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Integration of NHEJ into V(D)J Recombination Via Ku70/80 Binding to the RSS Heptamer and RAG1/2

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Integration of NHEJ into V(D)J recombination via Ku70/80 binding to the RSS heptamer and RAG1/2

By

Bo Cui

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The worth of that is that which it contains,
And that is this, and this with thee remains.

- William Shakespeare

ABSTRACT

The Ku autoantigen (Ku70/80) is a versatile DNA binding protein with prominent DNA-end and sequence-specific binding activities. It participates in the repair of DNA double stranded breaks (DSB) as a component of the nonhomologous end-joining (NHEJ) apparatus, which also includes DNA-PKcs, XRCC4 and DNA ligase IV. The Ku autoantigen is also integral to V(D)J recombination, a physiological process whereby the diversity of the immune repertoire is generated by the somatic assembly of the variable (V), diversity (D), or joining (J) gene segments in the immunoglobulin (Ig) or T cell receptor (TCR) variable chain gene in lymphocytes. As a sequence-specific DNA binding protein, the Ku autoantigen is also implicated in transcriptional regulation and control of DNA replication.

The overall goal of the present research project was to identify novel specific DNA binding sites for the Ku autoantigen and to link DNA sequence-specific binding by the Ku protein to specific cellular functions. The first principal objective was to directly compare DNA binding activities of Ku70/80 to disparate specific DNA sequences, NRE1 and A3/4, through reciprocal competitive electrophoretic mobility shift assays (EMSAs) and protein-DNA UV-crosslinking studies. The second principal objective was to identify novel classes of Ku70/80-specific DNA binding sites by the systemic evolution of ligands by exponential enrichment (*SELEX*) with recombinant human Ku70/80, and investigate the functional implications of the interaction of Ku70/80 with the specific DNA binding sites of interest evolved through *SELEX*.

In the present study, the sequence-specific binding activities of the Ku autoantigen to the negative regulatory element 1 (NRE1) in the long terminal repeat

(LTR) of the mouse mammary tumor virus (MMTV) and the A3/4 sequence, a sequence homologous to the various mammalian replication origins (*ors*), were investigated by reciprocal competitive EMSAs and protein-DNA UV-crosslinking experiments. The results demonstrated that the Ku protein binds to nonspecific DNA ends, NRE1 and A3/4 with apparently distinct affinities. Furthermore, its two subunits were shown to make differential contacts with nonspecific DNA ends, NRE1 and A3/4 in the absence or presence of Mg^{2+} and ATP. These findings establish A3/4 as a direct sequence-specific DNA binding site for the Ku autoantigen, which is distinct from either NRE1 or nonspecific DNA ends.

The sequence-specific binding activities of the Ku autoantigen were further investigated by *SELEX* with recombinant human Ku70/80 to evolve preferred DNA binding sites for the Ku autoantigen. The heptamer of the recombination signal sequence (RSS) that mediates V(D)J recombination was identified as a novel class of direct DNA binding sites for the Ku autoantigen. The RSS, comprised of the heptamer and nonamer DNA sequences spaced by 12 or 23 base pairs (bp), is known to serve as recognition motifs for the cleavage reaction by RAG1/2 that initiates recombination with the generation of signal or coding ends. *In vitro* DNA binding assays using linear oligonucleotides and circular DNA substrates further confirmed the sequence-specificity of Ku70/80 binding to the RSS heptamer. Additionally, plasmid and chromatin immunoprecipitation (ChIP) analysis demonstrated that endogenous Ku70/80 binds to the RSSs *in vivo*, which correlates with chromatin accessibility to the associated gene segments. *In vitro* DNA cleavage and *in vivo* V(D)J recombination assays further showed that the presence of Ku70/80 facilitates RSS cleavage by RAG1/2. Moreover, *in vitro*

protein binding assays and coimmunoprecipitation assays demonstrated a direct interaction of RAG1/2 and the Ku autoantigen.

The recombination of Ig and TCR variable region locus is initiated through specific cleavage of the RSSs by RAG1/2. Subsequent joining of the cleaved V, D, or J gene segments is mediated by the generic NHEJ machinery. Long-standing models for V(D)J recombination hypothesize that post-cleavage DNA-end recognition by the Ku autoantigen provides for the entry of other NHEJ factors to mediate the appropriate ligation of coding or signal ends. The findings from the present study suggested a central role of the Ku autoantigen in V(D)J recombination by integrating NHEJ into V(D)J recombination through its specific binding to the RSS heptamer and RAG1/2.

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I would like to express my deepest gratitude to my wife, whose love and encouragement give special meaning to every new journey I embark on, to my son and niece for their bounding spirits and warm hearts and to my parents for their expectations and encouragement.

Finally, this work is dedicated to the memory of those who died in the stormy summer night of 1989 in Beijing China. I looked in the face of death and ran away.

TABLE OF CONTENTS

LISTOF FIGURES

ABBREVIATIONS

Ab	antibody
A-MuLV	Abelson murine leukemia virus
AP	apurinic
β-ME	β-mercaptoethanol
bp	base pair
BSA	bovine serum albumin
BSAP	B-cell-specific activator protein
CASTing	cyclical-amplification and selection of targets
ChIP	chromatin immunoprecipitation
CHO	Chinese hamster ovarian
CIP	calf intestinal alkaline phosphatase
CSR	class switch recombination
CT	calf thymus
DHFR	dihydrofolate reductase
DMEM	Dulbecco's modified Eagle's medium
DNA-PK	DNA-dependent protein kinase
ds	double-stranded
DSB	double-stranded break
DTT	dithiothreitol
EMSA	electrophoretic mobility shift assay
EtBr	ethium bromide
FBS	fetal bovine serum
fmol	fentomole
GR	glucocorticoid receptor
h	hour
H7	heptamer
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HLH	helix loop helix
hPTTG	human pituitary tumor-transforming gene
HR	homologous recombination
HSE	heat shock element
HSF1	heat shock factor 1
HSR	heat shock response
IB	immunoblotting
Ig	immunoglobulin
Igh	immunoglobulin heavy chain
Igκ	immunoglobulin light chain kappa
IL	interleukin
IRBP	inverted repeat binding protein
KARP-1	Ku86 autoantigen related protein-1
kb	kilobase
Kd	dissociation constant
kDa	kiloDalton
low	lower

LPS	lipopolysaccharide
LTR	long terminal repeat
Mb	megabase
Mbr	major breakpoint region
MEF	mouse embryonic fibroblast
min	minute
Mlp	myosin-like protein
MMTV	mouse mammary tumor virus
MOI	multiplicity of infection
Mγ	molecular weight
μg	microgram
μl	microlitre
μM	micromole
N9	nonamer
N_{18}	18 random nucleotides
NE	nuclear extract
ng	nanogram
NHEJ	nonhomologous end joining
NLS	nuclear localization signal
NMR	nuclear magnetic resonance
NRE1	negative regulatory element 1
N-region	non-templated region
NS	nonspecific DNA
nt	nucleotide
Oct-1	octamer transcription factor 1
PARP	poly(ADP-ribose) polymerase
pBS	pBluescript
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pmol	picomole
PMSF	phenyl-methylsulfonyl fluoride
rag	recombination activating gene
RAG	protein encoded by recombination activating gene
RIPA	radioimmunoprecipitation assay
RNAP II	RNA polymerase II
RND	randomized nucleotides
RSS	recombination signal sequence
SAAB	selected and amplified binding site
SAP	SAF-A/B, Acinus and PIAS
scid	sever combined immune deficiency
SDS	sodium dodecyl sulfate
sec	second
SELEX	systemic evolution of ligands by exponential enrichment
Sf9	Spodoptera frugiperda
SLE	systemic lupus erythematosus
SRE	switch regulatory ATTT elements

ss	single-strand
$T_{1/2}$	half-life
TBE	Tris-borate-EDTA
TCR	T cell receptor
TdT	terminal deoxynucleotidyl transferase
TRF1	telomeric repeat binding facor 1
up	upper
UV	ultraviolet
V(D)J	variable (diversity) joining
vol	volume
vWA	von Willebrand factor A
XRCC	x-ray cross complementing
xrs	x-ray sensitive
yKu	yeast Ku protein

1. INTRODUCTION

1.1. The Ku protein: an overview

The Ku protein was originally recognized as an autoantigen in the sera of patients with the scleroderma-polymyositis overlap syndrome (Mimori et al. 1981). It is a predominantly nuclear protein with approximately 4 X 10^5 copies per cell and is comprised of a 70-kiloDalton (kDa) subunit (Ku70) and an 80-kDa subunit (Ku80) (Mimori et al. 1986; Mimori and Hardin 1986; Higashiiura et al. 1992). Ku70/80 is a versatile DNA binding protein: it binds nonspecifically to double-stranded (ds) DNA ends (Mimori and Hardin 1986), DNA nicks, gaps or bubbles, and to single-to-double-strand transitions in DNA (Blier et al. 1993; Falzon et al. 1993). It also binds to specific DNA sequences (Messier et al. 1993; Giffin et al. 1996; Ruiz et al. 1999). Furthermore, Ku70/80 is a component of the DNA-dependent protein kinase (DNA-PK) holoenzyme, acting as the DNA binding component of the complex (Gottlieb and Jackson 1993).

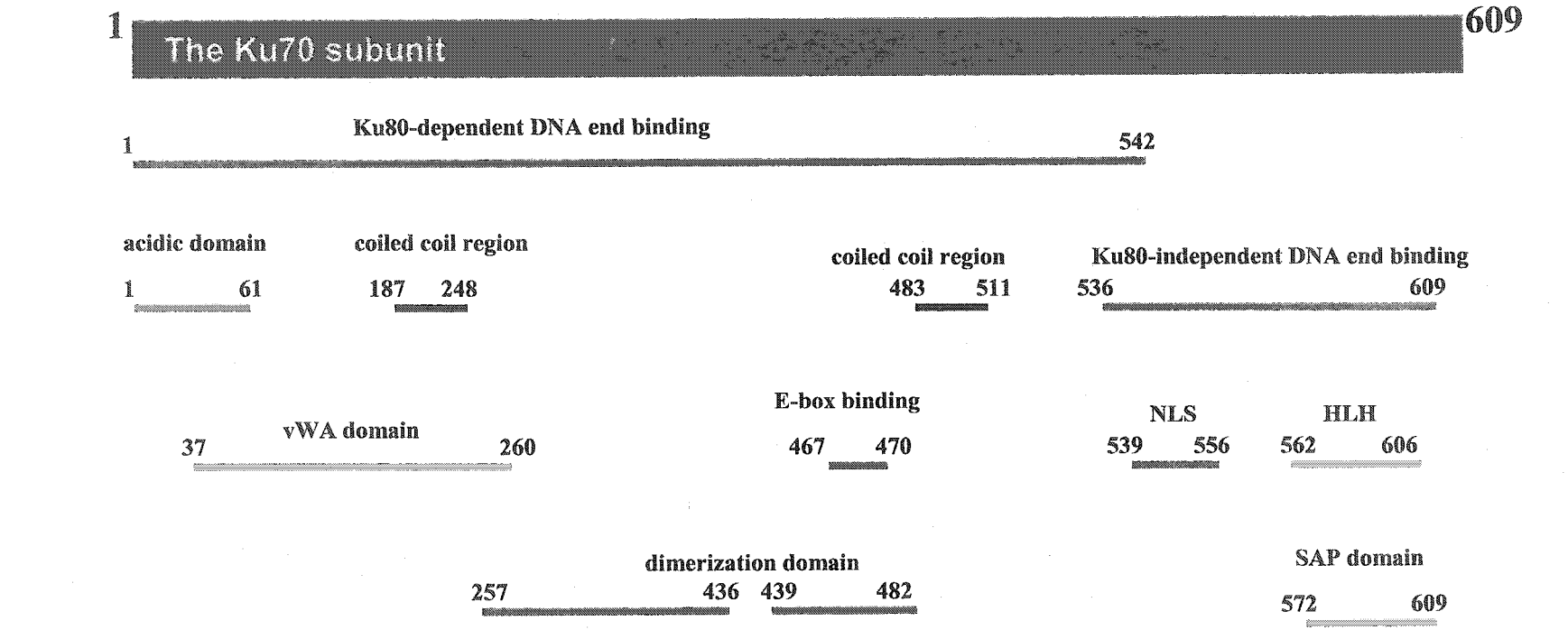
As a versatile DNA binding protein, Ku70/80 is implicated in multiple cellular processes, including the repair of DNA ds breaks (DSBs) by nonhomologous end joining (NHEJ) (Smider et al. 1994; Errami et al. 1996; Baumann and West 1998; Ramsden and Gellert 1998), V(D)J recombination during lymphocyte development (Taccioli et al. 1993, 1994; Smider et al. 1994; Errami et al. 1996), DNA replication (Shakibai et al. 1996; Ruiz et al. 1999; Cosgrove et al. 2002; Schild-Poulter et al. 2003a; Park et al. 2004), telomere length maintenance (Porter et al. 1996; Boulton and Jackson 1996; Gravel et al. 1998; Bailey et al. 1999), transcriptional regulation (Giffin et al. 1997;

Camara-Clayette et al. 1999a; Willis et al. 2002) and maintenance of genomic stabilities (Difilippantonio et al. 2000; Ferguson et al. 2000; Li et al. 2002).

Ku70/80 is well-conserved in organisms from yeasts to humans (Feldmann and Winnacker 1993; Beall et al. 1994; Mages et al. 1996; Boulton and Jackson 1996; Feldmann et al. 1996; Jacoby and Wensink 1996; Yagura and Sumi 1999). The human Ku70 subunit (Figure 1A) is composed of 609 amino acids and has a molecular weight (M_γ) of 69,851 Da (Appendix I) (Chan et al. 1989; Reeves and Sthoeger 1989). The human Ku80 subunit (Figure 1B) consists of 732 amino acids and has a M_γ of 82,713 Da (Appendix II) (Yaneva et al. 1989; Mimori et al. 1990; Stuvier et al. 1990). The gene encoding Ku70 is localized to chromosome 12 in human and chromosome 15 in mouse and the gene encoding Ku80 is localized to chromosome 2 in human and chromosome 1 in mouse (Cai et al. 1994; Koike et al. 1996). Moreover, an alternate form of Ku80, known as KARP-1 (Ku86 autoantigen related protein-1) is expressed from the human Ku80 gene locus with an extra 9 kDa of polypeptide appended to the amino- (N-) terminus of Ku80 (Myung et al. 1997; Braastad et al. 2002).

The Ku protein exists principally as a heterodimer in the cell (Mimori and Hardin 1986; Griffith et al. 1992; Ochem et al. 1997; Jin and Weaver 1997). The heterodimerization of Ku70 and Ku80, which apparently does not require the putative coiled coil motifs in each subunit, contributes to the steady state levels of one another. Ku80 has a half-life ($T_{1/2}$) of 1.5 h by itself and a $T_{1/2}$ of greater than 16 h when dimerized with Ku70 (Satoh et al. 1995). In Ku70-deficient mouse embryonic stem cells, there was a concurrent substantial decrease in the level of Ku80 (Gu et al. 1997a). A similar decline in the level of Ku70 was observed in Ku80-deficient cells (Boubnov et al. 1995;

Figure 1. The modular structure of the Ku protein. The modular structure of the Ku protein is schematically shown for the Ku70 subunit (A) and the Ku80 subunit (B). The numbers indicate the positions of amino acid residues in each subunit of the Ku autoantigen. **(A) The modular structure of Ku70.** The Ku70 subunit consists of 609 amino acids and has two separate DNA binding domains, the Ku80-dependent DNA end binding domain (amino acid residues from 1 to 542) and the Ku80-independent DNA binding domain (amino acid residues from 536 to 609) (Chan et al. 1989; Reeves and Sthoeger 1989; Wang et al. 1998a). The N-terminal 61 amino acid residues are very acidic (31% D+E). The domain from amino acid residues 257 to 482 is involved in heterodimerization with Ku80 (Wu and Lieber 1996; Wang et al. 1998a, 1998b; Osipovich et al. 1997; Cary et al. 1998; Gell and Jackson 1999; Walker et al. 2001). Two possible coiled coil regions are localized in amino acid residues from 187 to 248 and from 483 to 511 (Chan et al. 1989; Reeves and Sthoeger 1989). The putative coiled coil region from amino acid residues 187 to 248 shares sequence and secondary structural similarities to the *c-myc* and *v-myc* oncogene products. The nuclear localization signal (NLS) is localized in amino acid residues from 539 to 556 (Koike et al. 1999a, 2000; Bertinato et al. 2000). Amino acid residues from 467 to 470 (^{467}EKLR^{470}) constitute a putative E-box binding motif (Blackwell and Weintraub 1990). The von Willebrand factor A-like (vWA) domain covers amino acid residues from 37 to 260 (Aravind and Koonin 2001; Monferran et al. 2004). D43 constitutes a putative Mg^{2+} binding site in the subunit. Amino acid residues from 572 to 609 form the so-called SAP (SAF-A/B, Acinus and PIAS) domain, which corresponds to the carboxy- (C-) terminal Ku-80 independent DNA binding domain and contains a helix-loop-helix (HLH) structure (Aravind and Koonin 2000). **(B) The modular structure of Ku80.** The Ku80 subunit consists of 732 amino acids (Mimori et al. 1990; Stuvier et al. 1990). Amino acid residues from 261 to 492 are involved in heterodimerization with Ku70 (Wu and Lieber 1996; Wang et al. 1998a, 1998b; Osipovich et al. 1997; Cary et al. 1998; Gell and Jackson 1999; Walker et al. 2001). The DNA end binding domain covers amino acid residues from 210 to 531 (Osipovich et al. 1997; Cary et al. 1998). Ku80 also contains a vWA-like domain from amino acid residues 9 to 235 (Aravind and Koonin 2001) and a coiled coil region from amino acid residues 140 to 175 (Mimori et al. 1990; Stuvier et al. 1990). D15 and D137 constitute putative Mg^{2+} binding sites in the subunit (Aravind and Koonin 2001). The NLS spans from amino acid residues 561 to 569 (Koike et al. 1999b; Bertinato et al. 2000). The amino acid residues from 677 to 689 are a putative ATP binding site (Cao et al. 1994). The C-terminal 28 amino acid residues are involved in interaction with the catalytic subunit of DNA-PK (DNA-PKcs) (Gell and Jackson 1999; Singleton et al. 1999).


A.
1
The Ku70 subunit
609
1
Ku80-dependent DNA end binding
542
acidic domain
1
61
coiled coil region
187
248
coiled coil region
483
511
Ku80-independent DNA end binding
536
609
vWA domain
37
260
E-box binding
467
470
NLS
539
556
HLH
562
606
dimerization domain
257
436
439
482
SAP domain
572
609
B.
1
The Ku80 subunit
732
coiled coil region
140
175
210
DNA end binding
531
NLS
561
569
ATPase
677
689
vWA domain
9
235
dimerization domain
261
492
Interaction with DNA-PKcs
704
732

Singleton et al. 1997).

Predominantly a nuclear protein, each subunit of the Ku autoantigen possesses its own NLS (Koike et al. 1999a, 1999b, 1999c, 2000, 2001; Bertinato et al. 2000). Ku70 or Ku80 translocates either separately to the nucleus utilizing its own NLS or as a heterodimer. The Ku protein is rapidly mobile throughout the nucleus (Rodgers et al. 2002).

Ku70/80 is apparently also distributed in the cytoplasm. In murine pre-B cells, Ku70/80 is present in both the nucleus and the cytoplasm (Grawunder et al. 1996) while, in resting B cells, it is relatively in low abundance in the nucleus and confined to the cytoplasm in association with CD40 (Zelazowski et al. 1997; Morio et al. 1999). Ku70/80 translocates to the nucleus upon stimulation with IL-4 and anti-CD40 antibodies. Ku70/80 is also present on the cell surface (Prabhakar et al. 1990; Romancer et al. 1994). A vWA-like domain has been proposed for both Ku70 and Ku80 (Aravind and Koonin 2001; Monferran et al. 2004); proteins containing vWA domains usually function as adhesion molecules in the cell (Whittaker and Hynes 2002). In multiple myeloma cells, Ku70/80 translocates from the cytoplasm to the cell surface upon stimulation with CD40 ligand and functions as an adhesion molecule (Teoh et al. 1998). Ku80 is also membrane-associated in lymphocytes, mediating their adhesion to the endothelium upon hypoxia (Lynch et al. 2001).

Though an abundant nuclear protein, Ku70/80 is present at very low levels or absent in cells ready to undergo apoptosis such as mature human neutrophils (Ajmani et al. 1995; Choi et al. 2002). In human embryonic kidney 293T cells and HeLa cells, Ku70 is associated with Bax (Sawada et al. 2003a), which is a member of Bcl-2 family and

central to mitochondria-dependent apoptosis. The association of Ku70 with Bax blocks the translocation of Bax from the cytosol to the mitochondria and inhibits Bax-mediated apoptosis (Sawada et al. 2003a, 2003b; Cohen et al. 2004). Of interest are the findings that Ku80-deficient somatic cells undergo apoptosis after a limited number of cell divisions (Li et al. 2002) and, in Ku70- or Ku80- deficient mice, developing neurons succumb to massive apoptosis (Gu et al. 2000).

1.2. The Ku protein binds to dsDNA ends and is involved in DNA DSB repair

Ku70/80 possesses DNA-end binding activity (Mimori and Hardin 1986). It binds initially to broken DNA ends and translocates to and stalls randomly on internal sites of DNA molecules in a so-called "bind-and-slide" mechanism (DeVries et al. 1989; Paillard and Strauss 1991; Yaneva et al. 1997). Nonspecific protein-DNA complexes are stabilized primarily through electrostatic interactions and the protein-DNA interface is more hydrated (Vivadiu et al. 2000; Luscombe et al. 2001). In addition, there is lack of DNA backbone contacts by nonspecific DNA binding proteins. Crystallographic structural studies of a truncated form of the Ku protein indicated that Ku70/80 binds to duplex DNA ends with a preformed ring-like structure through which DNA is threaded and it does not form specific contacts with any bases of the DNA duplex, suggesting a nonspecific nature of its DNA end binding activity (Walker et al. 2001).

Ku70/80 required a minimum length of 16 to 18 base pairs (bp) of DNA for adequate DNA end binding activity (Falzon et al. 1993) and generated a footprint of 20 to 30 bp on DNA (Mimori and Hardin 1986). A 25-bp blunt-ended dsDNA accommodated one Ku protein molecule while a 50-bp blunt-ended dsDNA accommodated two Ku

protein molecules (Arosio et al. 2002). Ku70/80 bound to 30- to 32-bp dsDNA ends with dissociation constant (Kd) values from 1.5 and 2.0 X 10^{-11} M to 2.4 X 10^{-9} M and a $T_{1/2}$ of 14 to 15 h (Blier et al. 1993; Falzon et al. 1993). Following binding to DNA ends, Ku70/80 translocated along DNA with no preferential binding to internal specific sequences (Paillard and Strauss 1991) and exhibited sporadic distribution along the length of DNA fragment behaving like a bead threaded on a DNA string (Yaneva et al. 1997). A significant proportion of Ku70/80-bound DNA molecules demonstrated loop formation, probably due to the association of Ku70/80 bound to two separate sites on a single DNA molecule (Cary et al. 1997).

DNA end binding activity of Ku70/80 is essential for DNA DSB repair following ionizing irradiation damage to the cell. Ku80-deficient *x-ray sensitive* mutant 6 (*xrs*-6) cells showed no detectable DNA-end binding activity, increased sensitivity to ionizing irradiation, and defective repair of DNA DSBs (Taccioli et al. 1993), which were restored upon transfection of Ku80 cDNA into these mutant cells (Taccioli et al. 1994). Similarly, Ku80-deficient Chinese hamster ovarian (CHO) fibroblasts XR-V15B from the *XRCC* (*X-ray cross complementing*) group 5, which has an internal deletion from amino acid residues 372 to 417 of the Ku80 subunit (Cary et al. 1998), and Ku70-deficient mouse embryonic cells displayed no measurable DNA end binding activity and exhibited X-ray hypersensitivity (Taccioli et al. 1994; Smider et al. 1994; Errami et al. 1996; Gu et al. 1997a). Ku70- or Ku80-deficient mice also exhibited defective DNA DSB repair and increased radiosensitivity (Gu et al. 1997b; Nussenzweig et al. 1997). In addition, the level of Ku70 in the nucleus increased approximately 3 fold following ionizing irradiation, paralleled by a 5- to 7-fold increase in the level of p53 (Brown et al. 2000).

Expression of KARP-1 was also upregulated in response to ionizing irradiation in a p53-depedent manner (Myung et al. 1998).

1.3. Crystallographic structure of Ku70/80 bound to structured duplex DNA ends

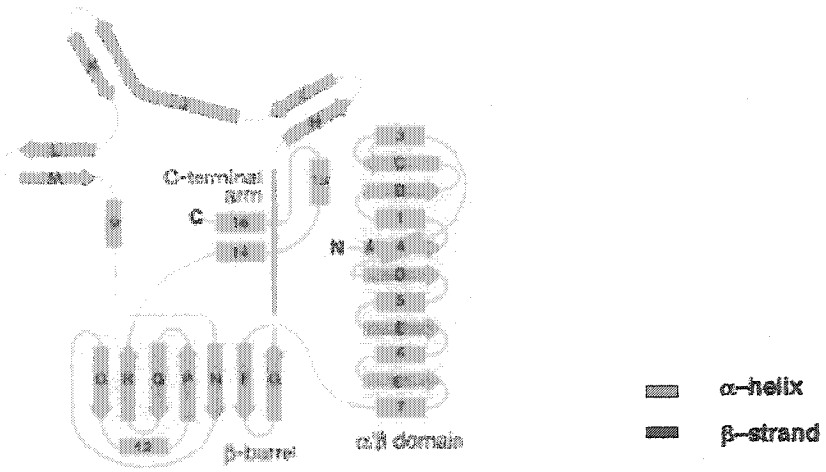
The crystallographic structure of Ku70/80 bound to 14-bp structured duplex DNA ends with one end free and the other end blocked was resolved at 2.5 Å, which showed that Ku70/80 possesses an overall dimension of approximately 120 X 70 X 60 Å (Figure 2A and 2B) (Walker et al. 2001). Though the two subunits do not bear extensive sequence homology, they exhibit structural similarities: each consists of an N-terminal α/β domain, a central β-barrel domain and a helical C-terminal arm.

The N-terminal α/β domain, which is present in both Ku70 and Ku80, is composed of a six-stranded β-sheet. It lies at the periphery of the Ku70/80 heterodimer and in each subunit makes scarce contribution to the dimer interface between Ku70 and Ku80. It corresponds to the vWA-like domain in each subunit (Aravind and Koonin 2001). The central β-barrel domain in each subunit consists of seven β strands and centers on the protein dyad axis and is the DNA binding domain in each subunit. It forms a symmetrical round barrel in each subunit and the two β-barrel domains together form a relatively flat base that holds approximately two turns of DNA. The Ku protein exhibits minimal contact with the DNA backbone that sits on the cradle-like β-barrel domains.

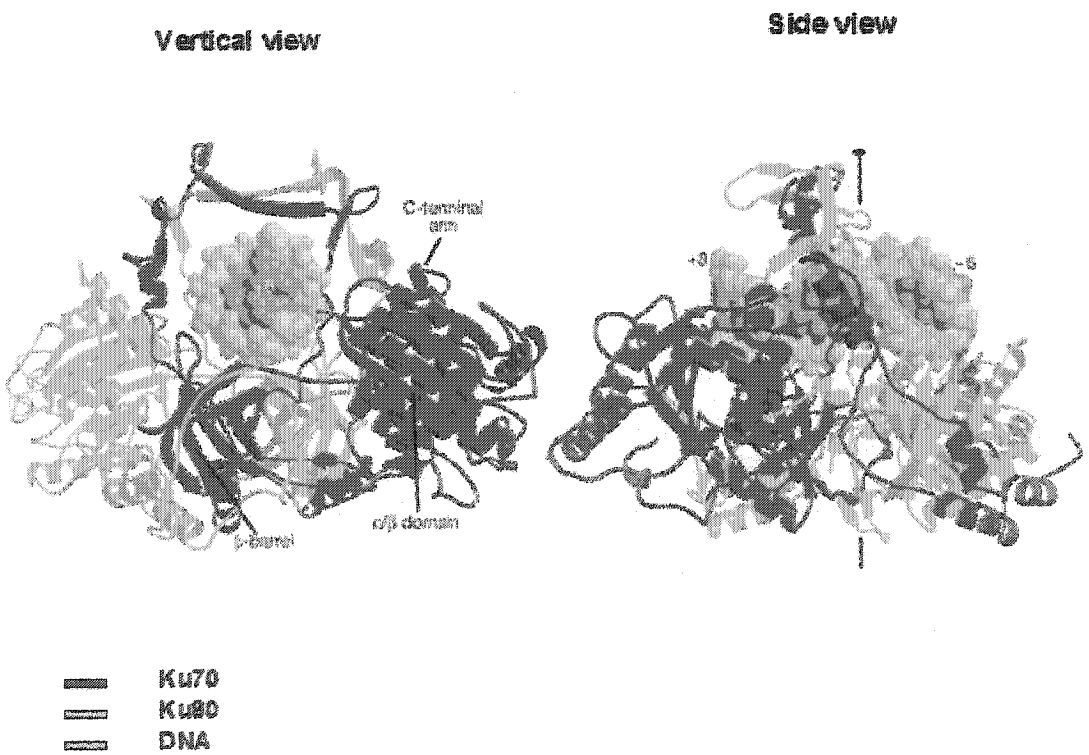
Furthermore, a bridge-like structure arises from each subunit and encircles approximately 3 to 4 bp of DNA. Much of the DNA surface facing away from the cradle is exposed except the 3 to 4 bp of DNA encircled by the bridge-like structure, which acts as a mechanical barrier to exclude promiscuous binding of the Ku protein to unbroken

Figure 2. The structure of the Ku protein. (A) The topographical diagram of Ku70 and Ku80. Each subunit of the Ku autoantigen is comprised of an N-terminal α/β domain, a central β-barrel domain, and a helical C-terminal arm. The N-terminal α/β domain consists of amino acid residues from 34 to 250 in Ku70 and from 6 to 238 in Ku80. The central β-barrel domain consists of seven β-strands in each subunit, which are composed of amino acid residues from 257 to 276 (strands F and G) and from 343 to 436 (strands N, O, P, Q and R) in Ku70 and from 247 to 267 (strands F and G) and from 339 to 423 (strands N, O, P, Q and R) in Ku80. The C-terminal arm of the Ku protein consists of amino acid residues from 455 to 528 in Ku70 and from 447 to 536 in Ku80. The α–helices are shown in green and the β–strands in blue. **(B) The structure of the Ku protein bound to a structured 14-bp duplex DNA molecule.** The N-terminal α/β domain is composed of a six-stranded β-sheet in each subunit and lies at the periphery of the Ku70/80 heterodimer. The α/β domain defines the long molecular axis (120 Å) of the Ku autoantigen and is distant from the protein dyad axis. The amino edge of the β-sheet is proximal and the carboxy edge is distal to the DNA-binding grove. The central β-barrel domain consists of seven β-strands and forms part of the DNA binding channel. A bridge-like structure extends from amino acid residues 277 to 341 in Ku70 and from 267 to 336 in Ku80 and encircles approximately 3 to 4 bp of DNA that sits on the flat base formed by the two β-barrel domains. The C-terminal arm of the Ku protein is composed of 3 α helices in each subunit. The view down the DNA helix is shown on the left and the side view on the right. Ku70 is shown in red and Ku80 in yellow and the duplex DNA in gray. **(C) The structure of the C-terminal DNA binding domain of Ku70.** The amino acid residues from 539 to 556 constitute the NLS for Ku70 (Koike et al. 1999a, 2000; Bertinato et al. 2000). The lysine residues in the NLS (K539, K542, K544, K553 and K556) are targets for acetylation (Cohen et al. 2004). The C-terminal DNA binding domain of Ku70 consists of a helix-extended loop-helix structure. *Helix a* consists of amino acid residues from 562 to 570, *helix b* from 578 to 587 and *helix c* from 596 to 606. *Helices b* and *c* run in parallel and are connected by an extended loop region. Amino acid residues 582, 586, 591, 595 and 596 form a clustered positive surface area in the DNA-binding domain and contact the major groove of DNA and the phosphate backbone. *Helix b* was shown to interact with Bax (Sawada et al. 2003a, 2003b). Amino acid residues from 583 to 609 were found to interact with homeodomain proteins (Schild-Poulter et al. 2001). The putative SAP domain spans from amino acid residues from 572 to 609 (Aravind and Koonin 2001). **(D) The structure of the C-terminal region of Ku80.** Amino acid residues from 543 to 592 in Ku80 exhibit no defined structure in solution. The C-terminal region from amino acid residues from 594 to 709 consists of six α-helices: α1 from amino acid residues 594 to 602; α2 from 611 to 625; α3 from 629 to 649; α4 from 652 to 668; α5 from 672 to 681 and α6 from 608 to 704. The six α-helices are organized into a globular domain with a potential ligand binding pocket between helix α2 and α4. Amino acid residues from 709 to 732 in Ku80 exhibit no defined structure in solution. Figures in (A) and (B) were adapted from Walker et al. (2001). Figure in (C) was taken from Zhang et al. (2001) and in (D) from Harris et al. (2004).

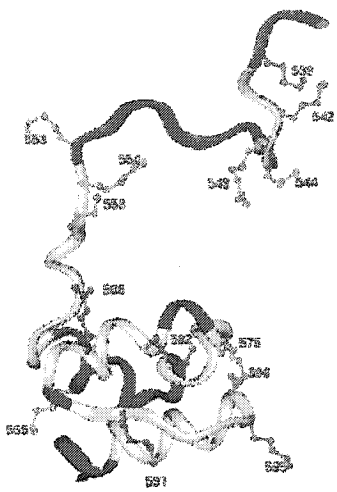
A.


C-terminal arm
β-barrel
α/β domain
α–helix
β–strand

B.


Vertical view
Side view
C-terminal arm
β-barrel
α/β domain
Ku70
Ku80
DNA

C.



D.

DNA molecules. The structure of Ku70/80, however, does not preclude its binding to certain structured DNA molecules like cruciform DNA or DNA hairpins in a similar fashion to its binding to DNA ends (Uliel et al. 2000). Moreover, the arrangement of the Ku protein on one side of the DNA helix allows access by other proteins to the contralateral surface of the DNA helix (Yoo et al. 1999).

1.4. The Ku70 subunit has a separate DNA binding domain in the C terminus

The C-terminal 73 amino acid residues from 536 to 609 in Ku70, which constitute a major epitope recognized by autoantibodies from the sera of patients with systemic lupus erythematosus (SLE) and the scleroderma-polymyositis overlap syndrome, possess Ku80-independent DNA binding activity (Chou et al. 1992; Wang et al. 1998a, 1998b). Deletion of amino acid residues from 536 to 560 abrogated while deletion of amino acid residues from 600 to 609 markedly reduced the DNA binding activity of the C-terminal domain (Chou et al. 1992; Wang et al. 1998a).

Nuclear magnetic resonance (NMR) spectroscopic study of the C-terminal DNA-binding domain of Ku70 revealed the presence of a helix-extended loop-helix structure (Figure 2C) (Zhang et al. 2001), which corresponds to a proposed DNA-binding motif called SAP (Aravind and Koonin 2000). It spans from amino acid residues 572 to 609 and consists of helix a, b and c with an extended loop between helix b and c. In the crystallographic structure of the Ku protein unbound to any DNA, the SAP domain is packed against the base of the Ku80 α/β domain with *helix c* exposed to the solvent (Walker et al. 2001). When the Ku protein is bound to DNA ends, the SAP domain is disordered and appears to have moved away from the base of the Ku80 α/β domain into

the solvent. The significance of the presence of a separate DNA binding domain in the C-terminus in Ku70 remains to be established. Proteins containing the SAP motif in general organize the interphase chromosomes by binding to the scaffold attachment regions (Aravind and Koonin 2000). The motif is also found in apurinic (AP)-endonuclease and poly(ADP-ribose) polymerase (PARP).

1.5. The Ku protein is a functional component of the DNA-PK holoenzyme

The Ku protein is also part of the DNA-PK holoenzyme, acting as the DNA binding component of the complex and targeting the catalytic subunit of the holoenzyme (DNA-PKcs) to DNA (Gottlieb and Jackson 1993; Suwa et al. 1994; Lees-Miller et al. 1995; Giffin et al. 1996). The activity of DNA-PKcs is DNA-dependent. The association of DNA-PKcs and Ku70/80 is dependent upon and stabilized in the presence of DNA. The C-terminal 28 amino acid residues of Ku80 interacts with the kinase homology domain (amino acid residues from 3002 to 3850) of DNA-PKcs, a region with which the nuclear tyrosine kinase, *c-Abl*, is also associated (Gell and Jackson 1999; Singleton et al. 1999; Jin et al. 1997; Kharbanda et al. 1997). The domain in Ku80 that interacts with DNA-PKcs is probably masked in the absence of DNA binding by Ku70/80. DNA binding by Ku70/80 is hypothesized to induce conformational changes in Ku80 that unmasks the binding site for DNA-PKcs (Gell and Jackson 1999).

The activation of DNA-PKcs requires the presence of Ku70/80 and DNA-PKcs on the same DNA molecule. The recruitment of DNA-PKcs to Ku70/80-bound DNA ends results in the translocation of Ku70/80 from DNA ends to internal positions on DNA molecules and the activation of its kinase activity (Yoo and Dynan 1999; West et al.

1998). No DNA-PKcs activity is detectable in Ku80-deficient *xrs*-6 cells (Finne et al. 1995).

DNA-PKcs phosphorylates Ku70/80 at S6 in Ku70, S577 and S580 and T715 in Ku80; however, phosphorylation of Ku70/80 did not interfere with its DNA binding activity (Chan and Lees-Millers 1996; Chan et al. 1999). DNA-PKcs was phosphorylated at T2609, T2612, T2620, T2638 and T2647 in response to ionizing irradiation (Chan et al. 2002; Ding et al. 2003; Soubeyrand et al. 2003). Phosphorylation and dephosphorylation of DNA-PKcs may be associated with the regulation of its kinase activity *in vivo* (Douglas et al. 2001). Autophosphorylation of DNA-PKcs, which was required for the rejoining of DNA DSBs, resulted in the inactivation of its kinase activity and the disruption of the DNA-PKcs-Ku70/80 complex (Chan and Lees-Millers 1996; Merkle et al. 2002). The kinase activity of DNA-PKcs remained intact with simultaneous mutations of T2609, T2612, T2620, T2638 and T2647; furthermore, the interaction between DNA-PKcs and Ku70/80 remained unchanged (Ding et al. 2003). However, the mutant DNA-PKcs was unable to carry out NHEJ due to a failure to undergo autophosphorylation.

DNA-PKcs was also capable of binding to DNA ends in the absence of Ku70/80 (Yaneva et al. 1997; Hammarsten and Chu 1998). However, binding to DNA by DNA-PKcs alone was infrequent (19%) and was markedly increased in the presence of Ku70/80 (77%) (Cary et al. 1997). Furthermore, atomic force microscopic studies revealed that DNA-PKcs was bound predominantly to DNA termini whereas Ku70/80 was distributed at both DNA ends and internal regions of DNA (Yaneva et al. 1997). DNA-PKcs contained two open channels that bound to dsDNA and an enclosed cavity

that bound to single-stranded (ss) DNA (Leuther et al. 1999). The structure of DNA-PKcs allowed it to mediate the synapsis of two DNA ends (DeFazio et al. 2002).

Though DNA-PKcs can bind to DNA in the absence of Ku70/80, its activation requires the presence of DNA ends (Hammarsten and Chu 1998). Moreover, the activation of DNA-PKcs depends on the structural features of the DNA molecules to which it binds. DNA-PKcs was efficiently activated when the DNA substrates contained unpaired ssDNA ends (Hammarsten et al. 2000; Mårtensson and Hammarsten 2002). The Ku70 subunit possesses ATP-dependent DNA helicase activity and preferentially unwinds fork-like DNA substrates (Tuteja et al. 1994; Ochem et al. 1997; Matheos et al. 2002). One probable scenario is that, after its binding to DNA ends, the Ku protein unwinds the dsDNA ends with its helicase activity, recruits DNA-PKcs to the unpaired ssDNA ends and subsequently activates DNA-PKcs (Gell and Jackson 1999). Hairpin-ended and nicked DNA generated during V(D)J recombination, on the other hand, failed to activate DNA-PKcs even though a Ku70/80-DNA-PKcs complex is assembled on the hairpin DNA ends (Smider et al. 1998; Hammarsten et al. 2000; Mårtensson and Hammarsten 2002). Ku70/80 bound to cisplatin-damaged DNA also failed to activate DNA-PKcs (Turchi and Henkels 1996). Translocation of Ku70/80 on cisplatin-damaged DNA was inhibited and DNA-PKcs-DNA complexes formed at the cisplatin-damaged DNA ends were inactive (Turchi et al. 2000).

DNA-PKcs is required for the repair of DNA DSBs by NHEJ in response to ionizing irradiation (Fulop and Phillips 1990; Biedermann et al. 1991; Finnie et al. 1995; Tacciolli et al. 1998; Baumann and West 1998). Its exact role in NHEJ, however, remains elusive. Autophosphorylation of DNA-PKcs, which inactivates its kinase activity,

is required for the rejoining of DNA DSBs (Chan and Lees-Miller 1996; Chan et al. 1999). Furthermore, binding to duplex DNA ends by Ku70/80 recruits other proteins such as octamer transcription factor 1 (Oct-1) and increases the phosphorylation of these proteins by DNA-PKcs in response to ionizing irradiation (Schild-Poulter et al. 2001; Schild-Poulter et al. 2003b). Additionally, DNA-PKcs was shown to stimulate the rate of DNA DSB repair by NHEJ (DiBiase et al. 2000).

DNA-PKcs is also implicated in V(D)J recombination (Bosma et al. 1983; Lieber et al. 1988; Hendrickson et al. 1991; Blunt et al. 1996; Araki et al. 1997). Mutation of DNA-PKcs is responsible for the *s*evere *c*ombined *i*mmune *d*eficiency (*scid*) phenotype in *scid* mice, which has a mutation in the DNA-PKcs gene resulting in the truncation of its C-terminal kinase domain (Blunt et al. 1996; Araki et al. 1997). DNA-PKcs and a newly identified protein, Artemis, function together to resolve DNA hairpins generated during V(D)J recombination (Moshous et al. 2001; Ma et al. 2002; Rooney et al. 2002).

1.6. The Ku protein is a component of the NHEJ apparatus

In higher eukaryotic cells, DNA DSBs are repaired primarily by NHEJ and homologous recombination (HR) (Jeggo 1998; Haber 1999). Compared with DNA DSB repair by HR, DNA DSB repair by NHEJ is accomplished without an apparent requirement for sequence homology of broken DNA ends. DNA DSB repair by HR is mainly active during late S and G2 phase while NHEJ contributes to DNA DSB repair throughout the cell cycle and is the primary DNA DSB repair apparatus during G1 and early S phase (Hendrickson 1997; Takata et al. 1998; Rothkamm et al. 2003; Guirouilh-

Barbat et al. 2004). Coincidentally, the activity of DNA-PKcs peaks at G1 and early S phase (Lee et al. 1997).

The components of the NHEJ pathway include Ku70/80, DNA-PKcs, DNA ligase IV, XRCC4 and probably additional cellular factors (Baumann and West 1998; Labhart 1999; Wood et al. 2001; Dai et al. 2003; Mahajan and Mitchell 2003). Ku70/80 plays an essential role in the repair of DNA DSBs by NHEJ. Ku70- or Ku80-deficient cell lines or mice exhibit defective DNA DSB repair and increased sensitivity to ionizing irradiation (Taccioli et al. 1993, 1994; Smider et al. 1994; Errami et al. 1996; Gu et al. 1997a). Ku70/80 binds to and protects broken DNA ends against degradation (Mimori and Hardin 1986; Liang and Jasin 1998; Lee et al. 1998; Tomita et al. 2003). DNA ligase IV is involved in the rejoining of broken DNA ends (Wilson et al. 1997; Critchlow and Jackson 1998; Frank et al. 1998; Adachi et al. 2001). XRCC4, a protein of 336 amino acid residues, interacts with DNA ligase IV and stimulates its activity (Li et al. 1996a; Critchlow et al. 1997; Grawunder et al. 1997; Junop et al. 2000; Lee et al. 2003). Ku70/80 forms a complex with XRCC4-DNA ligase IV on DNA ends and inward translocation of the Ku protein from DNA ends stimulates the activity of DNA ligase IV (McElhinny et al. 2000; Chen et al. 2000; Calsou et al. 2003; Kysela et al. 2003).

