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In Vivo Association of Ku with Mammalian Origins of DNA Replication

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Ku is a heterodimeric (Ku70/86-kDa) nuclear protein with known functions in DNA repair, V(D)J recombination, and DNA replication. Here, the in vivo association of Ku with mammalian origins of DNA replication was analyzed by studying its association with *ors8* and *ors12*, as assayed by formaldehyde cross-linking, followed by immunoprecipitation and quantitative polymerase chain reaction analysis. The association of Ku with *ors8* and *ors12* was also analyzed as a function of the cell cycle. This association was found to be approximately fivefold higher in cells synchronized at the G1/S border, in comparison with cells at G0, and it decreased by approximately twofold upon entry of the cells into S phase, and to near background levels in cells at G2/M phase. In addition, in vitro DNA replication experiments were performed with the use of extracts from $Ku80^{+/+}$ and $Ku80^{-/-}$ mouse embryonic fibroblasts. A decrease of ~70% in in vitro DNA replication was observed when the $Ku80^{-/-}$ extracts were used, compared with the $Ku80^{+/+}$ extracts. The results indicate a novel function for Ku as an origin binding-protein, which acts at the initiation step of DNA replication and dissociates after origin firing.

INTRODUCTION

According to the replicon model (Jacob *et al.*, 1963) origins are defined by specific DNA sequences (replicators) and an initiator protein or complex of proteins, that bind to this sequence (reviewed in Berezney *et al.*, 2000). Once the origin is activated, the initiator protein unwinds the DNA duplex, allowing the entry of the replication machinery and synthesis of the first primers for chain elongation (reviewed in Ritzi and Knippers, 2000).

Considerable progress has been made in *Saccharomyces cerevisiae* toward our understanding of the regulation of initiation of DNA replication in relation to the cell cycle (reviewed by Quintana and Dutta, 1999). The origin recognition complex (ORC) was first described in yeast and led to the subsequent identification of ORC homologs in humans, invertebrates (*Caenorabditis elegans*), plants (*Arabidopsis thaliana*), fission yeast, and flies (*Drosophila melanogaster*) (Gavin *et al.*, 1995; Gossen *et al.*, 1995). In budding yeast, ORC is bound to the replication origins (or ARS elements) throughout the cell cycle (Diffley *et al.*, 1994; Aparicio *et al.*, 1997; Liang and Stillman, 1997). A prereplication complex (preRC) assembles during G1 phase of the cell cycle in preparation for initiation of DNA replication at the origin. In *S. cerevisiae*, this complex consists of ORC proteins, Cdc6p, and the family of MCM proteins, licensing factors. Afterward, activation of cell cycle-regulated protein kinases guides the "licensed" origin into S phase. The preRC gradually dissociates by releasing Cdc6p and MCM proteins; this postreplication complex persists until the next G1 phase when another round of replication can occur. ORC was recently shown to play a critical role in replication initiation by positioning nucleosomes adjacent to yeast origins of replication, which influences the preRC assembly (Lipford and Bell, 2001), reinforcing the hypothesis that chromosomal context can significantly affect origin function (Newlon *et al.*, 1993; Friedman *et al.*, 1996).

Chromosomal proteins often interact with DNA to control maintenance, propagation, and expression of the genome. Identification and isolation of proteins interacting with origins of replication are essential for understanding the mechanism of initiation of DNA replication. In *S. cerevisiae*, the Ku-like protein (OBF2) was shown to be required for the assembly of a stable multiprotein complex at essential sequences within the eukaryotic origin of replication (Shakibai *et al.*, 1996). Ku is an abundant heterodimeric nuclear protein, composed of ~70- and ~86-kDa subunits, originally identified as an autoantigen recognized by sera from patients with autoimmune diseases (Mimori *et al.*, 1981). Furthermore, Ku is the regulatory subunit of the DNA-dependent serine/threonine protein kinase (DNA-PK) (Carter *et al.*, 1990), and acts as the component of DNA-PK that confers binding to DNA (Dvir *et al.*, 1992). Ku is present in all eukaryotes, suggesting conservation of function. This mul-

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tifunctional protein has been implicated in many cellular metabolic processes, such as nonhomologous DNA double-strand break repair, site-specific V(D)J recombination of immunoglobulins and T-cell receptor genes, transcriptional regulation, telomeric maintenance, replicative senescence, cell cycle regulation, and DNA replication (Ruiz *et al.*, 1999, and references therein; reviewed in Tuteja *et al.*, 2000). Maintenance of the genome's integrity has been suggested to be accomplished by the Ku80 caretaker gene, through suppression of chromosomal rearrangements (Difilippantonio *et al.*, 2000). Most recently, Pucci *et al.* (2001) proposed a differential DNA-binding activity of Ku in human neoplastic tissues that might be associated with tumor progression. Ku is not only a double-stranded DNA end-binding protein but also has sequence-specific DNA binding (Griffith *et al.*, 1992; Ruiz *et al.*, 1999), ATPase (Ochem *et al.*, 1997), and helicase activities (Tuteja *et al.*, 1990, 1993, 1994). The role of Ku in cell cycle regulation has been largely investigated in the past decade. Both Ku70 and Ku80 (or Ku86) subunits are coexpressed in human cell lines throughout the cell cycle. The catalytic subunit of DNA-PK (DNA-PKcs) is also present in the nucleus in interphase cells, but unlike Ku, none of DNA-PKcs was localized at the periphery of condensed chromosomes during mitosis (Koike *et al.*, 1999). These data along with knockout data of Ku70, Ku86 and DNA-PKcs (Gao *et al.*, 1998) suggest that there is an important function of Ku in growth control, which is separate from the DNA-PK activity. Furthermore, a role for Ku in tumor suppression, has been suggested (Nussenzweig *et al.*, 1997; Li *et al.*, 1998), because Ku70 and Ku80 deficiencies facilitated neoplastic growth.

Evidence involving Ku in DNA replication is accumulating. Ku has been shown to associate with several origins of replication, such as the adenovirus type 2 origin (de Vries *et al.*, 1989), B48 human DNA, lamin B2 region (Toth *et al.*, 1993), A3/4 sequence present in the minimal origin of the monkey *ors8* and *ors12* (Ruiz *et al.*, 1999; our unpublished results), the Chinese hamster dihydrofolate reductase origin *oriβ* (Ruiz *et al.*, 1999), and the human *dnmt1* (DNA-methlytransferase) origin (Araujo *et al.*, 1999). Recently, Ku was found to bind to matrix attachment regions, which are implicated in the loop domain organization of chromatin (Galande and Kohwi-Shigematsu, 2000). Matrix attachment regions have been shown to colocalize with origins of replication (Largarkova *et al.*, 1998).

Our laboratory has purified an origin binding activity (OBA) (Ruiz *et al.*, 1995) through its ability to interact specifically with *ors8*, a mammalian (monkey) origin of replication. OBA binds specifically to A3/4 (Ruiz *et al.*, 1999), a 36-bp mammalian replication origin sequence that is capable of supporting autonomous replication in vivo and in vitro (our unpublished results). Furthermore, OBA has helicase activity and associates with proteins involved in DNA replication (our unpublished results), such as PCNA, DNA polymerases δ and ϵ, topoisomerase II, RF-C, and Oct-1. Microsequencing analysis of the DNA binding activity of OBA revealed that it was identical to the 86-kDa subunit of Ku antigen (Ruiz *et al.*, 1999). In addition, the affinity-purified OBA fraction contained the 70-kDa subunit of Ku and DNA-PKcs. Furthermore, our laboratory has previously isolated origin-enriched sequences, *ors*, from early-replicating CV-1 monkey cells (Kaufmann *et al.*, 1985; reviewed in Zannis-Hadjopoulos and Price, 1998,1999), which are capable of conferring autonomous replication to plasmids in vivo (Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991) and in vitro (Pearson *et al.*, 1991). In addition, in vivo mapping of *ors12* by competitive PCR demonstrated that it acts as a chromosomal origin of DNA replication (Pelletier *et al.*, 1999). Among the *ors*, *ors8* and *ors12* have been characterized in detail. They both contain an internal minimal origin fragment, 186 bp for *ors8* (Todd *et al.*, 1995) and 215 bp for *ors12* (Pelletier *et al.*, 1997), AT-rich regions, inverted repeats, bent DNA, the ARS consensus sequence of yeast, the consensus for scaffold attachment regions of *Drosophila*, and various eukaryotic transcriptional regulatory elements (Rao *et al.*, 1990). These sequences and structural features have been associated with origins of replication (reviewed in Zannis-Hadjopoulos and Price, 1998, 1999).

In the present study, we quantitated throughout the cell cycle, the in vivo binding of Ku to replication origin-containing sequences (*ors8* and *ors12*), with the use of the formaldehyde cross-linking technique (Strahl-Bolsinger *et al.*, 1997). Immunoprecipitation of Ku-DNA cross-links was performed with antibodies against the 70- and 86-kDa subunits of Ku antigen and against the Ku70/86 heterodimer. Conventional, competitive, and real-time PCR were then performed with the use of the immunoprecipitated material as template. Ku was found to associate specifically with *ors8* and *ors12*, because DNA fragments from these regions were enriched in the immunoprecipitate compared with other portions of the genome not containing replication origins. Furthermore, higher binding of Ku to *ors8* and *ors12* was found at the G1/S border, in comparison with other stages of the cell cycle. The data suggest an involvement of Ku in mammalian DNA replication as an origin-binding protein.

