from the thymidine/mimosine block into prewarmed complete medium, harvested every 2 hours and fixed in 70% ice-cold ethanol. For flow cytometry analysis, cells were washed twice in ice-cold phosphate buffered saline (PBS), and resuspended in Vindelov's solution (3.4 mM Tris, 75 μ M propidium iodide, 0.1% NP40, 0.01M NaCl, 700 U/L RNase A (Invitrogen) (Vindelov, 1977), overnight (o/n) at 4°C. They were analyzed using a Beckman flow cytometer and the CellQuest program.

Nuclear extract preparation and quantification

Nuclear extracts (NE) were prepared by a modification of a previously described method (Dignam et al., 1983; Pearson et al., 1994). Briefly, cells cultured in complete medium were harvested at 60-70% confluency, following two washings with ice-cold PBS. They were resuspended in 5 \times packed cell volume (pcv) hypotonic buffer [10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT and Complete protease inhibitor tablet (Roche Molecular Biochemicals, Indianapolis, IN)] and left to swell on ice for 15 minutes. After centrifugation at 500 *g* for 5 minutes at 4°C, they were resuspended in 2 \times pcv hypotonic buffer and nuclei were isolated by passage of the cells through a 21G needle five times. The nuclei were pelleted by centrifugation at 2500 *g* for 10 minutes at 4°C, washed once with hypotonic buffer and resuspended in 1 \times packed nuclear volume in hypertonic buffer (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, and Complete protease inhibitor tablet (Roche Molecular Biochemicals)) for 30 minutes at 4°C with continuous shaking. The NE was obtained by centrifuging at maximum speed in a microcentrifuge for 15 minutes at 4°C. The concentrations of the NE were determined using the Bradford protein assay (BioRad, Hercules, CA).

Chromatin loading

Log phase cells (60-70% confluency), cultured in complete medium, were scraped off 100 mm plates into ice-cold PBS (Tatsumi et al., 2000). After centrifugation, they were resuspended in 1 ml of lysis buffer A (10 mM HEPES-KOH pH 7.9, 100 mM NaCl, 300 mM Sucrose, 0.1% Triton X-100) containing the Complete protease inhibitors (Roche Molecular Biochemicals) and incubated on ice for 10 minutes. Following centrifugation at 2000 *g* for 3 minutes at 4°C, they were washed again in buffer A, and resuspended in 100 μ l of buffer B (10 mM HEPES-KOH pH 7.9, 200 mM NaCl, 300 mM Sucrose, 0.1% Triton X-100, 5 mM MgCl₂) containing 1000 U of DNase I (Roche Molecular Biochemicals). Following incubation at 25°C for 30 minutes the chromatin enriched fraction was isolated by centrifugation at 2500 *g* for 5 minutes at 4°C. The Bradford protein assay (BioRad) was used to determine the protein concentration of the supernatant.

Western blot analysis and quantification

Western blot analysis was carried out according to standard protocols (Sambrook et al., 1989). Briefly, the indicated amounts were resuspended in SDS loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), boiled for 4 minutes and loaded onto a 5% stacking/8% separating SDS-PAGE gel. Following electrophoresis and transfer onto a PVDF membrane (Millipore, Billerica, MA), the membranes were immunoblotted with either a 1/1000 dilution of anti-Ku70 (C19) or a 1/100 dilution of anti-Ku80 (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA) antibody for 1 hour at room temperature (r.t.). Anti-goat horse radish peroxidase (HRP)-conjugated IgG was added (1/2000 dilution) for 20 minutes at r.t. A 1/1000 dilution of anti-p53 (FL393), -p21 (C19), -cdc6 (D12), or -cdk2 (H304; Santa Cruz Biotechnology) antibodies, along with the corresponding HRP-conjugated secondary antibody, was used to probe the membrane for their target proteins. Immunoblots were also probed with a 1/1000 dilution of either anti-actin (Sigma) or anti-MCM7 antibody (N-20; Santa Cruz Biotechnology) and 1/2000 dilution of anti-rabbit HRP-conjugated IgG, used as loading controls. Proteins were visualized using the enhanced chemiluminescence (ECL) kit according to the manufacturer's instructions (Amersham Biosciences, Arlington Heights, IL). Films of western blots were scanned and quantified using FujiFIlms Image Gauge V3.3.

Isolation of nascent strand DNA

Isolation of nascent strand DNA was done as described previously

Table 1. Sequences and annealing temperatures of primers used for the LightCycler

Primer name	Sequence (5'→3')	T _{Annealing} (°C)	Amplicon size (bp)
LB2-F	GGCTGGCATGGACTTTCATTTTCAG	66	231
LB2-R	GTGGAGGGATCTTTCTTAGACATC		
LB2C1-F	GTTAACAGTCAGGCGCATGGGCC	66	240
LB2C1R	CCATCAGGGTCACCTCTGGTTCC		
BG40.9-F	AATCTATTCTGCTGAGAGATCACAC	62	210
BG40.9-R	CCACTTGCAGAACTCCCGTGTAC		
BG72-F	GTCTAAAACACCAAAACGAATGGCAAC	55	326
BG72-R	CATTTCTCTGATGGCTAGTGATGATGAG		
<i>Myc11-F</i>	TATCTACACTAACATCCCACGCTCTG	62	192
<i>Myc11-R</i>	CATCCTTGTCCTGTGAGTATAAATCATCG		
<i>Myc1-F</i>	TTCTCAACCTCAGCACTGGTGACA	60	248
<i>Myc1-R</i>	GACTTTGCTGTTTGTCTGCAGGCT		

Names and sequences of primers used for real-time quantification of DNA using the LightCycler (Roche Diagnostics) are listed above. The names are identical to the region amplified (see Fig. 4A) and are followed by 'F' or 'R' to designate the forward and reverse primers, respectively. The T_{annealing} is the annealing temperature used in the cycling conditions for the LightCycler. The size of the PCR products are indicated in base pairs (bp).

(Giacca et al., 1997; Tao et al., 2000) with the following modifications: five to six 150 mm dishes containing logarithmically growing cells cultured in complete medium were used per experiment. At 30-50% confluency, the cells were washed twice in PBS, and lysed in 5 ml Hirt's lysis buffer/plate (50 mM Tris-HCl, pH 8.0, 0.6 M NaCl, 1 mM EDTA, 0.5% SDS) (Hirt, 1967). Following a 5 minute incubation at r.t., the lysate was decanted into a 50 ml screw cap tube and incubated o/n with 0.1 mg/ml proteinase K at 37°C. The lysate was extracted once with phenol/chloroform (1:1), and back-extracted once with chloroform:isoamyl alcohol (24:1). Following ethanol precipitation, the DNA was resuspended in 150-200 µl of RNase-free water and sheared by passage ten times through a 26G3/8 needle. Shearing increases the number of DNA ends on which the λ-exonuclease enzyme kinase (New England Biolabs, Beverly, MA) can bind and digest non-nascent DNA (see below). Twenty micrograms (µg) of sheared DNA were then heat denatured, phosphorylated with 10 units (U) T4 polynucleotide kinase (New England Biolabs) and 100 µM ATP for 30 minutes at 37°C, then heat inactivated and digested with 15 U of λ-exonuclease (New England Biolabs) o/n at 37°C. λ-exonuclease digests DNA, but not RNA, such as non-replicating DNA; nascent DNA is protected by its RNA primer.

To separate nascent DNA from Okazaki fragments, heat denatured DNA that had been treated with λ-exonuclease was subjected to electrophoresis on 1% non-denaturing agarose gel and stained with 0.01% methylene blue (Sigma). Regions containing either 0.5-1 kb or 1-2 kb DNA were excised and the DNA was extracted using a QiaExII extraction kit according to the manufacturer's protocol (Qiagen, Valencia, CA). DNA was eluted from the beads in 250 µl distilled water (dH₂O) and used in the LightCycler instrument (Roche Diagnostics, Basel, Switzerland) to quantitate the abundance of nascent DNA. Results were expressed as 'molecules/20 µg genomic DNA' to normalize between the two cell lines.

In vivo crosslinking and chromatin fragmentation

Ten plates (15 cm) of cells at 60-70% confluency were washed with pre-warmed PBS and treated with 1% formaldehyde for 10 minutes to crosslink proteins and DNA in vivo (Ritzi et al., 1998; Novac et al., 2001; Novac et al., 2002); they were then washed and scraped into ice-cold PBS, and resuspended in lysis buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1% Triton X-100, 2 mM EDTA, Complete protease inhibitors tablet) (Roche Molecular Biochemicals). Following passage through a 21G needle three times, the nuclei were harvested by centrifugation at 2500 g for 5 minutes at 4°C. Nuclei were then resuspended in one packed nuclear volume of lysis buffer (i.e. 100 µl of buffer per 100 µl of nuclei), and sonicated ten times for 30 seconds each until fragments of DNA of less than 1 kb were obtained. Chromatin size was monitored by electrophoresis.

For cell counting, one untreated plate was scraped into PBS

and resuspended well. The cells were then counted with a hemacytometer, and this number was used to derive the total number of treated cells.

Immunoprecipitations

Immunoprecipitations were carried out essentially as described by the manufacturers, with minor modifications (Santa Cruz Biotechnology; Roche Molecular Biochemicals). Briefly, sheared chromatin lysates from 2×10⁷ cells were pre-cleared by incubation with 50 µl of protein G agarose (Roche Molecular Biochemicals) to reduce background caused by nonspecific adsorption to the beads. Pre-cleared lysates were incubated for 6 hours with either 20 µg of anti-Ku80 (C-20; Santa Cruz), or anti-Ku70 (C-19; Santa Cruz), anti-Orc2 antibody (Quintana et al., 1997) (a generous gift from Anindya Dutta, University of Virginia Health Sciences Center, Charlottesville, VA) or normal goat serum at 4°C with constant shaking. Protein G agarose (50 µl) was added and incubated o/n at 4°C. The pelleted beads were washed successively twice with 1 ml of lysis buffer for 15 minutes each at 4°C, followed by 1 ml of WB1 (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.1% NP40, 0.05% sodium deoxycholate, Complete protease inhibitors tablet) (Roche Molecular Biochemicals), 1 ml of WB2 (50 mM Tris-HCl pH 7.5, 0.1% NP40, 0.05% sodium deoxycholate, Complete protease inhibitors tablet) (Roche Molecular Biochemicals) and 1 ml of sterile TE lacking any protease inhibitors. The beads were resuspended in 200 µl TE/1% SDS, incubated at r.t. for 15 minutes and centrifuged at 1000 g for 1 minute at r.t. Half of the supernatant was then incubated o/n at 65°C to reverse the crosslinks, followed by 100 µg of proteinase K at 55°C for 2 hours. The DNA was purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) and eluted in 100 µl 10 mM Tris-HCl (pH 8.0). The remaining half of the supernatant was boiled for 10 minutes in SDS-PAGE loading buffer and subjected to electrophoresis on a 5% stacking/8% separating SDS-PAGE gel for western blot analysis.

Real-time PCR quantification of immunoprecipitated DNA

Immunoprecipitated DNA was quantified using the LightCycler FastStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. Briefly, 2 µl of 10× Master SYBR Green mix was combined with 2 µl of 10 µM of each primer and 2 or 4 µl of either nascent or immunoprecipitated DNA in a total volume of 20 µl. The cycling conditions consisted of an initial 10 minute denaturation step at 99°C, followed by 40 cycles of the following cycling conditions: 99°C for 10 seconds, annealing temperature (as indicated in Table 1) for 10 seconds, and 72°C for 15 seconds. All primer sets used and their annealing temperature are listed in Table 1.

To calibrate each PCR run, a standard curve for each amplicon was derived from nonreplicating DNA of serum-starved HCT116 cells, extracted using Qiagen's cell culture DNA extraction midi kit (Qiagen), eluted in 500 μ l dH₂O, and quantified by spectrophotometry. Known amounts of the nonreplicating DNA were used in four reactions to derive a standard curve of DNA concentration versus fluorescence (Fig. 5C). By measuring the amount of fluorescence emanating from the sample tubes and plotting that against the standard curve, the concentration of nascent or immunoprecipitated DNA in each sample was determined. The number of DNA molecules present in each sample was calculated using the formula:

$$\frac{([\text{Amplicon}])(\text{Rxn vol.})(N_A)(\text{Dil. factor})}{(\text{MW}_{\text{Amplicon}})}$$

where amplicon concentration, [Amplicon], is given by the LightCycler instrument as μ g/ μ l; the reaction volume is 20 μ l; Avogadro's number (N_A) is 6×10^{23} molecules/mole; the dilution factor was derived by dividing the elution volume (100 μ l for ChIP and 250 μ l for nascent DNA) by the volume of sample added to each reaction (either 2 or 4 μ l); the molecular weight of the amplicon ($\text{MW}_{\text{Amplicon}}$; g/mole) was calculated by multiplying 660g/(mole \times bp-length) by the length of the amplicon (in bp) found in Table 1.