Other probable components of the NHEJ pathway include Artemis, a protein implicated in resolving coding-end DNA hairpins generated during V(D)J recombination (Moshous et al. 2001; Ma et al. 2002) and DNA polymerase μ (Aoufouchi et al. 2000; Mahajan et al. 2002; McElhinny and Ramsden 2003). Artemis-deficient mice exhibited increased sensitivity to ionizing irradiation (Rooney et al. 2002). Artemis-deficient cell lines were mildly radiosensitive, but they were proficient in the rejoining of DSBs

(Nicolas et al. 1996; Rooney et al. 2003). DNA polymerase μ is associated with Ku70/80 and DNA ligase IV (Mahajan et al. 2002). DNA polymerase μ-deficient fibroblasts, however, did not manifest increased sensitivity to ionizing irradiation (Bertocci et al. 2003). The exact role of Artemis or DNA polymerase μ in DNA DSB repair by NHEJ remains to be defined.

The NHEJ pathway plays an active role in DNA DSB repair following genotoxic insults. It is also implicated in retroviral infection and the transposition of retrotransposons (Downs et al. 1999; Daniel et al. 1999; Li et al. 2001; Izsvák et al. 2004). In addition, the NHEJ activities are required for the development of embryonic neurons. Deficiency of DNA ligase IV, XRCC4 or Ku70/80 leads to massive apoptosis of developing neurons (Barnes et al. 1998; Gao et al. 1998a; Chun and Schatz 1999; Frank et al. 2000; Gu et al. 2000; Terskikh et al. 2001). Furthermore, Ku70/80 and the NHEJ apparatus are required for V(D)J recombination during lymphocyte development (Grawunder and Harfst 2001; Gellert 2002). Deficiencies in the components of the NHEJ pathway have resulted in clinical phenotypes ranging from immunodeficiency, growth retardation to the development of malignancies (Nicolas et al. 1998; O'Driscoll et al. 2001; Riballo et al. 2001; Moshous et al. 2003).

1.7. Ku70/80 acts to maintain chromosomal stability

DNA DSBs are generated in the event of genotoxic insults like ionizing irradiation or arise during physiological processes such as V(D)J recombination or somatic hypermutation in the immunoglobulin (Ig) variable region genes (Roth et al. 1992a, 1992b, 1993; Papavasillou and Schatz 2000; Bross et al. 2000; Zan et al. 2003).

Repair of DNA DSBs is critical for maintaining genomic integrity (Vanasse et al. 1999a; Rogakous et al. 1999; Myung et al. 2001). It has been shown that as few as two DNA DSBs within a single cell were sufficient to cause chromosomal instability (Richardson and Jasin 2000).

DNA DSB repair by NHEJ maintains chromosomal stability throughout the cell cycle, especially during G1 phase. $Ku70^{-/-}$ mouse embryonic fibroblasts (MEFs) exhibited spontaneous fragmentation of chromosomes and nonreciprocal translocations involving two or more chromosomes (Ferguson et al. 2000). Similarly, $Ku80^{-/-}$ mouse cells displayed chromosomal breakage, translocations and aneuploidy (Difilippantonio et al. 2000). $Ku70^{-/-}$ mouse had a markedly increased incidence of T cell lymphomas (Gu et al. 1997b; Li et al. 1998). Ku80 was also essential for the survival of human somatic cells (Li et al. 2002). $Ku80^{-/-}$ cells only survived 8 to 10 cell doublings and, compared with its wild-type counterparts, $Ku80^{+/-}$ cells demonstrated elevated p53 levels, slowed cell proliferation and increased sensitivity to ionizing irradiation.

The *Drosophila Dmblm* is a homologue of the human BLM protein and a 3'-5' helicase of the RECQ family (Dutertre et al. 2002). *Drosophila* defective in the *Dmblm* gene exhibited a phenotype of partial sterility, increased chromosome nondisjunction and chromosome loss, which was partly rescued upon transfection with Ku70 cDNA, suggesting that Ku70 can partially replace *Dmblm* in maintaining genomic stability in *Drosophila* (Kusano et al. 2002). Ku70/80 is associated with human securin, hPTTG (human pituitary tumor-transforming gene), a protein involved in sister chromatid separation (Romero et al. 2001). Interestingly, DNA DSBs disrupted the hPTTG-

Ku70/80 complexes, suggesting a role of Ku70/80 in modulating sister chromatid separation subsequent to the generation of DNA DSBs from genotoxic events.

1.8. The Ku autoantigen maintains the integrity of telomeres

Ku70/80 is implicated in the maintenance of telomeres from yeasts to humans (Boulton and Jackson 1996; Bailey et al. 1999; Hsu et al. 2000; Baumann and Cech 2000; Peterson et al. 2001; Stellwagen et al. 2003). The yeast Ku protein (yKu) is associated with telomeric DNA *in vivo* (Gravel et al. 1998). YKu80 and yKu70 mutant strains displayed markedly shorter telomeres than their wild-type counterparts (Boulton and Jackson 1996; Porter et al. 1996). The maintenance of telomere length by yKu70/80 was independent of its DNA-end binding activity (Bertuch and Lundblad 2003). The deletion of the C-terminal 20 amino acid residues of yKu70 resulted in shortened telomeres in *Saccharomyces cerevisiae* while the DNA-end binding activity of the protein remained intact (Driller et al. 2000). YKu70/80 maintained telomere clusters near the nuclear periphery (Laroche et al. 1998; Hediger et al. 2002). YKu70 interacted with the yeast myosin-like protein 2 (Mlp2p), which, together with the yeast myosin-like protein 1 (Mlp1p), formed nuclear-pore-complex extensions (Galy et al. 2000). Mlp2p physically tethered yKu70 to the nuclear periphery, thus docking a nuclear subdomain, the telomeres, to the nuclear periphery.

The yKu proteins concentrated at the telomeres also participated in DNA damage repair in *Saccharomyces cerevisiae* (Gravel et al. 1998; Laroche et al. 1998; Mills et al. 1999). In response to DNA DSBs as a result of DNA damages, the telomere-bound yKu70/80 was delocalized from the telomeres to the sites of DNA DSBs and participated

in DNA DSB repair by NHEJ (Tsukamoto et al. 1997; Mills et al. 1999; Martin et al. 1999). Moreover, attenuated DNA DSB repair capacity and chromosomal aberrations were observed in mice deficient in the telomerase RNA gene, attesting a close relation between telomere and DNA repair (Wong et al. 2000).

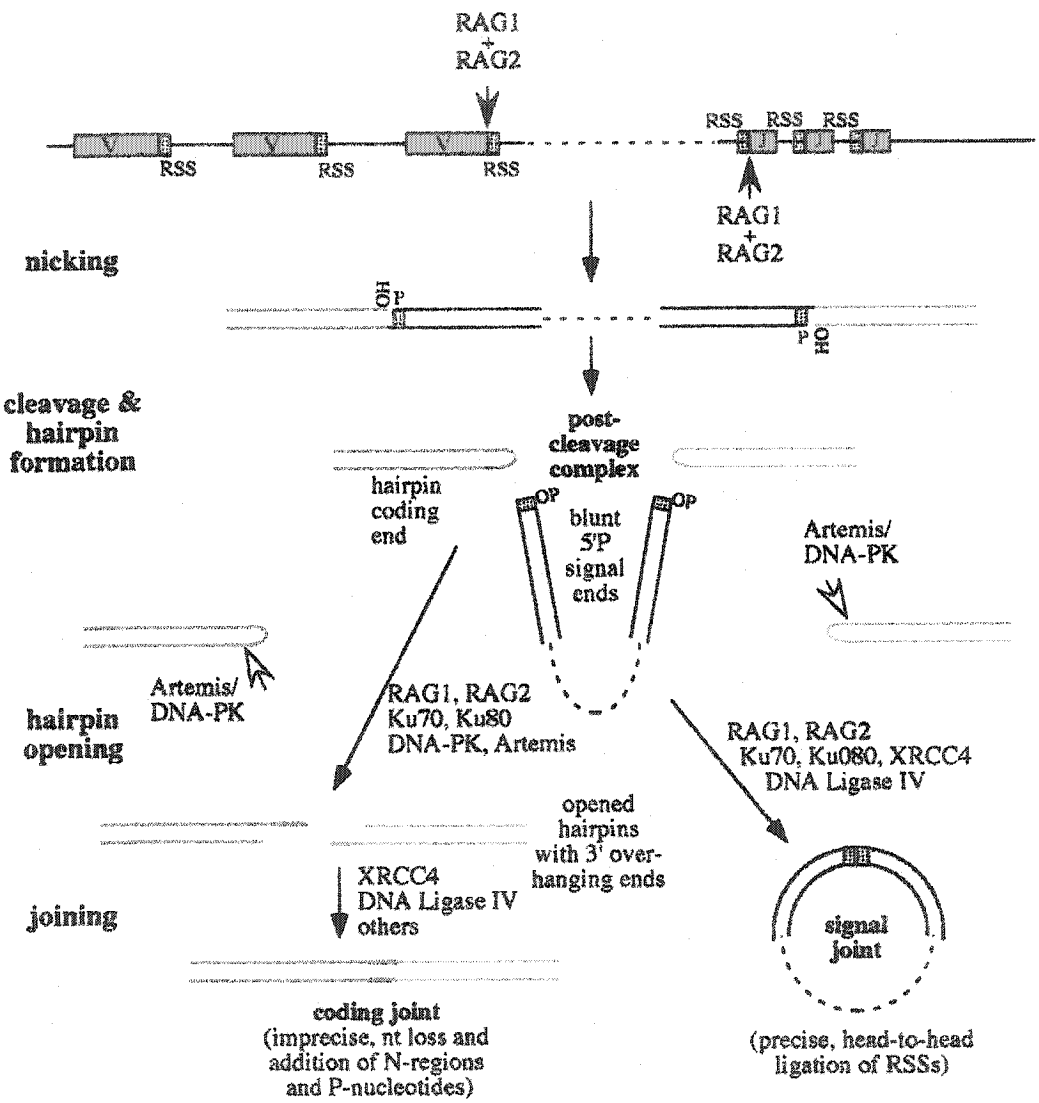
The Ku autoantigen is also essential for maintaining telomere in integrity in humans. Inactivation of a single allele of the Ku80 locus resulted in morphological abnormalities in telomeres such as telomere shortening, chromosomal fusions and translocations (Myung et al. 2004). Ku70/80 is localized to human telomeric DNA repeats independent of DNA-PKcs (Bianchi and de Lange 1999; Hsu et al. 1999). Ku70/80 binding to the telomeric DNA is mediated by its interaction with the telomeric-repeat binding factor 1 (TRF1) (Hsu et al. 2000), which is a negative regulator of telomere length maintenance (van Steensel and de Lange 1997; Bianchi et al. 1997). DNA-PKcs is also involved in maintaining the integrity of telomeres as high levels of telomeric fusions were observed in DNA-PKcs-deficient cells while the length of telomeres remained normal (Gilley et al. 2001; Goytisolo et al. 2001). End-to-end telomeric fusions were also observed in either $Ku70^{-/-}$ or $Ku80^{-/-}$ mouse cell lines though they were not as frequent as those seen in *scid* cells (Bailey et al. 1999).

1.9. The Ku protein is integral to V(D)J recombination during lymphocyte development

1.9.1. V(D)J recombination: an overview

V(D)J recombination is a physiological process whereby the diversity of the immune repertoire is generated by the somatic assembly of the V (variable), D (diversity), and J (joining) gene segments in the Ig variable chain gene in B lymphocytes and the T cell receptor (TCR) gene in T lymphocytes (Figure 3) (Blackwell and Alt 1989; Lewis

Figure 3. The V(D)J recombination pathway. RAG1/2 recognizes and binds to the nonamer of the recombination signal sequences (RSS) and nicks DNA at the coding region 5' of the RSS heptamer. The exposed 3'-hydroxyl group then attacks the other strand with the formation of hairpin-ended coding ends and blunt-ended signal ends (Max et al. 1979; Sakano et al. 1981; Roth et al. 1992b; McBlane et al. 1995). The hairpin-ended coding ends are opened by Artemis following activation by DNA-PKcs (Ma et al. 2002; Rooney et al. 2002). The coding ends and signal ends are subsequently ligated by the NHEJ apparatus including Ku70/80, DNA-PKcs, XRCC4, and DNA ligase IV with the formation of coding joints and signal joints, respectively (Grawunder and Harfst 2001; Gellert 2002). The figure was taken from Schlissel (2002). Refer to the text for a more detailed description.


RAG1
+
RAG2
V
RSS
V
RSS
V
RSS
RSS RSS RSS
J
J
J
RAG1
+
RAG2
nicking
OH
P
P
OH
cleavage &
hairpin
formation
post-
cleavage
complex
hairpin
coding
end
OP
OP
blunt
5'P
signal
ends
Artemis/
DNA-PK
Artemis/
DNA-PK
hairpin
opening
RAG1, RAG2
Ku70, Ku80
DNA-PK, Artemis
RAG1, RAG2
Ku70, Ku080, XRCC4
DNA Ligase IV
opened
hairpins
with 3' over-
hanging ends
XRCC4
DNA Ligase IV
others
joining
signal
joint
coding joint
(imprecise, nt loss and
addition of N-regions
and P-nucleotides)
(precise, head-to-head
ligation of RSSs)

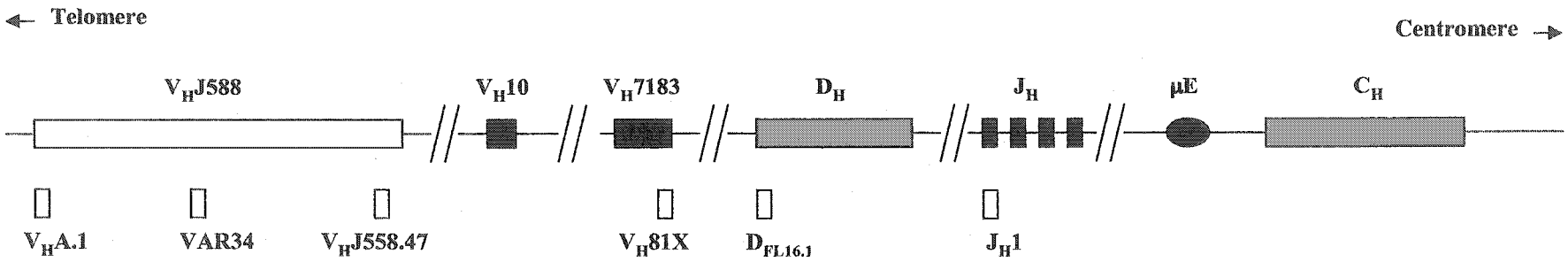
and Wu 1997, 2000; Sadofsky 2001; Bassing et al. 2002; Gellert 2002; Jung and Alt 2004). RAG1 and RAG2 encoded by the *r*ecombination *a*ctivating *g*enes 1 and 2 (*rag-1* and *rag-2*) specifically mediate the V(D)J recombination process (Oettinger et al. 1990). *Rag-1* or *rag-2*-deficient mice have no mature B or T lymphocytes due to their inability to initiate V(D)J recombination (Mombaerts et al. 1992; Shinkai et al. 1992).

RAG1/2 recognizes the highly conserved recombination signal sequences (RSS) flanking the gene segments near the site of rearrangement (Figure 3). These signals are comprised of a conserved heptamer sequence (5' -CACAGTG- 3') and an A/T-rich nonamer sequence (5' -ACAAAAACC- 3') separated by a spacer of 12 or 23 nucleotides (nt) (Max et al. 1979; Sakano et al. 1979). Following its binding to the RSS, RAG1/2 introduces a nick at the coding-heptamer border 5' of the heptameric sequence 5' -CACAGTG- 3' and the exposed 3'-hydroxyl group then attacks the other strand 16 Å away and generates a covalently sealed-hairpin coding end and a 5' phosphorylated blunt signal end (Roth et al. 1992b; McBlane et al. 1995; van Gent 1996b). Artemis, a protein belonging to the metallo-β-lactamase superfamily, resolves the coding hairpins in an ATP-dependent manner (Ma et al. 2002; Rooney et al. 2002). The subsequent rejoining of cleaved DNA ends requires the involvement of the NHEJ apparatus (Taccioli et al. 1993, 1994; Smider et al. 1994; Errami et al. 1996; Gao et al. 1998a; Chang and Brown 1999; Kurimasa et al. 1999).

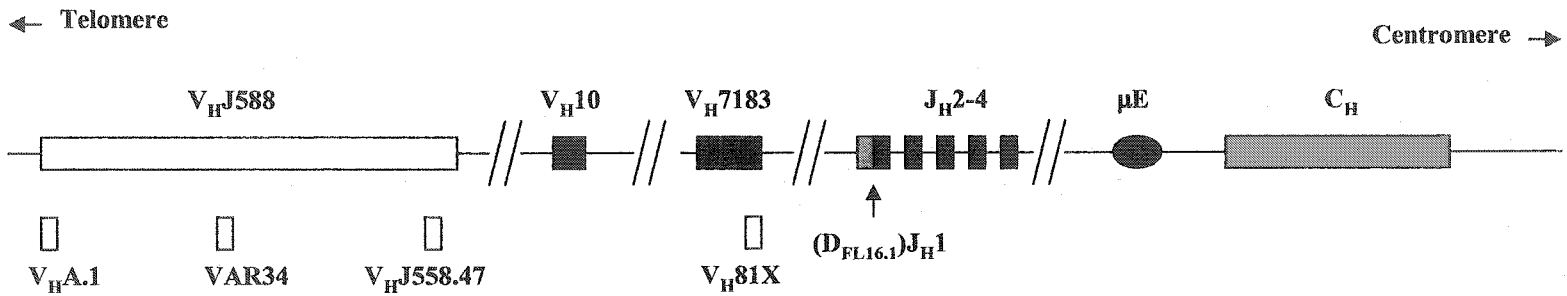
V(D)J recombination occurs between two gene segments flanked by RSSs in which one RSS contains a 12-bp spacer and the other a 23-bp spacer, the so-called 12/23 rule (Figure 4 A-C) (Early et al. 1980; Sakano et al. 1981; van Gent et al. 1996a; Eastman et al. 1996). In the Ig heavy chain, V_H and J_H gene segments are accompanied

Figure 4. Rearrangements of the Ig heavy chain (Igh) locus during B-cell development. The mouse Igh locus extends approximately 3 megabases (mb) and contains at least 134 V_H gene segments organized into 15 families, 12 D_H segments, 4 J_H segments, and 8 C_H genes (Shimizu et al. 1982; Mainville et al. 1996; Chevillard et al. 1998; Zhou et al. 2002). The μ heavy chain gene enhancer (Eμ) is located between the J_H segments and Cμ gene (Gillies et al. 1983). **(A) The germline configuration of the Igh locus on murine chromosome 12.** The V_H7183 family is the most μ-proximal V_H gene family and lies approximately 40 kilobases (kb) upstream from $D_{FL16.1}$ gene segment (Gillies et al. 1983). The V_HJ588 family is the largest family of the V_H genes and is clustered in the D-distal region of the Igh locus. The V_HA.1 gene segment is the 5' most and the V_HJ588.47 gene segment is the 3' most of the V_HJ588 family. The VAR34 gene segment lies midway between the V_HA.1 and V_HJ588.47 gene segments. **(B) D_H to J_H rearrangement.** As in pro-B cells, gene rearrangement has occurred between one D_H segment and one J_H segment with the intervening sequences being deleted. **(C) V_H to D_HJ_H rearrangement.** As in pre-B cells, the Igh locus undergoes V to DJ rearrangement and one V_H segment is brought together to the rearranged D_HJ_H segments with the intervening sequences being deleted.

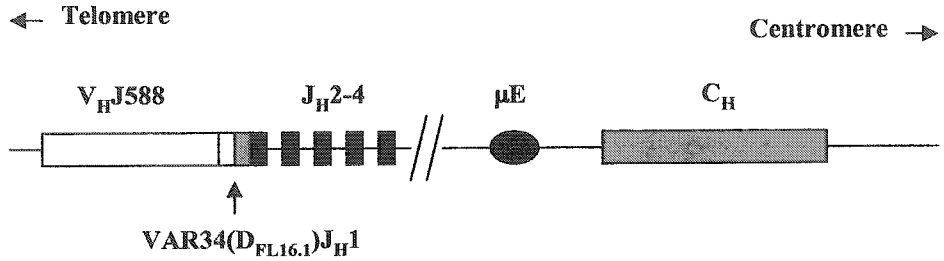
A.


Telomere
Centromere
V_HJ588
V_H10
V_H7183
D_H
J_H
μE
C_H
$V_HA.1$
VAR34
$V_HJ558.47$
V_H81X
$D_{FL16.1}$
J_H1

B.


Telomere
Centromere
V_HJ588
V_H10
V_H7183
J_H2-4
μE
C_H
$V_HA.1$
VAR34
$V_HJ558.47$
V_H81X
$(D_{FL16.1})J_H1$

C.


Telomere
Centromere
V_HJ588
J_H2-4
μE
C_H
VAR34$(D_{FL16.1})J_H1$

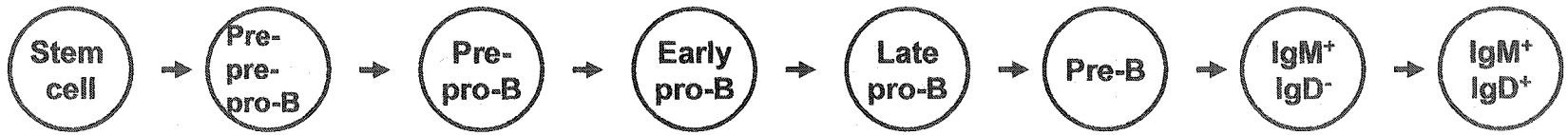
by 23-bp spacers while D_H gene segments are flanked by a 12-bp spacer at either side (Early et al. 1980; Sakano et al. 1981). The 12/23 rule prevents undue V-V, D-D, J-J or V-J recombinations. On the constraint of the 12/23 rule, rejoining can occur between two gene segments with the excision of the intervening sequences between and including the two RSSs, resulting in the formation of the so-called coding joints on the chromosome (Okazaki et al. 1987). In addition, an extrachromosomal circular heptamer-heptamer head-to-head structure forms from the excised product. Rejoining can also occur between two gene segments with the excision of the intervening sequences between the two RSSs with the retention of the RSSs on the chromosome, which results in the formation of the so-called signal joints. Furthermore, rejoining can occur between a signal end and a coding end, forming the so-called hybrid joints (Melek et al. 1998).

1.9.2 V(D)J recombination is a tightly regulated physiologic process

V(D)J recombination is temporally and developmentally regulated (Lieber et al. 1987; Constantinescu and Schlissel 1997; Haines and Brodeur 1998; Morshead et al. 2003). The Ig heavy chain, TCRβ and TCRδ are assembled from V, D and J gene segments while TCRα, TCRγ, Igλ and Igκ are assembled only from V and J gene segments (Lewis et al. 1994). RAG1 and RAG2 are the only lymphocyte-specific proteins required for V(D)J recombination (Oettinger et al. 1990; McBlane et al. 1995). The other proteins involved in the process are components from the NHEJ pathway, including Ku70/80, DNA-PKcs, XRCC4 and DNA ligase IV, and they are present in all cells.

Moreover, the expression of *rag-1* and *rag-2* is temporally regulated during B lymphocyte development (Figure 5) (Li et al. 1993, 1996a; Hardy et al. 1997; Allman et

Figure 5. Stages of B cell development in mice. Development of B cells from the stem cells to IgM^+IgD^+ B lymphocytes is shown. The precursors of the pre-pro-B cells (Hardy fraction A_0), which are $CD43^+$ HSA^- $AA4.1^+$ $B220^-$ $CD4^+$, exhibit progenitor activity for multiple hematopoietic lineages and express little detectable RAG1 or RAG2 (Li et al. 1996b; Allman et al. 1999). The pre-pro-B cells include $CD43^+HSA^-$ $AA4.1^+$ $B220^+$ $CD4^+$ cells (Fraction A_1) and the $CD43^+HSA^-$ $AA4.1^+$ $B220^+$ $CD4^-$ cells (Fraction A_2). The pre-pro-B cells from Fraction A_2 have initiated the expression of the *rag-1* and *rag-2* but they still lack D_H to J_H rearrangement. D_H to J_H rearrangement is initiated in early pro-B cells (Fraction B) coincident with the maximum of expression from the *rag-1* and *rag-2*. The early pro-B cells (Fraction B) and late pro-B cells (Fraction C) contain extensive D_H to J_H rearrangements but lack functional Igh expression (Hardy et al. 1991; Li et al. 1993). The pre-B cells (Fractions C' and D) maintain high levels of expression of the *rag-1* and *rag-2* and have initiated functional V_H to D_HJ_H rearrangement and expressed Ig heavy chains prior to Ig light chains. Fractions E and F contain B cells expressing both Ig heavy and light chains. Expression of *rag-1* and *rag-2* is still detectable at very low levels in IgM^+ IgD^- immature B cells but *rag-1* and *rag-2* remain undetectable in IgM^+ IgD^+ B cells.



Hardy fraction		A_0	A_1/A_2	B	C	C'/D	E	F
RAG1/2	-	-	-/+	+++	+++	+++	+	-
IgH	germline	germline	D-J	D-J	DJ	V-DJ	VDJ	VDJ
IgL	germline	germline	germline	germline	germline	V-J	VJ	VJ

al. 1999). Similarly, the expression of *rag-1* and *rag-2* is also temporally regulated during T lymphocyte development. In human pro-T lymphocytes, TCR-α, TCR-β, TCR-δ, and TCR-γ gene loci are in the germline configuration. Transcription from *rag-1* and *rag-2* is initiated at the pre-T2 stage (Ramiro et al. 1996). TCR-δ and subsequently TCR-γ undergo complete rearrangements at this stage (Ktorza et al. 1995; Blom et al. 1999). Dβ to Jβ rearrangement was also initiated at the pre-T2 stage and proceeds through the $CD4^+CD8\alpha\alpha^+$ pre-T3 stage. Vβ to DβJβ rearrangement is completed immediately prior to the $CD4^+CD8^+$ pre-T3 stage and the downregulation of the expression of *rag-1* and *rag-2* coincides with the expression of a functional TCR-β chain (Hoffman et al. 1996).

In addition, the expression of the *rag-1* and *rag-2* and V(D)J recombination are tightly regulated throughout the cell cycle. The initiation of V(D)J recombination is confined to G_0/G_1 phase (Schlissel et al. 1993). RAG2 also accumulates preferentially in the G_0/G_1 phase and is degraded at the transition from G1 to S phase (Lin and Desiderio 1993, 1994; Lee and Desiderio 1999; Mizuta et al. 2002; Ross et al. 2003). It has been speculated that the accumulation of RAG2 in G1 phase coordinates RSS cleavage with NHEJ activity (Lee and Desiderio 1999).

Though the expression of *rag-1* and *rag-2* is required for V(D)J recombination, it alone is insufficient to induce Ig or TCR gene rearrangement *in vivo* (Stanhope-Baker et al. 1996; Roth and Roth 2000). V(D)J recombination is restricted by the accessibility of the RSSs of various Ig gene segments to RAG1/2 (Yancopoulos and Alt 1985; Kwon et al. 1998, 2000; Cherry and Baltimore 1999; Sikes et al. 2002; Baumann et al. 2003) and is subjected to regulations by a multitude of growth factors, chromatin remodeling complexes or transcriptional factors such as IL-7 or Pax5 (Bain et al. 1994; Zhuang et al.

1994; Lin and Grosschedl 1995; Corcoran et al. 1998; O'Riordan and Grosschedl 1999; Allen et al. 1999; Egawa et al. 2001; Chowdhury and Sen 2001; Hesslein et al. 2003; Hardy 2003).

The Pax5 gene, which encodes the B-cell-specific activator protein (BSAP), is the critical transcription factor that controls and maintains the commitment to the B lymphocyte lineage (Nutt et al. 1999; Mikkola et al. 2002; Hesslein et al. 2003). In the absence of Pax5, B lymphocyte development was shown to be aborted at the stage prior to V_H to D_HJ_H rearrangement of the Igh locus (Urbánek et al. 1994; Nutt et al. 1997, 1999; Thévenin et al. 1998). There was a greater than 50-fold reduction of V_HJ558 gene rearrangements in $Pax5^{-/-}$ mice (Nutt et al. 1997), which normally are the most frequent rearrangements detected in $sIgM^+$ B lymphocytes (Li et al. 1993). However, Pax5 did not appear to regulate the accessibility of the D-distal V_HJ558 region as no differences in histone-acetylation were observed in pro-B cells from $Pax5^{-/-}$ mice and its wild-type counterparts (Hesslein et al. 2003).

The D-distal V_HJ558 family is also responsive to IL-7 signaling, which induces histone hyperacetylation of the region (Chowdhury and Sen 2001, 2003). In IL-7 receptor-deficient mice, V_HJ558 gene rearrangements were barely detectable in $sIgM^+$ B lymphocyte while D_H to J_H and V_H7183 to (D_H) J_H rearrangements occurred normally (Corcoran et al. 1998).

1.9.3. The Ku protein is integral to V(D)J recombination

Ku70/80 plays an indispensable role in V(D)J recombination. The formation of signal joints in $Ku70^{-/-}$ mouse embryonic stem cells was approximately 1% of the level seen in its wild-type counterparts and the signal joints recovered were imprecise (Gu et al.

1997a). The formation of coding joints also decreased from 10- to 100-fold. Ku70$^{-/-}$ mice were also severely impaired in the formation of both coding joints and signal joints (Gu et al. 1997b). Additionally, Ku80-deficient *xrs*-6 cells and V15B fibroblasts are defective in the formation of both signal joints and coding joints in *in vivo* transient V(D)J recombination assays using extrachromosomal recombination DNA substrates (Taccioli et al. 1993, 1994; Smider et al. 1994; Errami et al. 1996). More than 80% of the rarely formed signal joints recovered from these cells were imprecise with deletions from both RSSs; furthermore, no coding joints were recovered. Abnormalities of V(D)J recombination are also found in Ku80$^{-/-}$ mice. More than 80% of thymocytes from Ku80$^{-/-}$ mice were shown to be arrested at the double negative stage and B cells from the bone marrow were arrested at the immature CD43^{+} pro-B cell stage (Nussenzweig et al. 1997). Further, 90% of the rarely formed coding joints in Ku80$^{-/-}$ mice displayed short sequence homology (Bogue et al. 1997).

Ku70/80 participates in V(D)J recombination as a component of the NHEJ apparatus following the cleavage of the RSSs by RAG1/2. Ku70/80 remained associated *in vitro* with cleaved DNA containing the signal ends in a postcleavage complex that also included RAG1/2, DNA-PKcs and XRCC4 (Agrawal and Schatz 1997; Perkins et al. 2002). Signal joint formation was inhibited completely and coding joint formation was reduced 50% when the Ku protein was depleted from the nuclear extracts that were used to supplement RAG1/2 in *in vitro* V(D)J recombination assays (Cortes et al. 1996; Weis-Garcia et al. 1997).

Though Ku70/80 is essential for the formation of both coding and signal joints, somewhat surprisingly, it is apparently not required for the protection of coding ends or

signal ends against degradation by nucleases (Kabotyanski et al. 1998). The coding ends or signal ends detected in Ku80$^{-/-}$ *xrs*-6 cells were blunt and full length (Han et al. 1997) and the signal ends remained intact in Ku70$^{-/-}$ mouse thymocytes (Gu et al. 1997b). Furthermore, hairpin coding ends remained unprocessed and blunt signal ends accumulated in Ku80$^{-/-}$ mice, suggesting that Ku80 was not necessary for the protection or stabilization of coding ends or signal ends (Zhu et al. 1996). However, signal ends in *xrs-6* cells and Ku80$^{-/-}$ thymocytes were present in amounts similar to their wild-type counterparts where the processing of signal ends remained intact (Zhu et al. 1996; Han et al. 1997). In addition, Dδ1-Dδ2 signal ends and junctions were not detected in Ku80$^{-/-}$ thymocytes whereas they were found in wild-type or *scid* thymocytes (Nussenzweig et al. 1996).

Ku70/80 is also required for the insertion of non-templated nucleotides to the coding ends by the lymphocyte-specific enzyme, the terminal deoxynucleotidyl transferase (TdT), which further diversifies the specificity of the immune repertoire by creating non-templated (N)-region diversity. Both subunits of the Ku protein are associated with TdT, probably targeting it to the cleaved coding ends (Mahajan et al. 1999; Purugganan et al. 2001). The rare coding joints formed in Ku80$^{-/-}$ mice exhibited a lack of N-region diversity (Bogue et al. 1997).

DNA-PKcs-deficient cells exhibit a distinct phenotype from that of Ku70- or Ku80-deficient cells. In murine *scid* pre-B cells, signal joints were formed at approximately normal efficiency although half of the signal joints formed exhibited deletions from one or both signals while no formation of coding joints was observed (Lieber et al. 1988). In DNA-PKcs-deficient malignant glioma cell lines M059J, coding

joint formation was blocked while signal joint formation occurred at the wild-type levels with most of the signal joints formed being precise in *in vivo* transient extrachromosomal recombination assays (Kulesza and Lieber 1998). DNA-PKcs-null mice were able to carry out signal end rejoining whereas coding joint formation was defective (Gao et al. 1998b; Taccioli et al. 1998; Kurimasa et al. 1999). The impaired formation of coding joints in *scid* mice was due to a failure to open hairpin coding ends (Roth et al. 1992b; Chang and Brown 1999).

Artemis-deficient mice were also defective in coding joint formation with the accumulation of unresolved hairpin intermediates in thymocytes (Rooney et al. 2002). DNA-PKcs and Artemis formed a complex *in vitro* and DNA-PKcs activated Artemis for hairpin opening activity in an ATP-dependent manner (Ma et al. 2002). DNA ligase IV-deficient MEFs exhibited similar phenotypes to those of Ku70- or Ku80-deficient cells (Frank et al. 1998). No signal joints and only negligible level of coding joints were recovered in *in vivo* transient V(D)J recombination assays. DNA polymerase μ is also involved in V(D)J recombination (Mahajan et al. 2002; Bertocci et al. 2003). DNA polymerase μ-deficient mice displayed impaired Ig light chain rearrangement in the bone marrow B cells undergoing IgM^- to IgM^+ transition, exhibiting extensive nibbling of coding joints.

1.10. The Ku autoantigen binds to specific DNA sequences and is involved in the regulation of transcription and DNA replication

Though Ku70/80 has prominent nonspecific DNA-end binding activity and is integral to the NHEJ pathway for maintaining genomic stability, it has also been

implicated in transcriptional regulation and control of DNA replication through specific association with regulatory elements in various gene loci either independently or in association with other proteins (Messier et al. 1993; Giffin et al. 1996, 1997; Aoki et al. 1998; Ruiz et al. 1999; Schild-Poulter et al. 2001; Willis et al. 2002). The various specific DNA sequences that have been proposed for Ku70/80 or its associated supramolecular complexes are listed in Appendix III.

Our laboratory was the first to demonstrate convincingly that Ku can bind directly to specific DNA sequence, i.e. the negative regulatory element 1 (NRE1) in the long terminal repeat (LTR) of the mouse mammary tumor virus (MMTV), in the absence of DNA nicks, gaps or other structural transitions (Giffin et al. 1996, 1997). The MMTV LTR contains a complex array of DNA binding sites that interact with the steroid hormone receptors and transcriptional regulators including the glucocorticoid receptor (GR) and Oct-1 (Payvar et al. 1983; Bruggemeier et al. 1991; Truss et al. 1995). NRE1 is polypurine in nature and contains a 5'-GAGAAAGA-3' repeat. Direct binding by Ku70/80 to NRE1 was observed by using DNA microcircles free of DNA ends, nicks, gaps or structural features (Giffin et al. 1996). In addition, the binding of Ku70/80 to NRE1 was markedly preferred over its binding to DNA ends with a Kd of 0.84 ± 0.24 X 10^{-9} M for NRE1 (Giffin et al. 1996, 1997).

Moreover, the binding of Ku70/80 to NRE1 was not static. UV-crosslinking studies indicated that Ku70 interacted with NRE1 followed by a Mg^{2+}-dependent structural transition that led to the contact of Ku80 with DNA (Giffin et al. 1994, 1997; Giffin and Haché 1995; Torrance et al. 1998; Schild-Poulter et al. 2003a). Following its direct binding to NRE1, the Ku autoantigen recruited DNA-PKcs to the site and activated

its kinase activity, which subsequently phosphorylated GR and Oct-1 *in cis*, resulting in a marked repression of glucocorticoid-mediated activation of MMTV transcription (Giffin et al. 1996). Therefore, the repression of MMTV transcription by DNA-PKcs appeared to be dependent upon the induction of a structural transition upstream of NRE1 that coincided with the contact of DNA by Ku80 and required the activation of DNA-PKcs (Giffin et al. 1997, 1999). Furthermore, biochemical evidence indicated Ku70/80 conformation and accessibility differs when bound to DNA-ends, specific DNA sequences and structured ssDNA (Turchi and Henkels 1996; Giffin et al. 1999; Turchi et al. 2000; Soubeyrand et al. 2001).

Another disparate sequence-specific binding site for the Ku protein is the A3/4 sequence, a sequence homologous to the various mammalian replication origins (*ors*) (Appendix III). The binding of Ku70/80 to A3/4 was able to support *in vitro* DNA replication from the sequence, implicating the Ku protein in DNA replication (Ruiz et al. 1999). In addition, the deletion of Ku80 from MEFs reduced the level of DNA replication by approximately 70% compared with that of its wild-type counterparts (Novac et al. 2001). Furthermore, Ku70/80 was associated with *ors8* and *ors12 in vivo* at G1/S phase in CV-1 cells and with the dihydrofolate reductase (DHFR) replication origin-containing sequence in CHO K1 cell line (Novac et al. 2001; Matheos et al. 2003).

The Ku protein is also implicated in the regulation of transcription from the rat ribosomal RNA gene by RNA polymerase I (Hoff et al. 1994; Datta et al. 1995; Hannan et al. 1999; Yamamoto et al. 2000). Ku70/80 was involved in the initiation of transcription from the ribosomal RNA gene in closed circular DNA templates, which was markedly inhibited with anti-Ku70/80 antibodies (Hoff et al. 1994). The Ku protein was

shown to bind to the promoter region of the rat ribosomal RNA gene *in vitro* (Hoff et al. 1994; Datta et al. 1995; Hannan et al. 1999; Yamamoto et al. 2000). Furthermore, Ku70/80 was co-purified with a preassembled RNA polymerase I complex that also contained transcription factors and other DNA repair proteins (Zhang and Jacob 1990). It has been proposed that the Ku protein may regulate transcription from the ribosomal RNA gene by displacing negative transcription factors from the promoter region (Maldonado et al. 1996).

The sequence-specific binding activity of Ku70/80 was also implicated in the transcriptional regulation of heat shock response (HSR). During HSR, the trimeric form of heat shock transcription factor 1 (HSF1) binds to the heat shock element (HSE) and activates transcription from heat shock genes by RNAP II (Huang et al. 1997). HSF1 also formed a stable complex with Ku70/80 and, to a lesser extent, DNA-PKcs. The Ku protein was proposed to bind specifically to HSE and displace HSF1 from DNA (Kim et al. 1995; Tang et al. 2000); however, direct binding of the Ku protein to HSE was not observed when circular DNA containing HSE was employed (Giffin et al. 1997).

Furthermore, the Ku protein is involved in class switch DNA recombination (CSR) (Zelazowski et al. 1997; Manis et al. 1998; Casellas et al. 1998) in which a region-specific DNA recombination event replaces the Cμ Ig heavy chain with one of the other downstream heavy chain genes such as Cγ3, Cγ1, Cγ2α, Cγ2β, Cε or Cα (Honjo et al. 2002; Manis et al. 2002). Ku70-deficient B cells failed to undergo CSR to other classes of Ig (Manis et al. 1998; Casellas et al. 1998). In resting human IgM^+ IgD^+ B cells, the Ku protein formed a complex with the HoxC4 and Oct-1 homeodomain proteins (Schaffer et al. 2003). Binding by the Ku70/80-HoxC4-Oct-1 complex to the switch (S) regulatory

ATTT elements (SREs) in the Iγ and Iε promoters repressed CSR to Cγ and Cε, which was relieved in response to CD40 signaling. The binding specificity of the Ku70/80-HoxC4-Oct-1 complex probably is provided by HoxC4, which has been shown to bind to the homeodomain-specific ATTT/A motif (Saleh et al. 2000; Galant and Carroll 2002).

A multitude of other specific DNA sequences have also been proposed for the Ku protein (Appendix III); however, no apparent consensus sequences have been inferred from these diverse Ku70/80-specific DNA binding sites. Several factors have been confounding the study of sequence-specific DNA binding activity by Ku70/80. The Ku protein is known to bind nonspecifically to duplex DNA ends in a bind-and-slide mechanism (Mimori et al. 1986; DeVries et al. 1989; Paillard and Strauss 1991; Yaneva et al. 1997) and its apparent sequence-specific binding activity may arise from its stalled movement at internal sites on DNA following its translocation from DNA ends (Giffin et al. 1997). In addition, Ku70/80 interacts with various cellular proteins (Schild-Poulter et al. 2001; Sartorius et al. 2000; Lim et al. 2004) and binds to specific DNA sequences in an oligomeric protein complex in which the DNA binding specificity either originates from other components in the complex or arises from the combinatorial specificity of the entire oligomeric protein complex (Kim et al. 1995; Genersch et al. 1995; Chung et al. 1996; Huang et al. 1997; Nishishita et al. 1998; Ting et al. 1998; Tang et al. 2000; Woodard et al. 2001). Finally, because of its abundance in the cell and its apparent avidity for DNA ends, Ku70/80 can potentially contaminate other DNA-specific binding proteins (Quinn and Farina 1991; Inoue et al. 1997). Therefore, the demonstration of the sequence-specific DNA binding activity of Ku70/80 requires the exclusion of Ku70/80

entry to internal sites of DNA molecules from DNA ends, nicks or gaps (Giffin et al. 1996, 1997; Bliss and Lanes 1997).

1.11. In vitro evolution of preferred DNA binding sites for proteins by exponential enrichment

In vitro selection of high-affinity nucleic acid ligands for a DNA binding protein originated with the experiment by Oliphant et al. (1989) who developed a method of identifying high-affinity DNA binding sites for the yeast transcriptional activator protein, GCN4, from a pool of random ds oligonucleotides. After the coupling of GCN4 to the Sepharose columns, a pool of random ds oligonucleotides was passed over the columns. Elution of oligonucleotides bound to the GCN4-coupled columns revealed the presence of a total of 43 oligonucleotides, 40 of which contained a palindromic heptameric sequence TGA(C/G)TCA.

Though highly selective in nature, the experiment by Oliphant et al. was not reiterative. It was Tuerk and Gold (1990; Gold et al. 1997) who turned the random selection method into a repetitive selection and amplification process. They generated an initial pool of 4^8 random ss oligonucleotides by randomizing an eight-base region of a RNA molecule that was known to interact with T4 DNA polymerase. The random pool was subjected to selection by T4 DNA polymerase *in vitro*. The selected pool of ss oligonucleotides was amplified and subjected to further rounds of *in vitro* selection. After reiterative rounds of selection and amplification, the final selected pool of ss oligonucleotides contained high affinity binding sites for T4 DNA polymerase. Tuerk

and Gold termed the reiterative selection and amplification process the systemic evolution of ligands by exponential enrichment (*SELEX*).

Almost at the same time, Blackwell and Weintraub (1990) developed the same methodology and identified a consensus sequence, CANNTG, the E-box motif, as the selected and amplified binding site (SAAB) for the basic HLH proteins, MyoD and E2A. Pollock and Treisman (1990) also developed the same method in which they employed crude cellular extracts for the evolution of preferred DNA binding sites for a cellular protein or a supramolecular complex followed by immunoprecipitation with antibodies against the cellular protein of interest to separate the selected from unselected DNA sequences. Their method, which they called cyclical amplification and selection of targets (CASTing), allows the selection of high affinity DNA binding sites for a cellular protein or an oligomeric protein complex in the cell. The selection of affinity-enriched sequences from combinatorial libraries has since been widely used for the functional site recognition of proteins (Yoo and Dynan 1998; Bianchi et al. 1999; Burden and Osheroff 1999; Anwar et al. 2000; Zheng 2000 et al.; Ehret et al. 2001), multiple protein complexes (Wadman et al. 1997; Chalkley and Verrijzer 1999; Soldaini et al. 2000) and supramolecular targets (Morris et al. 1998; Blank et al. 2001; Wang et al. 2003).