Experimental Procedures

Cell Culture and Synchronization

CV-1 cells (monolayers) were cultured in minimal essential medium α (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) (termed regular medium) at 37°C, as previously described (Mah *et al.*, 1993). For synchronization to the G0/G1 phase, 80% confluent CV-1 cells were placed in serum-free medium for 48 h. For synchronization to G1/S, S (Stephens *et al.*, 1977), and M (Paulson and Taylor, 1982) phases, the procedure was modified as follows: 40% confluent CV-1 cells were treated with 2 mM thymidine (Sigma, St. Louis, MO) for 12 h, released for 9 h in regular medium without thymidine, and subsequently incubated for 12 h with 2 mM thymidine and 400 μM mimosine (Sigma). For S phase synchronization the cells were released, from the thymidine/mimosine block, for 2 h in regular medium. For synchronization to M phase the cells were released from the thymidine/mimosine block in regular medium supplemented with 1 μg/ml nocodazole (Sigma), for 14 h. Cell synchronization was monitored by flow cytometry. Mouse embryonic fibroblasts (MEFs) $Ku80^{+/+}$ and $Ku80^{-/-}$ cells (kindly provided by Dr. A. Nussenzweig), were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) at 37°C, as described in Nussenzweig *et al.* (1996).

In Vivo Cross-linking

In vivo cross-linking was performed as described in Ritzi *et al.* (1998) with some modifications. In brief, CV-1 cells, $Ku80^{+/+}$ and $Ku80^{-/-}$ MEFs, grown as described above, were washed twice with phosphate-buffered saline to remove all traces of serum and then formaldehyde (1%) in warm minimal essential medium α without serum was added for 10 min. Cells were then lysed (at 4°C) in lysis buffer (50 mM HEPES/KOH pH 7.5, 140 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, one capsule of protease inhibitors; Roche Molecular Biochemicals) and drawn into

and out of a 21-gauge hypodermic needle three times to effect cell lysis and dispersion of nuclei. Cell lysates were then layered over 4 ml of 12.5% glycerol in lysis buffer and nuclei were pelleted by spinning at 750 × *g* for 5 min in a benchtop centrifuge. The nuclear pellet was resuspended in 1 ml of lysis buffer.

Chromatin Fragmentation

Cross-linked or noncross-linked cell nuclei were sonicated 10 times for 30 s each time, and the chromatin size was monitored by electrophoresis (Hecht and Grunstein, 1999). This treatment generated fragments of ~20 kb. To further reduce the chromatin size into smaller fragments of 1.5 to 3.5 kb, DNA was then digested with *Sph*I, *Hin*dIII, *Pst*I, and *Eco*RI restriction endonucleases in NEB2 buffer (100 U of each; New England Biolabs, Beverly, MA) at 37°C for 6 h.

Immunoprecipitation and DNA Isolation

Sheared chromatin lysed extracts were incubated with 50 μl of protein G-agarose (Roche Molecular Biochemicals), to reduce background caused by nonspecific adsorption of irrelevant cellular proteins/DNA to protein G-agarose beads (as described in the protein G-agarose protocol). These cleared chromatin lysates were incubated, at 4°C for 6 h on a rocker platform, with either 50 μl of preimmune goat serum (Santa Cruz Biotechnology, Santa Cruz, CA), or 20 μg of anti-Ku70 (M-19) or anti-Ku86 (C-20) goat polyclonal antibodies (Santa Cruz Biotechnology), or anti-Ku70/86 heterodimer (clone162) mouse monoclonal antibody (NeoMarker), or anti-NF-κB p65 (C-20) goat polyclonal antibody (Santa Cruz Biotechnology) directed against the transcription factor nuclear factor-κB (NF-κB) p65, or anti-SC35 (Sigma) rabbit monoclonal antibody against the splicing factor SC-35. Protein G-agarose (50 μl) was then added and the incubation was continued for 12 h. The precipitates were successively washed two times for 5 min with 1 ml of each buffer: lysis buffer, WB1 (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.1% Nonidet P-40, 0.05% sodium deoxycholate), WB2 (as WB1 with no NaCl), and 1 ml of TE (20 mM Tris-HCl pH 8.0, 1 mm EDTA). The precipitates were finally resuspended in 200 μl of extraction buffer (1% SDS/TE). Half of the sample was then incubated at 65°C overnight to reverse the protein/DNA cross-links, followed by 2-h incubation at 37°C with 100 μg of proteinase K (Roche Molecular Biochemicals). The other half (nonreversed cross-link) was incubated at 50°C for 1 h with 100 μg of proteinase K. Finally, the samples were processed for DNA purification by passing them through QIAquick PCR purification columns (QIAGEN, Valencia, CA).

Blocking of Anti-Ku Antibodies with Ku70 and Ku86 Blocking Peptides

The anti-Ku70 and anti-Ku86 antibodies were neutralized with a sevenfold (by weight) excess of the Ku70 (sc-1486 P; Santa Cruz Biotechnology) or the Ku86 (sc-1484 P; Santa Cruz Biotechnology) blocking peptides, as previously described (Ruiz *et al*., 1999). The incubations were carried out overnight at 4°C, and the neutralized antibodies were then incubated with extracts of cross-linked CV-1 cells, as described above.

Western Blotting

Immunoprecipitates were resuspended in electrophoresis sample buffer (50 mM Tris-HCl pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and resolved on 8% SDS-polyacrylamide gels, transferred to Immobilon-P membranes (Millipore, Bedford, MA) and probed with anti-Ku70, anti-Ku86, anti-NF-κB p65, or anti-SC35 antibodies. Protein–antibody complexes were visualized by enhanced chemiluminescence with the use of the Amersham Pharmacia Biotech ECL system (Arlington Heights, IL), with the appropriate horseradish peroxidase-labeled conjugated antibodies (Santa Cruz Biotechnology).

PCR Analysis of Immunoprecipitated DNA

Conventional PCR reactions were carried out in 25 μl with 1/200th of the immunoprecipitated material with the use of the Ready-To-Go PCR Beads from Amersham Pharmacia Biotech. The PCR reaction contained 1 μM of each primer (for primers sets AF, AC, DF, BE, and ADA A), which were designed as 20- or 24-mers with ~50% GC content. Sequence for the CV-1 *ors*8 (accession no. M26221) and mouse genomic adenosine deaminase (ADA; accession no. L20424) amplicon primers used:

Primer	Sequence	$T_{ANNEALING}$ (°C)
*ors*8 A	5′GCTGAAGCATTTGCACTTCA3′	55
*ors*8 B	5′TTGCACTTCACTGAGCAGTCAT3′	
*ors*8 C	5′CATCTCCACTATAGCCATAT3′	55
*ors*8 D	5′CTACCATGCCTAATGCAAAA3′	
*ors*8 E	5′GACCCATAAAGGCAAAAGTACC3′	55
*ors*8 F	5′GCTTTCAGAGACGCCCCTGAAA3′	
ADA AF	5′CTGAGACTATCCTCCAGGTCTTCT3′	50
ADA AR	5′CATGGCTGCCTATGACCAACAGAA3′	

Genomic CV-1, $Ku80^{+/+}$, or $Ku80^{-/-}$ DNA (10 ng), used for the control reactions, was obtained from total cell lysates of noncross-linked cells. Typically, an initial denaturation for 2 min at 94°C was followed by 30 cycles with denaturation for 30 s at 94°C, annealing for 30 s at 55 or 50°C, polymerization for 30 s at 72°C, and a final extension for 5 min at 72°C. PCR products were separated on 2% agarose gels, visualized with ethidium bromide, and photographed with an Eagle Eye apparatus (Speed Light/BT Sciencetech-LT1000).

Competitive PCR Analysis of Immunoprecipitated DNA

The primers, competitors and PCR conditions used are described below.

Primer	Sequence	Product Size	$T_{ANNEALING}$ (°C)
*ors*8 11	5′TTGCACTTCACTGAGCAGTCAT3′	Genomic: 320 bp	55
*ors*8 330	5′GACCCATAAAGGCAAAAGTACC3′	Competitor: 267 bp	
B	5′CCCTTGATTAATGGTTGCTT3′	Genomic: 463 bp	60
B′	5′GCTGGTGGGGAATGTTAATG3′	Competitor: 400 bp	
E	5′GGAATTCTGTCTTAGGCAAT3′	Genomic: 250 bp	55
E′	5′TGATATTGCCAATCAGGATC3′	Competitor: 195 bp	

PCR reactions were performed with Ready-To-Go PCR Beads (Amersham Pharmacia Biotech), and primer concentrations were as for conventional PCR (see above). The difference lies in the addition of the appropriate competitor molecules to the reaction mixture. Each competitor was generated with the use of a third primer, as described by Forster (1994). *Ors*8 competitor (*ors*8c) was generated with primers *ors8* 330 and 8C (5′TGAGCAGTCATGAAGAAACCTAACTGAGATG). BB′ and EE′ competitors were generated as described in Pelletier *et al.* (1999).