Results

Ku80^{+/-} cells have decreased amounts of Ku protein

Initial characterization of Ku80^{+/-} cells revealed a 20-50% decrease in the expression of Ku protein in their nuclear extracts (NE) relative to wild-type HCT116 cells (Li et al., 2002). To confirm those findings and to test the sensitivity of the antibodies being used, we performed immunoblot analyses in these cell lines for both subunits of Ku, using anti-Ku80 (C20) and anti-Ku70 (C19) antibodies (Fig. 1A). Quantification of the immunoblots revealed a decrease of 75% ($P=0.01$) and 36% ($P=0.01$) in the nuclear expression of Ku80 and Ku70, respectively, in Ku80^{+/-} cells relative to the parental cell line (HCT116) (Fig. 1A,B). The specificity of the anti-Ku80 and anti-Ku70 antibodies had been previously verified, using blocking peptides (Novac et al., 2001). A stronger signal was obtained for Ku70 by comparison to Ku80, even in the wild-type HCT116 cells (Fig. 1A), suggesting a differential efficiency in the recognition of the two antibodies for their respective epitopes (Santa Cruz Biotechnology, personal communication) and not unequal loading, since immunoblotting for actin, as a loading control, showed no significant difference between the NE of HCT116 and Ku80^{+/-} (Fig. 1A,B).

A previous study using the Ku80^{+/-} cells indicated increased levels of p53 protein (Li et al., 2002), which can trigger the G₁/S checkpoint and prevent cells from entering S-phase. Because this may cause the reported 3 hour delay in cell cycle progression (Li et al., 2002) and indirectly affect the DNA replication assays used in this study, we examined the levels of p53 in our cell lines. Analysis of p53 protein expression levels in untreated human Ku80^{+/-} cells revealed no apparent difference relative to wild-type HCT116 cells (Fig. 1A). Similar analysis of the p53 target gene product, p21, also showed no significant difference between the two cell lines (Fig. 1A). Furthermore, because Ku can act as a transcription repressor (Kuhn et al., 1993; Kim et al., 1995; Camara-Clayette et al., 1999; Schaffer et al., 2003; Schild-Poulter et al., 2003;

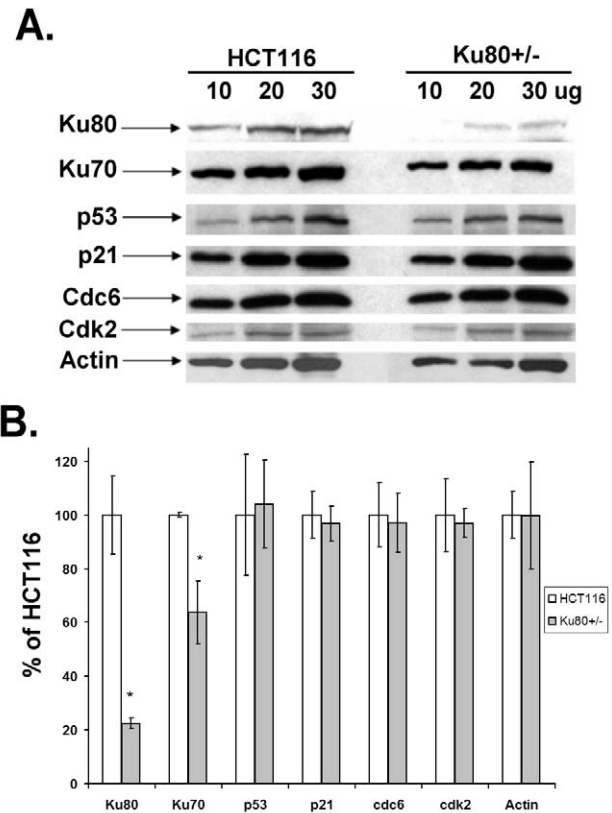


Fig. 1. Western blot assay of nuclear extracts (NE) from wild-type (HCT116) and Ku80^{+/-} cells. NE were prepared from HCT116 and Ku80^{+/-} by high-salt extraction of isolated nuclei and were immunoblotted for both subunits of Ku. The expression of p53 and p21 was examined to determine whether they were induced by the reduced Ku80 levels in Ku80^{+/-} cells. Expression of Cdc6 and Cdk2 were also inspected to verify that Ku80 deficiency did not reduce their expression. Nuclear actin was used as a loading control. (A) Increasing amounts (10, 20 and 30 μ g) of NE were subjected to electrophoresis and western blotting (as described in Materials and Methods). Immunoblotting with 1/100th dilution of anti-Ku80 (C-20), or 1/1000th dilution of anti-Ku70 (C-19), anti-p53 (FL393), anti-p21 (C19), anti-Cdc6 (D12), anti-Cdk2 (H304), and anti-actin antibody was carried out. (B) Histogram plots of quantifications of the Ku80, Ku70 and actin bands shown in A. Each error bar representing three experiments and one standard deviation (s.d.) is indicated. Signals obtained for HCT116 NE were set at 100%, and those of Ku80^{+/-} were expressed as a percentage of them. An asterisk (*) represents statistically significant differences ($P < 0.05$) in the expression of the indicated protein between HCT116 and Ku80^{+/-} cells.

Xu et al., 2004), the protein levels of Cdc6 and Cdk2, two regulators of progression through G₁- into S-phase, were examined by immunoblot analysis, to analyze whether Ku80 deficiency affected their expression, which might in turn affect the initiation of DNA replication. Cdc6 is required for the assembly of the pre-RC and loading of the MCM complex, the putative DNA replication helicase (reviewed by Bell and Dutta, 2002). Cdk2 phosphorylates Mcm4p (Detweiler and Li, 1998) and Orc1p (Findeisen et al., 1999), members of the MCM and ORC complex, respectively, and is required to trigger replication (reviewed by Bell and Dutta, 2002). Comparison of

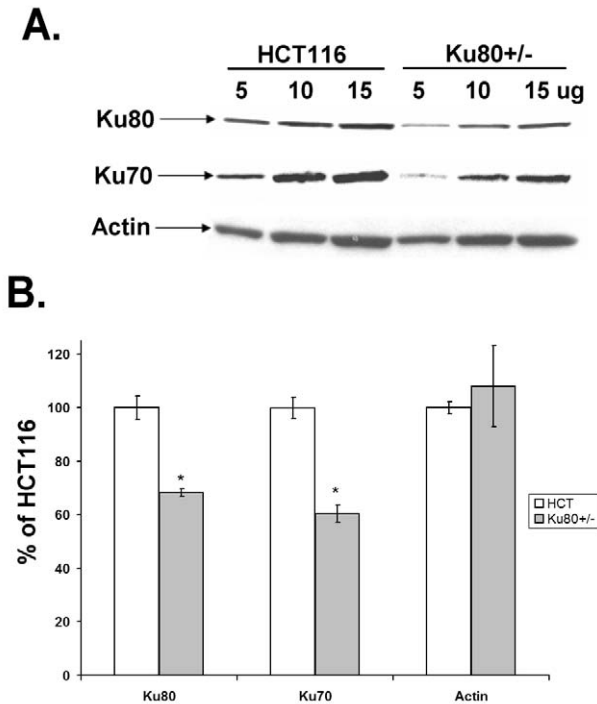


Fig. 2. Chromatin loading assay of Ku80, Ku70 and MCM7 in HCT116 and Ku80^{+/-} cells. Chromatin-enriched extracts were prepared (as described in Materials and Methods) and subjected to electrophoresis on 5% stacking/8% separating gels and western blotting. The abundance of Ku80 and Ku70 in the chromatin-enriched fractions was assessed. Actin was used as a loading control. (A) Immunoblotting with 1/100th dilution of anti-Ku80 (C-20), 1/1000th dilution of anti-Ku70 (C-19) or 1/1000th dilution of anti-actin antibody is shown. (B) Quantification of the Ku80 and Ku70 signals after normalization for actin. Each error bar represents three experiments and 1 SD is indicated. Signals obtained for HCT116 were set at 100% and those of Ku80^{+/-} were expressed as a percentage of them. An asterisk (*) represents statistically significant differences ($P < 0.05$) in the expression of the indicated protein between HCT116 and Ku80^{+/-} cells.

the two cell lines, however, indicated that the expression of Cdc6 and Cdk2 was not affected by Ku80 deficiency (Fig. 1A,B).

Decreased amounts of chromatin-associated Ku protein in Ku80^{+/-} cells

To analyze whether the decrease in the expression of Ku80 affected the association of either subunit of Ku with chromatin, increasing amounts of chromatin-enriched extracts from both HCT116 and Ku80^{+/-} cells were immunoblotted for Ku80 and Ku70 (Fig. 2A). As expected, the amount of both Ku80 and Ku70 associated with chromatin was found to be decreased in Ku80^{+/-} cells by comparison to that in HCT116 cells. Quantification of all the respective bands revealed that Ku80^{+/-} cells had 33% less Ku80 associated with chromatin than their parental cell line ($P = 0.0035$) versus a corresponding decrease of 40% for Ku70 ($P = 0.01$) (Fig. 2B). An immunoblot for actin was used as a loading control and its association with chromatin was similar in both cell lines (Fig. 2A).

Cell cycle analysis of Ku80^{+/-} cells

The effect of Ku80 deficiency on cell cycle progression was examined using asynchronous cultures of Ku80^{+/-} cells (Fig. 3). Flow cytometry was used to measure the fraction of both parental HCT116 and Ku80^{+/-} cells in each phase of the cell cycle (Fig. 3B). A significant ($P = 0.026$) decrease of 8.8% in the number of S-phase cells was observed in Ku80^{+/-} cells, accompanied by an increase of 10.6% in the number of G₁ cells ($P = 0.09$; Fig. 3B). Furthermore, no decrease in cell viability was observed in Ku80^{+/-} cells, as measured by trypan blue exclusion and flow cytometry (data not shown). By continuous bromodeoxyuridine incorporation for 48 hours, the number of cycling cells in Ku80^{+/-} cells was found to be nonsignificantly reduced by 2.3% compared with wild-type HCT116 cells ($P = 0.87$) (data not shown).

In mammalian cells, the majority of DNA replication origins are activated at the onset of S-phase (McAlear et al., 1989; Nakayasu and Berezney, 1989; Bell and Dutta, 2002; Zannis-Hadjopoulos et al., 2004). To determine the effect of Ku80 deficiency on the initiation of DNA replication, Ku80^{+/-} cells were synchronized to late G₁ using a thymidine/mimosine double block, as described in Materials and Methods (Krude, 1999; Novac et al., 2001; Novac et al., 2002). Cell cycle analysis of cells stained with propidium iodide (Fig. 3A) revealed that Ku80^{+/-} cells entered S-phase more slowly than wild-type HCT116 cells. Two hours following release from mimosine-induced cell cycle arrest, there was a 20% decrease in the number of G₁ HCT116 cells ($P = 0.0002$) (from 74.9% in G₁ to 54.5% in S-phase at S2, Fig. 3B), whereas Ku80^{+/-} cells displayed only a 4% decrease in the number of G₁ cells (from 75.3% at G₁ to 70.6% at S2, Fig. 3B). Four hours after release from mimosine-induced cell cycle arrest, Ku80^{+/-} cells showed a significant increase in the number of S-phase cells (36.4% at S4 versus 21.5% at S2, $P = 1 \times 10^{-6}$), reaching a percentage comparable to the S-phase cells of HCT116 S2 culture (36% for S4 of Ku80^{+/-} vs 35% of S2 of HCT116, $P = 0.46$, Fig. 3B). Combined, these data indicate a prolongation of 2 hours in the G₁ phase of Ku80^{+/-} cells relative to the parental HCT116 cells.