1.12. The principal objectives of the current research project

Ku70/80 is a prolific DNA binding protein with distinct modes of nonspecific DNA end binding activity and sequence-specific DNA binding activity (Mimori et al. 1986; DeVries et al. 1989; Paillard and Strauss 1991; Blier et al. 1993; Giffin et al. 1996, 1997) and its multiple cellular functions are directly related to its versatile DNA binding

activities. Our laboratory has shown that Ku70/80 binds to NRE1 and other putative specific sequences with different affinities (Giffin et al. 1997). We have also found that the two subunits of the Ku protein were crosslinked to the upper or lower strand of NRE1 differentially in the presence or absence of Mg^{2+} and ATP (Giffin et al. 1994, 1999; Giffin and Haché 1995; Torrance et al. 1998). However, there has been no detailed direct comparative study of Ku70/80 binding to different specific DNA binding sites, which is an important question to address as sequence-specific binding activities of Ku70/80 are implicated in transcriptional regulation (Giffin et al. 1996; Camara-Clayette et al. 1999; Willis et al. 2002) and control of DNA replication (Novac et al. 2001; Matheos et al. 2002, 2003). Furthermore, Ku70/80 was reported to bind to a variety of specific DNA sequences (Appendix III and the references therein); the diversity of these putative Ku70/80-specific DNA binding sites lends itself to the possibility that there exist yet unidentified novel classes of specific DNA binding sites for Ku70/80, which, through direct association with Ku70/80, regulate important physiological processes in the cell.

The overall goal of the present research project was to identify novel specific DNA binding sites for the Ku autoantigen and to link DNA sequence-specific binding by the Ku protein to specific cellular functions.

The first principal objective of the present research project was to directly compare DNA binding activities of Ku70/80 to disparate specific DNA sequences, NRE1 and A3/4, through reciprocal competitive electrophoretic mobility shift assays (EMSAs) and protein-DNA crosslinking studies. The comparative study would constitute the first detailed investigation of Ku70/80 binding to distinct specific sequences and should reveal subtle differences in specific DNA binding activities of the

Ku protein in terms of DNA binding affinities and differential contact of DNA by its two subunits.

The second principal objective of the current research project was to identify novel classes of Ku70/80-specific DNA binding sites by *SELEX* with recombinant human Ku70/80, and investigate the functional implications of the interaction of Ku70/80 with the specific DNA binding sites evolved through *SELEX*. This study would constitute the first direct purposeful identification *in vitro* of Ku70/80-specific DNA binding sites from a random array of distinct sequences, which, given the important roles of Ku70/80 in transcriptional regulation and control of DNA replication, would provide insight into novel functions of Ku70/80 in cellular processes that have been hitherto unappreciated. Moreover, the identification of preferred DNA binding sites in the selected DNA sequences itself should suggest sequence discrimination or preferences by Ku70/80 over random sequences, which would constitute further proof for the presence of sequence-specific binding activity in the Ku protein.

2. MATERIALS AND METHODS

2.1. Oligonucleotides, plasmids and vectors

2.1.1. Oligonucleotides

The oligonucleotides used in the experiments were purified on 8 to 14% nondenaturing polyacrylamide gels as needed and dissolved in TE buffer (10 mM Tris-HCl, 0.5 mM EDTA, pH 7.4). Their sequences are listed in the Appendix IV.

2.1.2. Plasmids

Plasmid pBR322-A3/4 was previously described (Frappier and Zannis-Hadjopoulos 1987; Ruiz et al. 1999) and kindly provided by Dr. M. Zannis-Hadjopoulos (McGill University, Montreal, Quebec, Canada). Plasmid pBS-NRE1 was previously described (Schild-Poulter et al. 2003a) and contained a single copy of NRE1 cloned into the *Sma*I site of plasmid pBluescript SK II (+) (pBS) (Stratagene). Plasmid pBS-H7 is described elsewhere in the text and contains a 62 bp insert with a single copy of the RSS heptamer cloned into the *Sma*I site of pBS. The extrachromosomal recombination DNA substrate pJH200, which was generously provided by Dr. M. Gellert (National Institute of Health, Bethesda, MD, USA), was previously described (Hesse et al. 1987; Lieber et al. 1988) and is detailed elsewhere in the text and. Plasmids pBS, pBS-NRE1, pBS-H7 and pJH200 were propagated in *Escherichia (E.) coli* DH5α while pBR322-A3/4 was grown in *E. coli* HB101. Large scale plasmid DNA was prepared by using a standard alkaline lysis protocol followed by cesium chloride/ethidium bromide gradient centrifugation (Maniatis et al. 1982).

2.1.3. Expression vectors

The baculovirus expression vectors VBB2-86Ku and VBB2-Kup70tH^6 were previously described (Ono et al. 1994) and generously provided by Dr. J.D. Capra (University of Texas Southwestern School of Medicine, Dallas, TX, USA). VBB2-86Ku encoded the Ku80 subunit and VBB2-Kup70tH^6 encoded a hexahistidine-tagged Ku70 subunit of the human Ku autoantigen. These expression vectors were propagated in *Spodoptera frugiperda* cell line *Sf9* (ATCC CRL1711) as described elsewhere in the text. The mammalian expression vectors pRAG1 (M2CD7) and pRAG2 (R2RCD2) encoding murine full-length RAG1 and RAG2, respectively, were previously described and kindly provided by Dr. M.A. Oettinger (Harvard University, Boston, MA). These expression vectors were grown in *E. coli* MC1061/P3 (Invitrogen) and large scale plasmid DNA was prepared by using a standard alkaline lysis protocol followed by cesium chloride/ethidium bromide gradient centrifugation (Maniatis et al. 1982).

2.2. Preparation of DNA substrates

2.2.1. Construction of the random library of ds oligonucleotides for SELEX by recombinant Ku70/80

The strategy for constructing the random library of ds oligonucleotides for DNA binding sites selection by recombinant Ku70/80 is described elsewhere in the text. The reverse primer P1 was radiolabeled at 37°C for 30 min with [γ-^{32}P]-ATP by T4 polynucleotide kinase (New England BioLabs) in a 20-µl reaction containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 1 mM ATP and 25 µg/ml bovine serum albumin (BSA) and purified using ProbeQuant^{TM} G-50 Micro columns

(Amersham Biosciences). As aforementioned, the ss 62-nt RND (ssRND) contains a central stretch of 18 random nt flanked by 22 nt of constant priming sequences at either side. After the annealing of [γ-^{32}P]-ATP labeled P1 (3 pmol) to the ssRND (1 pmol), second strand synthesis was carried out by extension with the exo$^{-/-}$ large Klenow fragment of DNA polymerase I (New England BioLabs) at 37°C for 1 h in a 50-µl reaction containing 10 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$ and 7.5 mM DTT in the presence of 33 µM all 4 dNTPs. Radiolabeled dsRND was subsequently purified on an 8% nondenaturing polyacrylamide gel and dissolved in 20 µl of TE buffer (pH 7.4).

2.2.2.. Construction of plasmid pBS-H7

Plasmid pBS (1 µg) was linearized by digestion with the endonuclease *Sma*I (New England BioLabs) at 25°C for 30 min in a 20-µl reaction containing 50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate and 1 mM DTT. Following purification by electrophoresis on 1% agarose gel, linearized pBS was dephosphorylated at 37°C for 1 h with calf intestinal alkaline phosphatase (CIP) (New England BioLabs) in a 20-µl reaction containing 100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$ and 1 mM DTT. The 62-bp insert containing a single copy of the RSS heptamer (100 ng) was phosphorylated by T4 polynucleotide kinase (New England BioLabs) and purified using ProbeQuant™ G-50 Micro columns (Amersham Biosciences). Fifty ng of the 62-bp insert were ligated into the *Sma*I site of pBS (200 ng) by T4 DNA ligase (New England BioLabs) at 16°C for 12 h in a 10-µl reaction containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 25 µg/ml BSA. After heat inactivation at 65°C for 10 min, 1/10 of the ligation reaction products were transformed into *E. coli* DH5α by electroporation using an *E. coli* Pulser

(Bio-Rad Laboratories). Plasmid DNA from transformed *E. coli* was prepared by a standard alkaline lysis protocol (Maniatis et al. 1982). The presence of the 62-bp insert in plasmids was initially assessed by restriction with the endonuclease *Sma*I. Plasmids (1 μg) resistant to digestion by *Sma*I were further digested at 37°C for 1 h with the endonucleases *Pst*I and *Not*I in a 20-μl reaction containing 100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT and 100 μg/ml BSA. Restriction with both *Pst*I and *Not*I generated a 92-bp fragment containing the 62-bp insert from positive plasmids versus a 30-bp fragment from pBS. The samples were resolved on 8% nondenaturing polyacrylamide gels and visualized with PhosphorImager and IMAGEQUANT software (Molecular Dynamics) following staining with ethidium bromide. The plasmid containing the 62-bp insert was sequenced with T7 primer using the T7 Sequenase™ DNA Sequencing Kit according to the manufacturer's recommended protocol (USB) and named pBS-H7.

2.2.3. Preparation of DNA microcircles for EMSAs

Covalently closed DNA microcircles free of nicks or other structural features were prepared as previously described (Giffin et al. 1996, 1997) with some modifications. Briefly, plasmids pBS or pBS-H7 were digested with the endonucleases *Not*I and *Bgl*I in a 20-μl reaction containing 100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT and 100 μg/ml BSA. The 266-bp *Not*I-*Bgl*I fragment from pBS and the 328-bp *Not*I-*Bgl*I fragment from pBS-H7 were purified by electrophoresis on a 1.2% agarose gel and radiolabeled with [α-^{32}P]-dCTP by end-filling with the exo$^{-/-}$ large Klenow fragment of DNA polymerase I (New England BioLabs) followed by purification by electrophoresis on a 4% nondenaturing polyacrylamide gel. Five pmol each of the [α-^{32}P]-dCTP labeled

fragments were incubated at 4°C for 36 h for intramolecular circularization in a 1-ml reaction containing 80,000 units of T4 DNA ligase (New England BioLabs), 200 units of T4 RNA ligase (New England BioLabs), 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 25 μg/ml BSA. The intramolecular ligation reaction products were digested by Exonuclease III (MBI Fermentas) at 37°C for 30 min in a 20-μl reaction containing 6.6 mM Tris-HCl (pH 8.0) and 0.6 mM $MgCl_2$ followed by digestion at 30°C for 30 min with the exonuclease BAL-31 (New England BioLabs) in a 20-μl reaction containing 600 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 12 mM $MgCl_2$ and 12 mM $CaCl_2$. The intramolecular ligation products resistant to nuclease digestion were purified by electrophoresis on a 4% nondenaturing polyacrylamide gel and dissolved in distilled H_2O for use in EMSAs. The labeling efficiency of covalently closely DNA microcircles was 100% as the formation of DNA microcircles depended upon the incorporation of [α-^{32}P]-dCTP.

2.2.4. Preparation of DNA substrates for in vitro DNA cleavage assays

Extrachromosomal recombination DNA substrate pJH200 is described elsewhere in the text. Five μg of pJH200 were digested at 37°C for 30 min with the restriction endonucleases *Sap*I and *Nco*I (New England BioLabs) in a 20-μl reaction containing 50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate and 1 mM DTT. Following purification by agarose gel electrophoresis, the *Sap*I-*Nco*I fragments (500 ng), which contain the VκL8-Jκ1 gene segments of Igκ light chain, were radiolabeled by end-filling with [α-^{32}P]-dCTP by the large Klenow fragment of DNA polymerase I (New England BioLabs) and purified by agarose gel electrophoresis and dissolved in H_2O at a concentration of 25 ng/μl.

2.3. Cells, infections and transfections

2.3.1. Cell culture

The *Sf9* cells were grown in TNM-FH made from Grace's insect medium TNM (Gibco–BRL) supplemented with yeastolate (3.3 mg/ml), lactalbumin hydrolysate (3.3 mg/ml) and 10% heat inactivated fetal bovine serum (FBS) (JRH Biosciences). They were seeded at a density of 8 X 10^5 cells/cm^2 in a 10-cm tissue culture dish and maintained at 27°C, and passed at confluency in 1 to 3-5 dilutions. Human embryonic kidney 293T/17 cells (ATCC CRL-11268), CHO fibroblasts V79 (ATCC CCL-93) and $Ku80^{-/-}$ V15B (ATCC CRL-2349) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) supplemented with 10% FBS (JRH Biosciences) at 37°C and were passed at confluency in 1 to 4-8 dilutions for 293T cells, 1 to 6-14 dilutions for V79 fibroblasts and 1 to 8-12 dilutions for V15B fibroblasts.

The adult BALB/c bone marrow-derived Abelson murine leukemia virus (A-MuLV)-transformed late pro-B cell line 220-8 and the fetal liver-derived A-MuLV-transformed RAG-$2^{-/-}$ pro-B-cell line 63-12 were previously described (Alt et al. 1981; Shinkai et al. 1992) and kindly provided by Dr. M.S. Schlissel (University of California, Berkley, CA, USA) and Dr. F.W. Alt (Harvard University, Boston, MA, USA). These cells were cultured in RPMI-1640 (Gibco–BRL) with 5% FBS (JRH Biosciences) in the presence of 50 μM β-mercaptoethanol (β-ME) (Sigma) and maintained at a density between 1 X 10^5 and 1 X 10^6 cells/ml. For the induction of the differentiation of RAG-$2^{-/-}$ pro-B-cell line 63-12, these cells were incubated with 10 μM ST1571 (Novartis), an inhibitor of the *Abl* kinase, for 16 h as described by Muljo and Schlissel (2003). Human

Jurkat T cells were maintained in RPMI-1640 (Gibco–BRL) with 10% FBS (JRH Biosciences) and 2 mM L-glutamine at a density between 1 X 10^5 and 1 X 10^6 cells/ml.

2.3.2. Propagation of the baculovirus expression vectors VBB2-86Ku and VBB2-Kup70tH^6 in Sf9 cells

For the propagation of the baculovirus expression vectors, *Sf9* cells were seeded at a density of 8 X 10^6 cells/cm^2 and allowed to attach for 1 h, and then gently washed with serum-free TNM. Thereafter, the baculovirus expression vectors, VBB2-86Ku and VBB2-Kup70tH^6, were separately infected into *Sf9* cells at a multiplicity of infection (MOI) of 1. One h after incubation, the inocula were discarded and 10 ml of fresh TNM-FH were added. Three days following incubation at 27°C, the media were collected and, after centrifugation at 1000 X *g* for 5 min, the supernatant was collected and stored at -80°C for future use.

The infection of *Sf9* cells with the baculovirus expression vectors, VBB2-86Ku and VBB2-Kup70tH^6, was carried out as previously described (Ono et al. 1994). *Sf9* cells were seeded at a density of 8 X 10^5 cells/cm^2 and allowed to attach for 1 h, and then gently washed with serum-free TNM. Thereafter, VBB2-86Ku and VBB2-Kup70tH^6 were co-infected into *Sf9* cells at an MOI of 5 to 10. Following incubation at 27°C for 1 h, the baculovirus innoculum was aspirated and discarded and fresh TNM-FH was added. Three days post-infection, *Sf9* cells were scraped off, washed 3 times with ice-cold 1 X phosphate-buffered saline (PBS) and stored on ice for the subsequent purification of recombinant Ku70/80.

2.3.3. Transient transfection of 293T cells

For the preparation of nuclear extracts from 293T cells co-expressing RAG1 and RAG2 for *in vitro* DNA cleavage assays and *in vitro* V(D)J recombination assays, 293T cells were co-transfected at a confluency of 60 to 70% with 2 μg each of pRAG1 and pRAG2 in 10-cm dishes using FuGene 6 Transfection Reagent according to the manufacturer's instructions (Roche Molecular Biochemicals). FuGene 6 Transfection Reagent was used in a ratio of 6 μl of the reagent to 1 μg of plasmid DNA. Briefly, 24 μl of FuGene 6 Transfection Reagent were added to 200 μl of serum-free DMEM and, after gently mixing, 2 μg each of pRAG1 and pRAG2 were added followed by incubation for 40 min at room temperature. Thereafter, the mixture was transferred dropwise to the tissue culture dish followed by 2 days of incubation at 37°C.

For plasmid immunoprecipitations, 293T cells were transfected as described above with 2 μg of pJH200. Following transfection, 293T cells were washed 3 times with DMEM and incubated at 37°C for 2 days.

2.3.4 Transient transfection of V79 and V15B fibroblasts

For the preparation of nuclear extracts for *in vitro* DNA cleavage assays from V79 or V15B fibroblasts co-expressing RAG1 and RAG2, V79 or V15B fibroblasts were transiently co-transfected at a confluency of 60 to 70% with 2 μg each of pRAG1 and pRAG2 in 10-cm dishes using ExGen 500™ according to the manufacturer's instructions (MBI Fermentas). Briefly, plasmid DNA was dissolved in sterile 150 mM NaCl at a ratio of 1 μg DNA per 100 μl of the salt solution. The mixture was gently vortexed for 10 sec and centrifuged at 800 X *g* for 5 min. ExGen500™ was added at a ratio of 8 μl of ExGen 500™ per 1 μg of plasmid DNA. After vortexing for 10 sec, the mixture was incubated at

room temperature for 10 min. V79 or V15B fibroblasts were rinsed twice with 1 X PBS and once with serum-free DMEM and were maintained in serum-free DMEM for 4 h after the addition of the mixture containing ExGen 500^{TM} and plasmid DNA. Thereafter, V79 or V15B fibroblasts were grown in DMEM supplemented with 10% FBS at 37°C for 2 days.

For transient *in vivo* V(D)J recombination assays, V79 or V15B fibroblasts were transfected as described above with 2 μg of pJH200 or co-transfected with 2 μg each of pRAG1, pRAG2 and pJH200 followed by incubation at 37°C for 18 to 48 h as needed.

2.4. Preparation of recombinant proteins, nuclear extracts and cell extracts

2.4.1. Purification of recombinant Ku70/80 from Sf9 cells.

Recombinant Ku70/80 was purified from infected *Sf9* cells by using the TALON™ resin according to the supplier's instructions with modifications (Clontech). Briefly, 100 μl of slurry TALON™ resin beads (50% w/v) were transferred to a 1.5-ml sterile Eppendorf tube and centrifuged at 700 X *g* for 2 min. After the removal of the supernatant, the 50% slurry beads were washed twice with 10 bed volumes (vol) of pre-chilled Extraction/Wash Buffer (pH 7.0) containing 50 mM sodium phosphate, 0.3 M NaCl, 2 mM DTT, 0.1% Nonidet P-40, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 mM phenyl-methylsulfonyl fluoride (PMSF). After spinning at 700 X *g* for 5 min to pellet the resin beads, the supernatant was discarded and the resin beads were stored on ice ready for use.

The harvested *Sf9* cells were centrifuged at 700 X *g* for 5 min and washed 3 times with 5 ml of ice-cold 1 X PBS. The *Sf9* cell pellets were then resuspended in 2 ml of pre-

chilled Extraction/Wash Buffer (pH 7.0). The *Sf9* cells were sonicated 3 times for 15 sec with a 30 sec interval on ice and centrifuged at 12,000 X *g* for 20 min at 4°C to pellet any insoluble material. The supernatant was subsequently transferred to the tube containing the pre-prepared TALON™ resin and rotated on the wheel at 4°C for 20 min. After centrifugation at 12,000 X *g* for 1 min to pellet the protein-resin complexes, the supernatant was removed and the pellet was washed twice with Extraction Buffer (pH 8.0) containing 50 mM sodium phosphate, 0.3 M NaCl, 2 mM DTT, 0.1% Nonidet P-40, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 mM PMSF. After the removal of the supernatant, 50 μl of Elution Buffer (pH 7.0) containing 150 mM imidazole, 50 mM sodium phosphate, 0.3 M NaCl, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM PMSF were added to the protein-resin complexes. After brief vortexing and centrifugation at 12,000 X *g* at 4°C for 2 min, the supernatant was collected and stored in 30% glycerol at -80°C for further use. The purity of the recombinant Ku protein was assessed by electrophoresis in 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel and its DNA binding activity was assessed by EMSAs as previously described (Giffin et al. 1997).

2.4.2. Preparation of HeLa, pro-B cell and Jurkat T cell extracts

HeLa S3 interphase nuclei and cytosol (Cellex Biosciences) were used to prepare nuclear and cytosolic extracts as described previously (Pearson et al. 1991; Ruiz et al. 1999). The nuclear and cytosolic extracts were mixed to reconstitute total cell extracts and dialyzed against Buffer I containing 26 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.8), 82 mM potassium acetate, 5.0 mM $MgCl_2$, 2.5 mM EDTA, 1.0 mM DTT, 1.0 mM PMSF, 1.0 μM pepstatin A, 1.0 μM leupeptin and 10% glycerol. The HeLa cell extracts were stored in aliquots at -80°C for future use.

Cellular lysates were also prepared for coimmunoprecipitation assays from A-MuLV-transformed pro-B cells 220-8 as described by Grawunder et al. (1996) with modifications. After washing with ice-cold 1 X PBS, 5 X 10^8 pro-B cells 220-8 were lysed in radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid and 0.1% SDS supplemented with a cocktail of protease inhibitors (Complete, Roche Molecular Biochemicals) by rotating in a microcentrifuge tube for 30 min at 4°C. The salt concentration of the lysates was then brought to 600 mM NaCl (Leu and Schatz 1995), the lysates were centrifuged at 14,000 × *g* at 4°C for 30 min and the supernatant was collected and stored at -80°C.

Jurkat T cell whole cell extracts for *in vitro* protein binding assays were prepared essentially as previously described (Romero et al. 1996; Schild-Poulter et al. 2001). Cell lysis was performed at 5 X 10^7 cells /ml at 4°C for 20 min in 150 mM NaCl, 1mM EDTA, 50 mM HEPES, 1 mM sodium vanadate, 10% glycerol, 0.875% polyoxylethylene-10-oleylether, 0.125% Nonidet P-40, 1% aprotinin, 1 mM PMSF, 1 μg/ml pepstatin and 1 μg/ml leupeptin. The lysates were centrifuged at 20,000 X *g* for 20 min and the supernatant was collected and stored in aliquots at -80°C.

2.4.3. Preparation of nuclear extracts from 293T, V79 and V15B cells

The nuclear extracts from 293T, V79 and V15B cells were prepared as previously described (Dignam et al. 1982; Cortes et al. 1996) with some modifications. Briefly, cells were harvested and washed with ice-cold 1 X PBS and resuspended in 5 vol of ice-cold Buffer A with 10 mM HEPES·KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% Nonidet P-40, 2 mM DTT and 1 mM PMSF. The samples were allowed to swell on ice

for 10 min and centrifuged at 1000 X *g* for 10 min at 4°C. The pellets were resuspended in 1.5 vol of Buffer C containing 20 mM HEPES·KOH (pH 7.9), 20% glycerol, 0.6 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Nonidet P-40, 0.5 mM DTT and 1 mM PMSF and incubated on ice for 30 min for high salt extraction. Cellular debris was removed by centrifuging at 12,000 X *g* for 5 min at 4°C and the supernatant fraction was dialyzed against 100 vol of Buffer D with 20 mM HEPES·KOH (pH 7.9), 20% glycerol, 0.1 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Nonidet P-40, 0.5 mM DTT and 1 mM PMSF for 6 to 8 h with 3 buffer changes in the interim. The dialyzed extracts were estimated to be 6 to 8 μg/μl in concentration by the Bradford method of protein quantification (BioRad) and stored in aliquots at -80°C.

2.5. Comparative study of Ku70/80 binding to NRE1, A3/4 and nonspecific DNA

2.5.1. Reciprocal competitive EMSAs using recombinant Ku70/80 or HeLa cell extracts and radiolabeled linear NRE1, A3/4 and nonspecific DNA

EMSAs were performed essentially as previously described (Giffin et al. 1996, 1997). Oligonucleotides used for EMSAs were kinased with [γ-^{32}P]-ATP using T4 polynucleotide kinase (New England BioLabs). The labeling efficiency of oligonucleotides was carefully monitored by spectrometry and scintillation counting to ensure that equal amounts of radioactivity and oligonucleotides were included in each reaction.

Reciprocal competitive EMSAs with recombinant K70/80 were performed as described (Giffin et al. 1997) by incubating the recombinant Ku protein (5 ng) with 25 fmol each of [γ-^{32}P]-ATP labeled NRE1, A3/4 or nonspecific DNA, NS1, at 20°C for 20

min in a 20-μl reaction containing 12 mM HEPES (pH7.9), 12% glycerol, 60 mM KCl, 0.12 mM EDTA and 1 μg BSA. Fifty ng or 1 μg of nonspecific competitor calf-thymus (CT) DNA sheared to an average size of less than 500 bp were included as needed. Unlabeled oligonucleotides NRE1, A3/4 or NS1 were added at a 50-fold molar excess as needed prior to the addition of Ku70/80. When the Ku70/80-specific mouse monoclonal antibody 162 (Ab-3, NeoMarkers) was used to supershift the Ku70/80-DNA complexes, the Ku protein was initially incubated with the radiolabeled DNA substrates for 5 min; thereafter, Ab 162 was added and the incubation was continued for 15 more min.

Reciprocal competitive EMSAs were also performed by incubating recombinant Ku70/80 (5 ng) or HeLa cell extracts (5 μg) with 25 fmol each of [γ-^{32}P]-ATP labeled NRE1, A3/4 or NS1 in the presence of 1 μg of CT DNA under standard *in vitro* binding reactions (Giffin et al. 1997). A 500 fold-molar excess of unlabeled NRE1 or A3/4 were included as needed prior to the addition of recombinant Ku70/80. Antibody 162 was included as needed.

Additionally, competitive EMSAs as described above were conducted by incubating recombinant Ku70/80 (5 ng) with 5 fmol of [γ-^{32}P]-ATP labeled A3/4 in the presence of 1 μg of CT DNA. Supercoiled plasmids containing NRE1 (pBS-NRE1) or A3/4 (pBR322-A3/4) or control supercoiled plasmids (pBS), which were purified through electrophoresis on 1% agarose gels, were included as competitor DNA at a 10-, 25- or 100-fold molar excess as needed prior to the addition of the Ku protein.

The samples from the above *in vitro* DNA binding reactions were all resolved by electrophoresis on 4% nondenaturing polyacrylamide gels (acrylamide:bisacrylamide =

40:1) in 0.5 X TBE (Tris-borate-EDTA) buffer. The gels were dried and exposed to an autoradiography film (DuPont) using Reflection intensifying screens (DuPont).

2.5.2. DNA-protein UV crosslinking

Ku70/80-DNA UV crosslinking studies were performed as previously described (Torrance et al. 1998; Giffin et al. 1999). Briefly, the upper strand of dsNRE1 and dsNS1, and the upper or lower strand of dsA3/4 were selectively labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase (New England BioLabs) and annealed with their corresponding unlabeled lower or upper strand followed by recovery through electrophoresis on an 8% nondenaturing polyacrylamide gel. Thereafter, dsNRE1, dsNS1 or dsA3/4 radiolabeled with [γ-^{32}P]-ATP either in the upper or lower strand was incubated with recombinant Ku70/80 under standard EMSA conditions as aforementioned or with the inclusion of 10 mM ATP and 10 mM $MgCl_2$. Following incubation with recombinant Ku70/80, the samples were directly exposed to UV for 12 min at 4°C in a Stratalinker 1800 (Stratagene). The UV-crosslinked products were resolved on 10% SDS-polyacrylamide gels and autoradiographed.

2.6. SELEX by recombinant Ku70/80

2.6.1. Titration of DNA end binding activity of recombinant Ku70/80 with CT DNA

The recombinant Ku70/80 employed for the *SELEX* was purified from *Sf9* cells as described elsewhere in the text. The random library of radiolabeled dsRND was prepared as aforementioned. *In vitro* DNA binding sites selection assays were initially performed by determining the concentration of nonspecific competitor CT DNA that optimally minimized DNA end binding activity of the Ku protein. EMSAs were carried out as

previously described (Giffin et al. 1997) in the absence of either Mg^{2+} or ATP. The recombinant Ku protein (12 fmol) and the radiolabeled dsRND (120 fmol) were included at a ratio of 1 molecule of the Ku protein to 10 molecules of dsRND. Varying amounts of highly sheared CT DNA from 25 ng/μl to 200 ng/μl were included prior to the addition of recombinant Ku70/80. EMSAs were carried out by incubating Ku70/80 (12 fmol) with 120 fmol of [γ-^{32}P]-ATP labeled dsRND at 20°C for 20 min in a 20-μl reaction containing 12 mM HEPES (pH7.9), 12% glycerol, 60 mM KCl, 0.12 mM EDTA and 1 μg of BSA in the presence of 25, 50, 100 or 200 ng/μl of highly sheared nonspecific CT DNA. The samples were resolved by electrophoresis on a 4% nondenaturing polyacrylamide gel, which was then wet exposed for 2 h to an autoradiography film (DuPont) using Reflection intensifying screens (DuPont). The concentration of 100 ng/μl of CT DNA was chosen for the competition of DNA end binding activity of Ku70/80 in subsequent *in vitro* DNA binding sites selection assays.

2.6.2. Selection of preferred DNA binding sites for Ku70/80

For each *in vitro* DNA binding sites selection reaction, 120 fmol or 7.23 X 10^{10} molecules of [γ-^{32}P]-ATP labeled dsRND from the initial random pool and the subsequently selected pools were used and each round of the *SELEX* by Ku70/80 was undertaken in quadruplets with a total of 2.89 X 10^{11} DNA molecules, which were more than four times the number of possible sequence variations in the random library. DNA binding sites selection assays were carried out by incubating recombinant Ku70/80 (12 fmol) with 120 fmol of [γ-^{32}P]-ATP labeled dsRND in the presence of 100 ng/μl of CT DNA at 20°C for 20 min. The Ku70/80-bound DNA was partitioned from the unbound DNA by electrophoresis on 4% nondenaturing polyacrylamide gels. The gels were wet

exposed to an autoradiography film for 8 h using Reflection intensifying screen (DuPont) and the bands corresponding to Ku70/80-bound DNA were recovered from the polyacrylamide gels and, after two phenol:chloroform extractions followed by ethanol precipitation, the recovered DNA was pooled in 20 µl of TE buffer (pH 7.4). A total of eight rounds of DNA binding sites selection by recombinant Ku70/80 were carried out followed in each round by amplification by polymerase chain reaction (PCR).

To confirm an enrichment of preferred DNA binding sites for Ku70/80 with the reiterative rounds of selection and amplification through the *SELEX* process, EMSAs were carried out by incubating Ku70/80 (5 ng) with 20 fmol of [γ-^{32}P]-ATP labeled dsRND from round 2, 4, 6 or 8 of the *SELEX* in the presence of 50 ng/µl of CT DNA. The samples were resolved by running on a 4% nondenaturing polyacrylamide gel. After drying, the gel was exposed to an autoradiography film (DuPont) for 8 h using Reflection intensifying screen (DuPont).

2.6.3. Amplification of the preferred DNA binding sites selected by Ku7080

One fourth of the pooled DNA from each round of the *SELEX* by Ku70/80 was used for PCR in a 25-µl reaction containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1 mg/ml of BSA, 1.5 mM $MgCl_2$, 150 ng each of primer P1 and P2, 20 µM all 4 dNTPs and 2 units of *Taq* polymerase (Amersham Biosciences). The PCR was run on a PTC-200 thermocycler (MJ Research) by incubating the reaction mixture at 94 °C for 5 min followed by 35 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min with additional incubation at 72°C for 5 min. The PCR products were purified on 8% nondenaturing polyacrylamide gels, dissolved in TE buffer (pH 7.4) and employed, after

being radiolabeled with [γ-^{32}P]-ATP by T4 polynucleotide kinase (New England BioLabs), for subsequent rounds of DNA binding sites selection by recombinant Ku70/80.

2.6.4. Cloning and sequencing of Ku70/80-selected DNA binding sites by SELEX

The Ku70/80-selected DNA sequences from the final round of the *SELEX* were phosphorylated by T4 polynucleotide kinase (New England BioLabs) and purified using ProbeQuant™ G-50 Micro columns (Amersham Biosciences). The cloning vector pBS (1 μg) was digested with the endonuclease *Sma*I and, following purification by electrophoresis on 1% agarose gel, the linearized pBS was dephosphorylated by CIP (New England BioLabs). Fifty ng of the phosphorylated Ku70/80-selected DNA sequences were ligated into the *Sma*I site of pBS (200 ng) by T4 DNA ligase (New England BioLabs). After heat inactivation of T4 DNA ligase, one tenth of the ligation reaction products were transformed into *E. coli* DH5α by electroporation and plasmid DNA was prepared by a standard alkaline lysis protocol (Maniatis et al. 1982). The presence of the Ku70/80-selected DNA sequences in the plasmids was initially assessed with the endonuclease *Sma*I for the absence of the restriction site in the plasmids followed by further digestion with the endonucleases *Pst*I and *Not*I. Restriction with both *Pst*I and *Not*I would generate a 92-bp fragment containing the 62-bp insert from positive plasmids versus a 30-bp fragment from the control vector pBS. The reaction products were resolved on 8% nondenaturing polyacrylamide gels and visualized using PhosphorImager and IMAGEQUANT software (Molecular Dynamics) after staining with ethidium bromide.

The sequencing of the cloned DNA sequences was done with T7 primer using Sequenase™ DNA Sequencing Kit according to the manufacturer's instructions (USB).

The reaction products were resolved on 8% denaturing polyacrylamide gels and visualized using PhosphorImager and IMAGEQUANT software (Molecular Dynamics).

2.7. Characterization of Ku70/80 binding to the RSS heptamer

2.7.1. Comparative EMSAs using recombinant Ku70/80 and linear radiolabeled RSS heptamer (H7), nonamer (N9), NRE1 or NS1

One pmol each of H7, N9, NRE1 or NS1 was radiolabeled with [γ-^{32}P]-ATP by T4 polynucleotide kinase (New England BioLabs) and purified with ProbeQuant^{TM} G-50 Micro columns (Amersham Biosciences). To compare Ku70/80 binding to the RSS heptamer and nonspecific DNA, EMSAs were performed as aforementioned by incubating recombinant Ku70/80 (5 ng) with 5 fmol of [γ-^{32}P]-ATP labeled H7 in the presence of 50 ng of CT DNA at 20°C for 20 min. A 25- or 50-fold molar excess of unlabeled H7 or NS1 were included as needed prior to the addition of recombinant Ku70/80. In addition, to compare Ku70/80 binding to the RSS heptamer and nonamer, NRE1 and nonspecific DNA, EMSAs were carried out as aforementioned by incubating recombinant Ku70/80 (5 ng) with 20 fmol each of [γ-^{32}P]-ATP labeled H7, N9, NS1 or NRE1 in the presence of 1 μg of CT DNA. The samples were resolved by electrophoresis on 4% nondenaturing polyacrylamide gels and visualized using PhosphorImager and IMAGEQUANT software (Molecular Dynamics).

To examine if the Ku70/80-RSS heptamer complexes were stable, EMSAs were also carried out by initially incubating recombinant Ku70/80 (5 ng) with 5 fmol of [γ-^{32}P]-ATP labeled H7 at 20°C for 5 min. Thereafter, a 10-, 100-, 1000- or 10,000-fold molar excess of unlabeled H7 or nonspecific DNA, NS or NS1, were added and the

incubation was continued for 20 min more. Ku70/80-specific antibody 162 was included where needed. In addition, EMSAs were performed by incubating recombinant Ku70/80 (5 ng) with 5 fmol of [γ-^{32}P]-ATP labeled H7 in the absence of any competitor DNA at 20°C for 30 min, 1, 2, 4, 8, or 16 h, or by initially incubating recombinant Ku70/80 (5 ng) with 5 fmol of [γ-^{32}P]-ATP labeled H7 at 20°C for 5 min, after which a 10,000-fold molar excess of unlabeled H7 were added and the incubations were continued up to 16 h. Ku70/80-specific antibody 162 was used to supershift the Ku70/80-DNA complexes as needed. The samples were resolved by electrophoresis on 4% nondenaturing polyacrylamide gels and visualized using PhosphorImager and IMAGEQUANT software (Molecular Dynamics).

2.7.2. Comparative EMSAs using recombinant Ku70/80 and linear radiolabeled H7, NS, or mutated RSS heptamers

Ku70/80 binding to the wild-type RSS heptamer and its mutated counterparts was examined by competitive EMSAs. One pmol each of H7 (-CACAGTG-) or the mutated heptamers $H7_{M12}$, (-gcCAGTG-), $H7_{M34}$, (-CAgcGTG-), and $H7_{M67}$, (-CACAGgc-), and NS were radiolabeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase (New England BioLabs) and purified with ProbeQuant™ G-50 Micro columns (Amersham Biosciences). EMSAs were carried out as aforementioned by incubating recombinant Ku70/80 (5 ng) with 10 fmol each of NS, H7, $H7_{M12}$, $H7_{M34}$, and $H7_{M67}$ in the presence of 50 ng or 1 μg of CT DNA. EMSAs were also performed by incubating recombinant Ku70/80 (5 ng) with 10 fmol of radiolabeled H7 in the presence of 50 ng of CT DNA. A 10-, 50- or 100-fold molar excess of unlabeled H7, $H7_{M12}$, $H7_{M34}$, or $H7_{M67}$ was included as needed. The samples were resolved by electrophoresis on 4% nondenaturing polyacrylamide gels.

After drying, the gels were exposed for 2 h for autoradiography and visualized with PhosphorImager and IMAGEQUANT software (Molecular Dynamics).

2.8. Studies of association of Ku70/80 with the RSS in vitro and in vivo

2.8.1. EMSAs using recombinant Ku70/80 and radiolabeled DNA microcircles

DNA microcircles were prepared as described above and EMSAs were carried out as aforementioned by incubating recombinant Ku70/80 (5 ng) and control DNA microcircles (10 fmol) or DNA microcircles containing the RSS heptamer (10 fmol) in the presence of 500 ng of CT at 20°C for 20 min. The samples were resolved by electrophoresis on a 4% nondenaturing polyacrylamide gel. After drying, the gel was exposed for 2 h for autoradiography and visualized with PhosphorImager and IMAGEQUANT software (Molecular Dynamics).

2.8.2. Plasmid immunoprecipitations

The procedures for plasmid immunoprecipitations were performed essentially as previously described (Yahata et al. 2001) starting with 1 X 10^6 cells. Briefly, 48 h following transfection of 293T cells with 2 μg of pJH200, cells were washed once with 1 X PBS and resuspended in serum-free DMEM. Following crosslinking for 10 min at 37°C with formaldehyde at a final concentration of 1%, the cells were rinsed with ice-cold 1 X PBS and collected into 1 X PBS containing a cocktail of protease inhibitors (Complete, Roche Molecular Biochemicals). After centrifugation, the cell pellets were resuspended in lysis buffer containing 1% SDS, 10 mM EDTA and 50 mM Tris-HCl (pH 8.1) supplemented with a cocktail of protease inhibitors and sonicated to reduce the DNA length to an average of 0.5 kb followed by centrifugation to remove insoluble material.

The supernatants were diluted in dilution buffer containing 1% Triton X-100, 2 mM EDTA, 150 mM NaCl and 20 mM Tris-HCl (pH 8.1) supplemented with a cocktail of protease inhibitors and precleared at 4°C for 2 h with 4 μg of sheared salmon sperm DNA and 60 μl of protein A-Sepharose beads (Sigma) in 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), 1 mM DTT and a cocktail of protease inhibitors (Complete, Roche Molecular Biochemicals). To the precleared lysates, a mixture of anti-Ku antibodies (Ab-3, 5 μg, and Ab-4, 5 μg) (NeoMarkers) or a control antibody against GAL4-DBD (*sc-510*, 5 μg) (Santa Cruz Biotechnology) was added, and the reaction was incubated overnight followed by the addition of 40 μl of protein A-Sepharose beads in sheared salmon sperm DNA (1.5 μg/ml) and a further incubation for 1 h. The Sepharose beads were then collected and washed sequentially for 10 min each in TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl at pH 8.1, and 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl at pH 8.1, and 500 mM NaCl), and TSE III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl at pH 8.1). The Sepharose beads were then washed 4 times with TE buffer (pH 8.0) and extracted 3 times with 100 μl in buffer E (1% SDS and 0.1 M $NaHCO_3$). The eluates were heated at 65°C overnight for the reversal of formaldehyde cross-links and the liberated DNA fragments were then purified using QIAquick columns (Qiagen).

For PCR, one fifth of the samples were used as input and 1/10 of the immunoprecipitated samples were employed for each PCR. The primer set R3 and R14 was described previously (Cortes et al. 1996) and the control primer set R7 and R8 was described elsewhere in the text. PCR was performed on a PTC-200 thermocycler (MJ

Research) by incubation at 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min with additional incubation at 72°C for 5 min. The PCR products were resolved on a 2% agarose gel stained with Vista Green (Pharmacia) and visualized with PhosphorImager and IMAGEQUANT software (Molecular Dynamics).

2.8.3. Chromatin immunoprecipitations (ChIP)

ChIP analysis of RAG2-/- fetal liver-derived Abelson pro-B cell line 63-12 was carried out as described above for plasmid immunoprecipitations. Pro-B cell line 63-12 was supplemented, as needed, with 10 μM (final) STI571 (Novartis) for 16 h (Muljo and Schlissel 2003). ChIP was performed starting with 2 x 10^7 cells. Following 10 min of crosslinking with 1.5% formaldehyde, cells were lysed and sonicated to shear the DNA to an average size of 1 kb. Following clearing of the lysates by centrifugation, supernatants were cleared for immunoprecipitation by a 2 h incubation with 60 μl of protein A Sepharose (Sigma) and 4 μg of sheared salmon sperm DNA in 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0) and 1 mM DTT. Immunoprecipitations were performed overnight with 4 μg of antibody 162, (AB-3, NeoMarkers) or 4 μg of control antibody against GAL4 (RK5C1, Santa Cruz). The protein-DNA complexes were recovered using 40 μl of protein A Sepharose beads in 1.5 μg/ml of sheared salmon sperm DNA. The precipitates were washed sequentially with TSE I, TSE II, and TSE III and twice in TE buffer (pH 8.0), then extracted 3 times in 100 μl of buffer E. Crosslinking was reversed at 65°C overnight and the liberated DNA was purified using QIAquick columns (Qiagen). One fortieth to one twentieth of the purified DNA was used for PCR reactions on a PTC-200 thermocycler (MJ Research) using the

appropriate primer sets (Appendix IV) for 30-35 rounds of amplification, and the products were analyzed on 1.2% agarose gels stained with ethidium bromide.

2.9. In vitro DNA cleavage assays

The [α-^{32}P]-dCTP labeled VκL8-Jκ1 fragments excised from the extrachromosomal recombination substrates pJH200 were prepared as aforementioned. The preparation of nuclear extracts from 293T cells, V79 or V15B fibroblasts co-expressing RAG1 and RAG2 is described elsewhere in the text. *In vitro* DNA cleavage assays were carried out as described previously (Cortes et al. 1996) with modifications. Briefly, 20 μg of nuclear extracts each from 293T, V79 or V15B cells were incubated at 30°C for 30 to 60 min with 50 ng of [α-^{32}P]-dCTP labeled VκL8-Jκ1 fragments in a 20-μl reaction containing 12.5 mM HEPES·KOH (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.05 mM EDTA, 5% glycerol and 0.5 mM ATP. The reactions were terminated by the addition of 2.5 μl of 10% SDS and 5 μl of 0.5 M EDTA. The samples were then treated with proteinase K (50 μg/ml) at 55°C for 1 h followed by two phenol:chloroform extractions and ethanol precipitation. The precipitates were dissolved in 20 μl of TE buffer (pH 7.4) and resolved on 5% nondenaturing polyacrylamide gels. Dried gels were visualized using PhosphorImager and analyzed with IMAGEQUANT software (Molecular Dynamics).