Real-time PCR Quantification Analysis of Immunoprecipitated DNA

PCR reactions were carried out in 20 μl with 1/200th of the immunoprecipitated material with the use of LightCycler capillaries (Roche Molecular Biochemicals) and the LightCycler-FastStart DNA Master SYBR Green I (Roche Molecular Biochemicals). The PCR reaction contained 3 mM Mg^{2+} and 1 μM of each primer of the appropriate primers set used; *ors*8 150, *ors*12 JJ′, *ors*12 MM′, EE′, BRCA, or CD4 intron. Because the optimal conditions for real-time PCR sometimes requires specific primer sets that differ from those with the use of conventional PCR, primer set *ors*8 150 was used to amplify a 150-bp genomic fragment of *ors8* (Figure 3A). Primer set *ors*12 JJ′ and *ors*12 MM′ were used to amplify a 360- or a 303-bp corresponding genomic fragment of *ors*12 (Figure 3B). Primer set EE′ was used to amplify a 250-bp genomic fragment, which was mapped ~5 kbp downstream of the origin of replication *ors*12 (Figure 3B). A control set of primers from the African Green Monkey BRCA2 gene (accession no. Z75666; Bignell, Micklem, Stratton, Ashworth, and Wooster, unpublished data; Pelletier *et al.*, 1999) and the CV-1 CD4 gene (accession no. AB052204; Matsunaga *et al.*, 2000) were also used. Primer sets BRCA and CD4 intron amplify a fragment of 459 and 258 bp, respectively, from genomic CV-1 DNA. Primers were designed as 20–22 mers with ~50% GC content. Sequence for the primers used was as follows:

Primer	Sequence	$T_{ANNEALING}$ (°C)
*ors*8 150F	5′-GACCCATAAAGGCAAAAGTACC-3′	45
*ors*8 150R	5′-GGAAGATATTAAGATAGATGG-3′	
*ors*12 J	5′-CAGACATCAGCAAGTGACGG-3′	50
*ors*12 J′	5′-TAGCCAATCTGCCCAATGTA-3′	
*ors*12 M′	5′-CATTCGTTCATCCATGTCTCC-3′	50
*ors*12 M′	5′-GTGAATGAGGCAGTTTGAGGA-3′	
E	5′-GGAATTCTGTCTTAGGCAAT3′	50
E′	5′-TGATATTGCCAATCAGGATC3′	
BRCA F	5′-GATCACAACTGCCCCAAAGT-3′	50
BRCA R	5′-TGTTGTTTTTCGGAGGGATG-3′	
CD4 intron F	5′-AGCTCTGTTCTGTATCTTTG-3′	50
CD4 intron R	5′-CCACAGGCACTTTTATCTTC-3′	

Genomic CV-1 DNA (9.3, 18.6, 27.9, 37.2, and 55.8 ng), used for the standard curve reactions (necessary for quantification of the PCR products) (Figure 5A), was obtained from total cell lysates of noncross-linked logarithmic 80% confluent cells. The quantification of the PCR products was assessed by the LightCycler (Roche Molecular Biochemicals), with the use of SYBR Green I dye as detection format (Pfitzner *et al.*, 2000). The quantification program used a single fluorescence reading at the end of each elongation step. Arithmetic background subtraction was used and the fluorescence channel was set to F1. Typically, an initial denaturation for 10 min at 95°C was followed by 35 cycles with denaturation for 15 s at 95°C, annealing for 10 s at 45°C (primer set *ors8* 150) or 50°C (primer sets *ors*12 JJ′, *ors*12 MM′, EE′, BRCA, or CD4 intron), and polymerization for 15 s at 72°C. The specificity of the amplified PCR products was assessed by performing a melting curve analysis cycle with a first segment set at 95°C for 0 s and a temperature transition of 20°C/s, a second segment set at 45°C or 50°C (depending on the annealing temperature of primer set used) with a temperature transition rate of 20°C/s, and a third segment set at 95°C with a temperature transition rate set at 0.2°C/s. PCR products were also separated on 2% agarose gels, visualized with ethidium bromide, and photographed with an Eagle Eye apparatus (Speed Light/BT Sciencetech-LT1000) (our unpublished results).

In Vitro Mammalian DNA Replication Assay

$Ku80^{+/+}$ and $Ku80^{-/-}$ MEFs nuclear and cytosolic extracts were prepared as previously described (Pearson *et al.*, 1991), from logarithmically growing cell monolayers. The protein concentrations of the nuclear and cytoplasmic extracts were 3.0 and 3.5 mg/ml, respectively. In vitro replication was performed as previously described (Matheos *et al.*, 1998), with slight modifications. Standard reactions included cytoplasmic (52.5 μg) and nuclear (21.0 μg) extracts from either $Ku80^{+/+}$ or $Ku80^{-/-}$ cells, 2 mM ATP, 100 mM each CTP, GTP, UTP, dATP, and dGTP, 10 μCi each of [α-^{32}P]dCTP and [α-^{32}P]dTTP, 2 U of pyruvate kinase, and 200 ng of input p186 plasmid (Todd *et al.*, 1995). A control reaction with pBR322, a plasmid lacking a mammalian origin of DNA replication, was also included to show origin-dependent DNA replication of the p186 plasmid. The reactions were performed at 30°C for 1 h. The reaction products were purified with the use of the QIAquick PCR purification kit (QIAGEN). Samples were digested with 0.8 U of *Dpn*I (New England Biolabs) for 45 min at 37°C in the presence of 1× NEB 4 buffer and 100 mM NaCl. The samples were separated on 1% agarose gel in 1× TAE buffer (16–20 h, 50–55 V).

Quantification was performed on *Dpn*I-digested products with the use of a Fuji BAS2000 phosphorimager analyzer. These results were typically corrected for the amount of DNA recovered from the in vitro replication assay by quantitative analysis of the ethidium bromide picture of the gel (not shown). This method of quantification for DNA recovery was also verified by quantifying and correcting for the amount of radionucleotide incorporated in unmethylated pBluescript DNA, included in each reaction (not shown). The amount of radioactive precursor incorporated into the DNA was expressed as a percentage of the wild-type reaction with $Ku80^{+/+}$ cell extracts.

RESULTS

Immunoprecipitation of Ku70, Ku86, SC-35, and NF-κB p65 Proteins from Lysed Cell Extracts

The Ku heterodimer as well as its Ku70- and Ku86-kDa subunits were separately immunoprecipitated, with anti-clone162, anti-Ku70, or anti-Ku86 antibodies, respectively, from extracts of monkey (CV-1) cells that had been previously treated or not with formaldehyde, to cross-link proteins bound to DNA in vivo. As negative control, antibodies against the spliceosome-specific protein, SC-35, a nuclear protein that does not bind to DNA (Fu and Maniatis, 1990), or the transcription factor NF-κB p65, a nuclear protein that binds DNA but does not associate with origins of DNA replication (Meyer *et al.* 1991), were used. Western blot analyses showed that CV-1 whole-cell-extracts (CV-1 WCE), prepared from either cross-linked or noncross-linked cells, contained all three proteins, Ku, SC-35, and NF-κB p65 proteins (Figure 1, A–E, lanes 1 and 2). In contrast, when normal goat serum (NGS) was used, neither Ku, NF-κB p65, nor SC-35 was immunoprecipitated in either the cross-linked

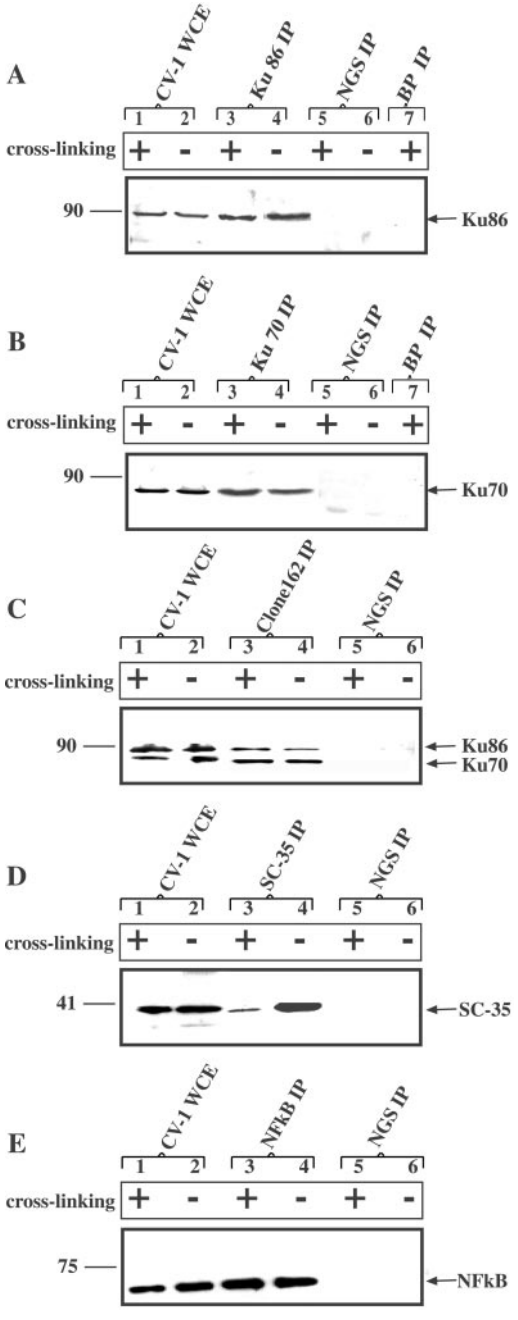

Figure 1. Immunoprecipitation assay showing that Ku, SC-35, and NF-κB p65 are present in both formaldehyde cross-linked or untreated cells. Western blots (as described in EXPERIMENTAL PROCEDURES) were probed with 1/100th dilution of anti-Ku 86 (A), 1/400th dilution of anti-Ku 70 (B), 1/100th dilution of anti-Ku 86 plus 1/400th dilution of anti-Ku 70 (C), 1/1000th dilution of anti-SC-35 (D), and 1/100th dilution of anti-NF-κB p65 (E). Lanes 1 and 2, 50 μg of cross-linked or not CV-1 WCE; lanes 3 and 4, 1/20th of immunoprecipitated Ku86, Ku70, SC-35, or NF-κB p65 material from cross-linked or untreated cells; and lanes 5 and 6, 1/20th of immunoprecipitated normal goat serum material from cross-linked or untreated cells. Lane 7, 1/20th of immunoprecipitated Ku86 or Ku70 material from cross-linked cells that was obtained after the pretreatment of the anti-Ku70 and anti-Ku86 antibodies with the corresponding blocking peptide.

or untreated cells (Figure 1, A–E, lanes 5 and 6). Furthermore, Western blot analyses with the use of anti-Ku70 and anti-Ku86 antibodies verified that the immunoprecipitated material from either the cross-linked or the untreated cells did contain Ku protein (Figure 1, A–C, lanes 3 and 4). Western blot analyses performed with anti-SC-35 antibody showed that the material immunoprecipitated from cross-linked cells contained ~10 times less SC-35 protein than the untreated ones (Figure 1D, lanes 3 and 4), indicating some nonspecific precipitation of this protein, whereas similar analyses performed with the anti-NF-κB p65 antibody showed that the material immunoprecipitated from either the cross-linked or untreated cells (Figure 1E, lanes 3 and 4) contained equivalent amount of NF-κB p65 protein. The specificity of the anti-Ku70 and anti-Ku86 antibodies used was assayed by blocking with the corresponding Ku70 and Ku86 peptides. Western blot analyses showed that neither the Ku70 nor the Ku86 subunits of Ku protein were immunoprecipitated from cross-linked CV-1 cells, when the Ku antibodies were treated with the respective blocking peptide before immunoprecipitation (Figure 1, A and B, lane 7).