Once cells entered S-phase, the rate of progression through S and entry into G₂/M was similar for both Ku80^{+/-} and HCT116 cells. The increase in the percentage of HCT116 cells entering the G₂/M phase for each of the synchronized cultures ($\Delta G_2/M$ in Fig. 3B) proceeded such that there was a 5.9% increase from S2 to S4, 30.5% from S4 to S6 and 14.6% from S6 to the S8 culture (Fig. 3B, $\Delta G_2/M$ column of HCT116). When comparing the Ku80^{+/-} cells, a similar pattern was observed, starting with the S4 culture: a 7.0% increase from S4 to S6, and a 28.6% increase from S6 to S8. Considering that Ku80^{+/-} cells were delayed by 2 hours in entering S-phase, the similarity in the kinetics of entry into G₂/M between the two cell lines indicates that S-phase proceeded as in the wild-type (HCT116) cells and that Ku80-deficiency did not influence the elongation step of DNA replication.

Synchronization of cells using drugs that arrest at different phases of the cell cycle can also be used to determine the step at which a protein functions. The double cell-cycle block with mimosine used here arrests cells in late G₁ (Krude, 1999; Novac et al., 2001; Biard et al., 2003) (Fig. 3A, late G₁ analysis), before origin firing. If Ku80 functions during S-phase, then treatment of Ku80^{+/-} cells with the

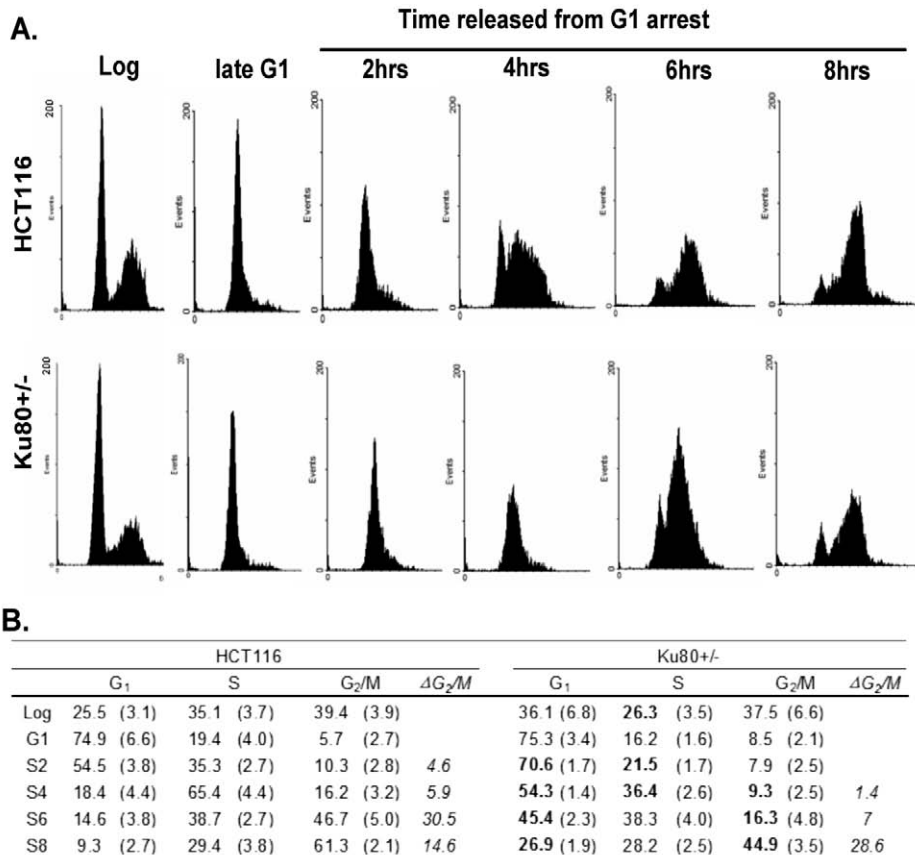


Fig. 3. Flow cytometric analysis of HCT116 and Ku80^{+/-} cells following synchronization to late G₁ and release into S-phase. Cells were synchronized to late G₁ by thymidine/mimosine double block (late G₁), and released into complete medium. They were harvested at 2 (S2), 4 (S4), 6 (S6) and 8 (S8) hours after release, and stained with propidium iodide to monitor their cell cycle distribution. An asynchronous culture of logarithmically growing cells (Log) was used for comparison. (A) Representative data from six experiments are shown. The top panel represents the wild-type (HCT116) cells, and the bottom panel is of the Ku80^{+/-} ones. (B) Quantification of the percentage of cells present in each phase of the cell cycle (G₁, S or G₂/M) during the synchrony (G₁) and release (S2-S8). Averages of six experiments and their standard deviations (in brackets) are shown. Cell cycle phases statistically different ($P < 0.05$) between Ku80^{+/-} and HCT116 cells are shown in bold.

thymidine/hydroxyurea double block, which arrests cells at the onset of S-phase (Merrill, 1998; Biard et al., 2003), would result in a delay in cell cycle progression similar to that of mimosine. However, when tested, hydroxyurea had no effect on the cell cycle progression of Ku80^{+/-} following removal of the drug (data not shown), further supporting the notion that Ku functions before origin firing.

Some cell cycle synchronization drugs, including mimosine and hydroxyurea, have been correlated with an increase in DNA breaks (Mikhailov et al., 2000; Szuts and Krude, 2004). To examine whether the different phenotypes of Ku80^{+/-} cells might be due to any induced DNA breaks, the association of Ku80, Ku70 and the single-stranded DNA binding protein, RPA70, to the DNA-containing chromatin fractions were measured in drug-treated and untreated Ku80^{+/-} cells (Fig. 4). If these drugs induced DNA breaks that elicited the nonhomologous end-joining (NHEJ) pathway, then an increase in the amount of Ku protein bound to the DNA and present in the chromatin-enriched fraction would be expected, whereas if

they induced the exposure of single-stranded DNA, then RPA70 would become enriched in the chromatin fraction. However, immunoblot analysis of the Ku and RPA70 proteins in chromatin-enriched extracts revealed no significant differences between the mimosine- (Fig. 4A, lanes 3 and 4) and hydroxyurea-treated (Fig. 4A, lanes 5 and 6) cells compared with an asynchronous culture (Fig. 4A, lanes 1 and 2; Fig. 4B), indicating that the differential effect of these two drugs on S-phase entry was not due to the induction of DNA breaks. Actin was used as a loading control (Fig. 4A).

Abundance of nascent DNA in 0.5-1 kb and 1-2 kb DNA fractions

In view of the prolonged G₁ phase observed in Ku80^{+/-} cells and to determine whether Ku80 deficiency had an effect on the frequency of initiation of DNA replication, the activities of three early-firing replication origins, the lamin B2, β-globin and *c-myc* origins, were examined. These three origins have been previously characterized in detail and their activities have been fine-mapped (Fig. 5A) (Giacca et al., 1994; Abdurashidova et al., 2000; Tao et al., 2000; Kamath and Leffak, 2001). In the case of the β-globin and *c-myc* origins, the dominant replication initiation sites were examined, hereafter referred to as BG40.9 and *Myc11*, respectively, as per their original designation (Tao et al., 2000; Kamath and Leffak, 2001). BG72 and *Myc1* are the corresponding non-origin containing regions. The lamin B2 origin was previously mapped to the initiating nucleotide (Giacca et al., 1994; Abdurashidova et al., 2000) and real-time PCR primers spanning the initiation site (LB2) as well as a region lacking origin activity (LB2C1), used as control, were previously described (Ladenburger et al., 2002). Real-time PCR primers for the β-globin and *c-myc* regions were designed and tested for the absence of nonspecific PCR products that would interfere with the quantification (Fig. 5B). None of the primer pairs produced nonspecific PCR products either in the absence of DNA or in DNA-containing samples (Fig. 5B). The melting temperatures of the different amplicons were ~82°C for BG40.9, ~81°C for BG72, ~94°C for *Myc11* and ~86°C for *Myc1*. For quantification purposes, a standard curve for all primers was constructed, using genomic DNA that was isolated from serum-starved HCT116 cells (Fig. 5C). The similarity in slopes of the standard curves indicates that the primer sets have comparable efficiencies, thus permitting comparisons of the results between the different primer sets.

Origin activity was measured by determining the abundance

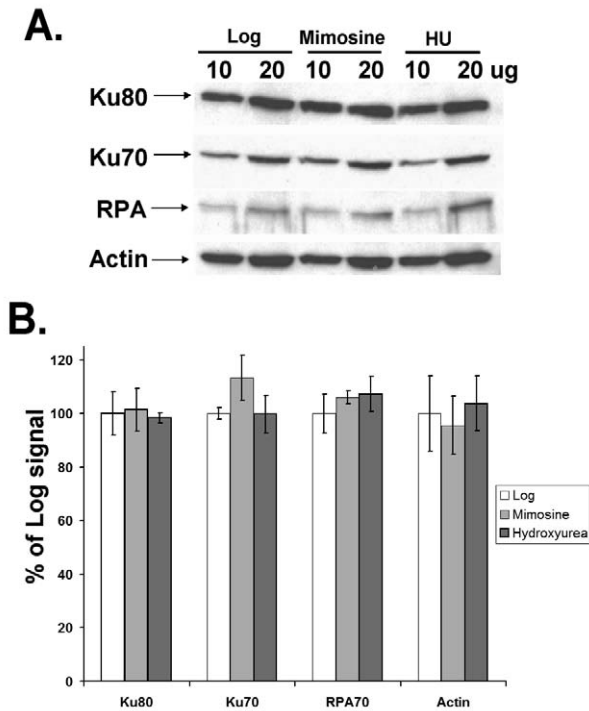


Fig. 4. Abundance of DNA repair proteins Ku80, Ku70 and RPA70 in chromatin-enriched fractions, following exposure of *Ku80*^{+/-} cells to mimosine or hydroxyurea. Chromatin fractions were prepared from HCT116 and *Ku80*^{+/-} cells that were either untreated or treated with thymidine/mimosine or thymidine/hydroxyurea double block. (A) Ten and 20 µg were subjected to electrophoresis and transferred onto PVDF membrane. They were then immunoblotted with 1/1000th dilution of anti-Ku80 (H300), 1/1000th dilution of anti-Ku70 (C-19), anti-RPA70, or anti-actin. (B) Quantification of the immunoblots after normalization for actin. Averages of three experiments and 1 s.d. are shown.

of nascent DNA using λ -exonuclease and quantitative PCR (Tao et al., 2000); λ -exonuclease digests any DNA lacking an RNA primer (Gerbi and Bielinsky, 1997), thus minimizing the background that would be caused by degraded genomic DNA. Previously, origin activity was measured by determining the abundance of origin-containing sequences within genomic DNA of 1-2 kb in length. Here, we assessed the abundance of origin-containing sequences within λ -exonuclease-treated DNA of 0.5-1 kb in length because it represents DNA closer to the origin, and compared the results with those obtained from the 1-2 kb fraction. Nascent DNA from HCT116 asynchronous cells was prepared by treating 20 µg genomic DNA with λ -exonuclease followed by size fractionation on a neutral agarose gel. Fractions containing DNA of 0.5-1 kb and 1-2 kb were analyzed by realtime PCR, whereas fractions containing DNA less than 500 bp in length were excluded to avoid the potential presence of Okazaki fragments. Within the origin-containing regions of lamin B2, β -globin and *c-myc*, the 0.5-1 kb fraction were 2-fold more abundant in nascent DNA compared with the 1-2 kb fraction ($P=0.02$; Fig. 6, see supplementary material Table S1). The respective origin-lacking regions of LB2C1, BG72 and *Myc1* were 3-, 2.4-, and 1.4-fold more abundant, respectively, in the 0.5-1 kb fraction relative to the 1-2 kb fraction. These results showed that

nascent DNA was more abundant in the 0.5-1 kb fraction of λ -exonuclease DNA. In both sets of DNA fractions, the origin-containing regions of LB2, BG40.9 and *Myc11* were more than eightfold more abundant than their respective origin-lacking regions of LB2C1, BG72 and *Myc1*, indicating that the nascent DNA preparation was of good quality.