2.10. In vitro V(D)J recombination assays

The extrachromosomal recombination DNA substrate pJH200 was previously described (Hesse et al. 1987; Lieber et al. 1998). The preparation of nuclear extracts from

293T cells or 293T cells co-expressing RAG1 and RAG2 is described above (see Section 2.4.3). The *in vitro* V(D)J recombination assays were performed as previously described (Cortes et al. 1996) with some modifications. Briefly, 20 μg of nuclear extracts from 293T cells or from 293T cells co-expressing RAG1 and RAG2 were incubated with 200 ng of pJH200 at 30°C for 12 to 16 h in a 20-μl reaction containing 12.5 mM HEPES·KOH (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.05 mM EDTA, 5% glycerol, 0.5 mM ATP and 50 μM each all 4 dNTPs. The reactions were terminated by the addition of 2.5 μl of 10% SDS and 5 μl of 0.5 M EDTA. Following treatment with proteinase K (50 μg/ml) and phenol:chloroform extractions followed by ethanol precipitation, the precipitates were dissolved in 20 μl of TE buffer (pH 7.4) and 1/10 each of the samples was used for PCR.

The primer set R5 and R14 was previously described (Cortes et al. 1996) and generated a 456 bp fragment spanning VκL8 and Jκ1 gene segments in pJH200 prior to recombination or in the absence of RAG1 and RAG2 and an additional 252 bp fragment spanning the RSSs in pJH200 following recombination in the presence of RAG1 and RAG2. The 20-μl PCR mixture contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 5.5 mM $MgCl_2$, 0.1 mg/ml of BSA, 100 ng of each primer, 20 μM each all 4 dNTPs, and 1 unit of *Taq* DNA polymerase (Boehringer Mannheim). The PCR was carried out by running 25 cycles at 94°C for 0.5 min, 65°C for 1 min and 75°C for 1 min with additional incubation at 72°C for 5 min. The PCR products were resolved on a 2% agarose gel stained with Vistra Green (Pharmacia) and visualized with PhosphorImager and analyzed using IMAGEQUANT software (Molecular Dynamics). Typically, ½ of each sample was analyzed in each PCR reaction.

In addition, the 252 bp fragments amplified from the recombined pJH200 were purified from the agarose gel and radiolabeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase (New England BioLabs) followed by purification with ProbeQuant^{TM} G-50 Micro columns (Amersham Biosciences). The endonuclease *Apa*L I recognizes and restricts the newly precisely formed interface of the two fused heptamers (-GTGCAC-). Restriction of the radiolabeled 252 bp fragments with *Apa*L I was performed at 37°C for 1 h in a 20-µl reaction containing 50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate, 1 mM DTT and 100 µg/ml BSA. Restriction of the 252 bp fragments with *Hind*III was carried out at 37°C for 1 h in a 20-µl reaction containing 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$ and 1 mM DTT. The products were resolved on 8% nondenaturing polyacrylamide gels and visualized with PhosphorImager and IMAGEQUANT software (Molecular Dynamics).

2.11. In vivo transient V(D)J recombination assays

For *in vivo* transient V(D)J recombination assays, V79 or V15B fibroblasts were transiently transfected as described above with 2 µg of pJH200 alone or co-transfected with 2 µg each of pJH200, pRAG1 and pRAG2. Two days following transfection, DNA substrates were recovered using a small scale alkaline lysis system, the Qiagen Miniprep kits, and were dissolved in 20 µl of TE buffer (pH 7.4).

One tenth of each of the samples was employed for PCR analysis. The primer set R3 and R14 as previously described (Cortes et al. 1996) generated a 460-bp fragment spanning VκL8 and Jκ1 gene segments in pJH200 in the absence of RAG1 and RAG2 and an additional 256 bp fragment in pJH200 in the presence of RAG1 and RAG2. The

control primer set R7 and R8 generated a 308 bp fragment more than 3 kb away from VκL8 and Jκ1 gene segments and amplified both recombined and unrecombined pJH200. The primer set was employed to confirm that equivalent amounts of DNA substrates were transfected into and recovered from V79 or V15B fibroblasts. When the primer set R3 and R14 was employed, PCR was run for 25 cycles of 95°C for 10 sec, 55°C for 0.5 min and 72°C for 1 min followed by incubation at 72°C for 10 min. When the primer set R7 and R8 was used, PCR was performed by incubating at 94°C for 5 min followed by 25 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min with additional incubation at 72°C for 5 min. The PCR products were resolved on 2% agarose gels stained with Vistra Green (Pharmacia) and visualized with PhosphorImager and analyzed using IMAGEQUANT software (Molecular Dynamics). Typically, ½ of each sample was analyzed in each PCR reaction.

2.12. In vitro NHEJ assays

The preparation of nuclear extracts from 293T cells is described above (see Section 2.4.3). V15B and V79 fibroblasts were transiently transfected as aforementioned with 2 μg of pJH200 alone or together with 2 μg each of pRAG1 and pRAG2. Eighteen h following transfection, extrachromosomal recombination DNA substrates were recovered from the transfected V15B and V79 fibroblasts using the Qiagen Miniprep kits and further incubated with 20 μg of nuclear extracts from 293T cells at 30°C for 12 h in a 50-μl reaction containing 12.5 mM HEPES·KOH (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.05 mM EDTA, 5% glycerol, 0.5 mM ATP and 50 μM each of all 4 dNTPs. The reactions were terminated by the addition of 2.5 μl of 10% SDS and 5 μl of 0.5 M EDTA.

Following treatment with proteinase K, the DNA substrates were purified by two phenol:chloroform extractions followed by ethanol precipitation and dissolved in 20 µl of TE buffer (pH 7.4). One fifth of the samples were employed for PCR using the primer sets R5 and R14, and R7 and R8. The PCR products were resolved on 2% agarose gels stained with Vistra Green (Pharmacia) and visualized with PhosphorImager and IMAGEQUANT software (Molecular Dynamics).

In addition, the 252 bp fragments amplified from the recombined pJH200 were purified from the agarose gel and digested with the restriction endonucleases *Apa*L I and *Hind*III as described above. The products were kinased by T4 polynucleotide kinase (New England BioLabs), resolved on an 8% nondenaturing polyacrylamide gel and visualized with PhosphorImager and IMAGEQUANT software (Molecular Dynamics).

2.13. In vitro protein binding assays

The preparation of whole cell extracts from Jurkat T cells is described elsewhere in the text. Ku70/80 used in the *in vitro* protein binding assays was immunoprecipitated from Jurkat T cell whole cell extracts. The immunoprecipitation of Ku70/80 was performed in WCE buffer containing 150 mM NaCl, 50 mM HEPES (pH 7.4), 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, 0.5 mM DTT and 1 mM PMSF with 0.5 µg/ml of anti-Ku70 antibody (clone N3H10, NeoMarkers) followed by incubation with protein A-Sepharose beads (Sigma). The Sepharose beads were washed extensively in binding buffer containing 25 mM HEPES (pH 7.9), 60 mM KCl, 0.5 mM EDTA, 0.2 mM DTT, 0.2 mM PMSF, 0.1% Nonidet P-40 and 12% glycerol. Each binding reaction contained immunoprecipitates from the equivalent of 200 µg of the whole cell extracts. Full-length

RAG1 and RAG2 were translated *in vitro* in the presence of ^{35}S methionine using the T7 coupled reticulate lysate system according to the manufacturer's recommended protocol (Promega). Binding of ^{35}S-labeled *in vitro* translated proteins to Ku70/80 immobilized on the protein A-Sepharose beads was performed in binding buffer for 2 h at 4 °C. In addition, 200 μg/ml ethidium bromide were included as needed. Following extensive washing in the same buffer, specifically bound RAG1/RAG2 was visualized by SDS-PAGE-autoradiography.

2.14. Coimmunoprecipitation assays

Cellular lysates from 5 X 10^8 A-MuLV-transformed pro-B cells 220-8 were prepared as aforementioned and the concentration of NaCl in the lysates was diluted to 60 mM with binding buffer containing 25 mM HEPES·KOH, 60 mM KCl, 0.5 mM EDTA, 0.2 mM DTT, 0.2 mM PMSF, 0.1% NP-40 and 12% glycerol. In addition, 10 to 20% of the diluted extracts were saved for use as a control for input. The diluted cellular lysates were precleared at 4°C for 2 h with 4 μg of sheared salmon sperm DNA and 60 μl of protein A-Sepharose beads (Sigma) (50/50 w/v slurry) and the supernatant was collected after brief centrifugation. A mixture of anti-Ku70 (M19, 5 μg) and anti-Ku80 (C20, 5 μg) antibodies (Santa Cruz Biotechnology), or anti-RAG1 (G109-256, 5 μg) or anti-RAG2 (G110-461, 5 μg) antibodies (BD PharMingen) alone or in combination were added to the precleared lysates. An anti-GAL4-DBD antibody (5 μg) (*sc-510*, Santa Cruz Biotechnology) was used as control antibody. After overnight incubation at 4°C, 60 μl of protein A-Sepharose beads (Sigma) (50/50 w/v slurry) were added followed by 1 h incubation at 4°C and extensive washing with 1 ml of binding buffer. In addition, 200

μg/ml of ethidium bromide were included in coimmunoprecipitation assays as described previously (Lai and Herr 1992). The immunoprecipitates were resuspended in SDS loading buffer and resolved on 8% SDS-polyacrylamide gels. For immunoblotting, the proteins in gels were transferred onto nitrocellulose membrane and immunoreactive proteins were visualized using enhanced chemiluminescence according to the manufacturer's instructions (Amersham Pharmacia Biotech).

All the experiments in the current study were repeated at least three times with reproducible results.

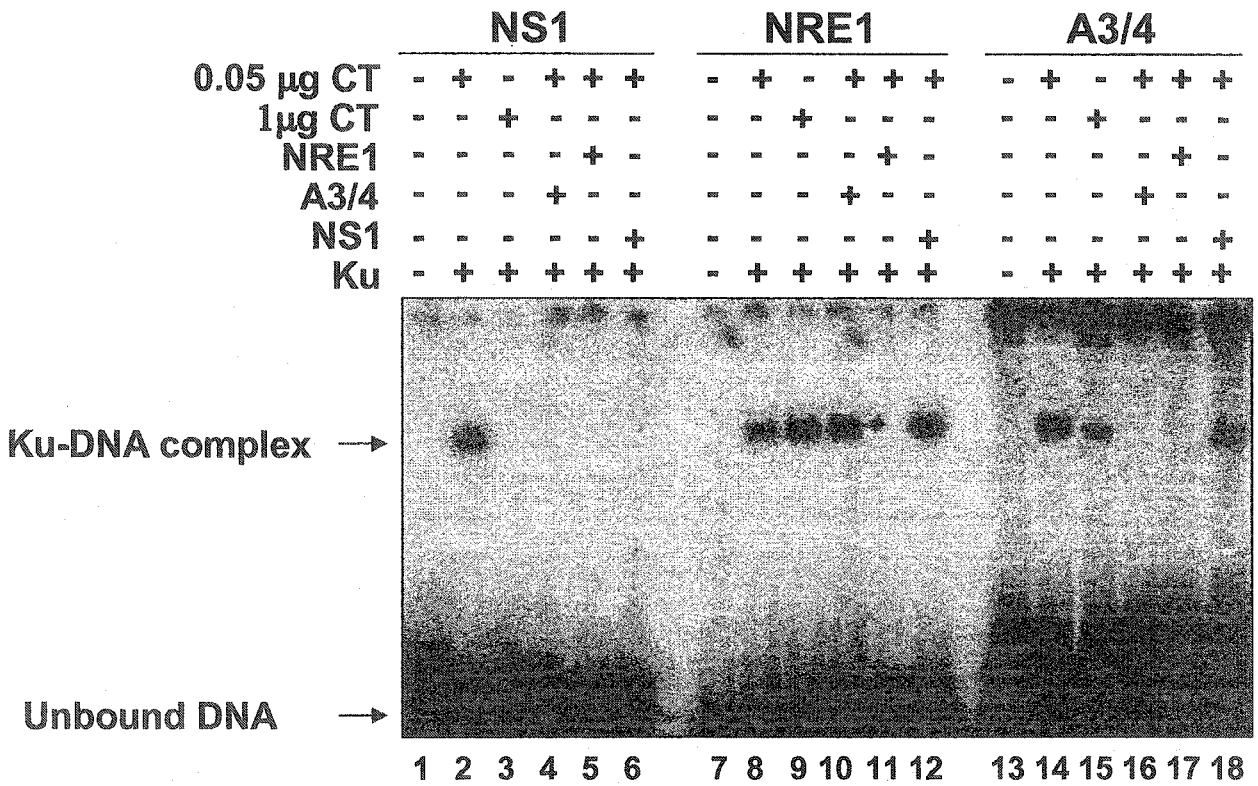
3. RESULTS

3.1. Ku70/80 binds differentially to NRE1, A3/4 and nonspecific DNA ends

Our laboratory has previously demonstrated that Ku70/80 binds directly and with high affinity to NRE1 (Giffin et al. 1996, 1997). To directly compare DNA binding activities of Ku70/80 to NRE1 and A3/4, competitive EMSAs were carried out using linear oligonucleotides and the recombinant human Ku70/80 expressed in and purified from the *Sf9* cells. The recombinant human Ku70/80 has been shown to bind to NRE1 and nonspecific DNA ends indistinguishably from human Ku70/80 purified from Jurkat T cells (Giffin et al. 1996, 1999; Torrance et al. 1998).

As shown in Figure 6, binding of Ku70/80 to [γ-^{32}P]-ATP labeled nonspecific oligonucleotide (NS1) was readily visible in the presence of 50 ng of highly sheared CT DNA (lane 2), but was effectively abolished when CT DNA was increased to 1 μg (lane 3) or when a 50-fold molar excess of unlabeled A3/4, NRE1 or NS1 were added (lanes 4 to 6). Similarly, binding of Ku70/80 to [γ-^{32}P]-ATP labeled NRE1 was readily visible in the presence of 50 ng of CT DNA (lane 8). By contrast, Ku70/80 binding to NRE1 was resistant to competition by 1 μg of CT DNA or a 50-fold molar excess of unlabeled A3/4 or NS1 (lanes 9, 10 and 12). Nonetheless, NRE1 binding by Ku70/80 was sensitive to competition by a 50-fold molar excess of unlabeled NRE1 (lane 11). Binding of Ku70/80 to [γ-^{32}P]-ATP labeled A3/4 was also readily visible in the presence of 50 ng of CT DNA (lane 14). Similar to its binding to NRE1, Ku70/80 binding to A3/4 was resistant to the competition by 1 μg of CT DNA or a 50-fold molar excess of unlabeled NS1 (lanes 15

Figure 6. Reciprocal competitive EMSAs using recombinant Ku70/80 and linear A3/4, NRE1 or nonspecific DNA ends. *In vitro* DNA binding assays were performed with recombinant Ku70/80 (5 ng) and 25 fmol each of [γ-^{32}P]-ATP labeled nonspecific oligonucleotide (NS1) (lanes 1 to 6), NRE1 (lanes 7 to 12) or A3/4 (lanes 13 to 18). Fifty ng or 1 μg of highly sheared CT DNA were added as indicated. Unlabeled NS1, NRE1 or A3/4 was added as indicated at a 50-fold molar excess. The samples were resolved through electrophoresis on a 4% nondenaturing polyacrylamide gel and autoradiographed.

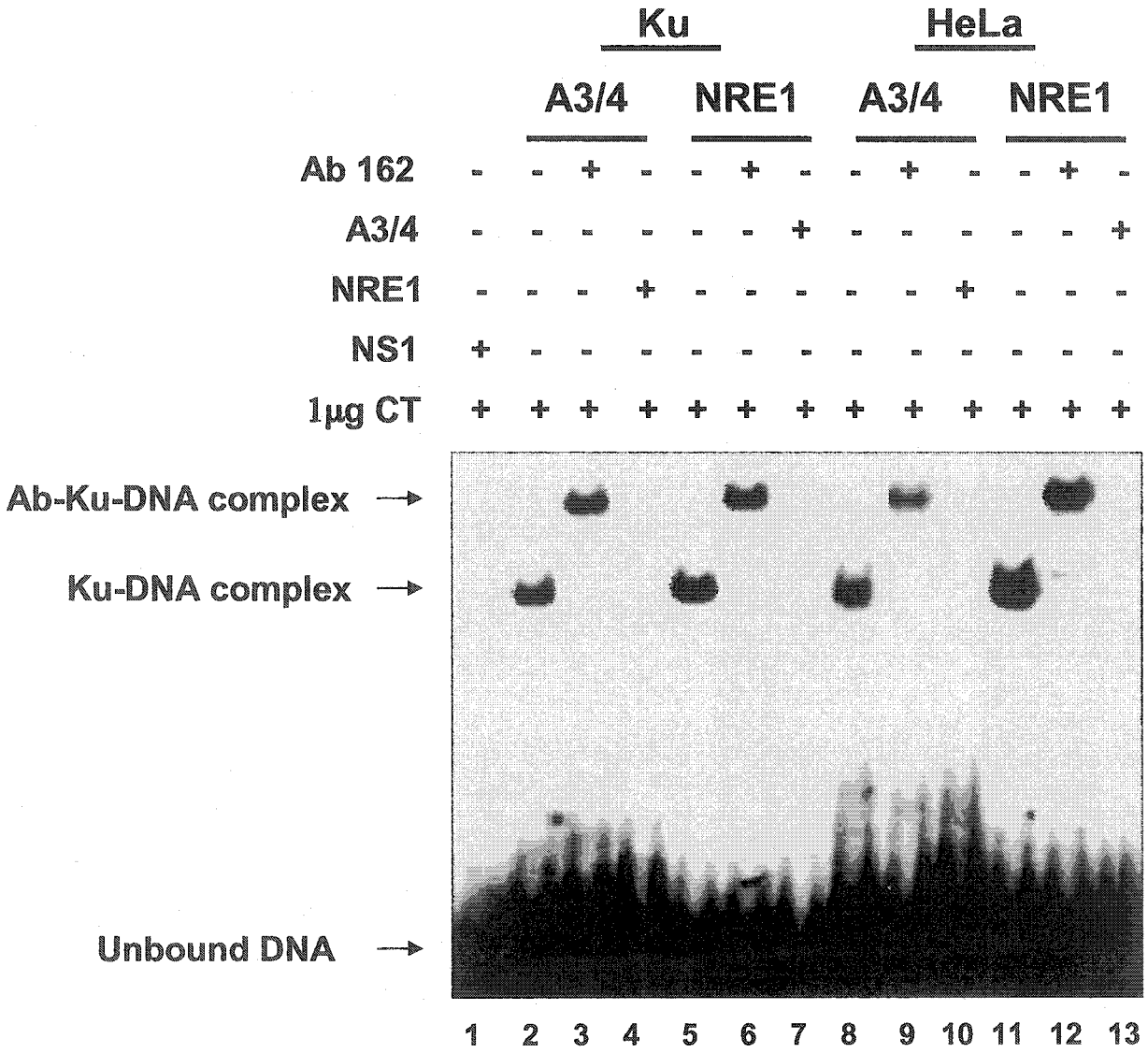

NS1
NRE1
A3/4
0.05 µg CT - + - + + + - + - + + + - + - + + +
1µg CT - - + - - - - - + - - - - - + - - -
NRE1 - - - - + - - - - - + - - - - - + -
A3/4 - - - + - - - - - + - - - - - + - -
NS1 - - - - - + - - - - - + - - - - - +
Ku - + + + + + - + + + + + - + + + + +
Ku-DNA complex
Unbound DNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

and 18), which was, however, effectively aborted by a 50-fold molar excess of unlabeled A3/4 or NRE1 (lanes 16 and 17).

These results suggested that Ku70/80 binds to NRE1 with a higher affinity than its binding to either A3/4 or NS1. While Ku70/80 binding to NRE1 was efficiently competed by a 50-fold molar excess of unlabeled NRE1, it was resistant to the competition by A3/4, CT DNA and NS1. By comparison, Ku70/80 binding to A3/4 was resistant to the competition by CT DNA and NS1, but was sensitive to the competition by either A3/4 or NRE1. In contrast, Ku70/80 binding to NS1 was sensitive to the competition by either CT DNA (1 μg), NRE1, A3/4, or NS1. These findings suggest that binding to A3/4 by Ku70/80 occurred with an affinity intermediate between its binding to NRE1 and nonspecific DNA ends.

To exclude the possibility that the differences in the apparent binding affinities to A3/4, NRE1 and NS1 by Ku70/80 were a reflection of the use of recombinant Ku70/80, competitive EMSAs were also performed using linear oligonucleotides and HeLa cell extracts. As shown in Figure 7, in the presence of 1 μg of highly sheared CT DNA, Ku70/80 binding to [γ-^{32}P]-ATP labeled NS1 was not visible (lane 1) while its binding to [γ-^{32}P]-ATP labeled A3/4 or NRE1 was readily observed (lanes 2 and 5). The binding of HeLa cell extracts to [γ-^{32}P]-ATP labeled A3/4 or NRE1 was easily visualized in the presence of 1 μg of CT DNA (lanes 8 and 11). That the binding observed reflected the activity of the Ku autoantigen in either the recombinant Ku70/80 (lanes 3 and 6) or endogenous Ku70/80 in HeLa cell extracts (lanes 9 and 12) was confirmed by the Ku70/80-DNA complexes supershifted by Ab 162, which is known to recognize the Ku70/80 heterodimer (Wang et al. 1997). The binding of Ku70/80 or HeLa cell extracts

Figure 7. Reciprocal competitive EMSAs using recombinant Ku70/80 or HeLa cell extracts and linear A3/4 or NRE1. *In vitro* DNA binding assays were performed in the presence of 1 μg of CT DNA with recombinant Ku70/80 (5 ng) or HeLa cell extracts (5 μg) and 25 fmol each of [γ-^{32}P]-ATP labeled A3/4 or NRE1. The binding of recombinant Ku70/80 to radiolabeled A3/4 (lanes 2 to 4) or NRE1 (lanes 5 to 7) and the binding of HeLa cell extracts to radiolabeled A3/4 (lanes 8-10) or NRE1 (lanes 11 to 13) were shown as indicated. Unlabeled NRE1 or A3/4 was added as indicated at a 500-fold molar excess. The antibody supershift experiments were performed with Ku70/80-specific antibody Ab 162 (lanes 3, 6, 9 and 12). The samples were resolved through electrophoresis on a 4% nondenaturing polyacrylamide gel and autoradiographed.

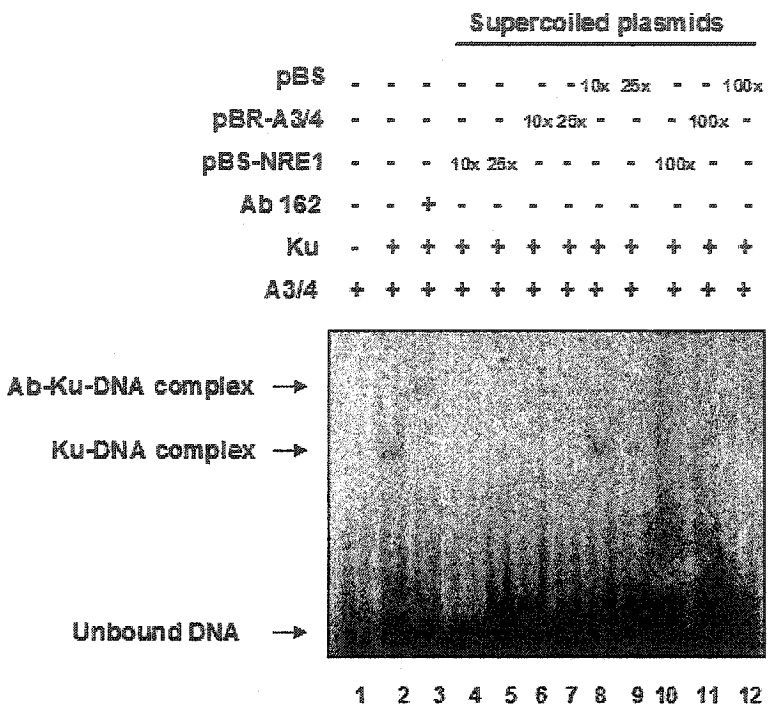

Ku
HeLa
A3/4
NRE1
A3/4
NRE1
Ab 162 - - + - - + - - + - - + -
A3/4 - - - - - - + - - - - - +
NRE1 - - - + - - - - - + - - -
NS1 + - - - - - - - - - - - -
1μg CT + + + + + + + + + + + + +
Ab-Ku-DNA complex
Ku-DNA complex
Unbound DNA
1 2 3 4 5 6 7 8 9 10 11 12 13

to [γ-^{32}P]-ATP labeled A3/4 was effectively abolished by a 500-fold molar excess of unlabeled NRE1 (lanes 4 and 10). Similarly, the binding of Ku70/80 or HeLa cell extracts to [γ-^{32}P]-ATP labeled NRE1 was aborted by a 500-fold molar excess of unlabeled A3/4 (lanes 7 and 13). These findings suggest that recombinant human Ku70/80 binds to A3/4 or NRE1 similarly to the endogenous Ku protein in HeLa cell extracts.

To investigate whether Ku70/80 interacted with A3/4 in a sequence-specific manner independent of the presence of DNA ends, competitive EMSAs were undertaken with recombinant Ku70/80 using as a competitor a supercoiled plasmid containing A3/4 (pBR-A3/4) or a supercoiled plasmid containing NRE1 (pBS-NRE1). Similar control reactions were performed using the supercoiled plasmid pBS. As indicated in Figure 8, the binding of Ku70/80 to [γ-^{32}P]-ATP labeled A3/4 was visible in the presence of 1 μg of CT DNA (lane 2) and its specificity was confirmed by the formation of a supershifted Ku70/80-A3/4 complex with Ab 162 (lane 3). Ku70/80 binding to [γ-^{32}P]-ATP labeled A3/4 was readily competed by a 10-, 25- or 100-fold molar excess of supercoiled plasmids containing NRE1 (lanes 4, 5 and 10). Similar results were observed when a 10-, 25- or 100-fold molar excess of supercoiled plasmids containing A3/4 were employed as competitor DNA (lanes 6, 7 and 11). However, Ku70/80 binding to [γ-^{32}P]-ATP labeled A3/4 was resistant to the competition by up to a 100-fold molar excess of the supercoiled control plasmids lacking either NRE1 or A3/4 (lanes 8, 9 and 12). These results were consistent with the earlier findings by Ruiz et al. (1999) and suggest that A3/4, like NRE1, is a direct DNA binding site for Ku70/80.

To investigate the contact of individual Ku70 or Ku80 subunit with NRE1, A3/4

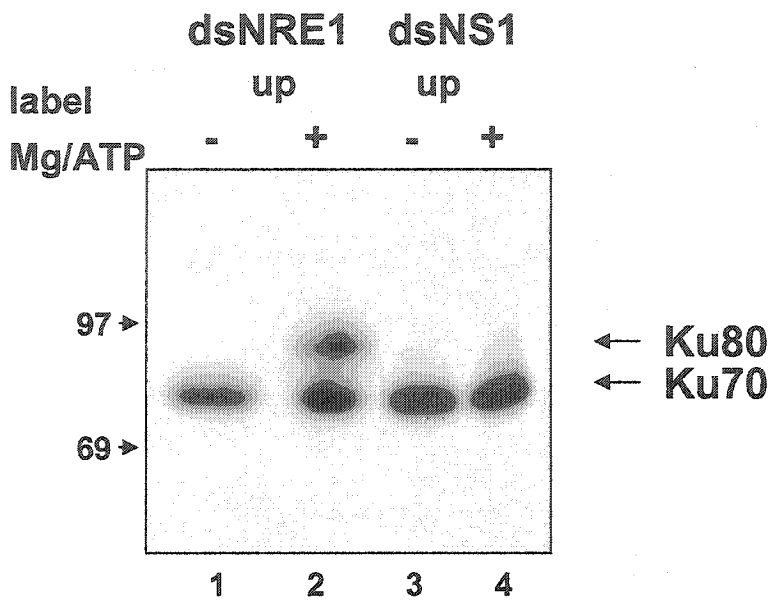
Figure 8. Competitive EMSAs using recombinant Ku70/80 and linear A3/4 and supercoiled plasmid DNA. *In vitro* DNA binding assays were performed with recombinant Ku70/80 (5 ng) and [γ-^{32}P]-ATP labeled A3/4 (5 fmol) in the presence of 1 μg of CT DNA. A 10-, 25- or 100-fold molar excess of supercoiled plasmids pBR-A3/4, pBS-NRE1 or pBS were added as indicated. The binding specificity of recombinant Ku70/80 to A3/4 was confirmed by the Ku70/80-A3/4 complex supershifted by the addition of Ab 162 (lane 3). The samples were resolved through electrophoresis on a 4% nondenaturing polyacrylamide gel and autoradiographed.

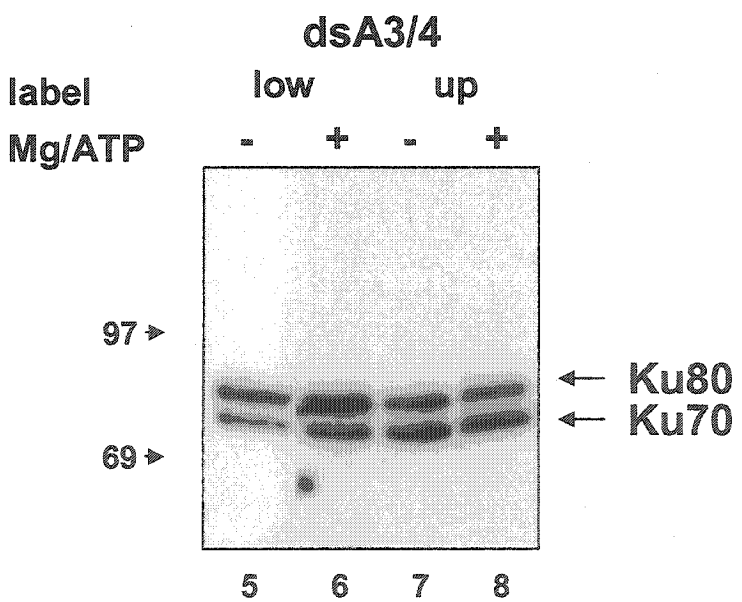

Supercoiled plasmids
pBS - - - - - - - 10x 25x - - 100x
pBR-A3/4 - - - - - 10x 25x - - - 100x -
pBS-NRE1 - - - 10x 25x - - - - 100x - -
Ab 162 - - + - - - - - - - - -
Ku - + + + + + + + + + + +
A3/4 + + + + + + + + + + + +
Ab-Ku-DNA complex
Ku-DNA complex
Unbound DNA
1 2 3 4 5 6 7 8 9 10 11 12

or nonspecific DNA, protein-DNA cross-linking experiments with recombinant Ku70/80 by UV irradiation were performed as previously described (Giffin et al. 1994, 1999; Torrance et al. 1998). As shown in Figure 9, under standard *in vitro* binding conditions in the absence of ATP and Mg^{2+}, only Ku70 was crosslinked by UV to the upper strand of NRE1 (lane 1). Upon the inclusion of ATP and Mg^{2+}, Ku80 was also crosslinked by UV to the upper strand of NRE1 (lane 2), suggesting a Mg^{2+} and ATP-dependent structural transition that led to the contact of Ku80 with DNA. For nonspecific DNA, regardless of the presence of Mg^{2+} and ATP, only Ku70 was crosslinked by UV to the oligonucleotides (lanes 3 and 4). These observations indicated a differential participation of individual Ku70 or Ku80 subunit in contacting different classes of DNA. Furthermore, a distinct pattern of Ku70/80-DNA interaction emerged when the Ku protein was UV-crosslinked to A3/4. Both Ku70 and Ku80 were crosslinked by UV to either the upper or lower strand of A3/4 regardless of the presence of Mg^{2+} and ATP (lanes 5 to 8). The results from a separate study in our laboratory also showed that, only Ku70 was crosslinked by UV to NS1 regardless of which strand was radiolabeled and whether Mg^{2+} and ATP were included in the binding reactions, which were consistent with the finding by other researchers (Mimori and Hardin et al. 1986; Gottlieb and Jackson 1993; Wang et al. 1994) that the Ku70 portion of the Ku heterodimer was the primarily subunit for DNA end binding.

To summarize, the findings from the reciprocal competitive EMSAs and UV-crosslinking studies showed that the Ku protein binds to NRE1, A3/4 and nonspecific DNA with distinct binding affinities and involves differential contacts of its two subunits with DNA. Furthermore, these findings established A3/4 as a novel class of direct DNA

Figure 9. UV-crosslinking of recombinant Ku70/80 to DNA. Recombinant Ku70/80 was incubated with NRE1, NS1 or A3/4 selectively radiolabeled with [γ-^{32}P]-ATP either in the upper (up) or lower strand (low) in the presence or absence of 4 mM ATP and 10 mM $MgCl_2$ as indicated. Following UV-crosslinking, the samples were resolved on a 10% SDS-polyacrylamide gel and autoradiographed.


dsNRE1
dsNS1
label
up
up
Mg/ATP
-
+
-
+
97
69
Ku80
Ku70
1
2
3
4


dsA3/4
label
low
up
Mg/ATP
-
+
-
+
97
69
Ku80
Ku70
5
6
7
8

binding site for Ku70/80, which is different from either NRE1 or nonspecific DNA ends.

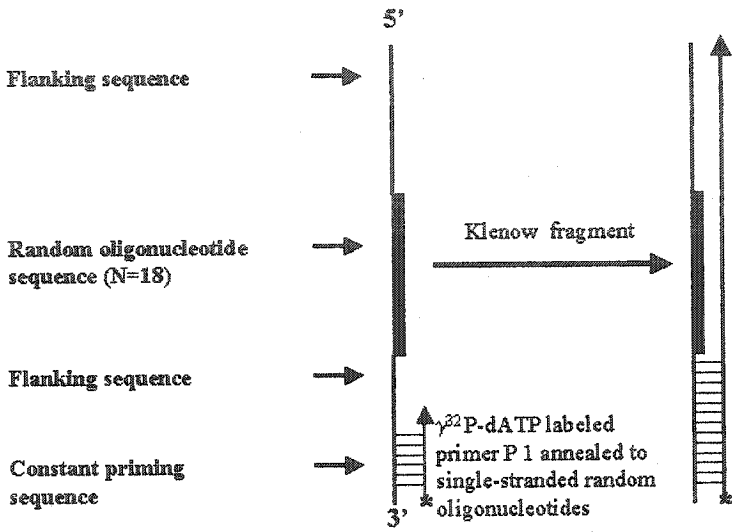
3.2. SELEX by Ku70/80 evolved preferred DNA binding sites for the Ku autoantigen including sequences homologous to the RSS heptamer and the E-box motif

SELEX has been widely used for the functional DNA binding site recognition of a variety of cellular proteins or supramolecular targets (Yoo and Dynan 1998; Bianchi et al. 1999; Burden and Osheroff 1999; Ehret et al. 2001). The strategy for *SELEX* by recombinant Ku70/80 is schematically shown in Figure 10. A random library of ds oligonucleotides containing a pool of approximately 6.87 X 10^{10} distinct nt sequences is generated and employed for the evolution of preferred DNA binding sites by recombinant Ku70/80 using standard *in vitro* DNA binding conditions as described previously (Giffin et al. 1997) followed by PCR amplification. After reiterative rounds of selection and amplification, the selected DNA binding sites are cloned and sequenced and analyzed for the presence of novel classes of direct, specific DNA binding sites for the Ku autoantigen.

The successful selection of preferred DNA binding sites for Ku70/80 by *SELEX* from an array of 6.87 X 10^{10} distinct nucleotide sequences would depend to a large extent on an exclusion of DNA entry by Ku70/80 from DNA ends. The inclusion of nonspecific competitor DNA such as highly sheared CT DNA in *in vitro* DNA binding assays has been shown to inhibit the DNA end binding activity of Ku70/80 while the sequence-specific binding activity of the Ku protein is conserved (Figure 6; Giffin et al. 1996; 1997; Schild-Poulter et al. 2003a). Therefore, we reasoned that by optimally minimizing DNA end binding activity of the Ku autoantigen during the *SELEX* with nonspecific competitor

Figure 10. Strategy for *in vitro* DNA binding sites selection by recombinant Ku70/80. (A) Generation of the random library of ds oligonucleotides. The initial random pool of ds oligonucleotides (dsRND) is generated from a pool of random ss oligonucleotides (ssRND) containing a stretch of 18 random nt (N_{18}) in the center flanked by 22 nt of constant priming sequences at either side by annealing [γ-^{32}P] ATP-labeled reverse primer P1 to ssRND followed by extension by the exo$^{-/-}$ large Klenow fragment of DNA polymerase I. The size of the initial random library so generated is 4 X 10^{18} or approximately 6.87 X 10^{10} distinct nt sequences. **(B) Strategy for *SELEX* by the recombinant Ku protein.** *In vitro* DNA binding assays as previously described (Giffin et al. 1997) are employed for the selection of high-affinity DNA binding sites by the recombinant Ku protein after optimal minimization of DNA end binding activity of Ku70/80 by titration with nonspecific competitor DNA. The Ku70/80-selected DNA binding sites are partitioned from the unselected sequences by EMSAs and are subsequently amplified by PCR for further rounds of selection by the Ku protein. A total of 8 rounds of selection and amplification are carried out. The amplified DNA containing preferred DNA binding sites for the Ku autoantigen from the final round of selection is then cloned for sequencing. The binding specificity of Ku70/80 to the selected functional DNA binding sites of interest is subsequently confirmed *in vitro* by EMSA using circular DNA templates free of DNA ends and other structural features.

A.



B.

Generate an oligonucleotide array

↓

Selection by *in vitro* DNA binding assay

↓

Partition bound from unbound DNA by EMSA

↓

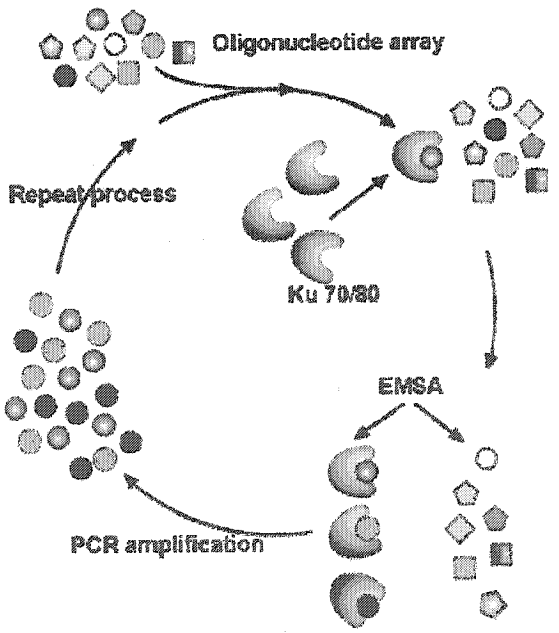
Amplify selected DNA by PCR

↓

Clone, sequence and analyze selected DNA binding sites

↓

Confirm sequence-specificity of Ku binding to selected DNA sequences by *in vitro* DNA binding assay using DNA microcircles



DNA such as CT DNA, the Ku protein would bind to high-affinity oligonucleotide sequences directly.

EMSAs were initially carried out by including varying amounts of nonspecific competitor CT DNA to determine the appropriate concentration of CT DNA that would optimally minimize the DNA end binding activity of the Ku protein for *SELEX*. As shown in Figure 11, Ku70/80 displayed marked DNA binding activity to radiolabeled dsRND in the presence of 25 ng/μl of CT DNA (lane 1), which was, however, completely negated when CT DNA was increased to 200 ng/μl (lane 4). In the presence of 100 ng/μl of CT DNA, the binding of Ku70/80 to radiolabeled dsRND was markedly inhibited but still slightly visible (lane 3). The concentration of CT DNA at 100 ng/μl was, therefore, chosen for *SELEX* by recombinant Ku70/80 as it minimized the DNA end binding activity by Ku70/80 and, at the same time, provided an initial marker for the recovery of Ku70/80-bound DNA.

Eight rounds of DNA binding sites selection assays with recombinant Ku70/80 were performed in the presence of 100 ng/μl of CT DNA and, as an example, the second round of the *SELEX* by the recombinant Ku protein is shown in Figure 12A. In addition, to determine if reiterative rounds of SELEX were associated with a selected pool of DNA sequences with increased binding affinities for recombinant Ku70/80, comparative EMSAs were carried out using a pool of selected DNA sequences from round 2, 4, 6 and 8 of *SELEX* even though 8 rounds of DNA binding sites selection were accomplished. EMSAs were carried out with recombinant Ku70/80 and [γ-^{32}P]-ATP labeled dsRND from round 2, 4, 6 and 8 of the *SELEX* in the presence of 1 μg of CT DNA. As indicated in Figure 12B, Ku70/80 binding to dsRND from round 2 of the *SELEX* was readily

Figure 11. Titration of DNA end binding activity of recombinant Ku70/80 with nonspecific competitor CT DNA. *In vitro* DNA binding assays were carried out with recombinant Ku70/80 (12 fmol) and [γ-^{32}P]-ATP labeled dsRND (120 fmol) from the initial pool of ds random oligonucleotides. Highly sheared nonspecific competitor CT DNA was included at the specified concentrations (lanes 1 to 4). The samples were resolved through electrophoresis on a 4% nondenaturing polyacrylamide gel. The gel was wet exposed to an autoradiography film for 2 h.

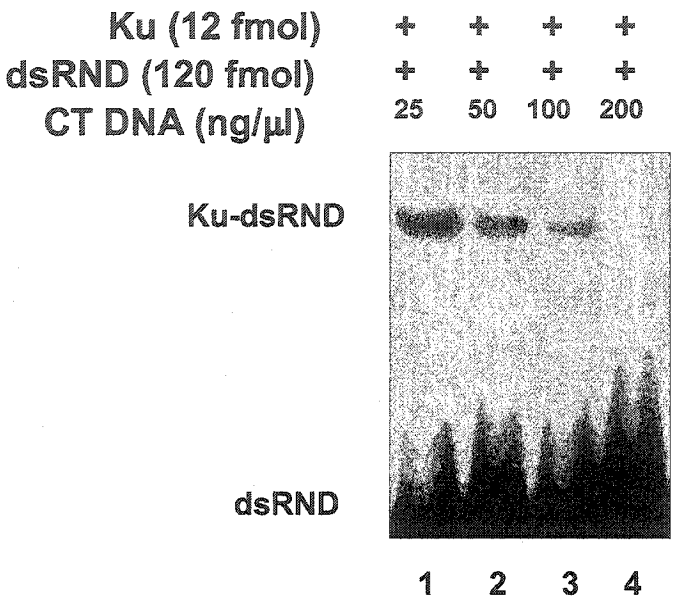
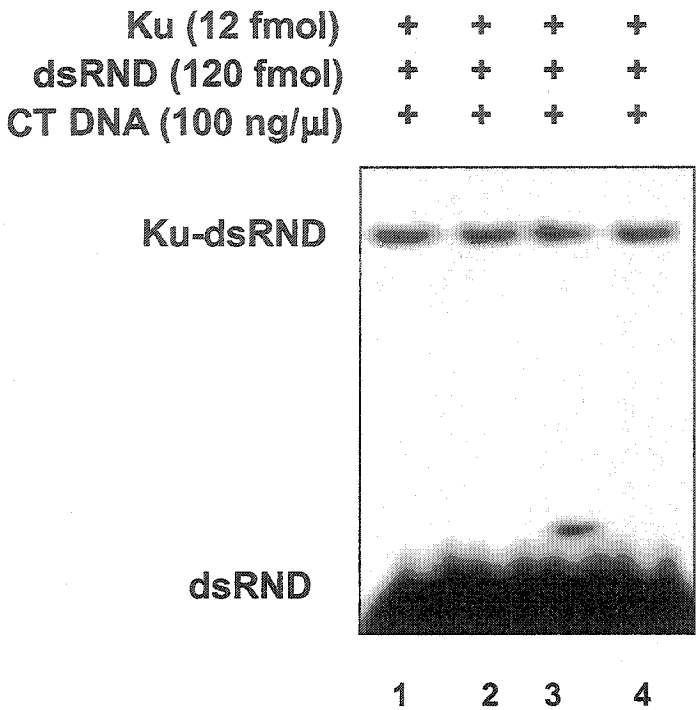
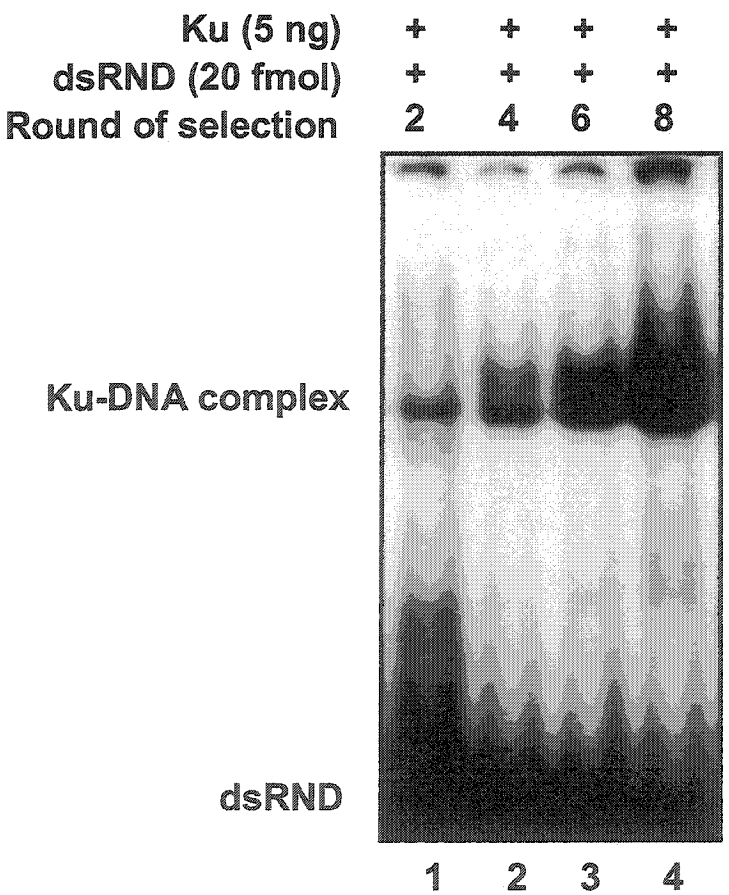

Ku (12 fmol)
dsRND (120 fmol)
CT DNA (ng/µl)
+ + + +
+ + + +
25 50 100 200
Ku-dsRND
dsRND
1 2 3 4

Figure 12. *SELEX* by recombinant Ku70/80. (A) The second round of *in vitro* DNA binding sites selection by recombinant Ku70/80. *In vitro* DNA binding assays were carried out by incubating recombinant Ku70/80 (12 fmol) with [γ-^{32}P]-ATP labeled dsRND (120 fmol) (Gibco) in the presence of 100 ng/μl of highly sheared CT DNA as nonspecific competitor DNA. The reaction was performed in quadruplets (lane 1 to 4). The samples were resolved through electrophoresis on a 4% nondenaturing polyacrylamide gel. The gel was wet exposed to an autoradiography film for 8 h. The bands containing Ku70/80-bound DNA were excised and recovered for subsequent DNA amplification by PCR and further selection by Ku70/80. A total of 8 rounds of *SELEX* by Ku70/80 were undertaken and the second round of the *SELEX* was shown as an example. **(B) Enrichment of high affinity DNA binding sites for recombinant Ku70/80 with reiterative rounds of *SELEX*.** *In vitro* DNA binding assays were undertaken with recombinant Ku70/80 (5 ng) and [γ-^{32}P]-ATP labeled dsRND (20 fmol) from round 2, 4, 6 and 8 of *SELEX* in the presence of 1 μg of CT DNA. The samples were resolved through electrophoresis on a 4% nondenaturing polyacrylamide gel. After drying, the gel was exposed to an autoradiography film for 8 h.