Ku70 and Ku86 Association with ors *8 and* ors *12 Detected by Formaldehyde Cross-linking*

The abundance of *ors8*- and *ors12*-containing genomic sequences bound to Ku protein, after formaldehyde cross-linking and immunoprecipitation, was measured by PCR. Four sets of primers, AC, DF, BE, and AF, were used to amplify four respective regions in *ors8* (Figure 2A). When the immunoprecipitated protein-DNA cross-links were reversed, by incubating at 65°C overnight, all four regions of *ors8* were amplified, giving the expected 197-, 212-, 320-, and 480-bp products, respectively (Figure 2B, lanes 1 and 3). In contrast, the immunoprecipitated material from the noncross-linked cells did not result in PCR amplification of any of the four *ors8* fragments (Figure 2B, lanes 2 and 4), indicating first that cross-linking was required before immunoprecipitation with anti-Ku antibodies, and second that the material that was immunoprecipitated with these antibodies from the noncross-linked cells did not contain detectable amounts of contaminating DNA, with the use of either the same amount of template as from the cross-linked cells, or 10 times more (our unpublished results) for the PCR reaction. The genomic DNA of noncross-linked cells gave the expected amplification products with the corresponding primer sets (Figure 2, B and C, lane 5). A similar procedure was used with the monkey *ors12* (Kaufmann *et al.*, 1985), human *c-myc* (Vassilev and Johnson, 1990) and human *dnmt1* (Araujo *et al.*, 1999) origins of replication and specific origin-containing fragments from each locus were similarly amplified (our unpublished results).

PCR Amplification across Ku Binding Site in ors8 *Is Blocked If Cross-link Is not Reversed before PCR*

When proteinase K was added without reversing the protein-DNA formaldehyde cross-link, the 480- and 320-bp *ors8* fragments were not amplified, whereas the 197- and 212-bp fragments were (Figure 2C, lanes 1–4). The region containing the OBA/Ku binding site of *ors8* (Ruiz *et al.*, 1999), located within the 186-bp minimal *ori*, was not part of the amplified fragments when primer sets AC (197-bp

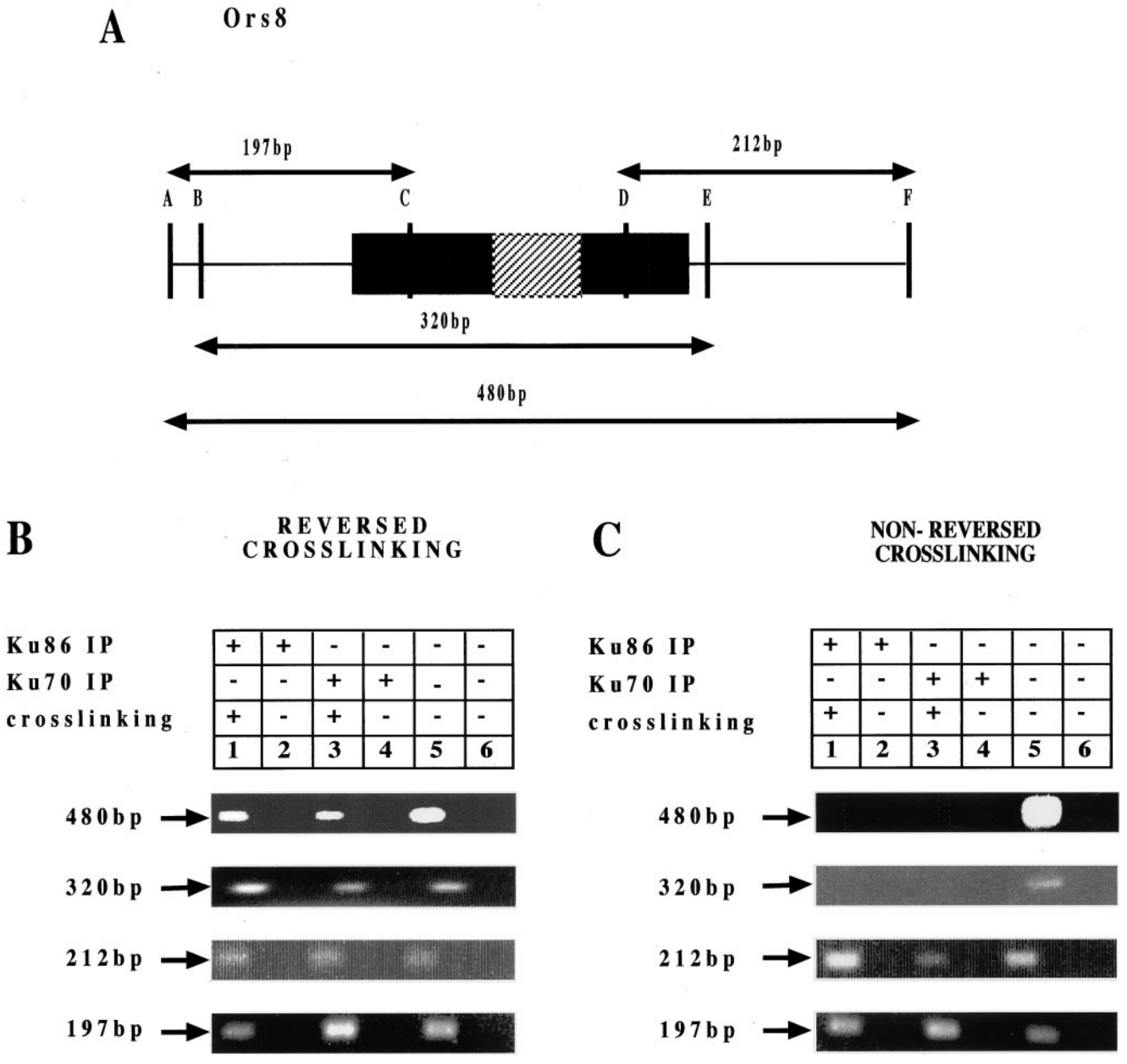

Figure 2. Ku association with *ors8* and *ors12* origins shown by PCR amplification. (A) Map of *ors8*, including location of primers A–F and their amplification products. The box represents the 186-bp minimal origin of *ors*8 and the hatched portion of the box represents the 59-bp Ku binding site that contains the A3/4 sequence homologous sequence. (B) PCR amplification products 197, 212, 320, or 480 bp of *ors8* with the use of primer sets AC, DF, BE, or AF, respectively. Template DNA used the following: lanes 1 and 2, reversed cross-linked or not cross-linked Ku86 immunoprecipitate; lanes 3 and 4, reversed cross-linked or not cross-linked Ku70 immunoprecipitate; lane 5, CV-1 total genomic DNA from untreated cells; and lane 6, negative control to verify primer contamination; no template DNA added to PCR reaction. (C) As for B, but cross-links were not reversed.

product) and DF (212-bp) were used (Figure 2A). Most likely, as a result of the cross-linking, an adduct-like structure may have been left within the Ku binding region that inhibited the amplification of the 480- and 320-bp fragments. Amplification of these fragments containing Ku binding sites was possible with total genomic DNA from noncross-linked cells (Figure 2, B and C).

Competitive PCR with DNA Immunoprecipitated with Anti-Ku70, Anti-Ku86, Anti-SC-35, and NGS

To analyze whether the DNA that was immunoprecipitated with the anti-Ku antibodies after cross-linking with formaldehyde was enriched in origin-containing sequences, and to quantify this association, competitive PCR was performed,

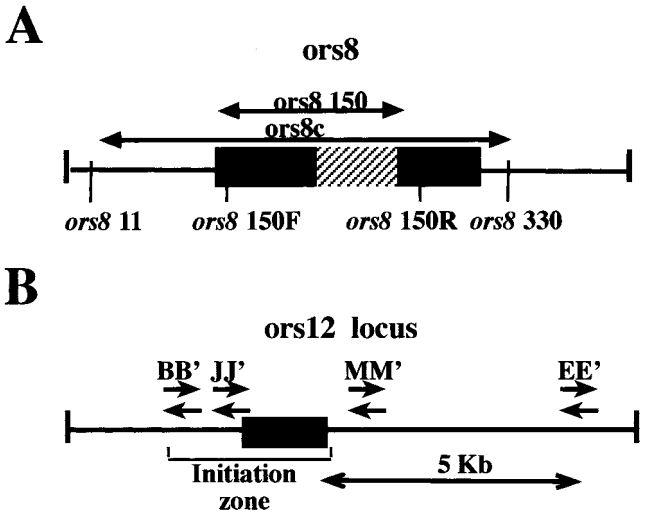

Figure 3. Map of *ors8* origin and *ors12* locus in CV-1 cells. (A) *Ors*8 origin showing location of expected target amplification products of *ors*8, generated by primer set *ors*8c and *ors*8 150 (Figure 2A, legend) The black box represents the 186-bp minimal origin of *ors*8. (B) *Ors*12 locus showing location of expected target amplification products of *ors*12, generated by primer set BB′, JJ′, MM′, or EE′. Primer set EE′ amplifies a fragment located 5 kb from *ors*12. The black box represents the 215-bp minimal origin of *ors*12.