Decreased replication initiation events in *Ku80*^{+/-} cells

Activities of the lamin B2, β -globin and *c-myc* replication origins were assessed by measuring the abundance of short nascent DNA (0.5-1 kb) in these regions. At all three origins examined, *Ku80*^{+/-} cells had decreased abundance of nascent DNA molecules compared with their wild-type counterparts (Fig. 7, supplementary material Table S2). At the region spanning the lamin B2 origin (LB2), there was 4.5-fold decrease in nascent DNA ($P=0.014$, Fig. 7A), whereas regions spanning the β -globin and *c-myc* origins had a 3.4- and 4.3-fold decrease, respectively ($P=0.002$, Fig. 7B; and $P=0.011$, Fig. 7C, respectively). The origin-lacking regions (considered as background) for all three origins were 20- to 39-fold less than their respective origins with no pronounced differences between the wildtype and deficient cells (Fig 7A-C). This indicated similar extraction efficiencies between the two cell lines.

Association of replication initiator proteins to origins

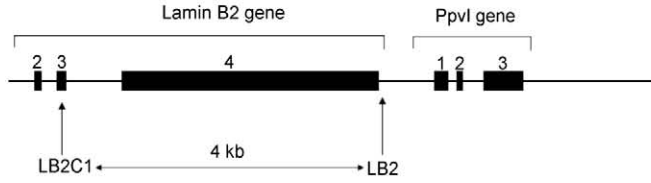
Several studies have used the localization of the ORC complex to mark origins and even predict their location (Wyrick et al., 2001; Ladenburger et al., 2002). To determine whether the Ku and Orc2 proteins associated with the lamin B2, β -globin and *c-myc* replication origins, a chromatin immunoprecipitation (ChIP) assay was used. The ChIP assay has been used previously (Novac et al., 2001; Novac et al., 2002) to quantitatively measure the association of DNA replication proteins with origins as well as to determine the specificity of the interaction between the tested protein and origins. Prior to DNA quantification, immunoblots of the immunoprecipitated lysates verified that the anti-Ku80 and anti-Ku70 antibodies immunoprecipitated their respective target protein (Ku80 and Ku70, respectively; Fig. 8A, lanes 2-3). Owing to the heterodimeric nature of the Ku protein (Ku70/Ku80), the immunoprecipitate of anti-Ku80 antibody contained Ku70 protein, and vice versa (Fig. 8A, lanes 2-3). Immunoprecipitation of Orc2 brought down the target protein as well as both subunits of the Ku heterodimer (Fig. 8A, lane 4). The Ku precipitates also contained the Orc2 subunit, indicating that these proteins are part of a complex (Fig. 8A, lanes 2-3). No proteins were immunoprecipitated by the normal goat serum (NGS), indicating the specificity of the immunoprecipitation reactions.

To determine the binding specificity of Ku and Orc2 to replication origins *in vivo*, their association with the origin-containing regions LB2, BG40.9 and *Myc11* was compared with that of their respective origin-lacking regions of LB2C1, BG72 and *Myc1* (Fig. 8B, supplementary material Table S3). In asynchronous cultures of wild-type HCT116 cells, the abundance of origin-containing DNA fragments in Ku and Orc2 immunoprecipitates was far greater than those lacking them (Fig. 8B). Thus, the abundance of LB2 DNA with Ku80,

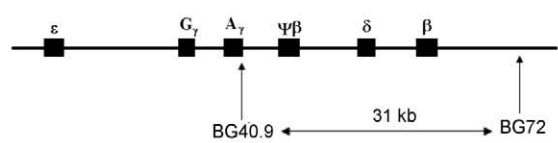
Ku70 and Orc2 was 7.5-, 16- and 17-fold greater, respectively, than that of LB2C1. Likewise, the abundance of BG40.9 was 28-, 36- and 97-fold greater, respectively, than that of BG72

with Ku80, Ku70 and Orc2 proteins. Similarly, *Myc11* was 94-, 53- and 31-fold more abundant than *Myc1* in the three precipitates, respectively. By contrast, the abundance of LB2,

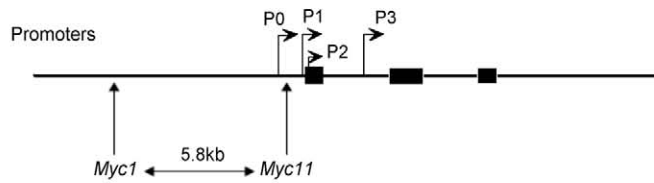
A. Lamin B2 Origin



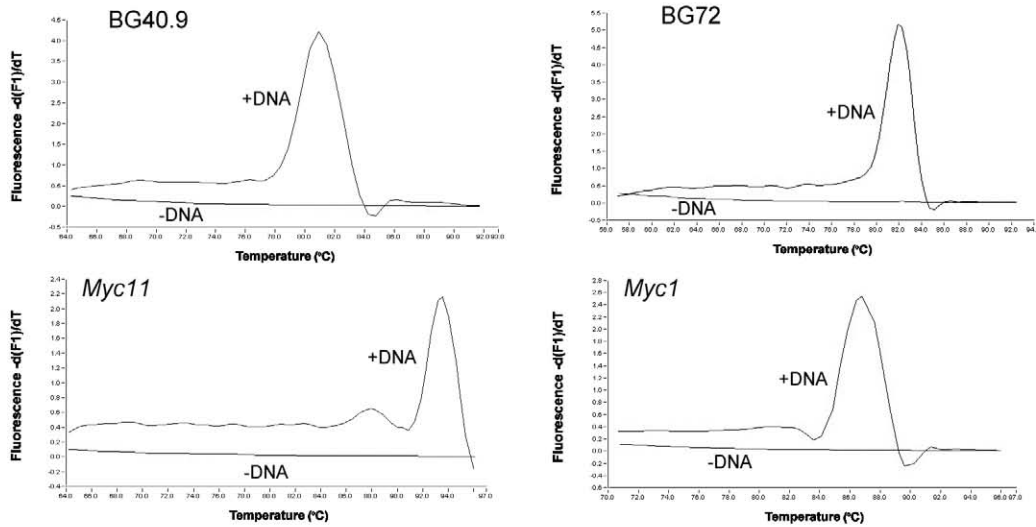
β -Globin Origin



C-Myc Origin



B.



C.

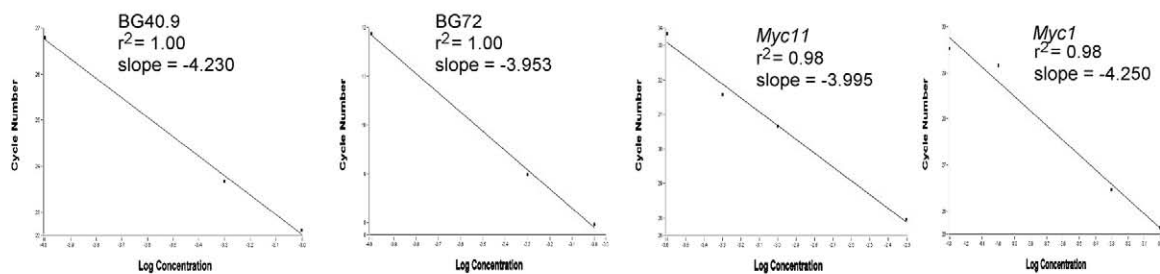
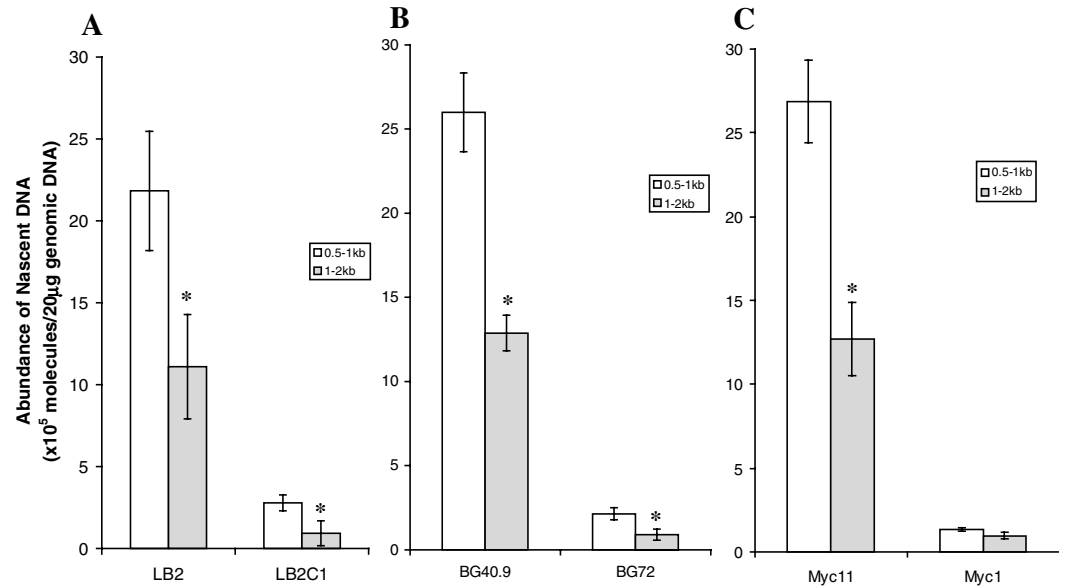


Fig. 5. See next page for legend.

Fig. 6. Abundance of nascent DNA within the 0.5-1 kb and 1-2 kb DNA fractions at three origins in HCT116. Nascent DNA from an asynchronous culture of HCT116, prepared as described in Materials and Methods, was fractionated on a non-denaturing 1% agarose gel following heat denaturation. Regions containing DNA spanning 0.5-1 kb and 1-2 kb were cut out and purified. Abundance of nascent DNA within those fractions was determined using realtime PCR, at three origins: lamin B2 (A), β -globin (B) and *c-myc* (C) origins. As described in Fig. 5, LB2, BG40.9 and *Myc11* are the origin-containing regions for these origins, whereas LB2C1, BG72 and *Myc1* are distal origin-lacking regions lacking origin activity. The average of three experiments and 1SD is indicated. An asterisk (*) represents a statistically significant difference ($P < 0.05$) between the 0.5-1 kb and 1-2 kb DNA fractions in the abundance of nascent DNA at the specified DNA regions.



BG40.9 and *Myc11* in NGS precipitates was 3.8- to 12.5-fold less than in Ku80 precipitates, while the origin-lacking regions, LB2C1, BG72 and *Myc1*, were 1- to 3-fold less abundant in NGS than Ku80 precipitates. The lower abundance of DNA present in NGS immunoprecipitates indicates that the ChIP conditions were stringent enough to prevent substantial nonspecific association of DNA with the immunoglobulins or agarose beads.

Abundance of origin-associated Ku in Ku80^{+/-} cells

In light of the decreased activity of the lamin B2, β -globin and *c-myc* origins observed in Ku80^{+/-} cells (Fig. 7), as well as the decreased association of Ku with these origins (Fig. 8), we examined whether Ku's recruitment to these origins was altered. ChIP analysis was performed using cells synchronized to late G₁ by mimosine (Fig. 9, see supplementary material

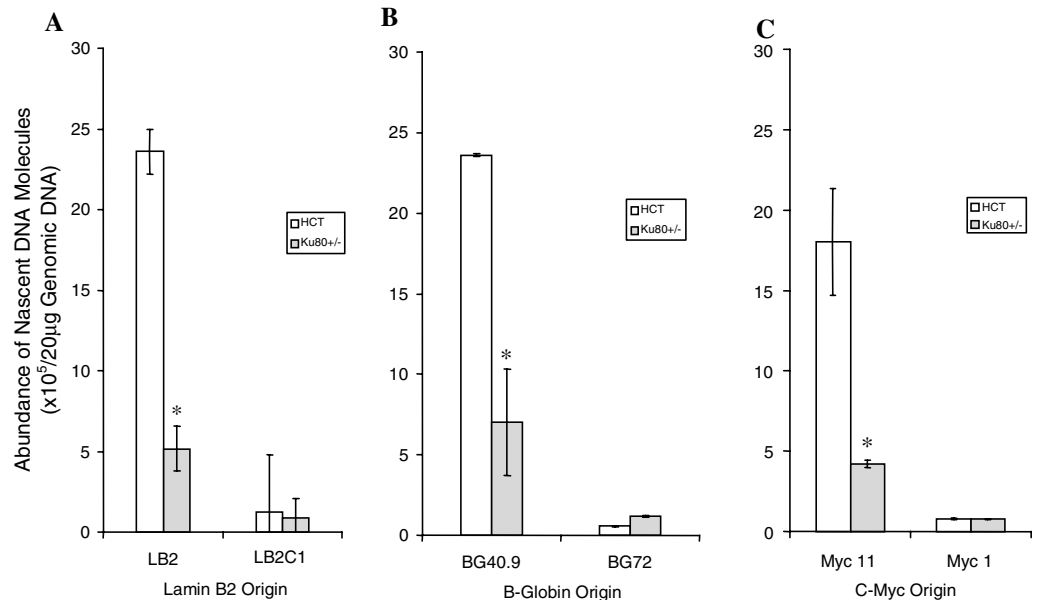
Table S4), when the association of Ku with replication origins is maximal (Novac et al., 2001). Analysis of the lamin B2 origin-containing region (LB2) revealed that Ku80^{+/-} cells had a 1.5-fold decrease in the association of Ku80 to this region relative to wildtype (HCT116) cells ($P = 0.003$, Fig. 9A). Likewise, the in vivo association of Ku70 to LB2 was decreased by 2.1-fold in Ku80^{+/-} cells ($P = 0.002$, Fig. 9A). Similar results were also obtained for the β -globin and *c-myc* origin-containing regions. Specifically, for the β -globin origin, the association of Ku80 with BG40.9 was decreased by 2.3-fold in Ku80^{+/-} cells ($P = 0.025$), and that of Ku70 was decreased by 1.5-fold ($P = 0.014$, Fig. 9B). The association of Ku80 with the *c-myc* origin, *Myc11*, was decreased by 2.5-fold in Ku80^{+/-} cells ($P = 0.03$), and that of Ku70 was decreased by 1.7-fold ($P = 0.02$, Fig. 9C).