A.


Ku (12 fmol) + + + +
dsRND (120 fmol) + + + +
CT DNA (100 ng/µl) + + + +
Ku-dsRND
dsRND
1 2 3 4

B.

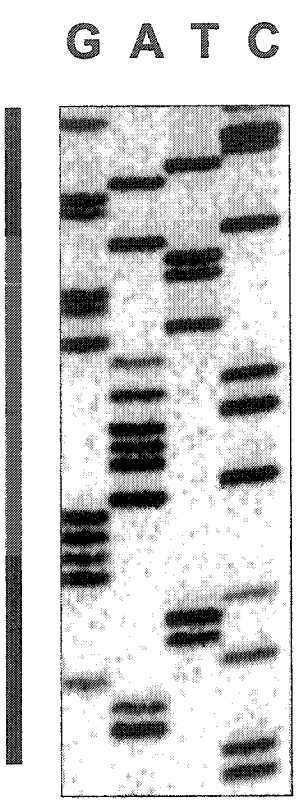

Ku (5 ng) + + + +
dsRND (20 fmol) + + + +
Round of selection 2 4 6 8
Ku-DNA complex
dsRND
1 2 3 4

observed in the presence of 1 μg of CT DNA (lane 1). By comparison, Ku70/80 binding to dsRND from rounds 2 to 8 of the *SELEX* (lanes 1 to 4) became increasingly stronger. These results demonstrated that, with successive rounds of the *SELEX* by recombinant Ku70/80, the selected pool of ds oligonucleotides displayed an increasingly higher affinity for the Ku protein (lanes 1 to 4), which suggested an enrichment of high affinity DNA binding sites for the Ku autoantigen as the *SELEX* progressed.

Seventy-two Ku70/80-selected DNA binding sites from the final round of the *SELEX* were sequenced and listed in Appendix V. Somewhat surprisingly, of the initial five clones sequenced, clone 01 contained a perfect match to the heptameric sequence – CACAGTG-, which is a recombination signal sequence that mediates V(D)J recombination (Figure 13A). In addition, clone 5 contained a 5/7 match (-ggCAGTG-) to the RSS heptamer. These preliminary results suggested that the *SELEX* by the Ku protein probably evolved a novel class of direct DNA binding sites, i.e. the RSS heptamer, for the Ku autoantigen. Further sequencing studies and analysis of the 72 sequences showed that approximately one third (30.56%) of the sequenced DNA binding sites bear homology to the RSS heptamer (Figure 13B and Appendix VI), which were aligned with the program MultAlin available at http://www.toulouse.inra.fr/multalin.html. Given the size of the initial random library of approximately 6.87 X 10^{10} distinct sequences, the probability of arbitrarily finding a definite heptameric sequence is one out of every 4096 sequences or approximately 0.024% and the probability of arbitrarily finding a definite heptameric sequence when variations at any two positions of the heptamer are allowed is one out of every 256 sequences or approximately 0.39%. Therefore, when variations at any two positions of the heptamer were allowed, the final pool of Ku70/80-evolved DNA

Figure 13. Preferred DNA binding sites for Ku70/80 evolved via *SELEX* contain sequences homologous to the RSS heptamer. (A) The evolved DNA binding sites for Ku70/80 contain the RSS heptamer. The Ku70/80-selected DNA binding sites from the final round of the *SELEX* were cloned and sequenced. As an example, the sequence from clone 001 was shown with the flanking sequence indicated in black, the N_{18} sequence indicated in red and blue and the sequence identical to the RSS heptamer underlined and indicated in red. **(B) Selected DNA sequences homologous to the RSS heptamer.** The selected DNA binding sites containing the sequences bearing homology to the RSS heptamer were manually identified and aligned with the program MultAlin available at http://www.toulouse.inra.fr/multalin.html. **(C) The nucleotide frequency at each position of the heptamer.** The nucleotide frequency at each position of the heptamer was calculated and shown in the bar graph. The vertical axis indicates the percentage of each nucleotide and the horizontal axis shows the position of nucleotides at the heptamer.

A.

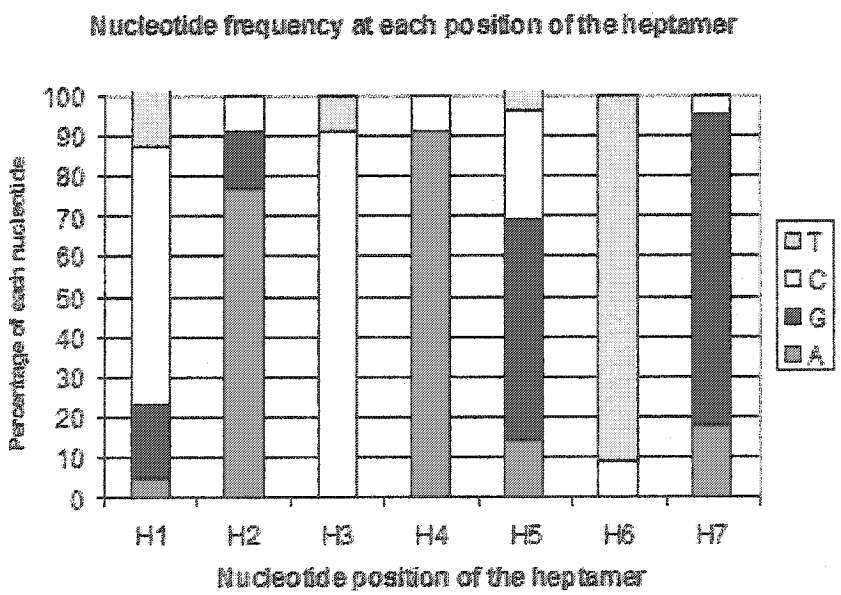


Clone 01 5'-CTTCGGGGACAAACACAGTGGTTACGGA - 3'

B. **Selected DNA sequences homologous to the RSS heptamer**

Sequence number	Aligned sequences
001	GGGACAAACACAGTGGTT
019	GGTACAGTGCCGCACCAT
038	GACAGTGGCTGATGCCTG
005	GACTAGGCAGTGCGCATA
024	GGCAGTGTAGCGACTTGA
010	TGCAGTGGACACCTCCTA
042	ACACACAAAAACCAGTGC
032	ACAGGATAGTGCTTGGTA
049	CCCGTACAATGATAGCGA
066	GGGGAGAGAACACCGTCT
060	GGATAATCCCACTGCCAT
039	ATGCATAGTGTTGTGTGT
051	ATATGCACCACACCATGG
021	GGGGACAAAACACACTGT
033	ACAAGTGCACAATGTTAC
041	CAACACAACACACTGCTA
069	GCACACTGCTAATGGCCC
009	GGAGGACACATCGGCCCC
036	ACGACACACTATCTGCCC
059	AGGTTGCACACTATACCC
018	CACAGTATTGAGTGCGGT
037	GGTTCGGTACACAGCATG
Consensus sequence:	CACAGTG
RSS heptamer	CACAGTG

C.



binding sites was enriched more than 70 fold for the heptameric sequence compared with the initial random pool of ds oligonucleotides.

The analysis of the frequency of nucleotides at each position of the heptamer from the sequences bearing homology to the RSS heptamer, -$C^1A^2C^3A^4G^5T^6G^7$-, showed that C^1 was 64%, A^2 77%, C^3 91%, A^4 91%, G^5 55%, T^6 91% and G^7 77% conserved (Figure 13C). Only two out of the twenty-two sequences bearing homology to the RSS heptamer show sequence variations at the third, fourth and sixth positions, respectively, with C^3 replaced by T only, A^4 by C only and T^6 by C only. On the other hand, positions 1, 2 and 7 of the heptamer tolerate greater sequence variations. These observations suggest that the third, fourth and sixth positions of the RSS heptamer were most important for binding by the Ku autoantigen as only slight variations were observed in these three positions.

The Ku70/80-selected DNA binding sites by *SELEX* also include a subgroup of sequences that contain the E-box motif (Appendix VII). The E-box motif is a palindromic sequence consisting of -CANNTG- and is involved in cell-type specific gene transcription regulation by interacting with a variety of E-box binding proteins (Massari and Murre 2000). The E-box motif is also present in Ig enhancers and is probably implicated in somatic hypermutations in the Ig variable region gene (Michael et al. 2003). The calculated frequency of sequences containing the E-box motif from the initial library of random oligonucleotides is one out of 64 sequences or approximately 1.56%. The percentage of the Ku70/80-selected sequences containing the E-box motif is approximately 7.14%, showing an approximately 4-fold enrichment over random sequences, which suggests that the E-box motif is a weaker putative specific DNA

binding site for Ku70/80 compared with the RSS heptamer. Interestingly, a reexamination of the reported putative specific DNA binding sites for the Ku protein (Appendix III and the references therein) revealed the presence of the E-box motif in several of these putative specific sequences like the negative calcium regulatory element (nCaRE) (Okazaki et al. 1991; Chung et al. 1996) (Appendix VII).

It is of interest to note that the hexameric E-box motif CANNTG bears homology to the RSS heptameric sequence CACAGTG, which also contains an embedded sequence CAGTG. These sequences can be shown as $CA(N_{1-3})TG$. Also of note is the presence of a potential E-box binding motif (^{467}EKLR^{470}) in Ku70 (Blackwell and Weintraub 1990; Fisher et al. 1992), which lies at the end of helix α14 (amino acid residues from 456 to 468) and the start of the following loop (amino acid residues from 469 to 480) followed by helix α15 (amino acid residues from 481 to 494) in the C terminal arm of Ku70 (Walker et al. 2001) and is adjacent to the putative leucine zipper region (amino acid resides from 483 to 511) (Chan et al. 1989; Reeves and Sthoeger 1989). Though the current study did not address the question whether the E-box motif was a direct DNA binding site for the Ku protein, it is worth pointing out that Ku70/80 was found to bind to a hairpin DNA substrate lacking free ends but containing a cryptic E-box motif (-CACGTG-) (Arosio et al. 2002). The characterization of Ku70/80 binding to the E-box motif and its functional implications and the role of the putative E-box binding motif in Ku70, however, have to await further investigations.

In summary, the *SELEX* by recombinant Ku70/80 appeared to efficiently select high affinity DNA binding sites for the Ku protein, which include the RSS heptamer and the E-box motif with a consensus of $CA(N_{1-3})TG$. The percentage of the RSS heptamer

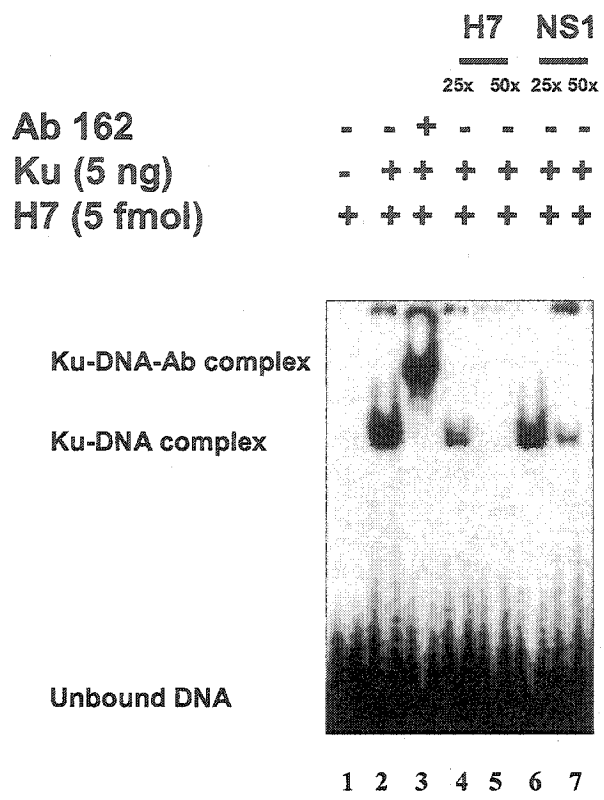
and that of the E-box motif in the selected pool of sequences suggest that the heptamer is a higher affinity DNA binding site than the E-box motif for the Ku autoantigen. The RSS heptamer and the E-box motif mediate important cellular processes (Massari and Murre 2000; Jung and Alt 2004); direct binding by the Ku autoantigen to these sequences, as suggested by the findings from the *SELEX* by Ku70/80, has important implications for its specific sequence-directed functions in the cell.

3.3. Ku70/80 binds preferentially to the RSS heptamer in vitro

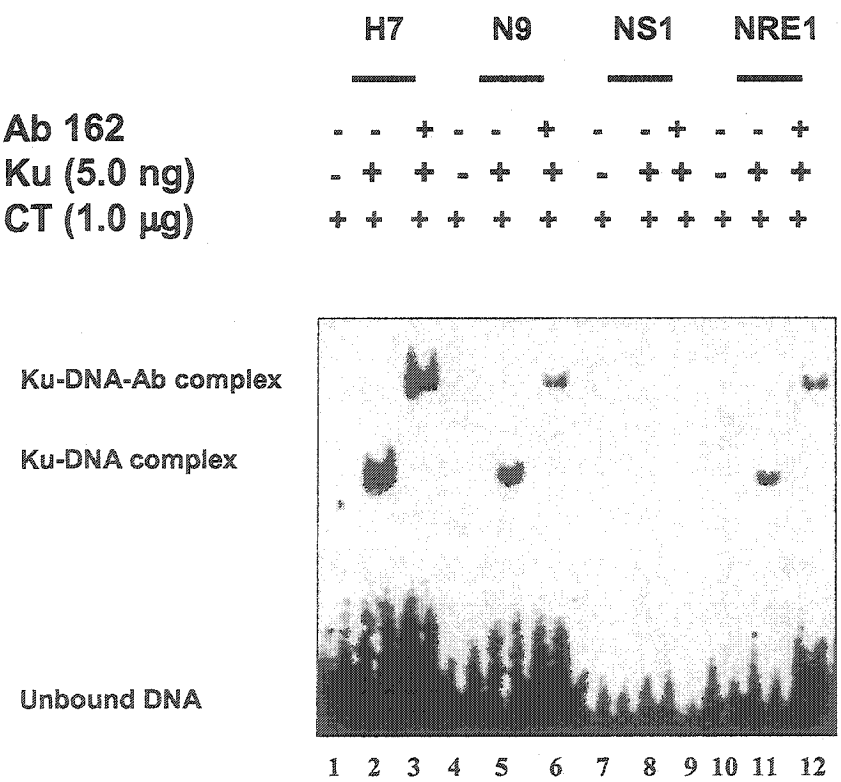
The results from the *SELEX* by recombinant Ku70/80 suggest preferential binding of Ku70/80 to the RSS heptamer over random DNA sequences. To further confirm preferential binding of Ku70/80 to the RSS heptamer over nonspecific DNA, competitive EMSAs were carried out using linear radiolabeled oligonucleotides (H7) which contained the wild type RSS heptamer and recombinant Ku70/80. As shown in Figure 14A, Ku70/80 binding to [γ-^{32}P]-ATP labeled H7 was easily visualized in the presence of 50 ng of CT DNA (lane 2) and the specificity of DNA binding by Ku70/80 was confirmed by the supershifted Ku70/80-DNA complex with Ab 162 (lane 3). Additionally, Ku70/80 binding to [γ-^{32}P]-ATP labeled H7 was sensitive to the competition by a 25-fold molar excess of unlabeled H7 (lane 4), but was resistant to the competition by a 25-fold molar excess of unlabeled NS1 (lane 6). While Ku70/80 binding to [γ-^{32}P]-ATP labeled H7 was effectively abolished by a 50-fold molar excess of unlabeled H7, it remained visible in the presence of a 50-fold molar excess of unlabeled NS1 (lane 7). These findings indicated that, compared to nonspecific DNA, the RSS heptamer is a preferred DNA binding site for the Ku autoantigen.

Figure 14. Preferential binding by Ku70/80 to the RSS heptamer *in vitro*. (A) Preferential binding of Ku70/80 to the RSS heptamer over nonspecific DNA. *In vitro* DNA binding assays were performed by incubating Ku70/80 (5 ng) with [γ-^{32}P]-ATP labeled H7 (5 fmol). Either a 25- or 50-fold molar excess of unlabelled H7 or NS1 were used as indicated to compete the binding of Ku70/80 to [γ-^{32}P]-ATP labeled H7. Ab 162 was used to supershift the Ku70/80-DNA complexes (lane 3). The samples were resolved through electrophoresis on a 4% nondenaturing polyacrylamide gel and autoradiographed. **(B) Comparison of Ku70/80 binding to the RSS heptamer, nonamer, NRE1 and nonspecific DNA.** *In vitro* DNA binding assays were performed by incubating Ku70/80 (5 ng) with 25 fmol each of [γ-^{32}P]-ATP labeled H7, N9, NS1 or NRE1 as specified. One μg of CT DNA was included as nonspecific competitor DNA and Ab 162 was added as specified. The samples were resolved through electrophoresis on a 4% nondenaturing polyacrylamide gel and autoradiographed.

A.


H7
NS1
25x 50x 25x 50x
Ab 162 - - + - - - -
Ku (5 ng) - + + + + + +
H7 (5 fmol) + + + + + + +
Ku-DNA-Ab complex
Ku-DNA complex
Unbound DNA
1 2 3 4 5 6 7

B.


H7
N9
NS1
NRE1
Ab 162 - - + - - + - - + - - +
Ku (5.0 ng) - + + - + + - + + - + +
CT (1.0 µg) + + + + + + + + + + + +
Ku-DNA-Ab complex
Ku-DNA complex
Unbound DNA
1 2 3 4 5 6 7 8 9 10 11 12

The results from the competitive EMSAs with NRE1, A3/4 and NS1 showed that Ku70/80 binds to different specific DNA sequences and nonspecific DNA with distinct binding affinities. It was also of interest to directly compare Ku70/80 binding to the RSS heptamer, the RSS nonamer, NRE1 and nonspecific DNA. *In vitro* DNA binding assays were carried out using recombinant Ku70/80 and linear radiolabeled H7, N9, NS1 or NRE1 in the presence of 1 μg of CT DNA, which was known to abolish DNA end binding activity of the Ku protein. As shown in Figure 14B, in the presence of 1 μg of CT DNA, Ku70/80 binding to [γ-^{32}P]-ATP labeled NS1 was effectively aborted (lane 8) while Ku70/80 binding to [γ-^{32}P]-ATP labeled H7 (lane 2), N9 (lane 5) or NRE1 (lane 11) was readily visible. Furthermore, Ku70/80 binding to the RSS heptamer (lane 2) was apparently of a higher affinity than its binding to either the RSS nonamer (lane 5) or NRE1 (lane 11).

In summary, these preliminary results demonstrated that Ku70/80 binds preferentially to the RSS heptamer *in vitro* over nonspecific DNA ends and it also binds to the RSS heptamer with a higher affinity than its binding to either the RSS nonamer or NRE1, suggesting that the RSS heptamer is probably in a different category of specific DNA binding sites from NRE1.

3.4. Ku70/80 is stably associated with the RSS heptamer

Our laboratory has shown that Ku70/80 remains stably associated with NRE1 once its binding to NRE1 has occurred (Rodda et al. 1995; Giffin et al. 1997; Torrence et al. 1998). Using crude Jurkat nuclear extracts for EMSA, the Ku protein was found to have a half time of one hour for release from dsNRE1 (Rodda et al. 1995). It was of

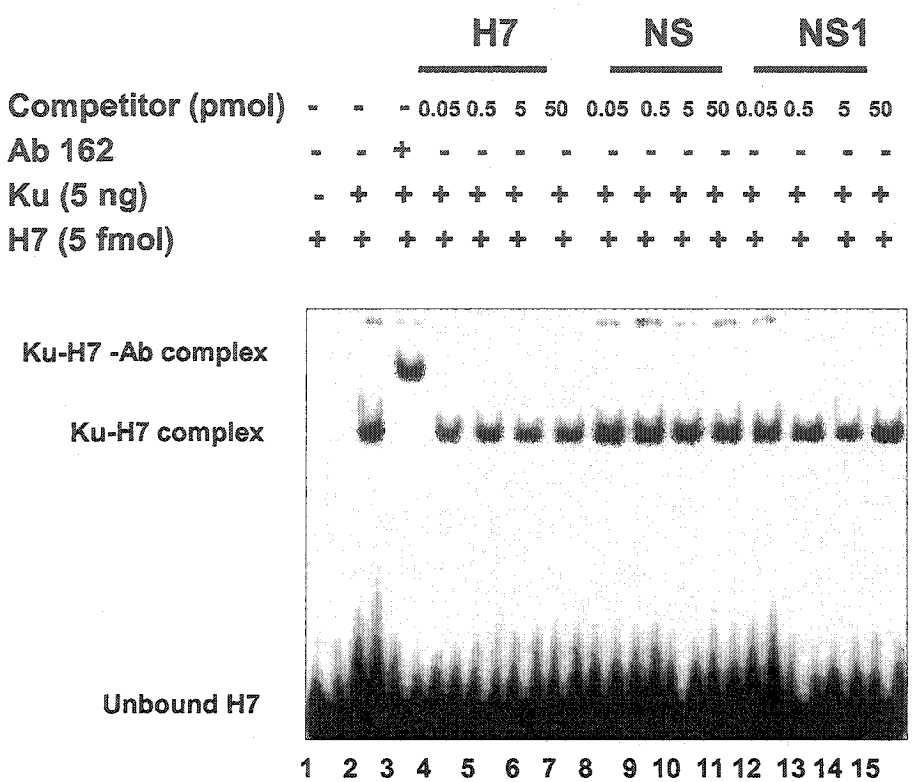
interest to know if the Ku70/80-RSS heptamer complexes were stable following Ku70/80 binding to the heptamer. As aforementioned, *in vitro* DNA binding assays were performed by first incubating recombinant Ku70/80 with [γ-^{32}P]-ATP labeled H7 for 5 min. Thereafter, unlabeled competitor DNA was added as needed and the reaction was continued for 20 more min. These assays differed from the standard *in vitro* DNA binding assays as previously described (Giffin et al. 1996, 1997) in which the target DNA substrates and competitor DNA substrates were mixed before Ku70/80 was added.

As shown in Figure 15A, Ku70/80 binding to [γ-^{32}P]-ATP labeled H7 was readily visualized (lane 2) under standard *in vitro* DNA binding conditions and its specificity was confirmed by using Ab 162 (lane 3). The addition of a 10-fold molar excess of unlabeled H7 resulted in a slight inhibition of Ku70/80 binding to [γ-^{32}P]-ATP labeled H7 (lane 4); however, a further increase of unlabeled H7 to up to a 10,000 fold caused no additional inhibition of DNA binding by the Ku protein (lanes 5 to 7). By comparison, the addition of from a 10- to up to a 10,000-fold molar excess of nonspecific DNA, NS or NS1, resulted in no noticeable inhibition of Ku70/80 binding to [γ-^{32}P]-ATP labeled H7 (lanes 8 to 11, and lanes 12 and 15) compared with Ku70/80 binding to the heptamer in the absence of any competitor DNA (lane 2). These results indicated that, once the Ku autoantigen was bound to the RSS heptamer, the Ku70/80-RSS heptamer complexes were stable and resistant to competition by other DNA molecules.

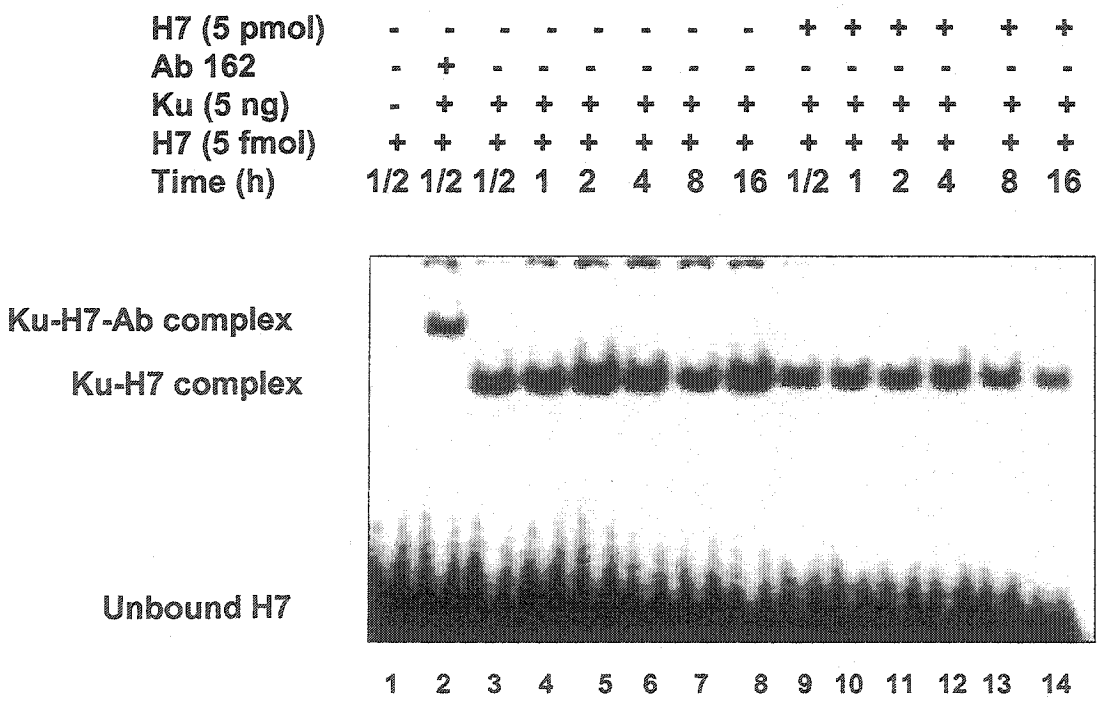
The binding of the Ku protein to [γ-^{32}P]-ATP labeled H7 was further investigated over the course of 16 h in the presence or absence of a 10,000-fold molar excess of unlabeled H7. *In vitro* DNA binding assays were performed by first incubating Ku70/80 with [γ-^{32}P]-ATP labeled H7 for 5 min; thereafter, a 10,000-fold molar excess of

Figure 15. Stable association of Ku70/80 with the RSS heptamer. (A) The Ku70/80-RSS heptamer complex was resistant to competition. Ku70/80 (5 ng) was pre-incubated with 5 fmol of [γ-^{32}P]-ATP labeled H7 for 5 min at 20°C. Thereafter, a 10-, 100-, 1000-, or 10,000-fold molar excess of unlabeled H7 or nonspecific DNA, NS or NS1, were added as indicated and further incubated for 20 min. The Ku70/80-specific antibody 162 was used to supershift the Ku70/80-DNA complexes (lane 3). The samples were resolved through electrophoresis on a 4% nondenaturing polyacrylamide gel and autoradiographed. **(B) The Ku70/80-RSS heptamer complex was stable over time.** Ku70/80 (5 ng) was incubated with 5 fmol of [γ-^{32}P]-ATP labeled H7 at 20°C for the length of time indicated. In parallel, a 10,000-fold molar excess of unlabeled H7 was added after 5 min of incubation of Ku70/80 with [γ-^{32}P]-ATP labeled H7 and the reactions were continued for the length of time indicated. The Ku70/80-specific antibody 162 was used to supershift the Ku70/80-DNA complexes (lane 2). The samples were resolved through electrophoresis on a 4% nondenaturing polyacrylamide gel and autoradiographed.

A.


H7
NS
NS1
Competitor (pmol) - - - 0.05 0.5 5 50 0.05 0.5 5 50 0.05 0.5 5 50
Ab 162 - - + - - - - - - - - - - - -
Ku (5 ng) - + + + + + + + + + + + + + +
H7 (5 fmol) + + + + + + + + + + + + + + +
Ku-H7 -Ab complex
Ku-H7 complex
Unbound H7
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

B.


H7 (5 pmol) - - - - - - - - + + + + + +
Ab 162 - + - - - - - - - - - - - -
Ku (5 ng) - + + + + + + + + + + + + +
H7 (5 fmol) + + + + + + + + + + + + + +
Time (h) 1/2 1/2 1/2 1 2 4 8 16 1/2 1 2 4 8 16
Ku-H7-Ab complex
Ku-H7 complex
Unbound H7
1 2 3 4 5 6 7 8 9 10 11 12 13 14

unlabeled H7 were added as needed and the reactions were continued for up to 16 h. In the absence of unlabelled H7, Ku70/80, if unbound, would be expected to associate with [γ-^{32}P]-ATP labeled H7 if it was capable of doing so. However, in the presence of a 10,000-fold molar excess of unlabelled H7, Ku70/80, if still unbound to DNA, would simply by probability preferentially bind to unlabeled H7, and Ku70/80 that happened to be dissociated from [γ-^{32}P]-ATP labeled H7 would also by chance preferentially re-associate with unlabeled H7. As shown in Figure 15B, in the absence of unlabeled H7, Ku70/80 binding to [γ-^{32}P]-ATP labeled H7 continued to increase until 2 h after incubation (lanes 3 to 5). In the presence of a 10,000-fold molar excess of unlabeled H7, Ku70/80 binding to [γ-^{32}P]-ATP labeled H7 remained comparable at different time points over time up to 8 h into the reaction (lanes 9 to 13) and, at 16 h of incubation, the binding to [γ-^{32}P]-ATP labeled H7 was approximately half of Ku70/80 binding to [γ-^{32}P]-ATP labeled H7 at 30 min of incubation (lanes 3 and 14).

The findings from the above *in vitro* DNA binding assays established that Ku70/80 binds preferentially to the RSS heptamer and remains stably associated with the heptamer with the formation of the Ku70/80-RSS heptamer complexes.

3.5. The 3rd and 4th positions of the heptamer are important for Ku70/80 binding

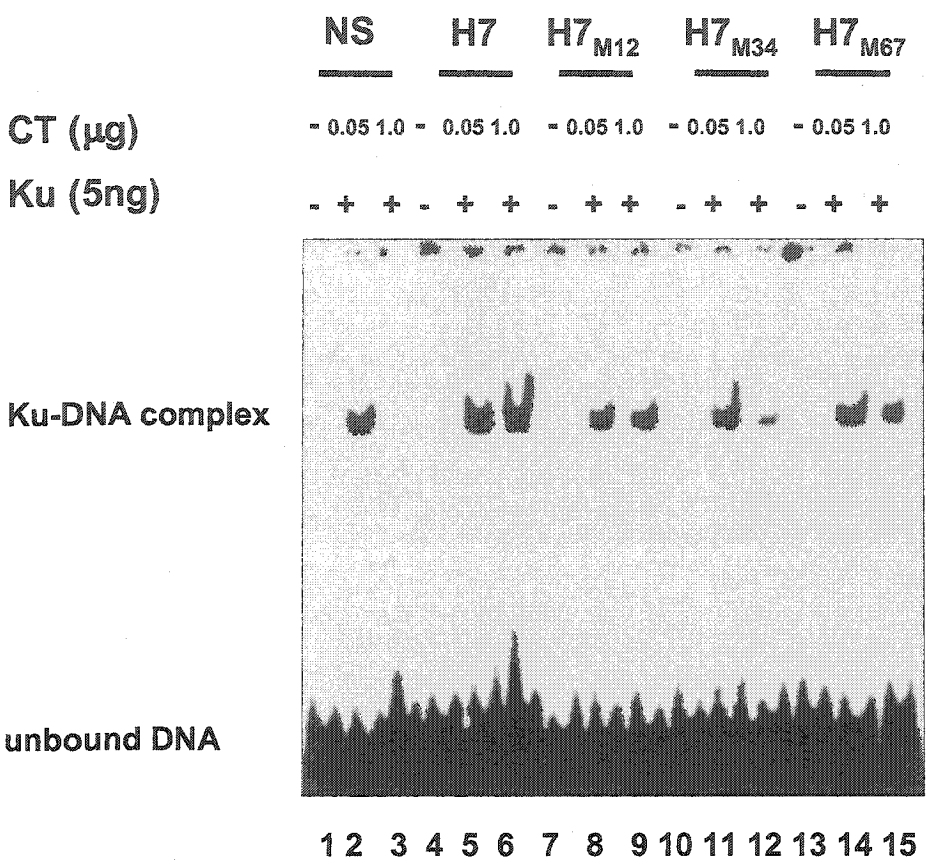
The frequency of nucleotides at each position of the heptamer from the selected sequences bearing homology to the RSS heptamer suggests that the 3rd, 4th and 6th positions of the heptamer were important for binding by Ku70/80 as only slight variations were observed in these three positions (Figure 13C). In contrast, the calculation of position information of naturally occurring RSS heptamers showed that the first three

nucleotides were almost strictly conserved followed in order by positions 4, 6, 5 and 7 (Cowell et al. 2003). To investigate if Ku70/80 binding to the wild-type or mutated heptamers corresponded to the findings from the *SELEX* by the Ku protein, *in vitro* DNA binding assays were performed by incubating Ku70/80 with either nonspecific DNA (NS), the wild-type or mutated heptamers in the presence of 50 ng or 1 μg of CT DNA. As shown in Figure 16A, noticeable binding by Ku70/80 to [γ-^{32}P]-ATP labeled NS was observed in the presence of 50 ng of CT DNA (lane 2), which, however, was abolished with 1 μg of CT DNA (lane 3). By comparison, Ku70/80 binding to [γ-^{32}P]-ATP labeled H7 was resistant to the competition by either 50 ng or 1 μg of CT DNA (lanes 5 and 6). On the other hand, in the presence of 50 ng of CT DNA, mutations in positions 1 and 2 ($H7_{M12}$), positions 3 and 4 ($H7_{M34}$) or positions 6 and 7 ($H7_{M67}$) of the heptamer similarly reduced DNA binding by Ku70/80 (lanes 7, 11 and 14) compared to its binding to the wild-type heptamer (lane 5). However, in the presence of 1 μg of CT DNA, binding by Ku70/80 to each mutant heptamer was reduced compared to its binding to the wild-type heptamer and the reduction in Ku70/80 binding to $H7_{M34}$ was most noticeable (lane 12) compared with mutations in other positions of the heptamer (lanes 9 and 15). These results indicated that, consistent with the results from the *SELEX* by recombinant Ku70/80, these two positions of the heptamer were important for binding by the Ku autoantigen *in vitro*.

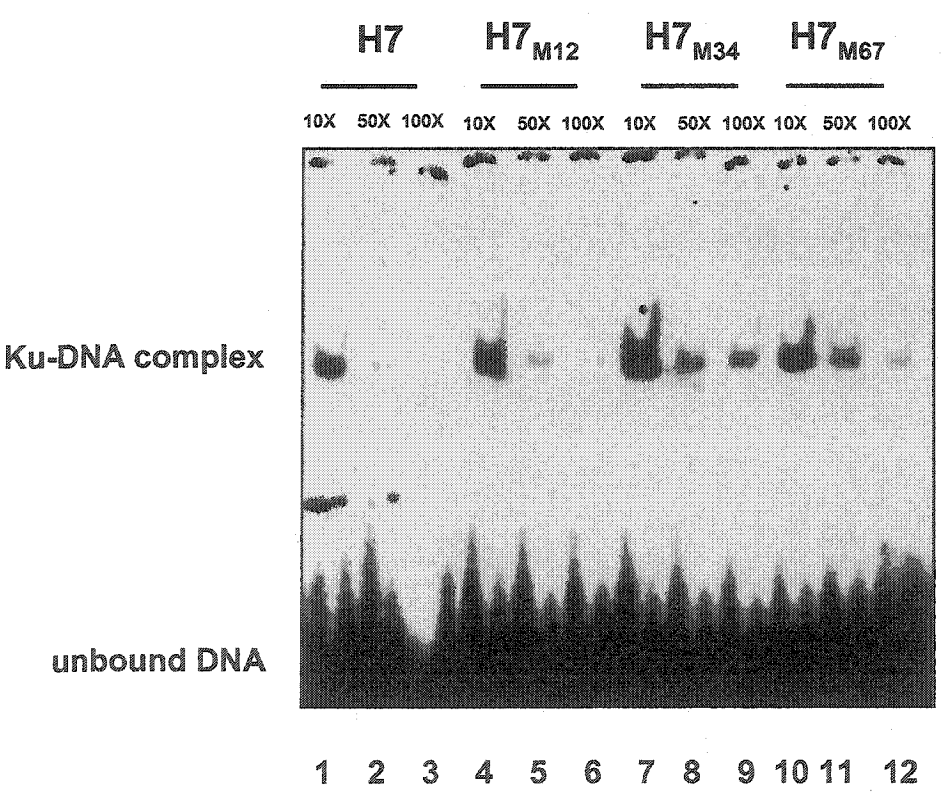
Competitive EMSAs were also carried out to examine Ku70/80 binding to [γ-^{32}P]-ATP labeled wild-type heptamer competed by either a 10-, 50- or 100- fold molar excess of the unlabelled wild-type or mutated heptamers. As shown in Figure 16B, binding by Ku70/80 to [γ-^{32}P]-ATP labeled H7 was readily visualized in the presence of a 10-fold

Figure 16. The 3rd and 4th positions of the RSS heptamer are important for binding by the Ku autoantigen. **(A)** *In vitro* DNA binding assays were performed by incubating Ku70/80 (5 ng) with 10 fmol each of [γ-^{32}P]-ATP labeled nonspecific DNA sequence (NS), the wild-type heptamer (H7, -CACAGTG-), or the mutated heptamers ($H7_{M12}$, -gcCAGTG-, $H7_{M34}$, -CAgcGTG-, or $H7_{M67}$, -CACAGgc-) in the presence of either 50 ng or 1 μg of CT DNA as indicated. The samples were resolved through electrophoresis on a 4% nondenaturing polyacrylamide gel and autoradiographed. **(B)** *In vitro* DNA binding assays were performed by incubating Ku70/80 (5 ng) with 10 fmol of [γ-^{32}P]-ATP labeled H7. A 10-, 50- or 100- fold molar excess of unlabeled H7, $H7_{M12}$, $H7_{M34}$, or $H7_{M67}$ were included as indicated. The samples were resolved through electrophoresis on a 4% nondenaturing polyacrylamide gel and autoradiographed.

A.


NS
H7
H7M12
H7M34
H7M67
CT (µg)
- 0.05 1.0 - 0.05 1.0 - 0.05 1.0 - 0.05 1.0 - 0.05 1.0
Ku (5ng)
- + + - + + - + + - + + - + +
Ku-DNA complex
unbound DNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

B.


H7
H7M12
H7M34
H7M67
10X 50X 100X 10X 50X 100X 10X 50X 100X 10X 50X 100X
Ku-DNA complex
unbound DNA
1 2 3 4 5 6 7 8 9 10 11 12

molar excess of either the wild-type or the mutated H7 (lanes 1, 4, 7 and 10). Ku70/80 binding to [γ-^{32}P]-ATP labeled H7 was effectively competed by a 50-fold molar excess of unlabeled H7 (lane 2). It was reduced to a negligible level by a 50-fold molar excess of unlabeled $H7_{M12}$ (lane 5) and virtually abolished by a 100-fold molar excess of unlabeled $H7_{M12}$ (lane 6). The reduction in Ku70/80 binding to [γ-^{32}P]-ATP labeled H7 was less noticeable when a 50-fold molar excess of unlabeled $H7_{M34}$ or $H7_{M67}$ were present (lanes 8 and 11). Furthermore, an increase of $H7_{M34}$ to a 100-fold molar excess over [γ-^{32}P]-ATP labeled H7 resulted in no further reduction in Ku70/80 binding to the heptamer while such an increase in $H7_{M67}$ reduced Ku70/80 binding to H7 to a negligible level (lane 12).

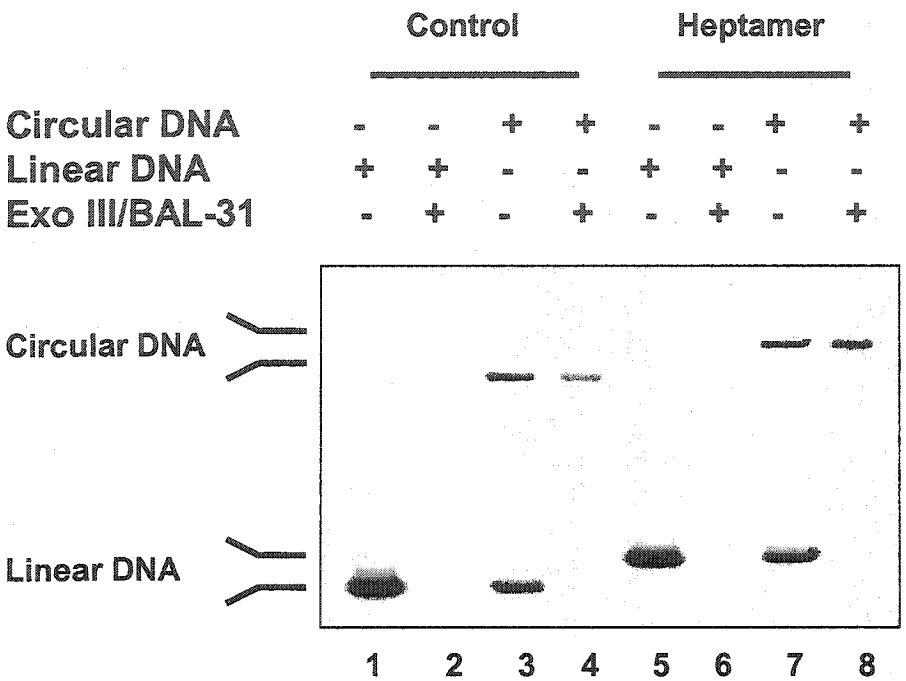
The above findings demonstrate that the 3rd and 4th positions, and, to a lesser extent, the 6th position, of the RSS heptamer are important for binding by the Ku autoantigen *in vitro* while the first two positions are less critical. These findings are consistent with the observations of the nucleotide frequency at each position of the heptameric sequences from the *SELEX* by Ku70/80 but differ from the theoretical calculation by Cowell et al. (2003), who suggested that the first three nucleotides are important for DNA-protein interaction as they are almost strictly conserved. Furthermore, in naturally occurring RSS heptamers in the Igh or TCR variable region locus, the first three nucleotides (-^{1}CAC^{3}-) are approximately 100% conserved while the remaining nucleotides are only 63 to 86% conserved (Akira et al. 1987; Hesse et al. 1989; Akamatsu et al. 1994).