with the use of specific primers of *ors8* and *ors12*. This was compared with DNA obtained by immunoprecipitation with an anti-SC-35 antibody or NGS, both used as negative controls (see competitive PCR raw data, Figure 4B). Competitive PCR was also used to standardize the differences among primers and competitors with respect to their amplification efficiencies. CV-1 genomic DNA, obtained from different regions that are either containing replication origins or not, was used to normalize the reaction products (Figure 3, A and B). The linearity of each competitor was verified by plotting the ratio of competitor DNA product to target DNA product (ordinate) versus the number of competitor molecules used (abscissa) (Figure 4A). In logarithmically growing CV-1 cells, the immunoprecipitated DNA obtained with either anti-Ku86 or anti-Ku70 antibodies was enriched in *ors8* sequence by approximately fivefold, in comparison with anti-SC-35–immunoprecipitated DNA (Figure 4C, *ors*8c). Similar results were obtained with *ors12* sequence, where DNA that was immunoprecipitated with either anti-Ku86 or anti-Ku70 antibodies was enriched in origin sequence by approximately six- and fivefold, respectively, in comparison with anti-SC-35–immunoprecipitated DNA (Figure 4C, *ors*12 BB′). When NGS was used, *ors8* and *ors12* sequences were amplified by approximately eight- and sixfold less, respectively, than when DNA was immunoprecipitated with the use of anti-Ku86 and anti-Ku70 (Figure 4C). In contrast, a sequence situated ~5 kb downstream of *ors12* was amplified by primer set EE′ by approximately fourfold less than the sequence amplified by primer set BB′, which contains *ors12* when anti-Ku70 or anti-Ku80 antibodies were used for the immunoprecipitation (Figure 4C, *ors*12 EE′). In addition, the DNA abundance in the region amplified by primer set EE′, corresponded to $\sim 3.0 \times 10^4$–4.5×10^4 molecules, when the immunoprecipitation was performed with either Ku antibodies, anti-SC-35 antibody, or NGS (Figure 4C, *ors*12 EE′).

Real-time PCR with DNA Immunoprecipitated with Anti-Clone162, anti-NF-κB p65, and NGS

The association of Ku heterodimer (immunoprecipitated with anti-clone162 antibody), NF-κB p65, and NGS with origin-containing sequences *ors*8 and *ors*12 and nonorigin-containing sequences EE′, BRCA, and CD4 intron was assayed by the real-time PCR quantification method with the use of the LightCycler (Roche Molecular Biochemicals). Genomic CV-1 DNA was used to build the standard curves necessary for the quantification of the immunoprecipitated DNA in different genomic regions (Figure 5A). In agreement with the results obtained with the use of the competitive PCR quantification methodology (see above), the association of Ku with *ors*8 and *ors*12 in logarithmically growing CV-1 cross-linked cells was approximately 3- and 4-fold higher, respectively, than that of NF-κB p65, and 3.5-fold and 5-fold higher, respectively, than NGS (Figure 5B, *ors*8 150, *ors*12 JJ′, *ors*12 MM). In comparison, the association of Ku with three genomic regions that do not contain an origin of DNA replication, EE′, BRCA, and CD4 was lower: the region amplified by primer sets EE′ was ~3.5-fold lower and those amplified by primer sets BRCA and CD4 intron were ~5-fold lower, respectively, than with the *ors*8 and *ors*12 origin-containing regions (Figure 5B). Finally, the amount of DNA immunoprecipitated with anti-NF-κB p65 and NGS in origin-containing regions was similar to that in nonorigin-containing regions (Figure 5B).

Cell Cycle-dependent Association of Ku with Origins of Replication

Competitive PCR was also used to quantitatively assess whether Ku associated with replication origins as a function of the cell cycle. CV-1 cells were synchronized to G0, G1/S, S, and M phase (see EXPERIMENTAL PROCEDURES) and synchronization was monitored by fluorescence-activated cell sorting analysis (Figure 6A).

The association of Ku with *ors8* and *ors12* was the highest at the G1/S boundary, decreased by approximately twofold at the start of S phase, remained low at G2/M, by decreasing approximately another twofold, and reached background levels in serum-starved G0 cells (Figure 6B). Background was considered to be the DNA that was brought down nonspecifically by anti-SC35 antibody (estimated as $\sim 2.2 \times 10^4$–4×10^4 molecules/1.5×10^7 cross-linked CV-1 cells), presumably as a result of the cross-linking with SC-35, a protein that does not bind to DNA (Figure 1D). If the association of Ku with *ors8* and *ors12* was set at 100% (Figure 4C), the background was determined to be 15% (Figure 6B).

The amount of Ku present in the different phases of the cell cycle was also analyzed, by Western blotting analyses and no significant differences were found (our unpublished results), in agreement with previous observations by Koike *et al.* (1999). Similarly, Western blot analyses showed that approximately similar amounts of Ku70 and Ku86 immunoprecipitated at each cell cycle stage, when cells were previously treated with formaldehyde (Figure 6C).

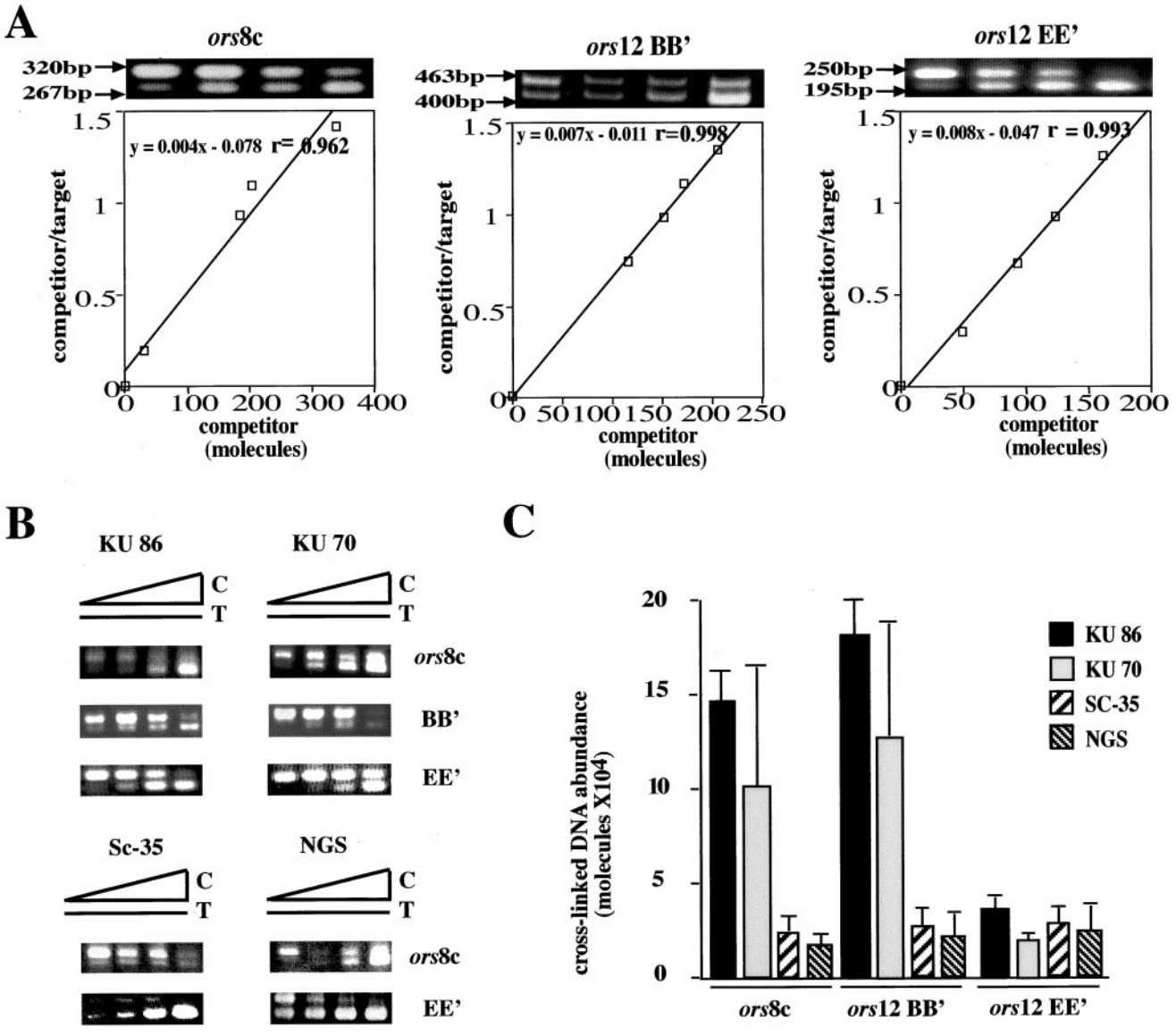

Figure 4. Competitive PCR with logarithmically growing CV-1 cells shows that Ku associates with origins of replication. (A) Competitive PCR (competitor *ors*8c, BB′, or EE′) with increasing number of competitor molecules (from left to right) and constant number of CV-1 genomic DNA template molecules, showing linearity of competitors used. (B) Raw competitive PCR data (with the use of increasing amounts of competitor *ors*8c, BB′, or EE′) and constant amount of template DNA (1/50th of DNA recovered from immunoprecipitate), purified from Ku86, Ku70, SC-35, or NGS cross-linked immunoprecipitates. (C) Normalized total cross-linked DNA molecules detected by competitive PCR, from logarithmically growing CV-1 cells. Products were amplified with primer sets *ors*8c, BB′, or EE′, respectively. The quantification results are the result of at least five competitive PCR reactions with the template genomic DNA isolated from different groups of cross-linked CV-1 cells. Each bar represents five experiments and 1 SD is indicated.