The specificity of the immunoprecipitation was determined by comparing the amount of DNA brought down by each of the antibodies with that of normal goat serum (NGS). For HCT116 cells, the LB2 region was enriched by 20-fold in the anti-Ku80 immunoprecipitate relative to NGS (Fig. 9A). It was also enriched 46-fold in anti-Ku70 immunoprecipitates relative to that of NGS (Fig. 9A). These results indicated that the nonspecific association of DNA with the antibodies and agarose beads was negligible. Similarly, the enrichment of BG40.9 in Ku80 immunoprecipitates was 13-fold compared with that of NGS (Fig. 9B). Ku70 immunoprecipitates were 29-fold more abundant in BG40.9 sequence relative to NGS (Fig. 9B). As for *c-myc* locus, *Myc11* was 19-fold more abundant in Ku80 precipitates than in NGS (Fig. 9C), whereas Ku70 precipitates were 39-fold richer in *Myc11* relative to NGS (Fig. 9C). Immunoprecipitates in Ku80^{+/-} cells produced a similar pattern in which the abundance of DNA associated with the anti-Ku antibodies was greater than that of NGS by at least tenfold.

A comparison of the abundance of origin-containing DNA and their origin-lacking regions for each of the

Fig. 5. Maps of the lamin B2, β -globin and *c-myc* origin loci, and location and characteristics of the LightCycler primers. (A) Maps of the lamin B2 (top left), β -globin (top right) and *c-myc* (bottom left) regions. The location and names of the primers is indicated (arrows). For the lamin B2 locus, the exon (black boxes) numbers of the *lamin B2* and *ppv1* genes are indicated above. For the β -globin locus, the name of each globin gene (black box) is indicated above. For the *c-myc* locus, the promoters (arrows) and exons (black boxes) of the *c-myc* gene are indicated. The distance between the origin-containing and origin-lacking amplicons are indicated in kb (double-headed arrows). (B) The melting curves of the new primers designed for the β -globin and *c-myc* origins are shown. Two curves are shown with each primer pair, one containing (+DNA) and one lacking (-DNA) template DNA. None of the primer sets showed any nonspecific products. (C) Standard curves used for the quantification analysis by the LightCycler instrument for each of the four primer sets shown in B. The correlation coefficients (r^2) and slopes are indicated. The similarity in the slopes indicates similar primer efficiencies between the different primer pairs.

Fig. 7. Quantification of nascent strand abundance at three origins in *Ku80*^{+/-} and wild-type cells. Nascent DNA from the 0.5-1 kb fraction prepared from asynchronous cultures of HCT116 and *Ku80*^{+/-} cells as in Fig. 5. Origin activity of the lamin B2 (A), β -globin (B) and *c-myc* (C) origins were measured by quantification of the nascent strand abundance at each origin. LB2, BG40.9 and *Myc11* are located within the origins whereas LB2C1, BG72 and *Myc1* are origin-lacking controls, located distant to the origins (see Fig. 4A for maps). Each error bar represents three experiments and 1 SD is indicated. An asterisk (*) represents a statistically significant difference ($P < 0.05$) between HCT116 and *Ku80*^{+/-} cells in the abundance of nascent DNA at the specified DNA regions.



immunoprecipitates gives a measure of the specificity of binding of Ku to DNA replication origins. In the case of the wild-type HCT116 cells, the origin-containing LB2 region was 12.3- and 21-fold more abundant in Ku80 and Ku70

immunoprecipitates, respectively, than the control region, LB2C1 (Fig. 9A). Similarly, the abundance of the origin-containing region, BG40.9, was 6.9- and eightfold higher in the Ku80 and Ku70 immunoprecipitates, respectively, than the origin-lacking region, BG72 (Fig. 9B). Finally, for the *c-myc* locus, the abundance of the origin-containing region, *Myc11*, exceeded that of the origin-lacking region, *Myc1*, by 7- and 11-fold for the immunoprecipitates of Ku80 and Ku70, respectively (Fig. 9C). In the case of the *Ku80*^{+/-} cells, all origin-containing sequences were at least 2.6-fold more abundant than their origin-lacking regions for the anti-Ku immunoprecipitates. These results indicate that the in vivo association of Ku proteins with the three replication origins examined is specific.

Because all origin-lacking regions are more than 4 kb away from the origin-containing sequences, and the DNA used in the ChIP assay was sonicated to <1 kb in length, then the abundance of the origin-lacking regions represent

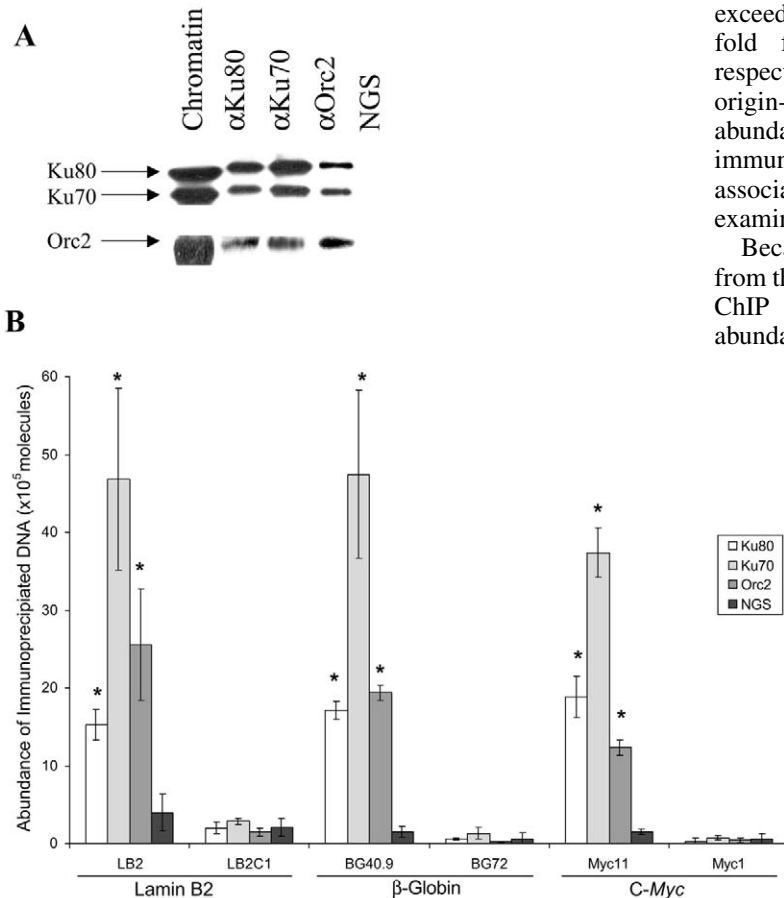


Fig. 8. Association of Ku70, Ku80 and Orc2 to the lamin B2, β -globin and *c-myc* origins in HCT116 cells. Asynchronous cultures of HCT116 cells were treated with 1% formaldehyde and harvested. Chromatin fractions were immunoprecipitated with either anti-Ku80, -Ku70 or -Orc2 antibodies, or normal goat serum (NGS) as control. (A) Western blot analyses of the immunoprecipitates with the immunoblotting antibody indicated on the left and the immunoprecipitating one above the figure. (B) The abundance of DNA for three origins (LB2, BG40.9 and *Myc11*) along with their origin-lacking controls (LB2C1, BG72 and *Myc1*) in each of the precipitates was determined using realtime PCR, thus measuring the association of each protein with the examined DNA region. The average of three experiments and 1 SD is shown. An asterisk (*) represents statistically significant differences ($P < 0.05$) in the association of the indicated protein between the origin-containing and origin-lacking regions.

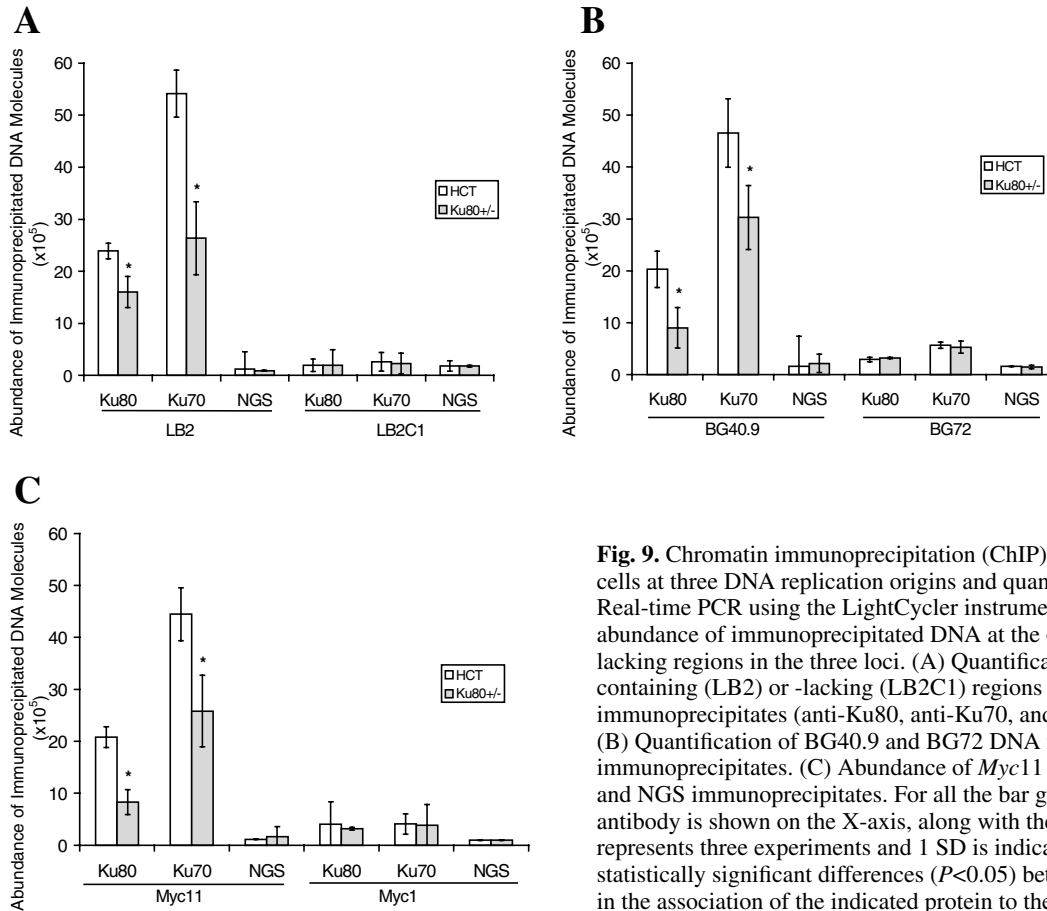


Fig. 9. Chromatin immunoprecipitation (ChIP) assay of HCT116 and Ku80^{+/-} cells at three DNA replication origins and quantification by Real-time PCR. Real-time PCR using the LightCycler instrument was used to quantify the abundance of immunoprecipitated DNA at the origin-containing and origin-lacking regions in the three loci. (A) Quantification of lamin B2 origin-containing (LB2) or -lacking (LB2C1) regions in the three different immunoprecipitates (anti-Ku80, anti-Ku70, and NGS) from 2×10^7 cells. (B) Quantification of BG40.9 and BG72 DNA regions in Ku80, Ku70 and NGS immunoprecipitates. (C) Abundance of *Myc11* and *Myc1* DNA in Ku80, Ku70 and NGS immunoprecipitates. For all the bar graphs, the immunoprecipitating antibody is shown on the X-axis, along with the region amplified. Each error bar represents three experiments and 1 SD is indicated. An asterisk (*) represents statistically significant differences ($P < 0.05$) between HCT116 and Ku80^{+/-} cells in the association of the indicated protein to the specified regions.