3.6. Direct binding of the Ku protein to the RSS heptamer

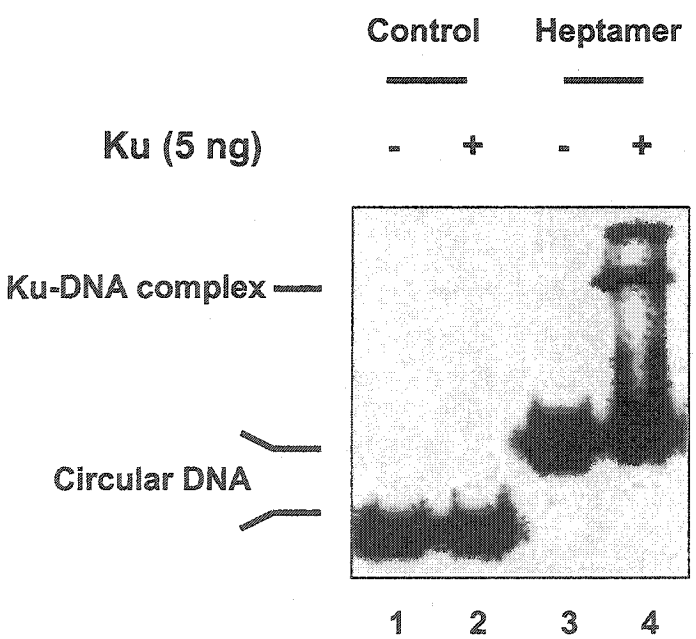
The enrichment of the RSS heptamer in Ku70/80-selected DNA binding sites by the *SELEX* suggests a specific binding of the Ku protein to the sequence. However, due to the linearity of the random ds oligonucleotides employed for the *SELEX* by recombinant Ku70/80 and the arbitrary cutoff of CT DNA concentration for the optimal minimization of DNA end binding activity by the Ku protein, nonspecific binding to DNA ends by Ku70/80 and its subsequent translocation to internal sequences on DNA remained a distinct possibility throughout the *SELEX* process. To demonstrate direct binding by Ku70/80 to the RSS heptamer, DNA microcircles containing the RSS heptamer were prepared as previously described (Giffin et al. 1996, 1997). As shown in Figure 17A, the intramolecular ligation of linear *Not*I-*Bgl*I fragments from the control plasmids (lane 1) and the RSS heptamer-containing plasmids (lane 5) resulted in the formation of DNA microcircles (lanes 3 and 7). Treatment of linear control DNA (lane 2) or linear heptamer DNA (lane 6) with Exonuclease III and BAL-31 nuclease resulted in the complete digestion of these linear DNA fragments. However, the circular control DNA (lane 4) or the circular DNA containing the RSS heptamer (lane 8) was resistant to digestion by the nucleases. *In vitro* DNA binding assays were carried out by incubating Ku70/80 with either the control DNA microcircles or the heptamer-containing DNA microcircles in the presence of 500 ng of CT DNA. As indicated in Figure 17B, the Ku protein was bound to the DNA microcircles containing the heptamer (lane 4) while it failed to bind to the control DNA microcircles (lane 2). These results demonstrated that the Ku autoantigen binds directly to the RSS heptamer *in vitro*.

Figure 17. Direct binding of Ku70/80 to the RSS heptamer. (A) Preparation of DNA microcircles. Linear DNA excised from the control plasmids or heptamer-containing plasmids (lanes 1 and 5) and the intramolecular ligation products of the linear control or heptamer DNA (lanes 3 and 7) were digested by Exonuclease III (Exo III) and BAL-31 nuclease as indicated. The samples were resolved through electrophoresis on a 6% nondenaturing polyacrylamide gel and wet exposed to an autoradiography film for 2 h. The DNA microcircles that were formed from intramolecular ligations resistant to nuclease digestion were recovered for EMSAs. **(B) Ku70/80 binding to DNA microcircles containing the RSS heptamer.** *In vitro* DNA binding assays were performed by incubating Ku70/80 (5 ng) with 10 fmol of [α-^{32}P]-dCTP labeled control DNA microcircles and DNA microcircles containing an insert of the RSS heptamer as indicated. The samples were resolved through electrophoresis on a 4% nondenaturing polyacrylamide gel and autoradiographed.

A.


Control
Heptamer
Circular DNA - - + + - - + +
Linear DNA + + - - + + - -
Exo III/BAL-31 - + - + - + - +
Circular DNA
Linear DNA
1 2 3 4 5 6 7 8

B.


Control
Heptamer
Ku (5 ng) - + - +
Ku-DNA complex
Circular DNA
1 2 3 4

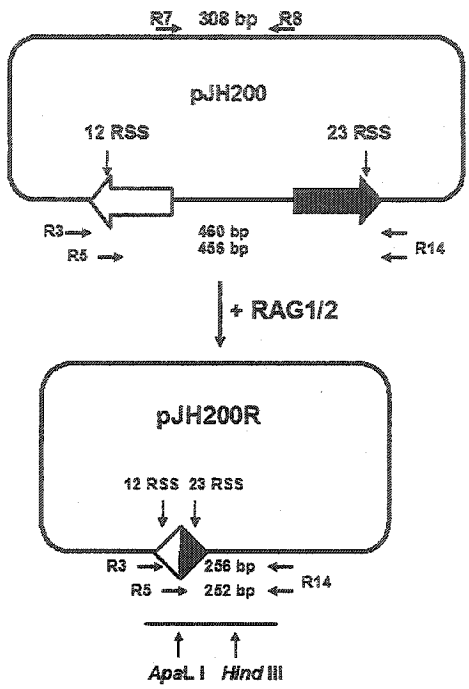
3.7. Ku70/80 is associated with the RSS in vivo

Though the RSS heptamer was the preferred *in vitro* DNA binding site for the recombinant Ku autoantigen as evolved through *SELEX* by Ku70/80 and demonstrated by EMSAs, little was known about the *in vivo* DNA binding activity of the Ku autoantigen. In addition, direct determination of Ku70/80 binding to the RSS heptamer *in vivo* required that the binding of endogenous Ku70/80 to the RSS heptamer be measured in living cells. For the determination of endogenous Ku70/80 binding to the RSS heptamer, the extrachromosomal recombination DNA substrates pJH200 (Figure 18A), which contained the endogenous VκL8 and Jκ1 gene segments of Igκ light chain (Hesse et al. 1987), were transiently transfected into 293T cells. As shown in Figure 18B, plasmid immunoprecipitation analysis of these transfected cells with anti-Ku antibodies or a control antibody to GAL4 showed that anti-Ku antibodies specifically recovered a 460 bp fragment spanning the endogenous VκL8 and Jκ1 gene segments (lane 2), but it failed to recover a 308 bp control DNA fragment approximately 3 kb across from the recombination insert from Igκ light chain (lane 5). On the other hand, an antibody to GAL4 failed to recover either the 460 bp or 308 bp fragments (lanes 3 and 6). These results suggest that the Ku autoantigen was specifically associated with the VκL8 and Jκ1 gene segments in the extrachromosomal recombination DNA substrates *in vivo*.

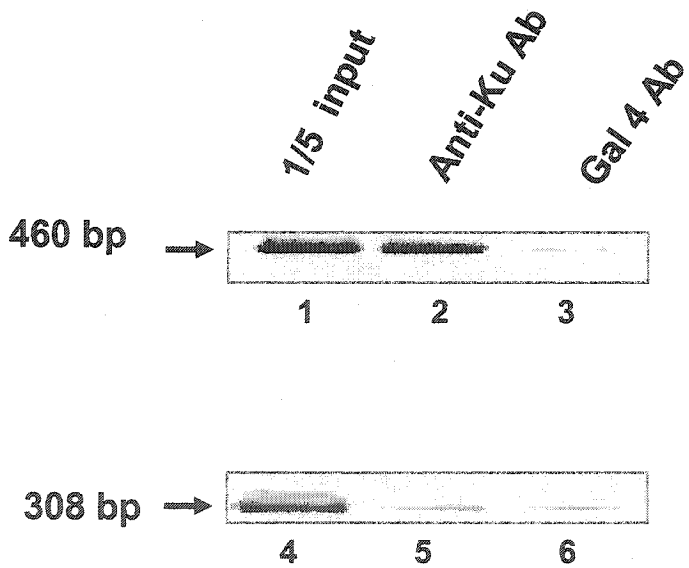
However, the Ku70/80 binding to the RSS heptamer *in vitro* and its binding to the RSS in the extrachromosomal DNA substrates *in vivo* may not reflect its *in vivo* binding activity to the endogenous RSSs residing within a highly organized chromatin structure whose accessibility is tightly regulated. To determine whether endogenous Ku70/80 was localized to the endogenous RSSs in the chromosome, A-MuLV transformed RAG2$^{-/-}$

Figure 18. *In vivo* association of Ku70/80 with the RSS. (A) The schematic representation of the extrachromosomal recombination substrate pJH200. pJH200 contains the endogenous VκL8 gene segment with its 12RSS in the orientation of the nonamer-12 bp spacer-the heptamer and Jκ1 gene segment with its 23RSS in the orientation of the heptamer-23 bp spacer-the nonamer. In the presence of the RAG1/2 protein, pJH200 undergoes deletional recombination with the intervening sequences between the two heptamers deleted from the plasmid substrates. The primer set R7 and R8 generates a 308 bp fragment more than 3 kb from the 12RSS or 23RSS and amplifies both unrecombined and recombined pJH200. The primer set R3 and R14 generates by PCR a 460 bp fragment spanning the region containing the 12RSS and 23RSS before recombination and a 256 bp fragment containing the fused RSSs in the configuration of the nonamer-12 bp spacer-the heptamer-the heptamer-23 bp spacer-the nonamer after recombination. The primer set R5 and R14 generates by PCR a 456 bp fragment spanning the region containing the 12RSS and 23RSS before recombination and a 252 bp fragment containing the fused RSSs following recombination. Primer R5 contains one nt mismatch to the unrecombined DNA substrate and a perfect match to the recombined DNA substrate and thus preferentially amplifies the recombined DNA substrates (Cortes et al. 1996). The endonuclease *Apa*L I restricts a precisely formed signal joint as a result of recombination. **(B) The Ku protein was associated with the RSS *in vivo*.** Immunoprecipitation with anti-Ku antibodies or an antibody to GAL4 as indicated was undertaken of 293T cells transiently transfected with pJH200. One tenth of the samples were amplified by PCR using the primer set R3 and R14 or the primer set R7 and R8. The PCR products were resolved on a 2% agarose gel stained with Vistra Green (Pharmacia). Binding of the Ku protein to the RSSs region was compared with 20% of the input which is the DNA substrates recovered after transfection.

A.


R7
308 bp
R8
pJH200
12 RSS
23 RSS
R3
R5
460 bp
456 bp
R14
+ RAG1/2
pJH200R
12 RSS 23 RSS
R3
R5
256 bp
252 bp
R14
ApaL I
Hind III

B.

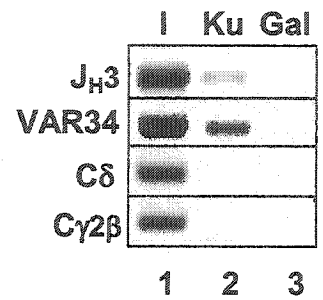

1/5 input
Anti-Ku Ab
Gal 4 Ab
460 bp
1
2
3
308 bp
4
5
6

pro-B cell line 63-12 (Shinkai et al. 1992) was employed for ChIP analysis of Ku70/80-RSS interaction. The *v-abl* nonreceptor tyrosine kinase expressed by A-MuLV allows pro-B cells to continuously grow in culture independent of IL-7; however, these cells failed to passs the pro- to pre-B cell transition step (Shinkai et al. 1992; Banerjee and Rothman 1998). This cell line undergoes the activation of the Igκ locus in response to external stimuli such as LPS and has been extensively used as a model cell line for studying the developmental regulation of the Igκ locus activation (Shaffer et al. 1997; Maes et al. 2001). In A-MuLV transformed $RAG2^{-/-}$ pro-B cells, the VAR34 gene segment in the more distal V_HJ558 family and J_H3 gene segment of the Igh variable region locus and Cδ and Cγ2β of the Igh constant region locus were acetylated while the 3:3.39 gene segment of the D_H proximal V_H7183 family of the Igh variable region locus was not acetylated (Chowhury and Sen 2001, 2003). The Igκ locus in these cells remained inaccessible, but underwent a rapid onset of transcription from the locus upon treatment with the *Abl* kinase inhibitor ST1571 (Muljo and Schlissel 2003). However, no RSS cleavage occurred in these cells because of a deficiency of RAG2 and a depressed expression of RAG1 (Shinkai et al 1992; Muljo and Schlissel 2003).

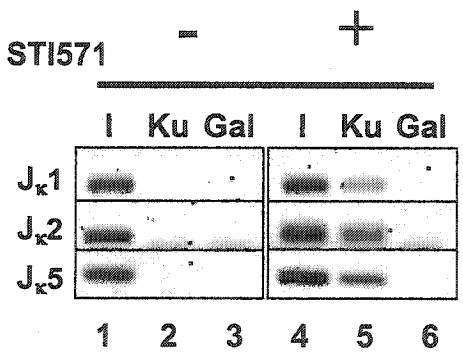
As shown in Figure 19A, an antibody against Ku70/80 specifically recovered DNA centered over the RSSs from the VAR34 and J_H3 gene segments of the Igμ heavy chain locus, but it failed to recover DNA from either the Cδ or Cγ2β locus, suggesting a strong specific association of the Ku protein with the endogenous RSSs from the Igh locus that was correlated directly with chromatin accessibility to these gene segments. For the Igκ locus, as shown in Figure 19B, in the absence of treatment with the *Abl* kinase inhibitor ST1571, an antibody against Ku70/80 failed to recover any DNA from

Figure 19. Association of endogenous Ku70/80 with the RSSs residing in the chromosome in pro-B cells. (A) ChIP analysis of Ku70/80 association with Ig heavy chain locus in RAG2$^{-/-}$ 63-12 pro-B cells. ChIP was performed with an antibody to Ku70/80 (lane 2) or an antibody to GAL4 (lane 3) and amplified with the primer sets for the indicated gene segments in the Ig μ heavy chain locus and the Igh constant region locus. Lane 1 is the input (I). **(B) ChIP analysis of Ku70/80 association with Igκ light chain locus in RAG2$^{-/-}$ 63-12 pro-B cells with or without ST1571.** RAG2$^{-/-}$ 63-12 pro-B cells were treated with 10 μM ST1571 as indicated. ChIP was performed prior to ST1571 treatment or 16 h following treatment with an antibody to Ku70/80 (lane 2) or an antibody to GAL4 (lane 5) and amplified with the primer sets for the indicated Jκ gene segments in the Igκ light chain locus. Lane 1 is the input (1). **(C) ChIP analysis of Ku70/80 association with genomic DNA in RAG2$^{-/-}$ 63-12 pro-B cells with or without ST1571.** RAG2$^{-/-}$ 63-12 pro-B cells were treated with 10 μM ST1571 as indicated. ChIP was performed prior to ST1571 treatment or 16 h following treatment with an antibody to Ku70/80 (lane 2) or an antibody to GAL4 (lane 5) and amplified with the primer sets for the indicated gene loci. Lane 1 is the input (I). (The ChIP experiments except primer design and data presentation were kindly performed by Mr. Dominick Vallée.)

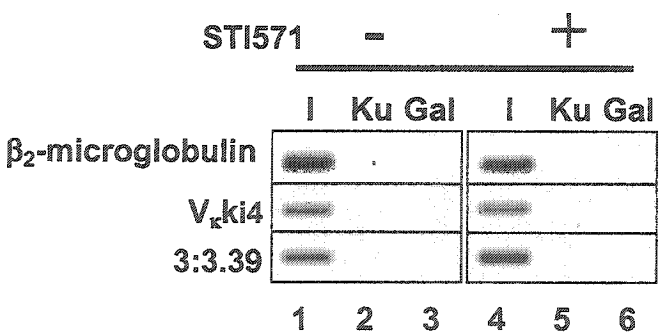
A.


I Ku Gal
J_H3
VAR34
Cδ
Cγ2β
1 2 3

B.


STI571 - +
I Ku Gal I Ku Gal
$J_κ1$
$J_κ2$
$J_κ5$
1 2 3 4 5 6

C.


STI571 - +
I Ku Gal I Ku Gal
$β_2$-microglobulin
$V_κ$ki4
3:3.39
1 2 3 4 5 6

the Jκ1, Jκ2 or Jκ5 gene segments (lane 2). However, upon treatment with ST1571, an antibody against Ku70/80 specifically recovered DNA centered over the RSSs from these gene segments (lane 5). On the other hand, as shown in Figure 19C, irrespective of ST1571 treatment, an anti-Ku antibody, like an antibody against GAL4, failed to recover DNA containing a remnant RSS from the Vκki4 pseudogene segment of the Vκ locus (Schäble et al. 1999), a segment of the β_2-microglobin gene or the 3:3.39 gene segment of the V_H7153 family.

These findings suggest that the endogenous Ku autoantigen binds to the chromatin segments containing endogenous RSSs in correlation with the chromatin accessibility of the various gene segments. In addition, Ku70/80 binding to the RSSs is independent of the presence of RAG1/2, suggesting a sequence-specific direct recruitment of Ku70/80 to the RSSs *in vivo*.

3.8. Ku70/80 facilitates RSS cleavage in vitro

V(D)J recombination is initiated with the recognition and cleavage of the RSS by RAG1/2, which alone has been shown to be sufficient for DNA cleavage (Oettinger et al. 1990; McBlane et al. 1995). The involvement of Ku70/80 in V(D)J recombination has been hypothesized to be confined to the postcleavage NHEJ step in which DNA end recognition by the Ku autoantigen provides for the entry of other NHEJ factors to mediate the appropriate ligation of cleaved coding or signal ends (Grawunder and Harfst 2001). The current finding that Ku70/80 is specifically associated with the RSS heptamer suggests a possible sequence-specific directed activity of the Ku protein in V(D)J recombination in addition to its nonspecific NHEJ activity.

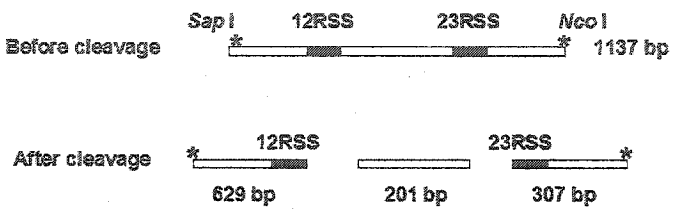
To investigate what effects, if any, Ku70/80 had on RSS cleavage by RAG1/2, *in vitro* DNA cleavage assays were carried out using nuclear extracts from 293T cells, V79 or Ku80-deficient V15B fibroblasts expressing full-length RAG1/2 and the [α-^{32}P]-dCTP labeled *Sap*I-*Nco*I fragment from pJH200 (Figure 20A). The fragment contains the endogenous VκL8 gene segment with its 12RSS and Jκ1 gene segment with its 23RSS. The cleavage by RAG1/2 at the 12RSS and 23RSS generates a radiolabeled 629 bp (12RSS) fragment, a radiolabeled 307 bp (23RSS) fragment, and an unlabeled 201 bp fragment. As shown in Figure 20B, when the *Sap*I-*Nco*I fragment was incubated with nuclear extracts from 293T cells, V79 or V15 fibroblasts, no cleavage of the *Sap*I-*Nco*I fragment was observed (lanes 2, 5 and 7). However, when the *Sap*I-*Nco*I fragment was incubated with nuclear extracts from 293T cells expressing RAG1/2, coupled cleavage of the 12RSS and 23RSS occurred efficiently (lane 3). By comparison, the coupled cleavage of the 12RSS and 23RSS by RAG1/2 was readily observable with nuclear extracts from V79 cells expressing RAG1/2 (lane 6), but hard to detect with nuclear extracts from Ku80-deficient V15B cells expressing RAG1/2 (lane 8). These results indicated that the presence of Ku70/80 in the nuclear extracts from cells expressing RAG1/2 enhances the efficiency of RSS cleavage by RAG1/2.

3.9. Establishment of an in vitro V(D)J recombination assay that mimics the in vivo V(D)J recombination reaction

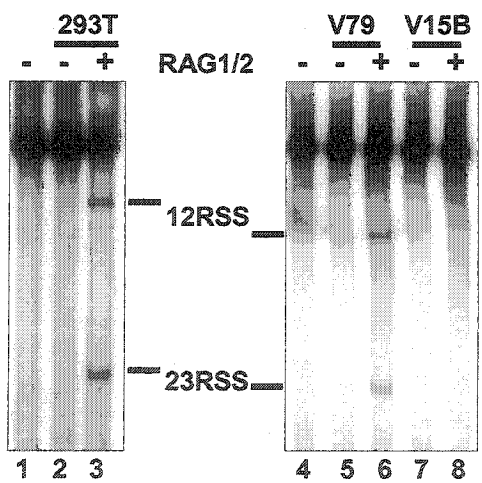
V(D)J recombination follows the 12/23 rule *in vivo* and requires the coupled cleavage by RAG1/2 of the 12RSS and 23RSS (Early et al. 1980; Sakano et al. 1981; van Gent et al. 1996a; Eastman 1996). V(D)J recombination has been reconstituted *in vitro*

Figure 20. Ku70/80 facilitates RSS cleavage by RAG1/2 *in vitro*. (A) The scheme of *in vitro* cleavage of recombinational DNA substrates. The *in vitro* cleavage DNA substrate was excised from the extrachromosomal recombination DNA substrate pJH200 with the endonucleases *Sap*I and *Nco*I and filled in with [α-^{32}P] dCTP and other dNTPs with the large Klenow fragment of DNA polymerase I. The radiolabeled *Sap*I-*Nco*I fragment contains the endogenous VκL8 gene segment with its 12RSS and Jκ1 gene segment with its 23RSS. Cleavage by RAG1/2 at the 12RSS and 23RSS generates a radiolabeled 629 bp fragment (12RSS), a radiolabeled 307 bp fragment (23RSS), and an unlabeled 201 bp fragment. **(B) The Ku protein facilitated RSS cleavage by RAG1/2.** *In vitro* cleavage assay was carried out by incubating nuclear extracts (20 μg each) from 293T, V79 or V15B cells alone or expressing RAG1/2 with the radiolabeled *Sap*I-*Nco*I fragment (50 ng). The products were resolved on 5% nondenaturing polyacrylamide gels and autoradiographed.

A.


Sap I
12RSS
23RSS
Nco I
Before cleavage
1137 bp
12RSS
23RSS
After cleavage
629 bp
201 bp
307 bp

B.


293T
V79
V15B
- - + RAG1/2 - - + - +
12RSS
23RSS
1 2 3 4 5 6 7 8

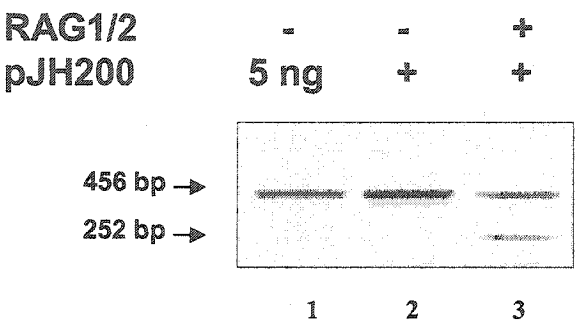
(Ramsden et al. 1997; Cortes et al. 1996; Weis-Garcia et al. 1997). However, the *in vitro* V(D)J recombination systems established by these researchers employed the divalent ion Mn^{2+}, which did not mimic the V(D)J recombination reaction *in vivo* in that, in the presence of the divalent ion Mn^{2+}, the 12RSS and 23RSS on a DNA substrate were cleaved independent of one another by RAG1/2 (McBlane et al. 1995; van Gent et al. 1995, 1996a). Mn^{2+} is known to cause a relaxation in the specificity of DNA binding and cleavage by RAG1/2 and allow uncoupled cleavage by RAG1/2 of individual RSSs while the coupled cleavage by RAG1/2 of a 12RSS and a 23RSS has been demonstrated in the presence of Mg^{2+} (van Gent 1995, 1996a; Santagata et al. 1998).

An *in vitro* V(D)J recombination system was established in the present study using nuclear extracts from 293T cells expressing full-length RAG1/2 and the extrachromosomal recombination substrate pJH200 in the presence of 10 mM Mg^{2+} (See Section 2.10). The extrachromosomal recombination substrate pJH200 contains the endogenous VκL8 gene segment with its 12RSS and Jκ1 gene segment with its 23RSS and it undergoes deletional recombination in the presence of RAG1/2 (Figure 18A) (Hesse et al. 1987). The primer set R5 and R14 generates by PCR a 456 bp fragment spanning the region containing the 12RSS and 23RSS prior to the deletional recombination of pJH200 and a 252 bp fragment spanning the RSSs with the two RSS heptamers fused together head-to-head following the recombination (Cortes et al. 1996). The precise formation of the signal joint between the two heptamers reconstitutes a new *Apa*L I restriction site (See Figure 18A).

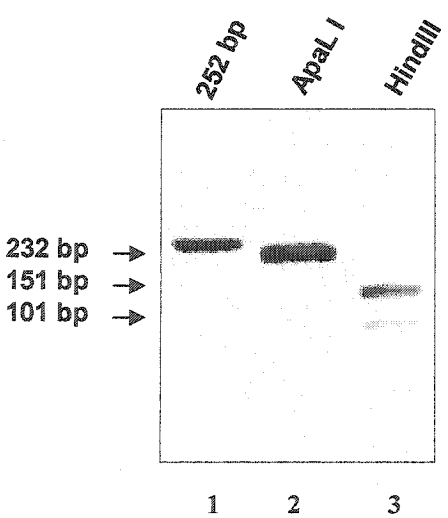
As shown in Figure 21A, the incubation of nuclear extracts from 293T cells expressing RAG1/2 with pJH200 *in vitro* resulted in the formation of signal joints as

Figure 21. Establishment of an *in vitro* V(D)J recombination assay using the divalent ion Mg^{2+}. (A) *In vitro* V(D)J recombination assay. *In vitro* V(D)J recombination was carried out by incubating nuclear extracts (20 μg) from 293T cells alone (lane 2) or from 293T cells transfected with pRAG1 and pRAG2 (lane 3) with pJH200 (50 ng) in the presence of 10 mM Mg^{2+} at 30°C for 16 h. The DNA was recovered and amplified by PCR using the primer set R5 and R14. Lanes 1, 5 ng of pJH200 as positive control; lane 2, nuclear extract from 293T cells plus 50 ng of pJH200; lane 3, nuclear extract from 293T cells containing full length RAG1 and RAG2 plus 50 ng of pJH200. The products were resolved on a 2% agarose gel stained with Vistra Green (Pharmacia). **(B) Precise *in vitro* signal joint formation.** The 252 bp fragment from the *in vitro* V(D)J recombination reaction in (A) was recovered and restricted with endonucleases *Apa*L I (lane 2) or *Hind*III (lane 3). The products were resolved on an 8% nondenaturing polyacrylamide gel and visualized after staining with ethidium bromide. Lane 1, undigested 252 bp fragment; lane 2, 252 bp fragment plus *Apa*L I; lane 3, 252 bp fragment plus *Hind*III.

A.


RAG1/2 - - +
pJH200 5 ng + +
456 bp
252 bp
1 2 3

B.


252 bp
ApaL I
HindIII
232 bp
151 bp
101 bp
1 2 3

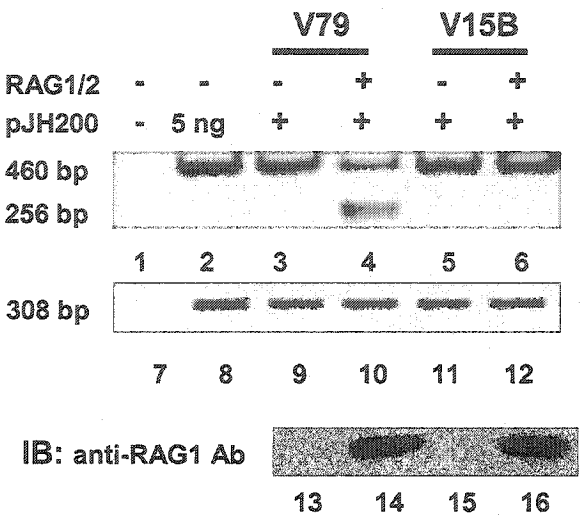
indicated by the presence of the 252 bp fragment (lane 3). In addition, the restriction endonuclease analysis of the recovered 252 bp fragment with *Apa*L I and *Hind*III confirmed the presence of an intact and precise signal joint interface (Figure 21B, lanes 2 and 3). These observations suggest that the new *in vitro* V(D)J recombination system established here mimics the *in vivo* V(D)J recombination reaction in terms of a coupled and precise cleavage of the 12RSS and 23RSS by RAG1/2 in the presence of the divalent ion Mg^{2+} and an intact and functional NHEJ apparatus with regard to the precise formation of signal joints. Establishing such an *in vitro* V(D)J recombination system has been a major challenge (Ramsden et al. 1997; Cortes et al. 1996; Weis-Garcia et al. 1997; Jung and Alt 2004) and the *in vitro* V(D)J recombination reaction described here is the first such system, which should allow the dissection of cellular proteins involved in the coupled cleavage of the 12RSS and 23RSS.

3.10. The Ku autoantigen facilitates DNA cleavage by RAG1/2 in vivo

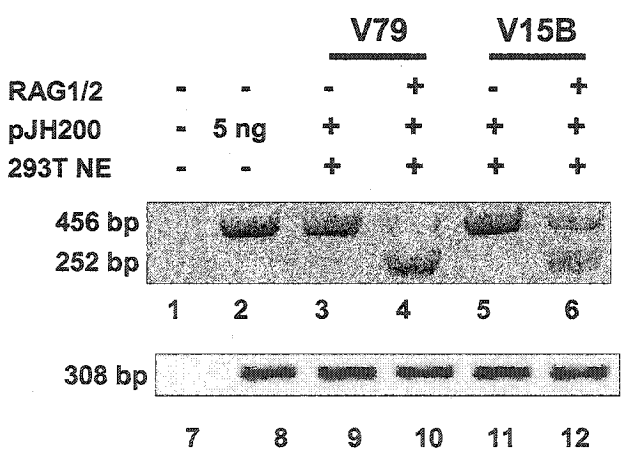
The Ku protein has been shown to be required for V(D)J recombination in Ku80-deficient cell lines and Ku70- or Ku80-deficient mice who have defective formation of both coding joints and signal joints (Taccioli et al. 1993, 1994; Smider et al. 1994; Errami et al. 1996; Gu et al. 1997a, 1997b; Nussenzweig et al. 1997; Bogue et al. 1997). *In vivo* signal joint formation was examined in V79 and Ku80-deficient V15B fibroblasts transiently transfected either with pJH200 alone or together with pRAG1 and pRAG2. As shown in Figure 22A, amplification by PCR of the recovered DNA from V79 cells transfected with both pJH200 and pRAG1 and pRAG2 revealed the presence of a 460 bp fragment corresponding to the region spanning the RSSs before recombination and a 256

Figure 22. The Ku autoantigen facilitates RSS cleavage but is not required for precise cleavage of the RSSs. (A) Ku70/80 is required for signal joint formation and facilitates RSS cleavage by RAG1/2 *in vivo*. The CHO V79 and V15B fibroblasts were either transiently transfected with pJH200 (2 μg) alone or pJH200 (2 μg) together with pRAG1 (2 μg) and pRAG2 (2 μg). Two days following transfection, the extrachromosomal recombination DNA substrates were recovered and amplified by PCR using the primer sets R3 and R14, and R7 and R8. The PCR products were resolved on a 2% agarose gel stained with Vistra Green (Pharmacia). Nuclear extracts from V79 or V15B cells with or without RAG1/2 were also resolved on a 10% SDS-polyacrylamide gel. Immunoblotting (IB) was carried out with an antibody to RAG1. Lanes 1 and 7, primer control; lanes 2 and 8, 5 ng of pJH200 as positive control; lanes 3 and 9, V79 cells transfected with pJH200; lanes 4 and 10, V79 cells transfected with pJH200 and pRAG1 and pRAG2; lanes 5 and 11, V15B cells transfected with pJH200; lanes 6 and 12, V15B cells transfected with pJH200 and pRAG1 and pRAG2; lane 13, IB with anti-RAG1 Ab of nuclear extracts from V79 cells; lane 14, IB with anti-RAG1 Ab of nuclear extracts from V79 cells expressing RAG1/2; lane 15, IB with anti-RAG1 Ab of nuclear extracts from V15B cells; lane 16, IB with anti-RAG1 Ab of nuclear extracts from V15B cells expressing RAG1/2. **(B) *In vitro* NHEJ of extrachromosomal recombination DNA substrates from V79 and V15B cells.** V79 and V15B cells were transfected as in (A). 18 h following transfection, the extrachromosomal recombination DNA substrates were recovered and re-incubated with nuclear extracts from 293T cells for 16 h. After purification, the DNA substrates were amplified using the primer sets R5 and R14, and R7 and R8. Lanes 1 and 7, primer control; lanes 2 and 8, 5 ng of pJH200 as positive control; lanes 3 and 9, V79 cells transfected with pJH200; lanes 4 and 10, V79 cells transfected with pJH200 and pRAG1 and pRAG2; lanes 5 and 11, V15B cells transfected with pJH200; lanes 6 and 12, V15B cells transfected with pJH200 and pRAG1 and pRAG2. **(C) Precise signal joint formation following *in vitro* NHEJ of extrachromosomal recombination DNA substrates from V79 and V15B cells.** The 252 bp fragments from (B) were restricted with the endonucleases *Apa*L I and *Hind*III and the products were resolved on a 5% nondenaturing polyacrylamide gel. Lane 1, undigested 252 bp fragments from V79 cells; lane 2, 252 bp fragments from V79 cells digested with *Apa*L I; lane 3, 252 bp fragments from V79 cells digested with *Hind* III; lane 4, undigested 252 bp fragments from V15B cells; lane 5, 252 bp fragments from V15B cells digested with *Apa*L I; lane 6, 252 bp fragments from V15B cells digested with *Hind*III.

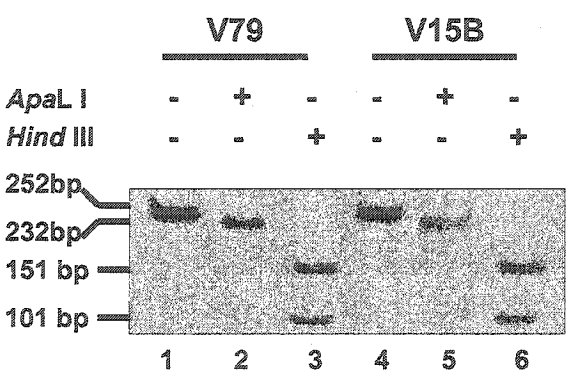
A.


V79
V15B
RAG1/2 - - - + - +
pJH200 - 5 ng + + + +
460 bp
256 bp
1 2 3 4 5 6
308 bp
7 8 9 10 11 12
IB: anti-RAG1 Ab
13 14 15 16

B.


V79
V15B
RAG1/2 - - - + - +
pJH200 - 5 ng + + + +
293T NE - - + + + +
456 bp
252 bp
1 2 3 4 5 6
308 bp
7 8 9 10 11 12

C.


V79
V15B
ApaL I - + - - + -
Hind III - - + - - +
252bp
232bp
151 bp
101 bp
1 2 3 4 5 6

bp fragment corresponding to the region spanning the RSSs after recombination with the two heptamers fused together head-to-head (lane 4). In the absence of RAG1/2, only the 460 bp fragment was amplified from DNA recovered from either V79 or V15B cells (lanes 3 and 5). On the other hand, amplification by PCR of the recovered DNA from Ku80-deficient V15B fibroblasts transfected with both pJH200 and pRAG1 and pRAG2 revealed the presence of only the 460 bp fragment from the unrecombined pJH200. The 256-bp fragment from the recombined pJH200 was not detected (lane 6), reflecting a lack of signal joint formation in V15B cells and a requirement for the Ku protein in the process.

Furthermore, the unrecombined substrates as represented by the 460 bp fragment from V15B cells (lane 6) existed in an amount higher than that from V79 cells (lane 4) while the total amount of both cleaved and uncleaved DNA substrates as represented by the 308 bp fragment remained comparable from V79 and V15B cells (lanes 10 and 12). The immunoblotting of nuclear extracts from V79 and V15B cells expressing RAG1/2 also showed that the level of RAG1 was comparable in these cell lines (lanes 14 and 16). These observations suggest that there was less cleavage of the extrachromsomal recombination substrates by RAG1/2 in the absence of the Ku protein, which is consistent with the findings from the *in vitro* DNA cleavage assays (Figure 20B).

Earlier studies of transient V(D)J recombination in Ku70- or Ku80-deficient cells showed that the rare coding or signal joints recovered from these cells had deletions (Taccioli et al. 1993, 1994), suggesting a possible role of Ku70/80 in the protection of cleaved DNA ends. Subsequent studies using ligation-mediated PCR, however, demonstrated that the coding or signal ends in Ku70- or Ku80-deficient cells remained

intact (Zhu et al. 1996; Han et al. 1997; Kabotyanski et al. 1998), suggesting that the Ku protein was apparently not required for the protection of cleaved signal or coding ends. To determine whether *in vivo* cleavage occurred in V15B cells expressing RAG1/2 with the generation of signal ends that could be further processed by an intact NHEJ apparatus, the extrachromosomal recombination substrates pJH200 were recovered from both V79 and V15B cells and were re-incubated *in vitro* with nuclear extracts from 293T cells under conditions used for *in vitro* V(D)J recombination except that the nuclear extracts from 293T cells had no RAG1/2.

As shown in Figure 22B, the re-incubation of recovered DNA substrates from V15B cells resulted in the appearance of the 252 bp fragment spanning the RSSs of the recombined pJH200 (lane 6), suggesting that RSS cleavage by RAG1/2 was intact in the absence of the Ku autoantigen and the signal ends generated from the *in vivo* cleavage in V15B cells expressing RAG1/2 were ready for processing by the NHEJ machinery. Furthermore, the total amount of both cleaved and uncleaved DNA substrates as represented by the 308 bp fragment remained comparable from V79 and V15B cells (lanes 10 and 12), suggesting that the DNA substrates in V15B cells suffered no excessive degradation. Restriction endonuclease analysis of the recovered signal joints (the 252 bp fragments) with *Apa*L I and *Hind*III (Figure 22C) further showed that the signal joints from *in vitro* NHEJ of the DNA substrates from V15B cells (lane 5), similar to those from V79 cells (lane 2), were precise, reflecting that the fidelity of RSS cleavage by RAG1/2 was not compromised by the lack of the Ku autoantigen.

The above findings confirm that Ku70/80 is required for *in vivo* signal joint formation during V(D)J recombination and facilitates RSS cleavage by RAG1/2.

Furthermore, the Ku protein is not required for the protection of signal ends in Ku80-deficient cells. The signal ends generated from *in vivo* cleavage in V15B cells could be processed further by the NHEJ apparatus. In addition, the extrachromosomal recombination DNA substrates in Ku80-deficient cells were shielded against degradation by nucleases.

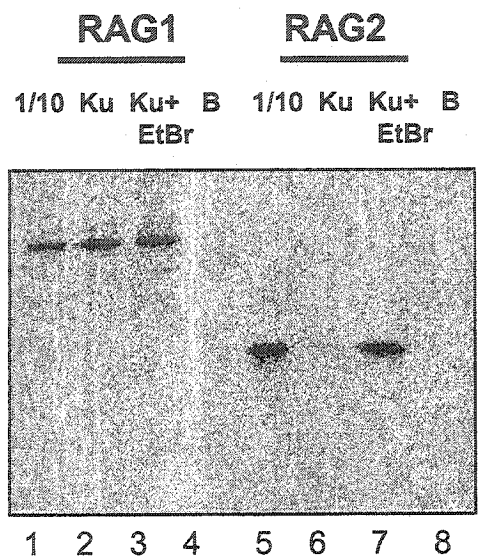
3.11. The Ku protein is associated with the RAG1/2 protein in vitro and in vivo

The findings that the Ku autoantigen binds specifically to the RSS heptamer and the fact that the binding of the RAG1/2 protein to the RSSs is mediated primarily through the nonamer (Nagawa et al. 1998) suggest that the Ku autoantigen and the RAG1/2 protein probably interact *in vivo* as a protein complex before their assembly on the RSSs or they form a complex following their binding to the RSS heptamer and nonamer, respectively. Earlier studies from our laboratory by Ms. Louise Pope and Dr. Caroline Schild-Poulter with *in vitro* translated ^{35}S-labeled RAG1 and RAG2 and the Ku protein immunoprecipitated from Jurkat T cell extracts (Figure 23A) showed that the Ku protein interacted with both RAG1 (lanes 2 and 3) and RAG2 (lanes 6 and 7) and the interaction between the Ku protein and RAG1 or RAG2 was not disrupted in the presence of ethidium bromide (lanes 3 and 7).

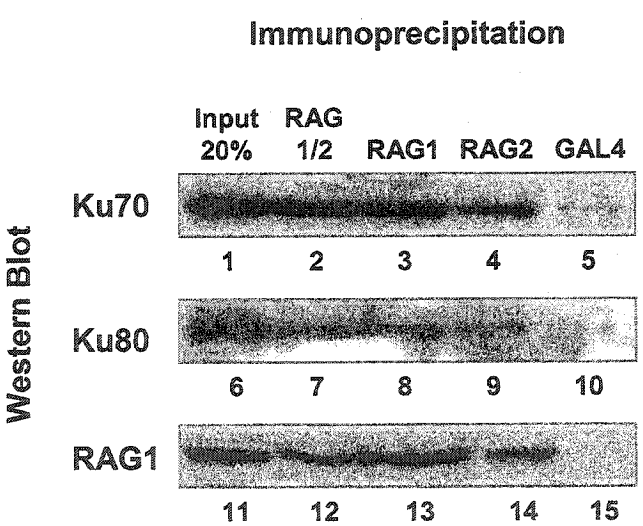
To further investigate if Ku70/80 was associated with RAG1/2 *in vivo*, immunoprecipitation studies were carried out using pro-B cells 220-8 with anti-RAG1 and anti-RAG2 antibodies alone or in combination. The presence of the Ku protein was detected by immunoblotting with anti-Ku70 and anti-Ku80 antibodies. As shown in Figure 23B, the Ku protein interacted with both RAG1 and RAG2 in pro-B cells (lanes 2

Figure 23. The Ku autoantigen is associated with the RAG protein *in vitro* and *in vivo*. (A) RAG1 and RAG2 interact with the Ku protein *in vitro*. The Ku protein was immunoprecipitated from Jurkat T cell extracts (lanes 2, 3, 6 and 7). *In vitro* S^{35}-labeled RAG1 and RAG2 were tested for binding to the immunoprecipitated Ku protein (lanes 2 and 6) or to protein A Sepharose beads (B) alone (lanes 4 and 8). Ethidium bromide (EtBr) (200 mg/ml) was included in the reactions in lanes 3 and 7. Binding to the Ku protein was compared to 10% of the input proteins (lanes 1 and 5). **(B) RAG1 and RAG2 interact with the Ku protein *in vivo*.** The RAG1/2 protein was immunoprecipitated from pro-B cells 220-8 with a mixture of antibodies to RAG1 and RAG2 (lanes 2, 7 and 12), or an antibody to RAG1 (lanes 3, 8 and 13) and an antibody to RAG2 (lanes 4, 9 and 14) or a control antibody to GAL4 (lane 5, 10 and 15). Ethidium bromide (200 mg/ml) was included throughout immunoprecipitation. The Ku protein was immunoblotted with an anti-Ku70 antibody (lanes 1 to 5) or an anti-Ku80 antibody (lanes 6 to 10) and the RAG1 was immunoblotted with an anti-RAG1 antibody (lanes 11 to 15). The binding of the Ku protein to RAG1/2 was compared with 20% of the input. **(C) RAG1 and RAG2 interact with the Ku protein *in vivo*.** The Ku protein was immunoprecipitated from pro-B cells 220-8 with an antibody to Ku70 (lane 2) or a control antibody to GAL4 (lane 3). RAG1 was immunoblotted with an anti-RAG1 antibody (lanes 1 to 3) and Ku70/80 was immunoblotted with an antibody to Ku70 or to Ku80 (lanes 4 to 6). The binding of RAG1 to the Ku protein was compared with 10% of the input.