Replication Activity and Ku Immunoprecipitation from Ku80$^{+/+}$ and Ku80$^{-/-}$ MEF Cells

Western blot analyses of Ku80$^{+/+}$ wild-type MEF cells, with the use of anti-Ku70 and anti-Ku86 antibodies, showed that both subunits of the Ku protein were immunoprecipitated with anti-clone162 antibody from logarithmically growing cells that were either cross-linked or not (Figure 7A, lanes 1 and 2). In contrast, neither subunit of Ku was detected when immunoprecipitation of either cross-linked or untreated Ku80$^{-/-}$ cells was performed with either clone162 antibody (Figure 7A, lanes 3 and 4) or anti-Ku86 (our unpublished results).

The abundance of origin-containing genomic sequence bound to Ku protein, after formaldehyde cross-linking and immunoprecipitation, was measured by conventional PCR (35 cycles). Primer set ADA A, which amplifies a 230-bp fragment of the adenosine deaminase amplicon (ADA) (Valerie *et al.*, 1993) was used to verify that genomic DNA from both noncross-linked Ku80$^{+/+}$ and Ku80$^{-/-}$ cells

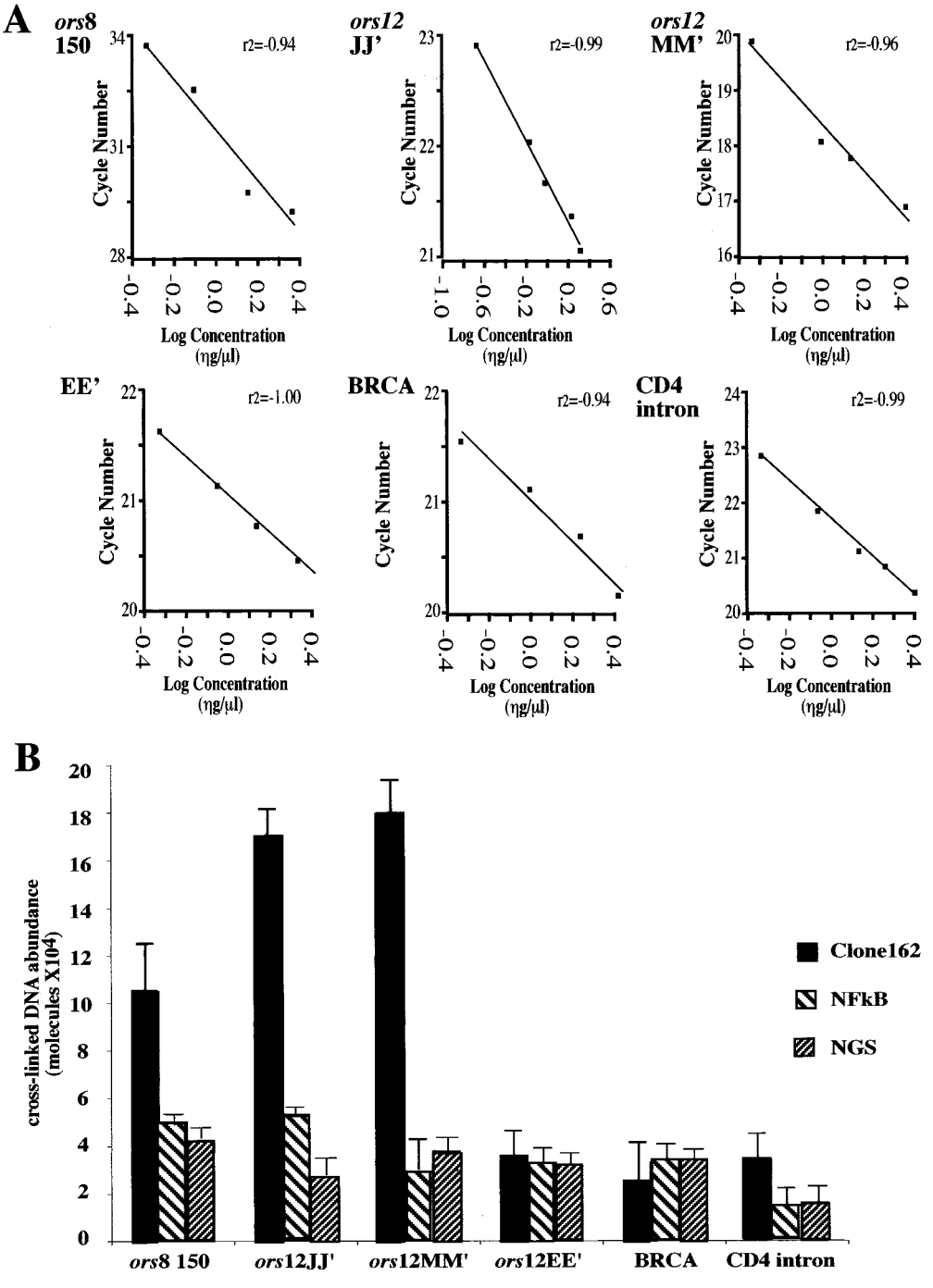

Figure 5. Quantification of DNA abundance in origin-containing sequences and nonorigin-containing sequences by real-time PCR. (A) Standard curves, with the use of genomic CV-1 DNA as template, used in the quantification of the PCR fragments amplified by the respective primer sets *ors*8 150, *ors*12 JJ′, *ors*12 MM′, EE′, BRCA and CD4 intron. (B) Total normalized cross-linked molecules detected by real-time PCR with the use of the LightCycler, from logarithmically growing CV-1 cross-linked Ku, NF-κB p65, and NGS immunoprecipitates, with primer sets for *ors*8 150, *ors*12 JJ′, *ors*12 MM′, EE′, BRCA, and CD4 intron. Each bar represents three experiments and 1 SD is indicated.

gave the expected amplification product (Figure 7B, lanes 1 and 2). A PCR reaction with the use of water instead of template DNA was performed to verify that the primers were free of contaminating DNA (Figure 7B, lane 3).

When the DNA immunoprecipitated by anti-clone162, anti-Ku70, or anti-Ku86 antibodies from cross-linked Ku80$^{+/+}$ cells were used as template DNA for the PCR reaction, the expected 230-bp fragment was amplified by the ADA A