'background' or DNA that was not digested by λ -exonuclease. Therefore, comparison of the abundance of these origin-lacking regions gives a measure of extraction efficiency between the two cell lines. The abundance of the origin-lacking regions was comparable for all immunoprecipitates and for both cell lines (Fig. 9). Thus, the differences observed between the origin-containing and their respective origin-lacking regions in the ChIP assays represent biological differences as opposed to technical ones.

When asynchronous cell cultures of HCT116 and Ku80^{+/-} were similarly analyzed by ChIP for the association of Ku80 and Ku70 to the three origins, no significant differences between the two cell lines could be detected (data not shown).

Discussion

Identification of Ku80 as the origin binding subunit (Ruiz et al., 1999; Schild-Poulter et al., 2003) and the availability of Ku80 deficient cells (HCT116 Ku80^{+/-}) (Li et al., 2002) allowed for assessment of Ku's role in mammalian DNA replication in vivo. Ku80^{+/-} cells express decreased levels of Ku80 and Ku70 relative to wild-type HCT116 cells (25% and 70% of HCT116, respectively, Fig. 1), and were reported to have a 3 hour increase in their doubling time (Li et al., 2002). The concomitant decrease in Ku70 protein expression was not surprising (Fig. 1), since Ku is a heterodimer and both subunits are required for each other's stability (Chen et al., 1996; Liang and Jasin, 1996). The decrease in the expression of both

subunits led to a significant decrease in their general association with chromatin (Fig. 2).

The general effect of Ku80 deficiency on cell cycle progression was assessed in asynchronous cultures of Ku80^{+/-} cells, and revealed a significant decrease in the number of S-phase cells and an increase in those in G₁ phase (Fig. 3B). This is similar to the pattern observed in *Orc2*^{Δ/-} cells (Dhar et al., 2001b), indicating that Ku80 deficiency affected cell cycle progression. *Orc2*^{Δ/-} cells express a truncated form of the *Orc2* protein, a member of the hexameric ORC complex, at 10% of the level present in wild-type cells (Dhar et al., 2001b). The truncated *Orc2* protein lacks the first 73 amino acid residues at the N-terminus, and results in the production of cells that have a prolonged G₁ phase and longer doubling time (Dhar et al., 2001b). Hence, the prolonged G₁ phase observed in Ku80^{+/-} cells may be characteristic of cells deficient for DNA replication initiators. Although the mechanism by which the cells induce this delay has not been determined, it does not seem to be mediated through p53 nor p21, as their expression was not significantly altered in Ku80^{+/-} cells (Fig. 1). Moreover, the lack of a difference in the expression levels of *Cdc6* and *Cdk2* between Ku80^{+/-} and HCT116 cells suggests that the effect of Ku on DNA replication is not indirect, through alteration of the level of expression of these initiator proteins.

Comparative analyses of the cell cycle kinetics of wild-type HCT116 and Ku80^{+/-} cells, by measuring the proportion of cells in each phase of the cell cycle following mimosine arrest and release, indicated that Ku80^{+/-} cells display a 2 hour delay

in S-phase entry (Fig. 3A). However, their progression through S-phase and entry into G₂/M mirrored that of wild-type HCT116 (Fig. 3B). This indicates that Ku is not involved in the elongation step of DNA replication, consistent with previous results (Matheos et al., 2002). Furthermore, when Ku80^{+/-} cells were synchronized with hydroxyurea (HU), no delay in entry into S-phase was observed (data not shown). HU inhibits ribonucleotide reductase, which converts ribo- to deoxyribo-nucleotides used for DNA synthesis (Huberman, 1981), whereas mimosine inhibits the expression of serine hydroxymethyltransferase (SHMT) (Lin et al., 1996; Oppenheim et al., 2000; Perry et al., 2005), an enzyme involved in the conversion of serine to glycine and 5,6,7,8-tetrahydrofolate to 5,10-methylenetetrahydrofolate. The latter folate is used in the production of purines, required for synthesis of the RNA primers during initiation of DNA replication, as well as for general DNA synthesis (reviewed by Rosenblatt and Fenton, 2001). These drugs have been reported to arrest cells at different phases of the cell cycle: mimosine at late G₁ (Biard et al., 2003; Lalande, 1990; Krek and DeCaprio, 1995) and during S-phase (Gilbert et al., 1995), and HU at the onset of S-phase (Maurer-Schultze et al., 1988; Merrill, 1998). A double cell-cycle block, using thymidine and then either mimosine or HU, arrested the cells either in late G₁ (Fig. 3A, late G₁) or at the onset of S-phase, respectively (Biard et al., 2003). Consequently, cells released from mimosine arrest have to initiate DNA replication, whereas those released from HU arrest only have to elongate already initiated, RNA-primed DNA chains. Because Ku is not involved in the elongation of DNA replication (Fig. 3B) and associates with origins at late G₁, the delay observed in mimosine-, but not HU-treated Ku80^{+/-} cells might be the result of its requirement to initiate DNA replication. Considering that most origins fire early in S-phase (McAlear et al., 1989; Nakayasu and Berezney, 1989), the G₁ delay in Ku80^{+/-} cells may reflect the postponing of entry into S-phase until all the origins are bound by Ku.

Because Ku is also an end binding protein involved in NHEJ and mimosine is associated with increased DNA breaks (Mikhailov et al., 2000), the G₁ delay observed in mimosine-treated Ku80^{+/-} cells might be attributed to the requirement of the cells to repair any DNA breaks before entry into S-phase. Such a scenario would infer an increase in the number of Ku heterodimers bound to DNA, which would result in enrichment of the DNA-containing chromatin with Ku. However, the chromatin fractions from untreated and mimosine- or HU-treated Ku80^{+/-} cells showed no detectable increase in Ku80's or Ku70's association (Fig. 4), suggesting that Ku is not required for the repair of mimosine- or hydroxyurea-induced damage. Furthermore, analysis of the abundance of RPA70 in these chromatin extracts did not reveal a detectable increase in its association with chromatin, indicating that any DNA damage induced by a 12 hour exposure to the drugs was not substantial.

Further support for a role of Ku in the initiation of DNA replication (Novac et al., 2001; Matheos et al., 2002; Matheos et al., 2003) is provided by the observed decrease in origin usage, reflected by a significant 3.4-4.5-fold decrease in nascent strand DNA abundance in Ku80^{+/-} cells (Fig. 7). Unlike Orc2^{Δ/-} cells, in which 10% of Orc2 protein was sufficient for initiation of chromosomal DNA replication, 25% of Ku80, present in Ku80^{+/-} cells, is apparently insufficient.

This might be a reflection of the multiple functions of Ku in vivo, which may result in less Ku being available for DNA replication. Alternatively, the differences in the results obtained with the Ku80^{+/-} and Orc2^{Δ/-} cells might be due to the different methods employed in the two studies to assess abundance of nascent DNA.

Previous studies have shown a specific association of Ku with the replication origins in monkey (CV1) and mouse embryonic fibroblast (MEF) cells (Novac et al., 2001), as well as in Chinese hamster ovary (CHO) cells (Matheos et al., 2003). In this study, ChIP analyses revealed the specific association of Ku with the lamin B2, β-globin and *c-myc* origins of DNA replication in vivo in human cells (Figs 8 and 9). Orc2 protein, a subunit of the ORC complex and a marker of origins (Ladenburger et al., 2002), was also found to be associated with these human origins in asynchronous cultures, further verifying them as bona fide origins (Fig. 8). Indeed, a footprint of Orc2 at the lamin B2 origin has previously been described (Abdurashidova et al., 2003), substantiating the use of Orc2 and the lamin B2 origin as positive controls in those experiments (Fig. 8). In these experiments, asynchronous cultures were used to eliminate the possibility of any artifacts arising from the use of cell synchronization drugs.

Analysis of the association of Ku with replication origins in Ku80^{+/-} cells revealed a significant 1.5-2.5-fold decrease for both subunits of the Ku heterodimer (Fig. 9). Although relatively moderate, this decrease correlated with a significant decrease in origin activity (Fig. 7). This relatively moderate decrease might also explain the inability to detect it in asynchronous cells, in which the majority of cells are not in late G₁ phase, thus masking the phenotype of those that are. In asynchronous cultures, only 25% of cells were present in G₁. Combined with the low affinity of the Ku80 antibody for its target protein (Santa Cruz Biotechnology, personal communication), this makes detection of a difference in the association of Ku with origins in Ku80^{+/-} and HCT116 cells difficult. However, 75% of cells arrest at late G₁ when synchronized with mimosine, thus facilitating the detection of differences between the two cell lines. Synchronization also avoided the potential bias that can arise by the prolonged doubling time of Ku80^{+/-} cells. If asynchronous cultures were used, the observed decrease in Ku's association with origins in Ku80^{+/-} cells might have been caused by (or attributed to) a decrease in the number of cells in late G₁. By synchronizing the cells, however, this observed decrease can be attributed to a decrease in the number of Ku molecules binding to replication origins.

The DNA association profile of Ku with origin-containing and -lacking regions shows sequence-specific binding. ChIP analyses revealed that the association of Ku with LB2, BG40.9 and *Myc11* was more than eightfold greater than its association with LB2C1, BG72 and *Myc1* (Fig. 9). Furthermore, the general association of Ku80 with chromatin was decreased by 33% in Ku80^{+/-} cells (Fig. 2), whereas its association with origins was decreased by up to 60% (Fig. 9). Such binding patterns suggest a sequence specific, nonrandom binding of Ku to origins of DNA replication. Others identified a sequence specific binding capacity for the Ku heterodimer by different techniques. In one study, Ku was footprinted on A3/4, a version of a mammalian DNA replication consensus sequence (Schild-Poulter et al., 2003). A separate study found that Ku bound

DNA microcircles containing the NRE1 element, a transcriptional repressor (Giffin et al., 1996), while another study found that mutation of a Ku binding site abolished its binding to the human oxidoreductase gene (Xu et al., 2004).

Although the exact role of Ku in initiation of DNA replication has not been determined yet, it is unlikely that the observed phenotypes in this study are solely due to its function in DNA repair. First, neither the expressions of p53 nor p21 proteins, two regulators of the G₁/S checkpoint in case of DNA damage, were affected by Ku80 deficiency (Fig. 1). Second, the progression through S-phase was indistinguishable between the Ku80^{+/-} and HCT116 cell lines (Fig. 3B). This is important since DNA repair can take place during any phase of the cell cycle, whereas DNA initiation occurs only at the beginning of S-phase. Several other studies have suggested that Ku deficiency leads to cell cycle arrest by a mechanism independent of its DNA repair function. Nussenzweig et al. (Nussenzweig et al., 1996) observed that Ku80^{-/-} mice are smaller than their wild-type counterparts and that this was not due to a decrease in the number of cells present in organs, but rather a decrease in the cell size. Arrington et al. (Arrington et al., 2000) observed that the inability of Ku80^{-/-} MEF cells to traverse the G₂/M cell cycle checkpoint after treatment with H₂O₂ was not due to Ku's DNA repair function. Furthermore, Rockwood et al. (Rockwood et al., 2003) identified a decrease in mutant frequency and chromosomal rearrangements in Ku80-null mice relative to their wild-type littermates, which they attributed to an increase in homologous recombination repair of DNA double-strand breaks (DSBs), arguing against the deleterious effect of Ku-deficiency in DNA repair. Finally, Park et al. (Park et al., 2004) described a role for Ku in DNA replication following ionizing radiation-induced DNA damage, in which it stabilizes the association of PCNA with chromatin, independently of DNA-PK kinase activity.