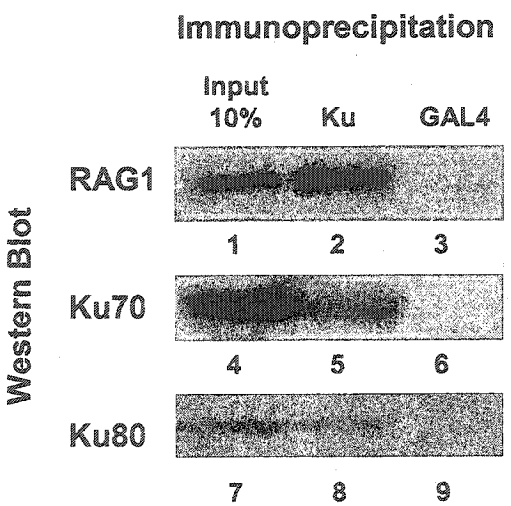
A.


RAG1
RAG2
1/10 Ku Ku+ EtBr B
1/10 Ku Ku+ EtBr B
1 2 3 4 5 6 7 8

B.


Immunoprecipitation
Input 20%
RAG 1/2
RAG1
RAG2
GAL4
Western Blot
Ku70
1 2 3 4 5
Ku80
6 7 8 9 10
RAG1
11 12 13 14 15

C.


Immunoprecipitation
Input 10%
Ku
GAL4
Western Blot
RAG1
1 2 3
Ku70
4 5 6
Ku80
7 8 9

to 4 and 7 to 9). In a separate experiment, the Ku protein was immunoprecipitated from pro-B cells 220-8 by an antibody to Ku70 (Figure 23C) (lanes 2 and 5) and RAG1 was immunoblotted with an anti-RAG1 antibody. The results showed that RAG1 also interacted with the Ku protein in pro-B cells. These findings demonstrated that the RAG1/2 protein interacted with the Ku protein both *in vitro* and *in vivo*. However, the functional implications of the interaction between RAG1/2 and Ku70/80 remain to be elucidated.

In summary, the findings from the above experiments demonstrated that the Ku protein binds differently to different categories of DNA substrates in terms of apparent binding affinities and the contacts with DNA of its individual subunits. Moreover, the *SELEX* by the recombinant Ku protein has evolved a novel class of functional DNA binding sites, the RSS heptamer, implicating the Ku autoantigen in sequence-specific-directed activities during V(D)J recombination. Additionally, the Ku protein is required for *in vivo* signal joint formation, but not for the protection of cleaved DNA ends that are generated during the V(D)J recombination process. Furthermore, the Ku autoantigen facilitates RSS cleavage by RAG1/2 both *in vitro* and *in vivo*. The Ku protein also interacts with RAG1/2 both *in vitro* and *in vivo*. These observations suggest that the RSS cleavage by RAG1/2 and the subsequent NHEJ by Ku70/80 and other proteins are a closely interwoven process and the current findings provide significant insight into the understanding of the roles of the Ku autoantigen in the V(D)J recombination process. Long-standing models for V(D)J recombination hypothesize that post-cleavage DNA-end recognition by the Ku autoantigen provides for the entry of other NHEJ factors to mediate the appropriate ligation of coding or signal ends. The findings from the present

study suggested a central role of the Ku autoantigen in V(D)J recombination by integrating NHEJ into V(D)J recombination through Ku70/80 binding to the RSS heptamer and the RAG1/2 complex.

4. DISCUSSION

4.1. Differential association of Ku70/80 with NRE1, A3/4 and the RSS heptamer

Though specific DNA binding activities have been reported for the Ku autoantigen or Ku-associated complexes, which implicate Ku70/80 in important cellular processes such as transcriptional regulation, DNA replication and Ig class switch recombination (Appendix III and the references therein), many of these putative specific DNA sequences have not been subjected to the same vigorous study as for NRE1 (Giffin 1996; 1997). Ku70/80 has prominent DNA end binding activities as well and, when bound to DNA ends, generates a footprint of 20 to 30 bp on DNA (Mimori and Hardin 1986). Ku70/80 binds to dsDNA ends with a preformed channel, which involves an extensive region in both subunits of the Ku protein (Walker et al. 2001). The structural basis for specific DNA binding by Ku70/80 is currently lacking; whether the SAP domain in Ku70 is implicated in such activities remains to be established. Furthermore, the importance of the putative Mg^{2+} binding sites in Ku70 or Ku80 for sequence-specific binding by the Ku autoantigen remains to be investigated (Aravind and Koonin 2001).

Our laboratory has shown that Ku70/80 binds differently to DNA ends and various specific sequences (Giffin et al. 1996, 1997; Schild-Poulter et al. 2003a). The findings from the current reciprocal competitive EMSAs using radiolabeled linear A3/4, NRE1 or nonspecific DNA and the UV-crosslinking studies indicated that binding by Ku70/80 to A3/4, NRE1 and nonspecific DNA ends is distinguishable by the different binding affinities of the Ku protein for these DNA substrates and by the differential contacts of DNA by Ku70 or Ku80 in the presence or absence of ATP and Mg^{2+}. These

results establish A3/4 as a novel class of direct DNA binding site for Ku70/80, which is distinct from either NRE1 or nonspecific DNA.

Additionally, our laboratory has shown that Ku70/80-NRE1 interaction is initiated with the contact of Ku70 to the upper strand of NRE1 followed by an ATP and Mg^{2+} dependent contact of Ku80 with NRE1 and subsequent recruitment of DNA-PKcs to DNA (Giffin et al. 1994, 1996, 1997, 1999; Schild-Poulter et al. 2003a). Ku70/80 binding to NRE1 induces structural transitions in flanking DNA sequences and supports the activation of DNA-PKcs. Ku70/80 binding to A3/4, on the other hand, is characterized by contacts of both strands of A3/4 by both the Ku70 and Ku80 subunit. Furthermore, Ku70 or Ku80 makes contacts with A3/4 regardless of the inclusion or exclusion of ATP and Mg^{2+}. Ku70/80 binding to A3/4, unlike its binding to NRE1, is incapable of activating of DNA-PKcs (Schild-Poulter et al. 2003a), suggesting that Ku70/80 is in a different conformation on A3/4 from that on NRE1 and DNA-PKcs is not required for A3/4 activity.

Moreover, the *SELEX* by Ku70/80 has evolved the RSS heptamer as a novel class of preferred direct DNA binding sites for the Ku autoantigen. The findings from EMSAs using radiolabeled linear H7, N9, NRE1 or nonspecific DNA further indicated that Ku70/80 binds to the RSS heptamer with a higher affinity than its binding to either NRE1 or the RSS nonamer, suggesting that the RSS heptamer is probably in a different class of direct DNA binding site for the Ku autoantigen than NRE1. The RSS heptamer is also probably in a different category of direct DNA binding site from A3/4 as the Ku protein binds to the circular DNA substrates containing the heptamer while it has failed to bind to the circular DNA substrates containing A3/4 (this study; Schild-Poulter et al. 2003a).

Our laboratory has previously demonstrated that Ku70/80 had a Kd of 0.84 ± 0.24 X 10^{-9} M for NRE1-containing DNA microcircles (Giffin et al. 1997). However, an earlier study by Falzon et al. (1993) showed that Ku70/80 had a Kd between 1.5 and 2.0 X 10^{-11} M for nonspecific DNA ends, suggesting a higher affinity of DNA end binding than its binding to NRE1. The same study also found that Ku70/80 was capable of binding to certain structured DNA substrates like DNA hairpins and dumbbell-shaped DNA constructs without an apparent requirement for DNA ends. Furthermore, Ku70/80 binding to these DNA substrates was able to activate DNA-PKcs (Falzon et al. 1993; Morozov et al. 1994). A reexamination of the DNA substrates employed in their study, however, revealed that these DNA substrates contained a cryptic RSS heptamer (-CACAGTG-), which may provide a direct DNA entry site for Ku70/80. Additionally, the $T_{1/2}$ of the Ku70/80-RSS heptamer complexes as revealed in the current study was comparable to the $T_{1/2}$ of 14 to 15 h of Ku70/80 for DNA substrates containing the cryptic heptamer (Falzon et al. 1993). Further, the dumbbell DNA construct containing a cryptic RSS heptamer is the first instance of a closed DNA structure shown to be capable of activating DNA-PKcs (Morozov et al. 1994).

Also of interest is the current finding that Ku70/80 binds comparably to the RSS nonamer and NRE1 (See Figure 14B). NRE1 is polypurine in nature and contains a 5'-GAGAAAGA-3' repeat (Giffin et al. 1996, 1997) while the RSS nonamer also contains a polypurine stretch in its sequence 5'-ACAAAAACC-3'.

The definition of Ku70/80 binding to the RSS heptamer by its DNA binding affinity and the differential contacts of its two subunits, however, has to await further studies through reciprocal competitive EMSAs and protein-DNA crosslinking assays as

have been undertaken for NRE1 and A3/4. Equally important is the determination whether the conformation of Ku70/80 on the RSS heptamer, like NRE1, is active in recruiting DNA-PKcs to the site and leads to its activation and what the consequences are, if any, of the activation of DNA-PKcs on V(D)J recombination.

4.2. The RSS offers a discriminating interface for the Ku autoantigen and the RAG protein

V(D)J recombination is initiated by RAG1/2, which recognizes, binds to and cleaves the RSSs with the constraint of the 12/23 rule (Oettinger et al. 1990; McBlane et al. 1995; Tillman et al. 2002). The RSS nonamer provides the major interface for DNA binding by the active cores of RAG1 (amino acid residues from 384 to 1008) and RAG2 (amino acid residues from 1 to 383) (Ramsden et al. 1996; Difillippantonio et al. 1996), which have been shown to be sufficient for V(D)J recombination *in vitro* (McBlane et al. 1995). Footprint analysis of RAG1/2-RSS interactions indicated that RAG1 made sequence-specific contacts primarily with the nonamer and only partial contacts with the heptamer while RAG2 made little contribution to RSS recognition (Nagawa et al. 1998). The nonamer binding domain (NBD) is confined to amino acid residues from 389 to 446 of the active core of RAG1 and mediates the binding of RAG1 to the RSS nonamer (Difilippantonio et al. 1996; Spanopoulou et al. 1996). In addition, RAG1/2 made contacts with the phosphate backbone of the RSS on only one side of the DNA helix (Swanson and Desiderio 1998), potentially allowing other proteins to gain access to the RSS from the contralateral side of the DNA helix.

In the absence of RAG2, RAG1 made extensive contacts with part of the spacer and the nonamer in the 12RSS and, in the presence of RAG2, RAG1/2 binding to the RSS extended over the spacer into the nonamer-proximal end of the heptamer (Swanson and Desiderio 1998, 1999). However, even in the presence of RAG2, RAG1/2 failed to make any contact with the heptamer in the 23RSS. Mutational studies of basic residues in RAG2, nevertheless, revealed its direct involvement in RAG1/2 binding to the RSS (Fugmann and Schatz 2001). Amino acid residues from 314 to 371 in the active core of RAG2 are involved in interaction with RAG1 (Aidinis et al. 2000). In addition, RAG2 was required for the formation of a stable functionally active cleavage complex by RAG1 with the RSS (Li et al. 1997), which required RAG1/2 binding to both the nonamer and the heptamer (Hiom and Gellert 1997).

Though the nonamer is critical for binding to the RSS by RAG1/2, the heptamer itself does not appear to provide a significant interface for binding by RAG1/2. RAG1 exhibited only a modest 2- to 4-fold preference for the heptamer over nonspecific DNA substrates (Hiom and Gellert 1997; Arbuckle et al. 2001). Moreover, mutations of the first three or the last four nucleotides of the RSS heptamer have caused only a moderate reduction in RAG1/2 binding to the RSSs (Hiom and Gellert 1997). Furthermore, DNA substrates with an intact heptamer and a scrambled nonamer competed for RAG1/2 binding to an intact RSS no more effectively than nonspecific DNA substrates (Ramsden et al. 1996). These observations indicate that the heptamer is less critical than the nonamer in mediating RAG1/2 binding to the RSSs.

Nevertheless, the RSS heptamer has been shown to be critical for DNA cleavage by RAG1/2. The first three positions in the heptamer adjacent to the coding sequence

were found to be critical for the recombination of an extrachromosomal recombination DNA substrate in transfected cells (Hesse et al. 1989). The heptamer, especially its first two positions, is also important for the formation of DNA DSBs (Ramsden et al. 1996). Mutation of the first position of the heptamer blocked the formation of hairpin coding ends even on pre-nicked RSS substrates. In addition, mutations in the first two positions of the heptamer selectively impaired hairpin formation without affecting binding to the RSS and nicking of the DNA substrates by RAG1/2 (Li et al. 1997; Nagawa et al. 1998). In *in vivo* transient V(D)J recombination assays, the effect of single nucleotide substitutions at either the first or third position of the heptamer on DSB formation *in trans* was as severe as the removal of the entire RSS (Steen et al.1997). Though with a reduced efficiency compared to a canonical RSS, the heptamer was sufficient to precisely target nicking and hairpin formation at the coding-heptamer border with a scrambled nonamer; however, with a scrambled heptamer, the nonamer targeted nicking with reduced specificity and even lower efficiency (Cuomo et al. 1996; Ramsden et al. 1996). These observations suggest that the heptamer, especially the first two nucleotides of the heptamer, though not critical for binding by RAG1/2, is important for cleavage by RAG1/2.

Furthermore, the analysis of the sequences of naturally occurring RSSs in humans indicated that the heptamer was less tolerant of sequence variations than the nonamer and offered a more discriminative binding interface than the nonamer (Ohno and Yomo 1990). The calculation of position-wise information for the heptamer, the nonamer or the spacer also revealed higher position-wise information for the heptamer

than either the nonamer or the spacer (Cowell et al. 2003), further attesting to a critical role of the heptamer in the interaction between the RSS and RSS binding proteins.

The heptameric sequence 5'-CACAGTG-3' and its complementary 5'-CACTGTG-3' are simultaneously complementary (7/7) and homologous (6/7) and presents a single entity of palindromic DNA binding interface (Ohno and Yomo 1990). Similar to the DNA bound by Tn5 transposase (Davies et al. 2000), the heptameric sequence probably displays unusual structural features with the disruption of the canonical donor-acceptor interactions in the major groove to form new interactions with the 5' neighbor of the opposite strand (Timsit et al. 1991). As a result, it exhibits a slippage of base pairing and a strongly locally modified structure adjacent to the cleavage site, which may constitute a specific recognition surface for DNA binding proteins. Moreover, the apparent requirement for the unpairing of base pairs at the border of the heptamer and the coding sequences for DNA cleavage by RAG1/2 (Cuomo et al. 1996; Ramsden et al. 1996) and the finding that significant interaction between the ss heptamer and RAG1 occurs only after the induction of conformational changes at the heptamer (Peak et al. 2003) strongly suggest the existence of additional cellular factors that distort or unwind the DNA helix 5' of the heptamer, which would facilitate DNA cleavage by RAG1/2.

In fact, there have been several attempts to identify RSS heptamer binding proteins (Matsunami et al. 1989; Muegge et al. 1993); however, the relevance of these putative heptamer binding proteins to V(D)J recombination remains unclear. The recombination signal binding protein-Jκ (RBP-Jκ) was initially recognized as a heptamer binding protein (Hamaguchi et al. 1989; Miyake et al. 1990; Furukawa et al. 1991),

which, however, was subsequently found not to have the appropriate specificity for the RSS heptamer (Tun et al. 1994).

Not coincidentally, the inverted repeat binding protein (IRBP) in *Drosophila melanogaster*, a homologue of human Ku70, was found to interact with the 31 bp inverted repeats of the P transposable element adjacent to the transposition cleavage site (Rio and Rubin 1988; Beall et al. 1994). The 31 bp inverted repeats are required for P element transposition (Mullins et al. 1989); however, the P element transposase does not interact with the repeats *in vitro* (Beall and Rio 1997).

Though a prominent DNA end binding protein, Ku70/80 also binds to specific sequences (Giffin et al. 1996, 1997; Ruiz et al. 2001). The findings from the present DNA binding sites selection studies demonstrate that Ku70/80 binds preferentially to the RSS heptamer over random DNA sequences. As the *SELEX* by recombinant Ku70/80 employed linear random oligonucleotides, the evolved DNA binding sites may constitute preferred pause sites for the Ku protein following its entry from DNA ends or the DNA sequences that enhance Ku70/80 binding to DNA ends. This problem was addressed by using covalently closed DNA microcircles free of nicks or gaps or structural transitions to demonstrate direct binding of Ku70/80 to the RSS heptamer. Additionally, endogenous Ku70/80 was shown to be specifically associated with the region containing RSSs from the VκL8 and Jκ1 gene segments of Igκ light chain in 293T cells transiently transfected with the extrachromosomal recombination DNA substrates pJH200. ChIP analysis of A-MuLV-transformed pro-B cells 63-12 further indicated that, in correlation with the accessibility of various gene segments in the chromatin, endogenous Ku70/80 was specifically associated *in vivo* with gene segments such as VAR34 of Ig heavy chain and

Jκ2 and Jκ5 of Igκ light chain that were poised to undergo Vκ to Jκ recombination. These findings convincingly demonstrated that the Ku autoantigen binds directly and specifically to the RSS heptamer, which is independent of the presence of DNA ends or the RAG1/2 protein.

Early studies have shown that RAG1/2 recognizes the RSS mainly through the nonamer (Oettinger et al. 1990; McBlane et al. 1995; Tillman et al. 2002). The results from the current study suggest that the Ku autoantigen is also involved in the specific recognition of the RSS through the heptamer. Furthermore, the Ku protein is shown to interact with the RAG protein both *in vitro* and *in vivo*. It can be envisioned that in the initial step of V(D)J recombination, the Ku autoantigen and the RAG protein may coordinately recognize the RSS heptamer and nonamer, respectively, and enforces the 12/23 rule. The present finding that Ku70/80 binds specifically to the RSS heptamer suggests a hitherto unappreciated sequence-specific function of the Ku autoantigen during V(D)J recombination, which is independent of its nonspecific NHEJ activity. There have been speculations about the intersection of the RSS cleavage machinery and the NHEJ apparatus during V(D)J recombination (Jung and Alt 2004), but the evidence for their coupling remains tenuous. The current finding of specific binding by the Ku autoantigen to the RSS heptamer and RAG1/2 provides important evidence for a linkage between RSS cleavage and the NHEJ apparatus during V(D)J recombination.

4.3. The Ku70/80-selected heptameric sequences are also present in naturally occurring RSS heptamers

The naturally occurring RSS heptamers in the Igh and TCR variable region locus tolerate slight variations in sequence (Akamatsu et al. 1994; Matsuda et al. 1998; Hassanin et al. 2000; Yu et al. 2002). The heptameric sequences evolved from the *SELEX* by recombinant Ku70/80 were identified on the basis of having 70% homology to the consensus RSS heptamer with variations at any two positions of the heptamer allowed. Some of the Ku70/80-selected heptameric sequences are also found in naturally occurring RSS heptamers.

The heptameric sequence, -CACAATG-, from clone 33, is found in a functional heptamer in human $V_{H4\text{-}31}$ (Matsuda et al. 1998) and J2 gene segments (Hieter et al. 1982) and in mouse $V_\lambda 1$, $V_\lambda 2$ and $J_\lambda 2$ gene segments (Ramsden and Wu 1991). The heptameric sequence, -CACCATG-, from clone 51, is found in a transcribed V_H gene segment ($V_{H7\text{-}81}$) in humans (Matsuda et al. 1998). The heptameric sequence, -CACAGCA-, from clone 37 (-GGTTCGG*TACACAG*CATG-), was seen in human D_{A1} gene segment (Ichihara et al. 1988). However, the heptameric sequence, -TACACAG- instead of -CACAGCA-, from clone 37, was found in a transcribed human V_H gene segment ($V_{H3\text{-}38}$) (Matsuda et al. 1998). The heptameric sequence, -CACACTG-, found in clones 21, 41 and 69, was also present at the 3' end of many human V_H gene segments (Givol et al. 1981) and mouse J_2 gene segment (Hieter et al. 1982). The heptameric sequence from clone 38, -GACAGTG-, was found in human D_{M2} gene segment and the heptameric sequence from clone 18, -CACAGTA-, was seen in human D_{A4} segment (Ichihara et al. 1988) and mouse $D_{FL16.1}$ gene segment (Connor et al. 1995).

Furthermore, the analysis of the frequency of nucleotides at each position of the heptamer from the Ku70/80-selected sequences bearing homology to the RSS heptamer

indicated that the third, fourth and sixth positions of the heptameric sequence 5'-CACAGTG-3' are important for binding by Ku70/80 as only slight variations are observed in these three positions. This observation was further confirmed by the findings from the competitive *in vitro* DNA binding assays with Ku70/80 and wild-type and mutated heptamers. By comparison, in naturally occurring RSS heptamers in the Igh or TCR variable region locus, the first three nucleotides (-CAC-) are approximately 100% conserved while the remaining nucleotides are only 63 to 86% conserved (Akira et al. 1987; Hesse et al. 1989; Akamatsu et al. 1994). The calculation of position-wise information of the naturally occurring RSS heptamers also showed that the first three nucleotides are almost strictly conserved (Cowell et al. 2003). In addition, the first three nucleotides of the RSS heptamer have been shown to be important for DNA cleavage by RAG1/2 (Akamatsu et al. 1994; Steen et al.1997; Nagawa et al. 1998).

The findings from the current study show that mutations in the wild-type heptamer did not abort Ku70/80 binding to the mutated heptamers, suggesting that sequence variations in the heptameric sequence might serve to modulate DNA binding affinities of Ku70/80. One implication of the variations in the RSS heptameric sequence is their probable effects on the efficiency of RSS cleavage by RAG1/2, the evidence for which, however, is thus far inconclusive (Ramsden and Wu 1991; Connor et al. 1995; Nadel et al. 1998; Feeney et al. 2000; Yu et al. 2002). The discrepancy between preferred nucleotides in the RSS heptamer for binding by Ku70/80 and those for cleavage by RAG1/2 suggests that the first two nucleotides might be required for unhindered access by RAG1/2 to the cleavage site or involved in the unpairing of base pairs upstream of the heptamer. Additionally, as the Ku autoantigen contacts only one side of the DNA helix

(Yoo et al. 1999) and the RSS heptamer itself presents an almost single entity of palindromic DNA binding interface (Ohno and Yomo 1990), the presence of another heptamer binding protein on the contralateral face of the DNA helix cannot be ruled out.

4.4. Coupling of NHEJ to V(D)J recombination through sequence-specific binding by the Ku protein to the RSS heptamer

V(D)J recombination is accomplished through distinctly defined stages including the recognition and cleavage of RSSs by RAG1/2, the resolution of DNA hairpins by Artemis and DNA-PKcs, and the re-ligation of cleaved DNA ends by XRCC4, Ku70/80, DNA-PKcs and DNA ligase IV; however, whether and how these disparate phases are interrelated with one another remain to be elucidated (Jung and Alt 2004). During V(D)J recombination, RAG1/2 mediates the recognition of the RSS mainly through the nonamer and initiates DNA cleavage 5' of the RSS heptamer while Ku70/80 is known to participate through its nonspecific NHEJ activity. The precise function of Ku70/80 in the process remains largely undefined. The current finding that Ku70/80 binds directly to the RSS heptamer independent of the presence of RAG1/2 suggests a specific-sequence-directed function of the Ku autoantigen during V(D)J recombination.

The RSS heptamer constitutes a critical interface for DNA cleavage by RAG1/2 (Cuomo et al. 1996; Ramsden et al. 1996; Nagawa et al. 1998). I show here that it offers, at the same time, a discriminative DNA binding site for Ku70/80. The close proximity of the DNA cleavage site for RAG1/2 and the DNA binding site for Ku70/80 bears direct relevance to RSS cleavage by RAG1/2. As revealed in the current study, the presence of the Ku autoantigen facilitated RSS cleavage by RAG1/2 both *in vitro* and *in vivo* whereas

its absence results in no observable effects on the fidelity of RSS cleavage. In addition, in the Ku80-deficient *xrs*-6 cells and mouse thymocytes, signal ends were present in amounts similar to those in their wild-type counterparts where the processing of DNA ends by the NHEJ apparatus remains intact (Zhu et al. 1996; Han et al. 1997), which suggests that RSS cleavage by RAG1/2 becomes less efficient with the absence of the Ku autoantigen. Furthermore, a separate study revealed that Dδ1-Dδ2 signal ends and junctions were not detectable in the Ku80-deficient mouse thymocytes while they were present in the wild-type or *scid* thymocytes (Nussenzweig et al. 1996), suggesting that the deficiency of the Ku autoantigen in the thymocytes resulted in a specific defect in the cleavage of these gene segments.

Though the Ku autoantigen possesses ATPase activity (Cao et al. 1994), its facilitation of RSS cleavage by RAG1/2 is unlikely due to the gross modulation of the accessibility of the chromatin at the cleavage site to RAG1/2, as no structural context unique to chromosomal DNA appears to be required for extrachromosomal recombination DNA substrates to undergo V(D)J recombination *in vivo* (Hesse et al. 1987) and the RSSs on these DNA substrates remain accessible to RAG1/2. DNA cleavage by RAG1/2 apparently requires the unpairing of base pairs at the border of the heptamer and the coding sequences (Cuomo et al. 1996; Ramsden et al. 1996). Similar to Tn5 transposase-bound DNA (Davies et al. 2000), structural distortions of the flanking coding region and the 5' portion of the heptamer likely precede DNA cleavage by RAG1/2 (Cuomo et al. 1996; Ramsden et al. 1996; Swanson and Desiderio 1998). The energetically unfavorable step of base unpairing at the border of the coding flank and the heptamer probably requires the action of a helicase (Ramsden et al. 1996). One probable

scenario is that, similar to its binding to NRE1 (Giffin et al. 1994, 1997; Giffin and Haché 1995; Torrance et al. 1998), the binding of Ku70/80 to the RSS heptamer induces structural transitions 5' of the heptamer or locally unwinds the DNA helix from its helicase activity (Tuteja et al. 1994; Ochem et al. 1997), which likely induces or facilitates RAG1/2 binding to the single-stranded heptamer as a result of the conformational changes (Peak et al. 2003) and thus enhances DNA cleavage by RAG1/2.

Another possible scenario is that Ku70/80 is involved in the juxtaposition of gene segments poised for V(D)J recombination. V(D)J recombination *in vivo* occurs between gene segments that may span more than 3 megabase pairs (mb) across the chromosome (Shimizu et al. 1982; Mainville et al. 1996; Chevillard et al. 1998; Kirschbaum et al. 1998, 1999; Röschenthaler et al. 1999; Thiebe et al. 1999; Schäble et al. 1999). The Igh and Igκ loci are positioned at the nuclear periphery by default but, when poised for recombination, move away from the nuclear periphery and undergo large scale compaction (Kosak et al. 2002). The Ku protein rapidly diffuses through the nucleus in the cell (Rodgers et al. 2002). In addition, two separate DNA ends bound by Ku70/80 on a single DNA molecule can be juxtaposed through the physical association of the Ku proteins (Cary et al. 1997). Similarly, two distant gene segments spanning megabases away on the chromosome can be brought in close proximity through the association of the Ku proteins bound to their respective heptamers, which would facilitate the formation of the synaptic complex between a 12RSS and a 23RSS.

On the other hand, Ku70/80, through its specific association with the RSS heptamer and the RAG1/2 protein, might also participate in the formation of synaptic complexes between a 12RSS and a 23RSS. The current model holds that RAG1/2 binds

to an individual 12RSS or 23RSS and forms a presynaptic complex *in vitro*, which, however, is catalytically incompetent (Hiom and Gellert 1997, 1998; Mundy et al. 2002; Jones and Gellert 2002). The assembled protein complex subsequently forms a synaptic complex by incorporating a second 23RSS or 12RSS, which is believed to trigger a conformational change in the synaptic complex rendering it catalytically competent for RSS cleavage (Hiom and Gellert 1997, 1998; Murphy et al. 1999). This sequential model of synaptic RSS complex formation assumes that RAG1/2 is inactive on individual RSSs and in the presynaptic complex. However, RAG1/2 is known to be active for DNA cleavage when bound to a single RSS *in vitro*, though with a reduced efficiency compared with the cleavage of an appropriately paired 12/23 RSS complex (Eastman et al. 1996; Sawchuk et al. 1997; Santagat et al. 1998). Furthermore, the formation of synaptic complexes between a 12RSS and a 23RSS and the coupled cleavage at the 12RSS and 23RSS by RAG1/2 likely involve other cellular factors in addition to RAG1/2 (Eastman et al. 1996; Sawchuk et al. 1997). The 12/23 rule was not strictly followed in *in vitro* DNA cleavage by purified RAG1/2 (Sawchuk et al. 1997), which, however, was remedied when complemented with whole cell extracts, suggesting the participation of other cellular components in the enforcement of the 12/23 rule during V(D)J recombination.

The current findings that Ku70/80 binds to the RSS heptamer prior to DNA cleavage by RAG1/2 and the facilitation by Ku70/80 of RSS cleavage by RAG1/2 suggest that the Ku autoantigen might be part of the presynaptic and synaptic RSS complexes. In the presynaptic RSS complex, Ku70/80 prevents undue cleavage by RAG1/2 in the absence of appropriately paired RSSs. With the formation of the synaptic

complexes facilitated by Ku70/80, RAG1/2 is rendered catalytically competent for RSS cleavage. This hypothesis is also supported by findings from a just published study by Sawchuk et al. (2004) that, along with DNA-PKcs, the Ku antigen participates in the enforcement of the 12/23 rule in RSS cleavage by RAG1/2. They found that the presence of the Ku autoantigen and DNA-PKcs suppresses the cleavage by RAG1/2 of uncoupled RSSs such as 12/12 RSS or 23/23 RSS.

The Ku autoantigen and other components of the NHEJ apparatus have been implicated in the maintenance of genomic stabilities (Ferguson et al. 2000; Difilippantonio et al. 2000). The entry into V(D)J recombination of the Ku autoantigen through direct binding to the RSS heptamer may ensure genomic integrity by precluding undue DNA cleavage by RAG1/2. It remains to be investigated whether Ku70/80 binding to the RSS heptamer results in the recruitment of other components of the NHEJ apparatus such as DNA-PKcs, XRCC4 or DNA ligase IV. One possible scenario is that the binding of Ku70/80 or the NHEJ apparatus to the RSS heptamer or cryptic heptameric sites denies DNA cleavage by RAG1/2 without a properly paired 12/23 synaptic complex by posing a steric hindrance to the recombinase complex. This scenario envisions that the binding of Ku70/80 to the RSS heptamer would negatively regulate DNA cleavage by RAG1/2 in the absence of a synaptic 12/23RSS complex. In the absence of Ku70/80 binding to the RSS heptamer, RAG1/2 would bind to an individual RSS including a cryptic RSS and initiates DNA cleavage without restraint; however, when Ku70/80 or its cognate supramolecular complex is present on the heptamer prior to or coincident with RAG1/2 binding to the nonamer on the RSS, it would prevent undue DNA cleavage by RAG1/2 *in cis* through mechanical hindrance. As a result, DNA cleavage by RAG1/2

would occur only *in trans* or following the displacement of Ku70/80 or its associated supramolecular complex from the RSS heptamer. In this regard, RAG1 was recently shown to possess E3 ubiquitin ligase activity in its N terminus (Yurchenko et al. 2003). RAG2 was recently found to be targeted for degradation by ubiquitination (Mizuta et al. 2002). One intriguing possibility, though highly speculative, is that RAG1 could also modulate DNA binding activity of Ku70/80 through ubiquitination by its E3 ubiquitin ligase activity.

It has been estimated that there are 10 X 10^6 sites in the genome with a DNA sequence compatible with a RSS function (Lewis et al. 1997). The cleavage of cryptic RSSs by RAG1/2 and subsequent chromosomal translocations have been long suspected to play an important role in the malignant transformation of lymphocytes (Tycko et al. 1989; Cheng et al. 1990; Vanasse et al. 1999a, 1999b; Kitagawa et al. 2002; Marculescu et al. 2002). The cleavage of these cryptic RSS sites has to be regulated, as unwarranted DSBs destabilize the chromosomal integrity (Richardson and Jasin 2000). Ku70/80 or other components of the NHEJ apparatus are known to be involved in the maintenance of genomic stability and $Ku70^{-/-}$ mice are predisposed to the formation of T cell lymphomas (Gu et al. 1997b; Li et al. 1998; Rooney et al. 2003, 2004). $Ku80^{-/-}$ $p53^{-/-}$ mice developed tumors resembling pro-B cell lymphoma involving a translocation between chromosomes 12 and 15 (Difilippantonio et al. 2000; Lim et al. 2000), which were similar to the pro-B cell tumors in $p53^{-/-}$ *scid* mice (Nacht et al. 1996; Vanasse et al. 1999a, 1999b). Recently, RAG1/2 was found to be capable of cleaving the BCL2 major breakpoint region (mbr) *in vivo* (Raghavan et al. 2004), the breakage of which and its subsequent fusion with IgH locus are responsible for human follicular lymphoma. It is tantalizing to speculate that the

Ku autoantigen may be involved in targeting RAG1/2 to the BCL2 mbr or that it may be involved in locally unwinding the BCL2 mbr for cleavage by RAG1/2 as the BCL2 mbr has been previously shown to be a sequence-specific DNA binding site for the Ku autoantigen (DiCroce and Krontiris 1995).

4.5. Coupling of Ku70/80 to V(D)J recombination through direct binding to the RAG1/2 protein

The current findings that the Ku autoantigen binds specifically to the RSS heptamer and facilitates DNA cleavage by RAG1/2 implicate the Ku protein in pre-NHEJ activities during V(D)J recombination. RAG1/2 contacts the RSS on only one side of the DNA helix (Swanson and Desiderio 1998) while the arrangement of the Ku protein on DNA ends on one side of the DNA helix also allows access by other proteins to the contralateral side of the DNA helix (Yoo et al. 1999). In addition, the close proximity of the RSS cleavage site by RAG1/2 and the Ku70/80 binding site lends a support to the possibility of negotiation between RAG1/2 and Ku70/80 with regards to RSS cleavage and subsequent NHEJ. Our initial findings that RAG1/2 interacts with Ku70/80 *in vitro* were further confirmed by the results from the coimmunoprecipitation studies of pro-B cells that Ku70/80 is associated with RAG1/2 *in vivo* independent of the presence of DNA. In addition, the *Sleeping Beauty* transposase, was found in association with Ku70/80 *in vivo* (Izsvák et al. 2004), thus providing a precedent for the interaction of a transposase with a component of the NHEJ apparatus. These observations together suggest that Ku70/80 and RAG1/2 probably exist as a higher order supramolecular

complex in the cell prior to their binding to the RSS or interact with one another following their independent binding to the RSS.

As a component of the NHEJ apparatus, Ku70/80 interacts with DNA-PKcs, XRCC4 or DNA ligase IV on nonspecific DNA ends (Gottlieb and Jackson 1993; Chen et al. 2000; Calsou et al. 2003; Kysela et al. 2003). In addition, Ku70/80 is associated with the lymphocyte specific TdT, which creates N-region diversity by inserting non-templated nucleotides to the cleaved coding ends (Bogue et al. 1997; Mahajan et al. 1999; Purugganan et al. 2001). The involvement of Ku70/80 in V(D)J recombination has long been hypothesized to be confined to the postcleavage NHEJ step in which DNA end recognition by the Ku autoantigen provides for the entry of other NHEJ factors to mediate the appropriate ligation of cleaved coding or signal ends (Grawunder and Harfst 2001). Direct binding by Ku70/80 to the RSS heptamer may provide an alternative entry point for other NHEJ components. It remains to be investigated whether Ku70/80 binding to the RSS heptamer also recruits these NHEJ components to the DNA poised to undergo recombination with the formation of a pre-ligation complex prior to DNA cleavage by RAG1/2. It is also of special interest to know if, similar to its binding to NRE1, Ku70/80 binding to the heptamer targets DNA-PKcs to the site and activates its kinase activity. The recent findings by Sawchuk et al. (2004) suggest that the Ku autoantigen and DNA-PKcs may be part of the pre-cleavage complex which enforces the 12/23 rule during V(D)J recombination. The interaction of the Ku protein and RAG1/2 and the association of Ku70/80 with the RSS heptamer raise the probability of the existence in the cell of an assembled supramolecular complex that includes RAG1/2 and Ku70/80 that coordinate the cleavage of the RSSs and the re-ligation of cleaved DNA ends.

Though an important participant in V(D)J recombination as a component of the NHEJ apparatus, the Ku protein is apparently not required for protecting either the coding or signal ends formed as a result of DNA cleavage by RAG1/2 (Zhu et al. 1996; Han et al. 1997; Kabotyanski et al. 1998). In addition, coding ends or signal ends recovered from the Ku80-deficient cells or thymocytes remained intact as assessed by ligation mediated PCR (Zhu et al. 1996; Han et al. 1997). The current study further demonstrated that the signal ends accumulated from RSS cleavage by RAG1/2 in the Ku80-deficient V15B fibroblasts are intact and can be further processed by an intact NHEJ apparatus. The observations indicate that, in the absence of the Ku protein, the cleaved coding or signal DNA ends are sequestered from nuclease attacks in the cell.

By contrast, the protection of cleaved coding or signal DNA ends appears to be provided by the RAG1/2 protein (Tsai et al. 2002), which was identified in a postcleavage complex *in vitro* containing signal ends, Ku70/80, DNA-PKcs and XRCC4 (Agrawal and Schatz 1997). Furthermore, in addition to DNA-PKcs and Ku70/80, RAG1/2 was associated with cleaved Jκ1 gene segment *in vivo* by ChIP analysis (Perkins et al. 2002). These observations suggest that RAG1/2 remains associated with cleaved signal ends following DNA cleavage, which might shield DNA ends against degradation by nucleases in the cell.

The association of RAG1/2 with signal ends following RSS cleavage may also differentially affect the processing of coding and signal ends by the NHEJ apparatus. Examination of V(D)J recombination products near T cell receptor α or δ locus in mouse thymocytes revealed that the coding ends were rapidly processed and rejoined while the signal ends accumulated in linear or circular forms (Roth et al. 1992a; Livák and Schatz

1996). Using a pre-B cell line that underwent rapid induction of RAG1/2 and active rearrangement at the Ig light chain loci at the nonpermissive temperature, coding ends and signal ends from the Jκ locus accumulated at similar rates (Ramsden et al. 1995). Coding ends existed either as unprocessed covalently closed hairpins or open, blunt, processed coding ends and can be rapidly resolved into coding joints at the permissive temperature whereas signal ends remained unjoined.

Besides its established role in RSS cleavage, RAG1/2 probably is involved in the joining phase of V(D)J recombination (Schultz et al. 2001; Fugmann and Schatz 2001; Tsai et al. 2002). RAG1 mutants (E547Q and E423Q) are proficient in DNA binding and cleavage, but they are defective in joining the coding or signal ends *in vivo* (Schultz et al. 2001). One probability is that these mutants are defective in the recruitment of the NHEJ apparatus to the cleaved DNA ends. Similar observations were also made with mutations in R401/402 or R440 of RAG1 and K34, H94/K97, R73 and R229 of RAG2, which exhibited a phenotype comparable to that of Ku70- or Ku80-deficient cells, implicating RAG1/2 in the joining phase of V(D)J recombination (Qiu et al. 2001; Huye et al. 2002). In addition, RAG1/2 markedly stimulated the formation of coding joints in a cell-free V(D)J recombination assay using purified RAG1/2 supplemented with partially fractionated HeLa cell extracts (Ramsden et al. 1997). Removing RAG1/2 prior to NHEJ markedly reduced coding joint formation *in vitro* (Leu et al. 1997).

These findings together suggest that RAG1/2 persisting on cleaved DNA ends might interact or recruit the NHEJ apparatus. In this regard, RAG1/2 was found to be associated with other components as high molecular weight complexes in the nucleus and probably acted as a central organizing molecule for an active V(D)J enzyme complex

(Leu and Schatz 1995). DNA-dependent ATPase activity has been reported for the Ku autoantigen (Cao et al. 1994). It is tempting to speculate that the association of the Ku autoantigen with the RAG1/2 protein might be involved in remodeling the RAG1/2 protein in the postcleavage signal end complexes to facilitate the initiation of NHEJ. In this regard, the *E. coli* ClpX, a member of the Clp/Hsp100 family of ATPases, remodels the Mu transposase stably bound to DNA and destabilizes the strand transfer complex (Burton et al. 2001). Recently, Ku70/80 was found not to be required for the addition of nucleotides by TdT to endonuclease-induced chromosomal and extrachromosomal breaks, but was needed for the addition of nucleotides by TdT to the coding joint junctions (Sandor et al. 2004), suggesting that Ku70/80 regulates the access of TdT to the RAG1/2 postcleavage complexes, which provides supports for the hypothesis that the Ku autoantigen is involved in disassembling or remodeling the postcleavage complexes. The findings from a recent study by Lee et al. (2004) suggest that the RAG1/2 protein persisted on cleaved DNA ends is probably involved in channeling DNA DSBs to the NHEJ machinery, further attesting a linkage between the RSS cleavage components with the NHEJ pathway.

4.6. A trans-cleavage V(D)J recombination model

Apart from its established role as a component of the NHEJ apparatus during V(D)J recombination, the current study established that the Ku autoantigen is specifically involved in the recognition of and binding to the RSS heptamer and facilitates RSS cleavage by RAG1/2. In addition, Ku70/80 directly interacts with RAG1/2 independent of the presence of DNA. The role of RAG1/2 in V(D)J recombination, on the other hand,

also extends beyond the recognition of and binding to the RSS nonamer and RSS cleavage. Considerable existing evidence implicates RAG1/2 in the NHEJ phase of V(D)J recombination (Schultz et al. 2001; Fugmann and Schatz 2001; Tsai et al. 2002). These observations suggest that the RSS cleavage machinery and the NHEJ apparatus are more interconnected during V(D)J recombination than previously thought and are coupled through the direct association of the Ku autoantigen with the RSS heptamer and the RAG protein. It remains a distinct possibility that a higher order supramolecular complex that includes Ku70/80 and RAG1/2 and probably other components of the NHEJ apparatus exists on the RSS which coordinates the RSS cleavage and NHEJ throughout the V(D)J recombination process.

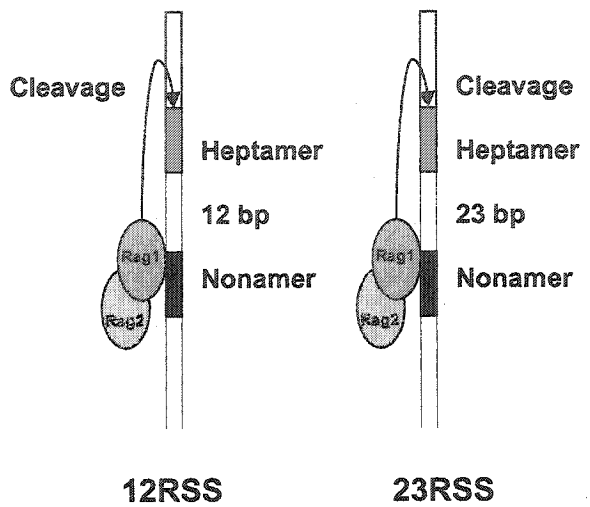
Substantial evidence suggests that V(D)J recombination occurs with the juxtaposition of a 12RSS and a 23RSS in a tightly regulated synaptic complex (van Gent et al. 1996; Hiom and Gellert 1998). Moreover, efficient RSS cleavage by RAG1/2 entails an appropriately coupled 12/23 RSS complex whose formation requires the presence of additional cellular factors (Meek et al. 1989; Eastman et al. 1996; Sawchuk et al. 1997; Steen et al.1997). In the absence of an appropriately formed synaptic complex, RAG1/2 cleaves a single RSS inefficiently (Eastman et al. 1996; Sawchuk et al. 1997). RSS cleavage by RAG1/2 is probably carried out *in trans*. RAG1 exists as a homodimer in solution and binds to the RSS as a dimer (Rodgers et al. 1999; Swanson 2002) and oligomeric forms of RAG1 may also exist (Leu and Schatz 1995; Godderz et al. 2003). Recent findings have shown that, when RAG1 binds to an individual 12RSS or 23RSS as a homodimer, DNA cleavage is not carried out by the RAG1 bound to the nonamer but by the other RAG1, suggesting a model of RSS cleavage by RAG1 *in trans* (Swanson

2001; Landree et al. 2001). This observation can be reasonably extended to the cleavage of a synaptic RSS complex in which the cleavage of a 12RSS or a 23 RSS is carried out by RAG1 bound to its partner RSS. DNA cleavage *in trans* has also been observed in transposases from bacteriophage Mu and Tn5 (Savilahti and Mizuuchi 1996; Namgoong and Harshey 1998; Naumann and Reznikoff 2000).