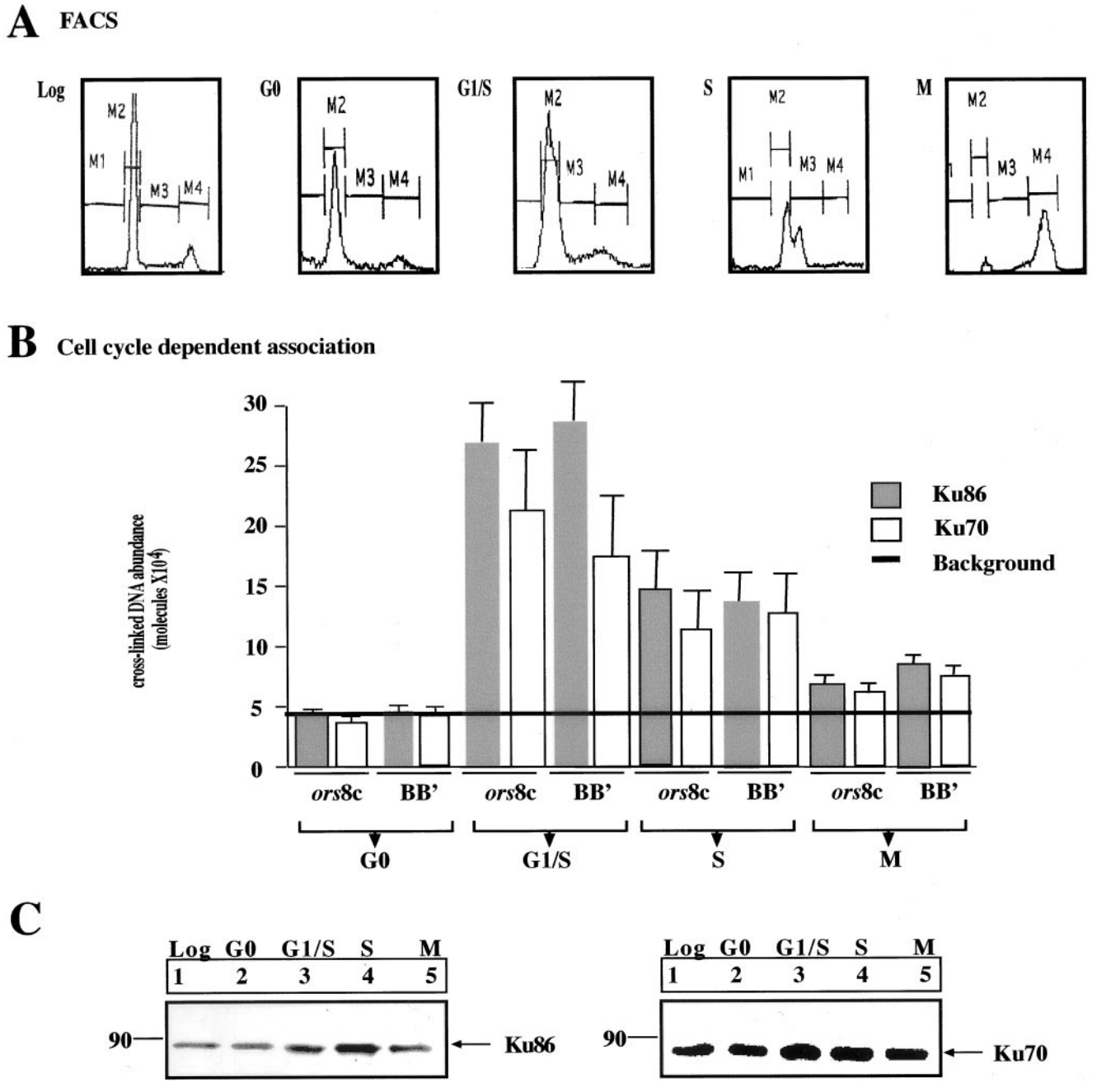

Figure 6. Cell cycle-dependent association of Ku with *ors8* and *ors12*. (A) Fluorescence-activated cell sorting analysis of DNA contained in logarithmically growing or synchronized CV-1 cells at G0, G1/S, S, or M phase of the cell cycle. (B) Total normalized cross-linked molecules detected by competitive PCR, from cross-linked Ku86 or Ku70 immunoprecipitates, at different points in the cell cycle, with primer sets *ors*8c or BB′. The thin black horizontal line represents contaminating DNA background calculated from logarithmically SC-35-immunoprecipitated DNA fragments amplified with *ors*8c or BB′ primers. As for Figure 4, the quantification was obtained from at least five different competitive PCR reactions with the template genomic DNA being from different groups of cross-linked cells. Each bar represents five experiments and 1 SD is indicated. (C) Western blot probed with 1/100th dilution of anti-Ku 86, or1/400th dilution of anti-Ku 70. Lanes 1 and 5, 1/20th of immunoprecipitated Ku86 or Ku70, from log, G0, G1/S, S, and M phases of the cell cycle.

primer set (Figure 7B, lanes 4, 6, and 8), whereas no product was detected when the DNA immunoprecipitated from the Ku80$^{-/-}$ cross-linked cells was used as template (Figure 7B, lanes 5, 7, and 9). When either the Ku80$^{+/+}$ or Ku80$^{-/-}$ cells were not treated with formaldehyde before immunoprecipitation with anti-clone162, anti-Ku70, and anti-Ku86 antibod-

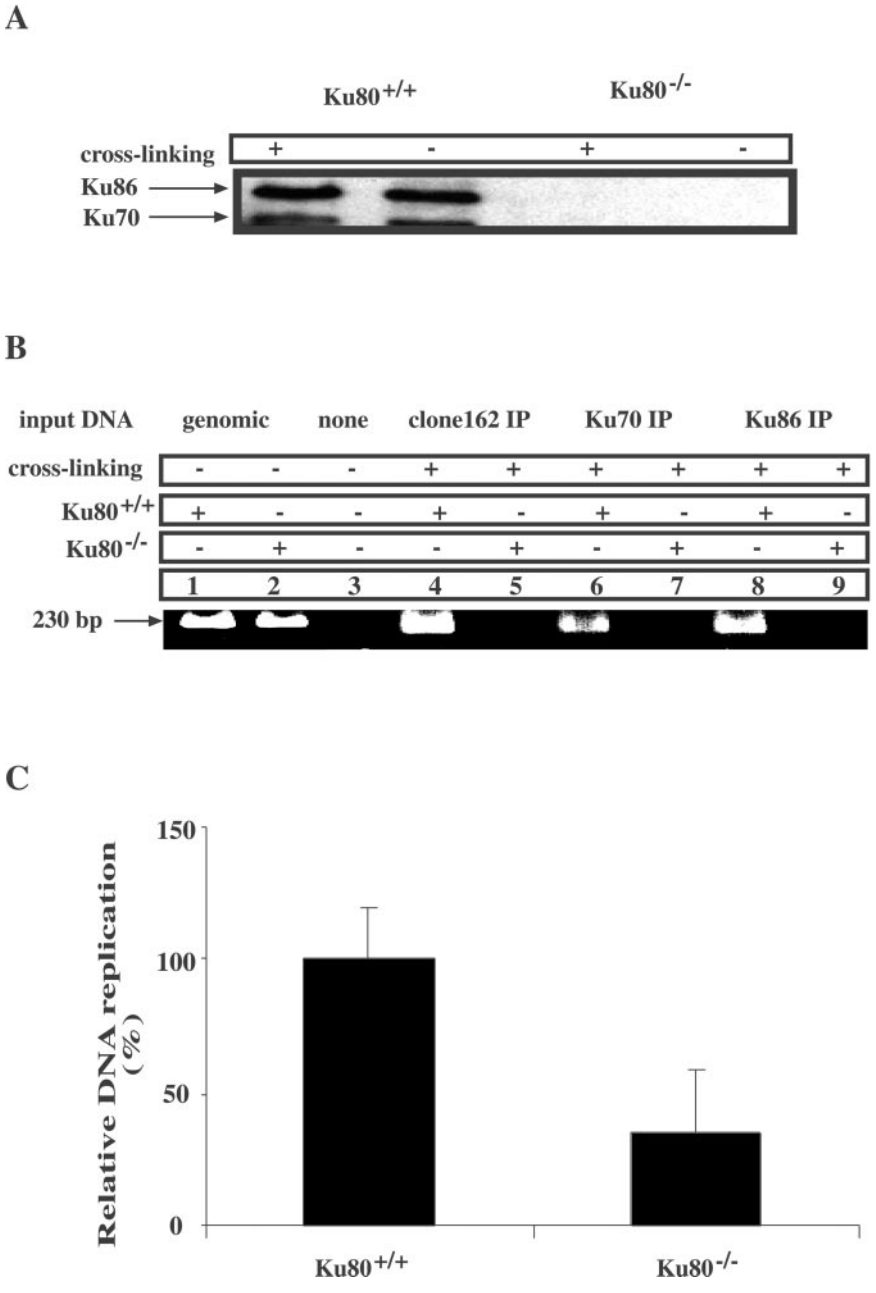

Figure 7. Ku is associated with the ADA-associated origin of the mouse genome in $Ku80^{+/+}$ cells, but not in $Ku80^{-/-}$ MEFs. $Ku80^{-/-}$ cell extracts have reduced replication activity. (A) Western blot probed with 1/100th dilution of anti-Ku86, or 1/400th dilution of anti-Ku70. 1/20th of immunoprecipitation with clone162 from cross-linked or untreated $Ku80^{+/+}$ or $Ku80^{-/-}$ MEFs. (B) PCR amplification with the use of primer set ADA A, which amplifies a genomic 230-bp fragment. Template DNA used was as follows. Lanes 1 and 2, total genomic DNA isolated from untreated $Ku80^{+/+}$ or $Ku80^{-/-}$ cells. Lane 3, negative control to verify primer contamination; no template DNA added to PCR reaction. Lanes 4, 6, and 8, Ku70, Ku86, or clone162 immunoprecipitate from $Ku80^{+/+}$ cells. Lanes 5, 7, and 9, Ku70, Ku86, or clone162 immunoprecipitate from $Ku80^{-/-}$ cells. (C) In vitro DNA replication assays were performed with $Ku80^{+/+}$ or $Ku80^{-/-}$ cells extracts and p186 as the template DNA. The in vitro replication products were purified, digested with *Dpn*I, and the *Dpn*I-resistant bands were quantitated with the use of a phosphorimager. The amount of radioactive precursor incorporated into the DNA is expressed as a percentage relative to the $Ku80^{+/+}$ cell extract reaction (100%). The quantification was obtained from at least three different in vitro reactions. Each bar represents three experiments and 1 SD is indicated.

ies, no PCR product was amplified by primer set ADA A (our unpublished results), indicating first that cross-linking was required before immunoprecipitation with anti-Ku antibodies, and second that the material that was immunoprecipitated with these antibodies from the noncross-linked cells did not contain detectable amounts of contaminating DNA for the PCR reaction.

Because Ku has been implicated in mammalian DNA replication (de Vries *et al.*, 1989; Toth *et al.*, 1993; Araujo *et al.*, 1999; Ruiz *et al.*, 1999; our unpublished results), in vitro DNA replication experiments were performed with the use of extracts prepared from both the $Ku80^{+/+}$ or $Ku80^{-/-}$ MEFs (Figure 7C) in a mammalian in vitro replication system (Pearson *et al.*, 1991; Zannis-Hadjopoulos *et al.*, 1994; Diaz-Perez *et al.*, 1996, 1998; Matheos *et al.*, 1998; Jilani *et al.*, 1999; Ruiz *et al.*, 1999). Approximately a 70% decrease in in vitro DNA replication was observed when the $Ku80^{-/-}$ extracts were used, compared with the $Ku80^{+/+}$ extracts.