From the data presented here and the published studies discussed, a model for Ku's role in initiation of DNA replication can be proposed: through its sequence-specific binding capacity, Ku recognizes and binds to origins of DNA replication, and unwinds them using its DNA helicase activity (Tuteja et al., 1990; Tuteja et al., 1994), thus permitting recruitment of other DNA replication proteins to the origin. These proteins include Orc2, DNA polymerases α , δ and ϵ , PCNA, topoisomerase II, RFC and RPA (Matheos et al., 2002). Alternatively, Ku may be necessary for stabilizing the interaction of DNA replication proteins with DNA (Park et al., 2004), as has been proposed in the budding yeast (Shakibai et al., 1996). The conservation of the DNA replication function of Ku through evolution, from the herpes simplex virus, to the yeast *S. cerevisiae* and to mammals, such as rodents and humans, infers its significance to cell survival.

This is the first study to show that decreased association of Ku in vivo with human replication origins correlates with a decreased frequency of initiation events at those origins. Further studies aiming to address the DNA replication function of Ku by purifying Ku-containing complexes, deciphering their contents and characterizing their DNA replication activities, will aid in the determination of the function of Ku in the process of initiation of DNA replication.

This research was supported by grants from the CIHR (M.Z.-H.) and the Cancer Research Society (G.B.P.). S.S. was a recipient of a

doctoral studentship from the CIHR/K.M. Hunter Charitable foundation and the FCAR/FRSQ, as well as the McGill Medicine studentship.

References

- Abdurashidova, G., Deganuto, M., Klima, R., Riva, S., Biamonti, G., Giacca, M. and Falaschi, A. (2000). Start sites of bidirectional DNA synthesis at the human lamin B2 origin. *Science* **287**, 2023-2026.
- Abdurashidova, G., Danailov, M. B., Ochem, A., Triolo, G., Djeliova, V., Radul, S., Vindigni, A., Riva, S. and Falaschi, A. (2003). Localization of proteins bound to a replication origin of human DNA along the cell cycle. *EMBO J.* **22**, 4294-4303.
- Arrington, E. D., Caldwell, M. C., Kumarvel, T. S., Lohani, A., Johi, A., Evans, M. K., Chen, H. T., Nussenzweig, A., Holbrook, N. J. and Gorospe, M. (2000). Enhanced sensitivity and long-term G₂ arrest in hydrogen peroxide-treated Ku80-null cells are unrelated to DNA repair defects. *Free Radic. Biol. Med.* **29**, 1166-1176.
- Baker, R. O., Murata, L. B., Dodson, M. S. and Hall, J. D. (2000). Purification and characterization of OF-1, a host factor implicated in herpes simplex replication. *J. Biol. Chem.* **275**, 30050-30057.
- Bell, S. P. and Dutta, A. (2002). DNA replication in eukaryotic cells. *Annu. Rev. Biochem.* **71**, 333-374.
- Biard, D. S., Miccoli, L., Despras, E., Harper, F., Pichard, E., Creminon, C. and Angulo, J. F. (2003). Participation of kin17 protein in replication factories and in other DNA transactions mediated by high molecular weight nuclear complexes. *Mol. Cancer Res.* **1**, 519-531.
- Camara-Clayette, V., Thomas, D., Rahuel, C., Barbey, R., Cartron, J. P. and Bertrand, O. (1999). The repressor which binds the -75 GATA motif of the GPB promoter contains Ku70 as the DNA binding subunit. *Nucleic Acids Res.* **27**, 1656-1663.
- Chen, F., Peterson, S. R., Story, M. D. and Chen, D. J. (1996). Disruption of DNA-PK in Ku80 mutant xrs-6 and the implications in DNA double-strand break repair. *Mutation Res.* **362**, 9-19.
- Chuang, R. Y. and Kelly, T. J. (1999). The fission yeast homologue of Orc4p binds to replication origin DNA via multiple AT-hooks. *Proc. Natl. Acad. Sci. USA* **96**, 2656-2661.
- Cosgrove, A. J., Nieduszynski, C. A. and Donaldson, A. D. (2002). Ku complex controls the replication time of DNA in telomere regions. *Genes Dev.* **16**, 2485-2490.
- Detweiler, C. S. and Li, J. J. (1998). Ectopic induction of Clb2 in early G₁ phase is sufficient to block prereplicative complex formation in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **95**, 2384-2389.
- Dhar, S. K., Delmolino, L. and Dutta, A. (2001a). Architecture of the human origin recognition complex. *J. Biol. Chem.* **276**, 29067-29071.
- Dhar, S. K., Yoshida, K., Machida, Y., Khaira, P., Chaudhuri, B., Wohlschlegel, J. A., Leffak, M., Yates, J. and Dutta, A. (2001b). Replication from oriP of Epstein-Barr virus requires human ORC and is inhibited by Geminin. *Cell* **106**, 287-296.
- Dignam, J. D., Lebovitz, R. M. and Roeder, R. G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**, 1475-1489.
- Downs, J. A. and Jackson, S. P. (2004). A means to a DNA end: the many roles of Ku. *Nat. Rev.* **5**, 367-378.
- Dutta, A. and Bell, S. P. (1997). Initiation of DNA replication in eukaryotic cells. *Annu. Rev. Cell Dev. Biol.* **13**, 293-332.
- Findeisen, M., El-Denari, M., Kapitza, T., Graf, R. and Strausfeld, U. (1999). Cyclin A-dependent kinase activity affects chromatin binding of ORC, Cdc6, and MCM in egg extracts of *Xenopus laevis*. *Eur. J. Biochem.* **264**, 415-426.
- Frappier, L. and Zannis-Hadjopoulos, M. (1987). Autonomous replication of plasmids bearing monkey DNA origin-enriched sequences. *Proc. Natl. Acad. Sci. USA* **84**, 6668-6672.
- Gerbi, S. and Bielinsky, A.-K. (1997). Replication initiation point mapping. *Methods* **13**, 271-280.
- Giacca, M., Zentilin, L., Norio, P., Diviacco, S., Dimitrova, D., Contreas, G., Biamonti, G., Perini, G., Weighardt, F., Riva, S. et al. (1994). Fine mapping of a replication origin of human DNA. *Proc. Natl. Acad. Sci. USA* **91**, 7119-7123.
- Giacca, M., Pelizon, C. and Falaschi, A. (1997). Mapping replication origins by quantifying relative abundance of nascent DNA strands using competitive polymerase chain reaction. *Methods* **13**, 301-312.
- Giffin, W., Torrance, H., Rodd, D. J., Préfontaine, G. G., Pope, L. and

- Haché, R. J. G. (1996). Sequence-specific DNA binding by ku autoantigen and its effects on transcription. *Nature* **380**, 265-268.
- Gilbert, D. M. (1986). Temporal order of replication of *Xenopus laevis* 5S ribosomal RNA genes in somatic cells. *Proc. Natl. Acad. Sci. USA* **83**, 2924-2928.
- Gilbert, D. M., Neilson, A., Miyazawa, H., DePamphilis, M. L. and Burhans, W. C. (1995). Mimosine arrests DNA synthesis at replication forks by inhibiting deoxyribonucleotide metabolism. *J. Biol. Chem.* **270**, 9597-9606.
- Goldman, M. A., Holmquist, G. P., Gray, M. C., Caston, L. and Nag, A. (1984). Replication timing of genes and middle repetitive sequences. *Science* **224**, 686-692.
- Hand, R. (1978). Eucaryotic DNA: organization of the genome for replication. *Cell* **15**, 317-325.
- Hirt, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26**, 365-369.
- Hua, X. H. and Newport, J. (1998). Identification of a preinitiation step in DNA replication that is independent of origin recognition complex and cdc6, but dependent on cdk2. *J. Cell Biol.* **140**, 271-281.
- Huberman, J. A. (1981). New views of the biochemistry of eucaryotic DNA replication revealed by aphidicolin, an unusual inhibitor of DNA polymerase alpha. *Cell* **23**, 647-648.
- Huberman, J. A. and Riggs, A. D. (1968). On the mechanism of DNA replication in mammalian chromosomes. *J. Mol. Biol.* **32**, 327-341.
- Jalouzet, R., Toublan, B., Wilhelm, M. L. and Wilhelm, F. X. (1985). Replication timing of the H4 histone genes in Physarum polycephalum. *Proc. Natl. Acad. Sci. USA* **82**, 6475-6479.
- Kamath, S. and Leffak, M. (2001). Multiple sites of replication initiation in the human beta-globin gene locus. *Nucleic Acids Res.* **29**, 809-817.
- Kim, D., Ouyang, H., Yang, S. H., Nussenzweig, A., Burgman, P. and Li, G. C. (1995). A constitutive heat shock element-binding factor is immunologically identical to the Ku autoantigen. *J. Biol. Chem.* **270**, 15277-15284.
- Klemm, R. D., Austin, R. J. and Bell, S. P. (1997). Coordinate binding of ATP and origin DNA regulates the ATPase activity of the origin recognition complex. *Cell* **88**, 493-502.
- Krek, W. and DeCaprio, J. A. (1995). Cell synchronization. *Methods Enzymol.* **254**, 114-124.
- Krude, T. (1999). Mimosine arrests proliferating human cells before onset of DNA replication in a dose-dependent manner. *Exp. Cell Res.* **247**, 148-159.
- Kuhn, A., Stefanovsky, V. and Grummt, I. (1993). The nucleolar transcription activator UBF relieves Ku antigen-mediated repression of mouse ribosomal gene transcription. *Nucl. Acids Res.* **21**, 2057-2063.
- Ladenburger, E. M., Keller, C. and Knippers, R. (2002). Identification of a binding region for human origin recognition complex proteins 1 and 2 that coincides with an origin of DNA replication. *Mol. Cell Biol.* **22**, 1036-1048.
- Lalande, M. (1990). A reversible arrest point in the late G1 phase of the mammalian cell cycle. *Exp. Cell Res.* **186**, 332-339.
- Landry, S. and Zannis-Hadjopoulos, M. (1991). Classes of autonomously replicating sequences are found among early-replicating monkey DNA. *Biochim. Biophys. Acta* **1088**, 234-244.
- Li, F., Chen, J., Solessio, E. and Gilbert, D. M. (2003). Spatial distribution and specification of mammalian replication origins during G1 phase. *J. Cell Biol.* **161**, 257-266.
- Li, G., Nelsen, C. and Hendrickson, E. A. (2002). Ku86 is essential in human somatic cells. *Proc. Natl. Acad. Sci. USA* **99**, 832-837.
- Li, J. J. and Herskowitz, I. (1993). Isolation of ORC6, a component of the yeast origin recognition complex by a one-hybrid system. *Science* **262**, 1870-1874.
- Liang, F. and Jasin, M. (1996). Ku80-deficient cells exhibit excess degradation of extrachromosomal DNA. *J. Biol. Chem.* **271**, 14405-14411.
- Lin, H. B., Falchetto, R., Mosca, P. J., Shabanowitz, J., Hunt, D. F. and Hamlin, J. L. (1996). Mimosine targets serine hydroxymethyltransferase. *J. Biol. Chem.* **271**, 2548-2556.
- Matheos, D., Ruiz, M. T., Price, G. B. and Zannis-Hadjopoulos, M. (2002). Ku antigen, an origin-specific binding protein that associates with replication proteins, is required for mammalian DNA replication. *Biochim. Biophys. Acta* **1578**, 59-72.
- Matheos, D., Novac, O., Price, G. B. and Zannis-Hadjopoulos, M. (2003). Analysis of the DNA replication competence of the xrs-5 mutant cells defective in Ku86. *J. Cell Sci.* **116**, 111-124.
- Maurer-Schultze, B., Siebert, M. and Bassukas, I. D. (1988). An in vivo study on the synchronizing effect of hydroxyurea. *Exp. Cell Res.* **174**, 230-243.
- McAlear, M., Ward, G. K., McKenzie, R., Price, G. B. and Zannis-Hadjopoulos, M. (1989). Biphasic activation of DNA replication. *Mol. Gen.* **8**, 11-14.
- Merrill, G. F. (1998). Cell synchronization. *Methods Cell Biol.* **57**, 229-249.
- Messer, W. (2002). The bacterial replication initiator DnaA. DnaA and oriC, the bacterial mode to initiate DNA replication. *FEMS Microbiol. Rev.* **26**, 355-374.
- Mikhailov, I., Russev, G. and Anachkova, B. (2000). Treatment of mammalian cells with mimosine generates DNA breaks. *Mutation Res.* **459**, 299-306.
- Mimori, T., Akizuki, M., Yamagata, H., Inada, S., Yoshida, S. and Homma, M. (1981). Characterization of a high molecular weight acidic nuclear protein recognized by autoantibodies in the sera from patients with polymyositis-scleroderma overlap. *J. Clin. Invest.* **68**, 611-620.
- Moon, K. Y., Kong, D., Lee, J. K., Raychaudhuri, S. and Hurwitz, J. (1999). Identification and reconstitution of the origin recognition complex from *Schizosaccharomyces pombe*. *Proc. Natl. Acad. Sci. USA* **96**, 12367-12372.
- Murata, L. B., Dodson, M. S. and Hall, J. D. (2004). A human cellular protein activity (OF-1), which binds herpes simplex virus type 1 origin, contains the Ku70/Ku80 heterodimer. *J. Virol.* **78**, 7839-7842.
- Nakayasu, H. and Berezney, R. (1989). Mapping Replication Sites in the Eucaryotic Cell Nucleus. *J. Cell Biol.* **108**, 1-11.
- Novac, O., Matheos, D., Araujo, F. D., Price, G. B. and Zannis-Hadjopoulos, M. (2001). *In vivo* association of Ku with mammalian origins of DNA replication. *Mol. Biol. Cell* **12**, 3386-3401.
- Novac, O., Alvarez, D., Pearson, C. E., Price, G. B. and Zannis-Hadjopoulos, M. (2002). The human cruciform-binding protein, CBP, is involved in DNA replication and associates *in vivo* with mammalian replication origins. *J. Biol. Chem.* **277**, 11174-11183.
- Nussenzweig, A., Chen, C., da Costa Soares, V., Sanchez, M., Sokol, K., Nussenzweig, M. C. and Li, G. C. (1996). Requirement for Ku80 in growth and immunoglobulin V(D)J recombination. *Nature* **382**, 551-555.
- Okuno, Y., McNairn, A. J., den Elzen, N., Pines, J. and Gilbert, D. M. (2001). Stability, chromatin association and functional activity of mammalian pre-replication complex proteins during the cell cycle. *EMBO J.* **20**, 4263-4277.
- Oppenheim, E. W., Nasrallah, I. M., Mastri, M. G. and Stover, P. J. (2000). Mimosine is a cell-specific antagonist of folate metabolism. *J. Biol. Chem.* **275**, 19268-19274.
- Park, S.-J., Ciccone, S. L. M., Freie, B., Kurimasa, A., Chen, D. J., Li, G. C., Clapp, D. W. and Lee, S.-H. (2004). A positive role for the Ku complex in DNA replication following strand break damage in mammals. *J. Biol. Chem.* **279**, 6046-6055.
- Pearson, C. E., Frappier, L., Price, G. B. and Zannis-Hadjopoulos, M. (1991). Plasmids bearing mammalian DNA-replication origin-enriched (ors) fragments initiate semi-conservative replication in a cell free system. *Biochim. Biophys. Acta* **1090**, 156-166.
- Pearson, C. E., Shihab-el-Deen, A., Price, G. B. and Zannis-Hadjopoulos, M. (1994). Electron microscopic analysis of *in vitro* replication products of *ors8*, a mammalian origin enriched sequence. *Somatic Cell Mol. Genet.* **20**, 147-152.
- Perry, C., Sastry, R., Nasrallah, I. M. and Stover, P. J. (2005). Mimosine attenuates serine hydroxymethyltransferase transcription by chelating zinc. Implications for inhibition of DNA replication. *J. Biol. Chem.* **280**, 396-400.
- Pierron, G., Durica, D. S. and Saner, H. W. (1984). Invariant temporal order of replication of the four actin gene loci during the naturally synchronous mitotic cycles of Physarum polycephalum. *Proc. Natl. Acad. Sci. USA* **81**, 6393-6397.
- Price, G. B., Allarakhia, M., Cossons, N., Nielsen, T., Diaz-Perez, M., Friedlander, P., Tao, L. and Zannis-Hadjopoulos, M. (2003). Identification of a *cis*-element that determines autonomous DNA replication in eukaryotic cells. *J. Biol. Chem.* **278**, 19649-19659.
- Quintana, D. G., Hou, Zh., Thome, K. C., Hendricks, M., Saha, P. and Dutta, A. (1997). Identification of HsORC4, a member of the human origin of replication recognition complex. *J. Biol. Chem.* **272**, 28247-28251.
- Quintana, D. G., Thome, K. C., Hou, Z. H., Ligon, A. H., Morton, C. C. and Dutta, A. (1998). ORC5L, a new member of the human origin recognition complex, is deleted in uterine leiomyomas and malignant myeloid diseases. *J. Biol. Chem.* **273**, 27137-27145.
- Ritzi, M., Baack, M., Musahl, C., Romanowski, P., Laskey, R. A. and Knippers, R. (1998). Human minichromosome maintenance proteins and human origin recognition complex 2 protein on chromatin. *J. Biol. Chem.* **273**, 24543-24549.