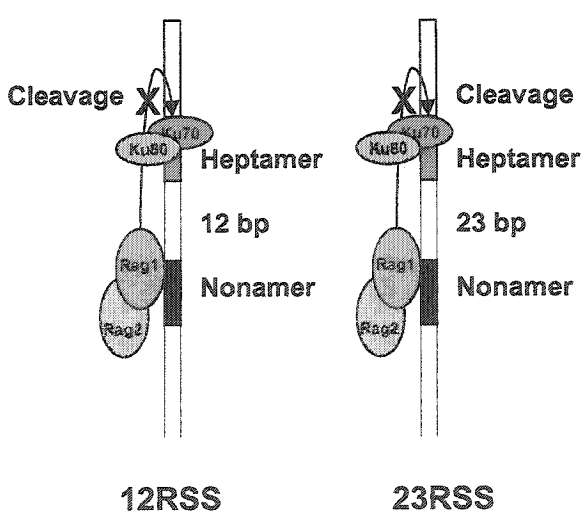
Based upon the above observations and the current findings, a *trans*-cleavage V(D)J recombination model is formulated (Figure 24), which envisions that, in the absence of the Ku autoantigen, RAG1/2 recognizes and binds to the nonamer of an individual 12RSS or 23RSS and cleaves singly 5' of the heptamer without restraint (Figure 24A). However, the cleavage of single unpaired RSSs by RAG1/2 is inefficient as suggested by the findings from Swanson (2001) and Landree et al. (2001). When the Ku protein is associated with the RSS heptamer in the absence of appropriately paired RSSs, RAG1/2 binds to the RSS nonamer but is restrained from cleaving DNA either from steric hindrance posed by the presence of Ku70/80 at the heptamer or due to an inactive conformation of RAG1/2 (Figure 24B). The presence of the Ku autoantigen on individual RSSs might exert greater control against uncoupled cleavage by RAG1/2, thus providing an important regulatory mechanism that prevents undue DNA cleavage. With the presence of the Ku autoantigen on the RSS heptamer, RAG1/2 bound to the nonamer of a 12RSS or a 23RSS and Ku70/80 that is present on the heptamer of a 23RSS or a 12RSS become associated with one another and form a precleavage synaptic complex, which triggers a conformational change in RAG1/2 rendering it competent for DNA cleavage *in trans* 5' of the heptamer (Figure 24C). This model suggests that RSS

Figure 24. A *trans* cleavage model for V(D)J recombination. **(A)** In the absence of Ku70/80 on the RSS heptamer and appropriately paired RSSs, binding of RAG1/2 to the RSS nonamer is followed by unrestrained DNA cleavage 5' of the heptamer. However, DNA cleavage by RAG1/2 in this scenario is random and inefficient. **(B)** In the presence of Ku70/80 on the RSS heptamer and in the absence of appropriately paired RSSs, binding of RAG1/2 to the RSS nonamer occurs normally but DNA cleavage by RAG1/2 is blocked by the presence of Ku70/80 close to the cleavage site. **(C)** When Ku70/80 is present on the RSS heptamer, RAG1/2 binds to the RSS nonamer and, with the formation of appropriately paired RSSs, RAG1/2 becomes competent for RSS cleavage *in trans*.

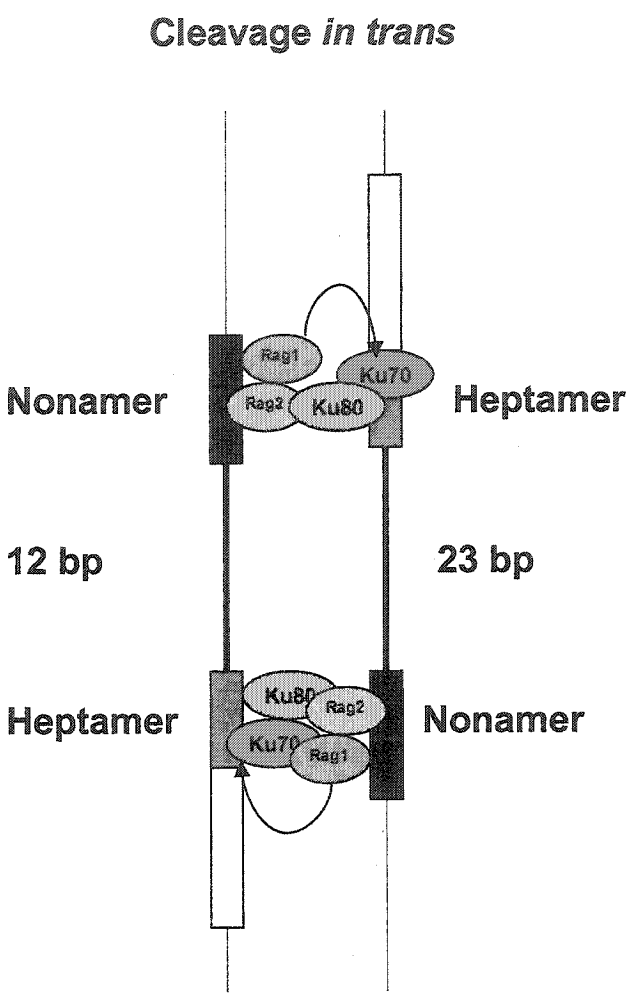
A.


Cleavage
Heptamer
12 bp
Nonamer
Rag1
Rag2
Cleavage
Heptamer
23 bp
Nonamer
Rag1
Rag2
12RSS
23RSS

B.


Cleavage
Ku80
Heptamer
12 bp
Nonamer
Rag1
Rag2
Cleavage
Ku80
Heptamer
23 bp
Nonamer
Rag1
Rag2
12RSS
23RSS

C.


Cleavage *in trans*
Nonamer
Heptamer
12 bp
23 bp
Heptamer
Nonamer
Rag1
Ku70
Rag2
Ku80
Ku80
Rag2
Ku70
Rag1

cleavage at individual RSSs by RAG1/2 or in the absence of the Ku autoantigen is uncoupled, inefficient and accessibility-determined and RSS cleavage at appropriately paired RSSs is coupled, efficient and facilitated by Ku70/80. The RAG1/2 *trans* cleavage V(D)J recombination model should provide a conceptually appealing framework for understanding and further elucidating the structural and functional organization of the higher order protein-DNA complexes within which V(D)J recombination takes place.

4.7. Future experiments

Our findings that the Ku autoantigen recognizes the RSS through the heptamer, interacts with the RAG protein *in vivo*, and facilitates RSS cleavage by RAG1/2 have important implications for further studies on the indispensable role of the Ku protein during V(D)J recombination. One important question to be addressed is if and how the Ku protein helps to enforce the 12/23 rule of V(D)J recombination together with the RAG protein. One way to address this issue is through *in vitro* RSS cleavage assays in which complex extrachromosomal recombination DNA substrates such as pJH200 that contain endogenous V, D or J gene segments are employed. Earlier studies have shown that the 12/23 rule of V(D)J recombination is not strictly enforced when RAG1/2 alone is employed for the cleavage of recombination DNA substrates containing endogenous V, D or J gene segments, which, however, is remedied upon supplementation with cellular extracts (Eastman et al. 1996; Sawchuk et al. 1997). To investigate if the Ku protein is involved in the enforcement of the 12/23 rule, *in vitro* RSS cleavage assays are to be performed in which RSS cleavage by purified RAG1/2 is carried out with or without recombinant Ku70/80 or whole cell extracts. The DNA recombination substrates

containing endogenous V, D or J gene segments are used and these substrates contain either 12/23 RSSs, 12/12 RSSs or 23/23 RSSs. The results from this study would provide an answer to the important question whether the Ku autoantigen and the RAG protein coordinately enforce the 12/23 rule of V(D)J recombination. Further, to take advantage of the *in vivo* recombination assay followed by the *in vitro* NEHJ assay developed in the current study, extrachromsomal recombination DNA substrates containing either 12/23 RSSs, 23/23 RSSs, or 12/12 RSSs are transfected into either V79 or Ku80-deficient V15B fibroblasts together with RAG1/2 constructs. Eighteen to 32 h following transfection, recombination DNA substrates are recovered by a standard alkaline lysis protocol and are subjected to *in vitro* NHEJ using 293T nuclear extracts followed by PCR analysis. The results from this study would provide insight into the question whether 12/12 RSSs or 23/23 RSSs are cleaved in V79 or V15B fibroblasts and the level of their cleavage, which may reveal how the 12/23 rule is enforced *in vivo* in the presence or absence of the Ku autoantigen.

RAG1/2 has been demonstrated to bind to the RSS *in vitro* and footprinting studies have shown RAG1 made sequence-specific contacts mainly with the nonamer and, in the presence of RAG2, RAG1/2 binding extended over the spacer into the nonamer proximal end of the heptamer (Nagawa et al. 1998; Swanson and Desiderio 1998, 1999). No convincing data are available that demonstrates structural distortion 5' upstream of the heptameric sequence -CACAGTG- in the presence of RAG1/2 alone. One important experiment to be carried out is the footprinting study of the interaction of the Ku protein with a 12RSS or 23 RSS DNA substrate, the results from which will provide further evidence for Ku70/80-specific binding to the RSS heptamer. An equally

important question to be addressed is whether the presence of the Ku protein alters the footprints of RAG1/2 on a 12 or 23 RSS and induces structural transitions 5' upstream of the heptamer. Experimentally, this can be addressed by DNA footprinting, sensitivity and hypersensitivity analyses using $KMnO_4$ or dimethysulfate (DMS) and S1 nuclease as has been done for NRE1 (Giffin and Haché 1995).

A recent study from the Cortes laboratory has shown that the Ku protein and DNA-PKcs and the RAG protein coordinately enforce the 12/23 rule of V(D)J recombination *in vitro* (Sawchuk et al. 2004). As the Ku protein is known to target DNA-PKcs to DNA ends (Gottlieb and Jackson 1993) and to specific DNA sequences like NRE1 (Giffin et al. 1996), it is of importance to investigate if Ku70/80 binding to the RSS also targets DNA-PKcs to the site and its effects on RSS cleavage. We have found that the Ku protein interacts with RAG1/2 both *in vitro* and *in vivo* by *in vitro* protein binding assays and coimmunoprecipitation studies. Similarly, we can also perform coimmunoprecipitations in 220-8 pro-B cells with antibodies against the RAG protein and immunoblot with antibodies to the Ku protein or DNA-PKcs. We may also immunoblot with antibodies to XRCC4 or DNA ligase IV to see if they are associated with the Ku autoantigen and the RAG protein in a precleavage complex. Furthermore, *in vitro* RSS cleavage assays and *in vitro* V(D)J recombination assays using pJH200 will be carried out using RAG1/2 transfected 293T nuclear extracts in the presence or absence of DNA-PKcs inhibitor such as wortmanin. The results would reveal whether inhibition of DNA-PKcs activity has any effect on RSS cleavage. In addition, *in vivo* V(D)J recombination assays followed by PCR analysis are carried out using DNA-PKcs

deficient cell line and its parental cell line to investigate whether DNA-PKcs deficiency results in reduced efficiency of RSS cleavage.

4.8. Summary and conclusions

The Ku protein has both specific DNA binding activity and nonspecific DNA end binding activity and is involved in multiple cellular activities including DNA DSB repair and V(D)J recombination, transcriptional regulation, control of DNA replication, maintenance of telomere integrity and genomic stabilities. The current study has demonstrated that the Ku autoantigen binds to different classes of DNA binding sites with different affinities, which also involves differential contacts of DNA by its two subunits. The *SELEX* by recombinant human Ku70/80 further corroborated sequence-specific activity of the Ku autoantigen and evolved a novel class of specific DNA binding sites with a consensus sequence of $CA(N_{1-3})TG$ for the Ku protein, which contains a consensus sequence identical to the RSS heptamer. The frequency analysis of nucleotides at each position of the heptamer and subsequent *in vitro* DNA binding assays further demonstrated that the third and fourth positions of the heptamer are important for binding by the Ku autoantigen. The current study also established an *in vitro* V(D)J recombination assay which mimics physiologic V(D)J recombination reactions with regards to the coupled cleavage of the 12RSS and 23RSS and the fidelity of RSS cleavage by RAG1/2. The presence of the Ku autoantigen during V(D)J recombination was further demonstrated to facilitate RSS cleavage by RAG1/2 both *in vitro* and *in vivo*. In addition, the absence of the Ku protein does not compromise the fidelity of RSS cleavage by RAG1/2 and does not result in an excessive degradation of cleaved DNA

ends. Moreover, the Ku autoantigen was found to be associated with the RAG protein both *in vitro* and *in vivo* independent of the presence of DNA. Based on these observations and the relevant findings from other researchers, a *trans* cleavage V(D)J recombination model is formulated, which envisions that RAG1 cleaves the RSS *in trans* in a synaptic 12/23 RSS complex and the presence of the Ku autoantigen in the synaptic complex regulates RSS cleavage by RAG1. The findings from the current study provide a significant insight into the role of the Ku autoantigen in V(D)J recombination and the *trans* cleavage V(D)J recombination model would establish a conceptual framework for further elucidating the roles of the various cellular players in V(D)J recombination.

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Appendix I

Amino acid sequence of the 70 kDa subunit of human Ku protein*

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DELTPFDMSIQCIQSVYISKIISSDRDLLAVVFYGTEKDKNSVNFKNIYVLQELDNP
GAKRILELDQFKGQQGQKRFQDMMGHGSDYSLSEVLWVCANLFSDVQFKMSH
KRIMLFTNEDNPHGNDSAKASRARTKAGDLRDTGIFLDLMHLKKPGGFDISLFY
RDIISIAEDEDLRVHFEESSKLEDLLRKVRAKETRKRALSRLKLKLNKDIVISVGIY
NLVQKALKPPPIKLYRETNEPVKTKTRTFNTSTGGLLLPSDTKRSQIYGSRQIILEK
EETEELKRFDDPGLMLMGFKPLVLLKKHHYLRPSLFVYPEESLVIGSSTLFSALLI
KCLEKEVAALCRYTPRRNIPPYFVALVPQEEELDDQKIQVTPPGFQLVFLPFADD
KRKMPFTEKIMATPEQVGKMKAIVEKLRFTYRSDSFENPVLQQHFRNLEALALD
LMEPEQAVDLTLPKVEAMNKRLGSLVDEFKELVYPPDYNPEGKVTKRKHDNEG
SGSKRPKVEYSEEELKTHISKGTLGKFTVPMLKEACRAYGLKSGLKKQELLEALT
KHFQD

* The GeneBank accession number for the amino acid sequence of the 70 kDa subunit of human Ku protein is P12956.

Appendix II

Amino acid sequence of the 80 kDa subunit of human Ku protein*

MVRSGNKAAVVLCMDVGFTMSNSIPGIESPFEQAKKVITMFVQRQVFAENKDEI
ALVLFGTDGTDNPLSGGDQYQNITVHRHLMLPDFDLLEDIESKIQPGSQQADFLD
ALIVSMDVIQHETIGKKFEKRHIEIFTDLSSRFSKSQLDIIIHSLKKCDISLQFFLPFS
LGKEDGSGDRGDGPFRLGGHGPSFPLKGITEQQKEGLEIVKMVMISLEGEDGLDE
IYSFSESLRKLCVFKKIERHSIHWPCRLTIGSNLSIRIAAYKSILQERVKKTWTVVD
AKTLKKEDIQKETVYCLNDDDETEVLKEDIIQGFRYGSDIVPFSKVDEEQMKYKS
EGKCFSVLGFCKSSQVQRRFFMGNQVLKVFAARDDEAAAVALSSLIHALDDLD
MVAIVRYAYDKRANPQVGVAFPHIKHNYECLVYVQLPFMEDLRQYMFSSLKNS
KKYAPTEAQLNAVDALIDSMSLAKKDEKTDTLEDLFPTTKIPNPRFQRLFQCLLH
RALHPREPLPPIQQHIWNMLNPPAEVTTKSQIPLSKIKTLFPLIEAKKKDQVTAQEI
FQDNHEDGPTAKKLKTEQGGAHFSVSSLAEGSVTSVGSVNPAENFRVLVKQKK
ASFEEASNQLINHIEQFLDTNETPYFMKSIDCIRAFREEAIKFSEEQRFNNFLKALQ
EKVEIKQLNHFWEIVVQDGITLITKEEASGSSVTAEEAKKFLAPKDKPSGDTAAV
FEEGGDVDDLLDMI

* The GeneBank accession number for the amino acid sequence of the 80 kDa subunit of human Ku protein is NP_066964.

APPENDIX III

Proposed Specific DNA Binding Sites for the Ku Autoantigen or its cognate complex

Proposed specific sequences for Ku70/80 or its cognate complex

1. **Enhancer of the T-cell receptor β-chain gene (E3A)** (Messier et al. 1993):

   5'-CCAGGAGTCACAACAGGATGTGGTTTG-3'
   -GGTCCTCAGTGTTGTCCTACACCAAAC-

2. **Osteocalcin fibroblast growth factor response element (OCFRE)** (Boudreaux and Towler 1996; Willis et al. 2002):

   5'-CCCGG***CAGCTG***CAGTCACCAACCACAGCATCCTTTGGGTTG-3
   -GGGCCGTCGACGTCAGTGGTTGGTGTCGTAGGAAACCCAAC-

   (*The osteocalcin promoter sequence encompassing the OCFRE is underlined. The bold italicized sequence is an E box motif.*)

3. **E-box/Ku86 sites in human xanthine oxidoreductase gene promoter (hXOR)** (Xu et al. 2004):

   5'- ATGTAGTTTCTGTGTCTTTGACA***CAGGTG***TGGAGAT-3'
   -TACATCAAAGACACAGAAACTGTGTCCACACCTCTA

   (The underlined sequence is a proposed Ku binding site and the bold italicized underlined sequence is an E box motif.)

4. **Enhancer 2 (Enh2) of mouse intracisternal A-particle (IAP) U3 region** (Falzon and Kuff 1989):

   5'-CTGCG***CATGTG***CCAAGGGTATCTTATGACT-3'
   -GACGCGTACACGGTTCCCATAGAATACTGA-

   (The bold italicized underlined sequence is an E box motif.)

5. **Human T-cell leukemia virus type I (HTLV-I) U5 repressive element (U5RE)** (Okumura et al.1994):

   5'-GAATGAAAGGGAAAGGGTGGAC-3'
   -CTTACTTTCCCTTTCCCACCTG-

   (*The underlined sequence is polypurine in nature.)*

6. **The -75 region of human glycophorin B (GPB) promoter** (Rahuel et al. 1992; Camara-Clayette et al. 1999a, 1999b):

   5'-CAT***CAGCTG***ATAGGC-3'
   -GTAGTCGACTATCCG-

   (The underlined sequence is a GATA box and the bold italicized sequence is an E box motif.)

7. **The +103 region of KEL promoter** (Camara-Clayette et al. 2001):

   5'-GCCACAGAAGATAGA***CAGATG***GTA-3'
   -CGGTGTCTTCTATCTGTCTACCAT-

   (The underlined is a GATA box and a proposed Ku binding site and the bold italicized sequence is an E-box motif.)

Proposed specific sequences for Ku70/80 or its cognate complex

8. **Negative calcium response element (nCaRE)** (Okazaki et al. 1991; Chung et al. 1996):

   5'-TTCTGCATACA***CAAATG***G -3'
   -AAGACGTATGTGTTTACC-

   (The bold italicized underlined sequence is an E box motif.)

9. **Negative vitamin D response element in the human parathyroid hormone-related peptide gene ($nVDRE_{hPTHrP}$)** (Nishishita et al. 1998):

   5'-TAAAGTGCTATAGATTCATATTTGGTTTAT-3'
   -ATTTCACGATATCTAAGTATAAACCAAATA-

10. **Proximal sequence element (PSE) of human U1 small nuclear RNA gene (Knuth et al. 1990;** Gunderson et al. 1990**):**

    5'-GGG***CAAGTG***ACCGTGTGTGAAAGAGTAG-3'
    -CCCGTTCACTGGCACACACTTTCTCATC-

    (*The bold italicized underlined sequence is an E-box motif.*)

11. **The -77 to -70 region of human transferrin receptor (TR) promoter** (Roberts et al. 1989; Roberts et al. 1994)

    5'-CAGGAAGTGACGCACAGCC-3'
    -GTCCTTCACTGCGTGTCGG-

12. **Negative regulatory element 1 (NRE1) in the long terminal repeat (LTR) of mouse mammary tumor virus (MMTV)** (Giffin et al. 1996; Giffin et al. 1997):

    5'-AACTGAGAAAGAGAAAGACGACA-3'
    -TTGACTCTTTCTCTTTCTGCTGT-

    (*The underlined sequence is a polypurine tract containing a repeat of GAGAAAGA.*)

13. **The -217 to -197 region of HIV-1 LTR** (Jeanson and Mouscadet 2002):

    5'-CTGAGAGAGAAGTGTTAGAGT-3'
    -GACTCTCTCTTCACAATCTCA-

14. **The purine box/NFAT sequence in the human IL-2 enhancer** (-259 to -284) (Aoki et al. 1998; Ting et al. 1998):

    5'-AAGAAAGGAGGAAAAACTGTTTCATA-3'
    -TTCTTTCCTCCTTTTTGATAAAGTAT-

15. **BCL2 major breakpoint region 37 (37MBR)** (DiCroce and Krontiris 1995; Ramakrishman et al 2000):

    5'-GATTCTAATTTTTAAGCAAAATATTATTTTATG-3'
    -CTAAGATTAAAAAATTCGTTTTATAATAAAATAC-

16. **A3/4** (Ruiz 1999; Novac 2001; Matheos 2002; Schild-Poulter et al. 2003a):

    5'-CCT***CAAATG***GTCTCCAATTTTCCTTTGGCAAATTCC-3'
    -GGAGTTTACCAGAGGTTAAAAGGAAACCGTTTAAGG-

    (*The bold italicized sequence is an E-box motif and the underlined sequence is a polypurine tract that bears sequence similarity to the NRE1.*)

Proposed specific sequences for Ku70/80 or its cognate complex

17. **The switch (S) regulatory ATTT elements (SRE) in the Iγ and Iε promoters** (Schaffer et al. 2003):

    5'-ATTT-3'
      -TAAA-

18. **Heat shock element (HSE)** (Kim et al. 1995; Giffin et al. 1997; Huang et al. 1997; Tang et al. 2000):

    5'-CCCGAAACTGCTGGAAGATTCTTGGCCC-3'
      -GGGCTTTGACGACCTTCTAAGAACCGGG-

19. **Hemin response element (HRE)** (Reddy et al. 1998):

    5'-ACCTTGGAGGCGAGGCGCAGGTAATGG-3'
      -TGGAACCTCCGCTCCGCGTCCATTACC-

    (The underlined sequence is polypurine in nature.)

20. **The -68 to -29 promoter region of CD34** (Taranenko and Krause 2000):

    5'-AGGAACTACGAGAGGGCTGGCCTCACCAAGACGCAACAG-3'
      -TCCTTGATGCTCTCCCGACCGGAGTGGTTCTGCGTTGTC-

21. **The downstream activation sequence (DAS) of the strict late U_L38 promoter of herpes simplex virus type 1** (Petroski and Wagner 1998):

    5'-CCGGAGCGGTAGC-3'
      -GGCCTCGCCATCG-

22. **The -117 to -77 region of human collagen III α1 (COL3A1) promoter** (Giampuzzi et al. 2000):

    5'-TCTTTTACTGCTGAGGGGATGGGTGCGGGCTCTCATATT-3'
      -AGAAAATGACGACTCCCCTACCCACGCCCGAGAGTATAA-

23. **The metal response element a (MREa) in the Wilson gene promoter** (Oh et al. 2002):

    5'-GGGCGCCTGCGCCCCCGTTCC-3'
      -CCCGCGGACGCGGGGGCAAGG-

24. **The octamer motif** (May et al. 1991)

    5'-ATTTGCAT-3'
      -TAAACGTA-

25. **Epstein-Barr virus-responsive enhancer (EBVRE) in the promoter region of CD23** (Lacy et al. 1994; Shieh et al. 1997):

    5'-AGCTAGGATTA**CAGGTG**CCCACCACCATGCCCAGCTA-3'
      -TCGATCCTAATGTCCACGGGTGGTGGTACGGGTCGAT-

    (The bold underlined sequence is an E-box motif.)

26. **Glucocorticoid receptor enhancing factor binding element (GREFE)** (Warriar et al. 1996):

    5'-GAAGTGCACACTTC-3'
      -CTTCACGTGTGAAG-

Proposed specific sequences for Ku70/80 or its cognate complex

27. **Rat rRNA gene promoter** (Hoff et al. 1994; Datta et al. 1995; Yamamoto et al. 2000):

5'-CTTTGCTATCTGTCCTTATTGTACCTGGAGATTATATGCTGACACGCTGTC-3'
-GAAACGATAGACAGGAATAACATGGACCTCTAATATACGACTGTGCGACAG-

Appendix IV

List of primers used in this study

EMSAs with NRE1 and A3/4:

NRE1 upper	5'-AACTGAGAAAGAGAAAGACGACA-3'
NRE1 lower	5'-TGTCGTCTTTCTCTTTCTCAGTT-3'
A3/4 upper	5'-CCTCAAATGGTCTCCAATTTCCTTGGCAAATTCC-3'
A3/4 lower	5'-GGAATTTGCCAAGGAAATTGGAGACCATTTGAGG-3'
NS1 upper	5'-AGCCGAAGCATGCTTAGACATGTTCTGAGC-3'
NS1 lower	5'-GCTCAGAACATGTCTAAGCATGCTTCGGCT-3'

***SELEX* by recombinant Ku70/80:**

RND	5'-GAGCTCGCACTAGCGGATCCGTN$_{18}$CGAAGCTTGGTCACGCTCCGCT-3'
P1	5'-AGCGGAGCGTGACCAAGCTTCG-3'
P2	5'-GAGCTCGCACTAGCGGATCCGT-3'
T7	5'-GTAATACGACTCACTATAGGGC-3'

EMSA with the RSS heptamer:

H7 upper	5'-GGGACAAACACAGTGGTTAGCAG-3'
H7 lower	5'-CTGCTAACCACTGTGTTTGTCCC-3'
H_{7M12} upper	5'-GGGACAAA*gc*CAGTGGTTAGCAG-3'
H_{7M12} lower	5'-CTGCTAACCACTG*gc*TTTGTCCC-3'
H_{7M34} upper	5'-GGGACAAACA*gc*GTGGTTAGCAG-3'
H_{7M34} lower	5'-CTGCTAACCAC*gc*TGTTTGTCCC-3'
H_{7M67}upper	5'-GGGACAAACACAG*gc*GTTAGCAG-3'
H_{7M67} lower	5'-CTGCTAAC*gc*CTGTGTTTGTCCC-3'
NS upper	5'-CCCTCGAGGTCGACGGTATCGAT-3'
NS lower	5'-ATCGATACCGTCGACCTCGAGGG-3'
N9 upper	5'-CTGCTGAGACAAAAACCTGTCCC-3'
N9 lower	5'-GGGACAGGTTTTTGTCTCAGCAG-3'
H7 insert upper	5'-GAGCTCGCACTAGCGGATCCGTAACCACTGTGTTTGTCCCCGAAGCTTGGTCACGCTCCGCT-3'
H7 insert lower	5'-AGCGGAGCGTGACCAAGCTTCGGGGACAAACACAGTGGTTACGGATCCGCTAGTGCGAGCTC-3'

Plasmid immunoprecipitations:

R3	5'-TGTTCCAGTCTGTAGCACTG-3'
R5	5'-CCAGTCTGTAGCACTGTGCAC-3'
R14	5'-TCCAGCTGAACGGTCTGGT-3'
R7	5'-TTACGGGACTCTCGGCAGAAGCTA-3'
R8	5'-ATCCCGCCATGGTATCAACG-3'

ChIP:

VAR34-5'	5'-CCTGGGATGTCACTGATATACACTCTG-3'
VAR34-3'	5'-GTAGTAGCCAGTAAATGAGTAACCAGAAGCC-3'
VH3:3.9-5'	5'GGCGATTCACCATCTCCAGAG-3'
VH3:3.9-3'	5'-GGGTGCTCTGCAGGAGGTTTT-3'
Cδ-5'	5'-GTAGGCCTCCTACCACCTCC-3'
Cδ-3'	5'-CGTGGAGCTACATAGGGCCC-3'
Cγ2β-5'	5'-GGGAGGAGGGATTCACCAGAGTTGAGGC-3

Cγ2β-3'	5'-CCTGGTATGGGCTTAGTTCCAGGATGATCC-3'
J3 Hept-5'	5'-TCCATCTGAGATAATCCTGGAGCCC-3'
J3 Hept-3'	5'-TGCAGAGAATCTTGGTCCTGAAGGC-3'
Kappa J1 Fw	5'-CTCTGTCAGAGAAGCCCAAGCGC-3'
Kappa J1 Rev	5'-ATAGAAGCCACAGACATAGACAACGG-3'
Kappa J2 Fw	5'-TCAGAAATGCTCAAAGAAGCAGGG-3'
Kappa J2 Rev	5'-GACTTAGTGAACAAGAGTTGAGAAGAC-3'
Kappa J5 Fw	5'-CTTCTGAGACCAGTTTTGTAAGGGG-3'
Kappa J5 Rev	5'-GTGTACTTACGTTTCAGCTCCAGC-3'
Kappa Ki4 Fw	5'-TGGAGAAATTGGGCTCATTCA-3'
Kappa Ki4 Rev	5'-GCAGCATCTTCAGCCTCCAT-3'
β_2 microglobulin Fw	5'-GTCCCAGGCTGAACGACCAG-3'
β_2 microglobulin Rev	5'-TGAGAGACCAGCTAGGGCGC-3'

APPENDIX V

The sequences of Ku70/80-selected DNA binding sites by *SELEX*

Sequence number	Sequences of evolved DNA binding sites	Sequence number	Sequences of evolved DNA binding sites
01	GGGACAAACACAGTGGTT CCC TGTTTGTGTCACCAA	13	CCCTGTAGGATCTGGTCA GGGACATCCTAGACCAGT
02	TAGATGTGAGGGCCTCAC ATCTACACTCCCGGAGTG	14	GTAACTCCATCCACCCCC CATTGAGGTAGGTGGGGG
03	AGCTAACTAGTTGACTCG TCGATTGATCAACTGAGC	15	ATAACGTCATGGTTCCCC TATTGCAGTACCAAGGGG
04	TAGCAGGCGATCGGTCGT ATCGTCCGCTAGCCAGCA	16	ATGGCCTGCCTCGTTGGA TACCGGACGGAGCAACCT
05	GACTAGGCAGTGCGCATA CTGATCCGTCACGCGTAT	17	GTTGGATACGATTGGCCC CAACCTATGCTAACCGGG
06	GGAATCGCGCTACCCTCA CCTTAGCGCGATGGGAGT	18	CACAGTATTGAGTGCGGT GTGTCATAACTCACGCCA
07	CAATAGTAGAAGGTGCCC GTTATCATCTTCCACGGG	19	GGTACAGTGCCGCACCAT CCATGTCACGGCGTGGTA
08	GAGGAGGCCTGTTAGGGT CTCCTCCGGACAATCCCA	20	ATGTCGCTATAATTCTGT TACAGCGATATTAAGACA
09	GGAGGACACATCGGCCCC CCTCCTGTGTAGCCGGGG	21	GGGGACAAAACACACTGT CCCCTGTTTTGTGTGACA
10	TGCAGTGGACACCTCCTA ACGTCACCTGTGGAGGAT	22	ACGTGTAACCTATCCCAT TGCACATTGGATAGGGTA
11	TTGAGGGCTGCTATAGGT AACTCCCGACGATATCCA	23	ATGGTTACCAACTACCCC TACCAATGGTTGATGGGG
12	GCAACTACCTGAGATTCC CGTTGATGGACTCTAAGG	24	GGCAGTGTAGCGACTTGA CCGTCACATCGCTGAACT

Sequence number	Sequences of evolved DNA binding sites	Sequence number	Sequences of evolved DNA binding sites
25	TAGCTCTGGCTCCACGTC ATCGAGACCGAGGTGCAG	39	ATGCATAGTGTTGTGTGT TACGTATCACAACACACA
26	ACAGGGATGCGTCTCCCC TGTCCCTACGCAGAGGGG	40	GCAATGTTGGCAACAGGT CGTTACAACCGTTGTCCA
27	AGTGCTGGAATACCATAT TCACGACCTTATGGTATA	41	CAACACAACACACTGCTA GTTGTGTTGTGTGACGAT
28	AGAGCAGTTATCGCTTGC TCTCGTCAATAGCGAACG	42	ACACACAAAAACCAGTGC TGTGTGTTTTTGGTCACG
29	ACCACCCGTACTGGTACA TGGTGGGCATGACCATGT	43	ATACTGCACGTGTTCCCC TATGACGTGCACAAGGGG
30	AGGCGGCGGACAACACAT TCCGCCGCCTGTTGTGTA	44	TGTGGTAACGTGCAAACC ACACCATTGCACGTTTGG
31	AGCAGCAAAGTTGACCCC TCGTCGTTTCAACTGGGG	45	GGAAGATCGCATTACCCC CCTTCTAGCGTAATGGGG
32	ACAGGATAGTGCTTGGTA TGTCCTATCACGAACCAT	46	CGGAGCGACGTGACCACA GCCTCGCTGCACTGGTGT
33	ACAAGTGCACAATGTTAC TGTTCACGTGTTACAATG	47	GGAGGCCGCACGTGCTAC CCTCCGGCGTGCACGATG
34	AGAGGTGGCGGAGGCCTA TCTCCACCGCCTCCGGAT	48	GTAGCTGGGAGTTGTTTA CATCGACCCTCAACAAAT
35	ACAAAGGACTCCATACCT TGTTTCCTGAGGTATGGA	49	CCCGTACAATGATAGCGA GGGCATGTTACTATCGCT
36	ACGACACACTATCTGCCC TGCTGTGTGATAGACGGG	50	GGGCATGTTACTATCGCT CCCGTACAATGATAGCGA
37	GGTTCGGTACACAGCATG CCAAGCCATGTGTCGTAC	51	ATATGCACCACACCATGG TATACGTGGTGTGGTACC
38	GACAGTGGCTGATGCCTG CTGTCACTGACTACGGAC	52	CTAGTGCGAGGGGGATCC GATCACGCTCCCCCTAGG

Sequence number	Sequences of evolved DNA binding sites	Sequence number	Sequences of evolved DNA binding sites
53	CGGGCGACGTATCCCGCT GCCCGCTGCATAGGGCGA	62	GAGCAGGACCAAGTCATC CTCGTCCTGGTTCAGTAG
54	ATGCGCACCCTTCACCCA TACGCGTGGGAAGTGGGT	63	GACAACAGGAGCCCCAGT CTGTTGTCCTCGGGGTCA
55	GGGGGAAACACTCCAGGT CCCCCTTT GTGAGGTCCA	64	GGACTGCAGGCGCGTCAT CCTGACGTCCGCGCAGTA
56	TAGTCACGAACGTCACCC ATCAGTGCTTGCAGTGGG	65	ATAGTGCCGTACATCGTA TATCACGGCATGTAGCAT
57	GGCCCACTTGTTGGCTGC CCGGGTGAACAACCGACG	66	GGGGAGAGAACACCGTCT CCCCTCTCTTGTGGCAGA
58	TTCGACCAAAACACACCA AAGCTGGTTTTGTGTGGT	67	TACACCACTAGACCATAT ATGTGGTGATCTGGTATA
59	AGGTTGCACACTATACCC TCCAACGTGTGATATGGG	68	GCAAATGATCGTGGCATT CGTTTACTAGCACCGTAA
60	GGATAATCCCACTGCCAT CCTATTAGGGTGACGGTA	69	GCACACTGCTAATGGCCC CGTGTGACGATTACCGGG
61	AGTGTTGGAAGTCCTCGA TCACAACCTTCAGGAGCT	70	TAACCTCCACGACGTAGG ATTGGAGGTGCTGCATCC
71	GAGCGTGACCAAGCTTCG CTCGCACTGGTTCGAAGC	72	GGGGAGGTTCCAAGCGTG CCCCTCCAAGGTTCGCAC

APPENDIX VI

Ku70/80-selected sequences homologous to the RSS heptamer

Sequence number	Aligned sequences
01	GGGACAAA*CACAGTG*GTT* CCC TGTTT*GTGTCAC*CAA
19	GG*TACAGTG*CCGCACCAT CC*ATGTCAC*GGCGTGGTA
38	*GACAGTG*GCTGATGCCTG *CTGTCAC*TGACTACGGAC
05	GACTA*GGCAGTG*CGCATA CTGAT*CCGTCAC*GCGTAT
24	*GGCAGTG*TAGCGACTTGA *CCGTCAC*ATCGCTGAACT
10	*TGCAGTG*GACACCTCCTA *ACGTCAC*CTGTGGAGGAT
42	ACACACAAAA*ACCAGTG*C TGTGTGTTTT*TGGTCAC*G
32	ACAG*GATAGTG*CTTGGTA TGTC*CTATCAC*GAACCAT
49	CCCG*TACAATG*ATAGCGA GGGC*ATGTTAC*TATCGCT
66	GGGGAGAGAA*CACCGTC*T CCCCTCT CTT*GTGGCAG*A
60	GGATAAT*CCCACTG*CCAT CCTATTA*GGGTGAC*GGTA
39	ATG*CATAGTG*TTGTGTGT TAC*GTATCAC*AACACACA
51	ATATGCACCA*CACCATG*G TATACGTGGT*GTGGTAC*C

Sequence number	Aligned sequences
21	GGGGACAAAA*CACACTG*T CCCCTGTTTT*GTGTGAC*A
33	ACAAGTG*CACAATG*TTAC TGTTCAC*GTGTTAC*AATG
41	CAACACAA*CACACTG*CTA GTTGTGTT*GTGTGAC*GAT
69	G*CACACTG*CTAATGGCCC C*GTGTGAC*GATTACCGGG
09	GGAGGA*CACATCG*GCCCC CCTCCT*GTGTAGC*CGGGG
36	ACGA*CACACTA*TCTGCCC TGCT*GTGTGAT*AGACGGG
59	*AGGTTG*CACACTA*TACCC* TCCAAC*GTGTGAT*ATGGG
18	*CACAGTA*TTGAGTGCGGT *GTGTCAT*AACTCACGCCA
37	GGTTCGGTA*CACAGCA*TG CCAAGCCAT*GTGTCGT*AC
Consensus	*CACAGTG* *GTGTCAC*
RSS heptamer	*CACAGTG* *GTGTCAC*

*The selected sequences with homologies to the RSS heptamer (5'-CACAGTG-3') are underlined and italicized. Sequence alignment was done with the alignment program MultAlin available at http://www.toulouse.inra.fr/multalin.html.

APPENDIX VII

Ku70/80-evolved sequences and published sequences containing the E-box motif

Sequence number or name	Aligned sequences
43	ATACTG*CACGTG*TTCCCC TATGAC*GTGCAC*AAGGGG
47	GGAGGCCG*CACGTG*CTAC CCTCCGGC*GTGCAC*GATG
57	GGCC*CACTTG*TTGGCTGC CCGG*GTGAAC*AACCGACG
68	G*CAAATG*ATCGTGGCATT C*GTTTAC*TAGCACCGTAA
33	A*CAAGTG*CACAATGTTAC T*GTTCAC*GTGTTACAATG
OCFRE	*CAGCTG*CAGTCAC *GTCGAC*GTCAGTG
IAP Ehn2	CTGCG*CATGTG*CCAAGGGTATCTTATGACT GACGC*GTACAC*GGTTCCCATAGAATACTGA
hXOR	ATCTCCA*CACCTG*TGTCAAAGACACAGAAACTACAT TAGAGGT*GTGGAC*ACAGTTTCTGTGTCTTTGATGTA
GPB promoter	CAT*CAGCTG*ATAGGC GTA*GTCGAC*TATCCG
KEL promoter	TACCATCTGTCTATCTTCTGTGGC ATGGTAGACAGATAGAAGACACCG
nCaRE	C*CATTTG*TGTATGCAGAA G*GTAAAC*ACATACGTCTT
U1 PSE	GGG*CAAGTG*ACCGTGTGTGAAAGAGTAG CCC*GTTCAC*TGGCACACACTTTCTCATC
A3/4	CCT*CAAATG*GTCTCCAATTTTCCTTTGGCAAATTCC GGA*GTTTAC*CAGAGGTTAAAAGGAAACCGTTTAAGG
EBVRE	AGCTAGGATTA*CAGGTG*CCCACCACCATGCCCAGCTA TCGATCCTAAT*GTCCAC*GGGTGGTGGTACGGGTCGAT
Consensus	*CANNTG* *TGNNAC*

Curriculum Vitae

Bo Cui, MD, M.Sc.

Graduate education

PhD candidate (current)	Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario, Canada **Supervisor: Dr. Robert J.G. Haché** **Thesis title: "Integration of NHEJ into V(D)J recombination via Ku70/80 binding to the RSS heptamer and RAG1/2"**
M.Sc. 1999	Department of Microbiology, Immunology and Parasitology, Louisiana State University Health Sciences Center, New Orleans, Louisiana, USA **Supervisor: Dr. Daniel J. Carr** **Thesis title: "A plasmid construct encoding murine interferon beta antagonizes the replication of herpes simplex virus type I in vitro and in vivo"**

Publications

Cui1, B., Schild-Poulter, C., Vallée, D., Pope, L., Farhat, N., Soubeyrand, S. and R.J.G. Haché. 2004. Sequence-specific DNA binding by Ku couples DNA repair to recombination signal cleavage. Submitted.

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Mei, Q-B., Tao, J-Y. and **B. Cui.** 1991. Advances in the pharmacological studies of *Radix Angelica Sinensis (Oliv.) Diels*. Chin. Med. J. **104**:776-781.

Conference presentations

Cui, B., Schild-Poulter, C., Farhat, N., Soubeyrand, S., and R.J.G. Haché. 2004. Coupling of Ku70/80 to V(D)J recombination through preferential binding to the recombination signal sequences and association with RAG1/2. *The 12th International Congress of Immunology*. Montreal, Canada

Cui, B., Schild-Poulter, C., Farhat, N., Soubeyrand, S., and R.J.G. Haché. 2003. Preferential binding of Ku70/80 to the recombination signal sequences (RSSs) as revealed by SELEX suggests a sequence-specific function of Ku70/80 during V(D)J recombination. *2003 Proceedings of the American Association for Cancer Research,* Washington, D.C., USA

Awards

2002-2004	Ontario Graduate Studentship in Science and Technology, Ontario, Canada
1994-1996	International Cultural and Technological Interchange Fellowship, East-West Center, Honolulu, Hawaii, USA

Membership

2003-2004	Associate member of American Association for Cancer Research
1996-2004	East-West Center Alumni Association
1990-1994	Member of Chinese Medical Association Shaanxi Chapter

Contributors

Mr. Ward Giffin conducted the Ku-DNA UV-crosslinking experiment. Ms. Nawal Farhat sequenced approximately two thirds of Ku70/80-selected DNA binding sites by *SELEX*. Ms. Louise Pope carried out the initial *in vitro* RAG1/2-Ku70/80 binding assays. Mr. Dominick Vallée performed the ChIP experiments in pro-B cells.

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