DISCUSSION

There is increasing evidence suggesting that Ku is involved in DNA replication, through binding to replication origins

(Ruiz *et al.*, 1999, and references therein). In the present study, we have investigated the association of Ku with specific genomic regions, containing origins of replication. These origins (*ors8*, *ors12*, *c-myc*, and *dnmt-1*) contain sequences homologous to the A3/4 sequence element that is present in mammalian replication origins (our unpublished results); *ors8* contains a sequence that is 85% homologous to A3/4 in the 186-bp minimal *ori*, *ors12* has a 94% homologous sequence close to its 5′ end, *c-myc* has a 88% homologous sequence (our unpublished results), and *dnmt-1* has a 86% homologous sequence (Araujo *et al.*, 1999). There are three types of in vivo DNA binding assays: genomic footprinting (Diffley and Cocker, 1994), immunolocalization (Lewis *et al.*, 1992), and cross-linking followed by chromatin immunoprecipitation (Aparicio *et al.*, 1997; Tanaka *et al.*, 1997). The formaldehyde cross-linking approach consists of using formaldehyde to covalently couple endogenous proteins to DNA, via 2-Å-long methylene bridges. Formaldehyde is a high-resolution easily reversible cross-linking agent that efficiently produces both DNA-protein and protein-protein cross-links in vivo. These characteristics reduce the risk of redistribution or reassociation of chromosomal proteins during the preparation of cellular extracts. Antibodies are then used to immunoprecipitate proteins coupled to their target DNA. This approach, unlike footprinting, permits the identification of the proteins bound to a specific region. The efficiency of this approach has been demonstrated in a number of studies (Jackson, 1978, 1999; Solomon and Varshavsky, 1985; Solomon *et al.*, 1988; Gohring and Fackelmayer, 1997; Nickerson *et al.*, 1997; Orlando *et al.*, 1997; Strahl-Bolsinger *et al.*, 1997; Tanaka *et al.*, 1997; Ritzi *et al.*, 1998; Treuner *et al.*, 1998; Homesley *et al.*, 2000). Because Ku has been shown to bind to DNA ends, nicks, and structural transitions (reviewed in Tuteja and Tuteja, 2000) as well as to specific internal sequences (Giffin *et al.*, 1996; Ruiz *et al.*, 1999), it was important to include a number of controls to ensure that the amplification signals obtained were due to specific protein–DNA interactions. First, immunoprecipitation with a nonspecific antibody, NGS, was performed and no DNA was amplified (i.e., no signal was detected) by conventional PCR. Second, the more sensitive competitive PCR method permitted quantification of the signal. The background signal arising from DNA that was immunoprecipitated with the anti-SC-35 antibody, directed against the non-DNA binding protein SC-35 was quantified. In addition, the DNA that was immunoprecipitated with anti-NF-κB p65 antibody, a DNA binding protein that does not associate with origins of DNA replication, was also quantified. These three negative controls permitted us to estimate the background nonspecific DNA as $\sim 2.2 \times 10^4$–4×10^4 molecules/1.5×10^7 cross-linked CV-1 cells. Immunoprecipitated material from cells that were not treated with formaldehyde was also analyzed by conventional (Figure 2, B and C) and competitive PCR (our unpublished results) and did not contain any DNA fragments from the origin regions under investigation. The three anti-Ku antibodies (anti-clone162, anti-Ku70, and anti-Ku86) used in immunoprecipitation, which recognize the Ku heterodimer or the two Ku subunits separately, respectively, gave similar results. The consistently slightly higher amount of molecules immunoprecipitated with anti-Ku86 (Figures 4C and 6B) might be due to a higher efficiency of the anti-Ku86 than the anti-Ku70 antibody in immunoprecipitation reactions or to a higher availability of Ku86 epitopes in the cross-linked Ku–DNA complexes. Interestingly, Ku binding to A3/4 is accomplished by the 86-kDa subunit (Ruiz *et al.*, 1999; Schild-Poulter *et al.*, unpublished data), whereas the 70-kDa subunit is mostly responsible for binding to DNA ends and other Ku-responsive sequences (Chou *et al.*, 1992; Schild-Poulter *et al.*, unpublished data). Both subunits of Ku are required for DNA binding activity of the protein (Griffith *et al.*, 1992; Ono *et al.*, 1994; Wu and Lieber, 1996; Ochem *et al.*, 1997) and are functionally dependent on each other, in that neither subunit can bind DNA alone (Wu and Lieber, 1996; Ochem *et al.*, 1997).

The abundance of origin-containing genomic sequences (*ors*8 and *ors*12) bound to Ku protein, after cross-linking and immunoprecipitation, was measured by PCR-based methods, namely, conventional, competitive, and real-time PCR (Figures 2, B and C, 4B, and 5B). Quantification of Ku association with replication origins, performed by both competitive and real-time PCR, gave similar results. Ku's association with origin-containing genomic regions of DNA replication was approximately fivefold higher than with nonorigin-containing ones. When binding of Ku to a genomic region of *ors12* that does not contain a detectable replication origin (amplified by primer set EE′; Pelletier *et al.*, 1999) was tested by the same methods, the immunoprecipitates containing this region were comparable to background DNA levels (Figures 4C, 5B, and 6B). In addition, other nonorigin-containing genomic regions were tested, such as those amplified by primer sets specific for the BRCA gene and the CD4 intron, and the DNA that was immunoprecipitated with anti-Ku, anti-NF-κB p65, or NGS antibodies was again comparable with background DNA levels (Figure 5B). These data suggest that Ku binds to genomic regions that contain origins of DNA replication.

Formaldehyde is an easily reversible cross-linking agent (Jackson, 1978) When proteinase K was added to the immunoprecipitated material before reversal of the protein-DNA cross-links, an adduct-like structure was likely left by the cross-linked protein complex, which blocked amplification of these genomic regions (Figure 2C). These data suggest that the Ku-containing complex is positioned near or at the A3/4 homologous region of origins (Araujo *et al.*, 1999; Ruiz *et al.*, 1999; our unpublished results).

Finally, the cell cycle studies indicated that the association of Ku with *ors8* and *ors12* was the highest at the onset of S phase, being approximately fivefold higher in cells synchronized at the G1/S boundary, compared with that in cells that were blocked at G0 by serum starvation. When the cells were released from G1/S boundary into S phase, Ku association decreased by twofold and further decreased by a factor of 2 in cells that were blocked at G2/M. The differences of Ku association with *ors8* and *ors12* in vivo during the cell cycle were not due to different amounts of Ku present in the cell extracts, and the association was the critical step in Ku being immunoprecipitated. The recovery of Ku subunits from cross-linked cells showed that approximately similar amounts of Ku were immunoprecipitated at each cell cycle stage (Figure 6C). In view of the recent finding, which is corroborated in this study, that the total amount of Ku protein does not change during the cell cycle (Koike *et al.*, 1999), the higher association of Ku with *ors8* and

ors12 at the onset of S phase is specific and occurs at a time when these origins become activated (Kaufmann *et al.*, 1985). Furthermore, it was also recently reported that the DNA-end binding activity of Ku remains constant during the cell cycle (Chou and Chou, 1999). Thus, the findings in this study suggest a role for Ku in the initiation of DNA replication, supporting our previous findings (Ruiz *et al.*, 1995, 1999; our unpublished results). Its higher association with origins at the G1/S phase of the cell cycle suggests that Ku acts at the level of initiation of replication and dissociates after origin firing.

Ku knockout mice and Ku-deficient cell lines have been recently generated (Nussenzweig *et al.*, 1996; Gu *et al.*, 1997). Ku80 knockout mice are viable but they exhibit defective V(D)J recombination, which result in the absence of T- and B-lymphocyte maturation (Nussenzweig *et al.*, 1996; Gu *et al.*, 1997). Furthermore, these mice are less than one-half the size of their heterozygous littermates and exhibit severe growth retardation (Nussenzweig *et al.*, 1996). $Ku80^{-/-}$ MEF cells have prolonged doubling times, and the nonproliferating cells arrest at G1 phase in early passages, indicating premature senescence (Nussenzweig *et al.*, 1996; Gu *et al.*, 1997). The knockout cells are radiosensitive and fail to resume the cell cycle after radiation-induced checkpoint arrest. These phenotypes correlate well with Ku's involvement in DNA repair, but are also compatible with a possible participation of Ku in DNA replication. Arrington *et al.* (2000) observed that H_2O_2-treated $Ku80^{-/-}$ MEFs were unable to traverse the G2 phase and that this defect was not due to deficiencies in DNA repair. Instead, they observed important differences in the expression of key cell cycle regulatory genes affecting progression through G2, which suggests a role for Ku in cell cycle regulated cellular processes, such as DNA replication.

Because previous reports had implicated the Ku protein in DNA repair and possibly DNA replication based on the phenotypes of the knockout mice and their cells (Nussenzweig *et al.*, 1996; Gu *et al.*, 1997; Featherstone and Jackson, 1999), the in vivo association of Ku with a known mouse origin-containing (ADA) genomic sequence was examined in both $Ku80^{+/+}$ and $Ku80^{-/-}$ MEFs, by measuring the DNA abundance in that region when immunoprecipitating with anti-Ku antibodies. Only the Ku wild-type ($Ku80^{+/+}$) MEFs showed specific PCR amplification of that region (Figure 7B). In contrast, in the Ku80 knockout cells, which do not contain detectable amount of Ku protein, anti-Ku antibodies did not immunoprecipitate a detectable amount of the ADA origin-containing sequence (Figure 7B). Furthermore, extracts from $Ku80^{-/-}$ MEFs, had an ~70% decrease in their replication activity, compared with the $Ku80^{+/+}$ extracts, in a mammalian in vitro replication system (Figure 7C). Taken together, these data suggest that the Ku protein plays an important role in the initiation of mammalian DNA replication, through its binding to Ku-responsive origins.

In *S. cerevisiae*, a Ku-like protein was shown to be required in vitro for the assembly of a complex at a replication origin, suggesting that Ku participates directly in the formation or establishment of a regulated complex involved in initiation of replication (Shakibai *et al.*, 1996). To date, several human proteins have been shown to be required for initiation of DNA replication and a replication–competent multiprotein complex has also been isolated from human cells, including proteins such as DNA polymerases α and δ, proliferating cell nuclear antigen, DNA primase, replication protein A, topoisomerases I and II, DNA ligase I, replication factor C, and DNA helicases I and IV (Malkas *et al.*, 1990; Wu *et al.*, 1994; Applegreen *et al.*, 1995; Coll *et al.*, 1996; Tom *et al.* 1996; Lin *et al.*, 1997; Jiang *et al.*, 1998; Malkas, 1998; Sekowski *et al.*, 1998). Our data indicate that the majority, not all, of mammalian replication origins are Ku-responsive, i.e., they contain an A3/4 homologous element(s) to which Ku binds specifically at the onset of S phase.

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