- Rockwood, L. D., Nussenzweig, A. and Janz, S. (2003). Paradoxical decrease in mutant frequencies and chromosomal rearrangements in a transgenic *lacZ* reporter gene in Ku80 null mice deficient in DNA double strand break repair. *Mutation Res.* **529**, 51-58.
- Rosenblatt, D. S and Fenton, W. A. (2001). Inherited disorders of folate and cobalamin transport and metabolism. In *The Metabolic And Molecular Basis Of Inherited Disease* (ed. C. A. Scriver, A. L. Beaudet, D. Valle and W. S. Sly), pp. 3897-3933. New York: McGraw-Hill Press.
- Rowles, A., Tada, S. and Blow, J. J. (1999). Changes in association of the Xenopus origin recognition complex with chromatin on licensing of replication origins. *J. Cell Sci.* **112**, 2011-2018.
- Ruiz, M. T., Pearson, C. E., Nielsen, T., Price, G. B. and Zannis-Hadjopoulos, M. (1995). Cofractionation of HeLa cell replication proteins with Ors-binding activity. *J. Cell. Biochem.* **58**, 221-236.
- Ruiz, M. T., Matheos, D., Price, G. B. and Zannis-Hadjopoulos, M. (1999). OBA/Ku86: DNA binding specificity and involvement in mammalian DNA replication. *Mol. Biol. Cell* **10**, 567-580.
- Sambrook, F., Fritsch, E. F. and Maniatis, T. (1989). Detection and analysis of proteins expressed from cloned genes. In *Molecular Cloning. A Laboratory Manual* (ed. C. Nolan), pp. 18.1-18.88. New York: Cold Spring Harbor Laboratory Press.
- Schaffer, A., Kim, E. C., Wu, X., Zan, H., Testoni, L., Salamon, S., Cerutti, A. and Casali, P. (2003). Selective inhibition of class switching to IgG and IgE by recruitment of the HoxC4 and Oct-1 homeodomain proteins and Ku70/Ku86 to newly identified ATTT cis-elements. *J. Biol. Chem.* **278**, 23141-23150.
- Schild-Poulter, C., Matheos, D., Novac, O., Cui, B., Giffin, W., Ruiz, M. T., Price, G. B., Zannis-Hadjopoulos, M. and Hache, R. J. G. (2003). Differential DNA binding of Ku antigen determines its involvement in DNA replication. *DNA Cell Biol.* **22**, 65-78.
- Shakibai, N., Kumar, V. and Eisenberg, S. (1996). The Ku-like protein from *Saccharomyces cerevisiae* is required *in vitro* for the assembly of a stable multiprotein complex at a eukaryotic origin of replication. *Proc. Natl. Acad. Sci. USA* **93**, 11569-11574.
- Sharova, N. P. and Abramova, E. B. (2002). Initiation of DNA Replication in Eukaryotes Is an Intriguing Cascade of Protein Interactions. *Biochemistry (Mosc)*. **67**, 1217-1223.
- Stephens, R. F., Pan, C. J., Ajiro, K., Dolby, T. W. and Borun, T. W. (1977). Studies of human histone messenger RNA. I. Methods for the isolation and partial characterization of RNA fractions containing human histone message from HeLa S3 polyribosomes. *J. Biol. Chem.* **252**, 166-172.
- Szuts, D. and Krude, T. (2004). Cell cycle arrest at the initiation step of human chromosomal DNA replication causes DNA damage. *J. Cell Sci.* **117**, 4897-4908.
- Tao, L., Dong, Z., Leffak, M., Zannis-Hadjopoulos, M. and Price, G. B. (2000). Major DNA replication initiation sites in the c-myc locus in human cells. *J. Cell. Biochem.* **78**, 442-457.
- Tatsumi, Y., Tsurimoto, T., Shirahige, K., Yoshikawa, H. and Obuse, C. (2000). Association of human origin recognition complex 1 with chromatin DNA and nuclease-resistant nuclear structures. *J. Biol. Chem.* **275**, 5904-5910.
- Taylor, T. J. and Knipe, D. M. (2004). Proteomics of herpes simplex virus replication compartments: association of cellular DNA replication, repair, recombination, and chromatin remodeling proteins with ICP8. *J. Virol.* **78**, 5856-5866.
- Todd, A., Landry, S., Pearson, C. E., Khoury, V. and Zannis-Hadjopoulos, M. (1995). Deletion analysis of minimal sequence requirements for autonomous replication of ors8, a monkey early-replicating DNA sequence. *J. Cell. Biochem.* **57**, 280-289.
- Tóth, E. C., Marusic, L., Ochem, A., Patthy, A., Pongor, S., Giacca, M. and Falaschi, A. (1993). Interactions of USF and Ku antigen with a human DNA region containing a replication origin. *Nucleic Acids Res.* **21**, 3257-3263.
- Tuteja, N., Tuteja, R., Rahman, K., Kang, L. Y. and Falaschi, A. (1990). A DNA helicase from human cells. *Nucleic Acids Res.* **18**, 6785-6792.
- Tuteja, N., Rahman, R., Tuteja, R., Ochem, A., Skopac, D. and Falaschi, A. (1994). Human DNA helicase II: a novel DNA unwinding enzyme identified as the Ku autoantigen. *EMBO J.* **13**, 4991-5001.
- Tuteja, R. and Tuteja, N. (2000). Ku autoantigen: a multifunctional DNA-binding protein. *Crit. Rev. Biochem. Mol. Biol.* **35**, 1-33.
- Vindelov, L. L. (1977). Flow microfluorometric analysis of nuclear DNA in cells from solid tumors and cell suspensions. A new method for rapid isolation and straining of nuclei. *Virchows Arch. B Cell Pathol.* **24**, 227-242.
- Vishwanatha, J. K. and Baril, E. F. (1990). Single-stranded DNA dependent ATPase from HeLa cells that stimulates DNA polymerase α -primase activity: purification and characterization of the ATPase. *Biochemistry* **29**, 8753-8759.
- Vogel, H., Lim, D.-S., Karsenty, G., Finegold, M. and Hasty, P. (1999). Deletion of Ku86 causes early onset of senescence in mice. *Proc. Natl. Acad. Sci. USA* **96**, 10770-10775.
- Wyrick, J. J., Aparicio, J. G., Chen, T., Barnett, J. D., Jennings, E. G., Young, R. A., Bell, S. P. and Aparicio, O. M. (2001). Genome-wide distribution of ORC and MCM proteins in *S. cerevisiae*: high-resolution mapping of replication origins. *Science* **294**, 2357-2360.
- Xu, P., LaVallee, P. A., Lin, J. J. and Hoidal, J. R. (2004). Characterization of proteins binding to E-box/Ku86 sites and function of Ku86 in transcriptional regulation of the human xanthine oxidoreductase gene. *J. Biol. Chem.* **279**, 16057-16063.
- Zannis-Hadjopoulos, M. and Price, G. B. (1998). Regulatory parameters of DNA replication. *Crit. Rev. Eukaryotic Gene Expr.* **8**, 81-106.
- Zannis-Hadjopoulos, M. and Price, G. B. (1999). Eukaryotic DNA replication. *J. Cell. Biochem.* **32/33**, 1-14.
- Zannis-Hadjopoulos, M., Sibani, S. and Price, G. B. (2004). Eukaryotic DNA replication proteins. *Front. Biosci.* **9**, 2133-2143.